Defined Bacterial Populations in the Rumens of Gnotobiotic Lambs

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SUMMARY

Five gnotobiotic lambs were fed on sterile diets until they were killed at 13 to 21 weeks of age. They were dosed orally with different combinations of 11 species of rumen bacteria. The biochemical reactions of each of the bacteria inoculated had been determined in pure culture *in vitro*, and they were chosen to perform the main reactions known to be associated with digestion in the normal mature rumen.

Two of the bacteria could not be reisolated, but the remainder had established readily in the rumen, forming stable, mixed, defined populations. The total numbers of bacteria in the rumen, and the viable counts of most of the individual species were comparable to those of normal sheep. The concentration of volatile fatty acids was lower, however, and in four of the lambs there was a higher proportion of butyric acid and a lower proportion of propionic acid than in normal sheep. Cellulolytic, ureolytic, and methanogenic activities appeared to be taking place and lactate-utilizing bacteria appeared to reverse the accumulation of lactate which resulted from the activity of lactate-producing bacteria.

Some of the bacteria also established at high levels in the caecum.

INTRODUCTION

The rearing of small omnivorous and carnivorous gnotobiotic animals is a well-established laboratory procedure (Coates, 1968) but ruminants present particular difficulties. Digestion in omnivorous or carnivorous animals can take place without the intervention of gut micro-organisms, but bacteria are essential for digestion in mature ruminants. A young ruminant, fed on milk, might be expected to behave as a monogastric animal and to thrive in the gnotobiotic state while its rumen is still undeveloped, but in the mature ruminant the main digestive action is provided by the micro-organisms of the rumen, regardless of the form of the diet.

The principal reactions of the main components of the rumen microflora, reviewed by Hungate (1966) and Hobson & Howard (1969), have been established largely from studies of pure cultures *in vitro*. Information on interactions between rumen bacteria has been obtained from mixed cultures which rarely comprise more than two bacterial species. In such studies 'animal factors' are missing and rumen conditions cannot be reproduced. *In vivo* studies in normal mature ruminants are complicated by the great variety and

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complexity of the rumen microflora. Establishing a relatively simple, stable, fully-defined, functioning population of bacteria in the rumens of gnotobiotic lambs should help to overcome these problems and provide information on the metabolism and interactions of rumen bacteria *in vivo* which would augment that derived from *in vitro* studies.

Between 1968 and 1973 a total of 29 gnotobiotic lambs were studied at Cambridge (Alexander & Lysons, 1971; Lysons, 1974; Lysons & Alexander, 1975). This paper reports on five of the lambs which were dosed orally with mixed populations of rumen bacteria. A short communication giving some results has been published (Lysons *et al.*, 1971).

Our main aims were to determine whether all the bacteria inoculated would establish in the rumen, and, if they did so, whether stable populations would persist; whether the total viable counts would be comparable to those found in the rumen of conventional lambs; and whether the bacteria would carry out the biochemical functions for which they were chosen. Attempts were also made to determine whether any of the bacteria established themselves in the caecum.

We thought that a comparatively simple flora should be capable of carrying out the main reactions known to be necessary for digestion (Hungate, 1966; Hobson & Howard, 1969), and 11 bacteria (Table 1) were chosen. Under natural conditions the rumen flora develops in stages as the diet of the young animal changes and it was decided, at least in the first experiments, to inoculate the bacteria in the approximate order in which we thought they might develop naturally. The first four bacteria in Table 1 are major components of the rumen flora of the young animal. Anaerobic bacteria such as those listed in the lower part of Table 1 become predominant as the animal is weaned. The exception is the facultative anaerobe *Streptococcus faecium* which frequently occurs in the young animal. It was given last to one lamb in order to perform an additional function.

Although anaerobic ciliate protozoa play a part in rumen reactions their fermentation reactions are similar to those of the bacteria. Protozoa are thought not to be essential to rumen function. As it is impossible to obtain ciliate protozoa reliably free from bacteria, either from the rumen or from culture, a protozoal population was not provided.

METHODS

Gnotobiotic lambs. The five lambs (Table 1) were obtained by hysterectomy and reared in cages inside flexible-film isolators using the equipment and techniques described by Alexander & Lysons (1971), and Alexander *et al.* (1973*a, b*).

Liquid feeds. The lambs were fed on sterile, canned, cows' milk (Carnation Food Co., London WC2) which was diluted, in the isolator, with 1 vol. sterile distilled water to 2 vols milk, giving approximately 20% total solids. Milk feeds were given for periods of between 5 and 10 weeks (Table 1) and solid feeds were given for varying periods before milk feeding was stopped until the animals were killed for post-mortem examination.

The lambs were given rumen bacteria while they were being fed milk plus solid diet, or solid diet alone (Table 1).

Solid feeds. Details of the two solid feeds, which were fed *ad libitum*, were given by Alexander *et al.* (1973*b*). Feed 1, a conventional lamb feed, was fed to lambs 9a and 10b. Feed 2, which combined some of the characteristics of a non-ruminant-type feed with those of a ruminant-type feed, was fed to the other three lambs.

