Composition of intestinal *Enterobacteriaceae* populations of healthy domestic pigs

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In this study, the *Enterobacteriaceae* microbiota, including their diversity as well as the distribution of haemolytic and virulence gene-harboursing *Escherichia coli* of 56-day-old healthy piglets, was characterized. Both the composition and the diversity of *Enterobacteriaceae* populations varied considerably between individual pigs and intestinal sections. *E. coli*, *Enterobacter cloacae*, *Citrobacter freundii* and *Klebsiella pneumoniae* dominated the *Enterobacteriaceae* microbiota. However, mucosa-associated *Enterobacteriaceae* were scarce or in some cases undetectable. The majority of *E. coli* clones from the jejunum were also found in the colon, with up to 10 different *E. coli* clones in one intestinal section. Other *Enterobacteriaceae* species were represented by only one clone localized to one intestinal section. While several piglets did not harbour virulence gene-positive or haemolytic *E. coli*, such strains dominated intestinal sections of other animals. This study reveals that the diversity of intestinal *Enterobacteriaceae* is clearly individual. In general, *Enterobacteriaceae* do not appear to be a consistent fraction of the microbiota of the jejunum. High numbers of adherent bacteria do not appear to be essential for successful intestinal colonization, and *E. coli* clones do not necessarily colonize distinct intestinal sections based on the particular phylogenetic affiliation. Furthermore, dominance of haemolytic or virulence gene-positive *E. coli* does not correlate with disease. Finally, probiotic *Enterococcus faecium* feed supplementation does not affect the *Enterobacteriaceae* microbiota.

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INTRODUCTION

The family *Enterobacteriaceae* consists of many genera and strains that colonize the small and large intestine, and includes members of the non-pathogenic autochthonous (commensal) microbiota as well as pathogens. Several pathogenic *Enterobacteriaceae*, especially *Escherichia coli* strains, cause diarrhoea, urinary tract infections, mastitis, arthritis and meningitis in both humans and animals (Alexander, 1994; Ewers *et al.*, 2007; Fairbrother *et al.*, 2005; Hermansson *et al.*, 1978; Kaper *et al.*, 2004; Nagy & Fekete, 2005).

A number of studies of pathogenic *E. coli* have been performed, partly because *E. coli* is economically one of the most important pathogenic bacteria in pig production (Barth *et al.*, 2007; Fairbrother *et al.*, 2005; Nagy & Fekete, 2005; Wieler *et al.*, 2001). However, few data are available on the composition of the commensal *E. coli* microbiota in

pigs. The *E. coli* microbiota in pigs appears to be individual, dynamic and age-dependent (Katouli *et al.*, 1995, 1999), and determinations of the *Enterobacteriaceae* microbiota are generally only snapshots of a highly dynamic intestinal steady state. It has also been reported that the *E. coli* microbiota in pigs is highly structured, and that different compartments of the gut are preferentially colonized by distinct phylogenetic *E. coli* clones (Dixit *et al.*, 2004).

Compared to *E. coli*, there are very little data about other non-pathogenic or facultatively pathogenic *Enterobacteriaceae* species. The few studies deal almost exclusively with those associated with sporadic infections, virulence determinants, antibiotic resistance or metabolism. To our knowledge, data about their distribution in the intestine, ratios to other *Enterobacteriaceae* species and diversity under physiological conditions have not been reported.

The aim of this study was to evaluate the *Enterobacteriaceae* populations in healthy domestic pigs, with consideration of their intestinal distribution, relative ratios and diversity. *E. coli* isolates were further evaluated for the presence of virulence genes, haemolysis and phylogenetic affiliation. In

Figures showing electron micrographs of mucosa-attached bacteria, the incidence of haemolysis among *E. coli* colonies, and the ECOR grouping of *E. coli* from pigs, are available as supplementary data with the online version of this paper.

addition, the effects of probiotic *Enterococcus faecium* feed supplementation on the *Enterobacteriaceae* microbiota were examined.

