APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1978, p. 582-591 Vol. 35, No. 3
0099-2240/78/0035-0582$02.00/0

Printed in U.S.A.

Bacteria Associated with the Gastric Epithelium of Neonatal Pigs

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Received for publication 20 September 1977

Light and electron microscopy showed lactobacilli and, to a lesser degree, streptococci to be closely associated with the squamous area of the pig stomach known as the pars esophagea. Several different types of extracellular layers were seen on bacteria attached to the epithelial surface. The total number of bacteria per square centimeter did not change with age up to 10 days, and there was no effect of weaning at 2 days. *Lactobacillus fermentum, L. salivarius,* and *Streptococcus salivarius* were isolated more frequently from sucking pigs than from those that were early weaned, whereas the reverse was true of *L. acidophilus* and *S. bovis.* All isolates recovered from washed macerated pars esophagea adhered to pig esophageal epithelial cells when tested in vitro.

Bacteria attached to epithelial surfaces have been demonstrated in the gut of a variety of different vertebrate animals (5, 8, 13, 14), including the pig (20), and it has been suggested that this attachment is an important factor in determining whether a particular organism colonizes the intestinal tract (11, 14). It, therefore, seemed likely that the presence of consistently high numbers of lactobacilli and streptococci in the stomach of baby pigs (2) was dependent on their ability to colonize the gastric surface. This paper presents cultural and microscopic evidence for the existence of such a microflora of lactobacilli and streptococci intimately associated with the squamous epithelium (pars esophagea) of the pig stomach.

MATERIALS AND METHODS

Pigs. Samples were obtained from the two groups of pigs (sucking and weaned at 2 days of age) described in a previous paper (2). Of the 21 early weaned pigs, 9 were scouring on the day they were killed. None of the sow-reared pigs scoured at any time during the experimental period.

Sampling procedure. Pigs were killed by an intracardiac overdose of pentobarbitone. They were dissected to expose the stomach, which after clamping at either end was removed. The stomach was slit along the line of greater curvature, the contents were removed, and the stomach wall was washed by gentle agitation in 100 ml of phosphate-buffered saline at pH 7.2. A circular sample (1 cm^2) was stamped out from the pars esophagea in the position shown in Fig. 1 and transferred to 10 ml of phosphate-buffered saline and shaken up and down manually ten times. This washing procedure was repeated twice. The washed disk was then transferred to a further 12 ml of phosphate-buffered saline and macerated with either a Griffith tube or a pestle and mortar. Decimal dilutions were made of the third wash and the macerate. These dilutions were surface inoculated onto reinforced clostridial medium containing horse blood (5%, vol/vol) and menadione (0.5 μg/ml). The plates were poured the day before and stored overnight in an atmosphere of 10% CO_2 in H_2. After inoculation, anaerobiosis was established by evacuating anaerobic jars containing cold catalysts, filling with 10% CO_2 in H_2, and repeating the procedure. The plates were incubated for 2 days at 37°C. On the basis of cell morphology and colony characteristics, representative colonies were selected and picked off into reinforced clostridial medium broth. One of each colony type of streptococcus and lactobacillus from each pig was subjected to a range of physiological tests for species identification.

Identification of isolates. Isolates were identified with miniaturized tests (16).

Microscopy. Transverse sections of the stomach fundus, duodenum, jejunum, and ileum were prepared for light microscopy as described below.

Samples of the pars esophagea were either taken from the area adjacent to the disk used for culture or from the disk area of pigs not used for culture. These samples were treated in three ways. (i) Samples were transferred to Shaudinn fluid, with transverse sections prepared and stained by the method of Gram (13) for examination by light microscopy. (ii) Samples were transferred to cacodylate-buffered glutaraldehyde fixative and processed as previously described (4) for conventional-transmission electron microscopy. Acrolein (5%, vol./vol) in the same buffer vehicle was used in place of glutaraldehyde on some occasions. Other samples were treated with the primary fixative described above but supplemented with 0.1% $HgCl_2$ for rapid fixation. These three methods of fixation were used in an effort to ensure adequate fixation by at least one. (iii) Samples were pinned to a sheet of dental wax, fixed by immersion in buffered 2.5% glutaraldehyde containing 0.5% Alcian blue 8GX for 2 h, and processed as described previously (4) for scanning electron microscopy.

