Ecological Factors Determining Establishment of Cellulolytic Bacteria and Protozoa in the Rumens of Meroxenic Lambs

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Ecological factors that control the establishment of cellulolytic bacteria and ciliate protozoa in the lamb rumen were studied in meroxenic animals. Axenic lambs received dilutions of rumen liquor from either conventional lambs and sheep (pool A) or meroxenic lambs (pool B).

The total number of bacteria established in the rumen was between 10^9 and 5×10^{10} g⁻¹. In lambs inoculated with dilutions (10^{-6} , 10^{-7} , 10^{-8}) of pool A, cellulolytic bacteria did not become established. However, subsequent inoculation with *Bacteroides succinogenes*, resulted in colonization in lambs that had received 10^{-6} and 10^{-7} dilutions of pool A. However, *B. succinogenes* became established in only one of three lambs that received the 10^{-8} dilution. Similar results were obtained for the protozoan *Entodinium* sp.

With pool B, lambs were inoculated earlier and cellulolytic bacteria were established directly from the 10^{-6} and 10^{-7} inocula. *Polyplastron multivesiculatum* establishment occurred readily when inoculated into the lambs which had received the 10^{-6} dilution of pool B.

Results obtained in this study suggest that establishment of cellulolytic bacteria and protozoa requires an abundant and complex flora and is favoured by early animal inoculation.

INTRODUCTION

Microflora and fauna in the bovine and ovine rumen have been the focus of many studies over the past thirty years. The characteristics of the predominant bacterial species are now well-known and have been summarized in several reviews (Hungate, 1966a; Hobson & Howard, 1969; Church, 1970; Bryant, 1977; Clarke, 1977; Prins, 1977). Nonetheless, the *in vivo* roles of the different bacteria and ciliate protozoa are, as yet, far from clear owing to the great diversity of the microbial population and the complexity of the metabolic reactions involved. A study of the main bacteria and ciliates and of bacteria–protozoa interactions is thus likely to be simplified by using a less complex environment than that found in the rumen of a conventional animal, i.e. by using an animal whose rumen contains a simplified flora. Such use of gnotoxenic conditions has been widely developed in studies of host–microflora relationships in monogastrics and so should help solve some of the problems concerning microbial ecology in the rumen.

Studies involving axenic or gnotoxenic ruminants, though dating back to 1915, remain limited in scope. The main ones (Mann & Stewart, 1974; Lysons & Alexander, 1975; Lysons *et al.*, 1971, 1976a; Cheng & Wallace, 1979; Barr *et al.*, 1980) reported the setting up in gnotoxenic lambs of different bacterial populations composed of rumen flora dominant in conventional animals and the verification *in vivo* of the activity of inoculated species. These studies showed the extreme difficulty of successfully establishing cellulolytic bacteria in the rumen of gnotoxenic animals. For instance, attempts to establish cellulolytic bacteria (*Ruminococcus* spp.) either failed (Mann & Stewart, 1974), or resulted in very low levels compared to those in conventional animals (Lysons *et al.*, 1976a). Difficulties in establishing cellulolytic bacteria were also reported by

Males (1973) who failed to establish *Bacteroides succinogenes* in sheep raised in isolation from birth. There have been no attempts to establish protozoa, whether axenic or not, in gnotoxenic ruminants.

Given the importance of cellulose hydrolysis in the digestive physiology of the ruminant, a study of the ecological factors that allow the establishment of cellulolytic bacteria and protozoa in the rumen would appear to be an essential starting point for any subsequent studies on the role of particular species, interactions between bacteria, and bacteria–protozoa relationships. The approach chosen here was to use ‘meroxenic’ lambs, i.e. axenic lambs inoculated with a flora containing a limited number of microbial types. Such flora, though imperfectly understood, can be obtained by simplifying the flora of conventional animals. Our long term aim is to determine both quantitatively and qualitatively the minimal flora that must be present in the rumen to allow cellulolytic bacteria and ciliate protozoa to be established.

METHODS

Production and husbandry of axenic lambs. Axenic lambs were obtained either by Caesarean section or by natural birth followed immediately by isolation in a sterilized isolator and decontamination with antibiotics (Gouet *et al.*, 1979).

During the first two weeks, the lambs were reared in rigid, transparent sterile isolators (70 × 50 × 110 cm) to ensure that they remained axenic. Animals were then transferred to a flexible isolator (80 × 200 × 150 cm) where they were put into a metabolism pen (Riou *et al.*, 1977). Each isolator was linked to a sterile ‘bank’ isolator where the sampling and animal care materials were kept along with solid feed. Further equipment (feeders, drinking troughs, urine evacuators etc.) was added in addition to that previously described (Riou *et al.*, 1977), to maintain the animals for three to four months. Until the end of the second, third or fourth week depending on the group (Fig. 2), UHT sterilized cow’s milk was given *ad libitum*. This was followed by a mixed diet (milk + complete solid feed) which was given up to weeks 10 to 11; thereafter solid feed alone was given. Two feed formulae were used (I and II). The composition of feed I was (% w/w): barley, 15; oats, 4; peanut meal, 21.7; dried pulp, 20; molasses, 1; roughly chopped lucerne, 5; meadow hay, 30; glucose, 3.3; vitamin and mineral supplement. Feed II comprised good quality dehydrated lucerne. Both feeds were in pellet form (7 mm) and sterilized by γ -irradiation (45 KJ kg⁻¹).