METHODS

Animal housing. Piglets (hybrids of Deutsche Landrasse and Duroc) were stalled in groups and fed according to the National Research Council (NRC) recommendations (National Research Council, 1998). Piglets had *ad libitum* access to feed, with (probiotic piglets) or without (control piglets) probiotic supplementation. The principal components of the liquid diet were wheat and soybean. The administration of antibiotics was prohibited for both sows and piglets for at least 3 months prior to the trial. In addition, the health status of piglets was monitored for signs of clinical illness. The study was approved by the local animal welfare committee of the Federal Ministry of Agriculture, Food and Consumer Protection, Germany (no. G0037/02).

The probiotic strain *Ent. faecium* SF68 (NCIMB 10415; Benyacoub *et al.*, 2003; Wunderlich *et al.*, 1989) was obtained from a commercial batch of the feed additive Cylactin (Cerbios-Pharma). The mean concentration of the *Ent. faecium* in the feed was $2.0 \pm 0.4 \times 10^5$ viable cells per gram food, as determined by colony hybridization (Macha *et al.*, 2004). Supplementation resulted in approximately 10^3 c.f.u. (g wet weight)⁻¹ of *Ent. faecium* SF68 in faeces of probiotic piglets. The absence of the probiotic strain in control piglets was confirmed in random faecal samples.

Cultivation of *Enterobacteriaceae*. *Enterobacteriaceae* were isolated from either the distal jejunum (50 cm proximal to the Plica ileocaecalis) or the proximal colon (apex of the ascending colon) of piglets from different litters. The distal jejunum was chosen as representative of the small intestine, since the more proximal jejunum generally contained no intestinal contents, and bacteria of different sections of the small intestine are thought to be similar (Dixit *et al.*, 2004). The proximal colon was chosen as part of the large intestine, as other authors have found a uniformity of bacterial adhesion at a number of sites of the large intestine, indicating that small biopsy samples are likely to be representative of wide areas of the gut epithelium (Hartley *et al.*, 1979; Swidsinski *et al.*, 2002). Piglets aged 56 days post-partum were euthanized by injection with sodium Pentobarbital, and clamped intestinal sections were removed for bacterial isolation from intestinal contents. *Enterobacteriaceae* were isolated from intestinal contents and mucosa and plated by serial dilutions on Gassner agar plates. Bacteria from the mucosa included bacteria associated to mucus and epithelial cells.

Mucus and epithelial cells were derived from an $\sim 2 \times 5$ cm section of intestinal tissue, as described elsewhere (Solano-Aguilar *et al.*, 2000). Briefly, sections of intestinal tissues were washed twice in PBS to remove visible faecal material. Tissues were transferred to an Erlenmeyer flask containing 25 ml PBS and incubated for 5 min (220 r.p.m., 37 °C). It was expected that these short washing steps would not affect mucosa-attached bacteria, as other authors have reported high numbers of mucosa-attached or epithelial cell-attached *E. coli* even after four to six changes of physiological saline washes (Hartley *et al.*, 1979; Swidsinski *et al.*, 2002). The resulting suspension was removed and plated to Gassner agar plates. For mucus extraction, tissues were incubated in 25 ml Hanks' balanced salt solution/DTT [HBSS-DTT; HBSS without Ca²⁺ and Mg²⁺ (Cambrex), 2 mM DTT, 0.01 mM HEPES] for 10 min (220 r.p.m., 37 °C). The suspension was passed through sterile 210 µm nylon mesh (Sefar) and plated to Gassner agar plates. For epithelial cell isolation, washed tissues were incubated with 25 ml HBSS-EDTA (HBSS without Ca²⁺ and Mg²⁺, 1 mM EDTA, 1 mM HEPES) for 45 min (220 r.p.m., 37 °C). The

suspension was passed through sterile 210 µm nylon mesh and plated to Gassner agar plates. Three additional controls were performed to determine the reliability of the procedure with respect to isolation of *Enterobacteriaceae* associated with epithelial cells: (1) solutions were sporadically tested for the presence of epithelial cells by light microscopy; (2) tissue sections were directly laid on Gassner agar plates after the last wash step to transfer attached bacteria; and (3) approximately 1 g mucosal tissue was homogenized in PBS after the second wash with a Dounce homogenizer, and homogenates were plated to Gassner agar plates.

