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Research paper

Intraepithelial lymphocyte numbers and histomorphological parameters in the porcine gut after *Enterococcus faecium* NCIMB 10415 feeding in a *Salmonella* Typhimurium challenge

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ABSTRACT

Salmonellae are among the most widespread sources of foodborne infections and *Salmonella* Typhimurium, in particular, is correlated with human disease caused by the consumption of contaminated pork. Intraepithelial lymphocytes (IEL) have early contact with intestinal antigens and play an important role in the detection of pathogenic bacteria. The objective of this study was to determine whether a presumed probiotic *Enterococcus faecium* strain could improve histomorphological and immune system-related parameters of gut function after a *Salmonella* challenge in weaned pigs. In particular the morphological parameters villus length and width, crypt depth and width as well as the actual enlargement of the intestinal epithelial surface were calculated and the number of IEL was evaluated in sections of the porcine gut.

Weaned piglets were challenged with *Salmonella enterica* serovar Typhimurium DT 104, and half of them also received *Enterococcus faecium* NCIMB 10415 in the diet. Animals were sacrificed at days post infection (DPI) 2 and 28. The effect of the factors "time post-infection/age" and "probiotic treatment" on jejunal morphology and IEL numbers and distribution was evaluated by light microscopy. The time post-infection had significant effects in both feeding groups. Animals sacrificed at DPI 28 had longer and wider villi, deeper and wider crypts, a higher villus enlargement factor, a higher ratio between villus and crypt enlargement factors as well as more IEL. Probiotic treatment resulted in longer villi, a higher ratio of villus surface/crypt circumference enlargement factors and significantly more IEL. The larger total number of IEL displayed by the probiotic group resulted from significantly higher numbers of IEL at the nuclear and apical levels of the intraepithelial compartment but not from the number of IEL situated at the basement membrane. The probiotic effects were only measurable 28 DPI. It is proposed that *Enterococcus faecium* NCIMB 10415 exerts an immune modulatory effect by increasing the numbers of intraepithelial lymphocytes.

Abbreviations: IEL, intraepithelial lymphocyte(s); S.Typhimurium, *Salmonella enterica* serovar Typhimurium; *E. faecium*, *Enterococcus faecium*; DPI, days post infection; CFU, colony forming units.

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1. Introduction and aim

Salmonellae are among the most widespread sources of foodborne infections and especially *Salmonella enterica* serovar Typhimurium (*S.*Typhimurium) that often contaminates pork (Hedemann et al., 2005; Kreuzer et al., 2012; Martins et al., 2012). In pigs, *S.*Typhimurium infection causes clinical symptoms with enterocolitis and regularly subclinical infections persist. These infected animals can act as a host reservoir and bring the pathogen into the food chain (Boyen et al., 2008; Collado-Romero et al., 2012). *Salmonella* infects epithelial cells of the small and large intestine and may cross this barrier via different mechanisms (Hulst et al., 2013; Schauser et al., 2004; Velge et al., 2012). It is crucial that mammalian hosts monitor and regulate microbial interactions with intestinal epithelial surfaces (Duerkop et al., 2009). Intestinal intraepithelial lymphocytes (IEL) are the first in line to have contact with the antigen present in the gut (Hershberg and Blumberg, 2005) and belong to a unique T-cell population (Chang et al., 2005). They are located between the enterocytes in the epithelium above the basement membrane and are phenotypically and functionally distinct from lymphocytes in the underlying lamina propria or draining lymph nodes (Lacković et al., 1999; Vega-López et al., 1993; Waly et al., 2001; Whary et al., 1995). IEL play an important role in the detection of pathogenic bacteria, are involved in the discrimination between pathogenic and commensal organisms and have been demonstrated to increase in numbers after oral infection by *S.*Typhimurium (Li et al., 2012). Under homeostatic conditions, IEL regulate the continuous turnover of epithelial cells by the eradication of infected epithelial cells and the control of epithelial repair (van Wijk and Cheroutre, 2009). The proliferation and apoptosis of the surface epithelium may also be influenced by probiotics which are claimed to improve intestinal health of pigs, reduce mortality and increase productivity rates (Cho et al., 2011; Resta-Lenert and Barrett, 2003). Probiotics are dietary additives, recognised to provide beneficial effects to the gastrointestinal tract, for example by modulating gut morphological features as well as the gut's immune system including intestinal lymphocytes (Baum et al., 2002; Roselli et al., 2009).

This study is part of a wider group of research activities with the aim to understand how nutritional factors influence the functioning of the intestines in the pig. Previous to the herein described challenge trial, several feeding experiments with a presumed probiotic *Enterococcus (E.) faecium* strain were carried out by our Collaborative Research Centre (SFB 852 "Nutrition and intestinal microbiota – host interactions in the pig" http://www.sfb852.de) and an earlier Research Unit (FOR 438). In different trials with pigs, a variety of observations were made. For example, in unchallenged piglets, *E. faecium* has been found to increase the absorptive and secretory capacity of jejunal mucosa (Klingspor et al., 2013) and reduce levels of cytotoxic T cells in the jejunal epithelium (Scharek et al., 2005), while mucosal morphology, villus height, crypt depth and the enlargement factor were not influenced (Martin et al., 2012; Reiter et al., 2006).