Between the second and third week, each animal was fitted under sterile conditions, with a permanent plastisol rumen cannula.

Origins and composition of inocula. Two pools, A and B, of rumen contents were made (Fig. 1). Pool A was a mixture of rumen contents from two conventional lambs and from four adult sheep. From this pool (which was distributed into tubes and kept at -80 °C in glycerined milk) three axenic lambs were inoculated with the 10⁻⁸ dilution [lambs A(10⁻⁸)1, A(10⁻⁸)2 and A(10⁻⁸)3], two with the 10⁻⁷ dilution [lambs A(10⁻⁷)1 and A(10⁻⁷)2] and one with the 10⁻⁶ dilution [lamb A(10⁻⁶)1]. Pool B was made from the mixture of rumen contents taken from two lambs made ‘meroxenic’ by inoculation with the 10⁻⁷ dilution of pool A. Pool B was kept at -80 °C in the same way as pool A. One axenic lamb was inoculated with the 10⁻⁸ dilution of pool B [lamb B(10⁻⁸)1], one with the 10⁻⁷ dilution [lamb B(10⁻⁷)1] and two with the 10⁻⁶ dilution [lambs B(10⁻⁶)1 and B(10⁻⁶)2]. Lamb B(10⁻⁸)1 was then inoculated (days 60 and 67) with a 7.5 × 10⁻⁸ dilution of the same pool B.

Inoculation with pools A and B. Lambs were inoculated each time with 1 ml of the selected dilution, either *per os* when the inoculation was given early on, or into the rumen, when inoculation followed cannula fitting. Pool A was inoculated between days 17 and 40, and pool B, between days 6 and 42. Times of each inoculation are given in Fig. 2. Animals inoculated with the same dilution of the same material were kept in the same isolator, except for lamb A(10⁻⁸)1 which was reared alone.

*Inoculation with *Bacteroides succinogenes*.* After receiving either pool A or pool B, lambs A(10⁻⁸)1 to 3, A(10⁻⁷)1 and 2, A(10⁻⁶)1 and B(10⁻⁸)1 were inoculated with *B. succinogenes* S85. This strain was grown in a liquid medium in which cellulose was supplied as a strip of filter paper (see below). Inocula were made with 10 ml of a 3 to 5 d broth culture in which filter paper had been degraded. Inoculation was carried out several times in lambs A(10⁻⁸)1 to 3 and B(10⁻⁸)1 (Fig. 2). Lambs A(10⁻⁸)1 to 3 were first inoculated with *B. succinogenes* alone, then with sterile rumen fluid from a conventional sheep and lastly, in the case of lambs A(10⁻⁸)2, A(10⁻⁸)3, with sterile cellulose powder (pulverized Whatman filter paper).

Inoculation with protozoa. Lambs A(10⁻⁸)2, A(10⁻⁸)3, A(10⁻⁷)1 and A(10⁻⁷)2 were inoculated with the ciliate protozoan *Entodinium* sp. and lambs A(10⁻⁶)1, B(10⁻⁶)1 and B(10⁻⁶)2 with *Polyplastron multivesiculatum* (Fig. 2). The protozoa were either axenic or extensively decontaminated with antibiotics and free from the bacteria commonly found in the rumen (Bonhomme *et al.*, 1982). The bacteria which remained (10² to 10⁴ ml⁻¹) in the protozoan cultures were essentially aerobic (bacilli, pseudomonads) or facultatively anaerobic (streptococci, enterobacteria). About one hundred protozoa were introduced into the rumen directly through the cannula at each inoculation.

Fig. 1. Origin and composition of inocula used for axenic lambs. Pool A was prepared from rumen contents of two conventional lambs (at age 4, 7, 9, 11, 15, 16, 18, and 21 d) and from four adult sheep. Pool B was prepared from rumen contents of two meroxenic lambs (at age 60, 72 and 80 d) that had previously received the 10^{-7} dilution of Pool A.

Fig. 2. Rearing and inoculation of lambs. Feeding regime is described in Methods: milk (□); solid food formula I (▨); solid food formula II (▤). Lambs were inoculated with: a dilution from pool A or pool B (↑); *B. succinogenes* (↓); *B. succinogenes* plus sterile rumen fluid (◡); *B. succinogenes* plus sterile rumen fluid plus cellulose powder (⊗); *Entodinium* sp. (▴) or *P. multivesiculatum* (⊙).

Sampling. Rumen contents were sampled through the cannula with a syringe before morning feeding. Samples were put into 30 ml flasks; the flasks were completely filled to reduce air volume, closed tightly and immediately taken out of the isolator, so that analysis could be carried out as promptly as possible.

