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Protective effect by *Bacillus smithii* TBMI12 spores of *Salmonella* serotype Enteritidis in mice

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Abstract

Avoiding food-borne diseases by competitive exclusion agents is a proactive strategy. In the current paper, we report the use of *Bacillus smithii* TBMI12 spores as potential competitive exclusion agents. One group of mice was pre-dosed for three successive days with 10^8 colony forming units of *B. smithii* TBMI12 spores followed by inoculation with 10^6 colony forming units of wild-type *Salmonella enterica* serotype Enteritidis cells. Microbial plate counts of the animals' livers and spleens showed that only 40% of the mice were infected with *S. enterica* serotype Enteritidis, while the control group was 100% infected. These results suggest that *B. smithii* TBMI12 spores may protect against infection by *S. enterica* serotype Enteritidis.

Keywords: spores, thermophilic bacterium, *in vivo* mouse trial

1. Introduction

Members of the genus *Salmonella* are motile, Gram-negative, facultative anaerobic, rod-shaped bacteria belonging to the *Enterobacteriaceae* family. *Salmonella* causes a variety of diseases including typhoid fever, bacteremia, enterocolitis and focal infections (Darwin and Miller, 1999). In the United States, approximately 95% of the human *Salmonella* infections are food-borne, corresponding to approximately 30% of deaths caused by food-borne infections (Mead *et al*., 1999). In the European Union, 37.2 cases of human salmonellosis were reported per 100,000 people. The most commonly reported serovars were *Salmonella enterica* serotype Enteritidis (*S.* Enteritidis) and *S. enterica* serotype Typhimurium, accounting for 52% and 9% respectively of reported cases (EFSA and ECDPC, 2006). Statistics reveal that *Salmonella* infection has a significant economic impact reflected in health care costs, direct losses to farmers in lost animals and reduced productivity (Humphrey, 2006).

In traditional agricultural practice, antibiotics are used to protect animals against *Salmonella* infections. The common practice of using antibiotics as a feed supplement resulted in the emergence of antibiotic-resistant bacteria. To avoid proliferation of antibiotic-resistant bacteria, the European Union has banned antibiotics in animal feed since 2006 (EC, 2003). In accordance with the abovementioned regulation, the use of vaccines, prebiotics, probiotics and synbiotics are of paramount importance for protecting animals against infections.

Autochthonous lactic acid bacteria are normally found in the gastrointestinal tract (GIT) and can persist there perpetually. Probiotic products based on lactic acid bacteria influence the natural microbiota of the gut. Other groups of allochthonous probiotic microbes are not usually common in the GIT, they enter the gut with food and stay there transiently. However, they are effective in preventing pathogenic infections. These groups of microbes include spore-forming bacteria, usually members of the genus *Bacillus* (Hong *et al*., 2005). Several studies have demonstrated that *Bacillus*

spp. (Kyriakis *et al*., 1999) and *Bacillus subtilis* spores (La Ragione *et al*., 2001) may protect against endemic and zoonotic pathogens. The ability to form spores may be advantageous for probiotic administration, because gastric acids can harm bacterial vegetative cells. Spores have higher resistance to toxic compounds such as antibiotics, and other environmental extremes such as temperature, mechanical force, and radiation. These environmental advantages may also facilitate the production, storage, transportation and administration of spore-based products (Wolken *et al*., 2003).

Our work-group has isolated thermophilic sporogenous bacterium *Bacillus smithii* TBMI12 (*B*. TBMI12) from the human gut and evaluated it as a potential probiotic. The objectives of the current investigation were: (1) to show whether *B*. TBMI12 spores are able to form a stable population in the GIT of mice; and (2) to determine whether mice colonised with *B*. TBMI12 are protected against infection by *S*. Enteritidis.

2. Materials and methods

Animals

A total of 25 adult BALB/c mice were obtained from the Institute of Molecular and Cell Biology (University of Tartu, Estonia) and maintained in the institute animal facility. Each mouse was housed individually in a polycarbonate cage with a solid floor. Before the experiment, the faecal pellets from each mouse were plated onto selective medium XLD (Oxoid) and SIM-7 (Michelson *et al*., 2006) to be sure that all mice were free from *Salmonella* and *B*. TBMI12. At the end of the experiment, all surviving animals were euthanised by cervical dislocation and subjected to immediate necropsy. The experiment was approved by the Estonian Ministry of Agriculture, Commission of Animal Trial. International regulations for animal experiments were also followed (Council of Europe, 1986).

