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Enumeration and Isolation of Anaerobic Microbiota of Piggery Wastes

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Media for enumeration of the microbiota of anaerobically stored piggery wastes were tested. Highest colony counts were obtained with 80 to 100% farm slurry supernatant included in the anaerobic roll tube media. Colony counts with these media numbered 2×10^9 to 12×10^9/g (wet weight), which represents about 20% of the microscopic counts. Lower percentages of slurry supernatant in the media gave lower colony counts. Addition of glucose, cellobiose, and starch or of Trypticase to media with 20% slurry supernatant did not increase colony counts. Higher values were obtained when hemicellulose preparations were added to these media. Incubation at 25°C gave the highest numbers. Incubation at 15 to 37°C gave counts of about 70 and 10%, respectively, of those at 25°C. Of the colonies picked for isolation, about 20% were obtained in pure culture. The isolates apparently belonged to the genera *Peptococcus, Ruminococcus, Peptostreptococcus,* and *Bacteroides.*

In intensive pig-fattening units, the animals are usually kept on slatted floors. The droppings are collected and stored underneath the floors, where the mixture of feces and urine is decomposed under the prevailing anaerobic conditions. This leads to accumulation of malodorous compounds, which may cause a serious odor nuisance to the surroundings when emitted with the ventilation air. Odor problems also arise when the slurry is spread on the land.

Wastes as stored in farm pits differ in composition. Notably, the dry matter contents show large variations (18). Variation in composition is a result of farm management; it is influenced by such factors as the rations fed, drinking water regimen, frequency and way of pit emptying, and storage conditions. In spite of all these variations, slurries from different piggeries may be considered as a group of wastes that are characterized by the anaerobic degradation of fibrous and proteinaceous matter leading to the accumulation of volatile fatty acids. These processes proceed in the presence of high NH_3 levels, derived from the decomposition of urea and protein. Table 1 contains measurements of the main features of farm slurry and of freshly voided pig feces.

The microbiota of farm slurry was studied as part of a research program on the origin of the obnoxious odors emitted from farm slurries. Because little microbiological work has been performed on pig slurries, no special techniques and media were available.

Anaerobic culturing techniques developed for the study of the microbiota of the rumen and of the intestinal tract have been adapted and used throughout this study. Typical rumen media, like M10 (4) and a 40% rumen fluid medium, were used as a starting point for the experiments.

MATERIALS AND METHODS

Slurry samples. Farm slurry samples were taken as grab samples from farm pits with storage of wastes under slatted floors. The sampling procedure has been described in detail elsewhere (16). Weights of the pigs ranged from 20 to 100 kg. The animals received commercial rations. The average age of the wastes cannot be established, but must be assumed to have ranged from several weeks to several months.

Anaerobic techniques. Media were prepared according to the methods of Hungate (10). However, Na_2CO_3, cysteine-hydrochloride, and Na_2S were added after boiling the media but before tubing and autoclaving. Tubes were gassed with an oxygen-free mixture of 96% CO_2 and 4% H_2. This gas mixture was used for all counting experiments. The tubes were provided with a screw cap to prevent blowing-out of the stoppers during autoclaving. Serial dilutions of farm slurry were made in the medium described as "dilution blanks" by Holdeman and Moore (9). For the first dilution, containing 10^{-2} g of slurry per ml, a preweighed sample of about 1 g of slurry, added to 99 ml of dilution medium, was mixed for 1 min under a stream of CO_2 in an electric blender. Subsequent dilutions were made by injecting 0.5 ml of liquid with a 1-ml disposable syringe into a stoppered tube (28 by 140 mm, Kontes) containing 49.5 ml of dilution medium. Tubes (22 by 140 mm, Kontes) containing 6 ml of molten agar medium were inoculated with 1 ml containing 10^{-8} g of slurry. Inoculation was done in

TABLE 1. *Characteristics of freshly voided feces and of anaerobically stored wastes from piggeries*

Characteristic	Feces (ref.)	Anaerobically stored wastes (ref.)
DM[a] (%)	17–25	2–14 (16)
Ash (% of DM)	17 (15)	20–40 (16)
Crude protein (% of DM)	19 (15)	
Fiber (% of DM)	40 (15)	
pH		6.7–7.8 (16)
Ammonia (g/liter)		2.3–9.1 (11)
Volatile fatty acids (g/liter)		4–20 (12)
Cresol (mg/liter)		100–350 (16)
Storage temperature (°C)		10–20

[a] DM, Dry matter.

eight replicate tubes. Roll tubes were prepared by spinning the tubes in a refrigerator until solidification of the agar and then were incubated in upright position at 25°C.

