Effects of Commensal Bacteria on Intestinal Morphology and Expression of Proinflammatory Cytokines in the Gnotobiotic Pig

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A germ-free neonatal pig model was established to determine the effects of bacterial colonization by different species on small intestinal morphology and proinflammatory cytokine gene expression. Two experimental groups of 16 pigs were aseptically delivered by cesarian section and allocated into 4 gnotobiotic isolators. Pigs were either maintained germ-free (GF), or were orally inoculated with either a single strain of nonpathogenic *Escherichia coli* (EC) or *Lactobacillus fermentum* (LF) or conventionalized with adult porcine feces (CV). After 13 days tissue samples were collected at 5 regions corresponding to 5%, 25%, 50%, 75%, and 95% of the small intestine (SI) length. In Experiment 2, the GF isolator became contaminated with *Staphylococcus epidermidis* (SE). In general, intestinal responses to bacterial colonization were similar among GF, LF, and SE pigs, and intestinal responses in EC pigs were more similar to CV pigs. Responses to bacterial colonization were most pronounced in the distal SI regions (50%–95%), suggesting that nonmicrobial factors may be more important in the proximal SI. Relative to CV pigs, the distal intestines of GF, LF, and SE pigs were characterized by long villi, shallow crypts, increased relative intestinal mass, and decreased lamina propria cellularity, whereas SI morphology was intermediate in EC pigs. Relative expression of proinflammatory cytokines interleukin-1β (IL-1β) and IL-6 generally increased distally in the SI and was highest in EC and CV pigs. We observed regional variation in SI morphology and proinflammatory cytokine expression, which differed with bacterial species. This study demonstrates that bacterial species differentially affect intestinal morphology and expression of proinflammatory cytokines and suggests that neonatal bacterial colonization patterns may have long-term effects on intestinal health and development. Exp Biol Med 231:1333–1345, 2006

Key words: pig; gnotobiotic; IL-1β; IL-6; intestinal morphology

Introduction

The gastrointestinal tract of an adult pig contains approximately 10^{14} prokaryotic and eukaryotic microorganisms, a number that is 10-fold higher than the number of mammalian cells in the body of the host (1). In addition, bacteria colonizing the intestinal tract represent an extremely diverse community (2, 3). As evidenced by early comparisons of germ-free and conventional animals, this diverse population of bacteria has a marked effect on host intestinal physiology, including morphology, mucus secretion, nutrient digestion, and metabolism and immune function (for review, see Refs. 4, 5).

Hooper et al. (6) used the germ-free mouse model to examine gene expression responses following monoassociation with *Bacteroides thetaiotaomicron*, a common component of normal mouse and human intestinal microbiota. Using DNA microarrays, laser-capture microdissection, and quantitative reverse transcriptase polymerase chain reaction (PCR), the group determined *B. thetaiotaomicron* modulated expression of 71 intestinal genes involved in nutrient absorption, mucosal barrier fortification, xenobiotic metabolism, angiogenesis, and postnatal intestinal maturation. Moreover, mice monoassociated with different prominent preweaning mouse and human ileal microbiota demonstrated bacterial species–specific host gene expression responses. Expression profiling in the gnotobiotic mouse model and the results of *in vitro* studies using bacterial coculture with intestinal epithelial cell lines also indicate that host responses are specific for different commensal bacteria (7–9). As a result, the composition of the commensal bacteria colonizing the neonatal intestine

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may have significant consequences relative to intestinal development, growth, and the digestion and absorption of nutrients.

While host responses to the intestinal microbiota have been primarily examined in rodents and, to a lesser extent, the chicken, few well-controlled experiments have been conducted in the pig, a model commonly used to investigate human neonatal intestinal development. *In vivo* models are critical to assess host developmental responses, particularly in an environment in which cellular responses are characterized by the interactions of cells of numerous lineages (enterocytes, intraepithelial lymphocytes, lamina propria lymphocytes, and reticuloendothelial cells). The objective of the current study was to determine the regional responses to intestinal colonization along the length of the small intestine (SI) and to differentiate host responses among bacterial species. To accomplish this we established a germ-free neonatal pig model and monoassociated pigs with a representative of gram-negative (*Escherichia coli*) or gram-positive (*Lactobacillus fermentum*) commensal bacteria commonly isolated from the neonatal pig intestine. Our observations confirm a marked effect of the commensal bacteria on intestinal physiology and demonstrate considerable variation in host responses dependent on small intestinal region and the species of colonizing organism.

Materials and Methods

Isolator Preparation. Four gnotobiotic isolators (Class Biologically Clean Ltd., Madison, WI), each capable of rearing up to 4 pigs from birth until approximately 10 kg body wt, were used in the experiment. Prior to assembling the isolators, internal removable components were autoclaved for 30 mins at 121°C. All experimental materials, including canned infant formula, porcine serum, can openers, graduated cylinders, and nursing bottles were placed within the stainless steel isolator, and the vinyl canopy was attached. A solution of 1:10:1 (base–water–activator) Clidox (Pharmacal Research Laboratories, Naugatuck, CT) was sprayed throughout the interior of the isolator. The isolators were further sterilized with a 5% solution of peracetic acid (35%; FMC Corp., Philadelphia, PA), sealed for 24 hrs, and vented for a minimum of 36 hrs prior to placing pigs inside. Isolators were maintained under positive pressure, and ventilation occurred through High Efficiency Particulate Air (HEPA; Class Biologically Clean Ltd.) filters to maintain sterility.

Germ-Free Pig Derivation. Experimental protocols were approved by the Animal Care Committee of the University of Saskatchewan and were performed in accordance with recommendations of the Canadian Council on Animal Care (1993). Pigs (Large White × White Duroc) were delivered by cesarian section and aseptically passed through a betadine-filled (10% povidone–iodine; Purdue Pharma, Stamford, CT) dip tank into a sterile HEPA-filtered transfer unit. Pigs were revived within the transfer unit, towel dried, and immediately fed using a baby bottle. The transfer unit was transported to the gnotobiotic research facility, and pigs were aseptically transferred into 1 of 4 gnotobiotic isolators.

Pig Maintenance. In the first 24 hrs postpartum, all animals were bottle fed to satiety at 3-hr intervals with sterile-filtered porcine serum (Gibco, Burlington, Canada), a source of porcine immunoglobulins. The porcine serum was mixed with Similac (Abbott Laboratories, Abbott Park, IL) in a 1:1 ratio prior to feeding. On Day 1, pigs were trough-fed a mixture of 2:1 Similac–water (4.7 g/100 ml protein; 12.2 g/100 ml lipid; 24.3 g/100 ml carbohydrate) *ad libitum*. For the remainder of the trial pigs were fed in 3 equal feedings at 8-hr intervals such that the troughs contained milk at all times. Isolator temperature was maintained at 34°C at experimental Day 0 and reduced in a stepwise fashion to 31°C by 13 days of age.

