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# Effects of *Enterococcus faecium* and *Bacillus cereus* var. *toyoi* on the morphology of the intestinal mucous membrane in piglets*

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**Abstract:** Eighty piglets aged 14, 28, 35 and 56 days – weaned at day 28 – were subjected to this investigation. Each age-group consisted of five animals which were fed an *Enterococcus faecium* NCIMB 10415 (Cylactin®) and *Bacillus cereus* var. *toyoi* (Toyocerin®) based diet. Five animals served as controls. Tissue samples were collected immediately after sacrifice at 8.30 h a.m. from duodenum, jejunum, ileum, cecum and colon to examine intestinal morphology and histochemistry. The results showed that with respect to villus height and crypt depth supplementation of probiotics in piglets feed seemed to influence the morphology and enlargement factor not at all or only to a certain extent. With respect to the number of goblet cells, the difference between probiotic fed animals and control animals was generally extremely low. The shape of the villi of the small intestinal segments greatly varied in all age groups of control and probiotic fed animals. However, this morphological variety does not depend on the mode of feeding.

**Key words:** probiotics; intestinal; morphology; piglet.

## Introduction

The use of probiotics for the prevention and treatment of diseases of the intestinal tract in humans (Fuller, 1989; Gorbach, 2000; Marteau & Seksik, 2001; O'Sullivan et al., 2005) and animals (Reuter, 2001; Ghabdan, 2002), especially pigs (Herich et al., 1999; Melin & Wallgren, 2002; Jadamus et al., 2002; Vahjen et al., 2002; Scharek et al., 2005) has become increasingly popular with the knowledge of problems associated with antibiotic use as feed additives (Bengmark, 2000). The benefit of probiotics on the host's intestinal tract is well established (Bocourt et al., 2004; Gibson, 2004; Huang et al., 2004; Marteau et al., 2004), however our knowledge about their mode of action and their influence on the morphology of the intestinal tract is very limited (Jahn, et al., 1996; Görke, 2000; Baum et al., 2002). To date, it is established that the composition of animal feed might influence the structure of the intestinal mucous membrane (Tuch & Amtsberg, 1973; Klein & Schmidts, 1997; Langhout et al., 1999; Wiese et al., 2003).

Based on the results of these earlier investigations, it was the aim of the present study to find out whether probiotic supplemented feed might render similar results and to give a detailed description of the associated morphological changes including villous height, crypt depth, enlargement factor for the villus and crypt surfaces, number of goblet cells as well as modifications of the mucous membrane surface occurring in the intestine of piglets aged 14, 28, 35 and 56 dpp (days post partum) under the influence of *Enterococcus faecium* and *Bacillus cereus*.

## Material and methods

A group of 20 sows (motherline: German Landrace × Duroc; father line: Landrace B × Hamshire) was randomly divided into two groups: (i) one (probiotic group) was supplemented with either the probiotic strain *Enterococcus faecium* SF 68 (NCIMB 10415, Cylactin®, Roche) (five animals) or with *Bacillus cereus* var. *toyoi* (Toyocerin®, Lohmann) (five animals) beginning with day 25 of pregnancy; and (ii) the other group of ten sows remained untreated (control group). Out of these 20 sows a total of 80 piglets aged 14, 28, 35, and 56 dpp were investigated. Each age group consisted of five animals which were fed an *E. faecium* or *B. cereus* based diet. Five animals served as control animals. All animals were weaned at the age of 28 days. After parturition through weaning of piglets the probiotic sows' feed was supplemented with barley, triticales, wheat bran, wheat and soy bean meal with a concentration of the probiotic $1.2 \times 10^9$ cfu/kg feed