Strains

B. TBMI12 was isolated from a healthy young man, and is a spore-forming, oxygen-tolerant microorganism that produces lactic acid as the major final fermentation product from carbohydrates (glucose, galactose, xylose, arabinose, sucrose, maltose and lactose). Sugar fermentation studies were performed in phenol red broth (Scharlau) with various carbohydrates (2% glucose, galactose, xylose, arabinose, sucrose, maltose or lactose). The cultivation temperature ranged from 30 °C to 56 °C. The isolated strain was identified by the 16S rRNA sequence, identifying it as *B. smithii* (GenBank no. EF010852). The strain was deposited in the Microbial Strain Collection of Latvia (international depositary authority: P737). A clinical isolate of wild-type *S.* Enteritidis (ID: 5-80) was kindly provided from the strain collection of the Department of Microbiology, Faculty of Medicine, University of Tartu, Estonia.

Preparation of spores and vegetative cells

B. TBMI12 spores were prepared by incubating the bacterial culture for 24 h at 56 °C on sporulation medium [10 g/l of yeast extract (Bacto) and 8 mg/l $MnSO_4$; 3-(N-morpholino)-propanesulfonic acid (MOPS, 40 mM, pH 5.5) was used to stabilise pH]. Spores were purified from vegetative cells using a standard method (Nicholson and Setlow, 1990) and maintained in deionised water at 4 °C. The inoculum of *B.* TBMI12 spores, suspended in 0.9% NaCl solution, contained 10^8 colony forming units (cfu) per 0.5 ml. Vegetative cells of *B.* TBMI12 were cultivated on SIM-7 solid medium overnight at 56 °C.

For the purpose of enumeration *S.* Enteritidis was cultivated on solid XLD medium overnight at 35±2 °C according to the manufacturer's protocol. To prepare inocula for mouse experiments, *S.* Enteritidis was aerobically grown for 6 h in Nutrient Broth (Difco) medium at 30 °C with agitation at 220 rpm. The *S.* Enteritidis culture was suspended in sterile Nutrient Broth medium at 10^6 cfu per 0.5 ml.

Intragastric dosing

A total of 25 mice were divided into three groups: A (control), B (treatment) and C. Mice were intragastrically dosed with 0.5 ml dosage using a sterile syringe with a blunt-ended needle. On the first and second experimental day, Group A mice (n=10) were administered 0.9% NaCl solution. On the third day, Group A was intragastrically inoculated with a single dosage of 10^6 cfu of *S*. Enteritidis cells. Group B mice (n=10) were treated daily for three successive days with 10^8 cfu *B*. TBMI12 spores. On the third day, four hours after receiving the last dosage of *B*. TBMI12 spores, mice were inoculated with a single dosage of 10^6 cfu *S*. Enteritidis cells. Group C mice (n=5) were intragastrically inoculated with 10^8 cfu *B*. TBMI12 spores on the first day and on the eighth day.

Enumeration of *S*. Enteritidis and *B*. TBMI12 in faeces and tissues

Freshly voided mouse faecal pellets were collected into sterile microtubes, weighed and suspended in 1 ml 0.9% NaCl solution. Homogenised faecal samples were serially diluted to 10^{-2} with 0.9% NaCl solution. Aliquots of 0.1 ml of each appropriate dilution were plated onto SIM-7 and XLD medium plates. Cfu/g of faeces was calculated based on the number of colonies.

For enumeration of the thermophilic bacterium *B.* TBMI12, the SIM-7 plates were incubated overnight at 56 °C, and colonies were counted to calculate cfu/g. Since mice do not

harbour thermophilic bacteria, high temperature was used as a selective factor. XLD selective medium was used to enumerate *S.* Enteritidis. The bacteria were grown overnight at 35±2 °C, whereas only the colonies with a phenotype specific to *Salmonella* were counted to calculate cfu/g.

