Bacillus cereus var. Toyoi modulates the immune reaction and reduces the occurrence of diarrhea in piglets challenged with Salmonella Typhimurium DT104

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ABSTRACT: A feeding trial with sows and their piglets was performed with the probiotic feed additive Bacillus cereus var. Toyoi in two consecutive experimental periods. Sows (n = 8) were allocated into treatment (Bc) and control (CO) groups. Sows of Bc group (n = 4) were fed 3.14 × 10^5 cfu/g Bacillus cereus var. Toyoi with the diet from d 87 of pregnancy on. Their piglets received Bacillus cereus var. Toyoi supplemented feed (8.7 × 10^5 cfu/g) starting on d 14 of life and further on after weaning (6.5 × 10^5 cfu/g), whereas sows and piglets of the CO group remained untreated. One day after weaning, piglets from both groups (n = 24 each) were challenged orally with Salmonella Typhimurium DT104 (3 × 10^9 viable bacteria). Health status, shedding of B. cereus in the feces, and performance of the piglets were monitored. At 24 h, 72 h, 6 d, and 28 d postinfection (PI), six piglets from each group were euthanized and cell counts of Salmonellae were determined in the colon contents, mesenteric lymph nodes, and tonsils. Peripheral blood mononuclear cells and jejunal intraepithelial lymphocytes (IEL) were analyzed by flow cytometry. The incidence of scours was lower in the Bc group than in the CO group (P = 0.004). In addition, the fecal shedding of Salmonella was significantly lower in the Bc group at 25 d PI (P = 0.004). Shortly after infection, the γδ T cells were significantly less frequent in the blood of Bc piglets. For both CD8-positive γδ T cells (P = 0.033) and CD8-negative γδ T cells (P = 0.028), significant differences were observed. Furthermore, 28 d PI piglets from the treated group showed lower numbers of γδ T cells in the jejunal epithelium (P = 0.036). To investigate the role of intestinal γδ T cells during the infection with S. Typhimurium, IEL were gained from six healthy 40-d-old piglets and infected in vitro with S. Typhimurium. CD8β cells and γδ T cells were detected by flow cytometry and the infection rates of both populations in the cell suspensions were compared. The infection rate (IR) of γδ T cells was higher in all six cell suspensions than the IR of CD8β expressing T cells (P = 0.002). In conclusion, Bacillus cereus var. Toyoi supplementation of sows and their piglets had a positive impact on the health status of the piglets after a challenge with Salmonella, likely due to an altered immune response marked by reduced frequencies of CD8+ γδ T cells in the peripheral blood and the jejunal epithelium.

Key words: Bacillus cereus var. Toyoi, gammadelta T cells, intraepithelial lymphocytes, pig, probiotics, Salmonella Typhimurium

INTRODUCTION

The use of probiotics in farm animals has gained interest due to globally increased efforts to avoid in-feed antibiotic growth promoters to maintain gastrointestinal health. Such feed additives may help to reduce the load of zoonotic microorganisms such as Salmonella serovars, which are among the most important zoonotic bacteria associated with pork products. A promising approach to overcome latent infections and reduce shedding of Salmonella might be through targeting the porcine immune system. Probiotics are considered as modulators of the immune system (Bermudez-Brito et al., 2012). However, probiotic bacteria encompass a diverse range of microorganisms, including both Gram-positive and Gram-negative strains, as well as yeast species (Quigley,
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Some can be regarded as members of the host’s autochthonous microbiota such as Enterococcus faecium, whereas others, e.g., Bacillus cereus var. Toyoi, are only transient in the intestinal tract. Therefore, the impact of probiotics on the immune system likely depends on the nature of the microorganism, its evolutionary relation to the host, and its capability to colonize the intestine.