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Table 1. Composition of the diets.[a]

| Ingredients | Sows: 25$^{th}$ day of pregnancy-date of parturition [g/kg] | Lactating sows (day of birth- weaning (28$^{th}$ day pp) [g/kg] | Prestart diet for piglets (15–28$^{th}$ day pp) [g/kg] | Basal diet (28–56$^{th}$ day pp) [g/kg] |
|---|---|---|---|---|
| barley | 543.0 | 400.0 | | 130.9 |
| wheat | 306.0 | 80.0 | 454.7 | 568.7 |
| triticale | | 149.5 | | |
| wheat bran | | 106.7 | | |
| soya bean meal | | 160.0 | 274.0 | 234.0 |
| milk powder | | | 120.0 | |
| oat bran | | | 100.0 | |
| 1$^{st}$ trial: Cylactin® | $1.6 \times 10^9$ cfu/kg feed | $1.2 \times 10^9$ cfu/kg feed | $1.7 \times 10^8$ cfu/kg feed | $2 \times 10^8$ cfu/kg feed |
| 2$^{nd}$ trial: Toyocerin® | $2.6 (\pm 0.3) \times 10^5$ cfu*/g dm** | $4.0 (\pm 0.6) \times 10^5$ cfu*/g dm** | $1.3 (\pm 0.4) \times 10^6$ cfu*/g dm** | $1.4(\pm 0.2) \times 10^6$ cfu*/g dm** |

[a] *Colony formatting units; ** dry matter.

Cylactin® or $4 \times 10^5$ cfu/g dry matter (dm) Toyocerin®. The controls were fed with the same diet without probiotic supplementation. From day 15, suckling piglets of the probiotic groups had free access to a prestart feed, a mixture of wheat, soy bean meal, milk powder and oat bran which contained the respective probiotic in the same concentration as in the sows' feed for the treated group. The control piglets got the same diet without probiotic supplementation. After weaning, the piglets were fed *ad libitum* with a basic diet of wheat, soy bean meal and barley. The feed of the treated group contained Cylactin® at a concentration of $1.7 \times 10^8$ cfu/kg feed or Toyocerin® at a concentration of $1.3 \times 10^6$ cfu/g dm. Control piglets were fed *ad libitum* with the same diet but without the probiotic supplementation. For more details see Table 1.

The sows together with their piglets were housed in a farrowing crate. The weaned piglets were placed in flat deck pens (2–3/box) with a slatted flour. The piglets were euthanized by an intravenous injection of an overdose of sodium pentobarbital (Eutha® 77, Essex, Tierarznei), in each case at 8.30 h a.m.

After opening of the abdominal cavity tissue samples were collected from the first two thirds of the duodenum descendens (1/duo), the ileum close to the ostium ileale (4), the apex ceci (5), the colon ascendens (distal to the ostium caecocolicum) (6/colon asc), and colon descendens (colon sigmoideum) (7/colon desc). Two samples were taken from the jejunum, one approx. 20 cm proximal (2/prox jej) and the other approx. 50 cm distal (3/dist jej) to its middle part. Intestinal segments were cut open lengthwise at the antimesenterial side, washed in ice cold Ringer solution and fixed in Bouin's fluid for light, respectively in cacodylate buffered 0.1 M 2% paraformaldehyde + 2.5% glutaraldehyde (pH 7.2) for scanning electron microscopic investigations. During the fixation, specimens were pinned to a piece of cork with the serosal side facing downwards. The Bouin-fixed samples were embedded in paraffin and cut at 5 $\mu$m thickness. For scanning electron microscopy, samples were postfixed in 1% osmium tetroxide, after dehydration in a graded series of ethanol and hexamethyldisilazane (HMDS, Roth) they were mounted on aluminium stubs, sputter coated with gold and finally examined using a scanning electron microscope (Nanolab 2000, Bausch & Lomb, Ottawa, Canada) at 10–15 KV.

*Histochemical investigations*

Neutral and acid mucous substances were traced with the help of the periodic acid Schiff (PAS) – Alcian Blue (ROMEIS, 1989).

*The morphometric procedure*

This was carried out with a computerized image analysis program "Lucia 32–G Corona 4.11" (Nikon GmbH, Tokyo, Japan) on Haematoxylin and Eosine stained tissue sections. The following parameters were determined:

(1) The villus height and crypt depth were only measured in those areas of sections which run vertically from the tip to the base of the villus or from the base of the adjacent crypt to the base of the villus. Fifty measurements were taken per intestinal segment and per animal at 125 × magnification.

