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Factors affecting the incidence and anti-salmonella activity of the anaerobic caecal flora of the young chick

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SUMMARY

Thirty-two different types of anaerobic bacteria isolated from chickens have been tested for anti-salmonella activity *in vitro*. Under the conditions of the test only *Bacteroides hypermegas* and a *Bifidobacterium* sp. were shown to inhibit the salmonellas and this was attributed to the production of volatile fatty acids (VFA's) coupled with a low pH. When these organisms were tested in newly hatched chicks no inhibition of *S. typhimurium* occurred. Possible explanations for this observation are considered.

The pH value and concentration of VFA's in the caecal material were determined in chicks from 0–84 days. *In vitro* tests with *S. typhimurium* indicated that, whilst the organism would be able to multiply at the pH and concentration of VFA's found during the first few days after hatching, the rapid increase inVFA concentration during the first 21 days would make this increasingly difficult. The significance of the developing caecal flora in relation to VFA production and pH is discussed.

Because certain feed additives are known to influence the carriage of salmonellas, the sensitivity of various caecal anaerobes to these compounds was determined *in vitro*, generally at 1, 10 and 100 μg/ml. The additives tested included flavomycin, furazolidone, nitrovin, tetracycline, tylosin, sulphaquinoxaline, virginiamycin and zinc bacitracin. All the organisms tested were inhibited by 100 μg/ml furazolidone; none were inhibited by 500 μg/ml sulphaquinoxaline.

Changes occurring in the VFA concentration, pH value and microflora of the caeca of chicks fed for 49 days or longer on a normal starter diet or the same diet containing 10 or 100 mg/kg nitrovin have been compared. When the chicks were fed on the diet containing 100 mg/kg nitrovin, the Gram-negative non-sporing anaerobes were eliminated as a significant part of the caecal flora. However, the VFA concentration combined with a low pH in chicks from 2 weeks onwards was still sufficient to inhibit salmonella multiplication. Other possibly interrelated factors which might lead to an increased salmonella carrier rate in the nitrovin-treated chickens are discussed.

INTRODUCTION

The mechanism by which the normal intestinal flora prevents the establishment of an invading pathogen is still a subject for discussion and research. Amongst the possibilities which have been considered are competition for limiting carbon

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sources, the presence of antibacterial compounds or the production of volatile fatty acids by the anaerobic flora, and it is now thought that all may be involved (Freter, 1974).

It was shown by Meynell (1963) and Bohnhoff, Miller & Martin (1964*a*, *b*) that the volatile fatty acids such as acetic, propionic and butyric acids produced by the anaerobic bacteria which predominate in the caeca and colon of man and other animals, are inhibitory for salmonellas. Most of their work was carried out in relation to *Salmonella typhimurium* and *Salmonella enteritidis* infections in mice. The acids exert their inhibitory activity in the undissociated state which is determined by pH, thus the effect will be much greater if the pH is below 6·0 than between 6·0 and 7·0. It was further shown by Meynell (1963) that the anti-salmonella activity was greater under anaerobic conditions whilst Bohnhoff *et al.* (1964*b*) reported that lactic or succinic acids could interfere with the antagonistic effect of the volatile fatty acids.

The problem of the salmonella carrier rate in healthy chickens and turkeys is receiving increasing attention. It is known that very young chicks are more susceptible to salmonella infections than older birds and the organisms are found more frequently in the caeca than other parts of the gut (Brownell, Sadler & Fanelli, 1969). Rantala & Nurmi (1973) showed that when the intestinal contents from an adult chicken were given orally to a 1-day chick, this prevented the establishment of *Salmonella infantis* when inoculated the following day. The authors also showed that the intestinal contents retained their activity when grown anaerobically in broth but the anti-salmonella activity was destroyed by aerobic culture, furazolidone 100 μg/ml and tetracycline 20 and 100 μg/ml. Activity was not destroyed by nitrovin 10 μg/ml or zinc bacitracin 10 μg/ml (Rantala, 1974; Nurmi & Rantala, 1974). Royal & Mutimer (1972) also demonstrated that cultures of poultry caecal contents grown anaerobically in nutrient broth buffered at pH 5·5 inhibited the growth of *Salmonella typhimurium*.

