BACILLUS SMITHII TBMI12 ENDOSPORES AS A POTENTIAL COMPONENT OF PROBIOTIC FEED ADDITIVE FOR PIGS

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Abstract. The purpose of this study was to show the safety of *Bacillus smithii* TBMI12 (*B. smithii* TBMI12) endospores for piglets. This paper describes safety and tolerance experiments with piglets. During the safety study, piglets from trial groups got a single dose of *B. smithii* TBMI12 endospores of 10^8, 10^9 or 10^{10} CFU. Statistically significant changes among the microbiota of gastrointestinal tract did not occur compared with control and placebo group animals. During the tolerance study, a normal (10^9 CFU) or overdose (10^{10} CFU) of endospores was administered every day to trial group piglets, but this did not damage the diversity of their microbiota. Both experiments showed that ingestion of *B. smithii* TBMI12 endospores had no negative influence on the weight gain of piglets. Based on those results, we suggest that endospores of *B. smithii* TBMI12 are safe for use as a component of a probiotic feed additive for pigs and this subject deserves further research.

Keywords: *Bacillus smithii*, probiotic, endospores, pigs.

Introduction

Using probiotics for improving human health has a long history. The oldest written record goes back to 76 B.C, when Plinius described the use of fermented milk products in curing gastro-intestinal infections (Bottazzi, 1983). By the beginning of the 20th century, microbiology had developed so far that it was possible for the famous researcher Metchnikoff to support the old empirical experiences scientifically. He postulated that the consumption of fermented milk would suppress the growth of proteolytic bacteria, thus prolonging the life span of the host (Metchnikoff, 1907). About a decade later, several products based on lactic acid bacteria were available in the market for the treatment of diarrhea (e.g. *Lactobacillus* LB Lactéol in 1907, *Escherichia coli* Nissle in 1917) or prevention of diseases (*Lactobacillus acidophilus* L-92 in 1910, *Lactobacillus casei* Shirota in 1935) (Jankovic *et al*., 2010). Due to discovering antibiotics, the field of probiotics was temporarily forgotten. However, the rise of antibiotic resistant bacteria encouraged the use of alternative methods against pathogens, such as vaccination, better hygiene or enhancing normal microbiota. In the veterinary field, probiotics were introduced in the 1970s as supplements to improve animal resistance against diseases and promotion of their growth (Fuller, 1989). Since 2006, antibiotics are forbidden in animal feed in the European Union (EC, 2003). According to the abovementioned regulation, vaccines, prebiotics, probiotics, synbiotics, bacteriophages, organic acids, plant extracts and other potential alternatives to antibiotic growth-promoters for animal production have achieved paramount importance (Tellez *et al*., 2011).

One group of probiotics is autochthonous lactic acid bacteria. They are normally found in the gastrointestinal tract (GIT) and can persist there perpetually. Products based on lactic acid bacteria enable to restore the natural microbiota of the gut. Another group − allochthonous probiotic microbes − are not usually common in the GIT. They enter the gut with food and stay there temporarily. However, they are effective in preventing infections caused by pathogens. This group of allochthonous probiotic microbes includes spore-forming bacteria, usually members of the genus *Bacillus* (Hong *et al*., 2005). Use of spores rather than vegetative cells has some advantages, since gastric juice can harm the vegetative cells of microbes, making the administration of probiotics problematic, but spores are less likely to be damaged. Spores have higher resistance to toxic compounds (e.g. antibiotics), extreme temperatures, mechanical force and radiation. On account of these advantages, the production, storing, transporting and administration of spore-based products is more feasible (Wolken *et al*., 2003).

Our work-group isolated the thermophilic sporogenous bacterium *B. smithii* TBMI12 and evaluated it as a potential probiotic. Our previous experiments had shown that *B*. TBMI12 endospores are able to form a stable population in the GIT of mice and that colonization with the bacteria provides remarkable protection against infection by *Salmonella* Enteritidis (Suitso *et al*., 2010). Encouraged by those promising results, we wanted to continue research on farm animals. Our ultimate purpose was to develop a probiotic feed additive and achieve approval from the European Food Safety Authority (EFSA). To claim the license, the safety of a product must be proved by conducting several studies based on EFSA standard protocols (EFSA, 2008).