Redox potential measurement. The redox potentials were determined for each sample, using the Schott pH meter (CG 817) with a 42a redox electrode.

Microbiological analysis. The total number of viable bacteria was determined for all the lambs, with the exception of lamb A(10^{-8})1, in addition to total counts of cellulolytic bacteria, enterobacteria, streptococci, lactobacilli and total aerobic bacteria.

In lambs inoculated with pool A material, counts were made of groups capable of metabolizing certain carbohydrate substrates. For lambs A(10^{-8})1 and 3, A(10^{-7})1 and 2, and B(10^{-6})1, bacterial counts were

determined before and after protozoan establishment, to determine whether the latter had any effect on the pre-established microflora. The cellulolytic bacteria were also counted in pool A and in pool B after storage in glycerinated milk and thawing, but no attempt was made to determine the total viable counts in these pools.

Bacteriological techniques and culture media. Except where indicated otherwise, techniques were based on those of Hungate (1966b) and Grubb & Dehority (1976). Rumen contents were diluted with the mineral solution of Bryant & Burkey (1953). Total viable counts of bacteria were determined using medium M2 (Hobson, 1966) and medium CCA (Allison *et al.*, 1979). Counts of groups capable of metabolizing individual carbohydrates were performed with media based on the Incubated Rumen fluid Agar medium (IRA) of Dehority & Grubb (1976) to which a single energy source was added: glucose, starch, cellobiose, maltose, xylose or pectin, all at 0.1% (w/v) except for pectin at 0.5%. Cellulolytic bacteria were counted in the rumen fluid glucose cellobiose agar medium of Bryant & Burkey (1953) without agar, in which glucose and cellobiose were replaced by cellulose as a strip of filter paper (Whatman no. 1) (Mann, 1968). Lactate-utilizing bacteria were counted in the medium of Giesecke (1968). The rumen fluid used in the M2 medium and in the medium for cellulolytic bacteria was strained through a double layer of cheesecloth and centrifuged at 20000 *g* for 20 min. Rumen fluid used for the CCA and IRA + carbohydrates media was strained, incubated for a week at 38 °C (Henning, 1979) and then centrifuged at 20000 *g* for 20 min.

Three sets of roll tubes or liquid culture were 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 culture, the count of cellulolytic bacteria was taken as the most probable number.

Counts of aerobic and facultative anaerobic bacteria were made in Petri dishes in the following media: medium C for total counts (Raibaud *et al.*, 1966), desoxycholate agar medium (DCA) (Difco) for enterobacteria, KF streptococcus medium (KF; Difco) for streptococci, Rogosa SL Agar medium (Difco) for lactobacilli.

Protozoan counts. In rumen content samples, protozoa were counted in a Dolfuss dish with a binocular magnifier after the sample had been diluted in the Hungate solution (Senaud *et al.*, 1980).

RESULTS

Redox potential

In axenic lambs, the E_h of the rumen was already strongly negative (−100 to −120 mV). After inoculation with the dilutions of pool A or of pool B, the E_h decreased progressively in all animals. Nonetheless, there were wide variations between samples from the same animal. The amplitude of these variations was greater in lambs A(10^{−8})1 to 3 and B(10^{−8})1 (−200 to −500 mV) than in animals A(10^{−7})1 and 2, A(10^{−6})1, B(10^{−7})1 and B(10^{−6})1 and 2 which contained more complex flora.

Bacterial counts prior to establishment of protozoa (Tables 1 to 3)

Total anaerobic bacterial counts were always two to three times greater in the CCA medium than in the M2 medium except in the cases of lambs A(10^{−8})2 and 3. Accordingly, figures from this medium were used to compare count differences between lambs.

In all lambs the total viable counts of anaerobic bacteria were high (between 10⁹ and 5 × 10¹⁰ g^{−1}) and these counts were highest in lambs inoculated with the more complex flora [A(10^{−6})1, A(10^{−7})1 and 2, B(10^{−7})1, and B(10^{−6})1 and 2]. In contrast, the aerobic counts were highest in lambs inoculated with more limited microflora [A(10^{−8})2 and 3, B(10^{−8})1]. This was particularly apparent in lamb B(10^{−8})1 where the total viable count increased from 1.5 × 10⁹ to 2.7 × 10¹⁰ g^{−1} and the aerobic count decreased from 1.2 × 10⁹ to 4 × 10⁷ g^{−1} after the inoculation of the 7.5 × 10^{−8} dilution from pool B. The aerobic microflora was essentially composed of *Escherichia coli* and streptococci, except in lambs A(10^{−8})2 and 3 where lactobacilli were present.

Counts on media containing individual carbohydrate substrates showed that the starch-, pectin- and glucose-utilizing populations accounted for the highest percentage compared to the total viable counts on CCA, and that lactate-utilizing bacteria made up only a small proportion of the flora. The percentage of organisms capable of metabolizing other substrates differed in different lambs (Tables 1 and 2).