Experimental Design. Two experiments were conducted. In each experiment 4 pigs were aseptically transferred into each of 4 isolators. Pig assignment to the isolators was balanced for litter of origin and sex. One isolator was maintained as germ-free (GF). Two isolators were designated as monoassociated: pigs in one isolator were orally inoculated with gram-positive *L. fermentum* (LF); pigs in the other, with a nonpathogenic gram-negative *E. coli* (EC). Pigs in the fourth isolator were used as a control and were orally inoculated with fresh fecal material from adult pigs (CV). *Lactobacillus fermentum* and *E. coli* inoculants were isolated from the cecum of a healthy adult pig. Both organisms were cultured for 18 hrs at 37°C in a tryptic soy broth (BBL, Sparks, MD), and a subsample from each culture was taken for enumeration. Fresh feces were obtained from conventionally reared pigs at Prairie Swine Centre Inc. (Saskatoon, Canada) and mixed 1:1 with sterile phosphate-buffered saline (0.01 *M* phosphate, 0.15 *M* NaCl; pH 7.4). Sealed tubes containing the appropriate 18-hr bacterial culture or feces were aseptically passed into the appropriate isolator. At 24 hrs and 30 hrs postpartum, the pigs were orally inoculated with 2 ml of their respective bacterial culture by adding the inoculants to the milk prior to bottle feeding. Pigs in the conventional isolator received 6 ml of inoculate (2 ml of each of the monoassociated inoculants and 2 ml of the fecal slurry at each feeding). Viable cell counts in the *L. fermentum* and *E. coli* inoculants were 10^8 colony-forming units (CFU)/ml and 10^9 CFU/ml, respectively.

Enumeration and Confirmation of Microbial Status. Sterile swabs were wiped peri-anally daily on experimental Days 0–4 and every second day thereafter for pigs in the GF group. The swabs were submerged in tubes of brain–heart infusion broth (Difco Laboratories, Sparks, MD) with 0.5% cysteine hydrochloride and were monitored for the development of turbidity. At the completion of the study cecal contents (1–2 g) were removed aseptically from each pig and immediately placed on ice for later (<3 hrs after collection) bacterial identification and enumeration.

Total aerobic and anaerobic bacteria were enumerated on blood agar base (BBL) with 5% defibrinated sheep blood and were incubated aerobically or anaerobically (GasPak anaerobic system, Becton Dickinson microbiology system; Franklin Lakes, NJ), respectively, for 24 hrs at 37°C. *Escherichia coli* and *L. fermentum* were enumerated aerobically on MacConkey agar (BBL) and microaerophilically (GasPak microaerophilic system) on Lactobacilli MRS agar (Difco Laboratories), respectively. Colonies demonstrating morphology and/or growth characteristics that were not consistent with the inoculating organisms were isolated and submitted to a clinical veterinary laboratory (Prairie Diagnostic Services, Saskatoon, Canada) for biochemical typing. In the case of Experiment 2, where a Staphylococcus contamination of the GF treatment group was confirmed, frozen digesta (–80°C) from LF and EC groups was cultured on mannitol salt agar (Difco Laboratories) to evaluate possible cross-contamination.

Tissue Collection. Pigs were euthanized by CO_2 asphyxiation, weighed, and exsanguinated on Day 13. An incision was made along the ventral midline of the abdomen to remove the intestinal tract, and the SI was dissected from the mesentery. The length of the SI was measured, and regions corresponding to 5%, 25%, 50%, 75%, and 95% in length beginning at the pyloric sphincter were identified. Two 10-cm segments at each region were excised, rinsed with cold physiologic saline (Bimeda-MTC, Cambridge, Canada), blotted dry on paper, weighed, snap-frozen in liquid nitrogen, and stored at –80°C for mRNA analysis. At each region a 1-cm segment of SI was placed in 10% formalin for 24 hrs. Thereafter, the tissue was placed in 70% ethanol until embedded in paraffin and stained with hematoxylin and eosin.

Intestinal Morphometric and Histological Analyses. Images of intestinal cross-sections were captured with a DVC digital camera (Digital Video Camera Co., Austin, TX) mounted on a BH-2 Olympus light microscope (Olympus America Inc., Melville, NY) and analyzed using Northern Eclipse Software (Empix Imaging Inc., Mississauga, Canada). Ten well-oriented villus length and crypt depth measurements per intestinal cross-section were measured, and the means of these measurements were calculated to yield a single value per pig. Intraepithelial lymphocytes (IELs) and goblet cells were morphometrically identified (confirmed by a veterinary pathologist) and enumerated per 200 enterocytes along the villus surface for each cross-section. The investigator was blinded to treatment assignment during all morphologic and histologic evaluations.

Quantitative Real-Time PCR (qPCR) Analysis. Whole-intestinal tissue was subjected to mechanical disruption under liquid nitrogen using a mortar and pestle. Total RNA was extracted from 20 to 30 mg of tissue using the RNeasy Mini Kit (Qiagen, Mississauga, Canada). Total RNA quantity and purity were determined by optical density (OD) at 260 and 280 nm wavelengths using a spectrophotometer (Ultrospec 2000; Pharmacia Biotech, Baie d'Urfe, Canada). One microgram of total RNA was used for a reverse transcription reaction (total volume: 21 µl) with oligo$(dT)_{12-18}$ primers and SuperScript II reverse transcriptase (SuperScript first-strand synthesis system for RT-PCR; Invitrogen, Carlsbad, CA). Negative controls (no reverse transcriptase added) were made by replacing the reverse transcriptase with diethyl pyrocarbonate–treated water. The resulting single-stranded cDNA was then used in qPCR (iCycler iQ Real-Time PCR detection system; Bio-Rad, Hercules, CA) for evaluation of relative expression.

Primers (Gibco, Carlsbad, CA) and TaqMan probe (5′ 6-Fam/3′ black hole quencher 1; Integrated DNA Technologies Inc., Coralville, IA) sets for porcine glyceraldehyde-phosphate dehydrogenase (GAPDH; accession number AF141959; product size: 148 bp), interleukin-1β (IL-1β; accession number M86725; product size: 113 bp), and IL-6 (accession number M80258; product size: 131 bp) were designed such that 1 primer spanned an intron to prevent amplification of genomic DNA. Primer/probe sets were designed using primer 3 (Whitehead Institute, Cambridge, MA) and oligo 6 (Molecular Biology Insights Inc., Cascade, CO) software. Target specificity of the PCR primers was confirmed by comparison against the Genbank database using BLAST (National Center for Biotechnology Information, Bethesda, MD). GAPDH was selected as the housekeeping gene because it has been described as one of the best overall control genes available for qPCR (10, 11). Primer and probe set sequences for the internal control and genes of interest are: porcine GAPDH forward primer 5′ GTT TGT GAT GGG CGT GAA C 3′, reverse primer 5′ ATG GAC CTG GGT CAT GAG T 3′, TaqMan probe 5′ CTC CAC GAT GCC GAA GTG GT 3′; porcine IL-1β forward primer 5′ GTG ATG GCT AAC TAC GGT GAC AA 3′, reverse primer 5′ CTC CCA TTT CTC AGA GAA CCA AG 3′, TaqMan probe 5′ AAT AAT GAC CTG TTA TTT GAG GCT GAT GGC 3′; and porcine IL-6 forward primer 5′ GTC GAG GCT GTG CAG ATT AGT 3′, reverse primer 5′ TTC TGT GAC TGC AGC TTA TCC 3′, TaqMan probe 5′ AGC ACT GAT CCA GAC CCT GAG G 3′.

