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Types and Distribution of Anaerobic Bacteria in the Large Intestine of Pigs

EDWARD G. RUSSELL
Department of Agriculture, Veterinary Research Institute, Parkville, Victoria, 3052, Australia

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An examination was made of various sites along the length of the swine large intestine, using strictly anaerobic culture methods. Sites were separated by differential washing into fractions described as lumenal content, lumenal surface layer, and intestinal wall tissue. Direct microscopic clump counts averaged 13.3 $\times 10^{10}$ organisms per g (dry weight) of material in the lumenal content, 14.0 $\times 10^{10}$ in the surface layer, and 5.1 $\times 10^{10}$ in the intestinal wall tissue. Both direct microscopic counts and viable culture counts were higher from the lumenal content and surface layer than from the intestinal tissue at all sites sampled in the intestine. Cultural counts averaged 56.2% of the direct microscopic counts in lumenal content and surface layer and 20.2% in intestinal tissue. Over 90% of the bacteria isolated were gram positive and consisted mainly of gram-positive cocci, lactobacilli, eubacteria, and clostridia. Of 192 isolates recovered, only 124 could be assigned to recognized species.

Studies of microbial fermentation (6, 28), the pathogenesis of swine dysentery (31, 47), and the growth-promoting effects of antibiotics (9, 14) have all aroused interest in the composition of the swine intestinal microbial flora. Furthermore, it has been shown that strictly anaerobic techniques are necessary to recover a significant percentage of human fecal flora (35) or swine intestinal flora (51). However, most studies carried out so far have not utilized strictly anaerobic techniques when isolating bacteria from swine feces or intestinal contents (4, 16, 17, 24, 30, 38, 39, 46, 53) or have concentrated on particular groups or organisms (1, 18, 32, 50, 55). Most of the investigations of the flora are not comparable with this present study or with each other, due to the wide variety of methods used.

Studies by Decuypere and Van der Heyde (10), Vervaeke and Van Nevel (51), and others have indicated that the distribution of bacteria varies along the length of the swine intestinal tract. Dubos et al. (13), using an anaerobic chamber (20, 29), observed similar variation in mice. A similar system was used by Attebery et al. (3) to achieve high bacterial recoveries from normal human feces.

The use of rumen fluid-containing media appears to give the highest and most representative recoveries from the ovine rumen (21) and human feces (35). In this study a rumen fluid-containing nonselective medium and strictly anaerobic conditions have been used to enumerate the predominant bacteria at various sites along the length of the large intestine. Additionally, an attempt has been made to fractionate the intestinal site into lumenal contents, mucus layer, and intestinal wall tissue and to isolate and identify bacteria from each site.

MATERIALS AND METHODS

Animals. Pigs used in these experiments were 20- to 25-week-old, specific-pathogen-free Large Whites from an agricultural college herd. They were fed a standard diet, designed and mixed at the college, containing 15% protein, 10% fiber, and 2,970 kcal of digestible energy per kg (12, 426 kJ/kg), with a complete vitamin and mineral premix but no antibiotics.

Four normal pigs considered to be in good health were selected for the experiments and processed on different days.

Sampling. After at least 1 week in the laboratory animal house, to allow the flora to stabilize after transport, pigs were sedated with azaperone (Stresnil, Ethnor) and killed by overdosing with sodium pentobarbitone (Euthatal, May & Baker). Each animal was opened immediately, and 25-cm sections of large intestine were tied off and cut from proximal, middle, and distal areas. The sections of intestine were immediately transported to an anaerobic chamber constructed according to the basic design of Aranki et al. (2). All further work was carried out in this chamber, in an atmosphere of 10% CO_2–10% H_2–80% N_2. A 1-cm^2 full thickness of colon was excised from each section, avoiding mesenteric blood vessels. Each piece was shaken in 10 ml of pH 7.3 prereduced phosphate-buffered saline (RPBS) in a weighed McCartney bottle until no lumenal content was visibly attached and then transferred to a second McCartney bottle containing 10 ml of RPBS. This was agitated vigorously on a whirlimixer (Super-mixer, Lab-Line Instruments, Chicago, Ill.) at full speed for 10 min to remove the