Enterobacteriaceae-like colonies on Gassner agar plates were randomly picked and replica-plated to CHROMagar Orientation plates (Merlino *et al.*, 1996). The differences in the numbers of tested isolates between pigs and intestinal sections were due to the substantial variation in the absolute numbers of mucosa-attached bacteria from each pig. Blue/green/yellow *Enterobacteriaceae*-like colonies on Gassner agar plates that appeared violet on CHROMagar Orientation plates were initially defined as *E. coli*. The haemolytic activity of these colonies ($n=3471$) was tested on sheep blood agar plates. After macrorestriction analysis of 643 *E. coli* isolates, each clone determined was finally verified as *E. coli* using LIM agar, Kligler agar, urea agar, citrate agar and malonate liquid culture. Blue/green/yellow *Enterobacteriaceae*-like colonies on Gassner agar plates which were blue or white on CHROMagar Orientation plates were initially defined as bacteria other than *E. coli*. After macrorestriction analysis, non-*E. coli* clones were finally tested using API 20E (bioMérieux) tests for further species identification. Four *E. coli* isolates initially isolated from the jejunum of piglet A were not recovered after storage and were therefore not included in the data shown.

Scanning electron microscopy and periodic acid–Schiff (PAS) staining. Intestinal segments were cut open lengthwise at the antimesenteric side, washed twice in PBS to remove visible faecal material, and fixed in 0.1 M cacodylate buffer, 2% paraformaldehyde, 2.5% glutaraldehyde (pH 7.2). Samples were post-fixed 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). For the PAS reaction after washing, segments were fixed in 4% paraformaldehyde and embedded in paraffin. Mucopolysaccharides were demonstrated using PAS reagents (Merck).

Virulence gene and ECOR (*E. coli* Reference Collection) group determinations using PCR. The presence of virulence genes associated with porcine pathogenic *E. coli*, *faeG*, *fanA*, *fasA*, *fedA*, *fimF41a* (F4, F5, F6, F18, F41 fimbriae), *stx2e* (shigatoxin 2e), *est-Ia*, *est-II* (heat-stable toxins 1 and 2) and *eltB-Ip* (heat-labile toxin), was determined by PCR for all *E. coli* clones isolated, as previously described (Moon *et al.*, 1999; Schierack *et al.*, 2006). The *E. coli* phylogenetic groups of all detected clones were determined by PCR, as described by Clermont *et al.* (2000).

Discrimination of *Enterobacteriaceae* strains by macrorestriction analysis and calculation of diversity. *Enterobacteriaceae* colonies were differentiated by macrorestriction analysis (PFGE), as previously described (Moser *et al.*, 2002). Bacterial DNA was digested with *XbaI* (Promega). If the macrorestriction patterns of isolates did not differ by more than one band, these isolates were considered to belong to a single clone. The diversity of the coliform bacteria was determined using Simpson's index of diversity (D_i) as described elsewhere (Hunter & Gaston, 1988; Katouli *et al.*, 1999). This calculation allows comparisons of the diversity of populations to be made, even where different numbers of isolates are included.

RESULTS

Comparison of *Enterobacteriaceae* populations between probiotic and control piglets

The 15 piglets included in this study showed no clinical symptoms of disease. There were also no obvious differences between animals of the probiotic and the control groups with respect to the numbers, composition, mucosal attachment, intestinal distribution or diversity of intestinal *Enterobacteriaceae*, or the occurrence of virulence gene-positive and haemolytic *E. coli* (Table 1, Figs 1 and 2, Supplementary Figs S2 and S3). Further analyses of the *Enterobacteriaceae* from both groups were therefore based on pooled samples, which were treated as a representative population.

