Epidem Inf. (1988), **101**. 337–345
Printed in Great Britain

The growth of salmonella in rumen fluid from cattle at slaughter

By T. MATTILA*, A. J. FROST and D. O'BOYLE

Department of Veterinary Pathology and Public Health, University of Queensland, St Lucia, Brisbane, Queensland 4067

(*Accepted 24 March 1988*)

SUMMARY

The pH of the rument contents of cattle was recorded at slaughter; pH ranged from 5·5 to 7·8 and was not correlated with the period from saleyard to slaughter. Volatile fatty acids (VFA) were measured in 43 rumen samples; acetic, propionic and butyric were the major acids present, and the total VFA ranged from 75·9 mM/l for samples between pH 6–7, to 7·1 mM/l for samples of pH 8–9. Ten *Salmonella* strains belonging to 8 serotypes were grown in these 43 rumen samples. Where acid levels of these samples were high and pH low, most *Salmonella* sp. were inhibited; as the pH rose (pH 7–8) all *Salmonella* serotypes grew, some vigorously; as the total acid declined and pH continued to rise, growth of salmonella ceased. Serotypes and strains of the same serotype differed in their ability to grow in rumen contents, particularly when the pH was low.

INTRODUCTION

It has long been recognized that during the transport of livestock from the farm to the abattoir and slaughter, the prevalence of salmonella infection may rise markedly, but the mechanisms involved are still not well understood. Grau & Brownlie (1965) showed that the rumen could often contain salmonella, and that in both cattle (Grau *et al.* 1968) and sheep (Grau *et al.* 1969) this was related to a period of starvation during transport followed by feeding. Later it was shown that up to 100% of cattle at slaughter could yield salmonella from the rumen, and this was associated with high levels of infection in the mesenteric lymph nodes (Samuel *et al.* 1980). The mechanisms may involve the presence, or a critical level, of volatile fatty acids (VFA) in the rumen; Meynell (1963) showed in rats that the levels of VFA in a reducing environment in the intestine influenced the survival of salmonella, and other pathogens may also be affected (Hentges, 1969). In chickens, high VFA concentration combined with a low pH from 2 weeks of age inhibited salmonella multiplication (Barnes *et al.* 1979). Salmonella did not survive in anaerobically fermented pig waste if the total VFA was greater than 30 mM/l and the pH around 4·0 (Henry *et al.* 1983). It has long been recognized that VFA are toxic for bacteria. Levine & Fellows (1940) cite Kahlenberg & True (1896) who considered that it was the free acid molecule and not the salt that is

* Present address: Food Research Laboratory, Technical Research Centre, 02150 Espoo, Finland.

primarily responsible for the toxicity. Thus the pH is critical, depending on the pK_a of the acid concerned. For acetic, propionic and butyric acids, pK_a is around 4·8, at which pH half of the acid is present as free acid.

The only investigation of VFA in the rumen and their effect on salmonella appears to be that of Chambers & Lysons (1979) who studied the influence of dietary changes in an experimental cow. This paper describes the growth of various *Salmonella* sp. in rumen fluid taken from cattle at slaughter, and relates growth to the levels of VFA and pH.

MATERIALS AND METHODS

Rumen samples and pH

Rumen contents were collected, and the pH recorded, from cattle slaughtered at a large Brisbane abattoir. Immediately after evisceration the rumen was opened and a sample taken in a sterile jar; pH was recorded immediately and at later periods using a Philips Stab electrode. The pH of the rumen contents was also recorded in a series of animals without collection of rumen samples. In most cases the farm of origin of the cattle was not known, but their period from saleyard to slaughter was recorded.

At the laboratory, the rumen samples were coarse filtered, centrifuged at 2500 ***g***, then again at 10000 ***g*** if required to clear the sample, and the supernate was then frozen at −20 °C. Before use as culture medium, samples were thawed and sterilized by gamma radiation.

Bacteria

S. typhimurium (culture 'a'), and *S. cholerae-suis* were type strains held in this laboratory. *S. anatum* (culture 'c') was isolated from a horse with enteritis. The remaining samples were isolated from cattle: *S. typhimurium*, *S. anatum*, *S. muenchen*, *S. adelaide*, *S. newport*, *S. saintpaul* and *S. chester*. The serotypes were confirmed by the Salmonella Reference Laboratory, Adelaide. Before use in growth tests bacteria were grown overnight at 37 °C in tryptone soya broth (Oxoid).