Primer and probe sets were optimized for annealing/extension temperature, $MgCl_2$, and primer/probe concentrations. The final qPCR reaction mix consisted of 12.5 µl Platinum Quantitative PCR SuperMix-UDG (30 U/ml Platinum Taq DNA polymerase, 20 m*M* Tris-HCl [pH 8.4], 50 m*M* KCl, 3 m*M* $MgCl_2$, 200 µ*M* dGTP, 200 µ*M* dATP, 200 µ*M* dCTP, 400 µ*M* dUTP, 20 U/ml uracil *N*′-glycosylase [UDG], and stabilizers; Invitrogen), 300 n*M* of each primer for each gene, 200 n*M* of the IL-1β and IL-6 probes, and 150 n*M* of the GAPDH probe. In triplicate reactions 1 µl cDNA was added to a final volume of 25 µl. The reactions were run in 96-well plates at 50°C for 4 mins to activate the UDG, then for 3.5 mins at 95°C to inactivate the UDG and activate the polymerase, followed by 45 cycles of 25 secs at 95°C and 60 secs at 60°C for GAPDH and IL-6 (63°C for IL-1β). During the primer optimization

Table 1. Mean Relative Length (m/kg BW) of Small Intestine (SI) and Relative Mass (g/kg BW) of a 10-cm Segment Collected at 5% to 95% of SI Length in Gnotobiotic and Conventional Pigs Derived by Cesarian Section and Reared in Isolators Until 13 Days of Age

Treatment group	SI length	SI location				
		5%	25%	50%	75%	95%
Experiment 1						
CV	2.95^{b}	0.92^{a}	0.87^{b}	0.83	0.87	0.96
LF	2.21^{a}	0.62^{b}	0.63^{a}	0.75	1.09	0.84
EC	2.48^{ab}	0.63^{b}	0.74^{ab}	0.81	1.11	1.05
GF	2.30^{ab}	0.66^{b}	0.70^{ab}	0.81	1.12	0.95
Pooled SEM	0.17	0.06	0.05	0.04	0.08	0.07
Experiment 2						
CV	2.13	0.74	0.71	0.73	0.75	0.78
LF	1.98	0.57	0.56	0.65	1.05	0.93
EC	2.10	0.63	0.69	0.75	0.93	0.77
SE	1.81	0.57	0.60	0.63	1.07	0.75
Pooled SEM	0.11	0.04	0.05	0.06	0.12	0.09

^{a,b} Means within the same column without a common superscript letter differ significantly ($P < 0.05$).

procedure 1.5% agarose gel (Invitrogen) analysis verified the amplification of one product of the predicted size. Relative expression levels of porcine IL-1β and IL-6 mRNA were calculated using the $2^{-\Delta\Delta CT}$ method (11) after confirmation that the efficiency of the qPCR reaction was similar for the 3 target genes over a range of template concentrations.

Statistical Analysis. Data from Experiments 1 and 2 were analyzed separately as a one-way analysis of variance (ANOVA) using the general linear model procedure (SPSS, Chicago, IL). The qPCR ΔC_T values were used to statistically analyze gene expression. Means were separated by REGWF multiple-range test using the 5% level of significance.

Results

Microbial Status. Culture of cecal contents indicated monoassociation of EC and LF groups by *Escherichia* sp. and *Lactobacillus* sp., respectively, in Experiment 1 and Experiment 2. LF colonization in the cecum was 6.1×10^7 and 3.5×10^7 CFU/g of contents in Experiments 1 and 2, respectively. EC colonization in the cecum was 1.7×10^9 and 3.5×10^9 CFU/g of contents in Experiments 1 and 2, respectively. No bacteria were cultured from cecal contents of GF pigs in Experiment 1; however, monoassociation of GF pigs by *Staphylococcus epidermidis* (SE) was detected in Experiment 2 (3.4×10^8 CFU/g of contents). *Staphylococcus* contamination of LF and EC groups was excluded in Experiment 2 by negative colony growth on blood agar incubated aerobically for the LF pigs and negative growth on mannitol salt agar of frozen digesta from LF and EC pigs.

Morphologic examination of colonies from cecal contents of CV pigs plated on aerobic blood agar (1.21×10^9 and 1.57×10^9 CFU/g of cecal contents in Experiment 1 and Experiment 2, respectively) and anaerobic blood agar (2.20×10^9 and 4.51×10^9 CFU/g of cecal contents in Experiment 1 and Experiment 2, respectively) suggested the presence of numerous different bacterial species. MacConkey agar counts indicated colonization at 9.78×10^8 and 1.57×10^9 CFU/g of CV cecal contents in Experiment 1 and Experiment 2, respectively. Bacterial growth on MRS agar indicated 8.12×10^8 and 4.87×10^8 CFU/g of CV cecal contents in Experiment 1 and Experiment 2, respectively.

Health Status and Body Weight (BW). In the second experiment, 1 CV pig became emaciated and was euthanized, and therefore it was excluded from the results. All remaining pigs appeared healthy. Mean BW (kg ± SE) at 13 days of age was 2.82 ± 0.10, 3.24 ± 0.31, 2.62 ± 0.02, and 2.12 ± 0.21 for GF, LF, EC, and CV, respectively, in Experiment 1. LF pigs were significantly heavier than CV pigs but not different from GF or EC pigs. In Experiment 2, mean body weight at 13 days of age (3.15 ± 0.48, 3.18 ± 0.38, 2.89 ± 0.13, and 2.61 kg ± 0.36 for ST, LF, EC, and CV, respectively) was not different.

Relative SI Length and Weight. To determine the impact of different bacteria on small intestinal growth, small intestinal length and mass were recorded and calculated relative to BW (Table 1). In Experiment 1 relative SI length was longer in the CV group ($P < 0.05$) than in the LF group and was numerically longer than in both the GF and EC groups. In Experiment 2 no significant differences in relative length of the SI were observed; however, as in Experiment 1, CV and EC tended to be longer than LF. SE pigs had the shortest small intestinal length/kg BW. Mean intestinal weight for proximal to mid–small intestinal (5%–50%) regions tended to be higher for CV pigs compared with monoassociated and GF pigs. At 75% of the SI, mean weight was noticeably lower in CV pigs compared with all other groups; however, weight at 95% of the SI tended to be similar for all treatment groups. Interestingly, relative intestinal weight for CV pigs remained constant along the

entire length of the SI, whereas relative intestinal weight for monoassociated and GF pigs increased in distal regions with the highest segmental weights observed at 75% of small intestinal length (Table 1).

Intestinal Histology and Morphology. Microscopic examination of cross-sections of intestine revealed relatively similar morphology among treatment groups in proximal regions (Fig. 1) compared with striking morphological differences in distal regions (Fig. 2). One to three large vacuoles were present in the cytoplasm of villus enterocytes in the distal SI in GF, LF, EC, and SE treatment groups. Vacuoles were present from villus tips to the top of crypts, although in EC pigs nonvacuolated cells appeared somewhat higher on the villus compared with other monoassociated groups. The vacuoles appeared empty and displaced the nucleus primarily but not exclusively to the basement membrane. Vacuolization was absent in proximal sections of SI of GF and monoassociated pigs and was absent from the entire intestine in CV pigs. Additionally, a subjective analysis of the width and number of cells in the distal lamina propria indicated a ranking of GF < LF = SE < EC << CV, where lamina propria cellularity was minimal in GF pigs and abundant in CV pigs. For GF and monoassociated pigs the area and cellular density of the lamina propria increased in proximal SI compared with distal SI, whereas as the opposite trend was evident in CV pigs. Thus, in proximal SI lamina propria volume and cellularity were similar among treatment groups. Peyer's patches were consistently observed at the 95% small intestinal location in all treatment groups and were occasionally observed in the 75% location. At the 95% region treatment differences in area of Peyer's patches exhibited a similar pattern as described in the lamina propria (data not shown). Peyer's patches in GF pigs were small, increasing marginally in diameter in LF and SE pigs. Peyer's patches in EC pigs were larger than in LF or SE pigs but remained approximately one half the size as observed in CV pigs.

