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The anti-parasitic effect of probiotic bacteria *via* limiting the fecundity of *Trichinella spiralis* female adults

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Summary

A potential protective effect of probiotic strains against zoonotic *Trichinella spiralis* infection was investigated in the framework of a new therapeutic strategy aimed at using probiotics to control parasitic zoonoses. The study was focused on the impact of six selected probiotic (bacteriocinogenic) strains on the intensity of *T. spiralis* infection and female fecundity *ex vivo* and *in vitro*. Bacterial strains of different origin (*Enterococcus faecium* EF55, *Enterococcus faecium* 2019 = CCM7420, *Enterococcus faecium* AL41 = CCM8558, *Enterococcus durans* ED26E/7, *Lactobacillus fermentum* AD1 = CCM7421, *Lactobacillus plantarum* 17L/1) were administered daily in a dose of 10^9 CFU/ml in 100 µl, and mice were infected with 400 *T. spiralis* larvae on day 7 of treatment. Female adults of *T. spiralis* were isolated on day 5 post infection (p.i.) and subsequently were used in fecundity test *ex vivo*. *E. faecium* CCM8558, *E. faecium* CCM7420 and *E. durans* ED26E/7 strains significantly reduced the number of adults in the intestine. The application of *L. fermentum* CCM7421, *L. plantarum* 17L/1, *E. faecium* CCM8558 and *E. durans* ED26E/7 caused a significant decrease in the number of muscle larvae. The treatment with *E. faecium* CCM8558 and *E. durans* ED26E/7 showed the highest inhibitory effect on female fecundity (94 %). The number of newborn larvae (NBL) was also significantly decreased after administration of *L. fermentum* CCM7421 and *L. plantarum* 17L/1 (80 %). A direct impact of probiotic strains on female reproductive capacity was examined *in vitro* in females isolated from untreated infected mice on day 5 p.i. A correlation was found between the inhibitory effect and the concentration of probiotic strains. The reduction effects of the strains manifested as follows: *L. fermentum* CCM7421 (93 %), *E. faecium* CCM8558, *L. plantarum* 17L/1, *E. faecium* EF55 (about 80 %), *E. faecium* CCM7420 and *E. durans* ED26E/7 (about 60 %).
Keywords: *Trichinella spiralis*; female fecundity; probiotic bacteria; *Enterococcus*; *Lactobacillus*

Introduction

The host gut represents a complex ecosystem where the interactions between intestinal microbiota, immune system, and pathogens occur. For healthy organisms is crucial to form the balance between the gut microbiota and the host organism (Berrilli *et al*., 2012). It has been recognized that the excretory/secretory molecules produced by helminths may lead to significant alterations in the composition of the gut microbiota (Walk *et al*., 2010; Li *et al*., 2012). Gut microbiota products and metabolites also significantly influence the survival and the physiology of many parasites and, consequently, the outcome of parasitic infections. This suggests that probiotic bacteria can successfully reduce the pathogenicity of many parasites, probably through multiple mechanisms (Berrilli *et al*., 2012; Travers *et al*.,

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2011). The main mechanisms of probiotic actions include: enhancement of the gut epithelial barrier, increase of adhesion to intestinal mucosa and simultaneous inhibition of pathogen adhesion, competitive elimination of pathogens, production of anti-microbial molecules, and modulation of the immune system (Goudarzi *et al*., 2014).

Several studies have investigated the ability of probiotics to influence the course of different parasitic infections (Travers *et al*., 2011). Positive effects of probiotic bacteria reducing the parasite burden and pathological changes in experimental trichinellosis due to the activation of local and systematic immune responses were previously described (Bautista-Garfias *et al.,* 1999, 2001; Martínez-Gómez *et al*., 2009, 2011; El Temsahy *et al*., 2015; Dvorožňáková *et al*., 2016), in ascariasis (Solano-Aguilar *et al*., 2004), and toxocarosis (Basualdo *et al*., 2007). Probiotic bacterial strains can positively affect protozoan parasitic infections such as cryptosporidiosis, giardiasis, coccidiosis (Gargala, 2008; Alak *et al*., 1999; Pérez *et al*., 2001; Shukla and Sidhu, 2011). Probiotic bacterial strains are also being tested in the host protection against blood parasites like *Babesia, Plasmodium,* and *Trypanosoma* (Bautista-Garfias *et al*., 2005; Galdeano and Perdigón, 2006; Martínez-Gómez *et al*., 2006; Eze *et al*., 2012).