Media. Medium 10 (M10) of Caldwell and Bryant (4) was used, but the fatty acid mixture was replaced by the mixture of Bryant (2). The 40% rumen fluid medium (R40) was similar to medium 98-5 of Bryant and Robinson (3). Rumen fluid was obtained from a fistulated grazing cow with free access to water.

Farm slurry supernatant (SS) was prepared from the same batch of slurry in which bacteria were counted. For each experiment, a new batch of farm slurry was used. An almost clear SS was obtained by centrifugation of the slurry at 25,000 × *g* for 30 min. It was found to be convenient to strain the slurry through cheesecloth before centrifugation to eliminate the coarsest particles.

Media containing SS were supplied with minerals, $Na_2S \cdot 9H_2O$, cysteine-hydrochloride, resazurin, Na_2CO_3, and agar in the same concentrations as in medium 98-5 (3). Rumen fluid was replaced by either 10, 20, 40, 60, or 80% SS. These media are referred to as SS10, SS20, SS40, SS60, and SS80. To the medium with 100% SS (SS100) no minerals were added. Carbohydrates, when included in the media, were supplied at a level of 1 g of each per liter.

The formulations of Sweet E broth and peptone-yeast extract-glucose (PYG) broth are given by Holdeman and Moore (9). The pH value of the media after autoclaving was 6.9. The hemicellulose preparation added to the media was a mixture of: xylan (Fluka), gum xanthan, locust bean gum, and gum arabic (the latter three purchased from Sigma). Incorporation of a mixture of 1 g of each of these polymers per liter gave the following composition on a monomer base (milligrams per liter): mannose (1,580), galactose (610), arabinose (355), xylose (350), glucuronic acid (285), glucose (260), rhamnose (155), and pyruvic acid (70). Hemicellulose (gum mixture), sometimes difficult to dissolve, was mixed with water, boiled while stirred, left overnight at room temperature, and added to the media, which were then boiled to expel oxygen.

Bacterial counts. Colony counts were performed after 5 or 6 weeks of incubation time unless otherwise stated. Only colonies visible with the unaided eye were counted. For the microscopic count, a Bürker-Türk counting chamber with a depth of 0.01 mm was used. In 20 to 40 squares of 0.0025 mm^2, all bacterial cells and clumps were counted in a dilution containing 10^{-2} g of slurry per ml.

Means were statistically compared by *t* tests (17).

Isolation and identification of strains. For the isolation of bacteria, roll tubes were inoculated with 10^{-9} g (wet weight) of slurry. Inoculation with highly diluted suspensions was chosen to increase the possibility of picking pure cultures. Colonies were transferred after 4 and 7 weeks to either Sweet E broth or SS100 medium enriched with hemicellulose but without agar. Media and methods for identification of strains were similar to those of Holdeman and Moore (9). Minor modifications were the following. Media to test acid formation from carbohydrates were tubed into 10-ml Vacutainer tubes (Becton, Dickinson & Co.) and inoculated with a syringe. Incubation was done at 25°C, and identification tests were performed after 7 to 10 days. The composition of volatile fatty acids in spent media was determined gas chromatographically. One microliter of culture fluid supernatant (centrifuged for 20 min at 25,000 × *g*) was introduced on a glass column (1 m by 4 mm [ID]) packed with 20% Tween 80 on Chromosorb W-AW (80 to 100 mesh). Carrier gas (N_2; flow, 80 ml/min) was saturated with formic acid. The column oven temperature was 115°C.