In previous experiments, we tested the two probiotic strains Bacillus cereus var. Toyoi and Enterococcus faecium NCIMB 10415 for their impact on the immune system of piglets. The two probiotic bacteria appeared to have contrasting effects. Feeding of B. cereus resulted in an increase in CD8+ lymphocytes (Scharek et al., 2007), whereas E. faecium appeared to reduce the presence of CD8+ T cells in the jejunal epithelium (Scharek et al., 2005). In challenge trials with S. Typhimurium DT104, piglets fed E. faecium showed a more severe course of infection than the control group and reduced performance (Szabó et al., 2009; Kreuzer et al., 2012). To test the hypothesis that probiotic-mediated alterations in CD8+ T cell populations are involved in the course of Salmonella infections, we performed a challenge trial with Salmonella with B. cereus var. Toyoi–supplemented animals. The aim of this study was to determine whether the lower frequency of CD8+ T cells was involved in the higher sensitivity to Salmonella, where an increase in CD8+ cells, as observed with B. cereus var. Toyoi, would lead to a strengthened defense against the pathogen.

MATERIALS AND METHODS

All experimental procedures were approved by the state animal welfare committee (LAGeSo, Berlin, Germany; approval No. 0481/09).

Animals, Diets, and Housing

Eight purebred Landrace sows, bred with Landrace boars, were purchased from a commercial breeding company (Bundeshybridzuchtprogramm, Dahlenburg-Ellringen, Germany). For practical reasons, the trial was performed in two consecutive experimental periods with 4 sows and 24 piglets in each period: sows in the treatment (Bc) group received $3.14 \times 10^5$ cfu/g Bacillus cereus var. Toyoi (ToyoCerin, Bacillus cereus var. Toyoi NCIMB 40112; Lohmann Animal Health, Cuxhaven, Germany) beginning on d 87 of pregnancy and continuing during lactation. Piglets in this Bc group received Bacillus cereus var. Toyoi supplemented prestarter feed ($8.7 \times 10^5$ cfu/g) from d 14 onward and further on after weaning ($6.5 \times 10^5$ cfu/g). Sows and piglets in the control (CO) group received no probiotics with the feed. The feed composition is shown in Table 1. To prevent cross-contamination, sows and piglets of the two treatment groups were kept in separate housing facilities of identical construction and environmental conditions until weaning of the piglets at the age of 28 d, with each litter in a separate pen. On d 28, six piglets (three male, three female) from each litter with similar body weight were chosen for the infection trial. The piglets were placed in commercial flatdeck pens, two piglets per pen balancing for gender and litter. Piglets from both groups were housed in one facility with the pens of both treatment groups separated by the stable gangway. Animals had free access to feed and water throughout the entire period. The piglets were infected perorally on d 29 with $3 \times 10^9$ viable Salmonellae. The oral infection was performed with Salmonella enterica subspecies enterica serovar Typhimurium definitive phage type 104 (S. Typhimurium DT104; Szabó et al., 2009). The strain was originally isolated from a swine with sepsis and is resistant to nalidixic acid, streptomycin, erythromycin, chloramphenicol, and tetracycline (NalR, StpR, EryR, CmaR, TetR, Identification Number of the Institute of Microbiology and Epizootics, FU Berlin: 3308).

Performance data (body weight, body weight gain, feed intake) were recorded in weekly intervals per pen. All piglets were observed daily for health status including fecal consistency. Fecal quality was monitored using a subjective scoring system ranging from 1 (normal or hard pellets), 2 (soft), and 3 (liquid) and scored each morning after feeding. Every day each animal excreting feces containing excess fluid was regarded as one event of scour. The events in each group were summed up until the end of the study. For one experimental period, a total of 117 events of scour per group would meet 100% (all animals of the group with scour on every day). Furthermore, the incidence of scour was monitored (number of new cases during the 4 wk period in the shrinking population). Rectal temperature was measured only in piglets at the time point of euthanasia.

Sampling

Two weeks before the infection of the piglets, fecal samples were taken from every sow to test for the occurrence of Salmonella. Enrichment cultures were made and a qualitative detection of Salmonella was performed on DIASALM agar (see 2.3).

