APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1978, p. 782-790
0099-2240/78/0035-0782$02.00/0

Vol. 35, No. 4
Printed in U.S.A.

Bacteria Isolated from the Duodenum, Ileum, and Cecum of Young Chicks

J. P. SALANITRO,[1]* I. G. BLAKE,[1] P. A. MUIRHEAD,[1] M. MAGLIO,[2] AND J. R. GOODMAN[3]

Shell Development Company, Biological Sciences Research Center, Modesto, California 95352,[1] and Departments of Anatomy[2] and Pediatrics,[3] University of California School of Medicine, San Francisco, California 94143

Received for publication 18 October 1977

Facultatively anaerobic and strictly anaerobic bacteria colonizing the intestinal tracts of 14-day-old chicks fed a corn-based diet were enumerated, isolated, and identified. Colony counts from anaerobic roll tubes (rumen fluid medium) or aerobic plates (brain heart infusion agar) recovered from homogenates of the duodenum, upper and lower ileum, and cecum varied appreciably among samples from individual birds. Anaerobic and aerobic counts from the duodenum and ileum were similar. Anaerobic counts were highest from the cecum (0.7×10^{11} to 1.6×10^{11}/g of dry tissue) and exceeded aerobic plate counts by a factor of at least 10^2. Facultatively anaerobic groups (*Streptococcus, Staphylococcus, Lactobacillus,* and *Escherichia coli*) comprised the predominant flora of the duodenum and ileum, although large numbers of anaerobes (9 to 39% of the small intestine isolates), represented by species of *Eubacterium, Propionibacterium, Clostridium, Gemmiger,* and *Fusobacterium,* were also recovered. Strict anaerobes (anaerobic gram-positive cocci, *Eubacterium, Clostridium, Gemmiger, Fusobacterium,* and *Bacteroides*) made up nearly the entire microbial population of the cecum. Scanning electron microscopy of the intestinal epithelia of chicks revealed populations of microbes on the duodenal, ileal, and cecal mucosal surfaces.

Bacterial colonization of the intestinal tract of chicks takes place soon after hatching when the young animals ingest food (24, 30). During the first 2 to 4 days, streptococci and enterobacteria colonize the small intestine and cecum. The work of Ochi et al. (24) also indicates that after the first week the composition of the flora stabilizes in that lactobacilli predominate in the small intestine (with smaller numbers of streptococci and enterobacteria), whereas the cecum is colonized mainly by anaerobes (*Bacteroides* and bifidobacteria) and fewer numbers of facultative bacteria. Other studies on the development of the intestinal flora in chickens (2, 15, 17, 32) show qualitatively similar results in that lactobacilli and fecal streptococci can be isolated from the duodenum and ileum, and *Clostridium, Bacteroides,* anaerobic lactobacilli, and *Escherichia coli* can be recovered from the cecum. Nearly all of this work on the intestinal microbiology of chicks has involved the use of selective plate media for recovery and enumeration of specific bacterial populations.

In this study we report on the enumeration and isolation of the predominant bacteria colonizing the intestinal tract of growing chicks (14 days old) by using nonselective anaerobic roll-tube culture media. Previous work in our laboratory demonstrated that use of a rumen fluid medium similar to one used for culturing ruminal bacteria enabled us to recover large numbers of chicken cecal anaerobes (25, 26). Data on the distribution of these bacteria along the intestinal tract indicate that several types of anaerobic and facultatively anaerobic bacteria can colonize the small intestine as well as the cecum. Electron microscopy of chick intestinal tract sections show the association of these indigenous bacteria with the epithelia.

MATERIALS AND METHODS

Animals and diet. One-day-old broiler cockerels (Hubbard cockerel × Hubbard hen), weighing approximately 40 g, were obtained from a commercial facility (Foster Poultry Farms, Livingston, Calif.) and fed a typical chick starter corn-soybean ration containing 62.5% corn-21% protein. Birds were housed three per cage in wire cages measuring 14 inches (length) by 14 inches (width) by 16 inches (height) (ca. 35.5 by 35.5 by 40.6 cm) and given feed and water without restriction for 14 days. Housing temperature was maintained at 35°C for the first 4 to 5 days and then kept at 25°C for the remaining growth period. The diet was supplemented with a coccidiostat, 0.0125% amprolium-0.004% ephopabate, as the only added drug.

Intestinal sampling and processing. Fourteen-day-old chicks weighing 200 to 230 g were sacrificed

by CO_2 asphyxiation. By using aseptic techniques, sections of the intestine were ligated and removed from the dissected body cavity of six individual animals. Approximately 5-cm lengths of intestinal tract with tissue and contents intact were taken from the duodenum, upper ileum (anterior to yolk stalk), lower ileum (anterior to the ileocecal valve), and cecum (one entire horn) of each animal. Intestinal sections were placed in sterile, preweighed, rubber-stoppered tubes (18 by 150 mm; Bellco), which were flushed with 100% CO_2, and the wet weight was obtained. An amount of anaerobic dilution solution (4) was added to make an initial 1:10 dilution. Tissues were homogenized for about 1 min under a steady flow of CO_2 with a Polytron probe (Type OT 10-24-350D; Brinkmann) set at medium speed. To minimize cross-contamination between samples, the probe was rinsed with 95% ethanol and at least three, 100-ml changes of sterile, distilled water.

