Effect of Food Intake on Growth and Survival of *Salmonellas* and *Escherichia coli* in the Bovine Rumen

By L. E. BROWNLIE AND F. H. GRAU

*Meat Research Laboratory, Division of Food Preservation,
Commonwealth Scientific and Industrial Research Organization,
Cannon Hill, Brisbane, Queensland, Australia*

(Accepted for publication 17 August 1966)

SUMMARY

When salmonella organisms were put into the rumen of cattle their subsequent growth or elimination depended on the dietary intake before and after the organisms were ingested. When the animals were receiving a regular daily ration of 6.8 kg. lucerne hay the organisms were rapidly eliminated from the rumen and viable organisms in the faeces were rarely detected. Decreasing the daily food intake to 2.3 kg. or interruption of feeding for one or more days retarded the elimination of salmonellas and *Escherichia coli* or permitted their growth in the rumen. Growth of salmonellas and *E. coli* type I occurred during starvation, and resumption of feeding after starvation caused further multiplication. Starvation for 2 or 3 days was generally followed by infection of the intestine, with salmonellas persisting in the faeces for at least a week.

INTRODUCTION

In an earlier study Grau & Brownlie (1965) showed that at five abattoirs in south-eastern Queensland salmonella organisms occurred in the rumen contents of 45% of healthy cattle at slaughter. *Escherichia coli* type I at about 10^5 organisms/ml. were also found in the rumen fluid, in agreement with other reports on the occurrence of *E. coli* (Mann, Masson & Oxford, 1954).

The work described in the present paper was undertaken to ascertain the fate of known numbers of salmonella organisms put into the rumen, and to determine concomitant changes in the population of *Escherichia coli*. The effect of deprivation of foodstuff was also examined, as it is common for cattle to be given little or no food for several days while being moved long distances or held at abattoirs. Deprivation of food may be a predisposing condition for infection in ruminants (Salisbury, 1958; Seddon, 1953).

METHODS

The salmonella organisms used were *Salmonella chester*, *S. typhimurium*, *S. oranienburg*, *S. muenchen*, *S. anatum* and *S. adelaide*, these having been isolated from the rumens of cattle at slaughter and identified by Dr K. F. Anderson (Salmonella Reference Centre, Adelaide). Cultures were grown in 0.8% (w/v) nutrient broth (Difco) for two 24 hr subcultures at 37°, and after suitable dilution in 0.1% (w/v) peptone (Oxoid) water the salmonellas were inoculated into the rumens of the animals.

The salmonellas were introduced into the stomach tube used for sampling (Raun & Burroughs, 1962) and flushed into the rumen with 50 ml. rumen liquor previously taken from the animal. The number of salmonellas given was estimated by serial dilution of the inoculum with peptone water and pour-plating with plate-count agar (Oxoid); colonies were counted after incubation at 37° for 24 hr. In some experiments the organisms were introduced into the rumen as a peptone water suspension of air-dried faeces contaminated with salmonellas, the numbers of which were estimated by the most-probable-number technique described below.

The experimental cattle were Polled Hereford females from 18 months to 3 years of age. In the experiments reported the animals were confined in a cattle crush, to prevent their ingesting faecal material. They were usually fed 6.8 kg. of lucerne hay, presented at the same time daily. In some experiments the animals were deprived of food, but not water, for various periods; while in others food was supplied in a limited amount (2.3 kg.) at the normal times. On the completion of an experiment the animals were treated with sulphadimidine (Mezobols, Parnell Laboratories, Carlton, N.S.W.) daily until faecal samples were consecutively negative. An animal was fed lucerne hay for a minimum of 2 weeks before an experiment was begun, or was grazed for about 6 weeks.