To obtain tissue samples from the gut immune system, three animals per group were sacrificed at 3, 24, and 72 h postinfection (PI). Piglets were sacrificed pen-wise (alternating male and female), with the pens in the treatment group being chosen randomly. Remaining animals (3 per group in each period) were sacrificed and housed in one pen. They were monitored for a period of 4 wk and sacrificed on d 28 PI. At each time point, three pigs per group were sedated with 20 mg/kg BW of ketamine hydrochloride (Ursotamin; Serumwerk Bernburg AG, Bernburg, Germany) and 2 mg/kg BW of azaperone (Stresnil; Jansen-
The remaining homogenate (dilution 1:10) was stored at 37°C for 24 h. For qualitative detection of Salmonella, 100 μL of the 1:10 dilution were spotted in triplicate to DIASALM agar plates (Diagnostic Semisolid Salmonella agar, containing 10 mg/L novobiocin) with 50 μg nalidixic acid/mL. To suppress the growth of Bacilli, Malachite Green (0.3 g/L; Sigma-Aldrich Chemie, Taufkirchen, Germany) was included in the agar. In addition, 1,000 μL of the 1:10 dilution were dropped on three XLD agar plates (333.3 μL per plate) to yield a detection limit of 10 Salmonella per gram feces or rectal contents. All plates were incubated at 37°C for 24 h.

**Bacterial Cell Counts**

To quantify Salmonella, 1 g of each wet sample, feces or rectum content, was mixed with 9 mL peptone water (1% peptone; Merck, Darmstadt, Germany) and homogenized with an Ultra Turrax (IKA, Staufen, Germany). Serial dilutions (1:10) in peptone water were made and 100 μL of the dilutions were spread to XLD agar plates (Roth, Karlsruhe, Germany) containing 10 mg/L novobiocin with 50 μg nalidixic acid/mL. To suppress the growth of Bacilli, Malachite Green (0.3 g/L; Sigma-Aldrich Chemie, Taufkirchen, Germany) was included in the agar. In addition, 1,000 μL of the 1:10 dilution were dropped on three XLD agar plates (333.3 μL per plate) to yield a detection limit of 10 Salmonella per gram feces or rectal contents. All plates were incubated at 37°C for 24 h and, in case of negative findings, the homogenate was dropped on DIASALM agar again and was incubated at 42°C for 20 h.

For quantitative detection of Salmonella, 100 μL of the 1:10 dilution were spotted in triplicate to DIASALM agar plates (Diagnostic Semisolid Salmonella agar, containing 10 mg/L novobiocin) with 50 μg nalidixic acid/mL in three replicates and incubated at 42°C for 20 h. The remaining homogenate (dilution 1:10) was stored at 37°C for 24 h and, in case of negative findings, the homogenate was dropped on DIASALM agar again and was incubated at 42°C for 20 h.

For quantification of Salmonella in the tonsils (tonsilla veli palatini), the tissue samples were flushed 3 times with sterile NaCl solution (0.85%) and minced with scissors; 3 g of the samples were then placed into a 50-mL plastic tube and diluted 1:10 in peptone water (1% peptone) and homogenized with an Ultra Turrax. The quantitative and qualitative detection of Salmonella was performed as described for the fecal samples.

For quantification of viable Salmonella in mesenteric lymph nodes, tissue samples were treated as described for the tonsils, but the XLD agar was not supplemented with Malachite Green.
Table 2. List of antibodies used in the study for staining of intraepithelial lymphocytes