Table 1. *Bacterial counts in rumen samples from lambs A(10⁻⁸)2 and A(10⁻⁸)3 inoculated with material from pool A*

Animals were inoculated according to the regime shown in Fig. 2; inoculation with *Entodinium* sp. was at 84 d. Counts are expressed as 10⁻⁸ × no. bacteria (g rumen contents)⁻¹.

Lamb no.	Age (d)	Total aerobic counts	Enterobacteria	Streptococci	Lactobacilli	Total anaerobic counts in medium: M2 CCA	Bacterial counts on medium containing individual carbohydrate substrate or lactate*:				
							Glucose	Starch	Cellobiose	Maltose	Lactate
A(10 ⁻⁸)2	84	7.4	1.2	2.5	0.4	80	51 (83)	60 (99)	50 (83)	NT	NT
	110†	10	1.6	3.0	10	85	60 (75)	74 (92)	50 (62)	25 (31)	22 (26)
A(10 ⁻⁸)3	84	6.4	4.8	1.4	0.8	31	21 (64)	33 (99)	13 (39)	14 (41)	NT
	110†	19	0.14	11	10	30	18 (90)	17 (85)	10 (50)	14 (70)	0.8 (4)

NT, Not tested.

* Numbers in parentheses represent percentage of total count in CCA medium.

† *Entodinium* sp. present in rumen.

Table 2. *Bacterial counts in rumen samples from lambs A(10⁻⁷)1, A(10⁻⁷)2 and A(10⁻⁶)1 inoculated with material from pool A*

Animals were inoculated according to the regime shown in Fig. 2; inoculation with *Entodinium* sp. was at 80 d for lambs A(10⁻⁷)1 and A(10⁻⁷)2. Counts are expressed as 10⁻⁸ × no. bacteria (g rumen contents)⁻¹.

Lamb no.	Age (d)	Total aerobic counts	Enterobacteria	Streptococci	Lactobacilli	Total anaerobic counts in medium: M2 CCA	Bacterial counts on medium containing individual carbohydrate substrate*				
							Glucose	Starch	Cellobiose	Maltose	Lactate
A(10 ⁻⁷)1	72	0.1	0.01	0.07	0	280	240 (52)	400 (86)	260 (56)	220 (47)	350 (76)
	96†	0.04	0.02	0.007	0	56	116 (93)	20 (16)	100 (80)	120 (97)	400 (87)
A(10 ⁻⁷)2	72	0.2	0.02	0.08	0	114	170 (81)	160 (76)	88 (42)	100 (47)	90 (72)
	96†	0.2	0.09	0.017	NT	NT	140 (66)	20 (9)	160 (76)	190 (90)	180 (88)
A(10 ⁻⁶)1	65	0.5	0.3	0.11	0	94	300 (100)	260 (87)	190 (63)	110 (37)	160 (77)
										280 (93)	NT
											9 (2)
											5 (4)
											6 (3)
											6 (3)
											5 (2)

NT, Not tested.

* Numbers in parentheses represent percentage of total count in CCA medium.

† *Entodinium* sp. present in rumen.

Table 3. *Bacterial counts in rumen samples from lambs B(10⁻⁸)1, B(10⁻⁷)1, B(10⁻⁶)1 and B(10⁻⁶)2 inoculated with material from pool B*

Animals were inoculated according to the regime shown in Fig. 2; inoculation with *P. multivesiculatum* was at 55 and 57 d, and 69 d for lambs B(10⁻⁶)1 and B(10⁻⁶)2, respectively. Counts are expressed as 10⁻⁸ × no. bacteria (g rumen contents)⁻¹.

Lamb no.	Age (d)	Total aerobic counts	Entero-bacteria	Strepto-cocci	Lacto-bacilli	Total anaerobic counts in medium:	
						M2	CCA
B(10 ⁻⁸)1	56	12	0	12	0	NT	15
	74	0.4	0	0.4	0	NT	270
B(10 ⁻⁷)1	46	1.6	0.3	1.5	0	33	100
B(10 ⁻⁶)1	44	4	0.7	4	0	83	250
	77*	1.7	0.8	1.5	0	46	75
B(10 ⁻⁶)2	54	4	0.15	4	0	80	230
	87*	1.5	0.5	1.4	0	26	44

NT, Not tested.

* *P. multivesiculatum* present in rumen.

Effect of ciliate protozoa on pre-established microflora

In lamb A(10⁻⁷)1 the total number of bacteria was four times lower after *Entodinium* sp. establishment (day 96), whilst this number remained constant in lamb A(10⁻⁷)2 (Table 2). Nevertheless, in both lambs, protozoa establishment brought about a change in the counts obtained in media with a single energy source. These changes showed the same tendency in both lambs, except for the glucose-utilizing population. The percentage of starch-utilizing bacteria dropped markedly, and to a lesser extent so did xylose- and pectin-utilizing bacteria. In contrast, the percentage of cellobiose- and maltose-utilizing bacteria increased (Table 2).

In lamb A(10⁻⁸)2, the introduction of *Entodinium* sp. had a much more limited effect; no great variations, either qualitative or quantitative, were observed in microflora on day 110. In lamb A(10⁻³)3, *Entodinium* sp. did not establish.