Smith & Tucker (1975) showed that, when chicks were experimentally infected with 10^9 *S. typhimurium* at 3 days, all of the chicks excreted the organisms for the first 2-3 weeks but after this the numbers steadily declined so that by about 8 weeks only a very small proportion of the chicks were excreting the organisms. When various permitted feed additives were incorporated the carrier rate was often extended; this was particularly the case when 100 mg/kg nitrovin was added to the feed.

Whilst clostridia can often be found in the caeca of chicks soon after hatching (Lev & Briggs, 1956; Smith, 1965), the non-sporing anaerobes tend to develop more slowly and only become fully established by about the 3rd week of life, when they outnumber all the other organisms present (Ochi, Mitsuoka & Sega, 1964; Barnes *et al.* 1972). In the experiments described below an attempt has been made to explain why the chick is so vulnerable to salmonella infection in the first week of life and why once the salmonellas are established in the caeca they may be more difficult to eliminate in the presence of certain feed additives, particularly nitrovin.

This work has involved (1) examining typical anaerobes isolated from chicken

caeca for their anti-salmonella activity, (2) testing the effect of various feed additives on the anaerobes *in vitro*, and (3) determining the changes in the volatile fatty acids, pH and caecal flora in chicks from 0 to 84 days fed on a control diet and on a diet containing nitrovin.

MATERIALS AND METHODS

Chickens

The chickens used in these experiments were Ross 1 male chicks obtained from the same supplier on three separate occasions. They were reared on wire-mesh floors in cages, those fed on different diets being kept in separate rooms. In all three experiments the control diet was a normal chick starter ration without any feed additives. The chicks were killed by neck dislocation.

In one experiment the chicks were only reared for 14 days, a bulk caecal sample from 10 chicks being taken at 0 days (before feeding), 3 and 6 days and from 5 chicks at 10 and 14 days. The samples were analysed for pH value, volatile fatty acids and non-volatile acids.

In the other two experiments the control diet was compared with the same diet containing either 10 or 100 mg/kg nitrovin. Bulk caecal samples from 10 chicks were taken for the first 7 days, from 5 chicks from 14-35 days and 3 chicks at 49, 63 and 84 days. All the samples were analysed for pH value, volatile fatty acids and non-volatile acids.

In the experiment using 10 mg/kg nitrovin the main bacteriological analysis was carried out at 31 days. When 100 mg/kg nitrovin was used, bacteriological analyses were carried out on every weekly sample from 7 to 84 days (except for the 63-day sample).

Chemical analysis

pH determination. The method of Ashcraft (1933) was used, the caecal material being homogenized in 2 volumes of freshly boiled and cooled glass-distilled water. The pH was measured electrometrically within 30 min of the sample being taken.

Volatile fatty acids. Samples of frozen caecal material (0·5–2·0 g) were homogenized in glass-distilled water to give a 4- or 5-fold dilution. Following the methods of Barnes *et al.* (1977) the volatile acids were separated from the caecal material by steam distillation and estimated by gas chromatography. Formic acid was not determined but its presence in significant amounts would have been suggested by any large discrepancy between the total of volatile acids as determined by gas chromatography and that given by the titration following steam distillation.

Succinic acid. The gas chromatograph column and conditions that were used for the estimation of volatile fatty acid were also used to estimate succinic acid as the methyl ester. A sample of caecal material (0·5–1·0 g) was homogenized in glass-distilled water and acidified to pH 2·0 by the addition of 50 % (v/v) H_2SO_4. After leaving at room temperature for 18–24 h the suspension was remixed and centrifuged at 2500 ***g*** for 20 min. To 2 ml of the supernatant, 2 ml of boron trifluoride methanol complex (B.D.H. Ltd) were added, mixed, and left at room

temperature for 18–24 h, then extracted with chloroform (1 ml). One μl of the chloroform extract was injected into the gas chromatograph and the amount of succinic acid present was calculated from the peak height of the methyl succinate compared with that from a succinic acid standard treated in the same way.