Trichinellosis is a serious food-borne parasitic zoonosis caused by the nematode of the genus *Trichinella*, which is characterized by an extremely wide host range and worldwide distribution (Bruschi, 2012; Goździk *et al*., 2017). In general, therapeutic approaches against trichinellosis can be divided into two groups: classic and alternative. The classic treatment includes the application of anthelmintics, primarily albendazole and mebendazole (Gottstein *et al*., 2009); however, the efficacy of these benzimidazole derivatives is limited by the following factors: 1) weak activity against encapsulated larvae, 2) low water solubility, 3) increasing anthelmintic resistance, 4) contraindication in children and pregnancy (Yadav and Temjenmongla, 2012). Therefore, the anti-parasitic potential of probiotic bacteria (El Temsahy *et al*., 2015), natural proteins (Othman *et al*., 2016), and substances such as myrrh, thyme or artemisinin (Attia *et al*., 2015; Abou Rayia *et al*., 2017) is being increasingly utilized in recent years.

The present study was designed to study the anti-parasitic effects of six different probiotic strains of lactobacilli and enterococci on the parasite burden in the host and on the fecundity of *T. spiralis* females.

Materials and Methods

Probiotic strains

The effects of the following bacteria were tested: bacteriocin-producing strains with probiotic properties (*Enterococcus faecium* EF55, *Enterococcus faecium* 2019 = CCM7420, *Enterococcus faecium* AL41 = CCM8558, *Enterococcus durans* ED26E/7, and *Lactobacillus plantarum* 17L/1) and probiotic strain *Lactobacillus fermentum* AD1 = CCM7421. All used strains are original isolates which were not previously used for this purpose.

Enterococcus faecium EF55 was isolated from the chicken crop and characterized at the Institute of Animal Physiology SAS – IAP SAS, Košice, Slovakia. The strain produces a thermo-stable bacteriocin EF55 (Strompfová *et al*., 2010).

Enterococcus faecium 2019 = CCM7420 is a rabbit-derived strain with probiotic properties, which produces enterocin 2019 (Ent 2019) (Pogány Simonová *et al*., 2013). It was isolated and characterized at IAP SAS, Košice, Slovakia and deposited in the Czech Culture Collection of Microorganisms, Brno, Czech Republic – CCM7420.

Enterococcus faecium AL41 = CCM8558 (isolated and characterized at IAP SAS, Košice, Slovakia and deposited in the Czech Culture Collection of Microorganisms, Brno, Czech Republic – CCM8558) is an environment-derived strain. The strain produces an enterocin M with a wide antimicrobial inhibitory spectrum and possesses probiotic properties (Lauková *et al*., 2012; Mareková *et al*., 2007).

Enterococcus durans ED26E/7 was isolated from traditional ewes milk lump cheese at the Research Dairy Institute, Žilina – RDI, Žilina, Slovakia; but identified, characterized and prepared for experiment at IAP SAS, Košice, Slovakia (Lauková *et al*., 2015).

Lactobacillus plantarum 17L/1 was isolated from stored ewes cheese (RDI, Žilina, Slovakia) but identified, characterized and prepared for experiment at IAP SAS, Košice, Slovakia (Lauková *et al*., 2013).

Lactobacillus fermentum AD1 = CCM7421 was isolated and characterized at IAP SAS, Košice, Slovakia and deposited in the Czech Culture Collection of Microorganisms, Brno, Czech Republic – CCM7421. It is a canine-derived strain possessing probiotic properties (Strompfová *et al*., 2008).

All used strains were evaluated according to the EFSA rules (Piskoríková, 2010). For the experiment they were prepared as follows: they were cultivated in MRS broth (Merck, Eppelheim, Germany) at 37 °C for 24 h. Broth cultures were centrifuged (30 min at 10,000*g*) and the sediment was resuspended in Ringer solution (Merck, pH 7.0) to a concentration of 10^9 colony forming units per ml (CFU/ml). The purity of the strains was checked by the standard microbiological method (ISO-International Organization for Standardization) by spreading dilutions in Ringer solution (Merck, pH 7.0) onto the selective medium ME-*Enterococcus* agar (ISO-15214, Difco, Detroit, USA) and/or MRS agar (Merck, Eppelheim, Germany). The cultures for application were stored at 4 °C.

Parasite

The reference isolate of *Trichinella spiralis* (ISS 004) (obtained and assigned codes from the Trichinella Reference Centre in Rome), maintained by serial passages in ICR mice at the Institute of Parasitology SAS, was used for the infection. Larvae were released by artificial digestion (1 % pepsin, 1 % HCl for 4 h at 37 °C; both from Sigma-Aldrich, Germany) of tissue following the protocol of Kapel and Gamble (2000) and kept in saline solution until inoculation of experimental mice.