For dry-weight determination on tissue samples, portions of the homogenate, e.g., 1 ml of a 1:10 dilution, were pipetted in duplicate into preweighed aluminum pans, placed in an oven at 180°C for 5 days, and dried to a constant weight. Microscopic bacterial cell counts were made from 1:1,000 dilutions of tissue homogenates as described previously (25). For determining viable bacterial cell counts, homogenates were serially diluted in anaerobic dilution solution under CO_2 from 10^{-3} to 10^{-10}, and 0.2- to 1.0-ml portions were added to roll tubes of M98-5 agar medium for anaerobic counts or plates of brain heart infusion agar (BHIA; Difco), pour plate method, for aerobic-facultative counts.

Culture methods and isolation of intestinal bacteria. Anaerobic methodology, composition, and preparation of M98-5 rumen fluid roll-tube medium were the same as reported previously (26). Four replicate roll tubes or plates were made for each selected dilution from a homogenate. Mean colony counts were determined after 7 and 3 days of incubation at 37°C for anaerobic roll tubes and aerobic plates, respectively. Percent recoveries of anaerobes from ceca with M98-5 medium were calculated from the ratio of roll-tube counts to microscopic bacterial cell count × 100. Depending upon the section, at least 25 to 50 colonies were picked from roll tubes. Colonies from tubes containing the highest dilution, usually 10^{-3} to 10^{-4} from the duodenum, 10^{-5} to 10^{-7} from the ileum, and 10^{-8} to 10^{-10} from the cecum, were stabbed into rubber-stoppered slants (13 by 100 mm) of the same medium, and 24- to 72-h cultures were examined for purity, morphology, and Gram-stain reaction (Kopeloff modification; 14). About 20 to 30 colonies from BHIA aerobic plates were picked from plates containing 20 to 50 colonies and transferred to BHIA slants and also examined for purity, morphology, and Gram-stain reaction.

Identification of intestinal strains. Roll-tube isolates were subcultured to serum bottles (5-ml capacity; Wheaton) containing 3 ml of prereduced, anaerobically sterilized peptone-yeast extract-glucose (PYG) medium. For medium composition and preparation of serum bottles see references 14 and 19. After 7 days of incubation at 37°C, the following features were determined from growth of the isolates in PYG medium and used to group and presumptively identify similar isolates: glucose fermentation (terminal pH), catalase, H_2 gas production, and fermentation acid and products from glucose. Additional carbohydrate fermentation and physiological tests performed on anaerobic isolates were made as described in the Virginia Polytechnic Institute (VPI) *Anaerobe Laboratory Manual* (14). Prereduced culture media for these tests, however, were dispensed in 5-ml serum bottles (Wheaton) containing 3 ml of medium per bottle according to the method described by Miller and Wolin (19). Test media were inoculated with 0.05 ml of culture and incubated for 7 days at 37°C before analysis. Fermentation and biochemical features were used to identify strains according to classification schemes of Holdeman and Moore (14) and by comparison with other published data on anaerobes. Similarly, facultatively anaerobic isolates, i.e., gram-positive and -negative rods and gram-positive cocci, from roll tubes and aerobic plates were subjected to several pertinent fermentation and biochemical tests for presumptive identification of strains.

Fermentation acids such as formic, acetic, propionic, butyric, lactic, and succinic were identified from PYG culture filtrates as described previously (27). Hydrogen in the gas phase of serum bottle cultures was determined by a thermal conductivity-gas chromatographic method (5). Ethanol in fermentation media was analyzed enzymatically by using alcohol dehydrogenase (Sigma kit no. 331-UV).

Electron microscopy of intestinal sections. The preparation of intestinal sections for scanning electron microscopy (SEM) was similar to that described by Savage and Blumershine (29). Six 14-day-old chicks were sacrificed, the body cavities were exposed, and cotton pads were soaked in cold (4°C) fixative and placed over the exposed intestinal tracts. The fixative consisted of 2% glutaraldehyde in 0.10 M phosphate buffer (pH 7.3). Cold fixative was injected into each lumen to fill each ligated section of about 3 cm in length from the duodenal, upper and lower ileal, and cecal areas. These were the same four sections as described above. After 5 to 10 min, 0.5-cm squares of tissue were cut from each intestinal loop and affixed with straight pins onto pieces of dental wax with the epithelial side exposed. Sections were placed in cold fixative for 1 h and then stored in fresh fixative for 24 h. After several changes with cold phosphate buffer, the sections were postfixed with 2% OsO_4 in phosphate buffer (pH 7.3) for 2 h, again washed free of fixative with buffer, and dehydrated through a graded series of ethanol-water to absolute ethanol. For SEM, the samples were dried in a critical-point drying apparatus using liquid CO_2, fixed to stubs with silver conducting paint, and coated with gold about 20-nm thick in a sputter coater. Coated specimens were viewed in a Cambridge S4 scanning electron microscope (Cambridge Instrument Co., Inc., Cambridge, England), and photographs were taken at 20 kV with type 55 Polaroid positive-negative film.

For transmission electron microscopy (TEM), sections of the intestinal tract were prefixed with glutaraldehyde-phosphate buffer as described for the SEM preparations. They were then cut into small 0.5-mm^3 pieces and postfixed in 1% OsO_4-0.10 M phosphate buffer (pH 7.3) for 30 min at room temperature. Sec-

tions were washed with buffer to remove fixative, dehydrated through a series of increasing concentrations of ethanol solution followed by propylene oxide, and embedded in Epon-Araldite mixture. Thin sections were cut with an ultramicrotome (LKB), mounted on copper grids, and finally stained with uranyl acetate and lead citrate. Sections were examined in an electron microscope (RCA-EMU-3F) operating at 50 kV.