In lambs B(10⁻⁶)1 and 2, the introduction of *P. multivesiculatum* brought about a drop in anaerobic and aerobic microflora. The total viable counts decreased threefold in lamb B(10⁻⁶)1 and fivefold in lamb B(10⁻⁶)2 (Table 3).

The aerobic and facultative anaerobic bacteria which were present in the cultures of protozoa inoculated in lamb rumens were not recovered in the rumen samples after protozoa establishment.

Establishment of cellulolytic bacteria

Pool A inoculated lambs. The number of cellulolytic bacteria present in pool A material was 6×10^7 bacteria g⁻¹, as estimated by the most probable number. The cellulolytic bacteria present in this pool did not become established in any of the lambs inoculated.

In lamb A(10⁻⁶)1, the cellulolytic population was high 6 d after inoculation with *B. succinogenes*. It reached a number comparable to that observed in conventional animals after 13 d, but then declined steadily. After day 109, the population increased again and regained its initial level (Fig. 3).

In lambs A(10⁻⁷)1 and 2, the cellulolytic population also increased following *B. succinogenes* inoculation. After 7 d this population reached about 10^7 bacteria g⁻¹ in the two lambs (Fig. 4).

In the three lambs A(10⁻⁸)1 to 3, *B. succinogenes* inoculated alone did not become established following several inoculations. However, in lamb A(10⁻⁸)1, this strain did become established when inoculation was done in the presence of sterile rumen fluid (60 ml) from a conventional sheep. The population reached 5×10^6 bacteria g⁻¹ 11 d after the last inoculation and then

Fig. 3. Numbers of cellulolytic bacteria (■) and *P. multivesiculatum* (●) in the rumen of lamb A(10^{-6})1. Inoculations were made with material from pool A (↑), with *B. succinogenes* (↓) and with *P. multivesiculatum* (⬇) as described in Fig. 2.

Fig. 4. Numbers of cellulolytic bacteria (■, □) and *Entodinium* sp. (●, ○) in the rumens of lambs A(10^{-7})1 (filled symbols) and A(10^{-7})2 (open symbols). Inoculations were made with material from pool A (↑), with *B. succinogenes* (↓) and with *Entodinium* sp. (⬇) as described in Fig. 2.

Fig. 5. Numbers of cellulolytic bacteria in the rumens of lambs A(10^{-8})1 (●), A(10^{-8})2 (■), A(10^{-8})3 (▲) and of *Entodinium* sp. in the rumen of lamb A(10^{-8})2 (□). Inoculations were made with material from pool A (↑), with *B. succinogenes* (↓) and with *Entodinium* sp. (⬇) as described in Fig. 2.

Fig. 6. Numbers of cellulolytic bacteria (■) and *P. multivesiculatum* (●) in the rumen of lamb B(10^{-8})1. Inoculations were made with material from pool B (↑) and with *P. multivesiculatum* (⬇) as described in Fig. 2.

Fig. 7. Numbers of cellulolytic bacteria (■) and *P. multivesiculatum* (●) in the rumen of lamb B(10⁻⁶)2. Inoculations were made with material from pool B (↑) and with *P. multivesiculatum* (↓) as described in Fig. 2.

Fig. 8. Numbers of cellulolytic bacteria (■) in the rumen of lamb B(10⁻⁸)1. Inoculations were made with material from pool B (↑) and with *B. succinogenes* (↓) as described in Fig. 2.

disappeared. Further experimentation on this lamb was not possible. In lamb A(10⁻⁸)2, establishment of this strain occurred when inoculation was done not only with sterile rumen fluid, but also with sterile cellulose (pulverized Whatman filter paper). In lamb A(10⁻⁸)3, *B. succinogenes* was not established even though this lamb had been reared simultaneously and in the same isolator as lamb A(10⁻⁸)2 and inoculated in the same manner (Fig. 5).

Pool B inoculated lambs. The number of cellulolytic bacteria present in pool B was 5×10^7 bacteria (g rumen content)⁻¹. In lambs B(10⁻⁶)1 and 2, *B. succinogenes* became directly established from pool B inocula. Its population oscillated between 10⁶ and 10⁸ bacteria g⁻¹ before *P. multivesiculatum* was introduced. Thereafter, the extent of variations between counts was even greater (Figs 6 and 7).

In lamb B(10⁻⁷)1, which could only be reared up to the age of 52 d, *B. succinogenes* was also established directly from pool B inocula. In the two counts taken 4 and 6 d after the last 10⁻⁷ dilution inoculation, the cellulolytic population reached 9×10^6 and 6.5×10^7 bacteria (g rumen content)⁻¹, respectively.

In lamb B(10⁻⁸)1, *B. succinogenes* did not become established from pool B inocula, and establishment from a pure culture of this same strain failed despite several inoculations (Fig. 8). However, it did become established after the lamb had been inoculated with 7.5×10^{-8} dilutions of pool B material and its population reached a high level (between 10⁷ and 2.5×10^8 bacteria g⁻¹). The change in feed (Fig. 2) on day 85 seems to have contributed to the stability of this population.