The objective of this study was to determine whether *E. faecium* could influence histomorphological parameters of gut function as well as IEL number and distribution in combination with a *Salmonella* challenge in weaned pigs. Because the results reported in this paper are part of a larger trial, several other parameters from the same experiment have already been reported in earlier publications. The following summarises these. Kreuzer et al. (2012) report that piglets treated with *E. faecium* gained less weight and that they had a higher incidence of the pathogen in tonsils. Conversely, *E. faecium* had no effect on fecal shedding of *Salmonella*. The specific (anti-*Salmonella* IgG) and nonspecific (haptoglobin) humoral immune responses as well as the cellular immune response (T helper cells, cytotoxic T cells, regulatory T cells, γδ T cells and B cells) in lymph nodes, Peyer's patches, the ileal papilla and in the blood do not seem to be affected by *E. faecium* treatment (Kreuzer et al., 2012). However, when analysing magnetically sorted lymphocytes from ileocecal mesenteric lymph nodes, differences in the activation of T helper cells between the treatment groups were found. It was concluded that a higher Th2 driven immune response in the control group could have been the reason for the better *Salmonella* clearance reported for the control group in this as well as in an earlier challenge study (Kreuzer et al., 2014). Siepert et al. (2014) have given a comprehensive summary of our previous and latest feeding experiments concerning several immunological aspects. They conclude that *E. faecium* may have an anti-inflammatory/immuno-suppressive effect in piglets, which becomes most apparent during the post-weaning period (Siepert et al., 2014).

2. Materials and methods

2.1. Study design and probiotic feeding in the Salmonella challenge trial

The experimental setup has already been described (Kreuzer et al., 2012). In short, sows of the German Landrace breed were fed daily either a diet supplemented with 4.3×10^6 colony forming units (CFU) of *E. faecium* NCIMB 10415/g feed from day 28 ante partum onward or with the control diet containing no *E. faecium*. *E. faecium* NCIMB 10415 is a commercial probiotic feed additive (Cylactin® LBC ME10, DSM Nutritional Products Ltd, Switzerland). Piglets of the probiotic group ($n = 16$) were offered creep feed supplemented with 5.1×10^6 CFU/g in the suckling period and 3.6×10^6 CFU/g post weaning (d 28). Piglets from the control group ($n = 16$) received the same feed without the probiotic. Details of the ingredients and chemical composition of the diets were the same as reported (Martin et al., 2012).

The piglets were allocated to boxes in pairs. At the age of 38 days all piglets were challenged intragastrically with *S. enterica* serovar Typhimurium DT104 ($1.4–2.1 \times 10^{10}$ CFU) using a stomach tube. Six piglets from each group were sacrificed at DPI 2 and ten piglets on DPI 28, respectively. The rationale behind the time points of sacrificing the piglets is based on earlier studies of our working group (Mafamane, 2009; Szabó, 2009). In summary, between 24 and 48 h post infection most animals show clinical symptoms such as