RESULTS

Culture counts of bacteria from the intestine and cecum. Anaerobic and aerobic intestinal bacterial populations recovered on rumen fluid (M98-5) roll-tube medium, and BHIA plate medium, respectively, are given in Table 1. Culture counts from dilutions of homogenates of the upper and lower ileal sections indicated that anaerobic population levels (10^6 to 10^7); anaerobic and aerobic counts in the duodenum were similar. Anaerobic and aerobic counts, however, were greatest in the cecum compared with those of other sections. In the cecum, anaerobic counts exceeded aerobic counts by at least a factor of 10^2. Large variations were noted in both culture counts when comparisons were made of the same section from different chicks. At least part of this variation was because the quantity of contents in the intestinal sections differed among animals at the time of sacrifice.

It is not known what proportion of the total number of bacteria present in the duodenal and ileal regions was actually cultured in the anaerobic and aerobic media. With most of these samples, bacterial cell counts were too low and/or tissue cells and debris interfered with accurate microscopic enumeration from dilutions of tissue homogenates. Anaerobic counts from chick ceca, however, accounted for 45% (mean of six samples; range, 24 to 77%) of the total microscopic bacterial count. These roll-tube culture count recoveries were similar to those reported for the same medium (M98-5) in previous studies in which the predominant cecal bacteria were isolated from older birds (25, 26).

Isolation and identification of intestinal strains. Colonies from roll tubes were isolated from four sections from each of six birds (14 days of age) on the same corn diet. Isolates were grouped and tentatively classified on the basis of morphology, Gram stain, ability to ferment glucose, and products formed from glucose fermentation (Table 2). Other carbohydrate fermentation, physiological, and biochemical tests used for aiding in identifying anaerobes were as described in the VPI *Laboratory Manual* (14).

(i) Facultatively anaerobic bacteria. The types of facultative anaerobes isolated from the small intestine and cecum included *Streptococcus, Staphylococcus, Lactobacillus,* and *Escherichia coli.* Two types of gram-positive, catalase-negative cocci were presumptively identified as belonging to group D enterococcus (*Streptococcus faecalis*) and viridans non-group D enterococcus on the basis of tests (bacitracin sensitivity, hippurate hydrolysis, bile-esculin hydrolysis, and tolerance to 6.5% NaCl) described by Facklam et al. (8). Strains resembling *S. faecalis* (about 10% of the streptococci) were further confirmed on the basis of other fermentation and physiological features (7). Catalase and coagulase-positive, β-hemolytic cocci were identified as *Staphylococcus aureus* (16); only a few strains of this species were isolated from the duodenum and ileum of only one animal.

Strains of catalase-negative, gram-positive rods that grew anaerobically (GasPak jar) or aerobically on blood agar plates or *Lactobacillus*-selective agar (BBL) were tentatively identified as *Lactobacillus* (10). These isolates, which grew well only in PYG media containing 0.05% Tween 80 or in chopped meat-glucose media, produced large amounts of lactic acid (40 to 75 μmol/ml; optical isomer mainly LDL; see

TABLE 1. *Culture counts of bacterial populations from the intestinal tract of chicks (14 days of age)*

Sample[a]	Intestinal section (colonies/g [dry wt] of tissue)							
	Duodenum		Upper ileum		Lower ileum		Cecum	
	Anaerobic[b] (× 10^4)	Aerobic[c] (× 10^4)	Anaerobic (× 10^7)	Aerobic (×10^6)	Anaerobic (× 10^7)	Aerobic (× 10^6)	Anaerobic (× 10^{11})	Aerobic (× 10^8)
A	6.5	1.6	1.2	0.8	2.4	8.9	0.7	4.4
B	2.1	14.9	13.1	75.5	0.6	0.3	1.3	11.9
C	2.6	7.5	0.7	8.5	39.5	14.4	1.6	6.4
D	1.5	1.7	0.2	1.7	0.1	0.6	1.5	0.5
E	68.6	ND[d]	13.0	ND	12.5	ND	1.0	1.9
F	17.7	41.7	6.2	1.8	39.0	19.3	0.9	17.7

[a] From individual animals.
[b] Roll tube counts in M98-5 medium.
[c] Plate counts in BHIA medium.
[d] ND, No data.

TABLE 2. *Presumptive identification features of intestinal bacteria isolated from chicks*[a]