Kinetics of protozoan establishment

Entodinium sp. In lambs A(10⁻⁷)1 and 2, the population of this ciliate reached a maximum 16 d after inoculation and then reached about 10⁶ protozoa ml⁻¹ (Fig. 4), i.e. a level comparable to that observed in conventionally reared lambs (Jouany 1978, Senaud *et al.*, 1980). In lambs A(10⁻⁸), *Entodinium* sp. only became established in lamb A(10⁻⁸)2, in which *B. succinogenes* was also established (Fig. 5).

Polyplastron multivesiculatum. In lamb A(10^{-6})1 the establishment did not succeed even though it was inoculated twice, on days 65 and 110. By contrast, its establishment was successful in the two lambs B(10^{-6})1 and 2. The population reached a maximum three weeks after inoculation (10^5 protozoa ml $^{-1}$) and fluctuated thereafter between 10^3 and 10^4 protozoa (ml rumen content) $^{-1}$.

DISCUSSION

Results of total anaerobic bacterial counts showed that even though the number of bacteria in the rumen of meroxenic lambs was high (10^9 to 10^{10} bacteria g $^{-1}$) and comparable to that found in conventional animals, there was a relationship between the composition of the inoculum given to the lambs and the size of the bacterial population which developed in the rumen. In animals inoculated with the 10^{-6} and 10^{-7} dilutions, the total viable count was higher than in animals that had received the 10^{-8} dilution, in animals inoculated with pool A or pool B. This effect was particularly pronounced in lamb B(10^{-8})1 where the total viable count was 20 times greater after inoculations with the 7.5×10^{-8} dilution. The ratio of facultative anaerobic microflora to strictly anaerobic flora was also a function of the inoculum that the lambs received. The more abundant and complex the flora in the rumen, the smaller the proportion of facultative anaerobic bacteria.

Cellulolytic bacteria did not become established from the 10^{-8} inocula because these bacteria were not present in such high dilutions. In contrast, the numerical factor cannot explain establishment of cellulolytic bacteria directly from the 10^{-6} and 10^{-7} dilutions of pool B and failure of direct establishment from the same dilutions of pool A, as there were approximately equal numbers of cellulolytic bacteria in both pools. It is likely that the early inoculation of lambs in group B by the first week provided more favourable conditions for establishment of cellulolytic bacteria and probably advanced the anatomical development and the physiological functioning of the rumen; in axenic lambs the rumen wall is thin, the papillae are small (Lysons *et al.*, 1976b) and the digestive tract is anatomically different from that of conventional lambs (Peyraud *et al.*, 1981).

Bacteroides succinogenes became established only in one of the lambs that had received inocula of 10^{-8} dilutions of pool A or pool B, whereas this strain became established more readily in lambs inoculated with the more complex and more abundant microflora of 10^{-6} and 10^{-7} dilutions. This suggests that establishment of cellulolytic bacteria requires a complex flora able to provide the necessary ecological conditions. These findings are in line with those obtained in gnotoxenic lambs by other authors. Mann & Stewart (1974) failed to achieve establishment of cellulolytic bacteria (*Ruminococcus* spp.) in the rumen of a hay-fed gnotoxenic lamb which had been inoculated with six other rumen bacteria. Also, in gnotoxenic lambs raised by Lysons *et al.* (1976a), establishment of *Ruminococcus* sp. occurred in most animals, but its population remained very low. Similarly, in sheep reared in isolation *B. succinogenes* succeeded only in one animal and only for a very short time (Males, 1973).

Difficulty in establishing cellulolytic bacteria in the rumen of animals with limited flora is probably linked to the very high and strict nutritional requirements of such organisms (Allison, 1965; Bryant, 1973; Dehority *et al.*, 1967). These conditions were probably not met in the rumen of lambs inoculated with dilutions of 10^{-8} . The pH (between 6 and 7) and the redox potential in these animals seemed favourable to development of cellulolytic bacteria, but the ammonia and volatile fatty acid concentrations were very low (20 to 40 mg l $^{-1}$ and 10 to 30 mM, respectively) and branched volatile fatty acids were generally absent (unpublished data). The creation of the necessary conditions to establish cellulolytic bacteria probably depends on a number of very complex requirements. These are not necessarily provided by the dominant bacteria of the flora which are nonetheless generally thought to play the main role in the rumen.

