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Colonization of the Porcine Gastrointestinal Tract by Lactobacilli

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Eight strains of lactobacillus isolated from the porcine gastrointestinal tract were tested for their ability to adhere in vitro to cells collected from stratified squamous epithelium in the digestive tracts of newborn piglets. Piglets were inoculated with individual strains, and their digestive tracts were sampled at intervals to determine the colonizing ability of the lactobacilli. The results of the in vitro test did not predict whether a lactobacillus strain would associate with stratified squamous epithelium in the piglet digestive tract, but epithelial association in vivo appeared to be an important factor in the maintenance of lactobacillus populations in the tract. None of the lactobacillus strains used as inocula was numerically dominant in the tract 7 days after inoculation of the piglets with a single dose of the bacteria.

Large populations of lactobacilli inhabit the proximal regions of the digestive tracts of pigs, fowl, and rodents. Some gastrointestinal strains of lactobacilli have the ability to adhere to and colonize the surface of stratified squamous epithelium in the esophagus, crop, or stomach. Other lactobacillus strains appear to be inhabitants of the gastrointestinal lumen. Lactobacilli, whether shed from epithelial surfaces or multiplying in the digesta, permeate all regions of the digestive tract in these animals (2, 3, 5, 6, 8).

The presence of lactobacilli in the proximal digestive tracts of piglets may be beneficial to the host animal. Gastric acidity during the first week of life appears to be largely due to lactic acid-producing bacteria residing in the upper regions of the digestive tract: little hydrochloric acid is secreted by the piglet during this period (4). The formation of milk curd in the stomach and of an acid barrier through which potential pathogens must pass may thus be markedly influenced by the presence of lactobacilli in the piglet digestive tract. These phenomena will not be adequately understood until the factors that regulate colonization of the digestive tract by lactobacilli have been investigated. We therefore carried out experiments to investigate the following questions relating to colonization of the porcine digestive tract by lactobacilli. (i) Can the ability of a lactobacillus strain to associate with epithelium be predicted by an in vitro adhesion test? (ii) Is epithelial association by lactobacilli a prerequisite for successful colonization of the digestive tract? (iii) Will a single oral inoculation of neonatal piglets with a specific strain of lactobacillus result in colonization of the digestive tract by that strain?

MATERIALS AND METHODS

Piglets. Twelve litters of conventional Landrace-Yorkshire-Pietrain crossbred piglets were used in these studies. The animals were maintained by standard Danish farming methods for pigs not specific pathogen free. Sows consumed a diet of barley grain supplemented with protein, usually in the form of soy meal. Piglets in our study received only maternal milk. Teeth and tails were trimmed at 1 day of age; males were not castrated. The sows and piglets did not receive any medication before or during the experiments. Two litters were used as uninoculated control animals for observing the natural acquisition of lactobacillus populations by piglets. The remaining litters were inoculated with specific strains of lactobacilli. Newborn piglets (before ingestion of colostrum) received about 10 ml of an anaerobic culture of a lactobacillus strain (ca. 10^{10} lactobacilli per piglet) in Lactobacilli MRS broth (Difco Laboratories, Detroit, Mich.). The piglets were then returned to the sow. Piglets were killed by intracardiac injection of pentobarbital and exsanguination at 1, 2, 4, or 7 days of age or, when litters were sufficiently large, at 6, 8, 10, or 13 days. Samples (about 1-cm long) of esophagus, duodenum, jejunum, and ileum were collected from each piglet. The entire pars oesophagea (an area of mucosa in the pig stomach with stratified squamous epithelium) was also collected, along with 0.5-g samples of stomach and rectal contents.

Lactobacillus strains. All lactobacillus strains tested were originally isolated from the porcine gastrointestinal tract (Table 1). *Lactobacillus fermentum* RF14 was resistant to erythromycin and tetracycline. The remaining strains were rifampin-resistant strains detected when cultures were plated onto rifampin-containing gradient plates (maximum concentration of rifampin, 100 μg/ml). *Lactobacillus salivarius* SS1 was resistant to erythromycin and to rifampin. (MICs of antibiotics for the strains are given in Table 1). The stability of rifampin-resistance was tested by culturing the strains without rifampin for eight successive subcultures in Lactobacilli MRS broth incubated anaerobically. The strains retained their rifampin resistance throughout this period.