Morphological group	Gram reaction	A/F	Glucose fermentation			Tentative identification
			Reaction	H_2 gas	Products	
I Coccus A (catalase negative)	+	F	a	−	L(fa)	*Streptococcus*
Coccus B (catalase positive)	+	F	a	−	L(Fa)	*Staphylococcus*
II Pleomorphic rod	+ (Var)	F(A)	a	−	L[b]	*Lactobacillus*
III Fermentative rod	−	F	a	−	Las(F)	*E. coli*
IV Chain-forming coccus	+	A	a	+	A(fle)	Anaerobic coccus
V Pleomorphic and fusiform rod						
a	+	A	a	−	AF1	*Eubacterium*
b	+	A	a	+	FL(as)	*Eubacterium*
c	+ (Var)	A	−	−	af	*Eubacterium*
d	+ (Var)	A	a	+	AS(f)	*Eubacterium*
e	+ (Var)	A	w	−	lb	*Eubacterium*
f	Var	A	w	+	a(f)	*Eubacterium*
g	Var	A	a	+	Bfa	*Eubacterium*
VI Pleomorphic rod	+	A	a	−	Pas	*Propionibacterium*
VII Spore-forming rod						
a	+ (−)	A	a	+	FAB(l)	*Clostridium*
b	−	A	a	+	Fab (le)	*Clostridium*
VIII Budding bacteria	−	A	w	+(−)	fba	*Gemmiger*
IX Fusiform rod						
a	−	A	a	+(−)	BLa(f)	*Fusobacterium*
b	−	A	a	+	BA	*Fusobacterium*
c	−	A	w	+	b	*Fusobacterium*
X Pleomorphic and fusiform rod						
a	−	A	a	+(−)	AF(e)	*Bacteroides*
b	−	A	−	+	a(s)	*Bacteroides*
c	−	A	a	−	Fa	*Bacteroides*
d	−	A	a	+	A	*Bacteroides*

[a] Data compiled from 983 roll tube isolates obtained from the duodenum, upper and lower ileum, and cecum of six chicks. Symbols: A, Anaerobic; F, facultatively anaerobic; +, positive; var, variable; −, negative; glucose fermentation (PYG medium): a, acid (final pH <5.5); w, weak (pH 5.6 to 6.2); −, not fermented (pH >6.2); gas (H_2) and glucose end products: e, ethanol; Ff, formic; Aa, acetic; Pp, propionic; Bb, butyric; Ll, lactic; Ss, succinic. Upper case letters refer to products formed in amounts of 10 μmol/ml of medium or greater, and lower case letters refer to amounts less than 10 μmol/ml. Symbols in parentheses are for a few isolates within a group.

[b] Produces large amounts of lactic acid with good growth only in PYG medium containing Tween 80 (0.05%).

reference 14 for method). Subsequent identification on the basis of sugar fermentation (fermented amygdalin, cellobiose, esculin, fructose, glucose, lactose, maltose, salicin, starch, and sucrose) and physiological tests indicated that these organisms were similar to *L. acidophilus* (14).

Gram-negative bacteria, which grew as pink colonies on plates of MacConkey medium (Difco), were identified in Enterotubes (Roche Diagnostics; 33) as *E. coli*. Aerobic isolates picked from BHIA plates were also similar to roll tube isolates of *Streptococcus, Lactobacillus,* and *E. coli*.

(ii) Anaerobic bacteria. Anaerobic gram-positive cocci, producing large amounts of acetate, could not be identified with certainty. However, these strains resembled *Ruminococcus obeum* (22) in sugar utilization and products from glucose. Gram-positive to gram-variable pleomorphic, fusiform rods (Table 2, group V) were tentatively identified as belonging to the genus *Eubacterium* (13). Group Va (eight strains) were characteristically very homogeneous, fermenting only arabinose, cellobiose, fructose, glucose, glycogen, lactose, maltose, melezitose, raffinose, rhamnose, starch (and hydrolysis), sucrose, and xylose, and were nonreactive in other tests (gelatin, nitrate, and indole). They consistently produced large amounts of acetate (41 to 50 μmol/ml) and formate (11 to 16 μmol/ml) with small amounts of lactate (1 to 5

μmol/ml), but no hydrogen gas. Although these acid products were similar to those of other strains of *Eubacterium* described (12, 21), they did not resemble known species of the genus in carbohydrate fermentation pattern. Group Vb organisms had features similar to those of *Eubacterium aerofaciens* (13), except that the strains isolated in this study fermented starch. Nonfermentative strains of *Eubacterium* of group Vc resembled *E. lentum* (13). Another homogeneous group of bacteria were the succinate-producing *Eubacterium* strains of group Vd. These organisms fermented many sugars (arabinose, cellobiose, esculin, fructose, glucose, lactose, maltose, melibiose, raffinose, rhamnose, ribose, salicin, starch, sucrose, trehalose, and xylose) and may be related to other succinate-producing *Eubacteria* isolated from human feces (12, 21) and chicken ceca (1, 25). Other groups of *Eubacteria* given in Table 2 could not be identified as belonging to known species. Pleomorphic gram-positive rods producing major amounts of propionic acid were identified as *Propionibacterium acnes* on the basis of comparison of similar characteristics from known species (20).

Of the spore-forming rods isolated (groups VIIa and b), only VIIb could be tentatively identified as being similar to *Clostridium beijerinckii* (31). Both groups of organisms were similar in morphology (fusiform-shaped with terminal spores) and in acids formed from glucose fermentation; however, VIIb differed from VIIa in fermenting amygdalin, arabinose, cellobiose, lactose, and ribose.

Gram-negative budding bacteria (group VIII), isolated from the duodenum and cecum, resembled known strains of *Gemmiger formicilis* from human feces (11) and chicken ceca (25). The isolates identified in this study produced formic, butyric, and acetic acids with small amounts of hydrogen and fermented (variable) glucose, lactose, raffinose, and trehalose.

The gram-negative, pleomorphic, fusiform-shaped, anaerobic rods isolated were presumptively identified (22) as *Fusobacterium* sp., producing butyric acid as a major fermentation product, and *Bacteroides* sp., producing acids other than succinic and lactic from glucose. Since only a few strains from each group were studied, none of the isolates could be identified as being similar to known species of *Fusobacterium* and *Bacteroides*.