Lactic acid. For some caecal samples total lactic acid was estimated by the ceric sulphate method of Elsden & Gibson (1954). Lactic acid was also estimated as the methyl ester by the method described above for succinic acid. Concentrations less than 5 μmol/g were not detected. L-lactic acid was estimated enzymically with lactic acid dehydrogenase (Lactic acid, u.v. method: Boehringer Corporation Limited, London).

Bacteriological methods

Media for use with the technique of Hungate (1950)

(i) Anaerobic dilution solution (ADS) (Bryant & Burkey, 1953).

(ii) Supplemented Medium 10 (SM10) broth and agar. (Barnes & Impey, 1974).

(iii) Lactate broth: SM10 broth in which the carbohydrates were replaced by 0·25% sodium lactate.

Media for use with traditional anaerobic techniques

(i) RCM broth: the medium was prepared according to the original formula of Hirsch & Grinsted (1954).

(ii) VL broth (modified from Beerens *et al.* 1963) contains (g/l): Tryptone (Oxoid), 10; NaCl, 5; beef extract (Lab-Lemco powder, Oxoid), 2·4; yeast extract (Difco), 5; cysteine hydrochloride 0·4; glucose, 2·5; agar (New Zealand), 0·6, pH 7·2–7·4. The medium is sterilized in 19 ml lots in 1 oz screw-capped bottles at 15 lb/in^2 for 15 min.

(iii) VL agar: VL broth containing agar (New Zealand) 12 g/l.

(iv) VLhlf agar: VL agar supplemented with (per litre) haemin 1 mg, liver extract 50 ml and chicken faecal extract 50 ml (Barnes & Impey, 1974).

(v) BGPhlf agar: as for VLhlf but containing glucose 1 g/l instead of 2·5 g together with Na_2HPO_4 4g/l.

(vi) Ethyl violet azide agar (Barnes & Goldberg, 1962). Used for the selective isolation of *Bacteroides hypermegas.*

(vii) China blue agar: modified from van der Wiel-Korstanje & Winkler (1970). The medium consists of VLhlf agar with the glucose increased to 10 g/l and containing china blue (3%) 10 ml/l. The china blue must be the ferri-ferrocyanide complex (No. 10365, Chroma-Gesellschaft, Stuttgart). Bifidobacteria appear as brown colonies but several other types of bacteria, including bacteroides, will grow on this medium.

(viii) Kanamycin vancomycin agar. VLhlf agar containing kanamycin 100 μg/ml and vancomycin 7·5 μg/ml. Used for the isolation of bacteroides (Finegold, Miller & Posnick, 1965).

Facultative anaerobes

(i) Rogosa agar (Difco): used for the lactobacilli. The plates were incubated for 2 days in an atmosphere of hydrogen and 10% carbon dioxide.

(ii) Thallous acetate tetrazolium agar (Barnes 1956) for faecal streptococci. The plates were incubated for 1 day and then left at room temperature for several days to detect some of the faecal streptococci which grow more slowly on this medium (Barnes *et al.* 1978).

(iii) MacConkey 3 agar (Oxoid): used for the detection of *coli-aerogenes* bacteria and any lactose negative enterobacteria such as *Proteus* spp. The plates were incubated for 1 day.

(iv) Brilliant green agar (modified) (Oxoid Code CM329) to which had been added 20 μg/ml nalidixic acid for enumeration of *Salmonella typhimurium* (strain resistant to nalidixic acid).

Bacteriological analysis of the caecal samples

These experiments involved the use of three different isolation techniques: (1) the isolation of strict anaerobes using the technique described by Hungate (1950); (2) the isolation of certain anaerobes which could be grown on pre-reduced agar plates incubated in an anaerobic jar (with catalyst) in an atmosphere of hydrogen + 10% carbon dioxide; (3) the isolation of the facultative anaerobes. All incubations were carried out at 37 °C.