Bacteria. The 11 bacteria listed in Table 1 were chosen for their biochemical activities, which had been determined in pure culture at the Rowett Research Institute. The strains inoculated had been in laboratory culture for some years, and were well characterized.

Table 1. *Sequence and intervals at which rumen bacteria were given orally to gnotobiotic lambs*

Lamb no. ...	Age (days)				
	9a	10b	16a	18a	12b
Solid feed first offered	39	16	21	21	44
Milk feed stopped	69	37	49	49	51
First inoculation	68	43	44	44	73
	Days after first inoculation				
	9a	10b	16a	18a	12b
<i>Streptococcus bovis</i> Strep26	0	0	0	0* 2*	0
<i>Lactobacillus</i> sp. LB17	0	0	0	0* 2*	NI
<i>Veillonella alcalescens</i> 692/1	1† 2† 3†	1† 2† 3†	0	0* 2*	0
<i>Megasphaera elsdenii</i> LC1	1† 2† 3†	3† 4† 5†	0	0* 2*	0
<i>Butyrivibrio fibrisolvens</i> B834	15	15	2	0* 2*	2 7
<i>Bacteroides ruminicola</i> 46/5(2)	22 23 24 28 29	22 23 24 29 30	10	13	7
<i>Bacteroides amylophilus</i> H12	22 23 24 28 29	22 25 26 28 29	NI	NI	NI
<i>Ruminococcus</i> sp. 426-3(1)	23 24 33	23 24 33	10 29	13	7 33
<i>Streptococcus faecium</i>	NI	NI	NI	NI	32
<i>Methanobacterium ruminantium</i> MB5151	NI	NI	NI	NI	32
<i>Anaerovibrio lipolytica</i> 5S	NI	NI	NI	NI	33

Day 0, first day of inoculation; NI, bacterium not inoculated. All inocula consisted of 10 ml culture medium, except *, inoculum consisted of 5 ml rumen contents from lamb 16a; and †, inoculum consisted of 100 ml culture medium.

Most were isolated from the rumens of sheep fed on rations containing concentrates; but the lactobacillus and megasphaera were isolated from calves being fed milk, and the strain of *Streptococcus bovis* was isolated from a red deer feeding on natural pasture. *Lactobacillus* sp. and *Ruminococcus* sp. had properties similar to *Lactobacillus plantarum* and *Ruminococcus albus*, respectively (Hungate, 1966).

The lactobacillus was inoculated first, to ferment sugars in milk which may have leaked from the oesophageal groove into the undeveloped rumen. *Streptococcus bovis*, which is a minor but consistent component of the adult rumen flora, was inoculated with the lactobacillus because it was thought that as a facultative anaerobe it would help in reducing the

rumen fluids to a redox potential, E_h , suitable for growth of the strict anaerobes which should later form the principal rumen flora. It was also amylolytic and fermented a number of sugars. Both these bacteria produced lactic acid as a major fermentation product. Lactic acid is undesirable in rumen contents and it is normally fermented to volatile fatty acids. The megasphaera and veillonella were both capable of fulfilling this latter function, producing mainly acetic and propionic acids. The megasphaera also produced some butyric and higher acids and hydrogen and fermented a number of sugars including maltose and sucrose. *Megasphaera elsdenii* produced considerable quantities of butyric acid when fermenting sugars (Hobson & Mann, 1961). *Butyrivibrio fibrisolvens* ferments a wide range of feed carbohydrates, including hemicellulose. The strain used here produced butyrate with acetate, formate and lactate. *Bacteroides amylophilus* ferments starch, dextrans and maltose to formic, acetic and succinic acids. The strain used was added for its amylolytic properties and also because it was proteolytic. The nitrogen requirements of the different bacteria were varied but would be supplied by this proteolytic activity and the deaminative and proteolytic activities of *Bacteroides rumenicola*. Deamination would also supply the branched-chain fatty-acid growth factors of the ruminococcus. *Bacteroides rumenicola* fermented a wide range of feed carbohydrates to lactic, acetic, formic and succinic acids. The formic acid would be expected to accumulate or to be a substrate for methanogenesis, while succinic acid would be decarboxylated to propionic acid by the veillonella. Cellulolytic activity was expected to be provided by the ruminococcus which was very active *in vitro* on prepared cellulose. Ruminococci can ferment some other carbohydrates including hemicellulose. The strain used here produced acetate, formate and ethanol as its major fermentation products.

It was thought that these eight species, if they established themselves in the rumen, should be capable of degrading feed components to produce the lower volatile fatty acids necessary for the animal's nutritional requirements. In addition to this basic flora, in one of the six gnotobiotic lambs, three further species were added to provide three other normal rumen functions: a strain of *Methanobacterium ruminantium* which produced methane from formate or hydrogen and carbon dioxide; a strain of *Anaerovibrio lipolytica* added principally for its lipolytic activity as it was capable of vigorous fermentation of only glycerol and fructose to, mainly, acetic, propionic and succinic acids; and a rumen strain of *S. faecium* which had ureolytic activity (Cook, 1976).