The main objective of the current study was to

investigate the safety of *B. smithii* TBMI12 endospores for piglets. In this research we tried to find answers to the following questions: (1) does ingestion of *B. smithii* TBMI12 endospores impair the diversity of microbiota or (2) have a negative influence on the weight gain of piglets? To answer these questions, two experiments were conducted: (1) a single dose safety study – to examine the safety of ingestion of a single dose of various amounts of endospores; and (2) a tolerance study – to examine the safety of prolonged ingestion of a suggested recommended dose and a 10-fold overdose of endospores.

Materials and methods

Microbes

B. smithii TBMI12 was isolated from a healthy young human, 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).

B. smithii TBMI12 endospores were prepared by incubating the bacterial culture for 24 h at 56 °C on a sporulation medium [10 g/l of yeast extract (Bacto) and 8 mg/l $MnSO_4$; to stabilize pH, 3-(N-morpholino)-propanesulfonic acid (MOPS, 40 mM, pH 5.5) was used]. Spores were purified from vegetative cells using a standard method (Nicholson and Setlow, 1990) and maintained in deionized water at 4 °C.

Animals and experimental design

Animal experiments took place at experimental facilities of the Veterinary Clinic of the Estonian University of Life Sciences. One month old piglets of the Estonian Yorkshire (Eesti Suur Valge) breed were used for the experiments. Animals got feed and water *ad libitum*. Piglets were visually observed every day for signs of changes in general health status (activity, eating and drinking behaviour).

For the single dose safety study, 29 piglets were labelled and divided randomly to five groups. Each group was located in a wall-separated pen. On day 0, 10 ml of one of following solutions was administered orally to the trial group piglets: the placebo group (n = 6) got dextrose solution (DE 15); Group8 (n = 5) got dextrose solution with 10^8 CFU; Group9 (n = 6) with 10^9 CFU and Group10 (n = 6) with 10^{10} CFU *B. smithii* TBMI12 endospores. Control group piglets (n = 6) were not treated. Faecal samples were collected directly from the rectum on the 0, 1st, 3rd, 5th and 7th day. Piglets were weighed on days 0, 3 and 7.

For the tolerance study, 45 piglets were divided randomly into three groups (n = 15 in each group). Trial group animals were in the same room divided into wall-separated pens (5 piglets in each). The control group was located in another room also divided into 3 pens. The piglets' feed was enriched with *B. smithii* TBMI12 endospores with the calculation that each animal from GroupN would get a normal dose (10^9 CFU) and GroupO animals an overdose (10^{10} CFU) every day during the 6-week trial. The control group piglets' feed was not enriched. Faecal samples were collected directly from the rectum on the 0, 21st and 42nd days. Piglets were weighed on days 0; 7; 14; 21; 28; 35 and 42.

The animal experiments were approved by the Commission of Animal Trial at Estonian Ministry of Agriculture. International regulations on animal testing were followed (Council of Europe, 1986).

Microbiological analysis

Freshly taken faecal samples were collected into sterile containers, weighed and suspended in Buffered Peptone Water ("Oxoid"). Homogenized faecal samples were serially diluted from 10^2 to 10^8 times and plated onto different kinds of media. For the detection of thermophilic bacilli, the SIM-7 (Michelson *et al*., 2006) media was used; for *Salmonall*a and *Shigella* – XLD ("Oxoid"); for coliform bacteria – VRB ("Oxoid"); for lactobacilli – LBS ("BBL"); for anaerobic bacteria – Anaerobic Agar ("Difco"); for clostridia – Differential Reinforced Clostridial Agar ("Difco"); for aerobic bacteria – TSA ("Oxoid"). Bacteria were grown usually at 37 °C, except thermophilic bacilli that preferred 56 °C. Lactobacilli, clostridia and anaerobic bacteria were incubated 48 hours in anaerobic conditions, created by the "Oxoid" atmosphere generating system AnaeroGen™. The rest of the microbes were incubated 24 hours in aerobic conditions. Colonies were counted and colony forming units per gram of faeces were calculated.

