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Some Facultatively Anaerobic Gram-negative Rods from the Rumen of the Calf and the Sheep

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SUMMARY: The coliform population in the rumens of twelve calves of ages 10-105 days was never greater than 10^6 /g., and consisted mostly of *Escherichia coli* and intermediate types. Only one well-capsulated strain of *Aerobacter aerogenes* was isolated, whereas such capsulated strains could be isolated with ease from the rumen of a starch-fed sheep. An unidentified small catalase-negative Gram-negative rod, which readily produced ammonia from urea, was isolated from the rumen contents of two of the older calves, at a dilution of 10^{-4} .

Mann, Masson & Oxford (1954*a*) showed that although the coliform population of the adult hay-fed sheep's rumen was numerically quite small, nevertheless it consisted mostly of *Escherichia coli* of intestinal type rather than *Aerobacter aerogenes* or *A. cloacae*, the common saprophytes found on plants and grains. *A. aerogenes* in the capsulated form has, however, since been isolated quite easily from the rumen of a starch- and concentrate-fed sheep (see below). It seemed of some interest therefore to study the distribution of coliform types in the rumen of the young ruminant, particularly the calf when reared by the usual method on milk and a 'starter' gruel, from the first tentative nibbling of straw at age 1-2 weeks to the establishment of the complete rumen microflora after discontinuance of milk in the ration. This has been now carried out in conjunction with the aureomycin feeding of calves described by Mann, Masson & Oxford (1954*b*) which resulted in the isolation of lactobacilli from the rumens of the younger calves only (reported by Mann & Oxford, 1954*a*). A description is now given of certain other unidentified non-coliform Gram-negative rods, also isolated from the calf rumen in the same experiment. These appeared to be of possible importance in the functioning of the rumen microflora because of their greater ability to produce ammonia from urea than is possessed by *A. aerogenes* or *A. cloacae*.

METHODS

Feeding of calves. This is fully described by Mann *et al.* (1954*b*). The calves (twelve in all) were fed 'starter' gruel by bucket. Hay and grass were available after the 3rd week, but fresh milk was discontinued after the 6th week. The three youngest calves examined (10-11 days old; group OC in Table 1) were slaughtered during March-May 1954 and were therefore separate from the main aureomycin-feeding experiment of May-August 1953.

Isolation of rumen Gram-negative rods in pure culture. The bottle-counting technique used for sheep rumen contents by Heald, Krogh, Mann, Appleby, Masson & Oxford (1953) and for the isolation of rumen lactobacilli by Mann & Oxford (1954*a*), although suitable for saccharolytic Gram-positive bacteria,

cannot be relied upon to give a true representation of rumen saccharolytic Gram-negative bacteria when these are in the minority. It was therefore replaced by the following method. A sample of rumen contents (10 g.) taken from the well-mixed total contents of a rumen as soon as possible after slaughter of the calf (i.e. within 2 hr.) was placed in a sterile McCartney bottle, diluted with an equal weight of sterile saline, gassed with sterile CO₂ and shaken mechanically for 20 min. to detach as many bacteria as possible from plant particles. The mixture was then centrifuged very lightly to bring down the larger plant particles, and serial ten-fold dilutions made from the supernatant in sterile saline. One ml. from each dilution was transferred to a sterile Petri dish, and incorporated at 45° in 15 ml. of Wright's nutrient agar containing glucose (0.2 %) and crystal violet (0.0005 %). This low concentration of crystal violet did not inhibit all Gram-positive bacteria, but did allow the maximum number of Gram-negative bacteria (coliforms and otherwise) to form distinct colonies in 48 hr. when the plates were incubated anaerobically at 38°. All the Gram-negative isolates obtained in this study proved however to be facultative anaerobes. They were purified by repeated plating on glucose + peptone + yeast extract + salt agar and maintained till required in Robertson's cooked meat medium.

Examination of Gram-negative rod isolates. Those which fermented lactose in MacConkey's bile salt medium with production of acid and gas and were also catalase-positive, were further studied by standard methods for coliform bacteria. Indole was tested for with Kovács' reagent, and both O'Meara's and Barritt's modifications of the Voges-Proskauer test were employed. Production of ammonia from urea was tested for by Christensen's (1946) method and also in the more highly buffered medium of Stuart, van Stratum & Rustigian (1945).

