SHORT COMMUNICATIONS

Establishment of a Limited Rumen Flora in Gnotobiotic Lambs Fed on a Roughage Diet

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(Received 20 May 1974)

The main impact of the development of techniques for rearing gnotobiotic animals has been on medical and veterinary research. The use of gnotobiotic lambs has, however, made possible a new approach to problems concerning the role of various rumen bacteria in the digestive process in ruminants.

Previous workers (Lysons *et al.* 1971, and unpublished) reared gnotobiotic lambs inoculated with a known, limited, rumen bacterial flora, but the diets of these lambs contained only up to 30 % roughage, and would be partly digestible in the gastro-intestinal system. Experiments were carried out to find whether full ruminal function could be established in lambs with a limited and simplified flora. It was thus the intention to establish in a gnotobiotic lamb a simplified rumen flora designed to digest cellulose, starch and other polysaccharides as in the conventionally reared animal, and to give this lamb an all-roughage diet digestible only with the aid of a rumen flora. The bacteria were chosen for their known activities and interactions and their relationship to the nutrient requirement of the lambs, according to the scheme of Lysons *et al.* (1971), but with some modifications because of the change in diets. *Streptococcus bovis* and *Bacteroides amylophilus* were omitted, and a strain of *Selenomonas ruminantium* was introduced which was both proteolytic and amylolytic. Also, since *S. ruminantium* produces propionate via the succinate pathway (Paynter & Elsdon, 1970), it seemed possible that extracellular succinate produced by *Bacteroides ruminicola* might be used by *S. ruminantium* and converted to propionate. When it became clear that *S. ruminantium* could not convert all of the extracellular succinate to propionate, *Veillonella alcalescens*, which is known to produce propionate from extracellular succinate *in vitro* (Johns, 1951), was introduced.

METHODS

Twin Finnish Landrace (Finnish Landrace X Polled Dorset Horn) lambs, A and B, were obtained by hysterectomy and reared in isolators on sterile food using techniques and apparatus similar to those of Alexander & Lysons (1971).

Feeding schedule. From birth to 22 days the lambs received 50 % (v/v, with water) sterile, evaporated cows milk (Carnation Foods Ltd, London). The milk was then replaced by a pelleted starter diet consisting mainly of a mixture of whole barley, dried grass and fish meal, with a commercial vitamin–mineral supplement (Isaac Spencer Ltd, Aberdeen). On day 53 this was replaced by coarsely ground, pelleted, dried grass, also with the vitamin–mineral supplement. All these feeds were sterilized by γ -radiation at 5 Mrad (Ethicon Ltd, Edinburgh).

Table 1. *Counts of bacteria, fermentation product concentrations and pH of rumen contents of gnotobiotic Lamb A inoculated with a limited bacterial flora*

Age (days) ...	19	26	40	48	71	78	85	91	99	110	123
$10^9 \times$ total viable count (TVC)*	0†	1.5	3.5	1.9	7.4	5.3	5.0	3.9	4.3	4.2	NS
Percentage of TVC contributed by:											
<i>Lactobacillus</i> LB17	—	93.8	88.9	52.4	1.7	1.3	2.5	4.1	1.5	0.4	NS
<i>Peptostreptococcus elsdenii</i> , LCI	—	4.0	0.4	0.4	0.7	0.7	0.9	1.3	1.2	0.8	NS
<i>Selenomonas ruminantium</i> , GLY4	—	2.2	10.7	10.2	4.8	5.6	5.9	10.3	7.8	0.2	NS
<i>Bacteroides ruminicola</i> , 46/5(2)	—	—	—	0.5	1.6	0.5	2.2	1.0	3.8	5.0	NS
<i>Butyrivibrio fibrisolvens</i> , B834	—	—	—	36.4	91.2	91.8	88.4	83.3	63.8	82.2	NS
<i>Veillonella alcalescens</i> , 692/1	—	—	—	—	—	—	—	—	21.9	11.3	NS
Fermentation products (μ mol/ml)											
Formate	2.0	4.8	5.3	2.1	NS	2.0	2.0	6.5	1.4	NS	2.0
Acetate	6.6	13.1	8.6	9.2	25.1	25.1	14.8	23.0	33.0	NS	27.9
Propionate	0.0	0.6	1.5	1.4	2.5	3.0	1.6	1.6	12.8	NS	10.5
Butyrate	1.2	3.5	1.9	2.3	7.6	10.9	5.3	6.7	8.5	NS	7.5
Valerate	0.0	0.7	0.9	0.6	1.4	1.8	1.2	1.1	2.3	NS	1.8
Lactate	4.0	5.5	9.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Succinate	0.0	2.4	12.8	6.0	7.9	11.9	12.2	8.2	11.5	2.5	1.9
pH of rumen contents	6.9	6.7	5.6	6.9	6.8	6.7	6.7	6.4	6.7	7.1	6.9

NS, Not studied.

Dates of introduction of bacteria were: *Lactobacillus*, day 19; *Peptostreptococcus*, day 20; *Selenomonas*, day 21; *Bacteroides*, day 40; *Butyrivibrio*, day 41; *Veillonella*, day 96. These bacteria were isolated in the authors' laboratory. When sampling dates coincided with inoculations, the rumen samples were taken shortly before inoculation.

* Colony-forming units/g rumen contents.

† Immediately before inoculation with *Lactobacillus*.

Bacterial inoculation. Lamb B was not inoculated and served as a control. Lamb A was inoculated *per os* with bacterial cultures grown for 24 h in 15 ml Mann's No. 2 medium (Hobson, 1969) except for the *Ruminococcus* (sp. 4263/1) which was grown in No. 2 base with 0.2 % (w/v) cellobiose (see Table 1 for strains and dates of inoculation).