The dates of deaths were registered. On day 14 post-inoculation, the surviving mice were euthanised. Livers and spleens were removed by sterile procedure, weighed, and homogenised by vortexing with glass powder in 5 ml 0.9% NaCl solution. A total of 0.1 ml of pure homogenate was serially diluted to 10^{-3} in 0.9% NaCl solution, and plated on SIM-7 and XLD medium. All operations were performed under aseptic conditions using sterile tools, solutions and a laminar flow hood.

Primers

Colonies with a phenotype specific to *Salmonella* were used for PCR analysis in order to verify *S.* Enteritidis. The primers Sef167 (5' AGG TTC AGG CAG CGG TTA CT 3') and Sef487 (5' GGG ACA TTT AGC GTT TCT TG 3') were used to amplify a 312 bp fragment of a unique target sequence in the *sefA* gene specific to *S.* Enteritidis (Soumet *et al.*, 1999). The 16S rRNA gene of *B.* TBMI12 was PCR-amplified using the primers PCRI (5' AGA GTT TGA TCA TGG CTC AG 3') and PCRII (5' TAC GGT TAC CTT GTT ACG ACT T 3'). For amplified 16S rRNA gene sequencing was also used primers PCRI, PCRII and SEQ2 (5' TTG CGC TCG TTG CGG GAC T 3') (Vedler *et al.*, 2000). The nucleotide sequences of the 16S rRNA genes were determined directly from the PCR fragment. DNA sequencing was carried out on an ABI PRISM 377 DNA sequencer (Perkin-Elmer, Waltham, MA, USA) using the DYEnamic ET terminator cycle sequencing premix kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

Statistical analysis

SigmaStat for Windows 2.0 was used for statistical analyses. The Mann-Whitney and Fisher Exact tests were used to calculate *p*-values.

3. Results

In this study, Group C mice were inoculated with *B.* TBMI12 spores to determine if this bacterial strain could form a stable population in the GIT of mice. After the first dosage, the number of *B.* TBMI12 detected from faeces quickly decreased. However, after the second dosage of *B.* TBMI12, the population was stabilised (Figure 1).

To test whether mouse GIT colonisation with *B.* TBMI12 prevents infection by *Salmonella*, mice in Group B were pre-dosed for three successive days with *B.* TBMI12 spores to ensure formation of a stable population in murine GIT.

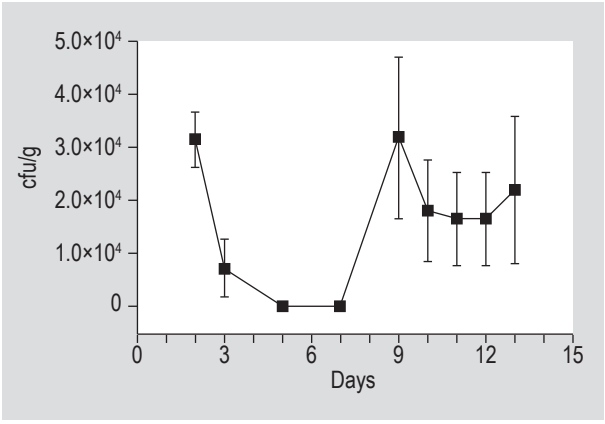

Figure 1. *B.* TBMI12 colonisation of the GIT of mice. Group C mice (n=5) were inoculated intragastrically with 10^8 cfu *B.* TBMI12 spores on the first day and on the eighth day. The average numbers of *B.* TBMI12 were determined by plate count of faeces. Error bars indicate standard error.

The experiment showed that colonisation was successful, and the population of *B.* TBMI12 was confirmed. However, the number of *B.* TBMI12 detected from the faeces of Group B mice decreased over a period of experiment from 1.2×10^5 to 2.8×10^3 cfu/g (Figure 2).