Experimental design

The experiment was performed on pathogen-free eight week old male BALB/c mice (VELAZ, Prague, Czech Republic; n = 110) weighting 18 – 20 g. Mice were kept under a 12-h light/dark regime at room temperature (22 – 24 °C) and 56 % humidity on a commercial diet and water.

Animals were divided randomly into 7 groups: Control (n = 15) – *T. spiralis* infection without the administration of bacterial strains; Group 1 (n = 15) – *Enterococcus faecium* EF55 + *T. spiralis*; Group 2 (n = 15) – *E. faecium* CCM7420 + *T. spiralis*; Group 3 (n = 15) – *E. faecium* CCM8558 + *T. spiralis*; Group 4 (n = 15) – *E. durans* ED26E/7 + *T. spiralis*; Group 5 (n = 15) – *Lactobacillus fermentum* CCM7421 + *T. spiralis;* Group 6 (n = 15) – *L. plantarum* 17L/1 + *T. spiralis*. Probiotic strains were administered *per os* daily at a dose of 10^9 CFU/ml in 100 µl and mice were infected *per os* with 400 *T. spiralis* larvae/mouse on day 7 of treatment. Samples of the small intestine and muscles were obtained on days: 5, 11, 18, 25 and 32 p.i. For *ex vivo* fecundity test, adult *T. spiralis* females were isolated from the small intestine of three mice from each group on day 5 p.i.

In vitro fecundity test included mice (n = 5) without probiotic treatment and infected *per os* with 400 *T. spiralis* larvae/mouse. Similarly, female adults of *T. spiralis* were obtained from the small intestine on day 5 p.i.

Intestinal worm burdens

The intestinal phase of infection was investigated on days 5, 11 and 18 p.i. The small intestine was cut into 5 – 10 cm long pieces, placed into a sieve and incubated in conical pilsner glasses in 37 °C NaCl (0.9 % saline) overnight. After incubation, gut pieces were discarded and the worms in the sediment were counted under stereomicroscope at 60 x magnification (Leica S8APO, Leica Microsystems, Germany).

Isolation of muscle larvae

The muscle phase of infection was examined on day 18, 25 and 32 p.i. Whole eviscerated carcasses were minced and artificially digested (1 % pepsin HCl for 4h at 37 °C; both from Sigma-Aldrich, Germany), according to Kapel and Gamble (2000). Samples were allowed to settle for 20 min before the supernatant was discarded and the sediment was poured through a 180 µm sieve into a conical glass and washed with tap water. The sediment was finally transferred to a gridded Petri dish and counted using a stereomicroscope at 40 x magnification (Leica S8APO, Leica Microsystems, Germany). Depending on the density of larvae either a sub-sample or the whole sample was counted.

Obtaining of female adults for fecundity tests

The adult *T. spiralis* females were obtained according to Cabaj (1990). The small intestine was washed with PBS medium (pH 7.2), split longitudinally, cut into 1 cm long pieces, placed into a sieve over 50 ml beakers containing RPMI 1640 medium (Sigma-Aldrich, Germany) with antibiotics (100U/ml penicillin; 100U/ml streptomycin) and incubated in a water bath at 37 °C for 2 h. After incubation, gut pieces were discarded and worms in the medium were centrifuged in centrifuge tubes (Falcon, France) at 67*g* for 5 min. The sediment was finally transferred to a Petri dish and the female worms were identified using a stereomicroscope at 40 x magnification (Leica S8APO, Leica Microsystems, Germany).

Ex vivo fecundity test

The females isolated from the gut of treated and infected mice (4 from each mouse) were rinsed with the incubation medium, transferred to separate wells of a 24-well tissue culture plate (Falcon, France) containing RPMI 1640 medium (Sigma-Aldrich, Germany) supplemented with 3 % foetal bovine serum plus antibiotics (100U/ml penicillin; 100U/ml streptomycin). The plates were sealed with plastic wrap and incubated for 20 h at 37 °C in 5 % CO_2. NBL were counted in each well using an inverted microscope at 60 x magnification (Leica DM IL LED, Leica Microsystems, Germany). Results were expressed as the average number of NBL *per* one female parasite.

In vitro fecundity test

T. spiralis female adults were obtained from the small intestine of untreated infected mice on day 5 p.i. (30 from each mouse) and incubated afterwards in RPMI 1640 medium (Sigma-Aldrich, Germany) enriched with 3 % foetal bovine serum and selected probiotic strain (*E. faecium* EF55; *E. faecium* CCM7420; *E. faecium* CCM8558; *E. durans* ED26E/7; *L. fermentum* CCM7421; *L. plantarum* 17L/1) at different concentration (10^7, 10^5, 10^3 and 10^1 CFU/ml) or without strains (control) for 20 h at 37 °C in 5 % CO_2. NBL were counted in each well using an inverted microscope at 60 x magnification (Leica DM IL LED, Leica Microsystems, Germany). Results were expressed as the average number of NBL *per* one female parasite.