adherent mucus layer. This piece of tissue was removed from the McCartney bottle and homogenized in another 10 ml of RPBS in a Griffith tube. Tenfold serial dilutions to 10^{-5} were made in RPBS from both washings and the final homogenate from each piece of tissue and were used for primary inoculations. The three fractions were designated lumenal content, lumenal surface layer, and intestinal wall. Histological sections were cut from duplicate pieces to determine the effects of these treatments. Direct microscopic clump counts were performed as described by Moore and Holdeman (35), and dry weights of specimens were determined by drying the weighed specimens at 160°C for 5 days and reweighing. Mean recoveries were compared by Student's t test at the 5% level of significance.

Culture methods and media. Primary cultures were obtained using inocula of 5 or 10 μl dropped on plates of rumen fluid-glucose-cellobiose agar and supplemented brain heart infusion agar prepared as described in the *Anaerobe Laboratory Manual* (26), with the addition of palladium chloride (0.03 g/liter) and dithiothreitol (0.01 g/liter) to both. Plates were spread using sterile glass spreaders and removed from the chamber in sealed GasPak (Baltimore Biological Laboratory, Cockeysville, Md.) jars. All media were prepared aerobically and reduced in the anaerobic chamber for at least 24 h before use. Resazurin was incorporated in all diluents and media as the oxidation-reduction indicator.

After 72 h of incubation at 37°C, numbers of colonies on plates were counted, and isolated colonies were picked nonselectively from both primary media into sweet E medium (26). The broths were incubated anaerobically for 24 to 72 h and checked for Gram reaction, purity on a brain heart infusion agar plate, and growth on brain heart infusion agar in a candle jar.

Peptone-yeast-glucose broths were inoculated for gas-liquid chromatographic analyses of fermentation products (23), and appropriate biochemical tests were carried out (26).

Identification of isolates. Isolates were assigned to a species only where all significant characteristics were consistent with published descriptions (11, 25-27, 34, 43, 55). Where isolates were found to differ in one or more significant characteristics, they were not assigned to a species.

RESULTS

Paraffin sections of sample pieces treated as described were stained with hematoxylin-eosin or Alcian Blue and showed removal of the lumenal content after the first shaking in RPBS. A thin Alcian Blue-positive mucosal layer was observed before and after this procedure but was removed by the vigorous second washing. It was not comparable in size or bacterial count to the mucus-overlaid epithelial layer observed in mice and rats (8, 45) and is here referred to as the lumenal surface layer. Many organisms were seen in the crypts of the intestinal wall. Direct microscopic clump counts obtained on the specimens are recorded in Table 1. Bacterial colony counts on the brain heart infusion agar and rumen fluid-glucose-cellobiose agar plates were not significantly different, but the count on the latter was invariably higher and has been used as the indicator of bacterial recovery. Recoveries ranged from 0.1×10^{10} to 11.7×10^{10} organisms per g (dry weight) of material (Table 2). The percent recoveries based on the ratio of viable counts to direct microscopic clump counts averaged 56.2% in lumenal content and surface layer and 20.1% in the intestinal tissue. Of 224 individual colonies subcultured to sweet E medium, 188 isolates were grouped by analysis of their end products from glucose fermentation

TABLE 1. *Direct microscopic clump counts from intestinal specimens*[a]

Section of colon	Bacterial count	
	Mean[b]	Range
Lumenal content		
Proximal	14.9	12.9-15.1
Middle	13.8	13.1-14.1
Distal	10.9	7.7-12.9
Lumenal surface layer		
Proximal	17.6	16.6-18.1
Middle	13.9	13.4-14.1
Distal	10.6	7.4-11.5
Intestinal wall		
Proximal	7.2	1.4-9.5
Middle	6.4	1.3-8.1
Distal	1.6	0.08-4.4

[a] Specimens from four pigs.
[b] Times 10^{10} organisms per g (dry weight) of material.

