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## EFFECT OF *Bifidobacterium animalis* ON MICE INFECTED WITH *Strongyloides venezuelensis*

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### SUMMARY

The administration of viable *Bifidobacterium animalis* was tested to induce resistance against *Strongyloides venezuelensis* infection in mice. Effects on parasite burden, worm length, egg output, and intestinal mucosal histology were evaluated. The oral administration of *B. animalis*, strain 04450B, starting 14 days before the inoculation of nematode larvae significantly decreased the worm burden and egg output. In probiotic treated animals, the percent reduction of adult worms in the intestine was of 33% and the reduction of egg production was of 21%, compared with those of the control group. The duodenum villous height and villous/crypt ratio were significantly higher in probiotic-treated mice, indicating that this group could be experiencing less intestinal damage. The present findings revealed that the administration of *B. animalis* for the amelioration of host response to nematode infections is biologically plausible and could have some potential for impacting public health. Meanwhile, further study is needed to delineate the nature and identity of the factor(s) involved in these beneficial effects.

**KEYWORDS:** *Strongyloides venezuelensis*; *Bifidobacterium animalis*; Probiotics; Mice.

### INTRODUCTION

In recent years, there have been remarkable advances in the understanding of the ecology, epidemiology, and morbidity related to helminthic infections, which have pointed to the need for new intervention tools, focusing especially on three main aspects: the improvement of the nutritional status of the host, the prevention or treatment of infection, and the improvement of immunocompetence<sup>20</sup>. Indeed, there has been a significant upsurge in research on the characterization of probiotic bacteria and the evaluation of the potential health benefits associated with the use of these microorganisms as functional food.

Probiotics are defined as live microbial food supplement microorganisms that confer health benefits when administered in adequate amounts<sup>15</sup>. In this way, the overall focus of probiotic research is to assess if dietary microorganisms can safely enhance immune function and gut health by improving the immune response against infectious agents.

The gastrointestinal helminth *Strongyloides stercoralis* currently infects an estimated 30-100 million people worldwide. This infection ranges from an asymptomatic clinical presentation to a severe life-threatening condition in certain population subgroups such as patients under immunosuppressive therapy and individuals with HIV/AIDS. In developing countries, where malnutrition is one of the most important causes of immunodeficiency, mainly among children, this clinical condition has been found to be associated with severe strongyloidiasis<sup>27</sup>.

Humans are the natural host of *S. stercoralis*, and up to now, the attempts made to develop an appropriated animal model have been unsuccessful, since this parasite cannot complete its life cycle in immunologically intact mouse strains<sup>10</sup>. Otherwise, *Strongyloides venezuelensis*, a nematode isolated from wild rats, is considered a suitable model of strongyloidiasis, as following the infection, its larvae migrate through the lungs before establishing themselves in the duodenal mucosa, evoking innate and acquired immune responses similar to those observed in human infection by *S. stercoralis*. In human hosts and in murine models, the immune response to *Strongyloides* spp. is characterized by intraepithelial and tissue increase of eosinophils, as well as by intestinal mastocytosis and production of Th2-type cytokines<sup>31</sup>. Moreover, *S. venezuelensis* is safe to be used in experimental assays, being a useful biological model to investigate several aspects of host-parasite relationship, including the efficacy of therapeutic agents<sup>24</sup>.

Lately, several probiotic microorganisms have been evaluated as single agents or combination therapies in a wide range of infectious and non-infectious diseases. Studies on probiotics have revealed that these microorganisms contribute to host defense by reinforcing non-immunological responses and stimulating both specific and non-specific host immunity<sup>14</sup>. Probiotic bacteria have successfully been tested in intestinal protozoan and helminth infections such as cryptosporidiosis<sup>29</sup>, giardiasis<sup>6</sup>, coccidiosis<sup>9</sup>, trichinellosis<sup>3</sup> and toxocariasis<sup>3,7</sup>.

The main organisms employed as probiotics are lactic acid bacteria,

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especially of the genus *Lactobacillus* and *Bifidobacterium*. They occur naturally as part of the normal gut flora, having a long history of safe use in food and fermented products<sup>14,30</sup>. Taking into account this particular aspect and all that has been mentioned above, *Bifidobacterium animalis*, one of the main well-characterized probiotic strains that is commercially available, was used in the present study to assess their positive effects on *S. venezuelensis* infection. The *S. venezuelensis* mouse model was chosen, considering that the infection leads to a local immune response at intestine mucosal surface, and an allergic response in the lung, both proposed to be modulated by probiotic administration.