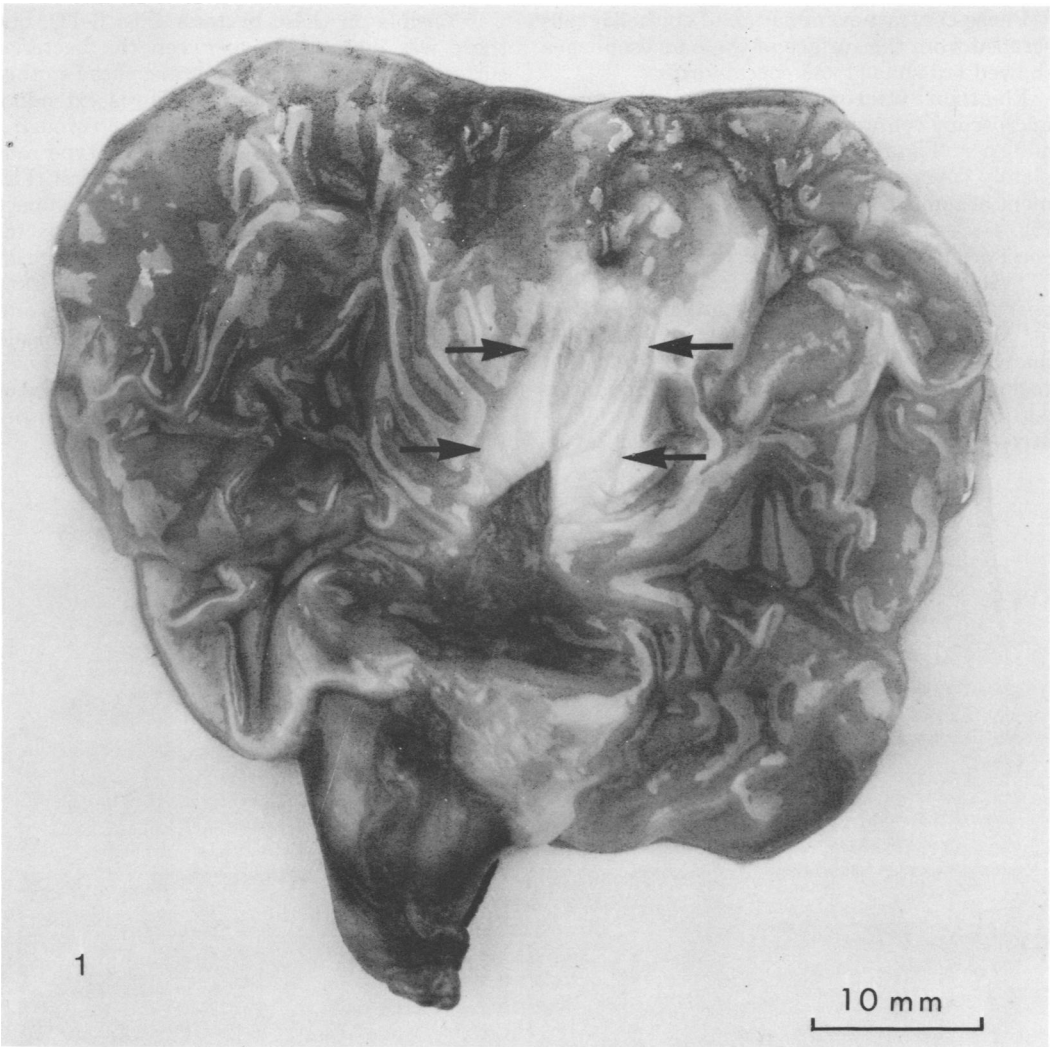


FIG. 1. *Pig stomach opened to show squamous epithelial surface of pars esophagea (arrows) (×2.2).*

Test for adhesion of bacteria to epithelial cells. Lactobacilli were grown overnight at 37°C in MRS medium (7), and streptococci were grown in yeastrel glucose broth. All isolates were tested in vitro for attachment to squamous epithelial cells from the esophagus of 16-week-old pigs. The test culture was centrifuged and resuspended in phosphate-buffered saline and the total count was adjusted by using a counting chamber to ca. 10^9 cells per ml. The epithelial cell suspension was prepared by gently brushing the esophageal surface and suspending the dislodged cells in phosphate-buffered saline. After washing once in phosphate-buffered saline, the concentration was adjusted by using a counting chamber to 10^6 cells per ml. A 1-volume amount of each suspension was mixed together to give a ratio of 1,000 bacteria to 1 epithelial cell. The mixture was incubated on a rotating platform (25 rpm) at 37°C for 30 min and then examined for adhesion by phase-contrast microscopy. Adhesive strains showed a concentration of organisms on the epithelial cells, whereas nonadhesive strains formed a uniform layer on and around the cells.