For pigs in all treatment groups villus length was greatest between 50% and 75% of small intestinal length (Fig. 3). Villus length was lowest in CV pigs compared with other treatment groups at all small intestinal regions in both experiments. In the distal small intestinal regions marked lengthening of villi was apparent in GF and monoassociated pigs such that villus length was up to 4-fold greater than that observed in CV pigs. The longest villi were observed in GF, LF, and ST pigs, whereas villi in EC pigs were generally of intermediate length between these groups and CV pigs. Crypts were deepest at 5% of small intestinal length, generally became shallower toward 75% of small intestinal length, and then again increased in depth at the 95% location of small intestinal length. Regional variation in crypt depth was not as dramatic as that observed for villus length. CV-treated pigs had the deepest crypts at all regions, followed by EC-inoculated pigs. Crypt depth was not different among GF, LF, and SE pigs.

Enterocyte vacuolization in the distal SI prevented differentiation of IELs in this region for GF and monoassociated pigs. In the proximal SI the number of IELs per 200 total enterocytes in the CV pigs was significantly ($P < 0.05$) greater than in GF, SE, LF, or EC pigs at most intestinal locations (Fig. 4). Goblet cells also were not differentiable in distal SI due to enterocyte vacuolization. In proximal regions the number of goblet cells per 200 total enterocytes in CV pigs generally was lower than in GF, SE, LF, or EC pigs (Fig. 4). Monoassociated pigs tended to have a higher goblet cell count relative to GF pigs (Experiment 1).

Small Intestinal Cytokine Expression. Abundance of cytokine mRNA was normalized to GAPDH (ΔC_T) and expressed relative to mRNA abundance in CV pigs at each small intestinal location (Tables 2 and 3). ΔC_T values for IL-1β were generally high (low expression) in GF, LF, and SE pigs relative to CV pigs at all sampled small intestinal locations. In EC pigs ΔC_T values for IL-1β were high (low expression) in the proximal SI and similar to other monoassociated groups; however, for both EC and CV pigs ΔC_T values started to decrease (increased expression) at 50% of small intestinal length, with the lowest values observed at 95% of small intestinal length. IL-6 expression patterns appeared relatively low over the length of the SI with a sharp increase (reduced ΔC_T) observed at 95% of small intestinal length. Relative to CV pigs, IL-6 expression patterns were similar to IL-1β patterns, although differences were less pronounced. The lowest ΔC_T values (highest expression level) were observed for EC pigs at 95% of small intestinal length for both IL-1β and IL-6.

Discussion

We successfully established a germ-free pig model facilitating investigation of intestinal development in the pig under gnotobiotic conditions. The experiments reported here were originally designed as replicate experiments; however, an *S. epidermidis* contaminant was cultured from the cecum of pigs in the germ-free treatment in the second replicate, necessitating separate analyses as Experiments 1 and 2. In both experiments we observed a consistent and marked effect of microbial colonization on intestinal morphology and proinflammatory cytokine expression, particularly in the distal SI. Intestinal responses in *E. coli* monoassociated pigs were more similar to conventionalized pigs, whereas intestinal responses in pigs monoassociated with *L. fermentum* and *S. epidermidis* were more like germ-free pigs.

Morphologically, the distal SI in germ-free and monoassociated pigs was characterized by relatively short crypts, extremely long villi populated by enterocytes with large cytoplasmic vacuoles, and a narrow lamina propria containing few cells. Reduced crypt depth and increased villus length agree with previous observations in the germ-free pig (12–15) and chicken (4). In rodent models, increased villus length also has been reported (16); however,

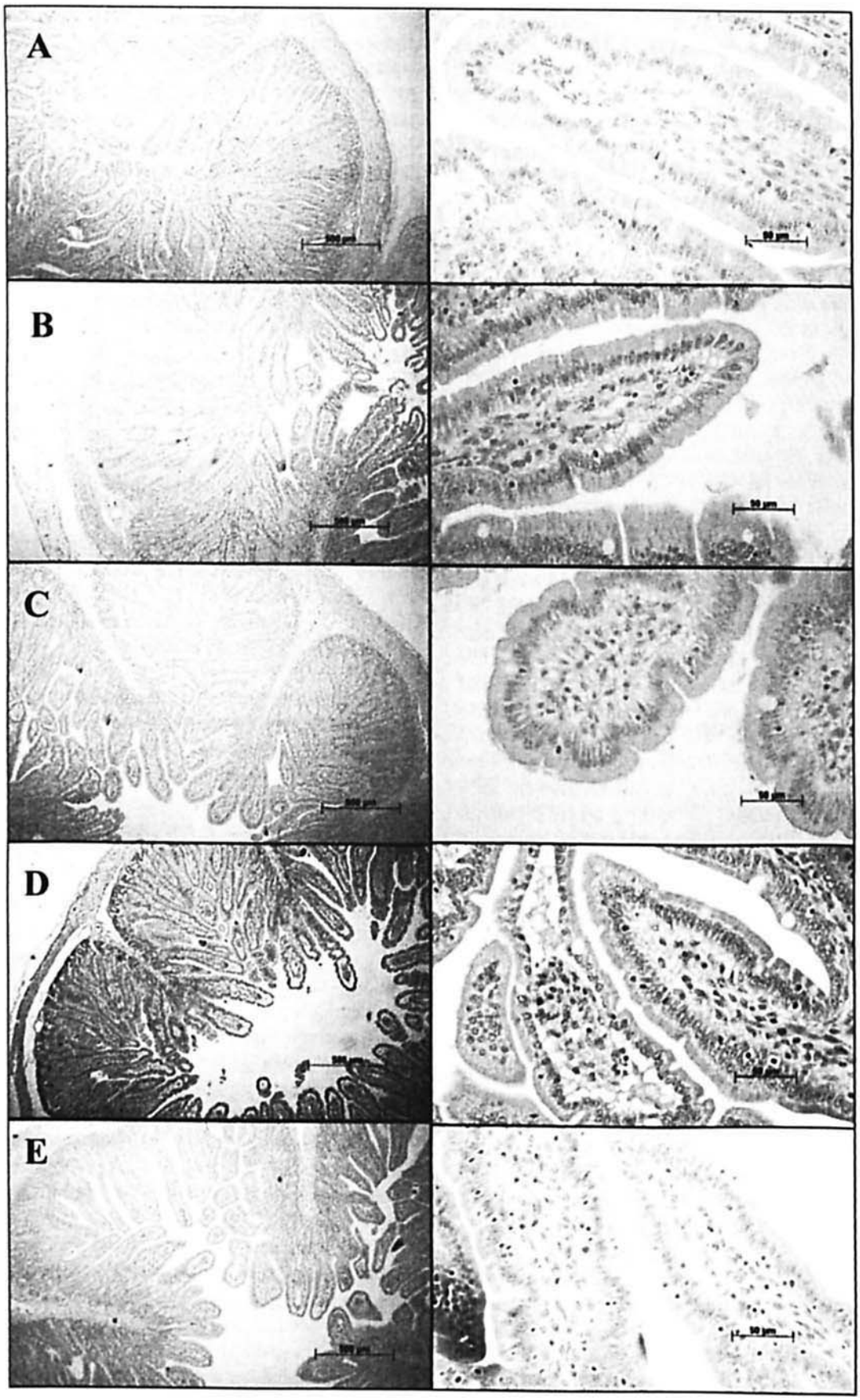

Figure 1. Representative photomicrographs of hematoxylin and eosin–stained cross-sections of tissue taken at 25% of the length of SI (pyloric sphincter 0%; ileo-cecal junction 100%) in pigs maintained as (A) germ-free, or inoculated with either (B) *L. fermentum*, (C) *E. coli*, (D) contaminated with *S. epidermidis*, or (E) conventionalized with adult porcine feces.