In vitro adherence assay. The lactobacillus strains were tested by the method of Fuller (1) for their ability to adhere to porcine squamous epithelial cells. Epithelial cells were brushed from esophagus and pars oesophagea of newborn piglets that had not yet suckled the dam (lactobacilli absent). The cells were suspended in phosphate-buffered saline (pH 7.3) to a density of approximately 5×10^5 cells per ml. Cells from overnight anaerobic cultures of lactobacillus strains in Lactobacilli MRS broth were washed and suspended in phosphate-buffered saline to a density of about 10^8 cells per ml. One-fifth of 1 ml of epithelial cell suspension was mixed with 0.05 ml of bacterial suspension and incubated at 37°C, with rotation, for 30 min. Adhesion of the lactobacilli to epithelial cells was observed by phase-contrast microscopy. The adhering strains in this study produced preparations in which more than 15 bacterial cells adhered per epithelial cell.

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TABLE 1. Characteristics of lactobacillus strains used as inocula

Lactobacillus strain	Adherence[a] to epithelial cells in vitro	Source	Antibiotic(s)[b] and MIC(s) (μg/ml)
L. fermentum RF14	+	Stomach	Em, 1,024 Tc, 256
L. acidophilus SS28	+	Duodenum	Rif, 256
L. acidophilus SS131	+	Ileum	Rif, 512
L. salivarius SS1	+	Stomach	Em, 1,024 Rif, 512
L. salivarius SS129	+	Jejunum	Rif, 1,024
L. salivarius SS258	+	Stomach	Rif, 512
L. crispatus SS151	−	Jejunum	Rif, 1,024
Lactobacillus sp. strain SS8	−	Ileum	Rif, 1,024

[a] Symbols: +, adhering strain; −, nonadhering strain.
[b] Em, Erythromycin, Tc, tetracycline; Rif, rifampin.

Fewer than three bacterial cells adhering per epithelial cell were seen in preparations from nonadhering strains.

Culture of specimens. Esophageal, duodenal, jejunal, and ileal samples, which contained only small amounts of digesta in piglets of this age, and the pars oesophagea were washed (and brushed in the case of esophagus and pars oesophagea) with 4.5 ml of sterile distilled water. Epithelial cells and digesta were suspended thoroughly, diluted in sterile distilled water, and used to inoculate agar medium selective for lactobacilli. Stomach and rectal contents were suspended in sterile distilled water as 10-fold (wt/vol) dilutions and diluted further in 10-fold steps before inoculation of agar medium. Total lactobacillus populations were enumerated on Rogosa SL agar (Difco), strain RF14 was enumerated on Rogosa agar containing 50 μg of erythromycin and 150 μg of tetracycline per ml, strain SS1 was enumerated on Rogosa agar containing 50 μg of erythromycin and 100 μg of rifampin per ml, and the remaining strains were enumerated on Rogosa agar containing 50 μg of rifampin per ml. Media were incubated anaerobically (GasPak jars; BBL Microbiology Systems, Cockeysville, Md.) for 48 h before examination. Results were expressed as lactobacilli per milliliter of washings (esophagus, pars oesophagea, duodenum, jejunum, and ileum) or per gram of contents (stomach and rectum).

Microscopic examination of epithelial surfaces. Samples of esophagus, pars oesophagea, duodenum, jejunum, and ileum were collected from some piglets. Some of these specimens were sectioned in a microtome-cryostat, Gram stained, and examined by light microscopy (7). Other specimens were fixed in a solution containing 3% glutaraldehyde, 0.1 M cacodylate, and 0.15 M calcium chloride. The specimens were processed through a series of acetone solutions (25, 40, 60, 90, and 100%), critical point dried, coated with gold, and examined by scanning electron microscopy.

TABLE 2. Populations of naturally acquired lactobacilli in digestive tract samples from 1-day-old piglets

Source of sample[a]	Lactobacillus populations[b]	
	Litter 1	Litter 2
Esophagus	5.2, 6.3	5.3, 6.3
Pars oesophagea	5.6, 6.3	6.0, 6.8
Stomach	7.3, 8.0	8.2, 9.7
Duodenum	4.7, 5.6	4.8, 5.6
Jejunum	2.0, 5.0	4.0, 5.6
Ileum	5.1, 8.0	5.0, 6.0
Rectum	8.8, 9.3	7.8, 8.1

[a] Stomach and rectal contents were examined; washings were examined from other specimens.
[b] Log_{10} lactobacilli per milliliter or per gram of sample from two piglets.