Inoculation. Lambs were dosed orally, at different times, with various amounts of 24 or 48 h cultures in non-selective broth medium (Table 1). Lambs 16a and 18a were reared at the same time: for convenience, and to find out whether the establishment of bacteria was similar, the first five bacteria were introduced into lamb 18a by transfer of 5 ml amounts of rumen contents from lamb 16a. No attempt was made to estimate the number of bacteria in any of the inocula.

Sampling of lambs. Details of the methods of sampling have been described by Alexander *et al.* (1973a, b).

Samples of rumen contents were withdrawn through a stomach tube into a bottle. They were always taken between 2 and 2½ h after the morning feed, and were removed from the isolator within a few minutes. Caecal contents were studied after removal of the caecum *post mortem*; examination was started within 30 min of death. Rumen gas was sampled by inserting a double-ended needle through the left flank of the animal into the dorsal sac of the rumen, and collecting the gas in an evacuated tube. Faeces were removed from the isolator daily.

Bacteriological examination of rumen samples. Except where noted, techniques were based

on those of Hungate (1950). Serial dilutions were made of rumen liquor in a basal non-selective liquid medium prepared under oxygen-free carbon dioxide. Viable counts of the bacteria were carried out on solid media in roll tubes and plates, or in broth cultures, three sets of roll tubes or liquid cultures being made from each dilution series. In roll tubes, the viable count was taken as the average of the three roll-tube counts at the appropriate dilution. In the broth cultures, the viable count was taken from the highest dilution at which growth was noted in all three tubes.

The non-selective medium used for total viable counts (Medium 1 of Kurihara *et al.*, 1968) contained glucose, maltose, cellobiose and lactate, and supported the growth of all the bacteria except *A. lipolytica* and *Meth. ruminantium*. The former does not usually ferment any of the sugars in the medium, although our strain showed slow faint growth on cellobiose in pure culture: the latter showed slight growth in the presence of hydrogen produced by the other bacteria. Gram-stained smears were made from 50 randomly-selected colonies and species counts were calculated.

Various other media were used in attempts to separate the bacteria, particularly those which comprised only a small proportion of the total population, so that more accurate counts could be made. The bacteria, and their selective media were: for *S. bovis*, azide agar (Oxoid); for the lactobacillus, Rogosa SL medium (Difco: Rogosa, Mitchell & Wiseman, 1951); for *Veillonella alcalescens* and *Meg. elsdenii*, veillonella agar (Difco: Rogosa *et al.*, 1958). These media were prepared in Petri dishes, inoculated, and incubated under carbon dioxide and hydrogen (5:95, v/v). Counts were made by spreading measured drops at serial dilutions over segments of the agar medium. Bacteria were recognized by examining Gram-stained smears from colonies. When colonies of a bacterium were detected but could not be counted accurately because of overgrowth of other bacteria, the count was recorded as being less than a given titre.

Butyrivibrio fibrisolvens occurred in high numbers in rumen samples and was counted on the non-selective medium, as no selective medium was available. The starch-fermenting *Ba. amylophilus* and *Ba. ruminicola* were counted on a starch medium (Medium 2 of Kurihara *et al.*, 1968), but it was difficult or impossible to distinguish between them. The amylolytic *S. bovis* also grew on this medium but could be distinguished by its orange colony. The counts of the cellulolytic ruminococcus were estimated from the highest dilution in which the filter-paper strips in all three tubes of the medium of Mann (1968) were seen to be attacked. *Streptococcus faecium* was counted on roll tubes of a medium similar to the non-selective one except that it contained mannitol as sole energy source and 6.5 % (w/v) NaCl which inhibited growth of *S. bovis*. *Anaerovibrio lipolytica* was counted as colonies producing clear zones in roll tubes of a linseed-oil medium (Hobson & Mann, 1961). *Methanobacterium ruminantium* was counted in a mineral medium under hydrogen and carbon dioxide (Henderson, 1973). Methanogenesis produces a decrease in gas pressure and dilutions in which this was shown in all three tubes were taken as positive. Gram-stained smears were prepared from all rumen samples to check the relative numbers and morphological types of bacteria present.

Bacteriological examination of caecal samples. Caecal bacteria were cultured in a similar manner to the rumen bacteria. In lambs 9a, 16a and 18a, the non-selective medium and the cellulose medium were used. In lamb 12b, both of these media were used, as well as the medium for *Meth. ruminantium*. In lamb 10b, all the non-selective and selective media were used. Gram-stained smears were examined as for rumen samples.

Total counts. These were made initially by a modification of the Breed (1911) smear technique, using Gram-stained smears. As well as the total number; attempts were made

to obtain differential counts of the individual species. Total counts were also made with a counting chamber and with a calibrated Coulter Counter (Hobson & Mann, 1970).

Contaminants. The methods used routinely for monitoring contaminants in the lambs were described by Elliott *et al.* (1974). In addition, all the rumen and caecal samples considered in this paper were examined for the presence of contaminants.