An abundant and diversified flora is also required for establishment of *Entodinium* sp.; this organism became established in only one lamb inoculated with a 10^{-8} dilution of pool A [A(10^{-8})2], whereas both lambs that received the 10^{-7} dilutions [A(10^{-7})1 and 2] became colonized. Moreover, in the latter, establishment was much faster. Failure of *P. multivesiculatum*

to develop in lamb A(10⁻⁶)1, although this lamb had complex rumen microflora, could be due to the introduction of inocula that were too low, or to the drastic effect of antibiotics. Other causes, however, are possible; in young lambs it seems that there are periods in which protozoa disappear from the rumen (Petkov & Enev, 1979; J. P. Jouany & G. Fonty, unpublished observations). The effect of protozoa on the bacterial flora in the rumen of meroxenic lambs is in line with that reported elsewhere for conventional animals (Eadie & Hobson, 1962; Eadie & Mann, 1970; Kurihara *et al.*, 1968) and is probably due to a predatory effect (Coleman, 1975). Because of the low number of animals used and the limited number of bacterial counts in this study, it is difficult to draw firm conclusions. However, interestingly, in both lambs A(10⁻⁷)1 and 2 the percentage of starch-utilizing bacteria was much lower after *Entodinium* sp. establishment, probably because the entodinium had ingested starch granules along with adherent bacteria (Kurihara *et al.*, 1968).

Clearly the establishment of cellulolytic bacteria and protozoa in the rumen depends on the presence of other micro-organisms, requires an abundant and diversified surrounding flora and is favoured by early animal inoculation.

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REFERENCES

- ALLISON, M. J. (1965). Nutrition of rumen bacteria. In *Physiology of Digestion in the Ruminant*, pp. 369–378. Edited by R. W. Dougherty. London & Washington: Butterworths.
- ALLISON, M. J., ROBINSON, J. A. & BOOTH, G. D. (1979). Characterisation of bacterial population from the pig gut based upon the energy sources used for growth. *Applied and Environmental Microbiology* **37**, 1142–1151.
- BARR, M. E. J., MANN, S. O., RICHARDSON, A. J., STEWART, C. S. & WALLACE, R. S. (1980). Establishment of ureolytic staphylococci in the rumen of gnotobiotic lambs. *Journal of Applied Bacteriology* **49**, 325–330.
- BONHOMME, A., FONTY, G., SENAUD, J. (1982). Essai d'obtention et de survie de ciliés entodiniomorphes du rumen en cultures axéniques. *Annales de Microbiologie de l'Institut Pasteur* **133B**, 335–341.
- BRYANT, M. P. (1973). Nutritional requirements of the predominant rumen cellulolytic bacteria. *Federation Proceedings* **32**, 1809–1813.
- BRYANT, M. P. (1977). Microbiology of the rumen. In *Dukes' Physiology of Domestic Animals*, 9th edn, pp. 287–304. Edited by M. J. Swenson. Cornell University Press: Ithaca & London.
- BRYANT, M. P. & BURKEY, L. A. (1953). Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *Journal of Dairy Science* **36**, 205–217.
- CHENG, K. J. & WALLACE, J. J. (1979). The mechanism of passage of endogenous urea through the rumen wall and the role of ureolytic epithelium in the urea flux. *British Journal of Nutrition* **42**, 553–557.
- CHURCH, D. C. (1970). Rumen microbiology. In *Digestive Physiology and Nutrition of Ruminants*, 1st edn, vol. 1, pp. 151–197. Corvallis, Oregon U.S.A.: O.S.U. Book Stores.
- CLARKE, R. T. J. (1977). Protozoa in the rumen. In *Microbial Ecology of the Gut*, pp. 251–275. Edited by R. T. J. Clarke & T. Bauchop. New York, London, San Francisco: Academic Press.
- COLEMAN, G. S. (1975). The interrelations between rumen ciliate protozoa and bacteria. In *Digestion and Metabolism in the Ruminant*, pp. 149–164. Edited by I. W. McDonald & A. C. I. Warner. Armidale, New South Wales, Australia: University of New England Publishing Unit.
- DEHORITY, B. A. & GRUBB, S. A. (1976). Basal medium for the selective enumeration of rumen bacteria utilizing specific energy sources. *Applied and Environmental Microbiology* **32**, 703–710.
- DEHORITY, B. A., SCOTT, H. W. & KOWALERK, P. (1967). Volatile fatty acids requirements of cellulolytic rumen bacteria. *Journal of Bacteriology* **94**, 537–543.
- EADIE, J. M. & HOBSON, P. N. (1962). Effect of presence or absence of ciliates on the total bacteria count in lambs. *Nature, London* **193**, 503–505.
- EADIE, J. M. & MANN, S. O. (1970). Development of the rumen microbial populations: high starch diets and instability. In *Physiology of Digestion and Metabolism in the Ruminant*, pp. 335–347. Edited by A. T. Phillipson. Newcastle upon Tyne, England: Oriel University Press.
- GIESECKE, D. (1968). Zur Kemzahlbestimmung der anaeroben lactilytischen Bakteriengruppe im Pansen. *Zentralblatt für Bakteriologie (I. Abteilung)* **109**, 101–106.
- GOUET, PH., RIOU, Y., CONTREPOIS, M. & DUBOURGUIER, H. C. (1979). Efficacité d'une technique simple de production d'agneaux axéniques et de veaux oligoxéniques par décontamination à la naissance. *Annales de recherches vétérinaires* **10**, 49–51.
- GRUBB, J. A. & DEHORITY, B. A. (1976). Variation in colony counts of total viable anaerobic rumen bacteria as influenced by media and cultural