## MATERIALS AND METHODS

**Animals:** Four-week-old male BALB/c inbred mice were purchased from the Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório (CEMIB), Universidade Estadual de Campinas, SP, Brazil. Twenty-eight animals were housed in plastic cages, fed a standard diet, and allowed free access to water. The experimental procedures were approved by the Animal Ethics Committee of College of Veterinary Medicine and Animal Science (UNESP).

**Bacteria:** *Bifidobacterium animalis* strain 04450B was kindly provided by Danisco Brasil Ltda. The probiotic was grown on De Man-Rogosa-Sharpie broth (Oxoid) adding  $\text{Na}_2\text{CO}_3$  (0.02%),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.01%), and L-cysteine hydrochloride (0.05%) under anaerobic conditions at 37° C for 16 hours. Following culture, the medium was centrifuged at 3000g, and the pelleted organisms were resuspended in 10% sterile milk to give a final concentration of  $2 \times 10^9$  CFU/mL.

**Nematode:** The strain of *Strongyloides venezuelensis* was isolated from a wild rat in the early '80s in Botucatu, São Paulo State, and has been maintained in the laboratory by repeated passages in Wistar rats. Filariform larvae (L<sub>3</sub>) were obtained from fecal cultures and adjusted to the appropriate number in distilled water in order to infect the animals<sup>23</sup>.

**Experimental procedure:** The animals were randomly allocated in groups of seven mice, distributed as follows: two probiotic treated groups (PB1 and PB2) and two non-treated control groups (C1 and C2). Daily, the animals of the groups PB1 and PB2 received one mL of milk containing  $2 \times 10^9$  UFC of *B. animalis* by gavage. The control groups of mice (C1 and C2) received the same volume (one mL) of skim milk. These procedures were repeated until the animals had been killed.

Fourteen days after the beginning of the experiment, 2,000 infective larvae (L3) of *S. venezuelensis* were subcutaneously inoculated in all animals of the four groups, according to AMARANTE & OLIVEIRA-SEQUEIRA<sup>1</sup>. For collection of fecal samples, the animals of probiotic treated (PB1 and PB2) and control (C1 and C2) groups were allocated in boxes where they stayed for three hours. The feces obtained from each group constituted a sample for fecal egg counts (FEC). Fecal egg counts were daily assessed by the modified McMaster technique<sup>16</sup> in which, each egg counted represents 100 eggs per gram of feces (EPG).

Six days after the inoculation of the nematode infective larvae, the mice of PB1 and C1 groups were killed. The small intestine (SI) was removed and a segment of one cm was sectioned (2-6 cm from the pyloric ring) and immersed in Boin's fixative for histological analysis. For the recovery and counting of adult worms, the remaining small intestine

was inverted over a thin wire support, placed in 20 mL tubes containing phosphate-buffered saline (PBS), and incubated for four hours at 37 °C<sup>1</sup>. The wire supports with intestines were then vigorously shaken and removed from tubes. Supernatants were discharged and the sediment containing the worms was preserved in formaldehyde 5%. The nematodes were counted under stereoscopic microscopy. To determine the worms' length, 20 partenogenetic females recovered from each animal were measured using a computerized image analysis system (QWin Lite 2.5, Leica), adapted in DMLB light photomicroscope (Leica).

The fecal egg counts were carried out for the remaining animals of groups PB2 and C2 until the 14<sup>th</sup> day, when they were killed. At the necropsy, the small intestine was removed and processed as previously described.

**Histological analysis:** The fixed fragments of duodenum tissues were embedded in paraffin, and cut into 3-5 µm thick sections. The sections stained with hematoxylin-eosin were used for histopathological analysis and for villous height and crypt depth measurements. Villous height was measured from the tip to the crypt junction, and the crypt depth was defined as the depth of the invagination between adjacent villi. The ratio of villous/crypt length (v/c) was also calculated. The villous and crypt lengths measurements were performed in 10-crypt units of each sample, as recommended by IERNA *et al.*<sup>19</sup>. These results were expressed as a mean of each group.

**Statistical analysis:** The two-tailed Student's *t*-test was employed to evaluate group differences in relation to parasitological parameters (worm burden and female length), and the differences related to histological parameters (crypt and villous length) were analyzed by Mann-Whitney *U* test. *p* values < 0.05 were considered statistically significant.