Molecular biological analysis

To extract DNA from faeces, the PSP Spin Stool DNA Kit ("Invitek") was used. DNA was amplified from the 16S rRNA gene in Eppendorf Personalcycler by using primers 341F-GC (5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG 3') and 907R (5' CCG TCA ATT CMT TTG AGT TT 3') (Muyzer *et al*., 1993). PCR products were analyzed by the denaturing gradient gel electrophoresis system DGGE 2001-220 ("C.B.S. Scientific"). Polyacrylamide gel (10%) was silver stained (Neilan *et al*., 1994), scanned and analyzed by the program "Quantity One" (Bio-Rad). The Shannon-Wiener index was calculated using a web-based calculator (Chang Bioscience).

Statistical analysis

Linear regression mixed models were used to analyze results of weight changes and microbiological results of faecal samples. The animal was included as a random factor. Isotropic spatial exponential correlation structure was used to account for correlation between repeated measurements within piglets. In the models of weight

gain, differences between the control and trial groups were compared based on changes in overall growth curves (modelled using polynomials of time). To analyze changes in the concentrations of sporogenous thermophilic bacilli in the faeces of the study groups' piglets over time, an n-day probe result was compared with 0-day ones. Differences between the control and trial groups were compared as changes of n-day results compared with 0-day ones. Model assumptions were verified by scatter and normality plots of standardized residuals, and logarithmic transformation of microbiological results was used. Statistical software R2.7.2 was used for linear regression mixed models.

Results

The single dose safety study showed that sporogenous thermophilic bacilli existed in the faeces of piglets already on day 0. The average natural level was 5.8 x 10^2 CFU/g. After ingestion of a single dose of *B. smithii* TBMI12 endospores, the number raised and remained higher than the natural level until the end of the trial (Fig. 1). Colonization of piglets' GIT by *B. smithii* TBMI12 did not cause remarkable changes in microbiota. Changes in time or between groups were not statistically significant among lactobacilli, aerobic and coliform bacteria. The number of anaerobic bacteria changed in several groups and was statistically significant ($P<0.05$). However, as changes were not unidirectional and took place in groups with and without endospores, we expect that those fluctuations were not caused by *B. smithii* TBMI12. Faecal samples did not contain *Salmonella* or *Shigella*. The welfare of test animals was also explored by clinical observation and the measurement of weight. The average body weight of piglets increased in the control group as well as trial groups. Differences of weight gain were not statistically significant (Fig. 2). Results of the safety study suggest that a single dose of *B. smithii* TBMI12 endospores does not change the normal microbiota of the GIT or influence the weight gain of piglets.

During the tolerance study, the number of thermophilic sporogenous bacilli in the faeces of piglets increased in trial groups. The change was statistically significant compared with the results of the control group and with the results of the same group at the beginning of the trial (Fig. 3). Despite this remarkable change in gastrointestinal microbiota, piglets were still healthy and the average weight gain was almost the same in all groups (Fig. 4). 16S rDNA DGGE profiles of intestinal microbiota were also analyzed. Ingestion of a normal or overdose of *B. smithii* TBMI12 endospores every day during the six-week trial did not cause a significant change of the Shannon-Wiener index of diversity (Table 1). Differences between the control group, normal dose and overdose groups were minor. Results suggest that small changes in DGGE profiles in time were caused by succession of microbiota due to aging of the piglets. The tolerance study showed that constant ingestion of *B. smithii* TBMI12 endospores has no negative effect on the weight gain of piglets or the diversity of normal microbiota of the GIT.

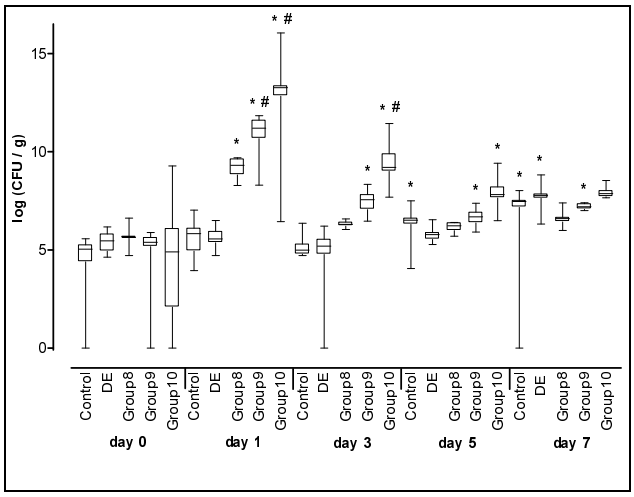

Fig. 1. **Concentrations** (log (CFU/g)) **of thermophilic sporogenous bacilli in faeces of piglets during 7 days after oral ingestion of 10 ml of dextrose solution** (DE; n = 6), **dextrose solution with 10^8 CFU** (Group8; n = 5), **with 10^9 CFU** (Group9; n = 6) **and with 10^{10} CFU *B. smithii* TBMI12 (Group10; n = 6) endospores on day 0. Control group piglets (n = 6) were not treated**

* Statistically significant difference compared with the result of the same group on day 0 ($P<0.05$).