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone/host species</th>
<th>Isotype</th>
<th>Cytochrome</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>MSA4</td>
<td>IgG2a</td>
<td>none</td>
<td>VMRD, Pullman, WA</td>
</tr>
<tr>
<td>CD4a</td>
<td>74–12–4</td>
<td>IgG2b</td>
<td>FITC</td>
<td>Southern Biotech, Birmingham, AL</td>
</tr>
<tr>
<td>CD5</td>
<td>9G12</td>
<td>IgG1</td>
<td>none</td>
<td>VMRD</td>
</tr>
<tr>
<td>CD8a</td>
<td>76–2–11</td>
<td>IgG2a</td>
<td>PE</td>
<td>Southern Biotech</td>
</tr>
<tr>
<td>CD8β</td>
<td>PG164A</td>
<td>IgG2a</td>
<td>none</td>
<td>VMRD</td>
</tr>
<tr>
<td>TcR1-N4</td>
<td>PGLBL22A</td>
<td>IgG1</td>
<td>none</td>
<td>VMRD</td>
</tr>
<tr>
<td>CD14</td>
<td>MIL-2</td>
<td>IgG2b</td>
<td>none</td>
<td>AbD Serotec, Oxford, UK</td>
</tr>
<tr>
<td>CD16</td>
<td>G7</td>
<td>IgG1</td>
<td>none</td>
<td>Acris, Herford, Germany</td>
</tr>
<tr>
<td>CD21</td>
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<td>IgG1</td>
<td>none</td>
<td>Biozol, Eching, Germany</td>
</tr>
<tr>
<td>IgG2a</td>
<td>pooled antisera from goat</td>
<td>IgG1</td>
<td>PE</td>
<td>Southern Biotech</td>
</tr>
<tr>
<td>IgG1</td>
<td>pooled antisera from goat</td>
<td>FITC</td>
<td></td>
<td>Southern Biotech</td>
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<tr>
<td>IgG1</td>
<td>pooled antisera from goat</td>
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<td>Southern Biotech</td>
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<td>IgG2b</td>
<td>pooled antisera from goat</td>
<td>FITC</td>
<td></td>
<td>Southern Biotech</td>
</tr>
</tbody>
</table>

For quantification of B. cereus, the samples of rectal content and fecal samples were frozen at -20°C. One gram of sample was homogenized in 9 mL peptone water (1% peptone) using an Ultra Turrax and the dilution was pasteurized for 15 min at 80°C. The solution was then chilled and 100 µL of serial dilutions were spread to PEMBA plates (Keul, Steinfurt, Germany) containing 2.5 µg Chloramphenicol/mL. Plates were incubated for 24–48 h at 30°C. Thus the detection limit was 100 cells in 1 g feces measured on a wet basis.

Detection of Immune Cell Populations

The isolation of the intestinal cells was performed as described previously (Solano-Aguilar et al., 2000) with slight modifications. For isolation of intraepithelial lymphocytes (IEL), a 20-cm section without discrete Peyer’s patch (PP) was taken from the midjejunum. The intestine was inverted, tied at one end, filled with PBS, and tied at the other end. The inverted intestinal sample was then incubated in Hanks balanced salt solution containing Dithiotreitol (HBSS-DTT; HBSS without Ca2+ and Mg2+, 2 mM DTT, 0.01 mM HEPES) and gently shaken for 5 min at 37°C. The medium was discarded and replaced with HBSS–EDTA (HBSS–EDTA; HBSS without Ca2+ and Mg2+, 1 mM EDTA, 1 mM Hepes) and incubated further for 35 min at 37°C with gentle shaking. After passage through a sterile 210-mm Nylon mesh, the cell suspensions were collected and centrifuged at 600 × g for 10 min at 4°C. The resulting cell pellets were resuspended in RPMI-1640 medium and kept on ice. Treatment of the original tissue sections was repeated, and both cell suspensions were combined and centrifuged again. Sediments were resuspended in 25% Percoll in HBSS and centrifuged at 600 × g for 30 min. The cell debris (top layer) was aspirated, the Percoll solution was removed, and the cell sediment was suspended in RPMI. The resulting cell suspensions (containing leukocytes and epithelial cells) were subjected to flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany). The staining procedure was performed as previously described (Mafamane et al., 2011). The following combinations of antibodies (Table 2) were used to stain the IEL: CD2/CD5, CD8α/γδ TCR, and CD8β/CD16.