Distribution of bacteria in the intestinal tract. The distribution of the ten major bacterial groups isolated from six chicks is given in Table 3. The majority (65 to 85%) of organisms isolated from the duodenum, ileum, and cecum were gram positive. Most of the bacteria from the small intestine were facultatively anaerobic, whereas strict anaerobes comprised most of the cecal microflora. Streptococci, lactobacilli, and *E. coli* accounted for about 60 to 90% of the bacteria in the duodenum and upper and lower ileum. *Staphylococcus* and *Propionibacterium* were infrequently isolated from the intestinal tract. It is worth noting that strict anaerobes (9 to 39% of the total isolated) were obtained from the small intestine and that the most diverse types were isolated from the duodenum. *Eubac-*

TABLE 3. *Distribution of intestinal bacteria in chicks*

Bacterial group[a]	% Total isolates[b] (frequency of isolation[c])			
	Duodenum	Upper ileum	Lower ileum	Cecum
I a) *Streptococcus*	36.6 (6/6)	8.9 (2/6)	16.8 (3/6)	0.7 (1/6)
b) *Staphylococcus*	0.4 (1/6)	—[d]	0.5 (1/6)	—
II *Lactobacillus*	19 (4/6)	33.8 (5/6)	59 (5/6)	—
III *Escherichia coli*	5.4 (1/6)	33 (3/6)	14.7 (2/6)	—
IV Anaerobic coccus	1.8 (2/6)	0.9 (1/6)	0.5 (1/6)	14.2 (5/6)
V *Eubacterium*	26.4 (4/6)	22.6 (2/6)	7.8 (2/6)	60.6 (6/6)
VI *Propionibacterium*	0.3 (1/6)	0.4 (1/6)	—	—
VII *Clostridium*	1.8 (2/6)	0.4 (1/6)	—	2.1 (1/6)
VIII *Gemmiger*	1.5 (1/6)	—	—	3.4 (5/6)
IX *Fusobacterium*	3.7 (4/6)	—	0.5 (1/6)	6.2 (5/6)
X *Bacteroides*	—	—	—	12.8 (5/6)
XI Unknown anaerobic species	3.1 (3/6)	—	0.2 (1/6)	—
% Facultatively anaerobic	61.4	75.7	91	0.7
% Anaerobic	38.6	24.3	9	99.3

[a] Bacterial groups given in Table 2.

[b] Number of isolates identified as belonging to a group per total number of isolates cultured from each tissue section.

[c] Number of animals from which bacterial group was isolated out of six samples from individual chicks.

[d] —, No strains isolated.

terium species were the most frequently isolated anaerobic groups from the small intestine and cecum. Strains of *Eubacterium* group Va accounted for more than half of all *Eubacteria* isolated in the duodenum and ileum, whereas those from the cecum were mainly groups Vd and f. Other kinds of anaerobes (e.g., anaerobic cocci, and strains of *Clostridium, Gemmiger,* and *Fusobacterium*) were recovered from the small intestine in smaller numbers (up to 9% of the total in the duodenum). The predominant anaerobes recovered from the cecum included a gram-positive coccus and *Eubacterium, Clostridium, Gemmiger, Fusobacterium,* and *Bacteroides* species.

Electron microscopy of the intestinal epithelia. SEM was used to visualize the bacterial populations inhabiting the epithelial surfaces of the intestinal tracts of chicks. Rod-shaped bacteria (similar to lactobacilli in morphology) were observed lying on the surface of the duodenal mucosa (Fig. 1A). These organisms seemed to "associate" loosely with the epithelium. Their infrequent appearance in SEM of duodenal sections indicated that they may be easily dislodged during preparation of specimens. Fixation of tissues in glutaraldehyde containing ruthenium red, a technique used for staining mucosubstances in chicken crop tissue (3), had no effect in preserving adherence of more bacteria to the epithelium. In the upper ileum, four to five different bacterial types (rods and cocci) could be seen lying on the epithelium (Fig. 1B). SEM observations of the lower ileum, however, revealed normal broad, finger-shaped villi, but bacteria were rarely seen on the epithelial surface. The largest populations of bacteria from all surfaces of the intestine were observed on the cecum (Fig. 1C). Rods and cocci adhered throughout the epithelial surface and in cecal folds. Occasionally it was noticed that bacteria appeared to be located in and around the rim areas of cecal "pores" (Fig. 1D).

TEM of thin sections from the chick duodenum, ileum, and cecum indicated that bacteria were observed almost exclusively in cecal tissue. Thin sections of ceca suggest that many organisms may lie in a layer of material (electron-dense particles) just above the microvilli (Fig. 2). In many tissue sections, bacteria, usually one to two cells wide, could be seen in this layer in close association with the brush border. Attempts to demonstrate microorganisms by TEM of the duodenum and upper and lower ileum by altering fixation times or methods of tissue handling failed to reveal any bacteria lining the brush border surface of epithelia.

DISCUSSION

The results of this study on the enumeration and isolation of bacteria from the intestinal tract of young (2-week-old) chicks partly substantiate the chicken microflora work of other investigators. Ochi et al. (24), Barnes et al. (2), and Smith (30) observed that lactobacilli were the predominant organisms (populations ranging from 10^5 to 10^8/g of contents) in the duodenum and ileum of the 2-week-old chick. Streptococci, lactobacilli, and *E. coli* comprised the most numerous of the facultatively anaerobic microflora in these regions. We also have determined that 60 to 90% of the small intestinal flora in the chick is represented by these three bacterial groups. The majority of the predominant bacteria recovered from the duodenum, ileum, and cecum were gram positive (ca. 65 to 85% of the isolates) as in other chicken microflora studies (25, 26).