Statistical analysis

Statistical differences were assessed using one-way ANOVA, followed by *post hoc* Tukey's test (a value of $P<0.05$ was considered significant), which allowed comparison between each two groups at each time point. The analyses were performed using the Statistica 6.0 (Stat Soft, Tulsa, USA) statistical package.

Ethical Approval and/or Informed Consent

The research related to animals has been complied with all the relevant national regulations and institutional policies for the care and use of animals. The experimental protocol was in compliance with current Slovak ethical rules for animal handling and it was approved by the Animal Care Committee of the Institute of Parasitology SAS and the State Veterinary and Food Administration of the Slovak Republic (Ro-3184/14-221).

Table 1. Numbers of adult worms isolated from the small intestine of mice with probiotic treatment and *T. spiralis* infection.

Day post infection	*T. spiralis* (Mean ± S.D.)	*E. faecium* EF55 + *T. spiralis* (Mean ± S.D.)	*E. faecium* CCM8558 + *T. spiralis* (Mean ± S.D.)	*E. faecium* CCM7420 + *T. spiralis* (Mean ± S.D.)	*E. durans* ED26E/7 + *T. spiralis* (Mean ± S.D.)	*L. fermentum* CCM7421 + *T. spiralis* (Mean ± S.D.)	*L. plantarum* 17L/1+ *T. spiralis* (Mean ± S.D.)
5	295 ± 24	293 ± 38	244 ± 25	235 ± 35	256 ± 7	209 ± 21	326 ± 48
11	229 ± 37	160 ± 39	*107 ± 25	*112 ± 14	*142 ± 29	210 ± 27	192 ± 8
18	2 ± 2	0 ± 0	0 ± 0	0 ± 0	1 ± 2	40 ± 12	1 ± 2

*P < 0.05 - statistically significant differences from *T. spiralis* infected group without treatment

Table 2. Numbers of muscle larvae isolated from mice with probiotic treatment and *T. spiralis* infection.

Day post infection	*T. spiralis* (Mean ± S.D.)	*E. faecium* EF55 + *T. spiralis* (Mean ± S.D.)	*E. faecium* CCM8558 + *T. spiralis* (Mean ± S.D.)	*E. faecium* CCM7420 + *T. spiralis* (Mean ± S.D.)	*E. durans* ED26E/7 + *T. spiralis* (Mean ± S.D.)	*L. fermentum* CCM7421 + *T. spiralis* (Mean ± S.D.)	*L. plantarum* 17L/1+ *T. spiralis* (Mean ± S.D.)
18	2 ± 4	40 ± 9	47 ± 2	24 ± 6	28 ± 16	4 ± 2	65 ± 6
25	50,080 ± 4,931	37,060 ± 4,150	**13,220 ± 1,842	27,970 ± 7,212	*23,250 ± 5,938	**18,380 ± 2,039	**15,540 ± 2,191
32	54,069 ± 8,020	42,580 ± 7,750	*23,810 ± 799	46,950 ± 3,818	**29,080 ± 2,204	*26,272 ± 2,566	32,070 ± 6,463

*P < 0.05; **P < 0.01 - statistically significant differences from *T. spiralis* infected group without treatment

Table 3. *In vitro* fecundity of *T. spiralis* females isolated from mice without treatment and subsequently cultivated with probiotic strains – Numbers of newborn larvae.

Concentration of probiotic strains (CFU/ml)	Control (Mean ± S.D.)	*E. faecium* EF55 (Mean ± S.D.)	*E. faecium* CCM8558 (Mean ± S.D.)	*E. faecium* CCM7420 (Mean ± S.D.)	*E. durans* ED26E/7 (Mean ± S.D.)	*L. fermentum* CCM7421 (Mean ± S.D.)	*L. plantarum* 17L/1 (Mean ± S.D.)
0	58 ± 8						
10^1		39 ± 13	46 ± 14	36 ± 16	43 ± 11	32 ± 14	38 ± 23
10^3		24 ± 15	**23 ± 7	37 ± 11	38 ± 21	*25 ± 13	37 ± 10
10^5		*34 ± 10	*29 ± 9	*32 ± 8	**26 ± 7	**16 ± 8	37 ± 13
10^7		**14 ± 9	**12 ± 7	**22 ± 10	**23 ± 9	***4 ± 3	**13 ± 5

*P < 0.05
**P < 0.01
***P < 0.001 - statistically significant differences from *T. spiralis* females incubated in medium without probiotic strains (control)