Isolation of salmonella

Rumen samples were cultured for salmonella by direct plating on selective media and also after enrichment as described by Samuel *et al.* (1980).

Growth of salmonella in rumen fluid

Bacterial suspensions were calibrated with a Multiskan microplate reader to give an initial turbidity of 0·15 before inoculation; this represents approximately $10^{6·5}$ organisms per ml. Bacteria were grown in the rumen fluid at 37 °C in Honeycomb plates consisting of 200 microwells. The amount of rumen fluid was 200 μl in each well to which 100 μl of bacterial suspension was added. Bacterial growth was monitored by a vertical pathway using an automated turbidometer (Bioscreen, Labsystems Oy, Helsinki, Finland). Growth was monitored every 10 min for 24 h by the automated analyser and the data were transferred to the computer. The data were analysed with the Bioscreen software (Labsystems Oy,

Table 1. *pH of rumen contents of 941 cattle at slaughter, grouped according to the time taken from saleyard to slaughter*

Days	Number of animals	Number of batches	pH range	Mean pH
1	163	17	5·5–7·7	7·29
2	129	9	6·4–7·7	7·12
3	106	6	6·8–7·7	7·37
4	219	7	6·4–7·5	7·28
5	137	12	6·1–7·9	7·37
>5	187	13	6·8–7·8	7·28
Total	941	64		

Helsinki, Finland). On examination of the growth curves it was considered that the most appropriate parameter for comparison of strains and of the nutrient qualities of the rumen fluid was the area under the growth curve.

A pilot study showed the turbidometric results to be highly repeatable. Four serotypes were grown in ten samples of rumen fluid and the experiment replicated five times. The results for each sample differed by less than 1%, similar to other systems studied with the automated turbidometer. For the present study, samples were cultured in duplicate.

Estimation of volatile and non-volatile fatty acids

The method used was as described by Holdeman *et al.* (1977), using a G.C. Varian 3700 with flame ionizing detector and a Shimadzu Chromatopac C-R3A data processor.

Estimation of total carbon

The samples were acidified and degassed under vacuum. Carbon was measured at 247·8 nM using an inductively coupled plasma atomic emission spectrometer (Labtest model 20100), by the Department of Agriculture, University of Queensland.

RESULTS

pH of rumen contents

The pH of the rumen of 941 cattle was measured at slaughter. They represented 64 groups, most of which had passed through saleyards before being transported to slaughter. Table 1 shows these animals grouped on the length of time from saleyard to slaughter, and the pH of the rumen contents. There was no significant correlation between pH and the time before slaughter and there was considerable variation in pH not only between batches, but also within each batch.

Samples of rumen contents, taken from a further 100 animals representing 10 groups, were examined in the laboratory. The pH was measured after filtration and again after thawing. The pH usually rose slightly after filtration and further again after freezing-thawing. These samples were cultured for salmonella, and 23 were positive. Salmonella were isolated from four of these on direct culture; these were from a single batch that had been held for 7 days before slaughter; 8 of 10 animals were positive. Eight serotypes were identified from the 23 samples.

Table 2. *VFA levels and pH of 43 rumen samples*

No. of samples	18 (pH 6–7)	9 (pH 7–8)	4 (pH 8–9)	12 (pH > 9·0)
VFA mM/l				
Acetic	32·4(42·6)*	22·5(41·8)	1·9(26·8)	6·2(50·0)
Propionic	25·3(33·3)	18·4(34·2)	3·0(42·2)	3·2(25·8)
Butyric	11·0(14·5)	6·3(11·7)	0·9(12·6)	1·3(10·5)
Total acids	75·9	53·8	7·1	12·4
Mean pH	6·6	7·3	8·7	9·6

* Percent of total acid.