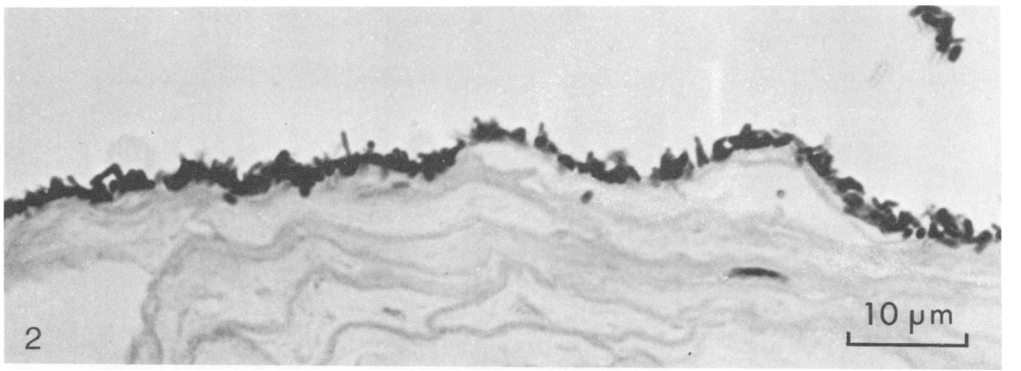
RESULTS

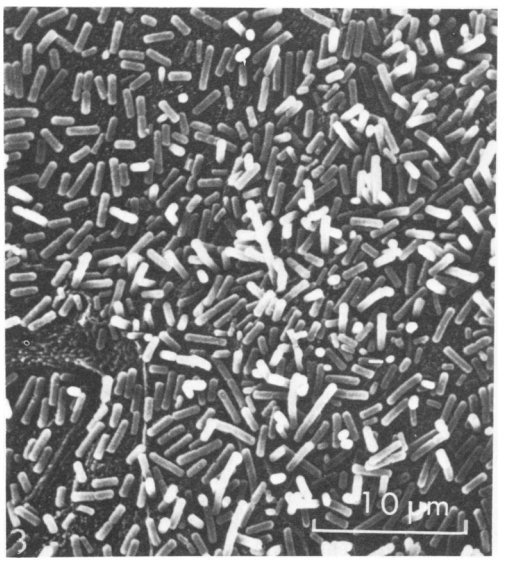
Light microscopy. No epithelium-associated bacteria were seen in the Gram-stained sections of the stomach fundus, duodenum, jejunum, and ileum. In the squamous area of the stomach (pars esophagea), gram-positive bacteria were seen on the surface of the epithelium. Under high magnification, these bacteria were seen to be rod shaped. The extent of the lining varied, but where it was most dense it had a palisaded appearance (Fig. 2) due to the attachment of the organisms at right angles to the epithelial surface.

Phase-contrast examination of epithelial cells scraped from the surface of the pars esophagea showed rod-shaped and coccal forms.

Electron microscopy. Scanning electron microscopy confirmed the presence of both rods and cocci. Figure 3 shows a piece of tissue completely covered with rods. The end-on attachment of some of these organisms can be seen. In Fig. 4 the surface is incompletely covered, and cocci are present as well as rods.

Transmission electron microscopy also demonstrated the end-on type of attachment (Fig. 5). Microcapsules can be seen associated with many of these bacteria. Micrographs of other regions showed several different microorganisms with gram-positive- and occasionally gram-negative-type cell walls (15). Extracellular layers were visible on these bacteria (Fig. 6–11), but there was often a gap between the bacterial surface and the plasma membrane of the epithelial cell that was bridged by fibrils extending from the bacterial capsule. Figures 6 through 9 show four examples of gram-positive-type rods with different types of capsule. In the first (Fig. 6) the extracellular layer is narrow and compact and gives rise to fibrils that run from the bacterium to the epithelial surface. The organism shown in Fig. 7 has a wide, solid capsule. Figure 8 shows an organism that could be a coccus or a transverse section through a rod. The capsule consists of a dense mat of fibers. Figure 9 shows a gram-positive-type coccus with a system of coarse fibers connecting bacterial cell to epithelium and also bacterial cell to bacterial cell.





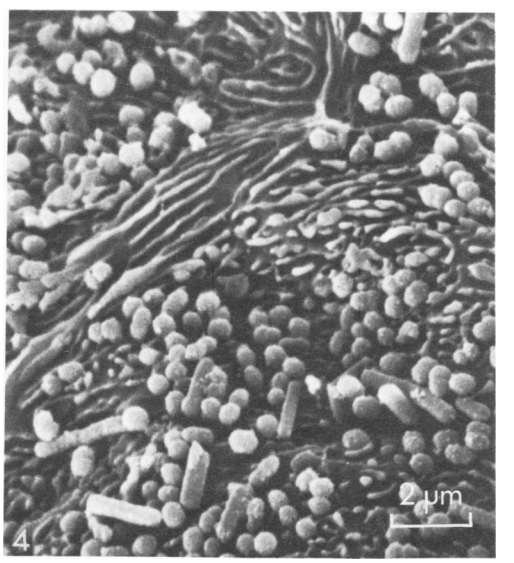


FIG. 2. *Transverse section through pars esophagea of pig stomach. Gram stain (×1,500).*

FIG. 3. *Scanning electron micrograph of surface of pars esophagea with rod-shaped bacteria attached (×2,000).*

FIG. 4. *Scanning electron micrograph of surface of pars esophagea with rod-shaped bacteria and cocci attached (×5,300).*