Total anaerobic flora

In order to isolate the predominant anaerobic flora the Hungate (1950) technique was used, all operations being carried out under a continuous flow of oxygen-free carbon dioxide. The bulk caecal samples were weighed without delay into a tube containing 9 ml anaerobic dilution solution (ADS) with glass beads. After mixing, tenfold dilutions were made in ADS and 1 ml of the required dilutions added to 9 ml pre-reduced molten SM10 agar and roll tubes prepared. After incubation for 1 week colonies were picked into SM10 agar slopes and further characterized as described by Barnes & Impey (1974).

Alternatively, dilutions were made in a total growth medium such as SM10 or lactate broths and the highest dilutions showing growth were examined microscopically. The organisms were then isolated by streaking on pre-reduced BGPhlf agar plates and also, in some cases, on China blue agar and Kanamycin vancomycin agar. The dilutions were also heated at 70 °C for 10 min before streaking on BGPhlf agar to detect the presence of clostridia.

Isolation of more oxygen tolerant anaerobes and facultative anaerobes

The caecal samples were weighed into 18 ml RCM broth + glass beads and were further diluted in 18 ml lots of RCM broth. Immediately before use the RCM broths were held in a boiling water bath for 20 min to expel oxygen and then cooled. To isolate the anaerobes 2 drops (1/15 ml) of the required dilutions were rapidly spread over half plates of the pre-reduced agar media, the plates being returned to the anaerobic jar without delay and incubated in an atmosphere of hydrogen + 10% carbon dioxide, with a cold catalyst, for up to 7 days. Isolated colonies were purified by streaking on pre-reduced BGPhlf agar plates and, after anaerobic incubation for several days, stock cultures were prepared by picking

colonies into SM10 agar slopes using the Hungate technique. Preliminary tests were then carried out as for the other anaerobes (Barnes & Impey, 1974).

For the facultative anaerobes 1 drop (1/30 ml) of each dilution was spread over one quarter of the petri-dish containing the required agar medium.

Direct microscopical counts

The method used was that described by Barnes & Impey (1970).

Anti-salmonella activity of the anaerobes

Organisms used. The anaerobes tested in these experiments are shown in Table 1. They had all been isolated from the caeca of chickens, in a number of experiments carried out by the authors.

For the detection of anti-salmonella activity the following strains were used. *Salmonella typhimurium* 3698/60 from Dr B. C. Hobbs, Central Public Health Laboratory, *S. typhimurium*, *S. haardt*, *S. livingstone*, *S. cerro* and *S. agona*, all isolated from chickens by the V.I. Centre, M.A.F.F., Norwich. Other organisms tested which were isolated from chickens included 4 strains of *Escherichia coli* (EBF 85/3, EBF 85/6, GCM/T1 and GCM/T3), *Clostridium perfringens* GCM/N62/14 and *Streptococcus faecalis* s.s. *zymogenes* EBF 30/27. *S. typhimurium*, a nalidixic-resistant mutant obtained from Dr H. Williams Smith, Houghton Poultry Research Station, was also used in some of the experiments.

In vitro *tests*

Most of the anaerobes were tested by taking a 24 h culture in SM10 broth and making a primary streak across two VLhlf agar and two BGPhlf agar plates which were incubated in an anerobic jar under hydrogen + 10% carbon dioxide for 3 and 7 days. The plates were then cross-streaked using five test organisms per plate. The aerobes used were 24 h cultures in heart infusion (Difco) broth whilst *Cl. perfringens* was grown in Robertson's cooked-meat medium. After anaerobic incubation for a further day any inhibition of the test organisms was recorded. Those anaerobes which could only be grown using the Hungate technique were tested on SM10 agar in a stoppered flat bottle. After incubation for 7 days the test organisms were streaked at right angles to the main streak and results recorded as previously.