TABLE 2. *Recovery of bacteria from intestinal specimens*[a]

Section of colon	Bacterial count		Avg % recovery[c]
	Mean[b]	Range	
Lumenal content			
Proximal	10.4	9.0-11.6	69.8
Middle	8.3	4.2-11.7	57.5
Distal	7.1	0.17-8.0	57.6
Lumenal surface layer			
Proximal	9.9	6.4-11.6	56.3
Middle	8.1	5.4-9.8	48.2
Distal	6.7	1.5-9.0	53.6
Intestinal wall			
Proximal	1.5	0.31-2.50	20.8
Middle	0.1	0.51-2.20	20.3
Distal	1.3	0.10-2.41	24.2

[a] Specimens from four pigs.
[b] Times 10^{10} organisms per g (dry weight) of material.
[c] Bacterial colony count/direct microscopic clump count × 100.

and Gram reaction into genera of anaerobic bacteria. They were further subdivided into species by biochemical tests. Four isolates were facultative anaerobes and were identified by biochemical tests. Thirty-two isolates failed to grow in sweet E medium. The results summarized in Table 3 show that gram-positive bacteria made up over 90% of the flora and consisted of gram-positive cocci, lactobacilli, eubacteria, and clostridia. The relative populations of different species are given in Table 4. Many strains of bacteria would not readily fit into any described species. Cultural reactions of these are presented in Table 5. None of these strains grew aerobically or microaerophilically even after several subcultures.

DISCUSSION

The direct microscopic clump counts from the lumenal content and lumenal surface layer are of the same magnitude as those obtained by Moore and Holdeman (35) from human feces and those obtained by Salanitro et al. (44) from swine feces. The direct counts from the intestinal wall are much lower and subject to more variation and resemble those taken from human intestinal samples (12, 37, 40). Direct counts of bacteria from the lumenal content are not significantly different from those from the lumenal surface layer in any pig. This is not in accord with histological studies carried out in this laboratory (R. B. Callinan and E. G. Russell, unpublished data), which have indicated that there are considerably fewer bacteria in the thin mucus layer than in the intestinal content. These studies have also shown organisms of various morphological types within the crypts of the intestinal wall, occurring there in lesser numbers than in the lumen. The lack of correlation between the direct counts of bacteria from the layers and their appearance in situ is evidence that it is not possible to completely separate the layers by simple washing, although it may appear so when sections of the washed tissues are examined histologically.

TABLE 3. *Distribution of bacterial groups isolated from intestinal specimens*

Bacterial group	% Distribution of strains in specimens from 4 different pigs				
	A	B	C	D	Mean
Gram-positive cocci	40.3	41.5	48.9	17.3	37.0
Lactobacillus	24.5	13.2	18.1	27.4	20.8
Eubacterium	29.9	31.0	17.7	27.8	26.6
Gram-negative rods		7.1	7.8	16.3	7.8
Clostridium	5.3	3.4	3.5	4.6	4.2
Other		3.8	4.1	6.5	3.6

TABLE 4. *Relative frequency of bacterial species in total intestinal flora of pigs*

Rank	% of flora	Organism(s)
1	8.33	*P. asaccharolyticus*
2	6.25	*Streptococcus* C
3	4.69	*L. leichmannii*
4	4.17	*M. elsdenii*
5–7	3.65	*E. rectale*, *Eubacterium* E, *L. delbreuckii*
8	3.13	*P. intermedius*
9–17	2.60	*B. ruminicola*, *Bacteroides* A, *E. aerofaciens*, *E. lentum*, *Eubacterium* G, *L. plantarum*, *Lactobacillus* C, *P. magnus*, *P. productus*
18–26	2.08	*E. tenue*, *Eubacterium* B, *Eubacterium* D, *L. acidophilus*, *L. brevis*, *L. minutis*, *P. albus*, *P. constellatus*, *Streptococcus* A
27–31	1.56	*Bifidobacterium* A, *E. coli*, *Clostridium* C, *E. ventriosum*, *Eubacterium* A
32–40	1.04	*Clostridium* A, *Clostridium* B, *Eubacterium* C, *Eubacterium* F, *G. anaerobia*, *P. granulosum*, *S. salivarius*, *Streptococcus* B, *Streptococcus* D
41–46	0.56	*B. fibrisolvens*, *E. hafniae*, *Clostridium* D, *Lactobacillus* A, *Lactobacillus* C, *Streptococcus* E