Samples of rumen fluid were obtained by the stomach-tube method, and after their transfer to the laboratory suitable samples of fluid or of peptone water dilutions of it, were subjected to enrichment as described by Grau & Brownlie (1965). Tetrathionate Tergitol brilliant green broth enrichments were incubated at 37° for 24 hr and streaked on the surface of brilliant green sulphadiazine agar and aged bismuth sulphite agar. The selective media were incubated overnight at 37° and colonies suspected of being salmonellas were suspended in saline and tested by slide agglutination with somatic antigens. The enrichments proven to contain salmonellas were eliminated from further incubation, those negative at the 24 hr streaking were retained in incubation for a total of 72 hr before restreaking. The enrichments positive for salmonellas after 24 hr incubation were counted cumulatively with those found positive at the 72 hr streaking. The most-probable-number of salmonellas, after 72 hr enrichment, was estimated from the number of positive enrichments in ten tubes at each dilution (McCoy, 1962). *Escherichia coli* type I was estimated by serial dilution in peptone water and pour-plating with eosin methylene blue agar (Oxoid), counting the lactose-positive colonies greater than 0.5 mm. in diameter after incubation at 44.5° for 48 hr. Only 2 of 200 such colonies tested did not give typical reactions of *E. coli* type I. Faecal samples were examined for salmonellas by enrichment of two 5 g. samples, each in 100 ml. of tetrathionate Tergitol brilliant green broth, following the procedure used for rumen samples. Representative isolates from faeces and rumen were identified by the Salmonella Reference Centre, Adelaide, and proved to be of the serotype inoculated.

pH measurements of the pH values of rumen fluids were made with a Radiometer pH meter and glass electrode after the bacterial samples were taken. The measurements were made about 30 min. after removal from the animal and no precautions were taken to maintain carbon dioxide in solution. Volatile fatty acids were determined in a 20 ml. sample of clarified rumen liquor by the methods outlined by Neish (1952) for clarification and distillation. Ammonia was determined on suitable samples of clarified rumen liquor by vacuum distillation from borate buffer pH 10 (Varner,

Bulen, Vanecko & Burrell, 1953) into a boric acid trap, titrated by the technique of McKenzie & Wallace (1954). Paper chromatography and thin-layer chromatography of acidified ether extracts of clarified rumen fluid were done by the methods of Gordon, Thornburg & Werum (1962) and Ting & Dugger (1965).

RESULTS

The effects of regular daily feeding with 6.8 kg. lucerne hay/day

Four experiments were made to test the effect of inoculation of salmonellas into the rumens of animals fed 6.8 kg. lucerne hay daily. These experiments involved three cattle and three different salmonella serotypes. The results of a typical experiment are shown in Fig. 1. In this experiment, air-dried faeces obtained from an animal shedding *Salmonella chester* was inoculated as a peptone water suspension. The inoculum contained 2.8×10^5 salmonellas, estimated by the most-probable-number technique. Salmonellas in the rumen decreased rapidly from 300/100 ml., and were not detectable 48 hr after inoculation. In this experiment salmonellas were present in the faeces 24 hr after inoculation, but not at later samplings. In three other experiments in which a similar inoculum of salmonellas, grown in nutrient broth, was used, the organisms were not detected in the rumen at or after 24 hr and were not detected at any sampling of the faeces.

The effects of regular daily feeding with 2.3 kg. lucerne hay/day

Three experiments with three animals and two salmonella serotypes were made to test the effect of feeding 2.3 kg. lucerne hay every day on changes in the numbers of salmonella and *Escherichia coli* type I organisms.

In the first experiment the inoculation with 3.5×10^5 salmonellas was on the first day of the smaller feed and the numbers decreased from 161/100 ml. rumen liquor until they were undetected at 48 hr when the experiment was terminated. In the second experiment the animal was given 1.4×10^6 salmonellas on the third day of decreased feeding. The numbers decreased from 693/100 ml. to 1/100 ml. 72 hr after inoculation, when the experiment was terminated. Salmonellas were not detected in the faeces in either experiment.