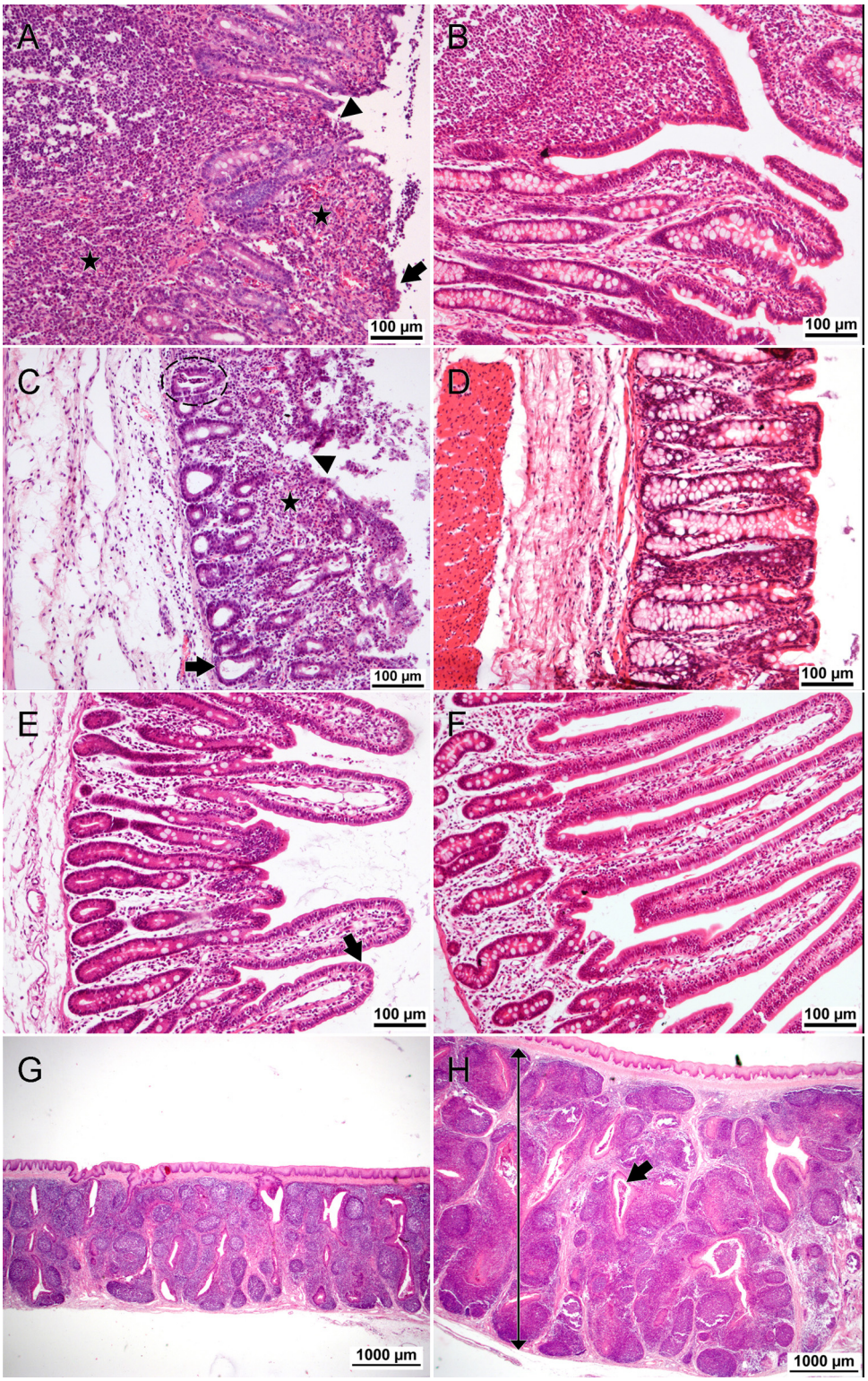

Fig. 1. Pathohistology: Pictures on the left side of the panel show H&E stained tissue sections of ileum (A), colon ascendens (C), jejunum (E) and tonsilla veli palatini (G) of animals 2 days post infection (2 DPI). The corresponding pictures on the right side (B, D, F, and H) represent tissues from animals 28 DPI. Differing degrees of *Salmonella* induced damage can be identified. (A) In the ileum villus atrophy up to a complete loss of villi (arrow), epithelial erosion (arrowhead) and massive inflammatory cell infiltration (asterisk) is noticeable. (B) Ileal tissue structure at 28 DPI has a normal appearance. (C) The example from the colon at 2 DPI shows a region with mucosal erosion (arrowhead). The lamina propria is markedly expanded due to infiltration with numerous inflammatory cells (asterisk). There are crypt abscesses (encircled), the crypts are lined by flattened epithelial cells (arrow) and only few goblet cells are

fever and diarrhoea. The late sampling date (28 days post infection) was chosen to examine the long time effects of probiotic feeding and *Salmonella* infection. The animals were infected 10 days later than in the first experiment (Szabó, 2009; Mafamane, 2009) because of a newly implemented adaptation period to the new environment. It should be noted that all animals in our study were challenged with *Salmonella* and so the "control group" in our study also includes infected animals. This definition of the term "control group" in animal infection studies was established earlier (Johnson and Besselsen, 2002; Sargeant et al., 2014). Experimental approval was given by the local authority (Landesamt für Gesundheit und Soziales, Berlin ID: G0348/09).

2.2. Sample collection and histochemical staining

Samples of mid-jejunum, ileum, colon ascendens and tonsilla veli palatini were taken immediately after slaughter and rinsed in ice cooled Ringer's solution. Gut samples were cut open on the mesenterial side, trimmed to 2 cm × 1 cm squares and pinned mucosal side up on cork pieces. Fixation of all samples was done for 26 h in Zamboni's fixation solution. Fixed tissues were processed according to standard histological protocols and stained with H&E or panoptic Pappenheim tissue stains (Romeis, 1989).

2.3. Morphometric parameters assessed by light microscopy

For each sample at each point in time, 4 slides were prepared and morphometric parameters were evaluated under a light microscope (Axioplan, Carl Zeiss, Jena, Germany). Digitalised live pictures were analysed on a monitor with the help of the computer assisted image analysis program NIS-Elements AR (Nikon Instruments Inc., U.S.A.). The following parameters were determined as previously described (Wiese, 2003; Wiese et al., 2003):

- villus length
- villus width
- crypt depth
- crypt width
- length of villus surface (equalling the length of the enterocyte brush border)
- total circumference of crypts demarcated by their basement membrane
- corresponding length of the lamina muscularis mucosae in the area measured