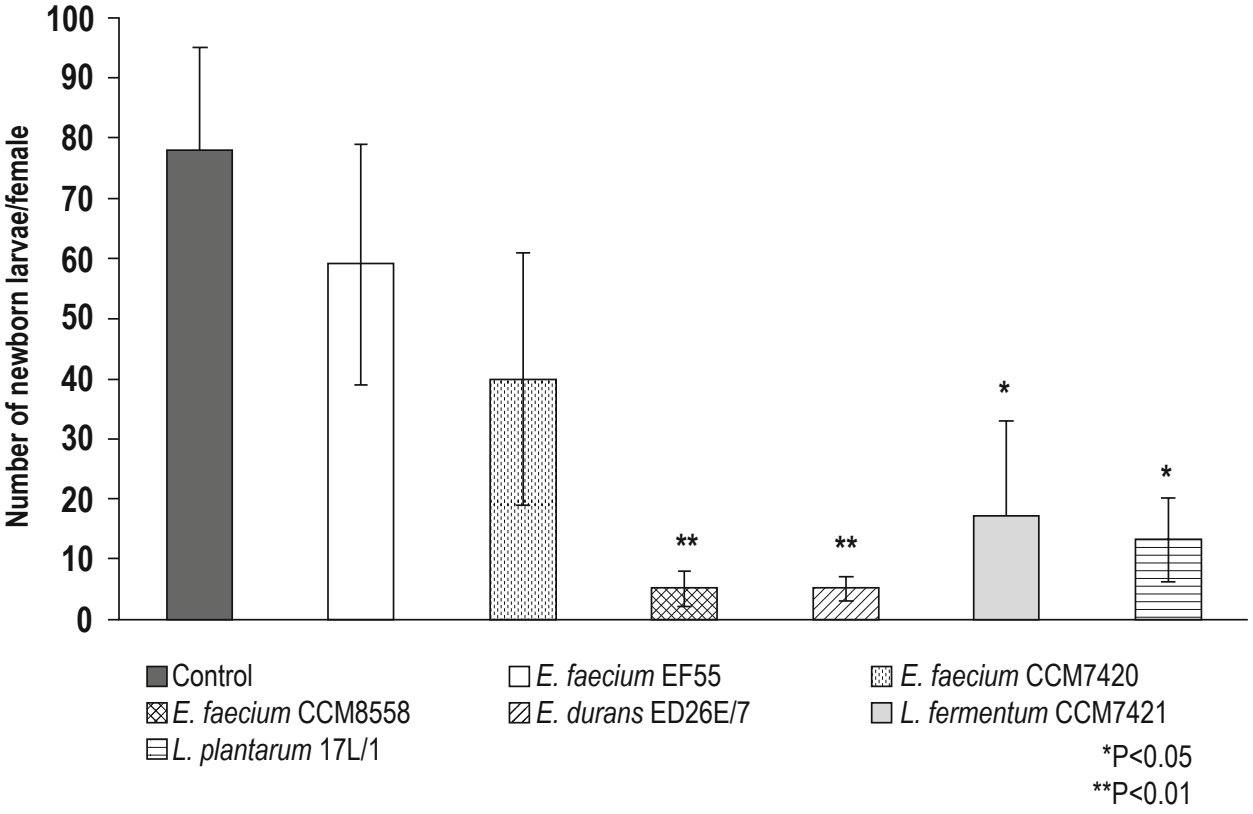

Fig. 1. *Ex vivo* fecundity of *T. spiralis* females isolated from mice treated with probiotic strains – Numbers of newborn larvae. *P<0.05; **P<0.01-statistically significant differences from *T. spiralis* infected group without probiotic treatment (control).

Results

Parasite burden – numbers of adults and muscle larvae

The highest numbers of *T. spiralis* adults (209 – 326) were found in the small intestine on day 5 p.i. in all groups (Table 1). A significant reduction of intestinal parasites occurred on day 11 p.i. in mice with administration of bacteriocin-producing strains *E. faecium* CCM8558 (107 ± 25), *E. faecium* CCM7420 (112 ± 14) and *E. durans* ED26E/7 (142 ± 29). Mice with this probiotic treatment absolutely eliminated adults from the small intestine till day 18 p.i. In evaluation of muscle phase of infection (Table 2), the occurrence of *T. spiralis* larvae was sporadic on day 18 p.i. (2 – 65). Numbers of muscle larvae reached the maximum in untreated mice on day 25 and 32 p.i. (50,080 ± 4,931 and 54,069 ± 8,020; respectively). Administration of strains *E. faecium* CCM8558, *E. durans* ED26E/7, *L. fermentum* CCM7421 and *L. plantarum* 17L/1 resulted in a significant larval count reduction with a higher efficacy on day 25 p.i. (13,220 – 23,250 larvae/mice).