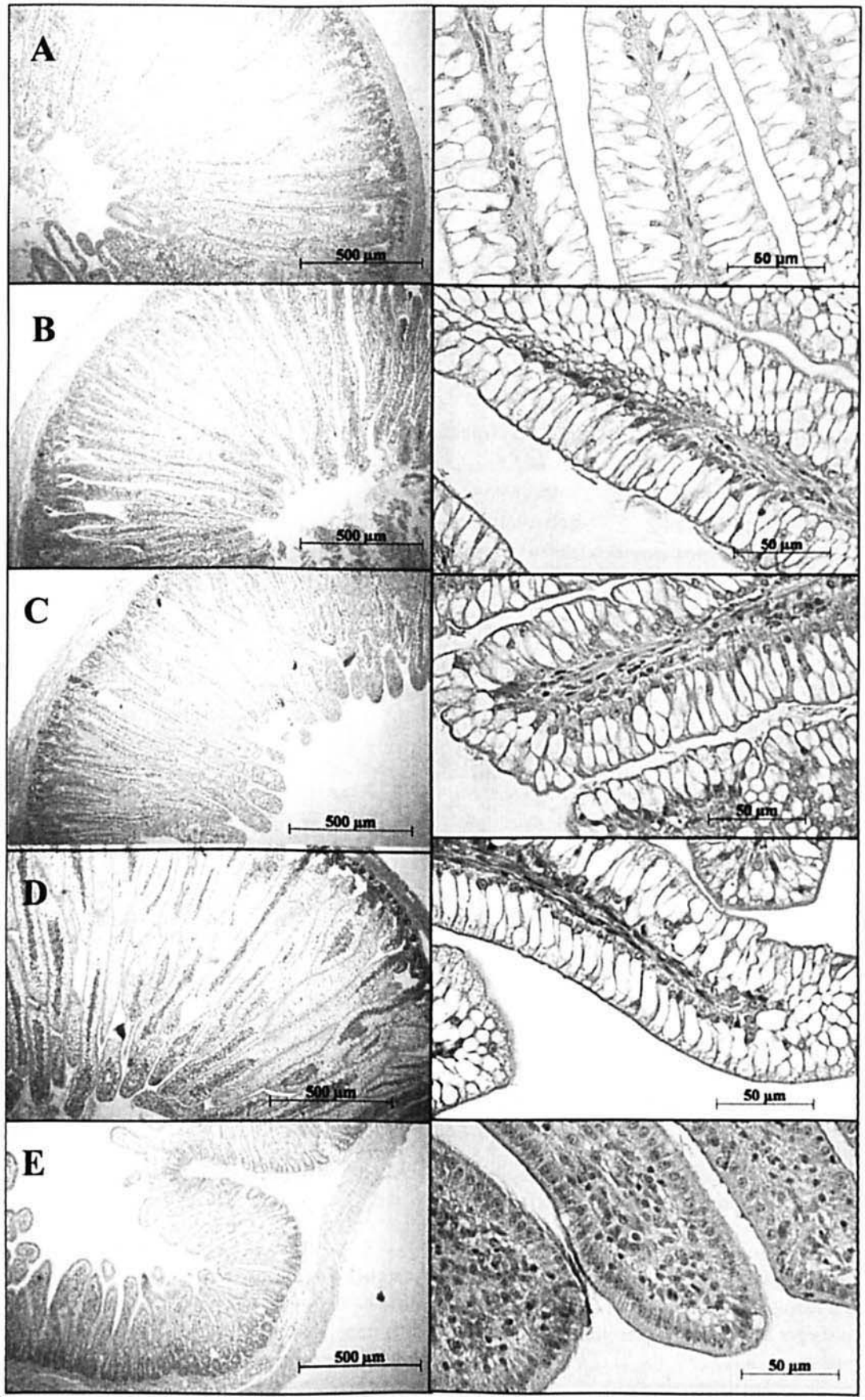

Figure 2. Representative light micrographs of hematoxylin and eosin–stained cross-sections of tissue taken at 75% of the length of SI (pyloric sphincter - 0%; ileo-cecal junction - 100%) in pigs maintained as (A) germ-free, or inoculated with either (B) *L. fermentum*, (C) *E. coli*, (D) contaminated with *S. epidermidis*, or (E) conventionalized with adult porcine feces.