On the third day, mice from Groups A and B were infected with *S.* Enteritidis. Group A mice were not pre-treated with *B.* TBMI12 spores. Instead of *B.* TBMI12 spores, Group A mice were given 0.5 ml of 0.9% NaCl solution. On the fourth day, *S.* Enteritidis cells were detected in the faeces of Group A mice. The plate count showed that 30% of Group A mice faeces contained *S.* Enteritidis cells on the first day after inoculation with a single dose of the pathogen. *S.* Enteritidis

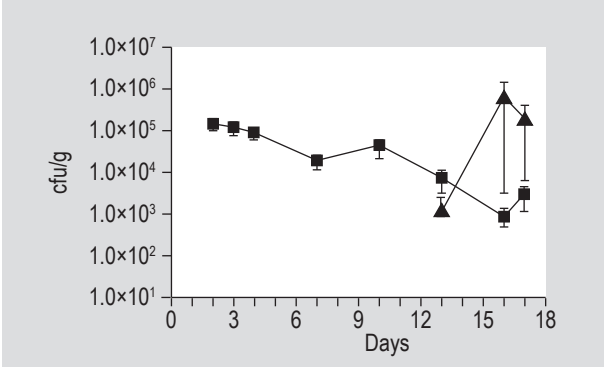

Figure 2. *S.* Enteritidis wt and *B.* TBMI12 colonisation in the GIT of mice. Group B mice (n=10) were treated on the first, second and third day with 10^8 cfu of *B.* TBMI12 spores. Four hours after the last dosage of *B.* TBMI12 spores, animals were inoculated with 10^6 cfu of *S.* Enteritidis cells. The average numbers of *S.* Enteritidis wt (—▲—) and *B.* TBMI12 (—■—) were detected by plate count of faeces. No *S.* Enteritidis were found prior to day 13. Error bars indicate standard error.

cells from the faeces of Group B mice were first detected on day 13 of the experiment. According to the plate count, the faeces of only 10% of Group B mice contained *S.* Enteritidis cells on day 13. During the experiment, the number of mice shedding *S.* Enteritidis in the faeces increased in both groups. However, only one mouse in Group A died of salmonellosis - on day 12 of the experiment. The cause of death was diagnosed by microbiological examination of liver and spleen. The remaining mice lived until the termination of the experiment, and were euthanised by cervical dislocation on the day 17. The plate counts of the liver and spleen confirmed that 100% of Group A mice and 40% of Group B mice were infected with *S.* Enteritidis (10/10 vs. 4/10; *P*=0.0005). Results are summarised in Figure 3.

As it is shown in Table 1, in mice treated with *B.* TBMI12 spores (Group B), *S.* Enteritidis counts in liver and spleen were somewhat lower compared to those in Group A. On day 10 and 17, the faecal counts of *S.* Enteritidis of Group B mice were lower and the *P*-value was not significant.

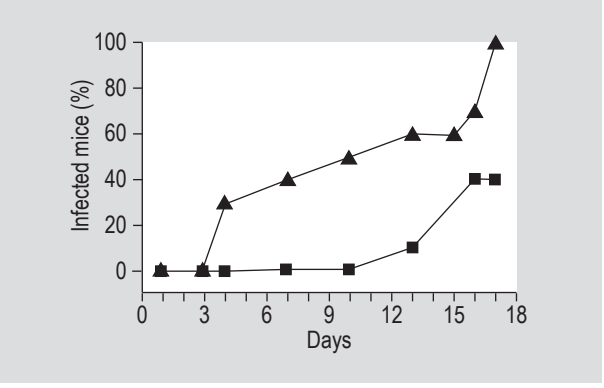

Figure 3. Comparison of Group A (—▲—) and Group B (—■—) *S.* Enteritidis infection, as detected by plate counts of faeces. Markers of day 17 indicate plate count of liver and spleen. Data is expressed as a percentage of infected animals. On the first and second day, Group A mice (n=10) were given 0.9% NaCl solution. On the third day, Group A was intragastrically inoculated with a single dosage (10^6 cfu) of *S.* Enteritidis cells. Group B mice (n=10) were pre-dosed daily for three successive days with 10^8 cfu *B.* TBMI12 spores. Four hours after the last dosage of *B.* TBMI12 spores, animals were inoculated with a single dosage (10^6 cfu) of *S.* Enteritidis cells.