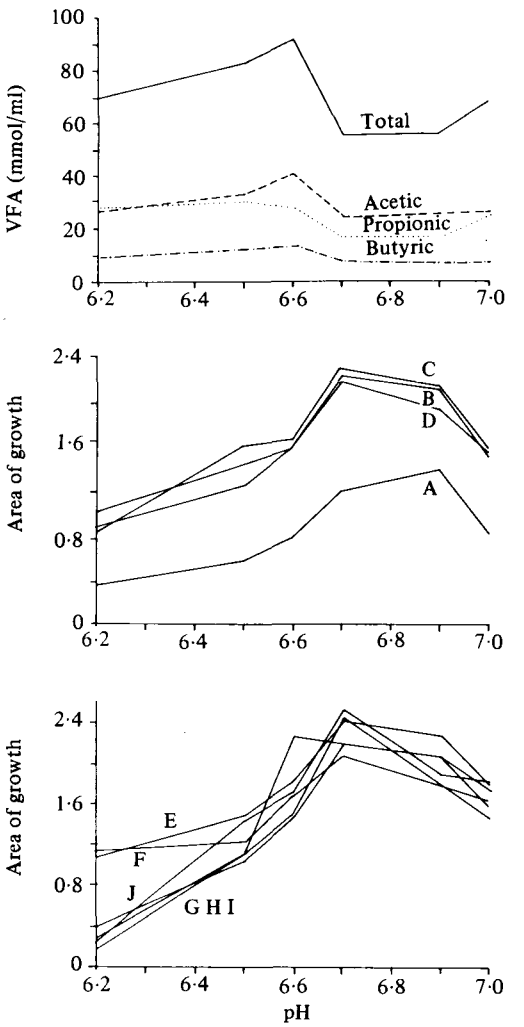

Fig. 1. Growth of ten strains of *Salmonella* spp. in rumen fluid pH 6–7, together with levels of VFA. Upper panel: levels of major acids. Middle panel: growth (total area under growth curve) of *S. typhimurium* (a, b) and *S. anatum* (c, d). Lower panel: growth of *S. muenchen* (e), *adelaide* (f), *newport* (g), *saintpaul* (h), *choleraesuis* (i) and *chester* (j).

https://doi.org/10.1017/S0950268800054273 Published online by Cambridge University Press

Table 3. *The growth of ten strains of* Salmonella *spp. in 43 rumen samples grouped according to pH*

No. of samples	18 (pH 6–7)	9 (pH 7–8)	4 (pH 8–9)	12 (pH > 9·0)
a *S. typhimurium*	0·84*	1·30	0·23	0·27
b *S. typhimurium*	1·49	1·66	0·28	0·10
c *S. anatum*	1·58	1·66	0·40	0·21
d *S. anatum*	1·67	1·67	0·48	0·15
e *S. muenchen*	1·49	1·69	0·50	0·27
f *S. adelaide*	1·75	1·95	0·68	0·34
g *S. newport*	1·41	1·95	0·37	0·13
h *S. saintpaul*	1·41	1·85	0·47	0·22
i *S. choleraesuis*	1·39	1·53	0·26	0·12
j *S. chester*	1·63	1·86	0·49	0·21

* Area under the growth curve (see text): mean area for growth in the 18 samples. Growth in TSB at pH 7 approximates 2·0–2·2. Less than 0·3 represents inhibition.

Levels of VFA

Forty-three of the 100 samples were analysed for fatty acids. They were selected to cover the range of pH recorded, extending to higher pH levels than seen in the first study (Table 1), due in part to the slight increase in pH after freeze-thawing of the samples. The major acids present are shown in Table 2, where the samples are grouped according to pH and the levels are presented as the mean for the group; other acids were detected, both volatile and non-volatile, at trace levels only. The levels of VFA in samples with pH 6–7 are shown in Fig. 1.

Growth of salmonella in rumen fluid

The 43 samples were sterilized by gamma radiation and used as growth media for the 10 strains of *Salmonella* spp.; the effect ranged from almost complete inhibition of growth to support of vigorous and rapid growth. All serotypes grew similarly in TSB, with a shorter lag phase and a steeper log phase than seen in any rumen sample. Table 3 shows the growth of the ten strains grouped according to pH of the rumen fluid. The area under the growth curve is shown through the range pH 6–7 in Figs. 1 where the upper panel shows the levels of VFA in these samples. Correlation of this growth with the levels of acid is shown in Table 4. At pH 6·2, the lowest used, there was considerable variation in the effect. Four serotypes were completely inhibited (Fig. 1, lower panel), and the laboratory strain of *S. typhimurium* ('a') grew poorly in any sample (Fig. 1, middle panel). However, *S. muenchen* and *S. adelaide* grew at the lowest pH, as did both strains of *S. anatum*. As the pH rose, these differences became less, such that beyond pH 6·6 there was little difference between strains, with the exception of the laboratory strain of *S. typimurium*, which still grew poorly.