(2) According to WIESE et al. (2003), the enlargement factors of the mucous membrane were determined as follows: (a) villi: length of the villous surface was related to the length of the associated lamina muscularis mucosae; (b) crypts: the total crypt area, referred to as the crypt volume (described by the area covering complete crypts and cross sections of crypts encircled by the basement membrane) was related to the length of the respective lamina muscularis mucosae. Fifteen fields of vision per animal and intestinal segment were measured at 62.5 × magnification.

(3) For quantitative determination of goblet cells within the epithelium of the intestinal villi and crypts, respectively, the number of PAS-Alcian-Blue-positive cells per mm surface length of villi and circumference of crypts were measured.

*Statistical analysis*

The statistical analyses were founded on the mean values per animal. In the first step a hierarchical mixed model with the fixed factors group, age, intestinal segments and the random factor piglet was applied. Because of strong interactions between age and intestinal segments, we analysed the data separately for each age group. In case of a group-effect (alpha = 0.05), t-test for independent samples were calculated in order to compare the groups within the single intestinal segments. Group differences are characterized by t-test values of $p < 0.05$. All statistical analyses were performed using SPSS 11.5 for Windows XP.

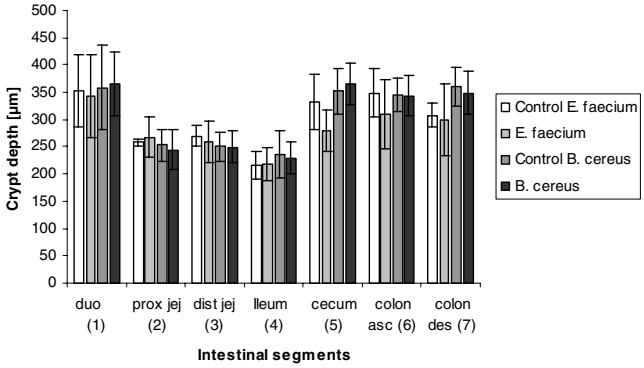


Fig. 1. Mean values of the crypt depth, e.g. considering 35-day old piglets of the *E. faecium*-group and the *B. cereus*- group and their corresponding control groups.

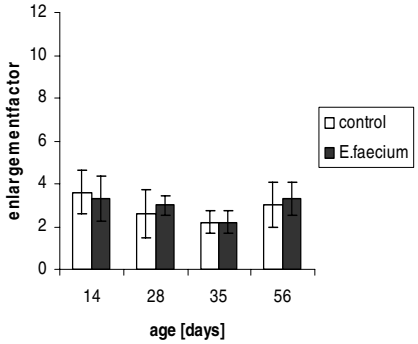


Fig. 2. Enlargement factor of villus surface in the duodenum of the *E. faecium* trial.

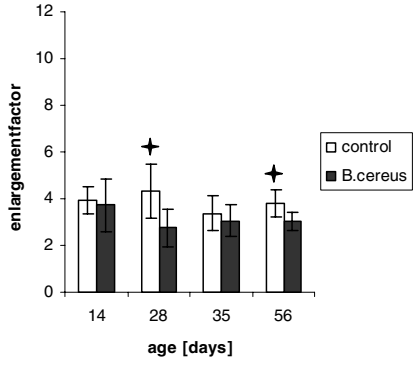


Fig. 3. Enlargement factor of villus surface in the duodenum of the *B. cereus* trial. ✦: demonstrates a significant difference ($p < 0.05$) between treated group and its corresponding control group regarding 28- and 56-day old piglets.

## Results

*Morphological aspects – light microscopy*

*Height of intestinal villi.* In both trials the villi of all small intestinal segments displayed minor but not significant differences between the controls and the probiotic groups.