Biochemical estimations. Volatile fatty acids (C_2 to C_5) were determined by gas-liquid chromatography (Whitelaw *et al.*, 1970). Formic acid was determined by the method of Grant (1947), and lactic acid by the method of Conway (1957). Rumen gas samples were analysed with a mass spectrometer (AEI MS10). Cellulose digestion was measured as the difference in the lignin:cellulose ratio between diet and faeces. Cellulose was estimated by the method of Barnett (1957). Lignin was estimated by the method of Ellis, Matrone & Maynard (1946) as modified by Waite, Johnston & Armstrong (1964). Urease activity in muslin-filtered rumen liquor was determined by the method of Hecker (1971).

RESULTS AND DISCUSSION

Examination of rumen samples

Pre-inoculation rumen samples. One or more samples were taken from lambs 9a, 10b, 16a and 18a before they were dosed with bacteria. The pH of the rumen fluid was between 6.5 and 7.2. There was a small concentration of volatile fatty acids (10 to 15 mM) of which about 90% was acetic acid, presumably from tissue metabolism. Lactic acid was not detected.

Lamb 9a. On the day after the first inoculation, *S. bovis* and the lactobacillus had established viable populations, with *S. bovis* comprising some 90% of a total count of 9×10^8 /ml rumen contents (Table 3), and the rumen pH had fallen (Table 2). By the next day the lactobacillus had increased to about 60% of the population and the pH had dropped further. On day 3 the lactobacillus count of 8×10^8 /ml was about 80% of the total, but the lactate-utilizing *Meg. elsdenii* was seen in smears, and over the next few days the rumen pH rose and the lactobacilli decreased in numbers and in proportion of the population (20% on day 5), while *S. bovis* and *Meg. elsdenii* increased. The total viable counts remained fairly constant at between 9×10^8 and 5×10^9 /ml. In all samples *V. alcalescens*, a very small, Gram-negative coccus, was difficult to distinguish from the Gram-negative background material, but the counts from day 10 onwards showed that it established during this first phase of the experiment. Viable counts during the next 7 days (Table 3) showed *S. bovis* to be dominant; and smears indicated that the population was stable over this period. However, a contaminant staphylococcus was noted in films on day 10 and this persisted throughout the experiment. *Butyrivibrio fibrisolvens* was first observed microscopically 4 days after its inoculation on day 15, but numbers were insufficient for a viable count on day 17. By day 24 all the bacteria had been inoculated at least once and both viable counts and microscopic observations showed that the flora had become predominantly Gram-negative, as in a sheep reared conventionally. Bacteroides were the principal bacteria detected by viable counts, although it was not possible to distinguish between the two species (Table 3). The total viable count remained at about 4×10^9 /ml, and the proportions of the constituents varied little until the lamb was killed, 38 days after inoculation. Attempts to reisolate the ruminococcus in cellulose medium were unsuccessful.

Total counts increased from 6.4×10^8 /ml on day 10 to an average of 3.3×10^{10} over the last 5 days. At that stage the viable count was about 12% of the total count which is similar to what might be expected in a normal rumen.

Table 2. *pH and concentrations of fermentation products in rumen samples taken from lambs 9a and 10b*

	Days after first inoculation	Concentration (mm)							Rumen pH
		Total VFA (C ₁ -C ₆)	Formate	Acetate	Propionate	Butyrate (iso-But)	Valerate (iso-Val)	Lactate	
Lamb 9a	0	10.3	ND	ND	ND	ND	ND	—	6.5
	3	13.4	—	10.6	—	—	2.8 (—)	80.0	4.3
	6	41.5	—	22.2	11.3	5.8 (—)	2.2 (—)	—	5.8
	8	42.4	2.1	19.0	12.0	6.5 (0.4)	1.8 (0.5)	15.0	5.1
	10	55.8	4.5	25.8	12.1	7.5 (0.9)	4.7 (0.2)	—	5.5
	11	48.7	7.0	27.0	8.1	4.4 (1.2)	1.0 (—)	—	5.9
	12	63.1	3.5	30.3	10.6	14.1 (1.0)	3.5 (—)	—	5.8
	13	54.0	7.0	17.7	8.1	17.5 (1.9)	1.8 (—)	10.5	5.2
	15	50.0	4.2	22.9	10.2	8.6 (1.1)	2.9 (—)	—	5.6
	16	59.1	3.5	29.6	12.8	8.3 (0.7)	3.2 (0.9)	—	5.7
	17	54.0	—	30.2	11.5	8.3 (1.6)	2.4 (—)	—	5.8
	18	48.0	—	30.0	3.9	10.3 (1.7)	1.6 (0.4)	—	5.8
	19	52.5	—	26.3	5.3	18.7 (—)	2.1 (—)	—	6.0
	22	51.0	2.5	25.9	7.7	13.2 (0.2)	1.4 (—)	—	6.3
	23	39.1	7.0	17.0	7.4	6.2 (0.1)	1.3 (—)	—	6.0
	24	40.0	4.2	16.4	9.2	7.9 (—)	2.3 (—)	—	6.2
	25	51.5	9.0	15.6	11.0	15.0 (—)	0.8 (—)	—	6.7
	33	47.0	1.4	17.8	14.6	11.4 (—)	1.5 (—)	—	7.0
	38	53.0	6.0	18.1	15.2	11.4 (—)	2.3 (—)	—	7.0
Lamb 10b	15	51.5	4.2	33.7	6.6	3.0 (4.0)	— (—)	15.0	6.4
	21	68.7	5.6	29.8	7.4	23.0 (2.2)	0.4 (0.1)	—	6.6
	29	49.0	2.1	19.6	6.0	20.7 (0.6)	— (—)	—	6.7
	35	46.5	2.1	20.0	6.5	16.2 (1.7)	— (—)	—	6.5
	42	28.3	6.3	10.9	1.6	7.9 (1.3)	— (0.2)	—	7.2
	49	49.5	—	17.8	3.3	21.1 (—)	2.4 (4.9)	—	6.8