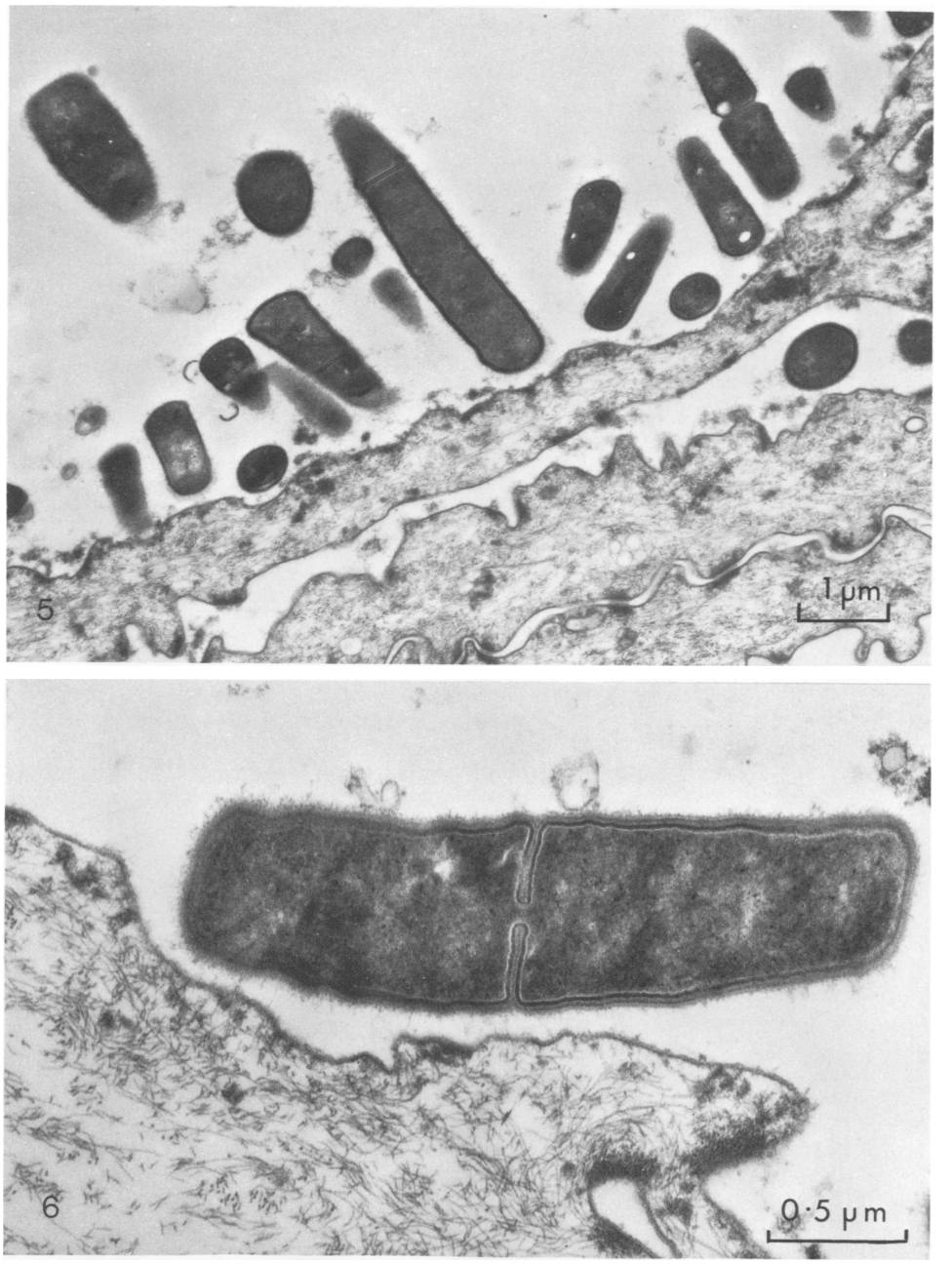


FIG. 5. *Bacteria attached to desquamating epithelial cell of pars esophagea. Many bacteria are attached at right angles to the epithelial surface and have fibrillar extracellular layer. Primary fixation with acrolein (×12,500).*

FIG. 6. *Gram-positive type bacterium with compact microcapsule. Primary fixation with acrolein (×37,500).*

The two morphological types seen in Fig. 10 and 11 both have a gram-negative type of cell wall. They were seen less frequently than the gram-positive organisms. The rod-shaped cell has a definite extracellular layer organized in the form of coarse fibers that appear to attach it to the epithelium. In the case of the coccus, there is no pronounced extracellular layer, although