Ex vivo fecundity test

In our experiments, female fecundity was significantly decreased after the administration of enterococci and lactobacilli in comparison to *T. spiralis* infected group without treatment (Fig. 1). The greatest inhibition in female reproductive capacity was caused by strains *E. faecium* CCM8558 and *E. durans* ED26E/7 with 94 % reduction of NBL. Similarly, the high reduction of female fecundity was recorded after treatment with *L. fermentum* CCM7421 and *L. plantarum* 17L/1 (78 % and 83 %). The application of strains *E. faecium* EF55 and *E. faecium* CCM7420 had only a modest inhibitory effect on the fecundity of females.

In vitro fecundity test

The strain concentration of 10^7 CFU/ml was the most effective among four examined concentrations of probiotic strains (Table 3). The highest decrease in the number of NBL was recorded after incubation of females with *L. fermentum* CCM7421 (93 %) followed by *E. faecium* CCM8558 (79 %), *L. plantarum* 17L/1 (78 %), *E. faecium* EF55 (76 %), *E. faecium* CCM7420 (62 %) and finally *E. durans* ED26E/7, which showed the 60 % reduction. The production of NBL was increased in relation to a decreasing concentration of bacteria. All tested probiotic strains at a concentration of 10^5 CFU/ml, except of *L. plantarum* 17L/1, had a significant inhibitory effect on female fecundity (in the range of 41 – 72 %). The females incubated with *E. faecium* CCM8558 or *L. fermentum* CCM7421 at a concentration of 10^3 CFU/ml were 60 % less fecund then control females. In comparison to the control, the lowest con-

centration of probiotic bacteria (10^1 CFU/ml) was also efficient to decrease the number of NBL.

Discussion

The available chemotherapy (benzimidazoles) of human trichinellosis is effective only against adult worms, not against muscle larvae. For trichinellosis as an important parasitic zoonosis with a worldwide distribution and epidemic occurrence (Devleesschauwer *et al.*, 2015), the development of new methods to control this disease is inevitable and the use of probiotic bacteria could be successfully employed (Martínez-Gómez *et al.*, 2011; El Temsahy *et al.*, 2015).

The nematode *T. spiralis* has been chosen as a model parasite to verify anti-parasitic properties of probiotic and bacteriocin-producing strains. Pathogenicity of *T. spiralis* is higher than other intestinal parasites due to the high production of NBL (Pozio *et al.*, 1992) and a strong immune response of the host (Pozio *et al.*, 1993; Bruschi *et al.*, 1999; Morales *et al.*, 2002). This study investigated the influence of tested probiotic bacteria on the adult worm and larvae burdens in mice.

T. spiralis infection affects the host in two phases, intestinal and muscular (Abou Rayia *et al.*, 2017). Parasite adults live in the epithelium of the small intestine, where the viviparous females produce a large number of NBL (500 – 1,500 larvae/female) from day 5 p.i. (Mitreva and Jasmer, 2006). These NBL migrate into the blood stream *via* intestinal lymphatics or mesenteric vessels, and finally reach the striated muscles that represent their predilection sites. There, they induce the formation of nurse cell and become encysted (Despommier, 1983). Gut microflora plays a crucial role in completing the life cycle of the parasite in the intestine, enabling the development into adults and their reproduction, and also in modulating the host immune response. Probiotic bacteria can provide an indirect protection, probably by modulating effect on newborn and muscle *T. spiralis* larvae (Travers *et al.*, 2011; Dvorožňáková *et al.* 2016).