Measurement was only done in well oriented parts of the sections, which means at least 4 villi were cut completely from tip to base and most crypts were cut longitudinally. Supplemental Fig. 1A gives an overview on how the parameters were measured. A minimum of 5 high power fields (total magnification ×50) were measured. To estimate the enlargement of the intestinal surface epithelium by villi and crypts, the villus and crypt enlargement factors were calculated as follows:

$$\text{Villus enlargement factor} = \frac{\text{length of the villus surface}}{\text{length of the corresponding lamina muscularis mucosae}}$$

$$\text{Crypt enlargement factor} = \frac{\text{summed up crypt circumferences}}{\text{length of the corresponding lamina muscularis mucosae}}$$

2.4. Counting of IEL by light microscopy

The number and distribution of jejunal IEL along the villi was evaluated within three different intraepithelial compartments, namely the apical, the perinuclear and the basal compartments as described elsewhere (Vega-López et al., 2001; see also Supplemental Fig. 1B). In brief, counting was done using a 400× magnification, centering a randomly chosen, well-orientated villus tip or villus base in the field of view. In case of short villi (tip and base overlapping in one field of view) only the tip was evaluated. In each sample 10 villi were analysed. IEL were identified by their morphology i.e. small lymphocytes (7–10 μm in diameter) with a round and intensely stained nucleus and little cytoplasm or larger lymphocytes (10–20 μm in diameter) with distinct cytoplasm located in the epithelium between enterocytes (Stokes et al., 1994; Vega-López et al., 2001; Wilson et al., 1986). As IEL were counted, the length of the corresponding enterocyte basement membrane was measured and finally the number of IEL could be calculated as IEL/100 μm basement membrane. The number was registered separately for tip and base of villi.

2.5. Statistical analysis

Statistical evaluations were carried out using the software package IBM SPSS 21 (IBM Deutschland GmbH, Ehningen, Germany). All variables were tested by two-way ANOVA with the factors "Age" (time post infection) and "Group" (feeding group). Since for some parameters age and feeding groups interacted significantly, we analysed each age group separately using the *t*-test for independent samples. IEL measurements at different localisations but from the same sample were compared by the *t*-test for paired samples. In order to achieve normality and homoscedasticity of the data expressed in relative frequencies (percentages), these were square root-arcsine transformed before parametric testing. However, for the convenience of the reader we report means of the

visible in comparison with the corresponding picture (D). (E) The jejunum at 2 DPI shows atrophic villi (arrow) which had regenerated fully by 28 DPI (F). (G and H) At day 28 post infection an increased thickness of the parenchyma, attributed to the tonsillar follicles and crypts (double arrow) which are filled with cellular detritus (arrow), is noticeable.

untransformed data. The level of significance was $\alpha = 0.05$. If groups were analysed separately or paired *t*-tests within groups were performed, the level of significance was adjusted to $\alpha = 0.025$ (Bonferroni adjustment). Results from separately analysed groups reaching a significance level between $\alpha \leq 0.05$ and $\alpha > 0.025$ were considered to be tendentially significant.

3. Results

3.1. Pathological findings

*S.*Typhimurium provoked damage of the intestinal mucosa, which was seen clearly in the ileum and colon. At DPI 2, villus atrophy was found with varying degrees of severity in the jejunum and ileum, as already described in our previous publication (Kreuzer et al., 2014). Neutrophil granulocyte infiltration, erythrocyte extravasation, as well as flattening, disintegration and degeneration of the surface epithelial layer plus the accumulation of cell detritus were found in the ileum, colon and occasionally in the jejunum of animals at DPI 2 (Fig. 1A, C and E). At DPI 28, the intestinal mucosa was found to recover with largely normal morphology (Fig. 1B, D and F). Tonsils of animals at DPI 2 exhibited follicular hyperplasia. Leukocytes, especially neutrophil granulocytes, were present in high numbers. The crypt epithelium was infiltrated with leukocytes, which were also present in the crypt lumen. Tonsils from DPI 28 animals were around 50% larger than those from DPI 2 animals. The enlargement was mainly attributed to the B cell areas where high densities of inflammatory cells were noticeable. The crypt epithelium was damaged and infiltrated by leukocytes. Crypts were filled with desquamated epithelium and inflammatory cells, mainly neutrophils (Fig. 1G and H).

3.2. Morphometric parameters and IEL counts assessed by light microscopy

The time post infection (age) had significant effects ($P < 0.05$) on most morphometric parameters and IEL numbers. Older animals (28 DPI) had longer and wider villi, deeper and wider crypts, higher villus enlargement factor, higher ratio between villus and crypt enlargement factors as well as more IEL (Table 1).

Feeding *E. faecium* had overall significant effects ($P < 0.05$) on IEL numbers, the probiotic group generally having higher numbers (Table 1). Analysing age groups separately by *t*-test showed that *E. faecium* had measurable effects only on parameters determined after day 28 PI: Probiotic fed animals tended to have longer villi ($P = 0.037$) and a higher ratio of villus surface/crypt circumference enlargement factors ($P = 0.046$) (Supplemental Fig. 2A–E and Table 1). Significantly more IEL ($P < 0.025$) were present in the jejunal epithelium of animals treated with *E. faecium*. The IEL located at the nuclear level of the intestinal epithelium presented the most prominent quantitative differences (Supplemental Fig. 2F–I and Table 1).