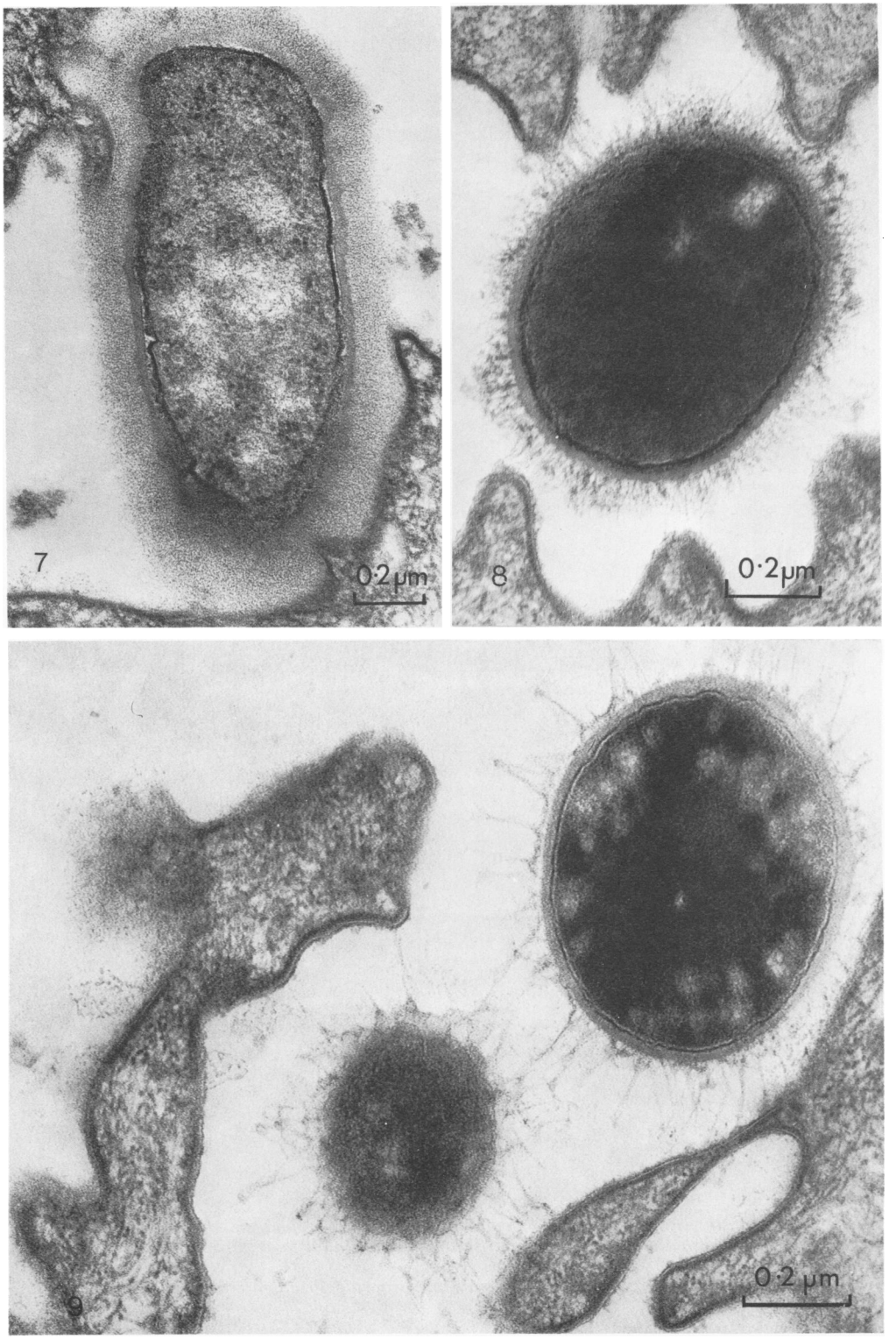


FIG. 7. *Gram-positive-type bacterium with thick microcapsule. Primary fixation with 6% glutaraldehyde containing 0.1% $HgCl_2$ (×51,500).*

FIG. 8. *Gram-positive type bacterium with fibrillar extracellular layer. Primary fixation with 6% glutaraldehyde (×70,100).*

FIG. 9. *Gram-positive-type coccus with coarse fibrils. Primary fixation with 6% glutaraldehyde (×78,750).*