Peripheral blood mononuclear cells (PBMC) were purified from heparinized blood by gradient centrifugation (Ficoll-Paque; Amersham Biosciences, Uppsala, Sweden) at 800 × g for 20 min at RT. The following combinations of antibodies (Table 2) were used to stain the cells: CD2/CD5, CD4α/CD8α/CD8β, CD8α/γδ TCR, CD8β/CD16, and CD21/CD14. The staining procedure was performed as described previously (Mafamane et al., 2011). The secondary antibodies (sec. ab) used to detect PBMC and IEL are shown in Table 2.

The cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany). To analyze the proportion of the stained cells, a lymphocyte gate was constituted following morphological criteria. The gate includes IEL or PBMC, respectively. Percentages of positive immune cells reflect the distribution of certain subpopulations within the relevant lymphocyte gate.

In Vitro Infection of IEL

Intraepithelial lymphocytes were isolated from six piglets aged 40 d according to the method described above (3.4). The IEL suspensions containing 3 × 10^6 cells/mL were infected with a green fluorescent protein (GFP)-expressing derivative of S. Typhimurium DT104 in a multiplicity of infection (MOI) of 10. After incubation of the cell suspensions for 1 h at 37°C, the cell suspensions were washed twice by centrifugation at 450 × g, and the cells were resuspended in 300 µL of PBS containing 0.2% BSA. Two samples, each containing 1 × 106 cells, were pipetted from every cell suspension to detect γδ T cells or CD8β-expressing T cells, respectively. The secondary antibodies were labeled with PE (Table 2) to allow a simultaneous detection of the GFP signal from the bacteria. The percentage of infected γδ T cells (infection rate, IR) was compared to the percentage of infected CD8β T cells from the same cell suspension. The mean fluorescence intensity (FI) of the GFP positive populations was also detected and compared.

Statistical Evaluation

The statistical analysis was performed with the software package SPSS (version 19.0; SPSS, Inc., Chicago,
IL). For feed intake, pen was considered as the experimental unit, whereas the individual pig was considered as the experimental unit for the other measurements. Means were compared by Welch’s $t$ test for 2 independent samples or by $t$ test for paired samples, where appropriate. The significance level was set at $\alpha = 0.05$. The Chisquare test was applied to analyze the incidence of scour. To achieve normality, the numbers of cfu/g were log-transformed and infection rates were transformed by arcsine square root transformation before analysis.

**RESULTS**

**Performance**

No differences in body weight, growth performance, feed intake, and feed conversion ratio were observed in both experimental periods. At the age of 28 d, all piglets (24 per group) showed similar bodyweights. The average weight of the piglets of the CO group was 7.0 kg (SD 0.7 kg), and that of the Bc group was 6.7 kg (SD 1.0 kg). At the end of the study, the individual differences increased, with piglets of the CO group ($n = 6$) showing an average bodyweight of 12.1 kg (SD 4.3 kg), compared to 10.7 kg (SD 2.5 kg) in the Bc group.

**Body Temperature and Scours (Incidence and Total Events)**

No differences in the body temperature of the piglets were observed at the 4 time points. The mean body temperature of all control piglets was 38.9°C. The piglets treated with *B. cereus* had a mean body temperature of 38.8°C.

During the 4 wk after infection with *Salmonella*, 17 control animals excreted liquid feces, compared to 7 animals in the Bc group. Thus, the incidence of diarrhea was significantly higher in the control animals ($P = 0.004$).

**Shedding of Bacillus Cereus**

Piglets of the Bc group had a consistently greater fecal shedding of bacteria belonging to the *Bacillus cereus* group in both experimental periods. In most samples of the CO group, less than 100 cfu of *B. cereus* were detected per gram wet feces. For visualization purposes, a value of 100 cfu was introduced into the data for the control group where the detection limit of 100 cfu per gram wet feces was not met. On Day 10, only 3 samples per group were analyzed, indicated with the numeral on the error bar.