Table 1. *Classification of calf rumen coliform bacteria*

Calves: A or A¹, fed aureomycin; C, control not fed aureomycin (see Mann *et al.* 1954*b*). N.B. *A. aerogenes* type II and *A. cloacae* were never isolated.

Calf reference number	Age of calf (days)	No. of isolates of				Total possible intestinal (VP - MR +) types	<i>A. aerogenes</i> type I (VP + MR -)
		<i>Escherichia coli</i>		Int.* I	Int. II		
		type I	type II				
OC1	11	2	0	0	0	2	0
OC2	11	1	0	0	0	1	0
OC3	10	0	0	0	0	0	0
1A	33	0	0	0	0	0	0
1A ¹	43						
1C	32						
2A	54	0	6	4	0	10	2
2A ¹	59	0	0	0	0	0	0
2C	60	2	1	0	0	3	0
3A	105	4	5	0	0	9	0
3A ¹	96	0	0	0	0	0	1
3C	86	2	7	0	0	9	2
Total no. of isolates:		11	19	4	0	34	5

* Int. = intermediate coliform types (MR +, VP -, citrate +).

RESULTS

Classification of coliform bacteria isolated from the rumens of calves of various ages from 10 to 105 days

The viable coliform population was usually 10^4 – 10^6 /g. rumen contents; sometimes much less (10^2 /g. in calf 3A), sometimes apparently nil, e.g. in calves 1A, 1A¹, 1C and 2A¹. The thirty-nine coliform isolates are classified in Table 1 according to the scheme used by Wilson *et al.* (1935, p. 156) for milk coliform bacteria. It will be seen from the table that *Escherichia coli* I and II and the intermediate types, all VP-negative and MR-positive, accounted for 87 % of the total isolates, whilst *Aerobacter aerogenes* (VP + MR –) accounted for only 13 % and *A. cloacae* was not found. All the *A. aerogenes* isolates attacked urea weakly and not consistently. Only one such isolate (out of five) showed well-marked capsulation, and, unlike the other four isolates, fermented adonitol and inositol.

Capsulated strains of Aerobacter aerogenes from the rumen of a concentrate-fed sheep

The rumen-fistulated sheep in question was fed the following daily ration: hay (300 g.), concentrates (500 g.), and potato starch (200 g.). The medium used for isolation of coliforms from rumen contents was MacConkey's agar. Capsulated isolates of *Aerobacter aerogenes* could invariably be obtained with ease from the rumen contents of the above sheep, usually at a dilution of 10^{-2} to 10^{-4} . Control experiments with rumen contents from two fistulated hay-fed sheep confirmed the conclusion of Mann *et al.* (1954a) that *A. aerogenes* could not be isolated from this source. Twenty-three encapsulated isolates of *A. aerogenes* were obtained in all from six separate samples of the starch-fed sheep's rumen contents. All produced markedly mucoid colonies on agar media containing a fermentable carbohydrate, and were indole and methyl red negative, nitrate-reducing, Voges-Proskauer, citrate, catalase and urease positive. No isolate liquefied gelatin, two only fermented starch, six fermented neither dulcitol nor adonitol, two (the starch-fermenters) attacked adonitol but not dulcitol, one attacked dulcitol but not adonitol, and the remaining fourteen fermented both these sugar alcohols.

Other urease-positive Gram-negative isolates from calf rumen

Of eleven catalase-negative non-coliform isolates, two from the rumen of calf OC1 attacked urea only weakly, whereas the remaining nine from much older calves (five from calf 2C and four from calf 3A¹) gave the typical reaction in Christensen's medium after 18 hr. incubation at 38°. They did not, however, produce the requisite lowering of pH, comparable with an authentic strain of *Proteus vulgaris*, in the more highly buffered medium of Stuart *et al.* (1945); but the growth was very poor in this instance. All these nine non-coliform urease-positive isolates seemed to be very similar and a short description is appended.