Analysis of rumen contents. Rumen contents were obtained by stomach tube about 2 h after feeding. Bacterial counts were made using selective or differential anaerobic media (Hobson, 1969). Rumen contents, faeces and buccal swabs were also monitored for contaminants using a variety of aerobic and anaerobic media (Elliott, Alexander, Wellstead & Lysons, 1974).

The concentrations of C_2 – C_4 volatile fatty acids (VFA) were estimated by the methods of Whitelaw, Hyldgaard-Jensen, Reid & Kay (1970). Formic, succinic and lactic acids, urea, ammonia and alcohol were determined by standard methods (Barker & Somers, 1966; Clark & Porteous, 1964; Conway, 1957).

Analyses by mass spectrometer (MS 10, AEI, Harlow, Essex) were used to obtain a qualitative estimate of hydrogen evolution from the rumen, by testing for the presence of hydrogen in expired air, which normally contains some gas eructed from the rumen.

RESULTS

Both lambs were free from contamination during the course of the experiment, so it can be assumed that the major differences between them are due to the activities of the known, limited rumen flora inoculated into Lamb A.

Lamb A initially grew well, eating up to 800 g/day. After about 98 days, however, appetite

decreased and the lamb lost weight from then on. On removal from the isolator at 125 days it weighed 22.5 kg. Total viable counts of bacteria, the percentages contributed by individual bacterial species to this total count, and rumen pH and fermentation product concentrations are shown in Table 1. The *Ruminococcus* did not establish and so does not appear in Table 1. Although most of the individual bacteria only account for between 0.2 and 11 % of the total viable count, this percentage represents quite high numbers, about 10^7 to 10^8 /g rumen content.

About half the rumen samples from Lamb A also contained up to 0.3 μ mol isobutyrate and isovalerate/ml; caproate was not detected. Ammonia and urea were present at about 2 μ mol/ml, there being a small increase in ammonia concentration after the introduction of *B. ruminicola* which deaminates amino acids. Alcohol was found only briefly after the introduction of *Ruminococcus*, and this may have been added with the inoculum. The amount of hydrogen detected in expired air from Lamb A increased significantly between days 77 and 81, and remained high upon repeated testing on days 85, 91 and 106, whereas no significant increase in the background hydrogen level was found in expired air from Lamb B.

Lamb B ate poorly and was in poor health throughout. The pH of the rumen contents was about 6.7 and 12 to 16 μ mol VFA/ml (mainly acetate) were present. Lamb B weighed only 13.5 kg when killed electively on day 103.

DISCUSSION

The total concentrations of fermentation products were generally related to the total number of bacteria and some specific relationships were apparent. Lactate increased between days 26 and 40, corresponding to a fall in the relative numbers of lactate-utilizing bacteria (*Peptostreptococcus elsdenii*). Then the concentration of lactate fell rapidly as the principal lactate-producing bacteria (*Lactobacillus*) were replaced by other genera. The predominance of *Butyrivibrio* from day 71 is reflected in the increased proportion of butyric acid in the fermentation products. Acetate is a common fermentation product of all the bacteria and its increase from day 71 is paralleled by a greater bacterial population.

Succinate was found in the rumen in significant amounts before the introduction of *B. ruminicola* on day 41, which presumably indicates succinate production either by *Selenomonas* or *Peptostreptococcus*. Hobson & Mann (1961) described one strain of *Selenomonas* which produced succinate in batch cultures, but Hobson (1965) studying a different strain of *Selenomonas* in continuous culture could not detect succinate. Some *P. elsdenii* strains are also regarded as being capable of producing succinate (Anaerobe Laboratory Manual, 1972). The present strains of these bacteria were not, however, tested for succinate production *in vitro*.

The high concentration of succinate from day 40 to day 99 suggests little, if any, succinate decarboxylation by the *Selenomonas* and that the small amount of propionate present was formed directly from carbohydrate. The increase in propionate from day 99 implicates the *Veillonella* (added at day 96) in succinate decarboxylation, although an immediate decrease in succinate concentration on establishment of the *Veillonella* was not apparent (day 99).

Assessment of fermentation balances shows that after day 48 the 'hydrogen' unaccounted for in acidic fermentation products increased, and this is undoubtedly the reason for the definite detection of hydrogen in the respired gases after this date. Although the presence of methanogenic or other hydrogen-accepting bacteria can, *in vitro*, shift fermentation products in mixed cultures towards the least reduced compounds (Ianotti, Kafkewitz, Wolin & Bryant, 1973), the absence of methanogenic bacteria here did not lead to utilization of all electrons in reduced end-products.

The failure of *Ruminococcus* to establish may indicate that, although it grows well on

filter paper, the cellulose of dried grass is not a suitable substrate. Alternatively (but considered less likely) there might have been a lack of some growth factor.

The experiment showed clearly that Lamb A did benefit from its limited bacterial flora, although it was about 10 kg lighter at 125 days than a conventionally reared lamb, and the rumen VFA concentrations were only half to two-thirds those of a normal lamb. This poor performance in comparison with conventionally reared lambs may reflect the absence of a vital microbial activity, but it seems more likely that the lack of cellulolysis resulted in accumulation of undigested fibre and this induced slow rumen turnover and correspondingly low microbial activity.

We thank G. Cushnie and the staff of the Veterinary Pathology Department for their maintenance of the animals and isolators, R. S. Reid for analysis of C₂-C₄ VFAs, R. Summers, Carol Grant and I. Skene for technical assistance, and Dr P. N. Hobson for helpful discussions and suggestions and for the mass spectrometric analysis.

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