The distribution of IEL within the epithelium was uneven ($P < 0.001$). Over all age and feeding groups, 60% of IEL were found in the basal region, 27% in the nuclear region and 12% in the apical region of the mucosal epithelium. We were able to detect significant differences ($P < 0.05$) for the factors “Age” and “Group” and a combination of both by ANOVA (Table 1). Separate analysis of the age groups by *t*-test revealed that significant differences ($P < 0.025$) between feeding groups were only found at day 28 PI. Probiotic fed animals had a smaller proportion of IEL in the basal level and a higher proportion at the nuclear level (Table 1). Separate analysis of the feeding groups by *t*-test (not shown in Table 1) revealed that significant differences ($P < 0.025$) between age groups were only found in probiotic fed animals. At day 28 PI these animals had a smaller proportion of IEL at the basal level (villus tip $P = 0.057$; villus base $P = 0.008$) and a greater proportion at the nuclear level (villus tip $P = 0.076$; villus base $P = 0.004$) compared to the corresponding locations at DPI 2. In Supplemental Fig. 3, the abundance of IEL in villus tip and villus base is shown for absolute as well as relative numbers (influence of probiotic treatment – Supplemental Fig. 3A and B; influence of time post infection – Supplemental Fig. 3C and D).

Evaluating the intravillus distribution of IEL, we compared measurements from villus tip and villus base via Paired Samples Test. Absolute numbers were found to be homogeneously distributed over all age and feeding groups, whereas relative numbers were not (Table 2). By analysing the test results for the influence of age, no differences could be detected. Also relative numbers in the probiotic feeding group showed no differences. However, in the villus tips of the control group, significantly ($P < 0.025$) less IEL at the basal level and more IEL at the nuclear and apical level were found compared to the villus base. By analysing the test results even further for the combined effects of age and feeding group, significant differences ($P < 0.025$) were only detectable in the 28 DPI control animals for the basal ($P = 0.148$ absolute, $P = 0.025$ relative) and nuclear ($P = 0.023$ absolute, $P = 0.025$ relative) IEL. See Supplemental Fig. 3 to visualise these descriptions.

4. Discussion

Salmonella Typhimurium infection is usually a self-limiting disease in pigs. Nevertheless, this pathogen represents a threat due to its potential to persist hidden in the porcine body for long periods (Boyen et al., 2008). Formerly, auxinic antibiotics were used as feed additives to prevent infection outbreaks in pig production. Increasing rates of microbial resistances against such antibiotics have led to the ban on their usage in the European Union since 2006 (Anadón, 2006). Since then, much interest has been raised in the pursuit of potential alternatives such as probiotics (Castillo et al., 2012). Probiotics are believed to have a beneficial effect on the gastrointestinal tract, for example via modulation of the immune system and regulation of the surface epithelium’s cellular growth and apoptosis (Kreuzer et al., 2012; Resta-Lenert and Barrett, 2003; Walsh et al., 2012; Yan et al., 2007).

Table 1
Morphological parameters and intraepithelial lymphocyte numbers: The data is separated and grouped for the factors "day post infection" (DPI) and "feeding group" (control and probiotic). The respective significant differences are indicated and the corresponding illustration can be found in Supplemental Figs. 2 and 3. The level of significance for ANOVA was $\alpha = 0.05$. As nearly all measurements were influenced by the time post infection, the age groups were additionally analysed separately via *t*-test and the level of significance was adjusted to 0.025 (Bonferroni adjustment). Intraepithelial lymphocyte (IEL) numbers are given in absolute numbers (IEL per 100 μm basement membrane) as well as relative numbers (IEL in the respective intraepithelial compartment as % of the total IEL in this area).