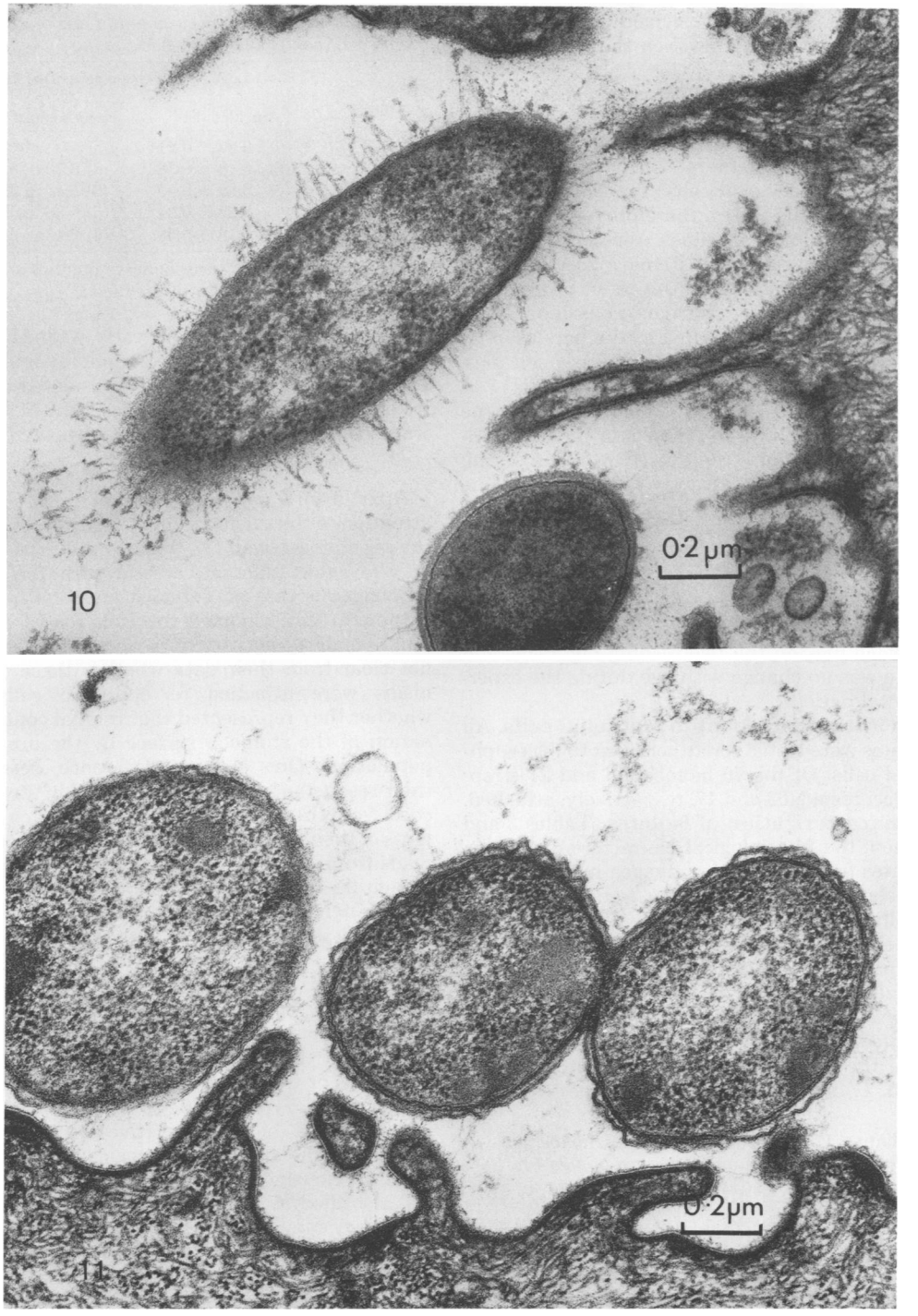


FIG. 10. *Gram-negative-type bacterium with coarse fibrils. Primary fixation with 6% glutaraldehyde containing 0.1% $HgCl_2$ (×58,500).*

FIG. 11. *Gram-negative-type coccus with poorly defined fibrils. Primary fixation with 6% glutaraldehyde containing 0.1% $HgCl_2$ (×58,500).*

some faint strands of electron-dense material can be seen running between the bacterial cell and the epithelium.

Bacterial counts. Microscopical examination of the macerates showed that neither method (Griffith tube or pestle and mortar) was completely effective in releasing all the bacteria attached to epithelial cells. The counts of bacteria in the macerates, therefore, should be regarded as minimum values. We considered organisms to be attached if the macerate count was equal to or exceeded the count of the third wash. By using this criterion, the proportion of pigs with bacteria attached to the pars esophagea was for sucking pigs, healthy weaned pigs, and scouring weaned pigs (15 of 20 [75%], 11 of 19 [92%], and 5 of 8 [63%], respectively). The mean $\log_{10}$ counts are shown in Table 1. Although the count is depressed in the scouring pigs, it was not significantly different from the count in the other two groups, and, therefore, in subsequent comparisons the results from healthy and scouring pigs were combined. A summary of the total viable count per square centimeter of the pars esophagea tissue is shown in Table 2. There was no significant difference between pigs on the two feeding regimens, and there was no change with age during the experimental period.

In vitro attachment to epithelial cells. All isolates were tested for attachment to pig esophageal cells. Of the 76 lactobacilli and 21 streptococci tested, 63 and 17, respectively, attached.

Characterization of isolates. Tables 3 and 4 show the physiological characteristics of the isolates from the pars esophagea macerate that attached in vitro. All isolates were either lactobacilli or streptococci. *Lactobacillus fermentum* was the commonest organism found attached to the pars esophagea epithelium. *L. acidophilus, L. salivarius* var. *salicinius, L. leichmannii, L. delbrueckii,* and two unclassified types were also isolated. *Streptococcus bovis* and *S. salivarius* were the only two species of streptococci isolated. *L. acidophilus* and its biotypes were isolated more frequently from early weaned pigs. All other species were more commonly found in suckled pigs. *S. salivarius* was isolated more frequently from sow-reared pigs, and *S. bovis* was isolated more frequently from weaned pigs.

TABLE 1. *Pooled mean values of numbers of bacteria from the pars esophagea of pigs 4 to 10 days of age*

Pigs	No. of pigs	Unbiased mean age (days)	$\log_{10}$ viable count per cm^2 (mean ± SEM)[a]
Healthy, sucking	20	6.40	6.00 ± 0.23
Healthy, early weaned	12	6.50	7.00 ± 0.29
Scouring, early weaned	9	6.89	5.32 ± 0.41

[a] SEM, Standard error of the mean.

TABLE 2. *Effects of weaning and age on the numbers of bacteria adhering to the pars esophagea*

Age of pig (days)	$\log_{10}$ viable count per cm^2 of:	
	Suckled pigs	Early weaned pigs
2	(6)[a] 6.87 ± 0.21[b]	
4	(6) 6.48 ± 0.38	(6) 6.30 ± 0.38
6	(6) 6.89 ± 0.49	(6) 6.44 ± 0.49
8	(6) 6.27 ± 0.21	(5) 6.36 ± 0.38
10	(4) 6.18 ± 0.37	(4) 6.15 ± 0.62

[a] Numbers in parentheses indicate number of pigs.
[b] Mean ± standard error of the mean.