*Depth of intestinal crypts.* Principally, crypts, especially in the jejunum, were less deep in the small intestine, than in the large intestine (Fig. 1). The slight increases in crypt depths were related to age in all groups and in all examined intestinal areas, but no distinct differences between the control and their corresponding probiotic groups were detectable. Crypts of *E. faecium*-fed animals compared to control piglets became generally deeper with increasing age in all intestinal segments. Regarding *B. cereus*-supplementation, however, this detail was only observed in the small intestine. With regard to crypt depth, no significant differences between either *E. faecium*- and *B. cereus*-fed animals, or between experimental and control animals were detectable.

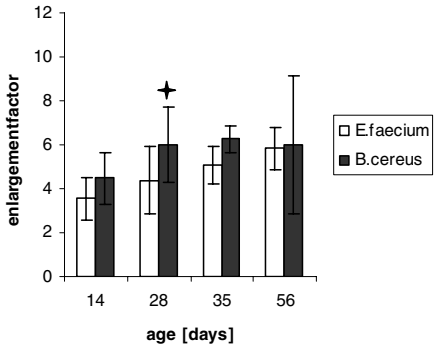


Fig. 4. Enlargement factor of crypt surface in the ileum. ✦: demonstrates a significant difference ($p < 0.05$) between *E. faecium* and *B.* cereus groups in 28-day old piglets.

*Enlargement factor*

*Intestinal villi.* Concerning the *E. faecium*-supplemented diet, no significant differences in all intestinal segments were detectable between control and probiotic-treated groups (Fig. 2). In the *B. cereus* trial, of the 28- and 56-day old piglets, however, the enlargement factor of the duodenum was higher in controls than in the probiotic group ($p < 0.05$; Fig. 3).

Significant differences between the two probiotic-fed piglet groups were only detected in the distal jejunum of 14-day old animals: regarding this location and age group the enlargement factor of the *B. cereus*-fed animals was much higher than in the *E. faecium*-fed animals.

*Intestinal crypts.* In the ileum, the enlargement factor of crypt surface displayed slight but not significant differences between control and their corresponding probiotic groups. However, there was a large significant difference ($p < 0.01$) between the *E. faecium* fed- and the *B. cereus* fed-group regarding the ileum of the 28-day old piglets (Fig. 4). In the colon ascendens (Fig. 5) and colon descendens (Fig. 6) of the 28-day old piglets, another significant ($p < 0.05$) difference between the two probiotic groups was detected. The enlargement factor of the crypts of all intestinal segments was higher in the *B. cereus*-fed than in the *E. faecium*-fed animals (Figs 4–6).

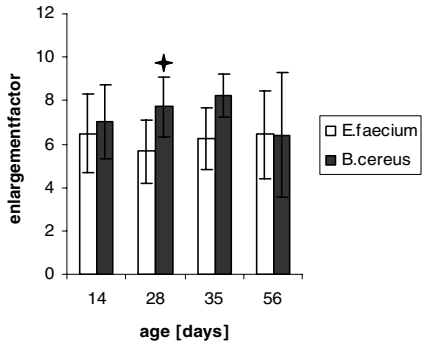


Fig. 5. Enlargement factor of crypt surface in the colon ascendens. ✦: demonstrates a significant difference ($p < 0.05$) between *E. faecium* and *B. cereus* groups in 28-day old piglets.

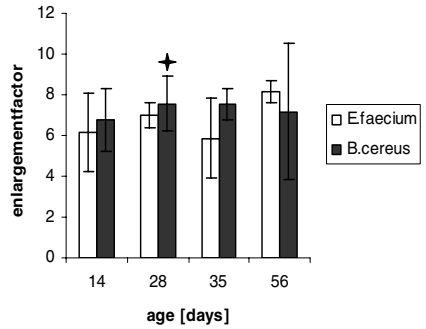


Fig. 6. Enlargement factor of crypt surface in the colon descendens. ✦: demonstrates a significant difference ($p < 0.05$) between *E. faecium* and *B. cereus* groups in 28-day old piglets.