Failure of previous studies to determine the presence of anaerobes in the small intestine of chicks prompted us to enumerate and identify the anaerobic flora in the duodenum and ileum by using nonselective media. These results clearly demonstrated that the chick small intestine is colonized by a significant anaerobic flora of diverse bacterial types (anaerobic cocci, *Eubacterium, Propionibacterium, Clostridium, Gemmiger,* and *Fusobacterium*). The presence of large numbers of anaerobes in the small intestine suggests that these species may be involved in a hitherto unrecognized function in the metabolism and microbial fermentation of this organ. They may also play a role in controlling populations of facultatively anaerobic and pathogenic bacteria in the small intestine; such a function has been suggested for the lactobacillary flora in the intestines of chicks (9) and rodents (23, 28).

It is not surprising that anaerobes comprised nearly the entire microbial population of the cecum. Highest numbers of gram-positive (anaerobic cocci, *Eubacterium,* and *Clostridium*) and gram-negative (*Gemmiger, Fusobacterium,* and *Bacteroides*) anaerobes were recovered from chick ceca. Lactobacilli, which have been recovered in large numbers from chick ceca or fecal material in other studies (2, 15, 18, 24, 30), were isolated (plates but not roll tubes) as minor components of the cecal flora.

Our observations of the associations of bacteria with the duodenal, ileal, and cecal epithelia by SEM suggest that the mucosal surfaces may not be as populous with intestinal microbes as has been shown for rodents (6, 29). We have noted that the epithelia of the duodenum and

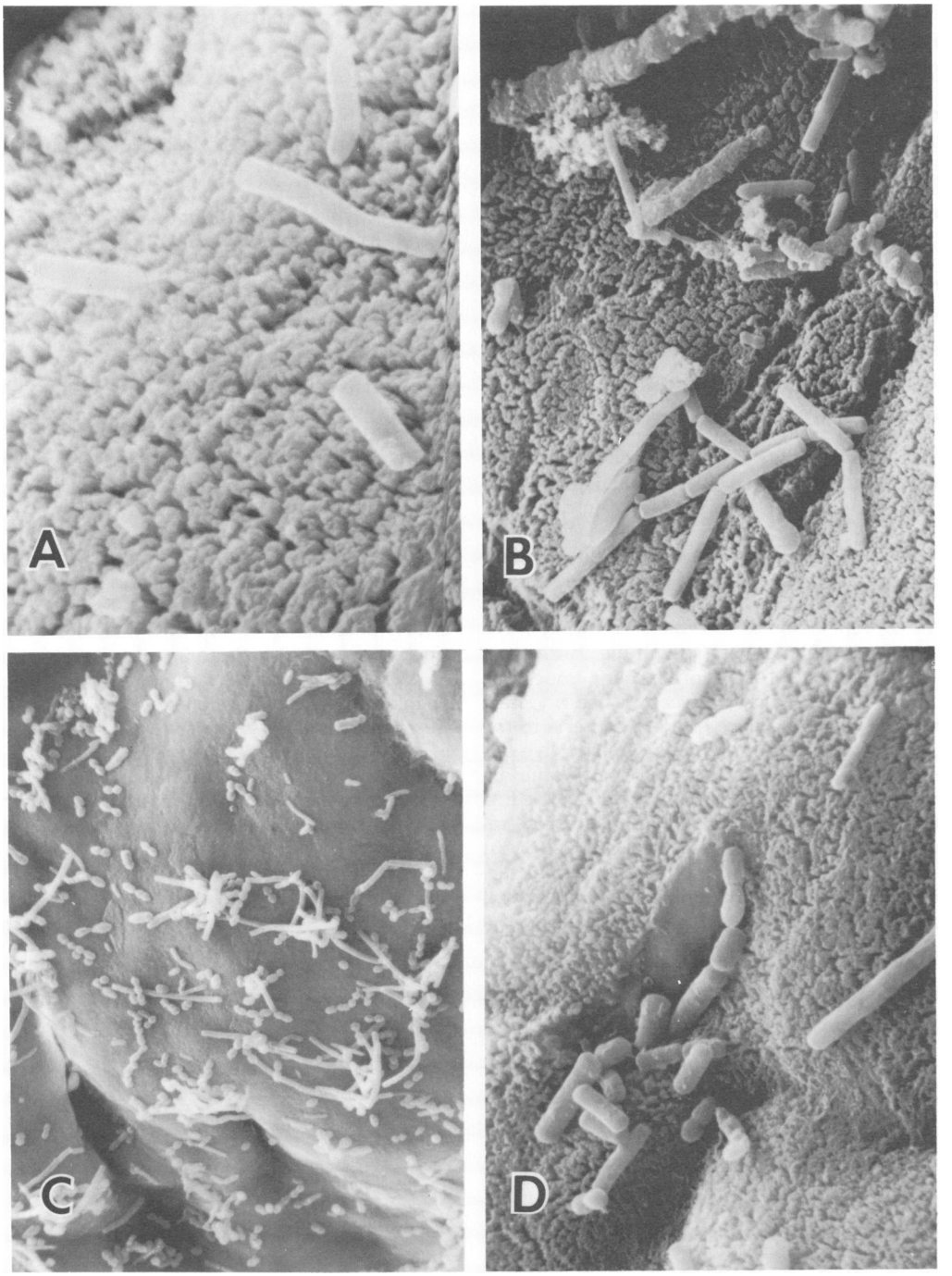

FIG. 1. *SEM of the intestinal epithelial surfaces of 14-day-old chicks. (A) Duodenum. ×10,000. Few rod-shaped bacteria "loosely" associated with the epithelium. (B) Upper ileum. ×5,500. View with several types of rod-shaped bacteria including fusiforms and cocci lying on the microvillar surface. (C) Cecum. ×1,600. Numerous rods and cocci lying on the epithelium. (D) Cecal surface. ×5,500. Rod-shaped bacteria in and near an epithelial pore opening.*