- methods. *Applied and Environmental Microbiology* **31**, 262–267.
- HENNING, P. A. (1979). Examination of methods for enumerating hemicellulose-utilizing bacteria in the rumen. *Applied and Environmental Microbiology* **38**, 13–17.
- HOBSON, P. N. (1966). Rumen bacteria. *Methods in Microbiology* **3B**, 133–149.
- HOBSON, P. N. & HOWARD, B. H. (1969). Microbial transformations. In *Handbuch der Tierernährung*, vol. 1, pp. 207–254. Edited by W. Lenkeit, K. Brenem & E. Craseman. Hamburg & Berlin: Paul Parey.
- HUNGATE, R. E. (1966a). *The Rumen and its Microbes*. New York & London: Academic Press.
- HUNGATE, R. E. (1966b). A roll tube method for cultivation of strict anaerobes. *Methods in Microbiology* **3B**, 117–132.
- JOUANY, J. P. (1978). *Contribution à l'étude des protozoaires ciliés du rumen, leur dynamique, leur rôle dans la digestion et leur intérêt pour le ruminant*. Thèse Doctorat d'Etat, no. d'ordre 256. Université de Clermont II. France.
- KURIHARA, Y., EADIE, J. M., HOBSON, P. N. & MANN, S. O. (1968). Relationships between bacteria and ciliate protozoa in the sheep rumen. *Journal of General Microbiology* **51**, 267–288.
- LYSONS, R. J. & ALEXANDER, T. J. L. (1975). The gnotobiotic ruminant and *in vivo* studies of defined bacterial populations. In *Digestion and Metabolism in the Ruminant*, pp. 180–192. Edited by I. W. McDonald & A. C. I. Warner. Armidale, New South Wales, Australia: University of New England Publishing Unit.
- LYSONS, R. J., ALEXANDER, T. J. L., HOBSON, P. N., MANN, S. O. & STEWART, C. S. (1971). Establishment of a limited rumen microflora in gnotobiotic lambs. *Research in Veterinary Science* **12**, 486–487.
- LYSONS, R. J., ALEXANDER, T. J. L., WELLSTEAD, P. D., HOBSON, P. N., MANN, S. O. & STEWART, C. S. (1976a). Defined bacterial population in the rumen of gnotobiotic lambs. *Journal of General Microbiology* **94**, 257–269.
- LYSONS, R. J., ALEXANDER, T. J. L., WELLSTEAD, P. D. & JENNINGS, I. W. (1976b). Observations on the alimentary tract of gnotobiotic lambs. *Research in Veterinary Science* **20**, 70–76.
- MALES, J. (1973). *Ration digestibility, rumen bacteria and several rumen parameters in sheep born and reared in isolation*. Dissertation, The Ohio State University.
- MANN, S. O. (1968). An improved method for determining cellulolytic activity in anaerobic bacteria. *Journal of Applied Bacteriology* **31**, 241–244.
- MANN, S. O. & STEWART, C. S. (1974). Establishment of a limited rumen flora in gnotobiotic lambs fed on a roughage diet. *Journal of General Microbiology* **84**, 379–382.
- PETKOV, A. & ENEV, E. I. (1979). Rumen digestion parameters in lambs fed with pelleted diet. *Annales de recherches vétérinaires* **10**, 440–441.
- PEYRAUD, J. L., THIVEND, P. & GOUET, PH. (1981). Role of digestive microflora on starch digestion in the preruminant lamb. In *Recent Advances in Germfree Research, Proceedings of the VIIth International Symposium on Gnotobiology*, pp. 285–288. Edited by S. Sasaki, A. Ozawa & K. Hashimoto. Tokyo: Tokai University Press.
- PRINS, R. A. (1977). Biochemical activities of gut microorganisms. In *Microbial Ecology of the Gut*, pp. 73–183. Edited by R. T. J. Clarke & T. Bauchop. London, New York, San Francisco: Academic Press.
- RAIBAUD, P., DICKINSON, A. B., SACQUET, E., CHARLIER, R. & MOCQUOT, G. (1966). La microflore du tube digestif du rat. I. Techniques d'étude et milieux de culture proposés. *Annales de l'Institut Pasteur* **110**, 568–590.
- RIOU, Y., GOUET, PH., DUBOURGUIER, H. C., CONTREPOIS, M., DARDILLAT, C. & LEVAIVRE, J. (1977). Techniques for obtaining fistulization and rearing of axenic or gnotoxenic lambs, kids and calves. *Annales de recherches vétérinaires* **8**, 13–24.
- SENAUD, J., GROLIERE, C. A., ZAINAB, B., GRAIN, J., JOUANY, J. P. (1980). Implantation et développement des protozoaires ciliés (*Polyplastron multivesiculatum*, *Entodinium* sp., *Isotricha prostoma*) dans le rumen de moutons recevant différents régimes alimentaires. I. Régime luzerne-orge. *Protistologica* **16**, 329–337.