## RESULTS

**Parasitological parameters:** Kinetics of fecal egg counts (FEC) in *S. venezuelensis* infected mice (probiotic-treated and control groups) are shown in Fig. 1. In both animal groups, eggs were first detected in feces five days after larval subcutaneous inoculation. In probiotic-treated animals, egg output peaked on day 7 when it reached 25,500 EPG. In the control group, the peak occurred on day 6, reaching 40,400 EPG. The patent period of infection was seven and eight days, respectively, for control and probiotic-treated groups. The total FEC at the end of the patent period was 60,367 and 76,433 EPG in probiotic treated and control groups, respectively.

On day 6 after infection, the worm burden assessment (Fig. 2) revealed that the number of partenogenetic females (5,235) recovered from all seven animals of *B. animalis*-treated group was lower than that from the control one (7,819). The mean number of females recovered from probiotic-treated mice was 654 (± 137) while in the control group it was 977 (± 183). In *B. animalis*-treated mice the minimum (216) and maximum (608) numbers of parasitic females recovered were lower than those from control animals, which ranged from 608 to 1,388 worms. All these differences were statistically significant (*p* < 0.05).

The female length recovered from probiotic-treated mice ranged from 1,860 to 4,082 µm (2,943 ± 128 µm), whereas those from control mice

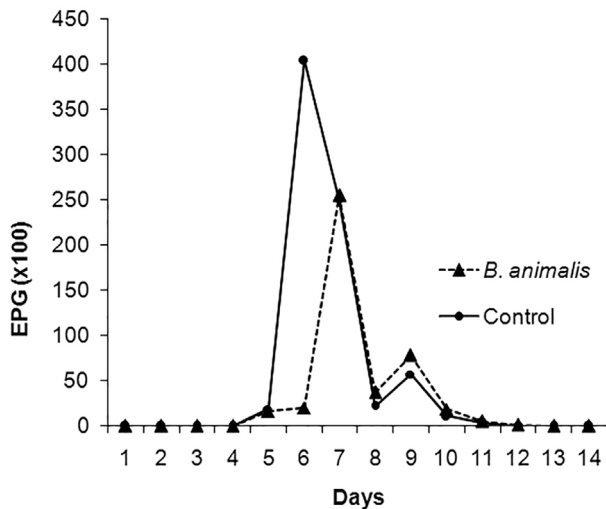


Fig. 1 - Kinetics of FEC in *S. venezuelensis* infected mice probiotic-treated and control groups. 177x133 mm (100 x 100 DPI).

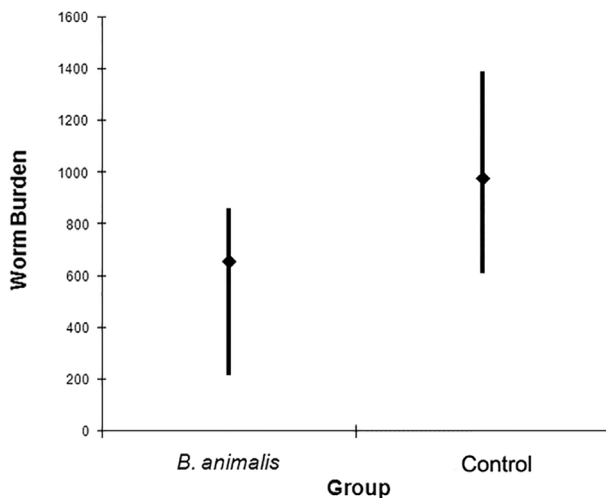


Fig. 2 - Mean, minimum and maximum numbers of *S. venezuelensis* partenogenetic females recovered from probiotic-treated and control groups of mice. 163x133 mm (100 x 100 DPI).

ranged from 2,319 to 3,781  $\mu\text{m}$  ( $3,025 \pm 151 \mu\text{m}$ ), showing no statistical differences ( $p = 0.25$ ).

**Histological analysis:** In the sections of duodenum collected at the 6<sup>th</sup> day after infection (dpi), mature worms were found in the lumina of the gut, lodged in spaces between mucosal epithelial cells and into the lamina propria (LP) of the villi. At this time, in mice of both PB1 and C1 groups, the presence of worms was accompanied by a diffuse cellular infiltration consisting of polymorphonuclear eosinophils and mononuclear cells. These inflammatory cells were sparsely distributed in the basal zone of LP, while at tip of the villi, these cells were clustered. Blood vessels at the tip of the villi showed some degree of congestion together with interstitial edema. Besides, the epithelial cells of the top of the villi showed a slight level of vacuolar degeneration. In the mice killed at 14<sup>th</sup> dpi, no worms were seen in the intestine, either in probiotic-treated or in control mice, but the same kind of cellular infiltration observed at 6<sup>th</sup> dpi was still present.