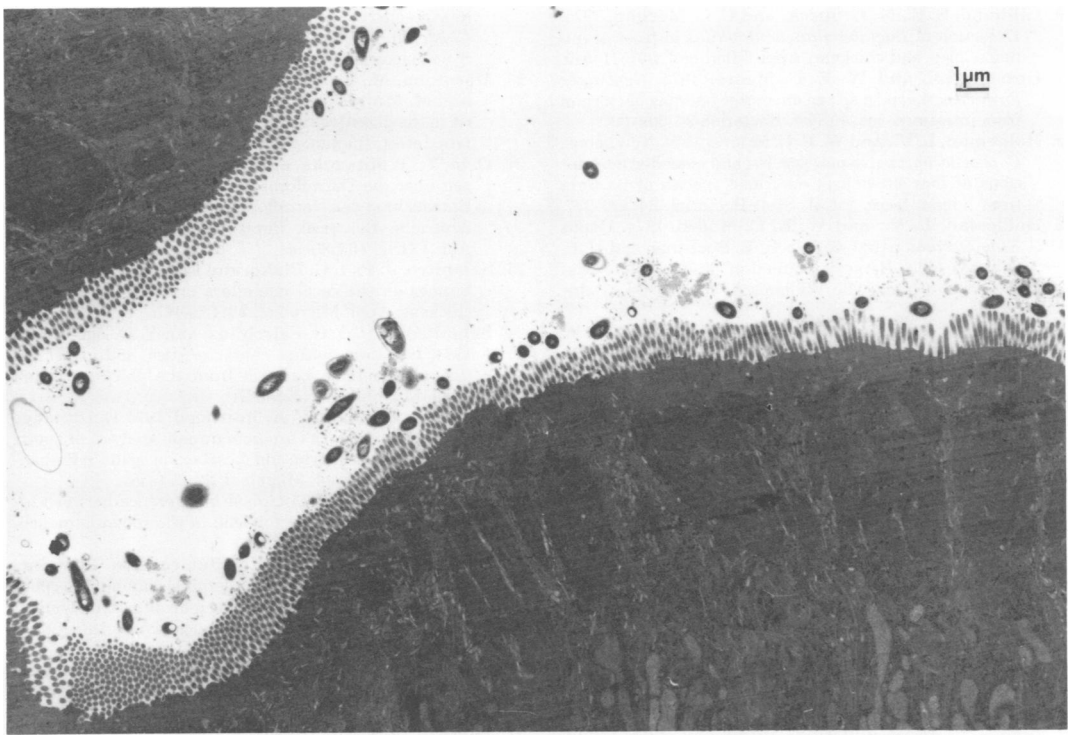

FIG. 2. *TEM of a chick cecum (14-day-old) showing bacteria entrapped in the mucin layers along the epithelial border × 6,000.*

parts of the ileum in chicks seem to be sparsely inhabited with bacteria. Another possible explanation is that the bacteria are associated with mucin layers which are easily removed from the epithelial surface when tissue sections are processed for electron microscopy. No structures (e.g., filaments) connecting bacteria to the epithelium or microbial penetration of the mucosa were seen in SEM or TEM of tissue sections from the duodenum, ileum, or cecum. TEM of thin sections of chick ceca showed that bacteria appear to be localized in the mucous layer lining the epithelium. Recent evidence (unpublished) in our laboratory suggests that diet composition has a profound effect on colonization of bacteria on the intestinal surfaces of young, rapidly growing chicks. It is also possible that the types and amounts of mucins produced in different animals are diet and species dependent. This could have a marked effect on the microbial composition and colonization of the various epithelial habitats.

ACKNOWLEDGMENT

We thank Netti Debbs for her technical assistance.

LITERATURE CITED

1. **Barnes, E. M., and C. S. Impey.** 1972. Some properties of the nonsporing anaerobes from poultry caeca. J. Appl. Bacteriol. **35**:241-251.
2. **Barnes, E. M., G. C. Mead, D. A. Barnum, and G. C. Harry.** 1972. The intestinal flora of the chicken in the period 2 to 6 weeks of age with particular reference to the anaerobic bacteria. Br. Poul. Sci. **13**:311-326.
3. **Brooker, B. E., and R. Fuller.** 1975. Adhesion of lactobacilli to the chicken crop epithelium. J. Ultrastruc. Res. **52**:21-31.
4. **Bryant, M. P., and L. A. Burkey.** 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36**:205-217.
5. **Carle, G. C.** 1970. Gas chromatographic determination of hydrogen, nitrogen, oxygen, methane, krypton, and carbon dioxide at room temperature. J. Chromatogr. Sci. **8**:550-551.
6. **Davis, C. P.** 1976. Preservation of gastrointestinal bacteria and their microenvironmental associations in rats by freezing. Appl. Environ. Microbiol. **31**:304-312.
7. **Facklam, R. R.** 1972. Recognition of group D streptococcal species of human origin by biochemical and physiological tests. Appl. Microbiol. **23**:1131-1139.
8. **Facklam, R. R., J. F. Padula, L. G. Thackers, E. C. Wortham, and B. J. Sconyers.** 1974. Presumptive identification of group A, B, and D streptococci. Appl. Microbiol. **27**:107-113.
9. **Fuller, R., and B. E. Brooker.** 1974. Lactobacilli which attach to the crop epithelium of the fowl. Am. J. Clin. Nutr. **27**:1305-1312.