From nonvolatile acids, methyl esters were prepared as described by Holdeman and Moore (9). Gas-chromatographic separation was achieved by a glass column (2 m by 2 mm [ID]) packed with 5% Carbowax 20M on Chromosorb W-HP (100 to 120 mesh). Operational temperature was 140°C. Nitrogen was applied as carrier gas (30 ml/min). The gas chromatographs used were equipped with flame ionization detectors.

Individual isolates were tested in PYG for growth at 37°C. Controls were incubated at 25°C. Cultures were observed for 5 weeks.

Isolated strains were maintained in Sweet E broth stored at 25°C. Transfer to fresh medium once per 3 months was sufficient to maintain viability.

RESULTS

Preliminary experiments. In preliminary experiments, colony counts on rumen media M10 and R40 were compared with those on media including SS (Table 2). The media with 20 and 40% SS were found to be superior to both M10 and the rumen fluid medium (R40). Colony counts on M10 and R40 gave mutually comparable results. To find an optimum level of SS, media containing different percentages of SS were tested (Table 3). Colony counts were nearly doubled when the percentage of SS was increased from 20 to 80% or higher.

Incubation temperature. Counts at 25°C with SS100 increased by about 35% when the incubation period was extended from 2 to 3 weeks. Longer incubation gave additional small increases of colony counts. With incubation at 15°C, colony numbers were nearly doubled when the incubation period was increased from 5 to 10 weeks.

Highest colony numbers were obtained when

TABLE 2. *Comparison of colony counts of farm slurry on media M10 and R40 with media including SS*

Expt[a]	Medium[b]	Colony count[c]
1	M10	3.47 ± 1.11^{x}
	R40	3.90 ± 0.45^{x}
2	M10	1.20 ± 0.86^{x}
	SS40	2.96 ± 0.98^{y}
3	M10	2.95 ± 0.29^{x}
	SS20	5.83 ± 0.79^{y}
4	R40	1.44 ± 0.36^{x}
	SS20	2.03 ± 0.48^{y}

[a] Only counts of the same experiment are comparable.

[b] Cellobiose, glucose, and starch were included with all media except the SS20 of experiment 4, which contained no carbohydrates.

[c] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols (x, y) denote significance at $P < 0.01$.

TABLE 3. *Effects of amounts of SS contained in media on colony counts*

Expt[a]	% SS	Carbohydrates[b] included	Colony count[c]
1	10	C, G, S	3.32 ± 0.67^{x}
	20	C, G, S	3.72 ± 1.59^{y}
	40	C, G, S	6.60 ± 0.89^{y}
2	20	C, G, S	5.83 ± 0.79^{x}
	40	C, G, S	$8.17 \pm 1.54^{y,p}$
	60	C, G, S	9.40 ± 1.20^{q}
	80	C, G, S	12.0 ± 2.30^{r}
3	20	None	1.48 ± 0.44^{x}
	100	None	2.62 ± 0.47^{y}
4	20	None	2.02 ± 0.40^{x}
	100	None	3.92 ± 0.63^{y}

[a] Only counts of the same experiment are comparable.

[b] C, Cellobiose; G, glucose; S, starch.

[c] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols denote significance at $P < 0.01$ (x, y) or $P < 0.05$ (p, q, r).

incubation was at 25°C (Table 4). Counts at 15°C were about 70% of the counts obtained at 25°C. Incubation at 37°C gave numbers amounting to less than 10% of the counts at 25°C.

Additions to media. The results of experiments with different carbohydrates added to SS20 and SS100 are presented in Table 5. The most frequently used additions to rumen media (cellobiose, glucose, and starch) tended to decrease colony counts when added to SS20. No significant effect (at $P < 0.05$) was obtained when starch or cellobiose was deleted from M10 (data not recorded). Addition of Trypticase to SS20 (Table 6) gave no general effect on numbers when counted after 10 weeks. Only the addition of 30 g of Trypticase per liter gave higher colony numbers than that of 2 g/liter ($P < 0.05$). However, counted after 17 days, 10 and 30 g of added Trypticase per liter gave higher numbers of bacteria than 2 g/liter ($P < 0.01$ and $P < 0.05$, respectively), whereas 10 g/liter showed increased counts compared with those with no Trypticase added ($P < 0.01$). Here, as in the case with the addition of cellobiose, glucose, and starch, the higher substrate levels tended to give some large colonies that might overgrow others.