During the first experimental period, 40 events (of 117 possible) of scour were counted in the CO group. Fifteen of those 40 events had a watery consistency. In contrast, only 14 events (of 117 possible) were counted in the Bc group and only 9 showed a watery consistency. During the second period, 61 events (of 117 possible) of scour were observed in control animals with 24 showing a watery consistency. In contrast, only 5 events of scour (of 117 possible) were noted in the Bc group, and none had a watery consistency. The data of both experimental periods were combined, and the number of animals suffering from scour at each time point after infection is shown in Fig. 1.

**Salmonella Shedding**

No *Salmonella* was cultivated from fecal samples of the sows, verifying that before infection, none of the piglet groups had contact with *Salmonella* species.

Shortly after oral infection of piglets, the shedding of *Salmonella* appeared to be similar in the piglets of both treatment groups (Fig. 3). However, *Salmonella* shedding was significantly lower in the group treated with *B. cereus* 25 d PI ($P = 0.004$).
Bacillus reduces Salmonella-diarrhoea in pigs

Translocation of Salmonella into Tonsils and Mesenteric Lymph Nodes

Comparison of the Salmonella cell counts in the tissue samples did not reveal significant differences between the groups for each time point (Fig. 4A and 4B). However, the overall load of Salmonella tended to be lower in the treatment group ($P = 0.071$).

Immune Response

The composition of the intraepithelial lymphocytes differed between the two groups of piglets during the course of infection in both experimental periods. For example, the percentage of CD8-positive γδ T cells was lower ($P = 0.036$) in the Bacillus cereus–treated group 4 wk after infection with Salmonella (Fig. 5). The majority of the γδ T cells in the epithelium expressed CD8. The mean percentage of CD8-negative (CD8neg) intraepithelial γδ T cells was below 4% (data not shown).

The analysis of peripheral blood mononuclear cells PBMC also revealed differences in the average relative numbers of γδ T cells, which were less frequent in the piglets treated with B. cereus 24 h PI Significant differences ($P = 0.028$) mainly arose from the CD8neg population of γδ T cells. One day after infection, the animals in the CO group showed 12.4% (SD 4.0) CD8neg γδ T cells in the lymphocyte gate, whereas the animals in the Bc group showed only 6.9% (SD 3.4).

Furthermore, although the mean percentages of CD14+ cells increased in both groups after the infection with Salmonella, no differences were observed between the groups (data not shown).

In Vitro Infection of IEL with Salmonella

With an MOI of 10, the IR of the different cell populations was clearly measurable (Fig. 6). Ex vivo IEL from all six animals showed a significantly higher IR of γδ T cells compared to the IR of CD8β T cells ($P = 0.002$; Table 3). The mean difference for the IR (γδ T cells – CD8β T cells) was 4.0 (SD 1.8). Additionally, the mean FI of GFP was greater in the GFP-positive γδ T cells compared to the GFP-positive CD8β cells in all 6 cell suspension ($P = 0.001$; Table 3). The mean difference for the FI (γδ T cells-CD8β T cells) was 10.8 (SD 3.8).