DISCUSSION

Apart from a paper in 1965 that merely drew attention to the existence of bacteria attached to the pig stomach wall (7), the only previous work on the association of bacteria with the pars esophagea is that of Tannock and Smith (20). Although they reported the isolation of small numbers of various bacteria and yeasts, it was not clear from their data whether these organisms were attached to epithelial cells or whether they represented the residual contamination of the stomach surface by the lumenal population. One striking difference between their results and ours is the absence of streptococci from the pars esophagea and stomach contents of their pigs. However, their isolations were made from 16-week-old pigs of unknown origin, so that direct comparison is not possible.

Although gram-negative organisms were occasionally seen in the electron micrographs, only streptococci and lactobacilli were isolated from the macerates of the pars esophagea. The reason for this may be that gram-negative organisms were present in small numbers and were suppressed on the plates by the more numerous organisms or the techniques used may not have been sufficiently anaerobic to allow their recovery. Certainly, the gram-negative coccus bears a strong resemblance morphologically to those cocci previously isolated from the pig gut (10) and classified as *Acidaminococcus fermentans* (18). They may be present in the stomach as a result of ingesting milk contaminated with feces. Alternatively, they may be *Veillonella* sp. or *Neisseria* sp. washed down from the mouth; small numbers of both of these species have been found associated with the buccal mucosa of humans (17).

The finding of both streptococci and lactobacilli attached to pig epithelium contrasts

TABLE 3. *Physiological characteristics of adhering lactobacilli isolated from the pars esophagea of suckled and early weaned pigs*

Determination	Data							
No. of isolates	7	10	7	1	2	32	1	1
Growth at 15°C	−	−	−	−	−	−	−	−
45°C	+	+	+	+	+	+	+	+
CO_2 from glucose	−	−	−	−	−	+	+	+
NH_3 from arginine	−	−	−	+	+	+	+	+
Acid from:								
Arabinose	−	−	−	−	−	−(28)[a]	+	−
Rhamnose	−	−	+	+	−	−	−	−
Xylose	−	−(5)	−	−	−	−(30)	+	−
Cellobiose	+	+	−	+	−	−	−	+
Melibiose	−(5)	−(5)	+	−	−	+(31)	+	+
Trehalose	+	−(2)	+	−	−	−(30)	+	+
Melezitose	−	−	−	−	−	−	−	−
Raffinose	−(5)	+(7)	+	−	−	+	+	+
Mannitol	−	−(7)	+	−	−	−	−	−
Sorbitol	−	−(9)	+	+	−	−	−	−
Amygdalin	+	+(5)	−	+	−	−(30)	+	−
Salicin	+	+(5)	+	−	−	−(31)	+	+
Identification	*L. acidophilus*	*L. acidophilus* biotypes	*L. salivarius* var. *salicinius*	*L. leichmannii*	*L. delbrueckii*	*L. fermentum*	Unidentified heterofermentative	biotypes
% Incidence in pigs								
Suckled (26)[b]	7.69	15.38	19.23	3.85	7.69	92.31	3.85	3.85
Early weaned (21)[b]	23.81	28.57	9.52	0	0	38.09	0	0

[a] Figures in parentheses show number of strains giving reaction indicated.
[b] Number of pigs in each group.

TABLE 4. *Physiological characteristics of adhering streptococci isolated from the pars esophagea of suckled and early weaned pigs*

Determination	Results	
No. of isolates	7	11
Tolerance of 40% bile	+	−
Hydrolysis of aesculin	+	+
Hydrolysis of starch	−	+
Growth in 6.5% NaCl	−	+
NH_3 from arginine	−	−
Growth at pH 9.6	−	−
Hydrolysis of gelatin	−	−
Reduction of TTC[a]	−	−
Tolerance of 0.04% K_2TeO_3	−	−
Acid from:		
Arabinose	−	−
Lactose	+	+
Melibiose	−	+
Melezitose	−	−
Raffinose	+	+
Mannitol	−	+
Glycerol	−	−
Identification	*S. salivarius*	*S. bovis*
% Incidence in pigs		
Suckled (26)[b]	19.23	19.23
Early weaned (21)[b]	9.52	28.57

[a] 2,3,5-Triphenyl tetrazolium chloride.
[b] Number of pigs in each group.

strongly with the chicken, in which only lactobacilli attach (10), but is similar to the mouse stomach squamous epithelium (19) and to human buccal mucosa, (14, 21) in which both genera are found. Indeed, the similarity to human oral flora goes further because in both habitats *S. salivarius* and *L. fermentum* are commonly found (21). The extracellular layers seen on the organisms attached to the pig stomach are similar to those described for bacteria adhering to other surfaces, such as human buccal cells (6), rat tongue (3), chicken crop (4), plant fragments in the bovine rumen (1), and solid marine surfaces (9). The consistent presence of these microcapsules on attached organisms in these varied habitats in itself argues strongly for their having an essential role in the attachment process.