Colony counts were higher when the hemicellulose preparation was included in the SS20

TABLE 4. *Effect of incubation temperature on colony counts*

Expt[a]	Medium	Carbohydrates[b] included	Incubation temp (°C)	Colony count[c]
1	SS80	C, G, S	15	4.82 ± 0.55^{x}
	SS80	C, G, S	25	7.10 ± 0.80^{y}
	SS80	C, G, S	37	0.31 ± 0.18^{z}
2	SS100	None	15	3.84 ± 0.20^{x}
	SS100	None	25	5.17 ± 0.63^{y}
	SS100	None	37	0.63 ± 0.15^{z}

[a] Only counts of the same experiment are comparable.

[b] C, Cellobiose; G, glucose; S, starch.

[c] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols (x, y, z) denote significance at $P < 0.01$.

TABLE 5. *Effect on colony counts of carbohydrates added to media with SS*

Expt[a]	Medium	Carbohydrates[b] included	Colony count[c]
1	SS100	None	6.06 ± 1.26^{x}
	SS100	C, G, S	5.86 ± 0.31^{x}
2	SS80	C, G, S	$7.10 \pm 0.80^{x,p}$
	SS80	G	$6.23 \pm 0.58^{x,q}$
	SS80	C	6.30 ± 1.04^{x}
3	SS20	None	2.02 ± 0.40^{x}
	SS20	C, G, S	1.21 ± 0.39^{y}
4	SS20	None	2.03 ± 0.48^{x}
	SS20	S	1.81 ± 0.67^{x}

[a] Only counts of the same experiment are comparable.

[b] C, Cellobiose; G, glucose; S, starch.

[c] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols denote significance at $P < 0.01$ (x, y) or $P < 0.05$ (p, q).

medium (Table 7). When hemicellulose was added to the SS100 medium, no significant increase in colony numbers was obtained in most experiments. An additional indication that the added mixture of gums was utilized as substrate is derived from the greater average colony size on the roll tubes containing this mixture.

In Table 8, microscopic counts and colony counts are compared. With the SS80 and SS100 media, colony counts reached about 20% of the microscopic counts.

Isolation and identification. In two experiments, bacteria were isolated from farm slurry and subsequently identified. In the first experiment, 42 colonies were transferred to Sweet E broth. Only 22 tubes showed good growth after a 3-week incubation period. On transfer to PYG broth, 7 isolates developed, and another 5 strains, all of them cocci, grew when 0.2% Tween 80 had been included in the PYG broth. In the second experiment, 100 colonies were inoculated into SS100 with the hemicellulose preparation but without agar. Growth in this medium was difficult to observe because of undissolved hemicellulose particles. From all of the latter tubes, 0.1 ml was transferred to PYG broth enriched with 0.2% Tween 80. Twenty-one tubes showed good growth. Inoculating 0.1 ml of liquid from the 79 hemicellulose-containing tubes that did not give growth in PYG without Tween 80 did not give positive results as contrasted with PYG with 2% oxgall, which enabled 5 more strains to grow. These 5 bacteria (4 cocci and 1 small rod) were not further identified. The strains growing in PYG or PYG with Tween 80 were checked for purity by microscopic examination and subsequently used for identification tests. The 33 isolates could be divided into three groups.