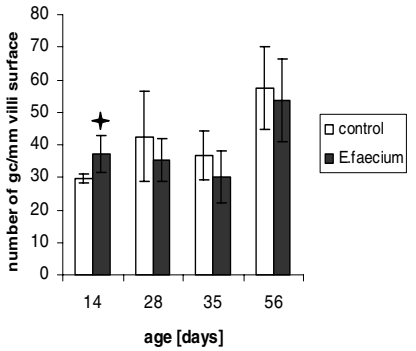


Fig. 7. Number of goblet cells (gc) in the villi of the duodenum. ✦: demonstrates a significant difference ($p < 0.05$) between *E. faecium* group and its control group in 14-day old animals.

*Histochemical investigations*

*Number of goblet cells in the villi.* Only in the 14-day old piglets of the *E. faecium*-fed group, the villi of the duodenum contained a significantly higher number of goblet cells than the corresponding control group ($p < 0.05$; Fig. 7). In the duodenal villi of the *B. cereus*-fed piglets, more goblet cells were observed than in *E. faecium*-fed animals, however, the differences were not significant (Fig. 7, 8). The villi of the other small intestinal segments displayed no significant differences

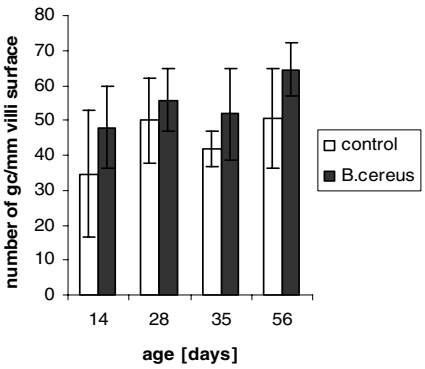


Fig. 8. Number of goblet cells (gc) in the villi of duodenum.

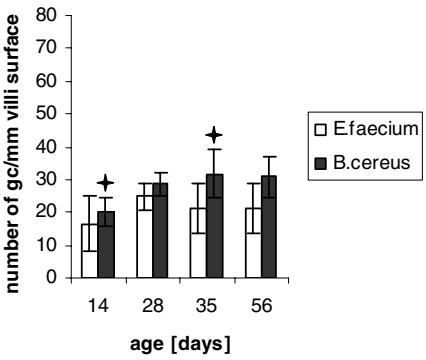


Fig. 9. Number of goblet cells (gc) in the villi surface of the proximal jejunum. ✦: demonstrates a significant difference ($p < 0.05$) between *E. faecium* and *B. cereus* supplemented groups of 14- and 35-day old piglets.

between the probiotic-fed groups and their corresponding controls. However, significant differences were detected between the two probiotic groups: In the villi of the proximal jejunum of 14- and 35-day old piglets of the *B. cereus* group, significantly more goblet cells were found than in the *E. faecium*-treated animals of the same age group ($p < 0.05$; Fig. 9). In the distal jejunum, a correspondingly significant difference ($p < 0.05$) was observed in the age groups of 14-, 28- and 35-day old piglets (Fig. 10).

*Number of goblet cells in the crypts.* With respect to the number of goblet cells per mm crypt circumferences, generally no significant differences were observed between the probiotic-treated groups and their corresponding control groups. When the two probiotic-supplemented groups were compared, some differences were detected but restricted to the crypts of the proximal jejunum: 28-day old *B. cereus*-treated piglets displayed significantly more goblet cells than the *E. faecium* group of the same age ($p < 0.05$). In the cecum, the results were similar, but the difference ($p = 0.051$) was not significant.

*Scanning electron microscopy*

The shape of the villi of the small intestinal segments varied greatly in all age groups of control and probiotic-

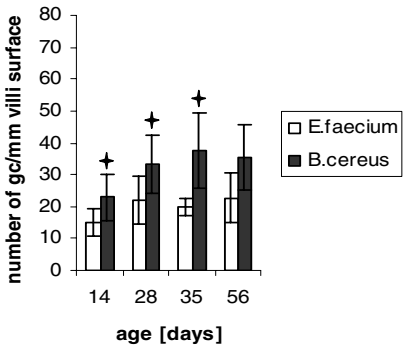


Fig. 10. Number of goblet cells (gc) in the villi surface of the distal jejunum. ✦: demonstrates a significant difference ($p < 0.05$) between *E. faecium* and *B. cereus* supplemented groups of the 14-, 28- and 35-day old piglets.