Anti-salmonella activity in vivo

Those organisms showing anti-salmonella activity *in vitro* were tested for *in vivo* activity with the collaboration of Dr H. Williams Smith, Houghton Poultry Research Station, Houghton, Huntingdon. Chickens were taken immediately after hatching and divided into three groups of initially 25 per group. One group was inoculated via the crop with 0·3 ml of uninoculated VLhlf broth. The other two groups were given either a 24 h VLhlf broth culture of *Bacteroides hypermegas* EBF 61/42 (total organisms $4{\cdot}3 \times 10^7$) or of *Bifidobacterium* sp. EBF 77/12B (total organisms $1{\cdot}0 \times 10^8$). The chicks were fed on a normal starter ration. On the following day they were infected via the crop with about 10^7 *Salmonella*

typhimurium (strain resistant to nalidixic acid). On the 8th day after hatching the chicks were killed and one caecum from each chick was sampled for salmonellas by streaking directly on to the Brilliant Green agar (see media). The other caecum was used to form a bulk sample with that from four other chicks in the same group. This was analysed under anaerobic conditions, dilutions prepared in ADS by the Hungate technique being used to determine the number of *B. hypermegas* by plating on ethyl violet azide agar whilst *Bifidobacterium* sp. were counted on China blue agar. The same dilutions were used to count the number of salmonellas and *coli-aerogenes* bacteria.

Effect of inhibitors on the anaerobes

The inhibitors listed below were incorporated in BGPhlf agar and the multipoint inoculation technique previously described by Barnes & Impey (1972) was used. With those anaerobes which could not be grown on agar plates the tests were carried out in SM10 broth (using the Hungate technique), the compound being dissolved or suspended in oxygen-free distilled water at 1000 μg/ml. It was further diluted in SM10 broth to give the required concentration. These broths were then inoculated with one drop (1/30 ml) of a 1- or 2-day culture of the test organism and incubated for 2 days. The following compounds were tested:-

Flavomycin (Hoechst UK Ltd.) 1, 10 and 100 μg/ml
Furazolidone (Furazon, Smith Klein & French Ltd.) 100 μg/ml
Nitrovin (Payzone, Cyanamid of GB Ltd.) 1, 10 and 100 μg/ml
Sulphaquinoxaline (May & Baker Ltd.) 5, 50 and 500 μg/ml
Tetracycline hydrochloride (Sigma) 1, 10 and 100 μg/ml
Tylosin (Tylan, Elanco Products Ltd.) 1, 10 and 100 μg/ml
Virginiamycin (2 UK units/μg, Smith Klein & French Labs Ltd.) 1, 10 and 100 μg/ml
Zinc bacitracin (53,000 units/g, Sigma) 1, 10 and 100 μg/ml

All of the antimicrobial compounds were dissolved in aqueous solution except for furazolidone, sulphaquinoxaline and nitrovin, which were suspended in a small amount of dimethyl formamide (DMF) and then further diluted in water. Control plates showed that the concentration of DMF used was not inhibitory.

In vitro *anti-salmonella activity of the volatile fatty acids*

The anti-samonella activity of the volatile fatty acids was shown by Meynell (1963) to be greater under anaerobic conditions, therefore VL broth which has a low redox potential was used for most of the tests, the medium being held in a boiling water bath for 20 min before use to expel any dissolved oxygen. For the qualitative tests the VL broth was used in 19 ml lots in 1 oz screw-capped bottles, the pH value ranging from 4·7–6·5 with or without the required amounts of volatile fatty acids. They were inoculated with 1 drop of an overnight culture of the test organism (*S. typhimurium* 3698/60, *S. haardt* or *S. agona*) grown in heart infusion (Difco) broth and growth recorded after 5 and 24 h.

For the quantitative estimations the VL broths were prepared in 100 ml lots in 250 ml screw-capped bottles having the required pH value and concentration

of volatile fatty acids. The broths were inoculated with *S. typhimurium* 3698/60 and counts made using heart infusion (Difco) agar at 0, 6, 24 and 48 hrs.