The villous height, crypt depth, and villous/crypt ratio in the upper small intestine of control and probiotic-treated mice on the 6<sup>th</sup> (PB1 and C1) and 14<sup>th</sup> (PB2 and C2) days after infection with *S. venezuelensis* are shown in Table 1. Probiotic administration did not affect crypt depth, but the associated villous height and villous/crypt ratio were significantly greater in probiotic-treated mice killed at 6<sup>th</sup> dpi (PB1) when compared to its respective control group (C1) and to probiotic-treated (PB2) and control (C2) groups, killed at 14<sup>th</sup> dpi.

**Table 1**  
Effect of probiotic treatment on villous height and crypt depth ( $\mu\text{m}$ ), and villous/crypt ratio in mice infected with *S. venezuelensis*

Groups	Villous height (Mean $\pm$ SEM)	Crypt depth (Mean $\pm$ SEM)	Villous/crypt ratio
PB1	477.1(86.4) <sup>a</sup>	236.8(33.2)	2.01 <sup>a</sup>
PB2	358.7(42.7) <sup>b</sup>	254.6(58.0)	1.41 <sup>b</sup>
C1	387.9(69.3) <sup>b</sup>	264.3(71.4)	1.47 <sup>b</sup>
C2	374.1(57.5) <sup>b</sup>	279.0(60.5)	1.34 <sup>b</sup>

Values followed by different letters in columns are significantly different ( $p < 0.05$ )

## DISCUSSION

Helminth infections are among the most common infections in humans. In developing countries, this infection is frequently associated with malnutrition, representing a significant cause of child developmental retardation. On the other hand, in most developing countries, there are public school feeding programs designed to avoid malnutrition focusing on complementary nutrition, child intestinal parasite control or both<sup>35</sup>. Therefore, the present study was an attempt to investigate the effect of probiotic supplementation on the modulation of helminth infection using a murine model.

Several clinical studies have demonstrated the therapeutic and/or prophylactic efficacy of specific probiotics against acute viral gastroenteritis and antibiotic-associated diarrhea. The studies on the protection afforded probiotics have been performed mainly with bacterial enteropathogens, but it has been suggested that they may also influence the degree of parasitic infection such as those due to *Giardia*<sup>5,18</sup>, *Eimeria*<sup>9,22</sup> and *Cryptosporidium*<sup>29</sup>.

In the present study, the administration of *B. animalis* to *S. venezuelensis* infected mice triggered a protective effect, characterized by a reduced number of worms and also by a reduced egg output. The percentage of adult worm reduction in the intestine, six days after *S. venezuelensis* infection, was 33% of mice treated with *B. animalis*. In the same way, the reduction of egg production in the probiotic-treated mice was 21%. Interestingly, the reduction of egg output was especially significant in the peak of elimination, when besides the reduction of 37%, peak delayed one day in probiotic treated mice in relation to control mice. Female length and pre-patent period were not affected by the probiotic administration, but the patent period was one day longer in probiotic-treated mice.

In relation to probiotic effects on helminth infections, the few available studies provide conflicting results. Reduction of worm burden

was reported in mice infected with *Trichinella spiralis*<sup>3,4,25</sup> and *Toxocara canis*<sup>2,7</sup>. The failure of probiotics in affecting parasite load was reported in rats<sup>12</sup> and mice<sup>33</sup> infected with *T. spiralis*, and finally, an enhancement of mice susceptibility to *Trichuris muris* was attributed to *Lactobacillus casei* administration<sup>11</sup>.

The overall evaluation of the effect of probiotic administration on the worm burden revealed that is difficult to compare results, because of differences in study design, animal model, probiotic dose and strain, and administration route. Nevertheless, most of the available studies revealed promising results, encouraging further investigations in order to confirm the real role of probiotics in host response to nematode infections.

Worm burden is the most frequently investigated aspect in terms of host response to helminth infections, but this response may also interfere with some of the physiological functions of the parasite, including reduction of parasite fertility, limited growth and structural damage<sup>21</sup>. Our data revealed no effect of probiotic administration on the growth of female recovery on the 6<sup>th</sup> day after infection ( $p = 0.26$ ). So, instead of being related to poor female growth, the observed reduction of egg output may be related to worm burden, because fecal egg counts are correlate highly with parasite load<sup>26</sup>. This is an important observation because egg production is the main means by which the parasite is known to spread. Reduction of fecal shed forms like cysts and oocysts associated with probiotic administration was previously reported in *Giardia*<sup>32</sup> and *Eimeria acervulina* infections<sup>8,9,22</sup>.