If the whole of the pars esophagea was covered with a confluent layer of bacteria with a cross sectional area of 1 μm^2 attached end on to the epithelium, it would have about 10^8 bacteria per cm^2 attached to it. The stratified squamous epithelium, of which the pars esophagea is composed, is continuously desquamating releasing cells with attached bacteria into the lumen to inoculate the food. Thus, these attached bacteria could prove to be an important mechanism for regulating the composition of the stomach microflora by supplying a continuous inoculum of specific lactobacilli and streptococci for the food as it enters the stomach, thus ensuring the dominance of lactic acid bacteria in the gastric contents. This situation is analogous to that in the chicken, in which the crop is also lined with stratified squamous epithelium and has large numbers of lactobacilli attached to it (13), which suppress the growth of *Escherichia coli* (11, 12). If this is the role of lactobacilli in the stomach of the sucking pig where numbers of *E. coli* are low, the mechanism seems to have broken down in the early weaned pig where the *E. coli* are considerably increased (2). Although the breakdown of this regulating influence is not reflected in the counts of bacteria per square centimeter of pars esophagea, the observed qualitative differences in the attached flora may be important in this context.

ACKNOWLEDGMENTS

We wish to thank C. Ogden, British Museum (Natural History), for the scanning electron micrographs, A. Turvey for light microscopy, D. E. Hobbs and S. O. Porcas for technical assistance, and H. D. Keal for care of the animals.

One of the authors (P.A.B.) was supported by a grant from the Meat and Livestock Commission.

LITERATURE CITED

1. **Akin, D. E.** 1976. Ultrastructure of rumen bacterial attachment to forage cell walls. Appl. Environ. Microbiol. **31**:562-568.
2. **Barrow, P. A., R. Fuller, and M. J. Newport.** 1977. Changes in the microflora and physiology of the anterior intestinal tract of pigs weaned at 2 days, with special reference to the pathogenesis of diarrhea. Infect. Immun. **18**:586-595.
3. **Brady, J. M., W. A. Gray, and W. Lara-Garcia.** 1975. Localization of bacteria on the rat tongue with scanning and transmission electron microscopy. J. Dent. Res. **54**:777-782.
4. **Brooker, B. E., and R. Fuller.** 1975. Adhesion of lactobacilli to chicken crop epithelium. J. Ultrastruct. Res. **52**:21-31.
5. **Brownlee, A., and W. Moss.** 1961. The influence of diet on lactobacilli in the stomach of the rat. J. Pathol. Bacteriol. **82**:513-516.
6. **Collan, Y., and P. Sainio.** 1970. Relationship of bacteria to exfoliated oral cells. An electron microscopic study. Acta Cytol. **14**:570-573.
7. **De Man, J. C., M. Rogosa, and M. E. Sharpe.** 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23**:130-135.
8. **Dubos, R., R. W. Schaedler, R. Costello, and P. Holt.** 1965. Indigenous, normal and autochthonous flora of the gastrointestinal tract. J. Exp. Med. **122**:67-76.
9. **Fletcher, M., and G. D. Floodgate.** 1973. An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. J. Gen. Microbiol. **74**:325-334.
10. **Fuller, R.** 1966. Some morphological and physiological characteristics of Gram negative anaerobic bacteria isolated from the alimentary tract of the pig. J. Appl. Bacteriol. **29**:375-379.
11. **Fuller, R.** 1973. Ecological studies of the lactobacillus flora associated with the crop epithelium of the fowl. J. Appl. Bacteriol. **36**:131-139.
12. **Fuller, R.** 1977. The importance of lactobacilli in maintaining normal microbial balance in the crop. Br. Poult. Sci. **18**:85-94.
13. **Fuller, R., and A. Turvey.** 1971. Bacteria associated

with the intestinal wall of the fowl (*Gallus domesticus*). J. Appl. Bacteriol. **34**:617–622.

14. **Gibbons, R. J., and J. van Houte.** 1971. Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. Infect. Immun. **3**:567–573.
15. **Glauert, A. M., and M. J. Thornley.** 1969. The topography of the bacterial cell wall. Annu. Rev. Microbiol. **23**:159–198.
16. **Jayne-Williams, D. J.** 1976. The application of miniaturized methods for the characterisation of various organisms isolated from the animal gut. J. Appl. Bacteriol. **40**:189–200.
17. **Liljemark, W. F., and P. J. Gibbons.** 1971. Ability of *Veillonella* and *Neisseria* species to attach to oral surfaces and their populations present indigenously. Infect. Immun. **4**:264–268.
18. **Rogosa, M.** 1969. *Acidaminococcus* gen. n., *Acidaminococcus fermentans* sp.n., anaerobic gram-negative diplococci using amino acids as the sole energy source for growth. J. Bacteriol. **98**:756–766.
19. **Savage, D. C., R. Dubos, and R. W. Schaedler.** 1968. The gastrointestinal epithelium and its autochthonous bacterial flora. J. Exp. Med. **127**:67–76.
20. **Tannock, G. W., and J. M. B. Smith.** 1970. The microflora of the pig stomach and its possible relationship to ulceration of the *pars oesophagea*. J. Comp. Pathol. **80**:359–367.
21. **van Houte, J., R. J. Gibbons, and A. J. Pulkkinen.** 1972. Ecology of human oral lactobacilli. Infect. Immun. **6**:723–729.