In the experiment illustrated in Fig. 2, a larger inoculum of *Salmonella oranienburg* (9.8×10^7) was used to follow changes in the salmonella numbers in the rumen over a longer period of time. Initially the salmonella numbers in the rumen decreased, but from about 35 to 40 hr after inoculation (i.e. 110 hr from last 6.8 kg. ration) there was a gradual increase until the animal was returned to a feed of 6.8 kg. lucerne hay/day; then the number decreased abruptly. The *Escherichia coli* type I numbers increased for about 70 hr after inoculation, then stabilized, and decreased when the animal was returned to 6.8 kg. lucerne hay/day. The period of salmonella growth during feeding with 2.3 kg. hay/day may be related to that period when the volatile fatty acids value was low and the pH value high. When the volatile fatty acids value increased and the pH value decreased on feeding 6.8 kg. lucerne/day, the numbers of salmonellas and *E. coli* decreased. A plot of pH value against the concentration of volatile fatty acids showed no points deviating from the approximate linear relation (Briggs, Hogan & Reid, 1957), in contrast to the later experiments which involved a starvation period

(Fig. 3). The faeces contained salmonellas from 24 hr after inoculation until after remedial treatment with Mezobols.

The effect of regular feeding on alternate days with 6.8 kg. lucerne hay

Three experiments were made to test the effect of alternate-day feeding; three animals and two salmonella serotypes were used. The results of one experiment are shown in Fig. 4: the inoculum was 2.1×10^8 *Salmonella muenchen* and was introduced during the second starvation period. The numbers decreased from an initial value of $5.1 \times 10^5/100$ ml. rumen liquor to $1.2 \times 10^4/100$ ml. at 24 hr after inoculation. The numbers then tended to be maintained, with some fluctuations. From 24 hr after inoculation, salmonellas were detected in the faeces for the duration of the experiment.

Fig. 1

Fig. 1. The effect on inoculated *Salmonella chester* (○) of regular daily feeding 6.8 kg. lucerne hay (↓). ●, Salmonellas not detected in 10×10 ml. rumen liquor samples; +, detected in faeces; -, not detected in faeces. ↑, Time of inoculation with faeces suspension containing 2.8×10^5 salmonellas. □, pH value.

Fig. 2

Fig. 2. The effect on *Salmonella oranienburg* (○) and *Escherichia coli* type I (Δ) on inoculation (↑) of 9.8×10^7 salmonellas into the rumen of an animal regularly fed (↓) 2.3 kg. lucerne hay/day. The animal was inoculated on the third day of diminished feeding. +, Salmonellas detected in the faeces; -, not detected in faeces. □, pH value; ▲, VFA, concentration of volatile fatty acids.

The *Escherichia coli* type I count increased during the second starvation period and also for 6–11 hr after each feed; in the period between feeds the numbers tended to decline. The *E. coli* numbers appeared to cycle roughly with the changes in volatile fatty acids. The plot of pH value against volatile fatty acids for this experiment (Fig. 3) shows two points off the approximate straight-line relation; these two points corresponded to the only two samples taken at 6 hr after feeding. Most of the *E. coli* growth occurred within this 6 hr period. The results of the other experiments were similar. In one experiment the salmonella fluctuations were greater and tended to parallel the *E. coli* fluctuations, except for a growth during the third starvation period.

The effects of withholding food for 2 to 3 days

Inoculation of salmonellas before feeding lucerne hay and before withholding food for 2 days. *Salmonella chester* (2×10^8) was inoculated into the rumen of one animal

immediately before feeding (Fig. 5). Food was then withheld for 2 days before the animal was given a single feed of 6.8 kg. lucerne hay. The number of salmonellas in the rumen was initially $3 \times 10^5/100$ ml. liquor, decreased to 916/100 ml. in 22 hr, remained constant for about 24 hr, and then increased a thousandfold at 48 hr after the last feed. Only a slight increase occurred on feeding after this starvation. On withholding food for a further 3 days the numbers declined about one log unit per day. On a subsequent feed the number increased about 30-fold.