—, Less than 0.1 mm; ND, not determined.

Tests of fermentation products were made more frequently on this lamb than on subsequent lambs. Rumen samples were taken almost daily.

Immediately before inoculation, the rumen pH was 6.5 and the concentration of volatile fatty acids (VFA) was 10.3 mm. On the first 3 days after inoculation the concentration of VFA remained about the same (average 12.4 mm), but lactic acid concentration was 60 mm on day 1 and 80 mm on day 3, and the pH dropped to 5.5, 4.6 and 4.3 on the three successive days. This coincided with the multiplication of the lactate-producing population dominated first by streptococci, then by lactobacilli. On day 4 the lactic acid concentration had dropped to 40 mm and the VFA concentration had increased to 22.5 mm, while the pH had risen to 5.0. This trend was followed on subsequent days and paralleled the increase in lactate-utilizing *Meg. elsdenii* and *V. alcalescens*. The lactate was probably produced mainly by *S. bovis* and *Lactobacillus* sp., but later also possibly by *Ba. ruminicola* (Stewart & Lysons, 1975). On day 6, and subsequently (Table 2), butyric acid could be determined with more confidence. Its rise in concentration occurred before *Bu. fibrisolvens* was introduced, so it must have come from another source, probably *Meg. elsdenii*. *Megasphaera elsdenii* produces more butyrate when it is fermenting sugars than when it is fermenting lactate and in lamb 9a butyrate was generally found in larger amounts when the lactate-producing bacteria were in low numbers. The concentration of butyrate did not increase noticeably after the establishment of *Bu. fibrisolvens*, and since this bacterium never became

Table 3. *Numbers of viable bacteria in rumen samples taken from lambs 9a, 10b, 16a, 18a and 12b*

Days after first inoculation	10 ⁻⁶ × count/ml rumen liquor							10 ⁻⁸ × total viable count/ml
	<i>Streptococcus bovis</i>	<i>Lactobacillus</i> sp.	<i>Veillonella alcalescens</i>	<i>Megasphaera elsdenii</i>	<i>Butyrivibrio fibrisolvens</i>	<i>Bacteroides ruminicola</i>	<i>Ruminococcus</i> sp.	
Lamb 9a								
10 to 17*	2000	100	40	< 100	NR			30
24 to 38*	1000	< 100	1000	800	< 100	2000	NR	40
Lamb 10b								
7	700	200	10	5				1
15	4000	100	80	1				40
21	1000	50	200	30	200			20
29	400	200	50	0.8	200	NR	0.1	9
35	300	80	200	30	40	200	0.001	10
42	800	100	200	20	NT	10	0.01	60
49	1000	20	700	60	200	900	0.001	20
Lamb 16a								
2 to 10*	2000	800	200	700	20 000			100
93 to 98*	300	60	900	500	30 000	4000	0.1	300
Lamb 18a								
2 to 13*	7000	NR	700	< 1	1 000			90
61 to 70*	300	30	700	700	20 000	2000	0.001	200
Lamb 12b								
19 to 27*	600		200	10	200	8	NR	7
55 to 64*†	700		200	2	900	200	0.001	20

Blank spaces occur where bacteria had not then been inoculated (see Table 1).

NR, Bacteria not reisolated; NT, not tested.

* Single counts or average of a number of counts made during the period.

† In lamb 12a, viable counts/ml rumen liquor of the extra bacteria in the period 55–64 days after first inoculation were: *Streptococcus faecium*, 1×10^7 ; *Methanobacterium ruminantium*, 10^4 . *Anaerovibrio lipolytica* was not reisolated. On day 75, the viable count of *Ruminococcus* sp. was 10^4 /ml and *Meth. ruminantium* was 10^6 /ml.

a major fraction of the population, most butyric acid production may have been due to *Meg. elsdenii*. The small amounts of valeric acid detected from day 3 onwards could also have been formed by this bacterium, since it is the only one present known to form valeric acid by fermentation (Hobson & Mann, 1961).

Propionate can be produced directly by *Meg. elsdenii* and *Bu. fibrisolvens*, and indirectly by *V. alcalescens* which decarboxylates succinate produced by bacteroides or ruminococcus. Acetate is a fermentation product of all the bacteria. Formate can also be formed by all the species and its accumulation in the rumen, in levels higher than normally found in conventionally-reared lambs, would be expected in the absence of methanogenic bacteria.