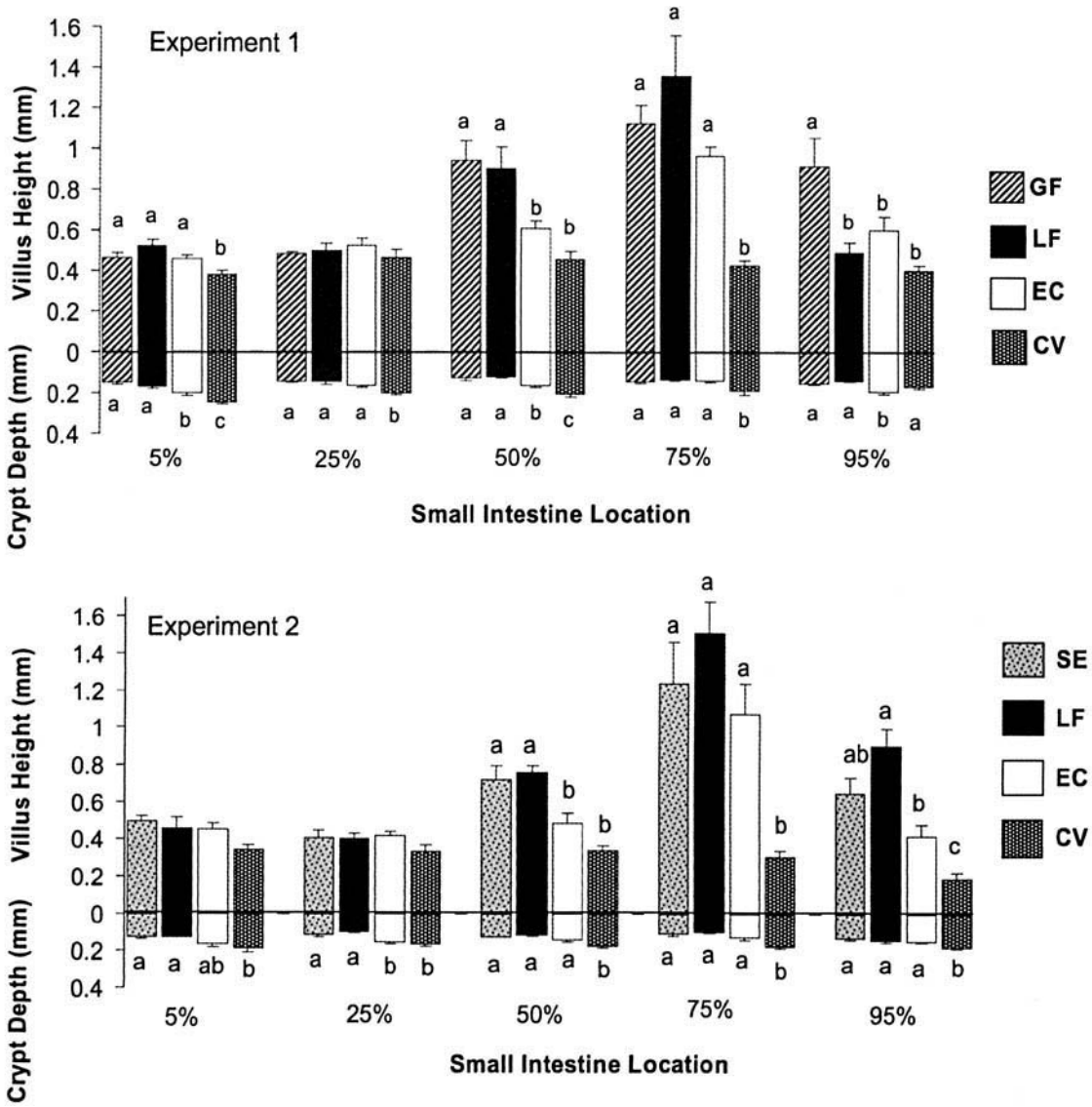

Figure 3. Mean (±SEM) villus height (upper bars) and crypt depth (lower bars) measured from 10 well-oriented villi and crypts from 5% to 95% of the length of the SI (pyloric sphincter = 0%; ileo-cecal junction = 100%) in pigs derived by cesarian section. In Experiment 1 (upper panel) pigs were germ-free (GF), or inoculated with either *L. fermentum* (LF; $n = 4$), a nonpathogenic *E. coli* (EC; $n = 4$), or conventionalized with fresh adult porcine fecal material (CV; $n = 4$). In Experiment 2 (lower panel) the GF group was contaminated with *S. epidermidis* (SE; $n = 4$). Bars within small intestinal location not sharing a common letter are significantly different ($P < 0.05$).

such findings have been inconsistent (5, 17). Surprisingly few reports have specifically characterized the regional morphologic response of the SI to bacterial colonization in gnotobiotic animals.

In the current study, regardless of microbial status, villi were longest in the jejunum and proximal ileum and shortest in the duodenum, whereas crypt depth was shortest at these regions and deepest in the proximal duodenum. These morphologic characteristics suggest that rates of enterocyte proliferation and exfoliation are highest in the proximal SI, as indicated by deeper crypts and shorter villi, respectively, with rates decreasing distally along the SI (18). This pattern of villus morphology has been observed in conventional neonatal pigs in which cell proliferation over the first 3 postnatal days, as measured by mucosal DNA content, was highest in the duodenum, intermediate in jejunum, and lowest in ileum (19). In agreement with our morphologic findings, Miniats and Valli (20) reported longer jejunal villi in germ-free pigs but did not measure villi in other regions. Conversely, Shurson et al. (15) reported that germ-free pigs had longer ileal and duodenal villi but shorter jejunal villi compared with their conventional counterparts. In the germ-free chicken (21) and rodent (17, 22, 23) villus length was reported to decrease progressively along the SI from the duodenum to the distal ileum. Interestingly, Ishikawa et al. (17) reported that conventionalization of germ-free rats resulted in a marked effect on ileal morphology (increased villus height, crypt depth, and number of mitotic figures), whereas no remarkable change was observed in duodenal and jejunal morphology. This regional response was

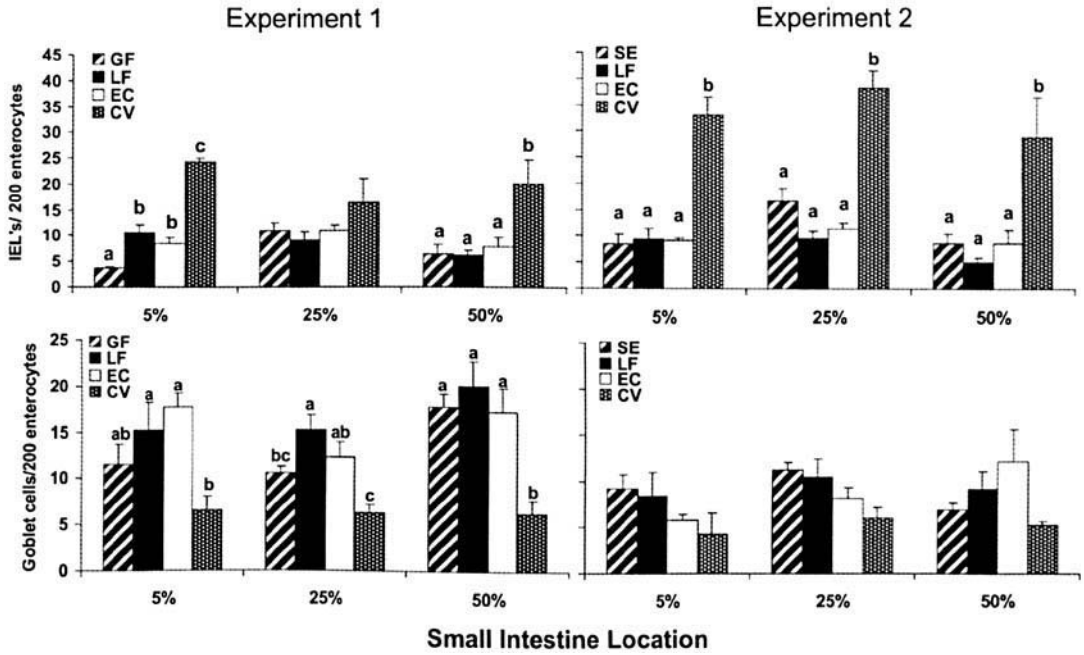

Figure 4. Mean (±SEM) number of IELs (upper panels) and goblet cells (lower panels) enumerated per 200 enterocytes in intestinal cross-sections obtained at 5%, 25%, and 50% of small intestinal length (pyloric sphincter = 0%; ileo-cecal junction = 100%) in pigs derived by cesarian section. In Experiment 1 (left panels) pigs were germ-free (GF), or inoculated with either *L. fermentum* (LF; $n = 4$), a nonpathogenic *E. coli* (EC; $n = 4$), or conventionalized with fresh adult porcine fecal material (CV; $n = 4$). In Experiment 2 (right panels) the GF group was contaminated with *S. epidermidis* (SE; $n = 4$). Bars within small intestinal location not sharing a common letter are significantly different ($P < 0.05$).

suggested to be the result of regional variation in microbial species diversity and abundance.