The faeces contained salmonellas at 24 hr after inoculation and then for the duration of the experiment.

Fig. 3

Fig. 3. The relation between pH value and volatile fatty acids in rumen liquor. ▲, Two days without food (see Fig. 7); ●, fed 6.8 kg. lucerne hay every alternate day (see Fig. 4); ■, fed 2.3 kg. lucerne hay daily (see Fig. 2).

Fig. 4

Fig. 4. The effect on *Salmonella muenchen* (○) and *Escherichia coli* type I (△) on inoculation (↑) of 2.1×10^8 salmonellas into the rumen of an animal fed (↓) 6.8 kg. lucerne hay every alternate day. The animal was inoculated during the second starvation period. +, Salmonellas detected in faeces; —, not detected in faeces. □, pH value; ▲, VFA, concentration of volatile fatty acids.

From low numbers *Escherichia coli* type I increased during starvation by over 10^5 times. A further increase of 30-fold occurred on feeding, but the numbers decreased during the second starvation. A further increase was obtained on giving the final feed.

Inoculation of salmonellas 24 hr after feeding lucerne hay and before withholding food for 2 days. In two experiments the animals were inoculated 24 hr after feeding and then deprived of food for 2 days; the results of one experiment are shown in Fig. 6. The inoculum contained 3.8×10^5 *Salmonella oranienburg* and the initial concentration of salmonellas in the rumen liquor was 300/100 ml. The salmonella count initially decreased, but at about 54 hr from the regular 6.8 kg. feed the salmonellas began to grow and continued to increase for 24 hr after the next feed, the total increase being about 3.5 log units; on continued feeding the salmonella concentration fell daily about one log unit.

The *Escherichia coli* type I numbers in the rumen increased at about 35 hr from the regular 6.8 kg. feed and showed a further sharp increase on feeding after the 2-day starvation. Salmonellas were detectable in the faeces 24 hr after inoculation and then

for the duration of the experiments. The results of the second experiment were similar except that *E. coli* began to increase in numbers at about 50 hr from the last feed and the count paralleled the changes in the numbers of salmonellas.

Inoculation of salmonellas before feeding and after food was withheld for 2 days. Two experiments using 2 animals and 2 salmonella serotypes were done to test the effect on inoculated salmonellas of feeding 6.8 kg. lucerne hay to cattle after food had been withheld for 2 days. Each inoculum was about 1000 salmonellas, and was given immediately before feeding the animals after starvation for 2 days. The results of one experiment are shown in Fig. 7. The numbers of *Salmonella anatum* increased over 24 hr

Fig. 5

Fig. 5. Changes in the numbers of *Salmonella chester* (O) and *Escherichia coli* type I (Δ) on inoculation (↑) of 2.07×10^8 salmonellas into the rumen of an animal before feeding 6.8 kg. lucerne hay (↓) and before 2 days without food. +, Salmonellas detected in the faeces; -, not detected in the faeces. □, pH value.

Fig. 6

Fig. 6. Changes in the numbers of *Salmonella oranienburg* (O) and *Escherichia coli* type I (Δ) on inoculation (↑) of 3.8×10^6 salmonellas into the rumen of an animal 24 hr after feeding and before 2 days without food. ↓, Fed 6.8 kg. lucerne hay; +, salmonellas detected in faeces; -, salmonellas not detected in faeces. □, pH value.

from an undetectable number to a maximum of 1610/100 ml. rumen liquor; most of the salmonella growth occurred in the first 6 hr. On subsequent feeding the salmonella numbers decreased by about 0.8 log units/day until undetectable. Salmonellas were detected in the faeces at 24 hr after inoculation and for 6 days subsequently; they were not detected after 8 days. Similar results were obtained in the other experiment.

The *Escherichia coli* type I count increased by about four log units during the starvation period. After feeding, the *E. coli* count increased rapidly about 2.5 log units to 8.45 log units/ml. and decreased again on further feeding.