4. Discussion and conclusion

The goal of the present study was to determine if *B.* TBMI12 spores can form a stable population in the GIT of mice. Our results showed that several dosages of *B.* TBMI12 spores are able to colonise and to persist in the GIT of mice. Another task was to verify colonisation of the mouse gut with *B.* TBMI12 in order to protect colonised mice against infection by *S.* Enteritidis. Thus, we used spores of *B.* TBMI12 for the prevention of salmonellosis and to reduce the number of carriers of non-typhoid *Salmonella* strain in the population. Our results demonstrated that colonisation successfully protected against infection by *S.* Enteritidis.

Many studies have been undertaken to ascertain the fate of ingested *Bacillus* spores. Several studies have shown that *B. subtilis* spores exist in the intestinal tract of mice. The probiotic effect of bacilli may be due to the presence of spores or some undefined extraintestinal life cycle, rather than arising from metabolically active cells (Spinosa *et al.*, 2000). However, Casula and Cutting (2002) detected spore germination in the jejunum and ileum of mice. Unfortunately the persistence of allochthonous probiotic bacteria is problematic. For example, commercial probiotic *Bacillus* strains can persist in the GIT of mice for 18 days after administration of 10^9 spores. At the end of this period, 10^3 cfu per gram were detected from faeces (Duc *et al.*, 2004). A problem concerning persistence was encountered in our study. A single dose of *B.* TBMI12 spores colonised the GIT of the mice for two days. However, the mice needed a second dosage of spores to form a stable population of *B.* TBMI12 in their gut. Treating mice with spores of *B.* TBMI12 for three successive days showed that the bacterium can colonise the GIT of mice for at least two weeks (the length of our experiment). We did not examine any mouse beyond two weeks. However, we demonstrated

Table 1. Comparison of *S.* Enteritidis plate counts (log_{10} cfu/g) of Group A and Group B mice. The Group B mice (n=10) were pre-dosed daily for three successive days with *B.* TBMI12 spores (10^8 cfu per dosage), whereas Group A mice (n=10) were given 0.9% NaCl solution. Four hours after the last dosage, all mice were inoculated with 10^6 cfu of *S.* Enteritidis cells.

	Liver		Spleen		Faeces, 10th day		Faeces, 17th day	
	Group A	Group B	Group A	Group B	Group A	Group B	Group A	Group B
Min	1.9	0	2.36	0	0	0	0	0
Max	8.46	7.03	8.6	5.42	7.86	0	5.41	6.31
Median	3.82	0	3.95	0	2.19	0	3.18	0
P-value	0.038		0.026		not significant		not significant	

that *B.* TBMI12 spores are able to colonise and persist in the GIT of mice.

The infectious dose of *Salmonella* is 10^5-10^{10} cfu, which is a relatively large inoculum required to overcome stomach acidity and to compete with the normal microbiota of the GIT. Reducing the number of normal microbiota by administration of antibiotics allows a decreased dose of *Salmonella* to infect 50% of mice (Bohnhoff *et al.*, 1964). Thus, restoring the normal bacterial community and increasing GIT bacterial number prevents infection with *Salmonella*. Lactic acid bacteria are administered as alternatives to normal intestinal microbes. Results have shown that those bacteria have antimicrobial activity against *S.* Enteritidis. For example, administration of lactic acid-producing bacteria culture condensate mixture to mice decreased both the viable *S.* Enteritidis cells found in faeces and the mortality rate of mice (Park *et al.*, 2005). Although spore-forming bacilli are not classified as lactic acid bacteria, they still may be successful competitive exclusion agents. Previous studies have demonstrated that administration of *B. subtilis* spores may exclude endemic and zoonotic pathogens from poultry (La Ragione and Woodward, 2003). As our current study was based on a mouse model, we cannot assume that *B.* TBMI12 spores have an antipathogenic effect on poultry. However, 60% of mice pre-dosed with spores of *B.* TBMI12 were not infected with *S.* Enteritidis. Our results also indicate that pathogenesis was slowed down. *S.* Enteritidis was detected in faeces on day 13 of the experiment. All mice that were not pre-dosed with *B.* TBMI12 spores had detectable *S.* Enteritidis in faeces the next day after inoculation.

In conclusion, our experiments showed that *B.* TBMI12 spores are able to form a stable population in the GIT of mice, and that colonisation with the bacteria provides remarkable protection against infection by *S.* Enteritidis.

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