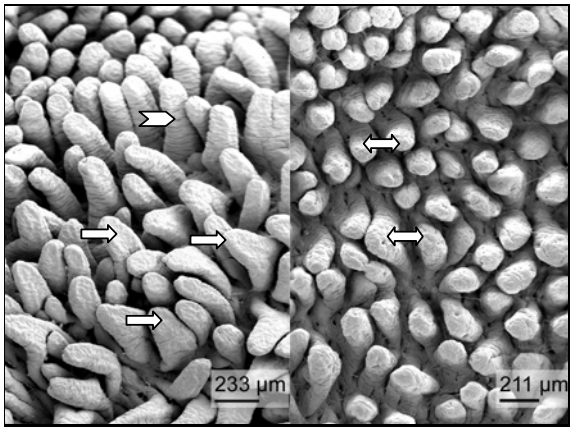


Fig. 11. REM- left side: tongue-like ( ⇨ ) and slender/finger-like ( ⊱ ) villi; right side: short and wide villi ( ⇔ ), both from 14-day old probiotic piglets.

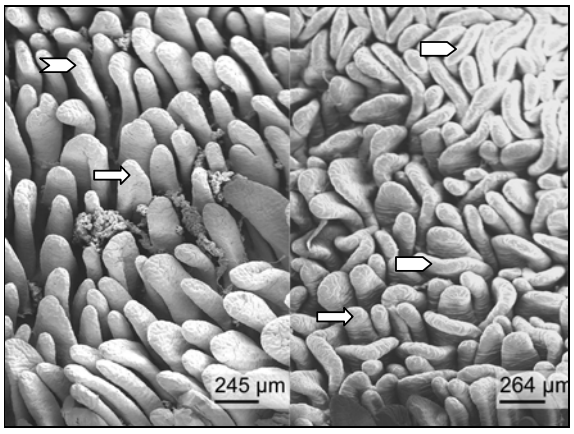


Fig. 12. REM- left side: tongue-like villi ( ⇨ ) and slender/finger-like ( ⊱ ) right side: tongue-like ( ⇨ ) and crest-like villi ( ⊏ ), both from 56-day old control-piglets.

supplemented animals. The shapes ranged from long and slender/finger-like villi (Figs 11,12), to short and wide villi, as well as tongue-like (Figs 11,12) and crest-like villi (Fig. 12). These morphological variations of the mucosal surface modifications however did not depend on the mode of feeding.

## Discussion

The results of our investigations demonstrate that the morphology of the mucous membrane in young piglet intestines is only moderately influenced by *E. faecium* and *B. cereus* diets. Clear differences between probiotic-fed animals and controls or between age groups were seen only occasionally in some individual animals or in some intestinal locations.

*Villi height*

Single individuals as well as animals belonging to the same age or feeding group differed extremely from the respective mean value of villi height. This variance was detectable both in the morphometric and in the scanning electron microscopic investigations. These results are contradictory to the investigations of Klein & Schmidts (1997) and Görke (2000) who described that the villi height in the jejunum of pigs increased after treatment with*B. cereus*, *S. boulardii* or yeast strain CNCMI-1079; or to the results of Di Giancamillo et al. (2003) who described similar findings for the ileum. Probably, these differing results are related to the fact that in the present study pigs of different origin and age group were examined. In this context, it has to be stressed that in our investigations the longest intestinal villi in all age groups of either probiotic-fed or control animals were detected in the jejunum. As mentioned by other investigators, e.g. Mitjans & Ferrer (2004), the jejunum displays the largest surface of intestinal mucosa, followed by the ileum and the duodenum.