RESULTS

Anti-salmonella activity of the caecal anaerobes

In vitro *tests*. The anaerobes shown in Table 1 were tested for any antibacterial activity by the cross-streak technique using VLhlf agar or BGPhlf agar (see Methods). Initially the anaerobes were tested against *Salmonella typhimurium* 3698/60, *Salmonella haardt*, *Escherichia coli* EBF 85/3, *Streptococcus faecalis* subsp. *zymogenes* EBF 30/27 and *Clostridium perfringens* N62/14. Where anti-salmonella activity was detected additional tests were carried out using six further strains of salmonellas (see Methods) and three more strains of *E. coli*.

Only two of the anaerobes tested, *Bacteroides hypermegas* and *Bifidobacterium* sp., showed any anti-salmonella activity and this was only detected on the unbuffered high glucose medium, VLhlf agar, the zones of inhibition being much larger after the anaerobes had been grown for 7 rather than 3 days. No activity was detected on the buffered low glucose medium (BGPhlf) agar. All of the *E. coli* strains were also inhibited but not *Strep. faecalis* s.s. *zymogenes* or *C. perfringens*.

Bacteroides hypermegas when grown in VL broth can produce as much as 30 μmol/ml propionic acid and 15 μmol/ml acetic acid, the terminal pH value being 4·5–4·6, whilst the *Bifidobacterium* sp. isolated from chickens produces mainly acetic acid with a terminal pH value 4·4–5·0. Growth tests (see Methods) carried out in VL broth with *Salmonella typhimurium* 3698/60 over a pH range of 4·7–6·5 and varying concentrations of acetic or propionic acids or a mixture of both (Table 2) confirmed that the inhibitory effect detected on the VLhlf agar plates was probably due to the combined effect of pH and concentration of volatile fatty acids present.

In vivo *tests*. Following the work of Rantala & Nurmi (1973), *Bacteroides hypermegas* EBF61/42 and *Bifidobacterium* sp. EBF77/12B were tested to see whether they had an *in vivo* activity in the young chick. The experiments were carried out with the collaboration of Dr H. Williams Smith (Houghton Poultry Research Station). Three groups of newly hatched chicks were compared (see Methods), one group being inoculated a few hours after hatching with VLhlf broth whilst the other two groups were inoculated with VLhlf broth cultures of *B. hypermegas* or *Bifidobacterium* sp. before being inoculated with a nalidixic acid resistant strain of *S. typhimurium* the following day. The results summarized in Table 3 show that, although both the test organisms established themselves in the caeca, no salmonella inhibition was observed, and when the chicks were killed at 8 days salmonellas were recovered from all the caeca in high numbers.

Effect of age on pH and volatile-fatty-acid production in the caecum

The changes in pH and volatile-fatty-acid (VFA) production occurring in the caeca of chicks during the first 21 days after hatching are shown in Fig. 1 and comprise the results obtained from three separate experiments carried out over

Table 1. *Reference strains of chicken caecal anaerobes used for testing anti-salmonella activity and sensitivity to feed additives*