Morphology. Films made from growth on Wright's nutrient agar showed

small rods or cocco-bacilli, $0.5-1 \mu. \times 0.3-0.5 \mu.$, with roughly parallel sides and rounded ends, sometimes in short chains but more often arranged in bundles, difficult to distinguish from aggregations of cocci; Gram-negative, non-motile, non-sporing.

Growth characteristics. The 24 hr. growth on agar consisted of almost colourless, smooth, transparent, punctiform colonies up to 0.8 mm. diameter with entire edge and convex elevation. After 7 days the colonies were still small (2 mm. diameter), pale brown in colour and differentiated into a low convex central zone and an effuse peripheral zone. There was no haemolysis on horse blood agar plates. In nutrient broth, after 48 hr., there was moderate uniform turbidity with granular deposit and thin granular pellicle. The growth range seemed to be from pH 6 to 9. The optimum growth temperature was $38-40^{\circ}$; growth was poor at 50° and at 30° , but still took place at 22° .

Biochemical reactions. The following compounds were fermented with production of acid and gas: arabinose, dextrin, galactose, glucose, glycerol, lactose, maltose, raffinose, rhamnose, sucrose, trehalose and xylose; only slight acidity was produced in litmus milk. The following compounds were not fermented: adonitol, aesculin, dulcitol, inositol, inulin, mannitol, salicin, sorbitol and starch. Methyl red-positive; methylene blue reduced; nitrate reduced to nitrite; Voges-Proskauer reaction negative; gelatin not liquefied; catalase not formed; H_2S not produced. Ammonia was readily produced from urea as previously stated. The organism, however, shows little resemblance in other respects to any species of *Proteus*, as will be seen from the above description.

DISCUSSION

It would seem that the rumen coliform bacteria of the milk-and-gruel-fed young calf closely resemble those of the hay-fed adult sheep both with respect to population ($> 10^6$ /g. rumen contents) and to type, which is intestinal rather than saprophytic. The urea-attacking *Aerobacter aerogenes* or *A. cloacae* were relatively in the minority, although other unidentified non-coliform Gram-negative bacteria with a more pronounced power of attacking urea were isolated from the rumens of two older calves. It has long been known that mixed rumen bacteria will rapidly produce ammonia from urea, even when the fodder does not contain this source of nitrogen (see Pearson & Smith, 1943). This is not surprising when it is remembered that urea is a normal constituent of ruminant saliva. What is surprising is that an association of preponderantly obligate anaerobic bacteria, as the rumen microflora is thought to be, should have this urea-splitting power so highly developed since, by and large, all the bacteria so far reported as attacking urea in pure culture belong to aerobic or facultatively anaerobic genera like *Proteus*, *Aerobacter*, *Bacterium* (Cooke & Keith, 1927), *Micrococcus* (*Staphylococcus*), *Sarcina*, with apparently no representatives so far among anaerobic genera like *Clostridium*, *Bacteroides*, *Veillonella*, *Propionibacterium*, members of which have at various times been isolated from the rumen. Urease-positive micrococci have in fact been isolated in pure culture from sheep rumen contents (Mann *et al.* 1954a) and also from

the rumens of the calves used in this study (Mann & Oxford, 1954*b*) but their numbers are usually, although not always, quite small. Hence an interest attaches to the isolation of any new urease-positive small Gram-negative rod from the calf rumen. There must clearly be other urease-positive bacteria in the rumen which have hitherto eluded isolation in pure culture.

A further interest attaches to rumen coliform bacteria of the saprophytic type in regard to their capsulation. Hobson & MacPherson (1953) noted that the final sediment obtained when strained sheep rumen contents was centrifuged consisted largely of capsulated Gram-negative bacteria, occurring in clumps in the case of the starch-fed sheep, rather than in relative isolation as with the hay-fed sheep. This suggests that the presence of starch in the ration may alter the 'free' rumen Gram-negative microflora. Although the population of capsulated *Aerobacter aerogenes* in the starch-fed sheep's rumen is quite small and can account for only a minute proportion of the total 'free' Gram-negative microflora, it is perhaps significant that this particular capsulated bacillus is apparently absent from the hay-fed sheep's rumen. This is not inconsistent with the findings of Wilson *et al.* (1935, p. 175) who reported that, in Britain at least, *A. aerogenes* can more easily be isolated from grains and feeding cakes than from hay.

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