TABLE 3. Populations of specific lactobacillus strains in piglet digestive tracts sampled 7 days after inoculation

Source of sample	Populations of strain[a]:		
	L. acidophilus SS28	*L. salivarius* SS129	*L. acidophilus* SS131
Esophagus	6.8, 7.6 (52.4)	2.0, 6.4 (16.5)	ND[b]
Pars oesophagea	6.7 (17.4)	3.0, 5.9 (0.27)	ND
Stomach	8.6, 8.9 (31.2)	4.6, 6.1 (0.08)	ND
Duodenum	6.5, 6.7 (33.5)	2.6, 3.7 (0.05)	ND
Jejunum	6.6, 6.8 (21.2)	ND, 4.8 (0.02)	ND
Ileum	8.1, 8.1 (21.4)	4.7, 6.6 (0.19)	ND
Rectum	6.3, 7.1 (0.09)	7.3, 8.0 (2.2)	5.2, 6.4 (0.02)

[a] Log_{10} lactobacilli per milliliter of washings or per gram of contents (stomach and rectum) from two piglets. Values in parentheses give the population of the inoculated strain as the mean percentage of the total lactobacillus population.
[b] ND, None detected (lower limits of detection: 2.0 for esophagus, pars oesophagea, duodenum, jejunum, and ileum; 3.0 for stomach and rectal contents).

RESULTS

In vitro adhesion to epithelial cells. Except for strains SS8 and SS151, all lactobacillus strains used as inocula adhered to porcine epithelial cells in vitro (Table 1).

Colonization of uninoculated piglets by lactobacilli. Uninoculated piglets were colonized by lactobacilli from the environment of the animals within 1 day of birth. Lactobacillus populations were detected throughout the gastrointestinal tracts of the animals (Table 2). These populations gradually increased, being about 10 times larger on day 10 than on day 1 of life. Rifampin- or tetracycline-resistant lactobacilli were not detected in uninoculated piglets.

Colonization of inoculated piglets. The lactobacillus strains used as specific inocula varied in their ability to colonize the digestive tracts of piglets. Although all strains were present as the numerically dominant lactobacillus type in piglets 1 and 2 days after inoculation, over time the population of the strain used as inoculum decreased in all regions of the

TABLE 4. Association of lactobacillus strains with stratified squamous epithelium in the porcine digestive tract 1 day after inoculation with lactobacilli

Lactobacillus strain[a]	Lactobacillus populations[b] associated with:	
	Esophagus	Pars oesophagea
Lactobacillus sp. strain SS8	4.7, 5.0 (78.2)	5.7, 5.7 (89.5)
L. crispatus SS151	5.3, 6.0 (82.8)	5.7, 6.2 (69.9)
L. salivarius SS129	3.6, 6.3 (88.4)	6.9, 7.4 (98.9)
L. acidophilus SS28	6.5, 6.5 (97.0)	6.6, 6.6 (100)
L. acidophilus SS131	4.2 (100)	3.4, 5.2 (78.2)
L. fermentum RF14	4.3, 5.9 (84.1)	6.9, 7.0 (100)
L. salivarius SS1	6.9, 7.0 (87.0)	7.0, 7.1 (96.3)
L. salivarius SS258	6.7, 7.6 (100)	7.3, 7.4 (93.8)

[a] Strains SS8 and SS151 did not adhere to porcine epithelial cells in vitro; the remaining cultures did adhere.
[b] Log_{10} lactobacillus populations per milliliter of washings from two piglets. Values in parentheses give the population of the lactobacillus strain as the mean percentage of the total lactobacillus population.