Once the complete flora was established the rumen pH remained relatively constant.

Lamb 10b. This lamb was inoculated with the same eight bacteria as lamb 9a (Table 1), but the first bacteria were not introduced until after milk feeding had stopped, because it was thought that the fermentation of residual milk sugars had contributed towards the initial drop in rumen pH in lamb 9a. Lamb 10b was also inoculated when younger and was kept alive longer after inoculation to allow more time for studying the flora and for the ruminococcus to establish itself.

Two to three days after the first inoculation, *Lactobacillus* sp. had not reached as

high a proportion of the population (Table 3) as in lamb 9a, and the rumen pH (Table 2) did not fall rapidly even though the delay in giving *Meg. elsdenii* meant that *V. alcalescens* was the only lactate-utilizing bacterium present until day 3. By day 15 (Table 3) the four bacteria inoculated had established in a pattern similar to that in lamb 9a. By day 29 (6 days after its inoculation) a small population of ruminococci could be detected in viable counts, but this did not increase even after a third inoculation on day 33. The number of viable ruminococci present may have been higher than estimated because this bacterium normally colonizes cellulosic fibres, which were not readily collected by the narrow-bored stomach tube, and from which it is not readily dislodged. It is also a difficult bacterium to detect visually in Gram-stained films.

The composition of the population remained relatively stable until the lamb was killed on day 51. Microscopic examinations suggested that *Bu. fibrisolvens* formed a larger proportion of the population than indicated by the viable counts, suggesting a high proportion of non-viable cells. *Bacteroides ruminicola* was identified in stained smears, but *Ba. amylophilus* was not identified either by direct observation or by culture. A contaminant streptococcus was detected on day 35 and remained part of the population.

Before inoculation, the rumen pH value was 7.2. On the day after inoculation, the rumen pH had fallen to 5.2 and the concentration of lactic acid was 40.0 mM. By the next day the pH was 6.3 and lactic acid was not determinable, and it remained so except on day 15. By day 7 the rumen pH was 6.6. Total volatile fatty acids increased after day 1, from their pre-inoculation concentration of 9.1 mM to 26.3 mM on day 7. The rest of the analyses are shown in Table 2.

As with lamb 9a, acetic acid concentration decreased as the flora became more complex. The proportion of propionate in the acids was less in this lamb than in lamb 9a, possibly reflecting a smaller proportion of bacteroides and megasphaera. However, higher butyrate concentrations were evident in most samples taken after the introduction of *Bu. fibrisolvens*.

Lamb 16a. Inoculation began six days before milk feeding stopped, but the lactate-utilizing bacteria were added with the lactate producers to prevent the increase in lactic acid and drop in pH noted in lamb 9a. Bacteria were introduced over a shorter period than in the two previous lambs (Table 1). *Bacteroides amylophilus* was not given to this and the two subsequent lambs, because it had proved difficult to identify and its amylolytic and proteolytic properties should have been covered by the other bacteria.

Streptococcus bovis dominated on day 1. The lactobacilli were dominant on day 2, but decreased in number on day 3. *Butyrivibrio fibrisolvens* then became dominant both in stained preparations and on viable counts: it remained so until the end of the experiment (Table 3). After the introduction of the remaining two bacteria (Table 1) the flora remained stable, the viable counts being similar to those recorded between days 93 and 98 (Table 3). Total viable counts were in excess of 10^{10} bacteria/ml rumen liquor, i.e. comparable with the highest counts recorded in sheep reared conventionally. The lamb was killed on day 98. A contaminant clostridium was detected at about day 50.

On the day of inoculation, the rumen VFA concentration was 12.9 mM and the pH 6.8. The pH dropped to 5.0 on day 1 when the lactate concentration was 29.0 mM, but had recovered to 6.0 by days 2 and 3 when the lactate concentration had diminished to 3.0 and 5.4 mM. On day 2, the VFA concentration was 46.5 mM and on day 3, 54.9 mM. Results selected to demonstrate the main trends (Table 4) show that the butyrate concentration was high and the propionate low from days 7 to 10, reflecting the high population of butyrivibrio. Although propionate remained low, there was a rise after the introduction of the succinate-producing bacteroides on day 10.