Group I consisted of 10 strains of non-saccharolytic gram-positive cocci. Only 2 of these grew at 37°C. The bacteria occurred in pairs or chains, and 1 organism showed tetrads. They fermented carbohydrates weakly or not at all. These bacteria resemble *Peptococcus* species or the non-saccharolytic species of the genus *Peptostreptococcus.*

Group II comprised 13 cocci that fermented carbohydrates. Four of these (1 grew at 37°C) were gram-positive to gram-variable cocci that fermented cellobiose, glucose, fructose, lactose, and mannose. The main acids produced were acetic and succinic acids. They were tentatively identified as *Ruminococcus* spp. Two other gram-positive cocci (1 grew at 37°C) were present in chains only. The cells had tapered ends and produced mainly acetic and succinic acids from all carbohydrates tested. These 2 strains were identified as *Peptostreptococcus productus.* Seven isolates (5 grew at 37°C) of gram-positive cocci occurred in pairs or chains and fermented glucose, fructose, maltose, and starch. The main acids formed were acetic, succinic, and (less) lactic. These isolates belong, probably, to the genus *Peptostreptococcus.*

Group III comprised gram-negative rods that

TABLE 6. *Effect on colony counts of Trypticase added to SS20*

Expt[a]	Trypticase added (g/liter)	Colony count[b]
1	0	1.24 ± 0.44^{x}
	10	1.48 ± 0.37^{x}
2	0	2.04 ± 0.34^{x}
	2	$1.77 \pm 0.27^{x,p}$
	10	2.06 ± 0.39^{x}
	30	$2.32 \pm 0.28^{x,q}$

[a] Only counts of the same experiment are comparable.

[b] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols denote significance at $P < 0.01$ (x, y) or $P < 0.05$ (p, q).

TABLE 7. *Effect on colony counts of addition of hemicellulose preparation to media including SS*

Expt[a]	Medium	Hemicellulose prepn	Colony count[b]
1	SS100	−	2.62 ± 0.47^{x}
	SS100	+	3.64 ± 0.31^{y}
2	SS100	−	5.17 ± 0.63^{x}
	SS100	+	4.96 ± 0.39^{x}
3	SS100	−	4.17 ± 0.93^{x}
	SS100	+	4.44 ± 0.77^{x}
	SS20	−	1.90 ± 0.34^{y}
	SS20	+	3.64 ± 0.75^{z}
4	SS20	−	2.03 ± 0.48^{x}
	SS20	+	3.30 ± 0.39^{y}

[a] Only counts of the same experiment are comparable.

[b] Count per 10^{-9} g (wet weight), mean with standard deviation of the mean. Different symbols (x, y, z) denote significance at $P < 0.01$.

TABLE 8. *Comparison of microscopic counts with colony counts of farm slurry*

Medium	Microscopic count[a]	Colony count[a]	%[b]
SS40	36	2.96	8
SS80	62	12.0	19
SS80	30	7.10	24
SS100	20	5.17	26
SS100	17	3.92	23

[a] Count per 10^{-9} g (wet weight).

[b] Colony count as percentage of the microscopic count.

often occurred in long pleomorphic cells. Of the 10 isolates in this group, 5 were able to grow at 37°C. The acids produced were acetic and succinic, and 7 isolates also formed propionic acid as a major product. The bacteria in this group can be described as saccharolytic *Bacteroides*.

DISCUSSION

Colony counts on SS-containing media. The data of Tables 2 and 3 show that it is advisable to incorporate about 80% SS in media for colony counts of the microbiota of farm slurries. The optimum quantity may vary with the degree of dilution and with the composition of the slurry under investigation. Lower amounts of SS in the media result in lower colony counts, indicating that the main substrates for many organisms are diluted too much to allow development to visible colonies. It may be expected that addition of the proper substrates to SS20 will give colony numbers of the same magnitude as obtained with media with high levels of SS. Addition of the compounds most frequently used as substrates in rumen media (cellobiose, glucose, and starch) to SS20 media did not increase colony counts (Table 5). The negative results of enrichment of the media with Trypticase (Table 6) suggest that protein degradation products are not limiting.