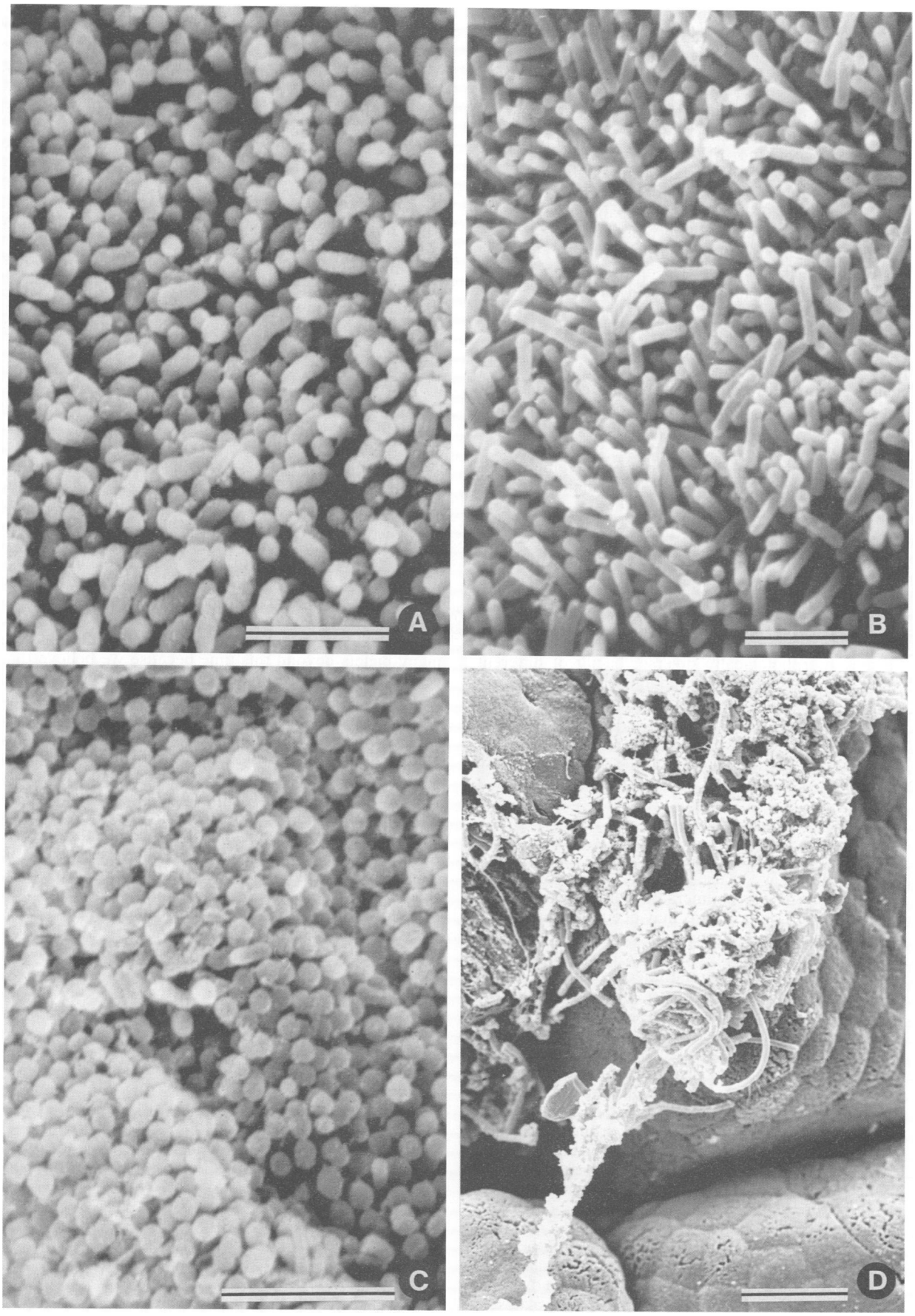

FIG. 1. Scanning electron micrographs. (A) Short bacilli predominating on the esophageal epithelial surface of a piglet inoculated 1 day previously with *L. salivarius* SS1. Bar, 5 μm. (B) Bacilli apparently attached end-on to the esophageal epithelium of a piglet inoculated 7 days previously with strain SS1. Bar, 5 μm. (C) Cocci predominating on the esophageal epithelium of an uninoculated piglet 4 days of age. Bar, 5 μm. (D) Bacilli in mucus associated with ileum of a piglet inoculated 4 days previously with *L. acidophilus* SS131. Bar, 10 μm.

digestive tract. *Lactobacillus acidophilus* SS28 was the best colonizer of the piglet digestive tract, exceeding 10^8 cells per g in stomach and ileal samples at 7 days after inoculation (Table 3) and in stomach and jejunum at 13 days after inoculation (data not shown). *Lactobacillus acidophilus* SS131, by contrast, was a poor colonizer of the digestive tract, being detectable only in rectal contents of piglets at 7 days after inoculation (Table 3). The remaining six strains were intermediate in colonizing ability, maintaining populations that constituted a small proportion of the total lactobacillus population in the tract at 7 days after inoculation (strain SS129 is given as an example in Table 3).