Table 4. *pH and concentration of fermentation products in rumen samples taken from lambs 16a, 18a and 12b*

	Days after first inoculation	Concentration (mm)							Rumen pH
		Total VFA (C ₁ -C ₅)	Formate	Acetate	Propionate	Butyrate (iso-But)	Valerate (iso-Val)	Lactate	
Lamb 16a	6	14.9	—	12.8	0.5	0.8 (0.7)	— (—)	—	7.1
	7	45.1	5.1	20.6	1.1	16.7 (0.6)	0.9 (0.1)	1.6	6.4
	8	45.1	6.3	26.0	0.3	11.6 (—)	0.8 (0.2)	5.2	6.1
	10	45.1	7.6	17.9	0.4	16.8 (1.8)	0.5 (—)	1.6	ND
	16	52.9	7.6	18.7	4.8	18.9 (—)	2.8 (—)	32.0	6.2
	32	52.9	—	22.6	6.2	18.0 (1.7)	4.1 (0.3)	—	6.6
	71	34.9	—	23.8	3.2	6.8 (0.2)	0.7 (0.2)	—	6.6
	98	54.2	—	27.6	5.3	17.7 (0.3)	3.0 (0.3)	—	ND
Lamb 18a	0	20.7	5.0	15.6	—	0.1 (—)	— (—)	—	7.1
	1	41.3	5.6	16.3	1.5	14.3 (—)	3.2 (0.2)	—	6.7
	13	46.5	3.2	23.7	1.5	16.3 (0.8)	0.9 (—)	—	5.8
	62	49.7	—	29.9	5.0	12.4 (0.9)	1.5 (—)	—	6.1
	70	77.3	—	35.2	15.3	20.0 (1.2)	4.9 (0.5)	—	5.4
Lamb 12b	1	47.8	2.0	20.3	2.0	18.5 (0.1)	4.8 (—)	—	5.3
	8	55.3	6.1	26.8	3.0	14.5 (0.3)	4.4 (0.2)	—	6.0
	19	39.8	2.7	29.4	0.9	5.8 (0.1)	0.6 (0.2)	—	7.1
	27	27.0	4.0	13.6	3.0	5.0 (0.2)	0.9 (—)	—	7.2
	55	14.8	4.6	6.9	1.2	1.6 (0.3)	0.2 (—)	—	7.2
	75	9.4	—	7.8	0.7	0.9 (—)	— (—)	—	6.8

—, Less than 0.1 mm; ND, not determined.

A comparison of dietary and faecal cellulose:lignin ratios indicated that about 20% of the cellulose present in the ration had been digested by this lamb.

Lamb 18a. The feeding regime and times of inoculation were the same as for lamb 16a, but the first bacteria were introduced by inoculation of rumen contents from lamb 16a. They were collected 6 and 8 days after lamb 16a had first been inoculated. One day after inoculation, *S. bovis* was dominant, with *Lactobacillus* sp. and *Meg. elsdenii* also present. This contrasted with the population in the inoculum. At this time, *Bu. fibrisolvens* was dominant in lamb 16a. However, cultural counts on days 2 to 13 (Table 3) showed that *Bu. fibrisolvens* rapidly became a major part of the flora and, eventually, the dominant bacterium in lamb 18a. As with lamb 16a, total viable counts in rumen liquor were high.

A number of contaminants, including clostridia (Elliott *et al.*, 1974; Lysons, 1974), developed in the rumen, and between days 65 and 69 were detectable in numbers up to about 10^7 /ml. The experiment was terminated on day 70.

Rumen analysis before inoculation was similar to those of the other lambs. Lactate was not detected (Table 4), but the lactobacilli became only a minor part of the population. The concentration of butyrate on day 1 was similar to that in lamb 16a and suggests that although *Bu. fibrisolvens* was not detected in Gram-stained films of rumen contents until day 2 it may have been active from the start. The preponderance of butyric acid in all the samples was similar to that in lamb 16a and reflected the continuing high numbers of *Bu. fibrisolvens*. The lamb was shown to have digested about 15% of dietary cellulose.

Lamb 12b. Milk feeding was stopped when this lamb was 51 days old, but the bacteria were not given until 22 days later. *Lactobacillus* sp. (Table 1) was omitted as the inocula were given well after milk feeding had stopped. This lamb was the one given the ureolytic, methanogenic and lipolytic bacteria (Table 3).

Streptococcus bovis established itself quickly and was dominant on day 1; *Meg. elsdenii* had increased in numbers on day 2. The flora remained stable with the streptococcus dominant until after the other bacteria were introduced on day 7 (Table 1). On day 8, *Bu. fibrisolvens* appeared to be the dominant bacterium, with *S. bovis*, *Meg. elsdenii* and *Ba. ruminicola* also present in high numbers. Microscopic observations suggested that *Bu. fibrisolvens* formed a larger proportion of the population than indicated by the cultural counts (Table 3). *Ruminococcus* sp. could not be detected after 27 days so a second inoculum was given on day 33, and a small population was subsequently detected. Of the three additional bacterial species (Table 3), two had established themselves by day 55. *Anaerovibrio lipolytica* was not reisolated in culture nor detected in smears. The lamb was killed on day 76.

Lactic acid was not found in the rumen of this lamb (Table 4) reflecting the absence of lactobacilli. Presumably the activity of *Meg. elsdenii* and *V. alcalescens* was sufficient to metabolize all the lactic acid produced by the streptococcus and bacteroides. The butyrate concentration remained high and the propionate concentration low until between days 19 and 27, reflecting the dominant flora. There was a decrease in the total VFA concentration on days 27, 55 and 75 (Table 4) which probably resulted from this lamb's poor appetite. It ate little in the last weeks of life and lost some weight (Lysons, 1974; Lysons & Alexander, 1975). The lamb was shown to have digested about 22 % of dietary cellulose.