Measured parameters (and letter of corresponding boxplot)		2 DPI		28 DPI		Two-way ANOVA			*t*-test	
	Group	N	Mean	N	Mean	Age	Group	Age × Group	2 DPI	28 DPI
Villus length in μm (A)	Control Probiotic	6 6	360.52 379.12	8 9	569.46 649.98	.000*	.139	.349	.768	.037
Villus width in μm	Control Probiotic	6 6	114.93 115.45	8 9	132.81 130.84	.041*	.926	.873	.962	.852
Crypt depth in μm (B)	Control Probiotic	6 6	211.88 227.73	8 9	272.37 267.04	.001*	.680	.408	.458	.734
Crypt width in μm	Control Probiotic	6 6	37.66 37.18	8 9	41.96 44.70	.005*	.557	.406	.888	.223
Enlargement factor villi (C)	Control Probiotic	6 6	4.71 4.97	8 9	6.53 7.92	.000*	.099	.247	.688	.057
Enlargement factor crypts (D)	Control Probiotic	6 6	5.23 5.58	8 9	6.22 5.67	.067	.728	.123	.328	.196
Ratio between enlargement factors (V:C) (E)	Control Probiotic	6 6	0.91 0.91	8 9	1.08 1.41	.003*	.125	.132	.981	.046
Basal IEL villus tip per 100 μm (H)	Control Probiotic	5 5	5.34 5.71	7 8	5.62 7.08	.114	.081	.289	.636	.058
Nuclear IEL villus tip per 100 μm (H)	Control Probiotic	5 5	2.07 2.39	7 8	2.37 4.40	.005*	.004*	.029*	.363	.004*
Apical IEL villus tip per 100 μm (H)	Control Probiotic	5 5	1.01 1.16	7 8	1.43 1.71	.096	.450	.815	.717	.479
Total IEL villus tip per 100 μm (F)	Control Probiotic	5 5	8.42 9.26	7 8	9.41 13.19	.007*	.011*	.090	.408	.007*
Basal IEL villus base per 100 μm (I)	Control Probiotic	4 4	5.97 6.10	7 8	6.55 7.12	.188	.559	.714	.918	.340
Nuclear IEL villus base per 100 μm (I)	Control Probiotic	4 4	1.93 2.58	7 8	1.97 4.58	.023*	.001*	.027*	.247	<.001*
Apical IEL villus base per 100 μm (I)	Control Probiotic	4 4	0.63 1.19	7 8	0.80 1.71	.187	.008*	.499	.128	.014*
Total IEL villus base per 100μm (G)	Control Probiotic	4 4	8.53 9.87	7 8	9.32 13.40	.049*	.016*	.198	.498	.003*
Basal IEL villus tip in %	Control Probiotic	5 5	63.10 61.82	7 8	59.37 54.35	.060	.269	.527	.769	.189
Nuclear IEL villus tip in %	Control Probiotic	5 5	24.60 25.83	7 8	25.40 32.74	.041*	.029*	.101	.732	.004*
Apical IEL villus tip in %	Control Probiotic	5 5	12.29 12.34	7 8	15.23 12.91	.468	.773	.717	.965	.608
Basal IEL villus base in %	Control Probiotic	4 4	70.15 62,02	7 8	70.79 53.75	.179	<.001*	.114	.099	<.001*
Nuclear IEL villus base in %	Control Probiotic	4 4	22.34 26.28	7 8	20.92 33.84	.143	<.001*	.032*	.207	<.001*
Apical IEL villus base in %	Control Probiotic	4 4	7.52 11.71	7 8	8.28 12.41	.704	.011*	.934	.077	.058

* $p < 0.05$ for Two-way ANOVA and *$p < 0.025$ for t-test.

4.1. Pathological findings

Salmonella penetrates the intestinal epithelium preferentially through the Peyer's patches or solitary lymphoid tissue of the small intestine (Reis and Horn, 2010). As described in several earlier investigations (Coombes et al., 2005; Letellier et al., 2001; Santos et al., 2011; Walsh et al., 2012), and in our study, *Salmonella* caused damage

Table 2
Paired Samples Test for intravillous distribution of IEL comparing measurements from villus tip and villus base: Absolute numbers were homogeneously distributed over all age and feeding groups whereas relative numbers were not. Separating the test results for the influence of age (DPI = days post infection), no differences could be detected. Separating the test results for the influence of the feeding group (group), relative numbers in the probiotic feeding group were evenly distributed. In the control group, significantly fewer basal level IEL and more nuclear and apical level IEL were found in the villus tip compared with the villus base. Separating the test results even further (not shown in Table 2 for the effects of age and feeding group together, significant differences ($P < 0.025$) were only detectable in the 28 DPI control animals for the basal ($P = 0.148$ absolute, $P = 0.025$ relative) and nuclear ($P = 0.023$ absolute, $P = 0.025$ relative) IEL.

Paired Samples Test		Basal IEL villus tip vs. base		Nuclear IEL villus tip vs. base		Apical IEL villus tip vs. base		Total IEL villus tip vs. base	
		Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
Over-all ($\alpha = 0.05$)		.055	.018*	.905	.332	.067	.008*	.667	
Separated for age ($\alpha = 0.025$)	2 DPI	.066	.142	.647	.943	.234	.070	.552	
	28 DPI	.206	.065	.703	.243	.173	.056	.891	
Separated for group ($\alpha = 0.025$)	Control	.051	.004*	.045	.020*	.040	.013*	.993	
	Probiotic	.587	.939	.486	.341	.769	.302	.592	

* $p < 0.05$ for over-all results of the Paired Samples Test and *$p < 0.025$ for the separated results of the Paired Samples Test.

of the intestinal mucosa at the preferred entry sites. Pathological alterations were particularly severe at day 2 PI, especially in the ileum and colon where the most characteristic inflammatory lesions were found. In the older animals, the intestinal mucosa appeared to have largely recovered towards its normal morphological and physiological status. However, it remains unclear whether the time the intestine needed to recover was the same in both groups, as no samples were taken between 2 and 28 DPI. The changes in the tonsilla veli palatini suggest that despite recovery of the intestine, immune reactions were still in progress at day 28 PI.