In Tables 4, 5 and 6 the growth of different salmonellae is correlated with the acids present in the medium and the initial pH. There were significant differences in the ability of the different rumen samples to support growth of a particular serotype, and for a single rumen sample, the serotypes differed in their ability to grow; there were also differences shown between strains of the same serotype (Fig.

https://doi.org/10.1017/S0950268800054273 Published online by Cambridge University Press

Table 4. *Correlations of VFA, pH and growth of different salmonella in rumen samples of pH 6–7 (n = 18). All the numbered correlations are significant (P < 0·01)*

	Acetic	Propionic	Butyric	Total acids	pH
a *S. typhimurium*	N.S.	−0·67	−0·49	−0·51	0·73
b *S. typhimurium*	N.S.	−0·61	−0·47	−0·47	0·54
c *S. anatum*	−0·50	−0·73	−0·63	−0·62	0·44
d *S. anatum*	−0·45	−0·72	−0·57	−0·58	0·46
e *S. muenchen*	N.S.	N.S.	N.S.	N.S.	0·50
f *S. adelaide*	N.S.	−0·53	N.S.	−0·40	0·55
g *S. newport*	N.S.	−0·56	N.S.	−0·40	0·70
h *S. saintpaul*	N.S.	−0·55	N.S.	−0·40	0·73
i *S. choleraesuis*	N.S.	−0·43	N.S.	N.S.	0·75
j *S. chester*	N.S.	−0·60	N.S.	−0·41	0·52

Table 5. *Correlations of VFA, pH and growth of different salmonella in rumen samples of pH 7–8 (n = 9). All the numbered correlations are significant (P < 0·01)*

	Acetic	Propionic	Butyric	Total acids	pH
a *S. typhimurium*	−0·65	−0·87	−0·63	−0·71	0·78
b *S. typhimurium*	−0·68	−0·84	−0·77	−0·70	N.S.
c *S. anatum*	−0·74	−0·87	−0·81	−0·76	N.S.
d *S. anatum*	N.S.	N.S.	N.S.	N.S.	N.S.
e *S. muenchen*	N.S.	N.S.	−0·73	−0·59	N.S.
f *S. adelaide*	−0·70	−0·84	−0·81	−0·79	N.S.
g *S. newport*	−0·82	−0·95	−0·80	−0·86	0·61
h *S. saintpaul*	−0·65	−0·78	−0·80	−0·76	N.S.
i *S. choleraesuis*	N.S.	N.S.	N.S.	N.S.	N.S.
j *S. chester*	−0·59	−0·61	−0·65	−0·55	N.S.

Table 6. *Correlations of VFA, pH and growth of different salmonellae in rumen samples of pH over 8 (n = 16). All the numbered correlations at significant (P < 0·01)*

	Acetic	Propionic	Butyric	Total acids	pH
a *S. typhimurium*	0·72	0·80	0·76	0·76	−0·81
b *S. typhimurium*	0·88	0·92	0·90	0·90	−0·94
c *S. anatum*	0·79	0·84	0·82	0·82	−0·90
d *S. anatum*	0·78	0·82	0·83	0·81	−0·89
e *S. muenchen*	0·81	0·87	0·84	0·84	−0·92
f *S. adelaide*	N.S.	N.S.	N.S.	N.S.	N.S.
g *S. newport*	0·87	0·91	0·89	0·90	−0·93
h *S. saintpaul*	0·83	0·87	0·85	0·86	−0·93
i *S. choleraesuis*	0·85	0·87	0·87	0·87	−0·93
j *S. chester*	0·80	0·86	0·83	0·84	−0·91

1, middle panel). The general trend was that as the level of VFA rose, growth was depressed, while as pH rose, growth was enhanced.