Ureolytic activity was not detected in rumen liquor before the introduction of *S. faecium*. After it had been introduced 5 ml samples of rumen liquor released 9 to 24 µg of ammonia from added urea over a 10 min period.

Other analyses. Rumen gas samples were taken from several lambs, both before and after inoculation. Unfortunately, the methods of collection led to contamination with air. Carbon dioxide was always in excess of that in normal air both before and after inoculation and in some of the samples hydrogen was similarly in excess, particularly after inoculation.

Methane was measured from the height of the *m/e* 15 peak, which had previously been shown to be proportional to the methane content of a gas mixture. However, as air constituted a large proportion of many samples the partial pressure of methane was very low and so the ratio of the heights of the *m/e* 15 to *m/e* 44 (CO₂) peaks was taken as a measure of the methane content of the gas. Thirteen gas samples were taken from lamb 12b in the period from 2 days before inoculation with bacteria until the end of the experiment. Up to, and including day 38, the height of the *m/e* 15 peak was similar to that in the background spectrum, as it was in samples from other lambs. The next sample, on day 53, and subsequent samples, to day 65, showed a 10-fold increase in the ratio of *m/e* 15 to *m/e* 44 peaks. Two samples taken at about this time from the air inside the isolator also showed *m/e* 15 peaks that were higher than the background. These results were taken, along with the cultural results, to indicate an active methanogenic flora.

It was not possible to study bacterial amylase, lipase and protease activity in the rumen since these enzymes were present at variable levels in the feed and in pre-inoculation rumen samples.

Examination of caecal samples

Total viable counts ranged from 6×10^8 to 1×10^{10} /ml caecal contents. In lambs 9a and 12b, Gram-positive cocci (presumably *S. bovis* and, possibly in 12b, *S. faecium*) were dominant in Gram-stained films. Smaller numbers of *Meg. elsdenii* and lactobacilli in lamb 9a and *Ba. ruminicola* in lamb 12b were also noted. *Methanobacterium ruminantium* was not detected.

The caecal flora of lamb 10b was studied in most detail. The total viable count was

5×10^9 /ml, with *S. bovis* dominant. The counts for other bacteria were lower: *V. alcalescens* and *Ba. ruminicola*, 2×10^8 /ml; *Lactobacillus* sp., 4×10^7 /ml; and *Meg. elsdenii*, $< 10^6$ /ml. The contaminant streptococcus previously noted in the rumen was also present.

In lamb 16a, *S. bovis* and *Ba. ruminicola* were again dominant in viable counts, with *Meg. elsdenii* (2×10^9 /ml) present in similar numbers. The clostridium was also present in high numbers.

In lamb 18a, the picture was much the same, and again the clostridial contaminant was present in high numbers (about 10^9 /ml). A species of *Neisseria* was also a contaminant at 5×10^8 /ml.

Neither *Bu. fibrisolvens* nor *Ruminococcus* sp. could be identified in any of the samples.

CONCLUSIONS

It has been reported (Lysons, 1975) that a single species of rumen bacteria, *Ba. ruminicola*, can establish itself in a gnotobiotic lamb. We have now shown that mixed defined bacterial populations can also be established *in vivo*. The functions of these defined floras are largely predictable from the reactions, *in vitro*, of their constituents. The most striking example was the development of the initial populations, where the addition of lactate-utilizing bacteria appeared to reverse the accumulation of lactate due to the activity of lactate-producing bacteria. When all the bacteria had been introduced, the rumen flora of the lambs became preponderantly Gram-negative, as in conventionally-reared lambs. The final flora exhibited a stability similar to that of the normal rumen. The general preponderance of *Bu. fibrisolvens* could be explained by its wide range of fermentative abilities. *Ruminococcus* sp. did not seem to grow in large numbers and this could be due to a limited ability to attack cellulose in the plant structure. The results indicated, however, that cellulolysis had taken place. It is also possible that some of the strains of bacteria had changed in the course of many passages in laboratory media which may have affected their ability to establish themselves and grow in the rumen.

The concentration of VFA in the rumen was less than that usually reported for sheep, although the total numbers of bacteria and the viable counts of most of the individual species were comparable to the highest recorded for sheep reared conventionally.

The mixture of VFA generally contained a higher proportion of butyric acid and lower proportion of propionic acid than is normally found. Only in lamb 9a, where the butyri-vibrio did not form a large proportion of the population, was the propionic acid concentration higher than butyrate and nearer the normal values. This would suggest that to obtain a more normal rumen VFA mixture the population should include other species of bacteria producing propionate or succinate which could establish themselves in high numbers. However, a detailed assessment of activity of the rumen bacteria is complicated by absorption of fermentation products through the walls of the rumen. This absorption depends on rumen development and the degree of development may have varied in different lambs (Lysons & Alexander, 1975; Lysons *et al.*, 1976).

Gnotobiotic lambs can, therefore, be used to provide a model for investigating the actions and interactions of selected mixtures of rumen bacteria *in vivo*.

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