The preservation of this variation in regional morphology observed here in the germ-free and monoassociated pig is interesting. Although bacterial colonization is lower in the proximal regions (ranging from 10^1 to 10^4 CFU/g of contents) compared with the distal regions (ranging from 10^9 to 10^11 CFU/g of contents) of SI in conventional pigs (24), regional colonization variation (species number or diversity) cannot explain the marked regional variation in small intestinal morphology observed here in the germ-free pig. Our observations suggest that regional variation in morphology, especially in the proximal SI, is not entirely dependant on microbial colonization but is also influenced by such nonmicrobial factors as bile salts, pancreatic secretions, and compounds of dietary origin which would be expected to be in higher concentration and have more contact with mucosal surfaces in the duodenum.

Table 2. Mean ΔC_T (IL-1β C_T − GAPDH C_T) and Fold Change[a] for IL-1β at 5% to 95% of Small Intestinal (SI) Length in Gnotobiotic and Conventional Pigs Derived by Cesarian Section and Reared in Isolators Until 13 Days of Age

Treatment group	SI location				
	5%	25%	50%	75%	95%
	ΔC_T (fold change)	ΔC_T (fold change)	ΔC_T (fold change)	ΔC_T (fold change)	ΔC_T (fold change)
Experiment 1					
CV	8.1^b (1.00)	9.9 (1.00)	5.8^b (1.00)	9.0^b (1.00)	7.8^b (1.00)
LF	9.6^c (0.35)	10.0 (0.51)	9.9^c (0.06)	11.3^c (0.20)	10.1^c (0.20)
EC	10.0^c (0.27)	10.7 (0.58)	9.0^c (0.11)	9.3^b (0.77)	7.6^b (1.16)
GF	9.7^c (0.33)	11.2 (0.40)	9.6^c (0.07)	11.5^c (0.17)	10.9^c (0.11)
Pooled SEM	0.37	0.46	0.33	0.28	0.45
Experiment 2					
CV	—	—	9.9 (1.00)	9.7^{bc} (1.00)	8.9^c (1.00)
LF	—	—	10.5 (0.66)	11.0^c (0.40)	10.5^d (0.32)
EC	—	—	10.1 (0.84)	8.7^b (1.97)	5.6^b (10.55)
SE	—	—	9.8 (1.06)	10.3^{bc} (0.64)	9.9^{cd} (0.49)
Pooled SEM	—	—	0.36	0.47	0.39

[a] Fold change calculated by $2^{-\Delta\Delta CT}$ method (Ref. 11).
[b,c,d] Means within the same column without a common superscript letter differ significantly ($P < 0.05$).

Table 3. Mean ΔC_T (IL-6 C_T – GAPDH C_T) and Fold Change[a] for IL-6 at 5% to 95% of Small Intestinal (SI) Length in Gnotobiotic and Conventional Pigs Derived by Cesarian Section and Reared in Isolators Until 13 Days of Age

Treatment group	SI location				
	5%	25%	50%	75%	95%
	ΔC_T (fold change)	ΔC_T (fold change)	ΔC_T (fold change)	ΔC_T (fold change)	ΔC_T (fold change)
Experiment 1					
CV	10.4 (1.00)	11.5 (1.00)	7.7^{b} (1.00)	10.9 (1.00)	8.2^{bc} (1.00)
LF	10.7 (0.83)	12.0 (0.67)	10.7^{c} (0.12)	10.9 (1.00)	8.4^{bc} (0.85)
EC	11.1 (0.62)	11.6 (0.90)	10.1^{c} (0.18)	10.6 (1.25)	7.4^{b} (1.71)
GF	11.6 (0.44)	11.7 (0.84)	11.0^{c} (0.10)	11.1 (0.86)	9.0^{c} (0.55)
Pooled SE	0.43	0.25	0.57	0.27	0.31
Experiment 2					
CV	—	—	10.6 (1.00)	9.5 (1.00)	8.6^{bc} (1.00)
LF	—	—	11.2 (0.66)	10.8 (0.40)	9.7^{c} (0.49)
EC	—	—	11.1 (0.74)	10.4 (0.52)	7.5^{b} (2.16)
ST	—	—	11.7 (0.49)	10.5 (0.49)	8.9^{c} (0.85)
Pooled SE	—	—	0.24	0.41	0.32

[a] Fold change calculated by $2^{-\Delta\Delta CT}$ method (Ref. 11).
[b,c] Means within the same column without a common superscript letter differ significantly ($P < 0.05$).

Germ-free and monoassociated pigs exhibited extreme vacuolization of enterocytes along the villi of the distal SI. Alexander et al. (25) and Kenworthy (12) noticed a similar "foamy" appearance in the extrusion area of the villi in germ-free pigs. These vacuoles also have been observed in the jejunum and ileum, but not duodenum of the colostrum-fed neonatal pig up to 3 days of age, and they were associated with a marked swelling of enterocytes and intracellular accumulation of protein consistent with macromolecular absorption (19). Colostrum appears to have an enhancing effect on macromolecule absorption and accelerates gut closure in pigs, whereas infant milk formula results in incomplete macromolecule absorption and delayed gut closure (26). Since piglets in our study were colostrum deprived (passive immunity provided as sterile porcine serum) we speculate that the vacuoles identified in villus enterocytes are most likely the result of incomplete macromolecule absorption. The observation of these vacuolated cells along the entire crypt to villus tip axis of the villi in 13-day-old germ-free and *L. fermentum*– and *S. epidermidis*–associated pigs suggested a remarkably slow rate of enterocyte replacement.

Among the monoassociated pigs in this study, intestinal morphological responses to bacterial colonization were species dependant. In *E. coli*–associated pigs shorter villi, deeper crypts, and a higher extension of nonvacuolated enterocytes along the villi suggested a higher intestinal enterocyte replacement rate than in germ-free pigs. In contrast, intestinal morphology was similar among germ-free pigs and pigs associated with *L. fermentum* or *S. epidermidis*. There are several possibilities that may explain changes in intestinal morphology in *E. coli*–associated pigs. First, Delneste et al. (8) have demonstrated that gram-negative *E. coli* induce a marked up regulation of immune response and inflammatory gene expression in cultured epithelial HT-29 cells. In contrast *L. johnsonnii* were relatively incapable of activating these cells. In addition, considerable variation has been reported in the intestinal inflammatory response to a range of bacterial species (27). Proinflammatory cytokines can enhance enterocyte renewal (28), consistent with the observed upregulation of IL-1β and IL-6 cytokine expression in *E. coli*–associated pigs. Second, morphology responses could reflect species variation in bacterial synthesis of toxic catabolites such as ammonia, which is known to increase enterocyte turnover by affecting cellular intermediary metabolism and DNA synthesis (29). Finally, increased crypt depth and villus shortening also could reflect a higher *E. coli* colonization rate relative to the *L. fermentum* and *S. epidermidis*. We did not measure small intestinal colonization in the current study, although *E. coli* cecal colonization was indeed 10- to 100-fold higher per gram contents. In similarly designed subsequent studies culture-based enumeration of *L. fermentum* and *E. coli* in small intestinal contents indicated a similar 10- to 100-fold difference in colonization abundance.