The beneficial claims of probiotics activities are poorly understood, needing scientific validation that can be addressed by assessing probiotic strains under controlled experimental conditions. In order to understand the immunomodulatory mechanism by which probiotic administration improves the host response against *S. venezuelensis* infection, some components of the intestinal response considered relevant against nematode infection were investigated.

In the present work, eosinophils and mononuclear were the main cells infiltrating the intestinal mucosa of mice, showing similar patterns of distribution in probiotic-treated mice and control group, either after six or fourteen days after the inoculation of the *S. venezuelensis* larvae. Increased eosinophils and mononuclear cell numbers are commonly associated with helminth infections, and in *S. stercoralis* infection of mice, their role as antigen-present cell for the induction of the primary and expansion of secondary Th2 immune response has been characterized<sup>28</sup>. According to the data obtained here, *B. animalis* administration appears to make no difference in patterns of effector cells infiltration and spatial distribution. Therefore, the effect of the probiotic administration in reducing the worm burden and the egg output in *B. animalis* treated mice cannot be attributed to a modulation of these inflammatory cells, at least at the intestinal level. It would be of great interest to know whether *B. animalis* has an effect upon these cells during lung migrating stages, since *S. venezuelensis* infection also increases eosinophil and mononuclear cell numbers in blood and bronchoalveolar fluid<sup>23</sup>. Besides, it appears that activated eosinophils are relevant for the destruction of migrating larvae, rather than for the elimination of adult worms<sup>13</sup>.

Villous atrophy and/or crypt hyperplasia are occasionally induced by nematode infection<sup>17</sup> and high apoptotic rates concomitant with low

cell proliferation was found associated with a *S. stercoralis* infection in humans<sup>34</sup>. On the other hand, it has been demonstrated that the administration of either *Bifidobacterium adolescentis* or *Bifidobacterium longum* increase the height of duodenum villi<sup>36</sup>. Thus, in the present work, the measurement of villous height and crypt depth was performed in order to verify if probiotic administration has some effect in repairing the intestinal epithelium during *S. venezuelensis* infection.

Data obtained here revealed that the duodenum villous height and villous/crypt ratio were significantly higher in probiotic-treated mice killed six days after *S. venezuelensis* infection, indicating that this group could be experiencing less intestinal damage. Fourteen days after infection, when most of the worms were expelled, no difference in villous length or villous/crypt ratio was found in probiotic-treated and control mice. A time declining effect of the administration of *Bifidobacterium longum* upon the mice's villous length was previously reported<sup>36</sup>.

Taken together, the present findings suggest that the administration of *B. animalis* to improve immunity to nematode infections is biologically plausible and could have some impact in public health. Considering that intestinal parasitism and malnutrition share a similar geographical distribution, studies with probiotics should focus on the development of functional foods for the treatment of both diseases.

## RESUMO

### Efeito da administração de *Bifidobacterium animalis* sobre a infecção por *Strongyloides venezuelensis* em camundongos

Os efeitos da administração de *Bifidobacterium animalis* viáveis sobre a infecção por *Strongyloides venezuelensis* foram avaliados em camundongos experimentalmente infectados. Os parâmetros analisados incluíram a carga parasitária, o comprimento dos vermes, a quantidade de ovos eliminados e a histologia da mucosa intestinal. A administração oral da cepa 04450B de *B. animalis*, iniciada 14 dias antes da inoculação de larvas do nematódeo, foi acompanhada de uma redução significativa do número de vermes que se estabeleceu no intestino e do número de ovos eliminados nas fezes. Nos animais tratados com o probiótico, o percentual de redução de vermes adultos no intestino foi de 33% e da produção de ovos foi de 21%, em comparação com os do grupo controle. O comprimento das vilosidades do duodeno e a relação vilus/crípta foram significativamente maiores nos animais tratados, indicando que nestes animais as lesões intestinais foram mais leves. Os resultados do presente trabalho revelaram que a administração de *B. animalis* com o propósito de modular a resposta do hospedeiro contra infecções por nematódeos é uma possibilidade biologicamente plausível com impacto potencial em saúde pública. No entanto, são ainda necessários mais estudos para esclarecer os mecanismos de ação destes microrganismos e identificar os fatores envolvidos na produção dos efeitos benéficos.

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