Sporadic absence of *Enterobacteriaceae* in the distal jejunum; comparative characterization of colonies from the mucosa with those from intestinal contents

In four cases, we were unable to isolate any *Enterobacteriaceae* from the distal jejunum of the animals. Although high numbers of *Enterobacteriaceae* from intestinal contents of other animals were detected, generally only low numbers of bacteria associated with the mucus and the epithelial-cell fractions from these same animals were isolated (Table 1). We therefore tested the applicability of the established protocol. Treatment of the mucosa with EDTA-containing solutions resulted in the release of single epithelial cells, and this was confirmed using light microscopy. The numbers of bacterial colonies per mucosal

sample (mucus plus epithelial cells) were similar using the physiological buffer and EDTA solutions compared to tissue homogenization with a homogenizer. If no bacteria were detectable with the physiological buffer and EDTA solutions and tissue homogenization with a homogenizer, no bacteria were detected after direct contact-transfer of tissues to Gassner agar plates. Using electron microscopy, it was shown that even the first washing step removed a considerable proportion of the secreted mucins, although remnants remained visible (see Supplementary Fig. S1A, B). Cell-bound mucins were not removed, as confirmed in parallel studies using PAS staining.

High numbers of bacteria associated with the mucosa were only found sporadically, including bacteria associated with the remaining secreted mucins, cell-bound mucins and glycocalyx. As shown in Table 1, the numbers of associated *Enterobacteriaceae* varied considerably, with large differences from animal to animal that appeared to be species-dependent. Even when strong attachment was observed, $<10^{-4}$ attached bacteria per epithelial cell was determined. From 11 animals, <100 colonies were isolated from the mucosa of the jejunum, corresponding to $<10^{-9}$ bacteria per epithelial cell. The low numbers of mucosa-associated *Enterobacteriaceae* were verified by scanning electron microscopy, which also showed only sporadically associated bacteria (see Supplementary Fig. S1C, D). Comparing the sparse colonies from the mucus with those from the epithelial-cell fractions, the diversity was similar, and often the same bacterial clones were detected in both fractions.

An obvious difference in the occurrence of clones between the intestinal contents and the mucosa was found in one jejunal sample. In all other samples, most clones were

Table 1. Association of *Enterobacteriaceae* with the mucosa

Association was calculated by enumeration of the colonies isolated from the mucosa (pool of cells associated with mucus and epithelial cells). Animals A–H were control piglets, and animals I–O were probiotic piglets.

Sample	Piglet (control)							
	A	B	C	D	E	F	G	H
Mucosa, jejunum	$1.7 \times 10^{3*}\dagger$	1.1×10^3	$1 \times 10^{4\ddagger}$	0	0	11	0	0
Mucosa, colon	6.7×10^5	$5 \times 10^2§$	$3 \times 10^3 $	0	2.7×10^4	9.2×10^3	17	6.4×10^2
	Piglet (probiotic)							
	I	J	K	L	M	N	O	
Mucosa, jejunum	0	3.8×10^3	4	26	0	36	5	
Mucosa, colon	3.1×10^6	1.2×10^3	1×10^3	$2.4 \times 10^3¶$	0	5.6×10^2	1.8×10^3	

*Total numbers of mucosa-associated bacteria.

†92 % *C. freundii*, 3 % *E. coli*, 5 % others, not defined.

‡95 % *Ent. cloacae*, 5 % *E. coli*.

§80 % *E. coli*, 20 % others, not defined.

||92 % *E. coli*, 8 % *Ent. cloacae*.

¶84 % *K. pneumoniae*, 16 % *E. coli*.