It is interesting to note the low level of recovery of organisms from the intestinal wall; only 20% of the bacteria were cultivated. Direct microscopic counts may have been less accurate here due to the particulate nature of the intestinal tissue. Many of the bacteria present were described as spiral-shaped or curved fusiform, and the known fastidiousness of these organisms may also partly account for the low percent recovery. The overall percent recoveries compare favorably with other studies on swine intestinal and fecal organisms (44) but are substantially below those achieved by investigations of human fecal flora (35). This may be attributed to the use of aerobically prepared media reduced in the cabinet rather than prereduced anaerobically sterilized media (15; L. V. Holdeman, personal communication).

The predominant organisms recovered from all pigs were gram-positive cocci, *Streptococcus*, *Peptococcus*, *Peptostreptococcus*, and *Megasphaera*. All strains of *Megasphaera elsdenii* stained gram positive on initial examination and have been included in that group, although older cultures are frequently gram negative. Gram-positive cocci have been classified according to Holdeman and Moore (26), Rogosa (42), and

TABLE 5. *Some characteristics of strains isolated which could not be identified as particular species*[a]

Characteristic	*Bacteroides* A	*Clostridium* A	*Clostridium* B	*Clostridium* C	*Clostridium* D	*Eubacterium* A	*Eubacterium* B	*Eubacterium* C	*Eubacterium* D	*Eubacterium* E	*Eubacterium* F	*Eubacterium* G	*Lactobacillus* A	*Lactobacillus* B	*Lactobacillus* C	*Streptococcus* A	*Streptococcus* B	*Streptococcus* C	*Streptococcus* D	*Streptococcus* E
Arabinose	a	–	a	w	a	w	–	w	a	–	–	–	–	a	–	–	a	a	–	–
Cellobiose	a	–	–	a	a	a	–	–	–	a	–	a	–	–	–	a	–	–	–	–
Esculin pH	–	–	–	–	a	–	–	–	–	–	–	–	–	–	–	–	–	a	–	–
Esculin hydrolysis	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
Fructose	a	–	–	a	a	a	–	w	a	a	–	–	–	–	–	a	–	–	–	a
Galactose	a	–	–	–	a	–	–	–	a	–	–	–	–	–	–	–	–	a	–	–
Glucose	a	–	–	w	a	w	w	a	a	–	–	–	a	a	a	a	–	a	a	a
Glycerol	a	–	a	a	–	·	·	a	–	a	a	–	a	–	–	a	–	a	–	–
Inositol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	a	–	–
Lactose	a	–	–	a	a	w	–	a	a	–	–	–	–	–	–	–	a	–	–	–
Maltose	a	a	a	a	a	a	–	w	a	a	–	–	–	a	–	–	–	a	–a	–
Mannitol	a	–	–	w	–	–	–	w	a	a	–	a	–	–	–	–	–	a	–	–
Melibiose	a	–	–	–	a	–	–	–	–	–	–	–	a	–	–	–	–	a	–	–
Raffinose	a	–	–	–	a	–	–	–	–	–	–	–	–	–	–	–	–	a	–	–
Salicin	–	a	a	a	a	–	–	a	–	a	a	–	–	a	–	–	a	w	–	–
Sorbitol	–	–	–	–	–	a	–	–	a	–	–	–	–	–	–	–	–	a	–	–
Starch pH	–	–	–	–	–	–	–	a	–	a	–	–	–	–	–	a	–	a	–	–
Starch hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Sucrose	a	–	–	a	a	a	–	w	–	a	–	–	–	–	–	–	–	a	–	–
Trehalose	–	a	–	–	a	a–	–	–	–	a	a	a	a	–	–	a	a	a	–	–
Xylose	a	–	–	a	a	a	–	a	a	–	–	w	a	–	–	–	–	a	–	–
Gelatin dig	–	+	+	–	–	–	–	–	–	–	=	–	–	–	–	–	–	–	–	–
Milk	–	–	–	ac	–	ac	–	ac	–	c	–	–	ac	c	–	–	–	–	–	–
Indole	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hemolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Motility	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
Lactic acid	·	·	·	·	·	·	·	·	·	·	·	·	DL	DL	L	·	·	·	·	·
Lecithinase	·	–	–	–	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Lipase	·	+	+	–	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
20% bile growth	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Gas from glucose	–	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
Acids from glucose	Apb	Lab	Lab	Lab	Lab	BL	BL	BL	LB	–	–	–	La	La	Laf	Af	–	Af	Afp	als
No. of strains	5	2	2	3	1	3	4	2	4	7	2	5	1	1	5	4	2	12	2	1