Probiotic organisms are able to modulate their physicochemical environment: nutrients, pH, availability of receptors on epithelial cells, the epithelial tight junctions, and peristalsis. Probiotic bacteria can also control their biotic environment by regulating intestinal motility and mucus secretion (Gupta and Garg, 2009; Travers *et al.*, 2011), two major components of the intestinal physiology participating in the host defence against worms (Khan, 2008). The attachment of probiotics to the gut epithelium is an important determinant to achieve their beneficial effect on the host organism. All administered strains from our study sufficiently colonize the small intestine during the infection (Dvorožňáková *et al.*, 2014). In the present study, three strains of enterococci, *E. faecium* CCM8558, *E. faecium* CCM7420 and *E. durans* ED26E/7, significantly reduced the number of adult parasites in the intestine on day 11 p.i., with reduction rates of 53 %, 51 %, and 38 %, respectively. On the other hand, *L. fermentum* CCM7421 and *L. plantarum* 17L/1 had no influence on worm burden during the intestinal phase of the infection. We assume that it could be caused by the worse adhesive capacity of lactobacilli compared with enterococci (Lauková *et al.*, 2004). A weak anti-adult effect of *Lactobacillus* strains documented in our experiment is opposite to other studies. After intraperitoneal application of *L. casei* ATCC7469, Bautista-Garfias *et al.* (1999) recorded 88.5 % reduction in the number of *T. spiralis* adults. Also, when the same *L. casei* strain was administered *per os*, the reduction effect was 58 % (Bautista-Garfias *et al.*, 2001). Similarly, intraperitoneally applied strain of *L. casei* Shirota implied a 78.6 % reduction of intestinal parasites (Martínez-Gómez *et al.*, 2011). El Temsahy *et al.* (2015) recorded the reduction of *T. spiralis* adults after treatment with *L. plantarum* P16456 by 98 %, 65.4 % and 69 % on days 5, 12, and 17 p.i., respectively. These differences between the present study and other studies could result from using of various strains of lactobacilli, different infective and therapeutic doses, application method and/or design of experimental studies. We observed an increase in larval burden between days 25 and 32 p.i. in all experimental groups, untreated or treated mice. This might be caused by the continuous larval migration to the muscles. The increase was the lowest in untreated mice (4,000 larvae/mouse), and similar (5,000 - 10,000 larvae/mouse) in all four treated groups. Only in mice treated with *L. plantarum* 17L/1 and *E. faecium* CCM7420 - the numbers of muscle larvae increased by 17,000 -19,000 larvae/mouse, respectively. We can assume that probiotic therapy delayed migration of the NBL and larval motility was disrupted. Bacterial strains produce lactic and acetic acid, hydrogen peroxide, proteinaceous enterocins and bacteriocins, which are important mechanisms in pathogens exclusion (Šušković et al., 2010; Lauková et al., 2012). These bacterial substances might also affect the larvae vitality and participate in their destruction, particularly through hydrogen peroxide. It could be documented by the reduced larval burden. In our study, the number of muscle larvae in treated mice has significantly decreased on days 25 and 32 p.i., particularly in mice with administration of *L. fermentum* CCM7421, *L. plantarum* 17L/1, *E. faecium* CCM8558, and *E. durans* ED26E/7. The percentage of larval count reduction on day 25 was as follows: 63 %, 69 %, 74 %, and 54 %, respectively. Lower reduction values, yet still significant, were recorded on day 32 p.i.: 51 %, 41 %, 56 %, and 46 %, respectively. Similar efficacy against *T. spiralis* larvae was also shown in another probiotic strains, e.g. *L. casei* ATCC7469, *L. casei* Shirota, and *L. plantarum* P164 in a variety of experiments (Bautista-Garfias *et al.*, 2001; Martínez-Gómez *et al.*, 2011; El Temsahy *et al.*, 2015). In this context, it is important to emphasize that the beneficial effects of probiotics cannot be generalized given that they are strain-specific (Gupta and Garg, 2009).

The parasite infectivity is a result of the interplay of four components: the number of females that develop into adults, their fecundity, the length of their survival in the gut, and the period during which the muscle larvae remain viable (Dvorožňáková *et al.*, 2011). The decreased numbers of *T. spiralis* muscle larvae

induced by bacterial strains in our study might be associated with a reduced female fecundity or destroying of NBL during their migration to the host muscles.

This is the first study that investigates the effect of probiotic strains on the fecundity of *T. spiralis* females. Our aim was to determine whether the reduced parasite burden is associated with a decreased fecundity induced by probiotic strains, or these strains prevented the NBL migration into the blood and the lymphatic circulation, or stimulated host immunity participated on this reduction. It could be discerned by results of *ex vivo* fecundity test at females isolated from the gut of infected and treated mice. The female reproductive capacity was significantly inhibited after administration of strains *E. faecium* CCM8558, *E. durans* ED26E/7 (about 94 %), *L. fermentum* CCM7421 and *L. plantarum* 17L/1 (about 80 %). In contrast to non-affected numbers of adults presented in the gut of mice treated with *Lactobacillus* strains, their reproductive capacity was suppressed. These strains did not affect the maturation of *T. spiralis* larvae into adults or their expulsion from the gut, but they contributed to the decreased muscle parasite loads in the host by the control of NBL production.