In vitro adhesion and colonization of the digestive tract. We did not observe a correlation between the results of the in vitro adhesion test and the behavior of lactobacilli in the digestive tract. Both in vitro adhering and nonadhering lactobacillus strains showed a similar ability to colonize the digestive tract, and all strains were detected in washings from the esophagus and pars oesophagea during the first few days of life (Table 4).

Microscopic examination of samples. Epithelium-associated populations were observed both by scanning electron microscopy and by light microscopy of Gram-stained sections of esophageal and pars oesophageal specimens. Adherent populations of bacteria were always seen in samples from inoculated and noninoculated animals from the first day of life. During the first few days after inoculation, the majority of bacterial cells associated with the esophageal and pars oesophageal epithelium were bacilli of a single morphological type. As time progressed, however, the microbial layer became morphologically diverse: bacilli of different lengths and diameters were observed in esophageal and pars oesophageal specimens, and cocci became increasingly common on esophageal epithelium (Fig. 1A to C). Bacteria adherent to duodenal, jejunal, or ileal epithelium were not observed, although rod-shaped organisms could sometimes be seen in mucus covering the mucosa (Fig. 1D).

DISCUSSION

To trace the colonization patterns of our lactobacillus strains, it was necessary to use antibiotic-resistant cultures that could be selectively enumerated with antibiotic-containing medium. Certain problems may occur in experiments of this type when antibiotic-resistant strains are used: the mutation leading to antibiotic resistance might affect other bacterial characteristics that could affect colonization ability, the mutated gene might revert to antibiotic sensitivity, and the resistance gene might be passed in vivo to other lactobacillus types in the digestive tract. We do not believe that our experiments were affected by these possibilities, however, since we measured resistance to two antibiotics (tetracycline and rifampin) in different experiments and obtained similar results. Resistance to erythromycin is common among lactobacillus strains isolated from Danish pigs (K. Pedersen and G. W. Tannock, unpublished data), yet the colonization ability of the bacteria is apparently not impaired. The reliability of the population levels obtained by selectively culturing lactobacilli on antibiotic-containing agar medium was supported by observations of the morphology of lactobacillus colonies cultured on Rogosa agar without antibiotics. During the first few days after inoculation of the piglets, one colony type (that of the strain used to inoculate the piglets) was dominant on Rogosa agar plates. A mixture of colony types was present on Rogosa agar when samples were cultured at later intervals, in agreement with the reduction in the number of antibiotic-resistant lactobacilli. Uninoculated piglets always harbored a mixture of lactobacillus colony types. Microscopic observation of digestive tract specimens also provided evidence of the reliability of our selective culture procedures: the morphology of the bacilli colonizing epithelial surfaces at various intervals following inoculation of the animals was different; mixtures of microbial types were more likely to be seen when antibiotic-resistant lactobacillus populations had decreased in size.

Comparison of two strains of lactobacillus that did not adhere to epithelial cells in vitro with six adhering strains showed that the in vitro test did not predict the outcome of in vivo experiments. Adhering and nonadhering strains were equally capable of associating with the esophagus and pars oesophagea of newborn piglets. The nonadhering lactobacilli may have been associating with adherent lactobacilli derived from the environment or may not have expressed the genes encoding adherence under in vitro conditions.

Epithelial association appears to be an important factor in colonization of the piglet digestive tract: our strains of lactobacilli continued to populate the gastrointestinal tract as long as their association with epithelium (of the esophagus in particular) continued to some extent. The strains tested in our study, although constituting the dominant lactobacilli on esophageal and pars oesophageal surfaces during the first 2 days of life of the piglet, were soon supplanted by lactobacilli from the environment. Most of the strains used as inocula, however, continued to cohabit the digestive tract with other lactobacillus types for at least 7 days. We did not examine many piglets beyond this time because we believe that any contribution that lactobacilli make to the well-being of the piglet is during the first week of life, when normal physiology and resistance to disease are developing.

Our experiments show that a single dose of a specific lactobacillus strain given to newborn piglets results in extensive colonization of the tissue surfaces and lumen of the proximal digestive tract with that strain during the first few days of the life of the animal. Thereafter, the lactobacillus strain is gradually supplanted by other lactobacillus types from the environment. We believe that a biological succession of lactobacillus types probably occurs in the digestive tract as the piglet develops. The factors that control such a succession in the digestive tract of the pig should now be investigated. The influence of a regime of multiple doses of a specific lactobacillus strain on this succession should also be studied.

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