DISCUSSION

All animals in the Bc group were highly colonized with the probiotic additive B. cereus var. Toyoi in both experimental periods, whereas piglets in the CO group...
had only very low numbers of members of the \textit{B. cereus} group, consistent with no cross-contamination between the groups. Based on the fecal cell counts of \textit{Salmonellae} shortly after infection, the Bc group showed equivalent levels of infection as the CO group. However, the group fed with \textit{B. cereus} showed significantly fewer events of scour. Therefore, the different pathologies observed shortly after infection were not due to differences in the infectious dose of \textit{Salmonella}. The intensity of diarrhea was unexpectedly high in all groups, but most severe in the CO group. As the diarrhea was not observed before infection and appeared shortly after the oral infections (1 d PI), \textit{Salmonella} challenge clearly appeared to trigger pathology. Possible explanations for the observed differences could be a different microflora between the groups or differences in the immune response and subsequently in the intestinal absorptive and secretory mechanisms (Lodemann et al., 2008). A strong correlation between the intestinal microbiota composition before infection and susceptibility to \textit{Salmonella}-induced pathology has previously been shown in mice. Interestingly, in general, the protection against colitis was not the consequence of reduced levels of \textit{S. Typhimurium}; rather, the resistance to inflammation appeared to be correlated to higher levels of distinct commensal bacteria, in particular to numbers of the \textit{Bacteroides} phylum (Ferreira et al., 2011). Furthermore, \textit{Salmonella} infections in mice have been shown to cause recruitment of neutrophils to the gut lumen. The production of elastase by these neutrophils affects the composition of the intestinal microbiota such that the colonization with \textit{Salmonella} is favored (Gill et al., 2012). However, in the present study, the frequency and function of granulocytes were not investigated.

Infection with \textit{Salmonella} triggers an immune response, which may not immediately hamper \textit{Salmonella} but may affect other microorganisms and thereby change the intestinal ecosystem to the benefit of \textit{Salmonella}. Concerning immunological parameters, the only consistent difference (observed in both experimental periods) was the reduced relative numbers of \(\gamma\delta\) T cells in the blood and in the jejunal epithelium of the piglets of the group fed with \textit{Bacillus cereus} var. Toyoi. The \(\gamma\delta\) T cells recognize antigens in a non-MHC-restricted manner (Takamatsu et al., 2006). They play a role in skin and mucosal epithelial wound repair (Jameson et al., 2002). The CD8-positive \(\gamma\delta\) T cells of the pig have been shown to have cytotoxic potential (Pauly et al., 1996; de Bruin et al., 2000). However, most of the recent knowledge about interactions of \textit{Salmonella} with the host immune cells is derived from experiments in mice. Murine CD8+ intraepithelial \(\gamma\delta\) T cells are described to be important for the clearance of \textit{Salmonella} (Li et al., 2012). Based on the data presented here, this doesn’t appear to be true for piglets. In the present study, elevated numbers of these immune cells did not correlate with a more rapid clearance of the pathogen; rather, the higher frequency of these cells in the jejunal epithelium of the control animals was associated with a stronger pathology and followed by a higher load of \textit{Salmonellae}. Pigs are known to have a higher amount of circulating \(\gamma\delta\) T cells compared to mice and humans (Yang and Parkhouse, 1996). Notably, the proliferating intestinal \(\gamma\delta\) T cells recirculate rapidly into the peripheral blood and are a major source of the T cell pool in the pigs’ blood (Thielke et al., 2003).
Table 3. Infection rate (IR) of γδ T cells and CD8β T cells and mean fluorescence intensity (FI) of infected cells in six ex-vivo IEL suspensions

<table>
<thead>
<tr>
<th>IR of γδ cells</th>
<th>IR of CD8β cells</th>
<th>FI of GFP+ γδ cells</th>
<th>FI of GFP+ CD8β cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1</td>
<td>7.3</td>
<td>99.0</td>
<td>87.7</td>
</tr>
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<td>18.5</td>
<td>12.7</td>
<td>109.6</td>
<td>99.1</td>
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<td>22.1</td>
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<td>34.5</td>
<td>30.0</td>
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<td>137.2</td>
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</table>

Furthermore, both porcine and human γδ T cells have antigen presentation functions which have to date not been shown for murine γδ T cells. A number of CD8+ γδ T cells co-express surface molecules usually associated with antigen presenting cells (APC), such as MHC class II, CD80/86, and CD40. They can take up ovalbumin and accumulate it in intracellular compartments, some of which also co-localize with intracellular MHC class II molecules (Takamatsu et al., 2002).