The adaptive response of the SI to bacterial colonization also is evident upon examination of relative small intestinal length and weight. In germ-free and monoassociated pigs, relative small intestinal length was reduced compared with conventional pigs. Few data are available on intestinal length in response to intestinal bacterial colonization; however, Furuse and Yokota (30) found that the absolute and relative lengths of the SI were lower in germ-free chickens compared with conventional chickens. The mechanisms affecting intestinal length are unknown; however, it can be hypothesized that increased small intestinal length in conventionalized pigs is a compensatory response to the decreased absorptive capacity associated with decreased surface area (decreased villus length) and/or

to direct competition with the microbiota for dietary nutrients.

In the proximal region of the SI, relative weights for segments from conventional pigs tended to be higher than germ-free and monoassociated pigs. This is consistent with previous reports indicating that compared with germ-free animals, conventionally reared animals experience intestinal "thickening" associated primarily with increased lamina propria cellularity (20), as well as thickening of the submucosa (containing large blood vessels, lymphatics, and neural complexes) and muscular layers (4, 15, 31). However, in the distal regions of the SI, differences in relative small intestinal weights tended to be increased in germ-free and monoassociated pigs versus conventional pigs. This paradoxical response appeared to be the result of extreme villus lengthening in germ-free and monoassociated pigs compared with the conventional pigs.

IELs are primarily T lymphocytes that participate in the regulation of the immune response (32). In the conventionally reared pig, the number of IELs is low at birth and then continuously increases throughout the first year of life. However, in the germ-free pig the number of IELs at 1 and 2 months of age were similar to that of the newborn conventional pig (33). In the present study we enumerated IELs in the proximal and mid-locations (5%–50%) of the SI in all treatment groups; however, in the distal locations (75% and 95%) it was not possible to differentiate IELs in germ-free and monoassociated pigs due to extreme vacuolization of enterocytes. In agreement with the observations of Rothkotter et al. (33) and others using rodents (34, 35), the number of intestinal IELs in conventionalized pigs was between 1.5- and 6.5-fold greater than in germ-free and monoassociated pigs, which is consistent with a trend toward higher inflammatory cytokine expression in proximal regions (Experiment 1) and suggests enhanced development of immune response. Distal small intestinal morphological changes and increased inflammatory cytokine expression in *E coli*–associated pigs did not affect IEL numbers in proximal SI compared with other monoassociated pigs and germ-free pigs.

Goblet cells synthesize and secrete mucus that covers the gastrointestinal epithelium, forming a boundary between the luminal contents and mucosa (36). There is ample evidence that intestinal microbiota affect goblet cell dynamics, including mucus secretion rate and composition either directly by the secretion of bioactive factors or indirectly by the activation of host immune cells (37–39). No consistent difference in goblet cell numbers among germ-free and monoassociated pigs was observed; however, surprisingly, goblet cell counts in SI tended to be highest in these groups relative to conventional pigs. Similar results have been observed previously in germ-free rodents (37, 40), dogs (5), and pigs (5). The relationship between goblet cell number and mucus secretion rate is unknown; however, the increase in number of goblet cells in germ-free and monoassociated pigs suggests microbial effects on goblet cell differentiation and/or renewal rate that may be distinct from effects on absorptive enterocytes.

IL-1β and IL-6 are proinflammatory cytokines synthesized by various cell types present in the intestinal wall of lymphoid and nonlymphoid origin. Macrophages, dendritic cells, lymphocytes, and neutrophils present in the lamina propria and in Peyer's patches can synthesize and secrete IL-1β and IL-6 (41). It has been recognized recently that the enterocyte is a constitutive component of the innate response of the host toward the luminal microbiota and is likely to be an important participant in intestinal immune networks. Enterocytes express a variety of cytokines common to antigen-presenting cell lineages, including the proinflammatory cytokines IL-1α, IL-1β, tumor necrosis factor-α (TNF-α), IL-6, and IL-8, and the anti-inflammatory cytokines transforming growth factor-β (TGF-β) and IL-10 (42, 43).

In the current study the abundance of IL-1β and IL-6 transcripts was highest in intestinal tissue collected at 95% of small intestinal length. In the pig there are two types of Peyer's patches: several separate patches in the jejunum and proximal ileum, and a long continuous patch in the distal ileum. In agreement with our observations Pabst et al. (44) observed development and growth of both types of Peyer's patches in the germ-free pig; however, the increase with age was significantly less than in age-matched conventional controls. It is likely that the observed upregulation of IL-1β and IL-6 at the 95% small intestinal location compared with more proximal regions in all treatment groups may be related to the contribution of densely organized Peyer's patch lymphoid cells.

Expression of IL-1β and IL-6 was similar among germ-free pigs and *L. fermentum* and *S. epidermidis* monoassociated pigs and remained low at all small intestinal regions relative to conventionalized pigs. These expression levels appear to be coupled with the increased volume of lamina propria in the conventional pigs and associated with increased numbers of lymphoid cells. In proximal SI, *E. coli* pigs also expressed low amounts of IL-1β and IL-6 relative to conventional animals; however, more distally, IL-1β and IL-6 expression levels increased and reached levels exceeding conventional pigs at the 95% small intestinal region. We hypothesize that the marked distal inflammatory cytokine response to *E. coli* may be the result of overgrowth of this species in the monoassociated state versus the conventional state and the increased propensity of this organism to translocate across the epithelial barrier. Based on studies in monoassociated mice, not all bacteria are as equally proficient at crossing the epithelial barrier. Steffen et al. (45) determined that indigenous gram-negative enteric bacilli, such as *E. coli*, translocated in large numbers to the mesenteric lymph node, whereas gram-positive bacteria translocated at intermediate levels and obligated anaerobic bacteria at only very low levels.

While the abilities to translocate across epithelial barriers differ among bacteria, so do their abilities to elicit

a host immune response. Using mouse peritoneal macrophages, Nicaise et al. (46) demonstrated that the production of IL-1 and IL-6 in conventional mice was significantly greater than in germ-free mice. Additionally, macrophage secretion of IL-1 and IL-6 in mice monoassociated with *E. coli* was comparable to levels produced by conventional mice, whereas mice monoassociated with *Bifidobacterium bifidum* did not increase production of IL-1 and IL-6 above levels observed in the germ-free mice. Conversely, gram-positive bacteria such as lactobacilli and staphylococci may trigger gene activation of the immunosuppressive cytokine TGF-β (47), which may have downregulated expression of IL-1β and IL-6 as observed in pigs monoassociated with gram-positive bacteria (48). Finally, the ability of intestinal enterocytes to respond differentially to bacteria has been demonstrated in studies employing cultured epithelial cell lines. Whereas *E. coli* was able to induce proinflammatory cytokine responses, these culture cell lines are unresponsive or elicited an anti-inflammatory responses when exposed to lactobacilli (8, 32).

In conclusion, we have established a gnotobiotic model that will allow systematic examination of the effect of a defined microbial population on neonatal intestinal development and function in a model species important in human biomedical and animal production research. We characterized regional variation in small intestinal morphological and inflammatory responses and suggest that factors of non-microbial origin are of major influence on these parameters in proximal intestine. We also identified that morphological and inflammatory responses in SI are differentially affected by bacterial species, supporting the contention that postnatal bacterial colonization patterns play an important role in neonatal intestinal development. Our findings highlight the need for an *in vivo* model to elucidate the complex host cell–cell interactions that govern the functional outcome of the SI. We intend to further refine the model for application in characterizing short- and long-term effects of microbial colonization in the neonate on intestinal development, including identification of microbial species and biochemical pathways that may impart a developmental benefit.

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