10. **Gilliland, S. E., M. L. Speck, and C. G. Morgan.** 1975. Detection of *Lactobacillus acidophilus* in feces of humans, pigs, and chickens. Appl. Microbiol. **30**:541-545.
11. **Gossling, J., and W. E. C. Moore.** 1975. *Gemmiger formicilis*, n.gen., n.sp., an anaerobic budding bacterium from intestines. Int. J. Syst. Bacteriol. **25**:202-207.
12. **Holdeman, L. V., and W. E. C. Moore.** 1974. New genus, *Coprococcus*, twelve new species, and emended descriptions of four previously described species of bacteria from human feces. Int. J. Syst. Bacteriol. **24**:260-277.
13. **Holdeman, L. V., and W. E. C. Moore.** 1974. Genus *Eubacterium*, p. 641-657. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
14. **Holdeman, L. V., and W. E. C. Moore (ed.).** 1975. Anaerobe laboratory manual, 3rd ed. Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg.
15. **Huhtanen, C. N., and J. M. Pensack.** 1965. The development of the intestinal flora of the young chick. Poul. Sci. **44**:825-834.
16. **Kloos, W. E., and K. H. Schleifer.** 1975. Simplified scheme for routine identification of human *Staphylococcus* species. J. Clin. Microbiol. **1**:82-88.
17. **Lev, M., and C. A. E. Briggs.** 1956. The gut flora of the chick. II. The establishment of the flora. J. Appl. Bacteriol. **19**:224-230.
18. **Mead, G. C., and B. W. Adams.** 1975. Some observations on the caecal microflora of the chick during the first two weeks of life. Br. Poul. Sci. **16**:169-176.
19. **Miller, T. L., and M. J. Wolin.** 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. **27**:985-987.
20. **Moore, W. E. C., and L. V. Holdeman.** 1974. Genus *Propionibacterium*, p. 633-641. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
21. **Moore, W. E. C., and L. V. Holdeman.** 1975. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl. Microbiol. **27**:961-979.
22. **Moore, W. E. C., J. L. Johnson, and L. V. Holdeman.** 1976. Emendation of *Bacteroidaceae* and *Butyrivibrio* and descriptions of *Desulfomonas* gen. nov. and ten new species in the genera *Desulfomonas, Butyrivibrio, Clostridium, Eubacterium,* and *Ruminococcus*. Int. J. Syst. Bacteriol. **26**:238-252.
23. **Morotomi, M., T. Watanabe, N. Suegara, Y. Kawai, and M. Mutai.** 1975. Distribution of indigenous bacteria in the digestive tract of conventional and gnotobiotic rats. Infect. Immun. **11**:962-968.
24. **Ochi, Y., T. Mitsuoka, and T. Sega.** 1964. Untersuchungen über die Darmflora des Huhn III Mitteilung: die Entwicklung der Darmflora von Küken bis zum Huhn. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **193**:80-95.
25. **Salanitro, J. P., I. G. Blake, and P. A. Muirhead.** 1974. Studies on the cecal microflora of commercial broiler chickens. Appl. Microbiol. **28**:439-447.
26. **Salanitro, J. P., I. G. Fairchilds, and Y. D. Zgornicki.** 1974. Isolation, culture characteristics, and identification of anaerobic bacteria from the chicken cecum. Appl. Microbiol. **27**:678-687.
27. **Salanitro, J. P., and P. A. Muirhead.** 1975. Quantitative method for the gas chromatographic analysis of short-chain monocarboxylic and dicarboxylic acids in fermentation media. Appl. Microbiol. **29**:374-381.
28. **Savage, D. C.** 1969. Microbial interference between indigenous yeast and lactobacilli in the rodent stomach. J. Bacteriol. **98**:1278-1283.
29. **Savage, D. C., and R. V. H. Blumershine.** 1974. Surface-surface associations in microbial communities populating epithelial habitats in the murine gastrointestinal ecosystem: scanning electron microscopy. Infect. Immun. **10**:240-250.
30. **Smith, H. W.** 1965. The development of the flora of the alimentary tract in young animals. J. Pathol. Bacteriol. **90**:495-513.
31. **Smith, L. D. S., and G. Hobbs.** 1974. Genus *Clostridium*, p. 551-572. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
32. **Timms, L.** 1968. Observations on the bacterial flora of the alimentary tract in three age groups of normal chickens. Brit. Vet. J. **124**:470-478.
33. **Tomfohrde, K. M., D. L. Rhoden, P. B. Smith, and A. Balows.** 1973. Evaluation of the redesigned Enterotube—a system for the identification of *Enterobacteriaceae*. Appl. Microbiol. **25**:301-304.