Six samples, three of which supported and three of which inhibited growth, were analysed for total carbon. For the first three, the levels were 4·27, 4·30, 3·68 mg/ml, the latter three were 2·68, 2·17, 1·78 mg/ml.

https://doi.org/10.1017/S0950268800054273 Published online by Cambridge University Press

DISCUSSION

The pH levels of rumen contents indicated it was rare at slaughter for the pH to be at the low levels expected in the well fed animal. In a fistulated cow, Chambers & Lysons (1979) found the pH to vary from 6·1 shortly after regular feeding to 8·5 after 48 h starvation, dropping again after feeding was resumed. The cessation of feeding together with the stress of transport means that at slaughter the dynamics of the rumen ingesta and its microflora may be at any stage from near maximum nutrition to near starvation; all stages were probably represented in the present study. The lowest pH range found (5·5 – 6·5) was in a group of feedlot cattle killed within 4 h of leaving the feedlot. Salmonella was not isolated from any of these samples, and the rumen fluid did not support the growth of salmonella *in vitro*. *Salmonella* spp. were isolated from 23 of the samples, all of which showed a pH > 7·0 when measured at slaughter.

The levels of VFA found in the rumen may show a wide variation. Hungate (1966) lists a series of references showing the ranges that can be expected, from a low of 51 mM/l total acid in cows starved for 24 h to greater than 200 mM/l in milking cows. VFA's are absorbed rapidly from the rumen so that the levels fluctuate around the feeding cycle. An average of around 75 mM/l and a pH < 7·0 could be considered normal, and in the present study, salmonella were inhibited in such samples. As the total acids dropped the pH rose, until at pH > 8·0 there was very little VFA present, probably indicating the animal had not been fed for at least 48 h. The relative concentrations of the major acids in sheep are maintained, regardless of the total acid at about 63% acetic, 21% propionic and 15% butyric (Hungate, 1966). In the slaughtered cattle the proportions differed, mainly due to the low levels of acetic acid, though at pH > 9·0 the relative levels of butyric acid also dropped (Table 1); at this stage, the total level of acid was, however, extremely low.

There was considerable variation in the ability of the various *Salmonella* spp. to grow in the same sample of rumen fluid, and there was also considerable difference between strains of the same serotype. This is shown in Fig. 1 where the area of growth for the eight Salmonella spp. is compared in rumen samples of pH between 6·2 and 7·0. Note from Fig. 1 that the highest growth of the salmonella was at pH 6·7, coinciding with a drop in total acid beginning at pH 6·6. Differences between serotypes in their ability to multiply in a potential reservoir such as the rumen may explain in part the prevalence of particular serotypes in the abattoir environment.

The correlations of growth of the serotypes with VFA levels and pH (Tables 4, 5 and 6) show the changing patterns of growth as these parameters varied. Between pH 6–7 (Table 4), there was a significant negative correlation between growth and the levels of propionic acid for all but one strain, less with levels of acetic and butyric acid. At pH > 8, there was minimal growth for most strains (Table 3), but within this group, growth was now correlated positively with the low levels of VFA. This inhibition of growth was not due to inhibitory factors, as disks impregnated with these rumen fluids had no effect on a lawn of salmonella growing on nutrient agar. Failure to grow was probably due to a lack of available

nutrients, as the levels of VFA were very low, as were the levels of total carbon in the samples analysed.

The rumen contents of cattle may therefore show great variation in their ability to support the growth of salmonella. Under conditions of *ad lib.* high energy nutrition, in spite of constant challenge, the animals usually remain free from salmonella (Frost *et al.* 1988). When feeding ceases, fermentation of the ingesta continues with high levels of VFA and low pH until the rate of fermentation equals the rate of absorption, after which the total levels of acid begin to decline (Hungate, 1966). This would probably occur in the early stages of transport of cattle for sale and slaughter, and would coincide with their exposure to a range of serotypes in the immediate environment (Grau *et al.* 1968).