\# Statistically significant difference compared with the control group on the same day ($P<0.05$).

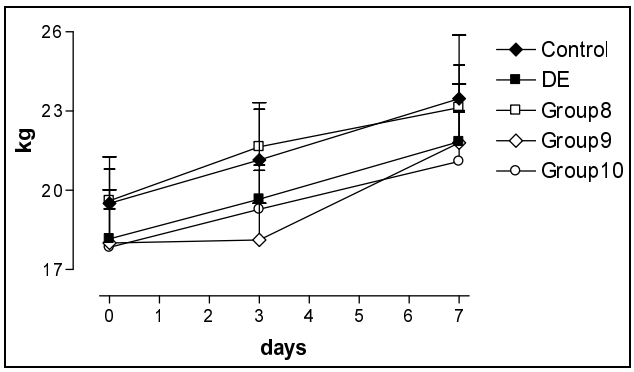

Fig. 2. **Average body weight of piglets** (+/- SEM) **during 7 days after oral ingestion of 10 ml of dextrose solution** (DE; n = 6), **dextrose solution with 10^8 CFU** (Group8; n = 5), **with 10^9 CFU** (Group9; n = 6) **and with 10^{10} CFU *B. smithii* TBMI12** (Group10; n = 6) **endospores on day 0. Control group piglets** (n = 6) **were not treated**

Discussion and conclusions

The main purpose of our work was to investigate the safety of *B. smithii* TBMI12 endospores for piglets to show their acceptability as a component of a probiotic feed additive. EFSA standard protocols were followed for trials to achieve internationally acceptable results. However, we still faced difficulties related to the taxonomic background of *B. smithii*. Despite their metabolic and morphologic similarity, this species was

separated from the *B. coagulans* (Nakamura *et al*., 1988) at the end of the 20th century due to minor differences on the molecular level. This makes estimation of the acceptability of these bacteria for use in a feed additive more complicated, since the juridical background is added to the scientific aspect. *B. coagulans* is qualified as a safe microorganism (QPS) by EFSA (EFSA, 2007), which makes it easier to claim licenses to produce and sell products containing the abovementioned bacteria. However, *B. smithii*, formerly known as *B. coagulans*, have to face full safety assessment − due to the new name it no longer has the QPS status.

Table 1. **Shannon-Wiener index of diversity based on 16S rDNA DGGE profile analysis of intestinal microbiota of piglets during tolerance study. Piglets' feed was enriched by *B. smithii* TBMI12 endospores with the calculation that each animal from GroupN would get a normal dose** (10^9 CFU) **and GroupO animals an overdose** (10^{10} CFU) **every day**

Group	Day 0	Day 21	Day 42
Control	2.4563498	2.433231	2.267759
Group N	2.3559363	2.173146	2.4455142
Group O	2.570983	2.1807802	2.4321086

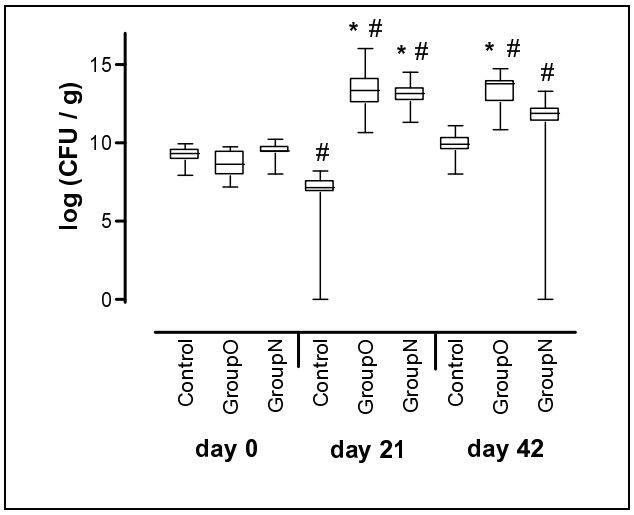

Fig. 3. **Concentrations** (log (CFU/g)) **of thermophilic sporogenous bacilli in faeces of piglets during the 6-week study period. Each animal from Group N** (n = 15) **got a normal dose** (10^9 CFU) **and Group O** (n = 15) **animals an overdose** (10^{10} CFU) **of *B. smithii* TBMI12 endospores every day during the experimental period. The control group's** (n = 15) **feed was not enriched with *B. smithii* TBMI12 endospores**