Regarding the chronological sequence of the microbiological and the immunological data in the present animal study, it does not appear that the different symptomatic was the result of a higher Salmonella cell count. During the first 3 d PI, the numbers of Salmonella in feces, lymph nodes, and tonsils were similar in both groups. However, scour was already more severe in the control group at 24 h PI and the percentage of circulating γδ T cells was significantly lower in the treatment group at that early time point. From studies in mice and pigs, it is known that Salmonella can survive within host-macrophages (Fields et al., 1986; Buchmeier and Heffron, 1989; Leung and Finlay, 1991; Verbrugghe et al., 2011). As porcine γδ T cells share characteristics with myeloid and professional antigen presenting cells, it would be of great interest to know whether they could be also carriers for Salmonellae in the pig as has been reported for CD18+ macrophages in mice (Vazquez-Torres et al., 1999). We attempted to address this question in vitro infection of IEL with S. Typhimurium and found that in comparison to CD8β T cells, the γδ T cells were indeed preferentially infected with S. Typhimurium. The percentage of infected lymphocytes, as well as the mean FI, was higher in the γδ T cells indicating a higher number of Salmonellae per γδ T cell. However, both populations of lymphocytes were infected with S. Typhimurium in vitro and the role of γδ T cells during the course of infection in the pig remains unclear.

It should be mentioned that the absolute numbers of γδ T cells were not determined. Only altered composition of the PBMC and IEL was detected. It remains possible that other lymphocyte populations, namely CD8β+ cytotoxic T cells and Natural Killer (NK) cells, were actually elevated in the Bacillus-treated group. This may coincide with the lower, although not significant, growth rate in these piglets. Indeed, some animals in the Bacillus group had a more rapid increase in cytotoxic T cells PI, whereas others showed an increase in NK cells (data not shown). Therefore, we cannot exclude the possibility that expansion of these other populations might be responsible for the relative reduction in γδ T cells observed.

It should also be noted that in the present challenge trial, the clinical symptoms after infection with S. Typhimurium DT104 were unexpectedly high and the performance of the piglets was poor. In swine, S. Typhimurium leads to a persistent infection with intermittent fecal shedding but often without clinical symptoms (Steinbach and Kroell, 1999). S. Typhimurium, especially definitive phage type 104 (DT104), is the most frequently serotype isolated from pork in Germany (Schroeter et al., 2004; EFSA, 2011). The severe clinical symptoms occurring after infection might suggest an additional stress factor afflicting the piglets in both groups. If so, this additional factor was present in both repetitions of the experiment, and the feed supplementation with B. cereus var. Toyoi did not significantly improve the performance of the animals in terms of better weight gain.

A recent analysis of the complete genome of B. cereus var. Toyoi indicated that the strain has the capacity to elaborate functional toxins (EFSA, 2012). Therefore, it may pose a hazard for the piglets. As B. cereus is a ubiquitous soil saprophyte, it appears doubtful that it constitutes a health risk for persons that do not handle the feed additive but consume contaminated products. Moreover, it cannot be excluded that the toxins it may produce are part of the probiotic mechanism of B. cereus var. Toyoi. With regard to the original hypothesis, that an increase in the fraction of CD8+ intraepithelial IEL by supplementation with B. cereus var. Toyoi plays a role in defense against S. Typhimurium could not be verified. Although, as expected, the health status of the piglets fed with B. cereus var. Toyoi was clearly improved, this effect was not associated with an increase in CD8+ cells; rather, an increased fraction of CD8+ γδ T cells after infection led to a more severe course of infection in the control group.

LITERATURE CITED

EFSA. 2012. Scientific Opinion on the safety and efficacy of Toyocerin® (Bacillus cereus) as a feed additive for sows, piglets, pigs for fattening, cattle for fattening, calves for rearing, chickens for fattening and rabbits for fattening. EFSA J. 10:2924.