4.2. Morphometric parameters were influenced by the time post infection (age) and probiotic feeding

The proximal small intestine is the primary site of digestion and absorption in the small bowel (Caspary, 1992; Sawaya et al., 2012). The size of the small intestinal surface area is commonly thought to be related to its functional capacity and has to be considered in nutritional studies (Mayhew and Middleton, 1985; Stenling and Helander, 1981). Villus and crypt related morphological parameters give an indication of the maturity and functional capacity of the enterocytes (Tang et al., 1999). An increase in the ratio between villus length and crypt depth corresponds to an increase in digestion and absorption (Gu et al., 2002; Navidshad et al., 2010). We suggest that the same principle holds true for the ratio between villus and crypt enlargement factors.

Nearly all measured morphometric parameters were influenced significantly by the time post infection. After day 28 PI, quantitative measurements revealed longer and wider villi, deeper and wider crypts, a higher villus enlargement factor and a higher ratio between villus and crypt enlargement factors compared to measurements after day 2 PI. We also compared our measurements of jejunal villus length at day 2 PI (≈370 μm 40 d/12 dpw (40 day old pigs 12 days post weaning)) with observations from our earlier trials without a *Salmonella* challenge (≈374 μm 34–40 d/8–12 dpw (Liu et al., 2014; Martin et al., 2012; Reiter, 2006)) and literature sources (≈420 μm 32–42 d/12–26 dpw (Arnal et al., 2014; Levesque et al., 2012; Nofrarías et al., 2006; Pluske et al., 1997)). The comparison shows that jejunal villus length at day 2 PI can be judged to be in a normal range or slightly shortened. Jejunal crypt depth and crypt enlargement factor measured at day 2 and 28 PI were in a normal range compared to our trials without *Salmonella* challenge (Liu et al., 2014; Martin et al., 2012; Reiter, 2006). Likewise, we compared our measurements of jejunal villus length from day 28 PI (≈570 μm control/≈650 μm probiotic 66 d/38 dpw) with observations from our earlier trials without *Salmonella* challenge (≈513 μm 54–56 d/26 dpw (Liu et al., 2014; Martin et al., 2012; Reiter, 2006)) and literature sources (≈527 μm 40–150 d/28–95 dpw (Agyekum et al., 2012; Arnal et al., 2014; Hedemann et al., 2005; Letellier et al., 2001; Levesque et al., 2012; Price et al., 2010; Suo et al., 2012)). The comparison at day 28 PI shows that jejunal villus length, especially that of the probiotic treatment group, can be judged to be lengthened to some extent. Also the villus enlargement factor measured at day 28 PI, especially that of the probiotic feeding group (7.92 66 d/38 dpw), was somewhat bigger than that (≈6.08 56 d/26 dpw) in observations from an earlier trial without *Salmonella* challenge (Reiter, 2006). The comparison with results from non-challenge trials showed that jejunal morphology was mainly normal and that the biggest changes can be attributed to an age dependant development. Probiotic treatment showed minor effects after DPI 28 in form of tendentially longer villi and a higher ratio of villus enlargement factor/crypt enlargement factor. As an increased rate of epithelial renewal is an important defensive mechanism in response to invading pathogens that damage epithelial cells (Tang et al., 1999), this could be interpreted as an advantage. Nonetheless, activation and mobilisation of immune cells to migrate actively towards or into the intestinal lumen requires an increased energy supply for these cells (Dugan et al., 1994; MacIver et al., 2013). This increased demand could have resulted in expanded proliferation of the epithelium.

4.3. IEL quantities and distribution were altered by time post infection and probiotic feeding

IEL are potentially the first immune cells to encounter *Salmonella* during infection via the oral route (Davies et al., 2004). They are responsible for maintaining the intestinal epithelial surface integrity and homeostasis (Mattapallil et al., 1998; van Wijk and Cheroutre, 2009; Yu et al., 2012).

The immune system continuously changes as the young pig matures. Quantitative and phenotypical alterations of immune cell populations, including increases in IEL numbers during development, have been described (Brown et al., 2006; Rothkötter et al., 1999; van Wijk and Cheroutre, 2009). IEL numbers have also been demonstrated to increase after oral infection with *S. enterica* serovar Typhimurium (Li et al., 2012). Thus, the increase of IEL in older animals observed in our study reflects both a maturation and an infection-driven process.

Vega-López et al. (2001) have studied the localisation and development of IEL in pigs. They have found over half of these IEL (52% duodenum, 53% ileum) localised at the epithelium's basement membrane, numerous (43% duodenum, 42% ileum) at the enterocyte nuclear level and relatively few (5%) apically in the epithelium. These proportions were homogeneously maintained along the villi's tip, middle and bottom sections (Vega-López et al., 2001). Similar proportions have been found in our trial examining the jejunum, although a slightly different numerical distribution pattern could be observed. This might be a consequence of the challenge situation, the different age of the piglets as well as the different localisation within the small intestine. Whether the differences between probiotic and control group concerning the distribution of IEL along the villi found in our study are of biological relevance is a matter of speculation. One may conclude that the villus tip with the more developed enterocytes has nearly reached an optimal functional and morphological state and that the villus base with the younger enterocytes is still in a process of regenerative change. This process seems to have happened faster and more effectively in the probiotic group.