[a] No strains produced acid from dulcitol, inulin, or rhamnose or reduced nitrate. w, Weak acid (pH 5.6 to 6.0); a, acid (pH below 5.5); F, A, P, B, L, or S, major product is formic, acetic, propionic, butyric, lactic, or succinic acid, respectively. Lowercase letter indicates minor product. –, Negative result; ·, not tested; +, positive result.

Deibel and Seeley (11). Antibiotic sensitivity testing results were generally in agreement with the scheme of Wren et al. (53) for differentiation of gram-positive cocci. Many obligately anaerobic cocci had fermentation reactions incongruous with any typing scheme. These are regarded as anaerobic *Streptococcus* species until further characterization can be carried out.

Gram-positive nonsporing rods were also isolated in large numbers. Lactobacilli have frequently been reported to be a major component of swine fecal flora (19, 49). Lactobacilli producing both D- and L-isomers of lactic acid (5) were assigned to *Lactobacillus acidophilus,* and those in which no L-isomer was detected were closest in characteristics to *L. leichmannii* and *L. delbrueckii* (43). The isolates of *L. acidophilus* resembled those isolated from pigs by Gilliland et al. (19) more closely than those isolated from other species in that esculin was not usually hydrolyzed. Many of the eubacteria isolated were initially characterized as sporeforming rods but did not stand heating at 80°C for 10 min. The proportion isolated corresponds with that in fecal flora reported by Salanitro et al. (44). Several species were identified, including *Eubacterium aerofaciens* (27, 35). Not all strains fermented sucrose, but all fermented cellobiose and salicin; thus, both *E. aerofaciens* I and II appear to be represented (35). Some strains decolorized readily, and central and terminal swellings were noticeable in many of them. *E. lentum* strains were characterized by their lack of fermentative ability. All strains isolated were nitrate negative, and growth was enhanced by the addition of Tween 80 to media. Isolates resembling *E. lentum* biochemically in their unreactivity were also obtained but were morphologically distinct, and their growth was not improved by addition of Tween 80.

Clostridium spp. have been reported to be a component of the fecal or intestinal flora (39, 53). Isolates comprised about 4.3% of the flora, but no strains were able to be classified to any known species by the criteria of Holdeman and Moore (26). The frequently reported *Clostridium perfringens* was not seen. Other gram-positive, nonsporeforming rods were isolated in relatively low numbers. Bifidobacteria and propionibacteria were the most common.

Gram-negative organisms comprised nearly 20% of the direct microscopic count but only 7.8% of the cultural count. Most species recovered were similar to *Bacteroides ruminicola* type 1, as described by Terada et al. (48). Many curved organisms were seen in direct smears, but no isolates were made. Large spiral organisms of the type associated with swine dysentery (22) were occasionally seen in stained direct smears. Other spiral organisms, smaller in length and more tightly coiled, were more common in direct smears and wet preparations. No spiral organisms were isolated.

The distribution of types of bacteria is quite similar to that reported for swine feces (44). This reinforces the evidence of Moore and Holdeman (36) that the fecal flora is representative of the intestinal flora. Given that the overall numbers were low, the distribution of bacterial genera among the pigs and from the different sites in the colon appeared to be random. There is some evidence that morphologically similar bacteria associated with the intestinal tissue surface in rodents and the aerobic bacteria in swine feces occur in microbial communities rather than randomly (7, 41). The demonstration of a similar organized distribution of bacteria in this situation requires overcoming the problem of localizing particular species of bacteria within the intestinal site.

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