Fig. 1. *Enterobacteriaceae* species. Bacterial species were determined from data derived from bacterial behaviour on plates and from macrorestriction analysis. All isolates tested in this study are shown, including bacteria from the mucosa as well as from intestinal contents. Animals A–H were control piglets, while animals I–O were probiotic piglets. From animals A and I, only data from the mucosa were available. *Number of tested colonies.

found to be associated with both the mucosa and intestinal contents, and the dominant clones of the intestinal contents were the dominant clones of the mucosa. Likewise, the diversity of *E. coli* from both fractions was similar. For these reasons, and due to the low numbers of mucosa-associated bacteria, data for one intestinal section (mucosa and intestinal contents) were pooled for further comparison between the jejunal and the colonic sections.

Identification and numbers of isolated *Enterobacteriaceae*

In four animals, *Enterobacteriaceae*-like isolates were not detected in the jejunum. Whenever *Enterobacteriaceae* were cultivated from the jejunum of the other animals, *E. coli* was always present. In four animals, *Enterobacter cloacae* and/or *Klebsiella pneumoniae* and/or *Leclercia adecarboxylata* or *Citrobacter freundii* were also detected. Isolates of these species in some cases dominated the *Enterobacteriaceae* microbiota. In the colon, *Enterobacteriaceae*-like bacteria, including *E. coli*, were always present. In three animals, *Ent. cloacae* or *K. pneumoniae* were also detected, although these species did not dominate (Fig. 1).

Diversity of *Enterobacteriaceae* other than *E. coli* and diversity of *E. coli* strains

In contrast to *E. coli*, only one clone of each non-*E. coli* species was detected in a single animal (with the exception of one animal). In that animal, one specific *Ent. cloacae* clone was detected in the jejunum and a second *Ent. cloacae* clone in the colon. When *E. coli* was present in one intestinal section, in general more than one clone could be recovered. The diversity of *E. coli* was on average higher in the colon ($D_i = 0.61 \pm 0.23$) than in the jejunum ($D_i = 0.39 \pm 0.26$). The *E. coli* diversity was only sporadically higher in the jejunum than in the colon. *E. coli* clones from the jejunum were also found in the colon of the same animal. The occurrence of *Enterobacteriaceae* other than *E. coli* did not affect the diversity of *E. coli* (Fig. 2).

Virulence genes in *E. coli* clones

Virulence gene-positive *E. coli* were isolated from eight animals. These *E. coli* were always found in the colon, and in three of the eight animals also in the jejunum. Up to four different clones originating from one animal were virulence gene-positive, with virulence gene profiles *stx2e*,

Fig. 2. Diversity of *E. coli* colonies. The diversity of *E. coli* was determined using macrorestriction analysis. Coloured sections indicate only *E. coli* clones isolated from intestinal sections within a given animal; the colours do not necessarily share identity with clones represented by the same colour in other animals. The distribution of virulence gene-positive *E. coli* is also shown within the same graphics, indicated by the colours shown. In animals H and O, two different clones were *est-II*-positive. Animals A–H were controls; animals I–O were probiotic piglets. From animals A and I, only data from the mucosa were available. *Number of tested colonies.

est-Ia, *est-II* or *est-Ia + est-II*. In some cases, virulence gene-positive *E. coli* were also found to dominate the *E. coli* population (Fig. 2).

Haemolytic *E. coli*

Haemolytic *E. coli* were detected in seven animals. Such strains were occasionally also found to dominate the *E. coli* microbiota (see Supplementary Fig. S2). However, the occurrence of haemolytic *E. coli* did not affect the diversity of the *E. coli* population. None of the haemolytic *E. coli* possessed any of the tested virulence genes.

ECOR groups of *E. coli* clones

Isolates of each of the four ECOR groups were found in the jejunum as well as in the colon. ECOR group A isolates were mostly detected, regardless of which intestinal section was investigated, followed by ECOR groups B2, D and B1. Isolates from ECOR groups A, B2 and D were able to dominate the *E. coli* microbiota (see Supplementary Fig. S3).