Of the products tested as added substrate, only the hemicellulose preparations increased colony numbers, though counts remained lower than those obtained with SS80 and SS100 (Table 7). Probably, only a limited fraction of the hemicellulose in the slurry is present in water-soluble form. The remaining insoluble fraction, including the residues of plant cell walls from the forage, is removed by centrifugation and, therefore, does not occur in the SS fraction that is used in the nutrient media used for counting. The relatively low concentration in the SS fraction of soluble components derived from hemicellulose apparently is sufficient for giving optimum growth of the microbiota. However, upon fivefold dilution, the concentration of the nutrients for many bacteria is too low to give visible colonies. Enrichment of the diluted SS medium with the hemicellulose preparation restored growth of these bacteria to a large extent. However, restoration is not complete, presumably because the added hemicellulose preparation does not contain the required components in optimum concentration.

Stimulatory effects of hemicellulose preparations on bacteria have been reported from rumen isolates by Dehority (6). Some workers found increased counts of rumen bacteria when fibrous material of the forage fed to ruminants was included in the media (5, 7). Salyers et al. (14) reported the fermentation of gums by *Bacteroides* spp. The use of commercial gums as a substitute for the hemicellulose of plant fiber in nutrient media has the advantage that a more homogeneous medium is obtained and that rather well-defined products are used (19). The combination of gums used in the present work was chosen as a trial mixture. The monomer composition does not reflect the composition of the hemicellulose fraction of plant material left in piggery wastes. It may be that other combinations of the gums used or including other gums in SS20 medium would have further increased colony counts.

Temperature. Highest colony counts were obtained at an incubation temperature of 25°C, with a small decline for counts at 15°C. These incubation temperatures do not deviate much from temperature generally found in stored farm slurries. Counts at 37°C, the expected optimum temperature of the microbiota of the incoming feces, show a sharp decrease. These results indicate that the intestinal microbiota of the pig does survive poorly in the wastes and is not likely to play a role in the decomposition of the slurry during storage. This conclusion is supported by the work of Allen and Brock (1), who found that only 1% of the aerobically growing intestinal microbiota of the rat was cultivable at 25°C.

When different slurries were inoculated into the respective SS100 media, considerable differences in colony counts were found. These differences were, probably, largely due to differences in dry matter content of the wastes. With the media SS80 and SS100, colony counts ranged from 2.6×10^9 to 12.0×10^9/g (wet weight). These numbers are considerably higher than the 6.5×10^8/ml reported by Hobson and Shaw (8), who used a medium with centrifuged fluid from an anaerobic digester with cellobiose, maltose, glucose, and lactate as substrates. But their counts have probably been negatively influenced by the incubation temperature of 38°C.

The colony counts with the media SS80 and SS100 amount to 20% of the microscopic counts. Comparable figures have been reported for the rumen microbiota (5). Recoveries of the intestinal microbiota are usually higher (13). However, farm slurries differ from both these habitats by not being a continuous system but a system without discharge, thus more approaching a batch culture, in which higher percentages of dead organisms are expected.

Isolates. The few samples from which organisms were isolated and the low number of isolates obtained for identification give a limited

impression of the microbiota of piggery wastes. Isolates that could be grown in PYG represent about 20% of the colonies picked for isolation and only 4% of the microscopic count. Colonies were picked after 4 and 7 weeks, so part of the picked colonies may have been dead. Recoveries increased somewhat when isolates growing only in PYG with 2% oxgall were included.

All organisms isolated were strict anaerobes. Earlier experiments had shown that counts on aerobically incubated media yielded 1×10^8 to 5×10^8 colonies per g (Spoelstra, unpublished results).

The tentatively identified anaerobic organisms consisted of gram-positive cocci (70%) and gram-negative pleomorphic rods (30%). Hobson and Shaw (8) also reported gram-positive cocci as the most important group of bacteria in piggery wastes. They also found clostridia to be an important group. However, in the present study no clostridia were isolated.

The identified organisms seem to belong to the genera whose representatives usually are isolated from the gastrointestinal tract of humans and animals and consequently have optimum temperatures around 37°C. However, the majority of the present strains did not grow at 37°C. This discrepancy causes some doubt about the identity of the isolated organisms, but it is also a reflection of the little attention that has been paid to the microflora of anaerobic ecosystems at lower temperatures.

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