The plot of pH value against volatile fatty acids for this experiment (Fig. 3) showed two points well below the approximate straight-line relation; these two points (at pH 6.8 and 6.3) corresponded to the samples taken at 6 and 11 hr after the feed which followed starvation. It was during this period that rapid growth of *Escherichia coli* and salmonellas occurred. It appeared that there were considerable amounts of non-volatile acids present. The major acids detected by chromatography of these rumen

liquors were lactic, succinic and pyruvic acids, none of which were detected in other rumen samples from the same experiment.

Inoculation of salmonellas before withdrawal for 3 days from lucerne hay or grazing. Three experiments to test the effect of no food for 3 days on the fate of inoculated salmonellas used two animals and three salmonella serotypes. In two of these experiments the animals were inoculated during grazing and then starved for 3 days, whilst in the other experiment the animal was fed the normal lucerne hay ration then inoculated and starved for 3 days.

Fig. 7. The effect on inoculated *Salmonella anatum* (O) of feeding 6.8 kg. lucerne hay/day (↓) after 2 days without food. ●, Salmonellas not detected in 10×10 ml. rumen liquor samples; +, detected in faeces; —, not detected in faeces. ↑, Time of inoculation with 1130 nutrient broth-grown salmonellas. Δ, *Escherichia coli* type I count; □, pH value; ▲, VFA, concentration of volatile fatty acids.

Figure 8 shows the results of one grazing experiment in which the inoculum was 4.3×10^8 *Salmonella oranienburg*, the initial salmonella concentration in the rumen then being $1.61 \times 10^5/100$ ml. rumen liquor. The animal was returned to grazing for 30 hr after inoculation and was sampled once during this period. The salmonella concentration in the rumen decreased rapidly during grazing and at the beginning of starvation was $10.5/100$ ml. rumen liquor. During the next 18 hr the salmonella concentration decreased to $1.05/100$ ml. rumen liquor. From this time on the salmonella and *Escherichia coli* type I numbers increased to $200-300/100$ ml. and $5.7 \times 10^5/ml.$, respectively. On feeding 6.8 kg. lucerne hay the numbers of salmonellas and *E. coli* increased rapidly to maxima of $3.75 \times 10^5/100$ ml. and $3.4 \times 10^7/ml.$, respectively. The animal took 24 hr to consume the 6.8 kg. lucerne hay, which would normally have been consumed in 2-3 hr. The salmonellas and *E. coli* decreased from the maximum concentrations during a further 3 days of food deprivation. Salmonellas were detected in the faeces at 24 hr after inoculation and for the duration of the experiment.

The results of the second grazing experiment were similar except that the time at

which growth occurred during the starvation period was 40 hr after removal from grazing, as against 18 hr in the previous experiment. In these two experiments the conditions of grazing were different; the experiment illustrated (Fig. 8) being on young green pasture and the second experiment on dry standing pasture.

The results of the experiment with lucerne hay were similar to those of the second experiment (dry standing pasture); the growth of salmonellas and *Escherichia coli* type I occurred during the starvation period at 48 hr from the last feed.

Fig. 8. Changes in the numbers of *Salmonella oranienburg* (○) and *Escherichia coli* type I (△) on inoculation (↑) of 4.3×10^8 salmonellas into the rumen of an animal allowed to graze for 30 hr ('Grazed'), then 3 days without food, and then fed one lot of 6.8 kg. lucerne hay ('Fed'). +, Salmonellas detected in the faeces; -, not detected in the faeces. □, pH value; ▲, VFA, concentration of volatile fatty acids.

DISCUSSION

It is apparent that when the animals were fed 6.8 kg. lucerne hay/day, or were allowed to graze freely, salmonellas were rapidly eliminated from the rumen. The *Escherichia coli* type I concentrations were usually under 10/ml. rumen liquor when the animals on this feeding regime were restrained in a cattle-crush to prevent ingestion of faecal material. Decrease of the food intake to 2.3 kg. hay/day, or deprivation of food for 1 or more days, resulted in maintenance or increases of the salmonella and *E. coli* numbers.