DISCUSSION

This study indicates that the intestinal *Enterobacteriaceae* population of pigs is highly individual. If *Enterobacteriaceae* were isolated, *E. coli* was always isolated and dominated most samples. The most common member of the *Enterobacteriaceae* of the porcine intestine was *E. coli*, consistent with a previous study of 642 Australian mammals in which *E. coli* was the most common of the 24 enteric species recovered (Gordon & FitzGibbon, 1999). However, in several samples, *E. coli* numbers were lower than numbers of *Ent. cloacae*, *K. pneumoniae* and *C. freundii*. The dominant species or single dominating *E. coli* clone of an intestinal section was not necessarily found in the other intestinal section. Many different clones of *E. coli* were found to colonize the intestinal tract of pigs, whereas other *Enterobacteriaceae* species were always found to be represented by a single clone in an intestinal section. Two possible explanations could be advanced for the latter observation: either only one clone of a species other than *E. coli* colonizes an intestinal section, or only one clone always dominates, such that other clones are not detectable.

However, both possibilities are conceivable, since *Enterobacteriaceae* species other than *E. coli* can also be highly diverse in animal populations. In a previous study, the genome diversity of intestinal *Enterobacteriaceae* from a variety of Australian mammals was estimated, and a high diversity among species was reported, with *C. freundii* exhibiting the highest diversity (Gordon & Lee, 1999).

E. coli, *Ent. cloacae*, *K. pneumoniae* and *C. freundii* can all be isolated as dominant *Enterobacteriaceae* in pigs. Information about the distribution in hosts of *Enterobacteriaceae* other than *E. coli* generally relates to pathogenicity. *Ent. cloacae*, *K. pneumoniae*, *C. freundii* and *L. adcarboxylata* are thought to be opportunistic pathogens. Most data about these species relate to human infection (de Baere *et al.*, 2001; Fattal & Deville, 2000; Kumar & Sharma, 1978; Omwandho *et al.*, 2006; Scheld & Tyson, 1979; Temesgen *et al.*, 1997; Tschape *et al.*, 1995). Data from pigs are not available, are very scarce or have not been related to infection or distribution in the intestine (Bertschinger *et al.*, 1977; Ross *et al.*, 1975). In this study it was shown that the porcine intestine is a source of all these different *Enterobacteriaceae* species. However, while they successfully colonize the intestine, they do not appear to affect the health of their hosts, despite high numbers.

The occasionally poor yield of mucosa-associated *Enterobacteriaceae* contrasts with previous reports, in which 10^3 – 10^9 *E. coli* per gram mucosal tissue was found even after four to six physiological saline washes (Hartley *et al.*, 1979; Swidsinski *et al.*, 2002). In only four mucosal samples were more than 10^3 *Enterobacteriaceae* per gram isolated, despite the high recovery of *Enterobacteriaceae* in intestinal contents. Although the secreted mucin layer could have been removed by the two short washes, bacteria attached to the remaining secreted mucin layer or to the surface of the epithelial cells were never detected in high numbers. We conclude that *Enterobacteriaceae* do not necessarily have to adhere to the mucosa in high numbers to successfully colonize the intestine.

Haemolysis by *E. coli* is thought to correlate with the presence of virulence genes and therefore with pathogenicity (Frydendahl, 2002). In a previous study, the majority of haemolytic *E. coli* clones isolated in a swine population were found to harbour no virulence genes and were therefore considered to be non-pathogenic (Schierack *et al.*, 2006). In this study, seven animals harboured haemolytic *E. coli* in the jejunum and/or the colon. Confirming our previous results, these haemolytic clones did not harbour virulence genes. Furthermore, even when present in high numbers in a given intestinal section (up to 78 % of all *E. coli* isolates), these *E. coli* did not affect the health status of the piglets, which showed no signs of disease. Haemolysis alone would therefore appear not to be an indicator of virulence, and does not correlate with the presence of other virulence-related markers.