*Crypt depth*

Generally, no distinct differences regarding crypt depth were detected in all investigated intestinal segments comparing probiotic and control groups. Görke (2000) observed the same. Di Giancamillo et al. (2003) however – while feeding pigs with yeast strain CNCMI-1079 (LEVUCELL® SB) – detected an increase of crypt depths in control animals. In our investigations, weaning did not seem to influence depths of crypts to a considerable extent. This fact is verified by Miller et al. (1986); Cera et al. (1988); Pluske et al. (1996) and Vente-Spreeuwenberg et al. (2004) who were studying the effect of feeding on piglet's intestines in weaning and post weaning stages. According to our own investigations and to other findings in the literature (Baum et al., 2002; Gu & Li, 2004; Mitjan & Ferrer, 2004; Vente-Spreeuwenberg et al., 2004), the main and time-consuming difficulty of the morphometric examination is to display enough straight villi, or straight and unbranched crypts within the intestinal mucosa material for measuring the villus surface and crypt volume. Therefore, we used the "enlargement factor" which – ac-

cording to Wiese et al. (2003) – produced more exact data in comparison to measurements of villus-heights and crypt-depths. However, the enlargement factors of the villous and cryptal areas differed not much from the results obtained from measuring villus-heights and crypt-depths in controls as well as in probiotic-fed animals. This occurrence may thus be rated as proof for the linear measurements employed in this study. Regarding intestinal villi morphology, the treatment of piglets with the probiotic *E. faecium* and *B. cereus* principally did not produce any difference between animals from control and probiotic groups. Only in *B. cereus*-fed 28- and 56-day old piglets, the factor of the villous surface increased compared to that of the control animals. And in 14-day old piglets the factor in *B. cereus* was higher than in *E. faecium*-fed animals. As far as the intestinal surface enlargement factors due to crypt formation are concerned, no clear differences between controls and probiotic-fed animals were detectable. However, in the ileum, colon ascendens and colon descendens of 28-day old *B. cereus*-fed piglets, the factors were (significantly) higher than in *E. faecium*-fed animals.

*Goblet cells*

The results produced with the enumeration of goblet cells in all animal groups principally resembled the other results obtained so far. This means that no major differences between probiotic-fed and control piglets were detected. In the present study, the number of goblet cells within the probiotic-fed piglets differed slightly from those found in the control animals. Fourteen-day old *E. faecium*-treated piglets displayed, for example, significantly more goblet cells in the duodenum than the control animals of the corresponding age group. Görke (2000) also noticed that piglets fed with a *S. boulardii* diet displayed more goblet cells in the cecal mucosa than the control animals. Baum et al. (2002) detected a decreased number of goblet cells with 2,6–sialylated mucins in the large intestine of post weaned *B. cereus*-treated piglets. Other authors examining different animal species like rats (Fernandez-Estivaritz et al. 2003; Kleessen et al., 2003), rodents (Wang et al., 2003), chicken (Langhout et al. 1999) or pigs (Lalles et al., 2001; Piel et al., 2005) also described that mode and type of feeding modifies the occurrence of intestinal goblet cells. Piglets fed a *B. cereus* supplemented diet were found to display more goblet cells in total than the *E. faecium*-treated animals. Differences were significant ($p < 0.05$) regarding the area of the proximal and distal jejunum.

*Shape of intestinal villi*

Intestinal villi shape was not dependent on age or on probiotic feeding. All types of shapes, such as long and slender, finger-shaped, or short thumb-shaped, or tongue-shaped, or even crest-like villi occurred throughout the small intestines. This fact has not found entry into general histology textbooks but has been described by other authors. Some attributed this phenomenon to the unweaned and weaned stages of young animals (Wiese et al., 2003), different functional stages (Verma et al., 1999; Megbungwan et al., 2004) or, contradictory to our findings, to the mode of feeding (Van Leeuwen et al., 2004).

*Conclusion*

The results of the present study showed that probiotic feeding supplementation – currently employed for therapy or prevention of intestinal diseases in juvenile pigs – did not influence the morphology of the intestinal mucosa to a considerable extent. Alterations were restricted to individual variations. Thus, it was established that *Enterococcus faecium* and *Bacillus cereus* did not affect the cellular features responsible for the specific functions of the intestine. Therefore both examined probiotics are recommendable as ideal replacements for the hitherto employed antibiotics that were rightly prohibited because of their noxious influence on the intestinal flora and their resistancy-inducing effect in animals and humans.

**Acknowledgements**

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