* Statistically significant difference compared with the same group on day 0 (P<0.05).

\# Statistically significant difference compared with the control group on the same day (P<0.05).

There are several probiotic products based on *B. coagulans* on the market nowadays. For example in the veterinary field, Lactopure is used to treat poultry, calves and swines. Neolactoflorene and Sustenex ® are meant for human use (Cutting, 2011). The latter product contains a *B. coagulans* strain GanedenBC[30] which has been granted the self-affirmed GRAS (generally regarded as safe) status by the FDA in the USA (Enders *et al*., 2009).

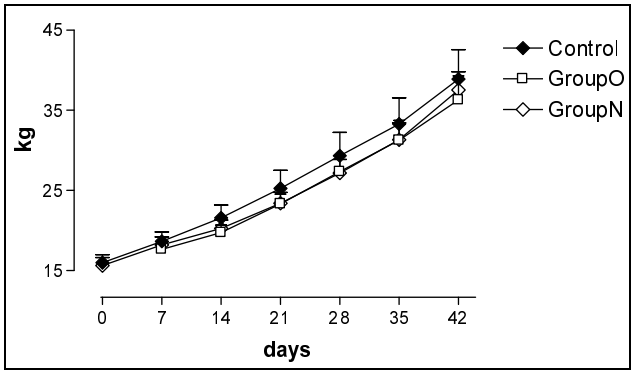

Fig. 4. **The change of average weight** (+/- SEM) **of piglets during the 6-week study period. Each animal from GroupN** (n = 15) **got a normal dose** (10^9 CFU) **and GroupO** (n = 15) **animals an overdose** (10^{10} CFU) **of *B. smithii* TBMI12 endospores every day during the experimental period. The control group's** (n = 15) **feed was not enriched with *B. smithii* TBMI12 endospores.**

The colonization of the GIT of piglets with *B. coagulans* might reduce mortality, increase daily weight gain by 16% and lead to better feed conversion ratio compared with the untreated group (Adami and Cavazzoni, 1999). However, the effect of a probiotic depends on composition and concentration. For example, dietary supplementation of a *B. coagulans*-based probiotic preparation at the level of 0.2% improved the growth performance of pigs. Meanwhile, the average daily gain of pigs fed with 0.1% probiotic-supplemented diet also increased compared with the control group, but without a significant difference (P>0.05) (Chen *et al*., 2006).

In the current study, the safety and tolerance experiments were conducted to prove the safety of *B. smithii* TBMI12 endospores. Trials with piglets were planned and performed based on methods suggested by EFSA (EFSA, 2008). Results of the current research showed that ingestion of a single dosage of *B. smithii* TBMI12 endospores did not disturb the balance of piglets' microbiota. Even colonization of the GIT with daily overdoses during the six-week trial did not harm the diversity of the normal microbiota. The effect on the weight gain of piglets can be considered neutral, as during the safety study, the body weight of piglets increased in the control, trial and placebo groups alike. Differences in the average body weight of piglets from the control, normal and overdose groups were not statistically significant during the whole tolerance study. Neither did clinical observation show any negative influence of the bacterium on the piglets. This supports our suggestion that despite lacking the QPS status, *B. smithii* TBMI12 is still safe.

We suggest that *B. smithii* TBMI12 endospores are

safe for piglets, since their ingestion does not cause significant changes in the microbiota or damage the biodiversity of the gastrointestinal tract and the influence on the weight gain is neutral.

Acknowledgements

This study was funded by the European Commission through Enterprise Estonia grant EU29512. We also appreciate financial support from AS Remedium, Eesti Töötukassa and Elingor OÜ.

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Received 25 February 2013
Accepted 16 April 2014