In agreement with our results, several studies have reported an increase in number of lymphocyte populations, especially IEL, after probiotic treatments (Babinska et al., 2005; Bai et al., 2013; Dalloul et al., 2003; Deng et al., 2012; Lee et al., 2010; Levkut et al., 2012; Pirarat et al., 2011; Roselli et al., 2009; Scharek et al., 2007). However, there are also reports on a reduction of intestinal IEL after feeding probiotics (Ashraf et al., 2013; Lee et al., 2010; Mafamane et al., 2011).

Interestingly, Mafamane et al. (2011) using flow cytometry found a reduced number of jejunal CD8αβ+ IEL in piglets treated with *E. faecium* in a similar *S.*Typhimurium challenged study. This does not necessarily contradict our results: $CD8^+$/$CD8\alpha\beta^+$ IEL have been found to be located at the basement membrane within the epithelial compartment (Helgeland et al., 1997; Vega-López et al., 2001). Since absolute numbers of IEL in this compartment were not reduced in our experiments, one may conclude that the $CD8\alpha\beta^+$ IEL population was unaffected in our study. However, when relative numbers were taken into consideration, a significantly smaller percentage of IEL in the basal intraepithelial compartment was detected at 28 DPI in the probiotic feeding group. Since flow cytometry results are usually given in relative numbers, our results are consequently in agreement with those of Mafamane et al. (2011). Additionally, Mafamane et al. have suggested a reduction in the absolute numbers of $CD8\alpha\beta^+$ cells. However, since histology is considered to be the most reliable way to count individual IEL (Peaudecerf and Rocha, 2011), this mainly demonstrates how difficult it is to compare absolute and relative numbers as well as different experimental setups and methods. It also raises the question whether flow cytometry of isolated cells alone can depict processes in complex tissues like the intestine.

Considering the prominent significant difference in IEL numbers at the nuclear level between probiotic and control groups, we assume that this population might be of great functional importance in piglets. This population was found to be $CD4^-CD8^-$ in pigs and $CD3^-CD8\alpha\alpha^+$ in rats (Helgeland et al., 1997; Vega-López et al., 2001). Using a simplified classification of IEL based on phenotype and gene expression (Hayday et al., 2001), the basal $CD8\alpha\beta^+$ IEL belong to the "conventional type", i.e., antigen experienced cells originating from peripheral T cells and homing to the gut mucosa. They have immunologic memory function and mount an adaptive response. The IEL found at the nuclear level belong to the "unconventional type". They have functions in-between adaptive and innate responses, with one being most interesting in the context of our study, namely the protection of epithelial integrity (Hayday et al., 2001; Peaudecerf and Rocha, 2011). Edelblum et al. (2012) demonstrated the ability of an IEL population fitting to the "unconventional type" to contact multiple epithelial cells over a short time and thus provide a potential mechanism by which they could prevent epithelial injury and infection (Edelblum et al., 2012). For non-challenged piglets, our research group already described that *E. faecium* enhanced intestinal barrier function (Klingspor et al., 2013). And since IEL profoundly regulate induction of epithelial apoptosis or growth (Swamy et al., 2010), we suggest that the nuclear IEL in the probiotic fed group possibly had a positive influence on epithelial integrity resulting in improved morphological features at 28 DPI.

5. Conclusion

In the present study we observed that in *Salmonella* challenged piglets the supplementary feeding of *Enterococcus faecium* NCIMB 10415 resulted in a significantly increased number of intraepithelial lymphocytes. The most prominent differences in IEL numbers were found in the population of IEL located at the enterocytes nuclear level. This IEL population may be a special subpopulation involved in the maintenance of epithelial integrity. The results indicate an immunomodulatory effect of *E. faecium*. The slight increase of jejunal villus length and villus/crypt enlargement factor ratio in probiotic fed animals may be the effect of a more efficient mucosal regeneration after the infection but no beneficial effect could be observed in the early infection phase. Additionally, we conclude that in future studies it may be necessary to combine the efficient

phenotypical characterisation of cell populations via flow cytometry with the in situ characterisation and quantification via histology.

Author contributions

J. Rieger: conception and design of the study; critical literature review; acquisition of data; analysis and interpretation of data; draft of manuscript; final approval

P. Janczyk: conception and design of the study; acquisition of data; draft of manuscript; final approval

H. Huenigen: conception and design of the study; analysis and interpretation of data; critical review; final approval

K. Neumann: analysis and interpretation of data; critical review; final approval

J. Plendl: conception and design of the study; analysis and interpretation of data; draft of manuscript; final approval

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2014.12.013.

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