In vitro test regarding the fecundity of females *ex vivo* showed the extreme reduction in their reproductive potential. However, it may not reflect the fecundity *in vivo* where total muscle larval recovery lead to the lower reduction effects what was caused by probiotic therapy. Considering the numbers of larvae, which reached and encysted in muscles, the actual reproduction of females *in vivo* finished at about 50 %. These differences between *in vivo* and *ex vivo* female fecundity could be caused by biochemical and physiological conditions within the host organism. For example, the physico-chemical conditions of the jejunum are more fecund than those in the ileum. This site is more appropriate and results in a higher reproductive success of *T. spiralis* (Sukhdeo, 1991). Other authors (Gagliardo et al., 2002) confirmed that the intestinal life cycle of *T. spiralis* (including reproduction) is supported entirely by the host epithelial cells. All these supporting mechanisms provided by the host are absent under *in vitro* conditions. Based on our results, the impact of our six probiotic strains on female fecundity was elucidated; however, an influence of other factors within the host organism such as gut physiology or immunomodulatory activity of probiotic bacteria cannot be excluded. It could be related to the colonization of the intestinal epithelium with probiotic strains. The adhesion of strains on gut mucosal surfaces and also the production of antibacterial agents as bacteriocins, hydrogen peroxide (Pridmore *et al.*, 2008; Gupta and Garg, 2009; Hertzberger *et al.*, 2014) might prevent the parasite to enter the host epithelial cells, a site where *T. spiralis* larvae molt, ecdyse, develop to adulthood and reproduce (Gagliardo *et al.*, 2002).

Nevertheless, the data obtained from *in vitro* fecundity test also revealed a direct inhibitory impact of probiotic bacterial strains on female fecundity. The highest efficacy was detected after incubation of *T. spiralis* females with *L. fermentum* CCM7421 (93 % reduction of NBL) followed by strains *E. faecium* CCM8558, *L. plantarum* 17L/1, *E. faecium* EF55 (about 80 %), *E. faecium* CCM7420 and *E. durans* ED26E/7 (about 60 %). This may be related to the fact that the genera *Lactobacillus* and *Enterococcus* belong to the lactic acid bacteria, which in the process of glucose fermentation produce primarily lactic acid but also other organic acids, e.g. acetate and butyrate (Lauková *et al.*, 1998; Araújo and Ferreira, 2013; Azat *et al.*, 2016). These acids can decrease the local intestinal pH and thus directly disrupt the growth of the acid-sensitive organisms, including parasites (Mukhopadhyay and Ganguly, 2014). Results of the study of El Temsahy (2001) revealed that the acidic gastric pH led to a significant decrease in the fecundity of *T. spiralis* females both *in vivo* and *in vitro*. This was obvious by observing the inability of females to give birth to NBL and morphological changes of the reproductive organs, mainly the uterus, which could cause of the impairment in embryogenesis.

The resistance to *T. spiralis* infection is related to the ability of the host to prevent the development of infective larvae by removing adult worms from the small intestine, limiting the fecundity of adult females, and destroying NBL (Vasconi *et al.*, 2015). Our study confirmed the anti-parasitic effect of six selected probiotic strains using an accelerated kinetics of worm expulsion from the gut (*E. faecium* CCM8558, *E. faecium* CCM7420 and *E. durans* ED26E/7), the reduction in female's reproductive capacity (all examined strains), and by reduction of muscle larvae (*L. fermentum* CCM7421, *L. plantarum* 17L/1, *E. faecium* CCM8558 and *E. durans* ED26E/7). All these anti-parasitic mechanisms were strain-dependent and were not acting solely, but in cooperation with other host defence mechanisms. This idea has been confirmed by differences in obtained results in anti-parasitic parameters, where decreased presence of adult worms in the gut has not resulted in decreased numbers of muscle larvae and *vice versa*. The inhibited female fecundity played an important role in infected mice treated with *E. faecium* CCM8558 and *E. durans* ED26E/7. However, *in vitro* conditions revealed a strong effect against NBL production in strains *L. fermentum* CCM7421, *L. plantarum* 17L/1, *E. faecium* CCM8558, and *E. faecium* EF55. This effect was suppressed in the host environment by interactions between bacterial strains, host immune response, and inflammatory processes. Immune mechanisms involved in killing of the NBL include oxidative processes, eosinophil major basic protein or complement activation (Wang, 1997). Mast cells, eosinophils, neutrophils and macrophages are all able to adhere to the larvae surface and destroy NBL during *in vitro* incubation (Mackenzie *et al.*, 1981). Probiotic strains tested in this study modulated the immune response and stimulated phagocytosis and oxidative burst of blood leukocytes thus participating in the killing of larvae (Dvorožňáková et al., 2016).

Therapeutic approaches with the use of probiotic strains could help to reduce the risks of trichinellosis or complement classical anti-parasite treatments. Our study demonstrates that probiotic bacteria can provide strain-specific protection against *T. spiralis* nematode throughout reduced female fecundity. Several addi-

tional mechanisms involved in the anti-parasite defence should be further studied and elucidated to justify the therapeutic use of probiotics.

Conflict of Interest

The authors declare there is no conflict of interest relating to the information presented in this manuscript.

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