The pH of the rumen should rise during this period, possibly owing to the buffering effect of saliva without the addition of new ingesta. As the pH rises towards 8, most of the VFA would be dissociated and non-toxic, and provided the levels of VFA and other nutrients are adequate, salmonella grow vigorously, and pass through to the intestine from which they may invade the mesenteric lymph nodes (Samuel *et al.* 1980; Frost *et al.* 1988). As the pH continues to rise and the nutrients drop, as shown by the low levels of VFA and of total carbon, growth of salmonella declines. Grau *et al.* (1968) showed that if the animals were fed after a period of starvation, the incidence and numbers of salmonella in the rumen increased. Chambers & Lysons (1979) suggested this change was due to a combination of slow and abnormal fermentation of the ingesta as rumen microbes recovered, and that the high pH was due to the buffering effect of saliva.

The present study demonstrates the great range in the nutrient qualities of rumen fluids in cattle at slaughter, their range in ability to support the growth of salmonella, and the growth response of different serotypes. The obvious managerial procedures that would flow from this work would be to minimize the time taken for the animals to travel from farm to slaughter or to feed animals often during transport and in lairage. Neither of these proposals is new but, when cattle are reared under range conditions, they are often impracticable. Further studies may indicate more simple measures which would maintain the inhibitory capacity of the rumen for salmonella for longer periods.

We thank Labsystems Oy, Helsinki, Finland for the Bioscreen analyser, and Dr D. P. Henry for the VFA analyses; we thank M. Moulds for her assistance.

REFERENCES

BARNES, E. M., IMPEY, C. S. & STEVENS, B. J. H. (1979). Factors affecting the incidence and anti-salmonella activity of the anaerobic caecal flora of the young chick. *Journal of Hygiene* **82**, 263–283.

CHAMBERS P. G. & LYSONS, R. J. (1979). The inhibitory effect of bovine rumen fluid on *Salmonella typhimurium*. *Research in Veterinary Science* **26**, 273–276.

FROST, A. J., O'BOYLE, D. & SAMUEL, J. L. (1988). The isolation of *Salmonella* from feed lot cattle managed under different conditions before slaughter. *Australian Veterinary Journal*. In press.

GRAU, F. H. & BROWNLIE, L. E. (1965). Occurrence of salmonellas in the bovine rumen. *Australian Veterinary Journal* **41**, 321–323.

GRAU, F. H., BROWNLIE, L. E. & ROBERTS, E. A. (1968). Effect of some preslaughter treatments

on the *Salmonella* population in the bovine rumen and faeces. *Journal of Applied Bacteriology* **31**, 157–163.

GRAU, F. H., BROWNLIE, L. E. & SMITH, M. G. (1969). Effects of food intake on members of salmonellae and *Escherichia coli* in rumen and faeces of sheep. *Journal of Applied Bacteriology* **32**, 112–117.

HENRY, D. P., FROST, A. J., SAMUEL, J. L., O'BOYLE, D. A. & THOMPSON, R. H. (1983). Factors affecting the survival of *Salmonella* and *Escherichia coli* in anaerobically fermented pig waste. *Journal of Applied Bacteriology* **55**, 89–95.

HENTGES, D. J. (1969). Inhibition of *Shigella flexneri* by the normal intestinal flora. *Journal of Bacteriology* **97**, 513–517.

HUNGATE, R. E. (1966). *The Rumen and its Microbes*. New York: Academic Press.

HOLDEMAN, L. V., CATO, E. P. & MOORE, W. E. C. (1977). *Anaerobe Laboratory Manual*, 4th ed. Virginia Polytechnic Institute and State University. Blacksburg, Virginia.

KAHLENBERG, L. & TRUE, R. H. (1896). On the toxic action of dissolved salts and their electrolytic dissociation. *Botanical Gazette* **22**, 81–124.

LEVINE, A. S. & FELLOWS, C. R. (1940). Action of acetic acid on food spoilage organisms. *Journal of Bacteriology* **39**, 499–513.

MEYNELL, G. C. (1963). Antibacterial mechanisms of the mouse gut. The role of Eh and volatile fatty acids in the normal gut. *British Journal of Experimental Pathology* **44**, 209–219.

SAMUEL, J. L., O'BOYLE, D. A., MATHERS, W. & FROST, A. J. (1980). Isolation of *Salmonella* from mesenteric lymph nodes of healthy cattle at slaughter. *Research in Veterinary Science* **28**, 238–241.