It is generally accepted that bacterial intestinal pathogens have to compete successfully with the autochthonous

microbiota of the host, adhere to the intestinal wall and proliferate to cause disease. Most porcine pathogenic *E. coli* adhere to specific membrane receptors on villous enterocytes of the small intestine, and autochthonous bacteria have been shown to block adhesion of pathogenic bacteria to these receptors (Cox & Houvenaghel, 1993; Fujiwara *et al.*, 1997; Schroeder *et al.*, 2006). Likewise, other bacteria introduced into the intestinal microbiota can also affect the course of infection by intestinal pathogens. For example, it has been shown both *in vivo* and *in vitro* that probiotic bacteria can prevent infection and disease caused by *Salmonella* and pathogenic *E. coli* (Kleta *et al.*, 2006; Schroeder *et al.*, 2006). The presence of probiotics or the integrity of a 'protective' intestinal mucosa-related microbiota have therefore been considered to be possibilities for the prevention of infection (Kuhbacher *et al.*, 2006). However, in this study, no or only very low numbers of *Enterobacteriaceae* in jejunal sections were detected. Even homogenized intestinal mucosa plated onto blood agar or CHROMagar plates was essentially bacteria-free, suggesting that this phenomenon might also apply to other bacterial species (e.g. lactobacilli or enterococci, which would be detectable on the CHROMagar plates). These observations suggest that the efficacy of the protective effects of specific established species in healthy individuals should be considered critically, as there are clear indications that no bacteria of otherwise abundant species are associated with intestinal sections.

Probiotic *Ent. faecium* supplementation showed no significant effect on the numbers and diversity of *Enterobacteriaceae* species, or on the total counts, diversity, haemolysis and distribution of virulence gene-positive *E. coli*. However, additional probiotic effects have recently been attributed to this strain (Lodemann *et al.*, 2006; Pollmann *et al.*, 2005; Taras *et al.*, 2006), and *Ent. faecium* has been reported to reduce the incidence of potentially pathogenic *E. coli*, as defined by serotypes associated with disease (Scharek *et al.*, 2005). Based on the results of this study, however, these probiotic effects are unlikely to be the result of general effects on the commensal *Enterobacteriaceae* microbiota. *Ent. faecium* might affect other intestinal bacterial species, as has been shown for other probiotics, e.g. lactobacilli, which increase the richness and diversity of intestinal bacteria (Kuhbacher *et al.*, 2006; Lan *et al.*, 2004).

E. coli populations in swine have been reported to be highly structured in their intestinal distribution (Dixit *et al.*, 2004). In this study, we found no association between the occurrence of *E. coli* clones in intestinal sections and a specific phylogenetic ECOR group. However, a few clones dominant in the jejunum were not detected in the colon when the diversity increased. Nonetheless, these clones might still successfully colonize the jejunum as well as the colon. We suggest that *E. coli* populations might not be as structured as previously proposed. Such associations between phylogenetically distinct animal/bacterial species and a specific biotope are based on the existence of spatially

determined habitats over long periods. This might not be an appropriate model for *E. coli*, since developments in animal production and breeding, changing feeding regimes and controlled housing conditions might have altered the intestinal milieu of swine and promoted colonization by more adaptive clones rather than distinct phylogenetic groups. In addition, *E. coli* can exchange genetic information, which also supports the establishment of *E. coli* in broadly varying habitats. We therefore consider it unlikely that specific clones are adapted to specific intestinal sections.

In conclusion, the *Enterobacteriaceae* microbiota of pigs is particular to the individual, and several different species can dominate. Attachment of bacteria to the mucosa in high numbers does not appear to be essential for successful colonization. While *E. coli* is the dominant species, it shows a high diversity. Finally, haemolytic *E. coli* and *E. coli* with many different virulence factor gene profiles can also establish in high numbers without affecting the health of their hosts, suggesting a low association between haemolysis or the presence of virulence genes and virulence or pathogenicity.

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