The period for the growth of salmonellas and *Escherichia coli* to take place during starvation varied with the type of feed. With animals fed lucerne hay salmonellas grew 48–54 hr after feeding and 20 or 40 hr after grazing; the *E. coli* increases were detected earlier. On feeding after this starvation further increases in numbers of salmonellas and *E. coli* were obtained; the period of most rapid growth was when non-volatile fatty acids were present in the rumen. The average net increases obtained during starvation and subsequent feeding were about 3×10^4 -fold for the salmonellas

and 10^7 -fold for *E. coli*, but the total increase in numbers of organisms within the rumen would have been greater considering dilution and passage. Further starvation or feeding caused the concentrations of these organisms to decrease by about one log unit/day.

Feeding on alternate days (i.e. starvation for one day) tended to stabilize the salmonella concentration after several cycles, while *Escherichia coli* type I increased from an initial low concentration to between four and five log units/ml. There was some evidence of cycling of *E. coli* and possibly of the salmonellas. Increased numbers of *E. coli* were detected within 6 hr after feeding when non-volatile acids were present in the rumen.

Decrease of the feed to 2.3 kg. lucerne hay/day after a period of 4–5 days on reduced feed led to a slow increase in salmonellas and *Escherichia coli*; a return to 6.8 kg. lucerne hay/day caused an immediate decrease in the numbers of these organisms. This contrasted with the observation that when the animal was starved entirely and then fed 6.8 kg. hay the numbers increased.

There was general agreement in the trends of the numbers of *Escherichia coli* type I and the salmonellas, although the methods of enumeration were independent. The method of estimating salmonellas by the most-probable-number technique (McCoy, 1962) although laborious, yielded consistent results.

Bergeim, Hanszen, Pincussen & Weiss (1941) established that volatile fatty acids at low pH values were bactericidal or bacteriostatic for several genera including salmonellas. Meynell (1963) discussed the findings of Bergeim and others on undissociated volatile fatty acids in relation to the inhibition of *Salmonella typhimurium* in the normal mouse gut. Wolin (1966) has shown *in vitro* that the concentration of volatile fatty acids and pH values found in the bovine rumen inhibit the growth of *Escherichia coli*. Although the concentration of volatile fatty acids and pH value may be correlated with changes in numbers of salmonellas in the bovine rumen in conditions of full feed and 2.3 kg. feed, our experiments involving starvation followed by a single feed and further starvation indicated that volatile fatty-acid changes may not be the full explanation. During the first starvation period growth of *E. coli* type I and salmonellas occurred at a pH value and volatile fatty-acid concentration which were attained again during the second starvation period, when the numbers of the bacteria were actually decreasing (e.g. Fig. 8). There did not seem to be any obvious relation between ammonia concentration in the rumen and growth or death of the organisms.

The growth or death of salmonellas and *Escherichia coli* type I in the rumen in relation to feeding or starvation may be important in the development of salmonellosis and infections with enteropathogenic *E. coli*, as well as in influencing the incidence of salmonellas found in cattle at slaughter, especially since a low dose of the order of 1000 salmonellas resulted in substantial growth in the rumen and in salmonellas being shed in the faeces for at least a week after dosage. Other experiments have shown that when soil from an abattoir holding-pen was added to the food of a starved animal the salmonella concentrations in the rumen were some hundreds/100 ml. rumen liquor and salmonellas were present in the faeces. Conditions which obtain during the transport and holding of cattle before slaughter would appear to predispose these animals to salmonella and *E. coli* infection.

The authors wish to thank Dr W. J. Scott for helpful discussions, Dr D. R. Lamond and Mr J. H. G. Holmes (C.S.I.R.O., Division of Animal Physiology) for advice and the provision of animals, and P. F. Everson, R. P. Holmes, D. J. Nicol and Beryl W. Stead for technical assistance.

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