	Glucose broth		Tested for anti salmonella activity	Tested against anti bacterial compounds
	Fermentation products*	Terminal pH		
Bacteroides vulgatus	APS tr i-b	4·7	× (8)†	×
B. hypermegas	APs	4·5–4·6	× (3)	× (3)
Bacteroides sp. EBF77/26A	ASpl	5·0	×	.
Bacteroides sp. EB77/78 (C)‡	AEl	5·2	× (2)	.
Bacteroides sp. Group 1 EB59/96 (A & C)	AE	5·2	×	.
Bacteroides sp. NE1/8 (C)	AE	5·2	×	.
Bacteroides sp. NE1/26	Eal	5·5	×	.
Bacteroides sp. NE3/254	AE	5·2	×	.
Fusobacterium plauti NE3/244 (C)	a, n-b, l	6·4	×	×
Fusobacterium sp. NE1/74	n-B, L	6·0	×	×
Fusobacterium sp. NE3/49	n-b	6·3	.	× (2)
Fusobacterium sp. NE3/82	Ln-b, p	5·2	.	×
Streptococcus intermedius	AL	4·6	×	.
S. pleomorphus (D)	La, tr n-b	4·6	×	.
Streptococcus sp. EBF77/14B (C)	La, tr e	4·8	×	.
Coprococcus sp. NE1/97 (E)	Bapls	5·0	×	×
Peptostreptococcus Group 2 NE3/225 (B)	Ale	4·8–5·0	×	.
Peptostreptococcus sp. NE3/239 (C)	LSa	4·6–5·1	× (2)	.
Peptostreptococcus sp. NE1/71 (C)	AE	5·2	×	.
Peptostreptococcus sp. NE3/250 (C)	Ale	5·6	×	.
Cocci or coccobacilli in chains NE1/51A	a	6·6	×	.
Bifidobacterium spp.	Ale	4·4–5·0	× (7)	× (5)
Eubacterium sp. NE3/191 (C)	LSa, tr e	4·7	×	.
Eubacterium sp. NE3/197 (C)	Ea, tr l	5·2	×	.
Eubacterium sp. NE3/198 (C)	Sa, tr p	4·8	×	.
Eubacterium sp. EBF 77/35	Ea, trl	4·6–5·0	× (2)	.
Eubacterium sp. NE3/230B	AS	5·0–5·2	× (3)	.
Gram-positive curved rod NE1/22	AEl	4·7	×	.
Gemmiger formicilis NE3/247 (F)	n-B al	5·0	×	×
'Budding' bacteria NE3/265	n-b al	6·6	.	×
'Budding' bacteria NE3/209	Als	4·8–5·0	×	×
'Budding' bacteria NE3/235	AL	5·0	×	.
'Budding' bacteria NE3/217	AEl	5·6	×	×
Clostridium symbiosum	Ln-b a	5·0	× (2)	×
C. malenominatum (C)	n-b	6·6	×	×

* Products: A, acetic; P, propionic; n-B, *n*-butyric; i-B, isobutyric; E, ethanol; L, lactic; S, succinic acids. Capitals, > 10 μmol/ml; lower case, < 10 μmol/ml. tr, trace.

† Number of strains tested in parentheses.

‡ (): A, Barnes & Impey (1968); B, Barnes & Impey (1970); C, Barnes & Impey (1974); D, Barnes *et al.* (1977); E, Holdeman & Moore (1974); F, Gossling & Moore (1975).

a 6-month period. It can be seen that in the newly hatched chick before feeding, the VFA's were very low whilst the pH value was high (above 6·5). During the first 7 days there was a rapid increase in the VFA's whilst the pH value fell so that by 7 days there was an average pH value of 5·7 and the VFA's were 70 μmol/g (wet weight). After this the pH tended to rise so that it was above 6·0 at 21 days

Table 2. *Effect of acetic and propionic acids on the growth of* S. typhimurium 3698/60 *in VL broth at pH values* 4.7–6.5

VFA's (μ mol/ml)	Incubation time (h)	Growth in VL broth* 4·7	5·1	5·6	6·0	6·2	6·5
0	5	±	+	+ +	+ +	+ +	+ + +
	24	+ +	+ +	+ + +	+ + +	+ + +	+ + +
Acetic acid 15	5	–	–	–	–	–	+
	24	–	–	+	+ + +	+ + +	+ + +
Acetic acid 30	5	–	–	–	–	–	–
	24	–	–	+	+ +	+ +	+ + +
Propionic acid 15	5	–	–	–	–	n.t.	±
	24	–	–	+ +	+ +	n.t.	+ + +
Propionic acid 30	5	–	–	–	–	n.t.	–
	24	–	–	–	+ +	n.t.	+ + +
Acetic acid 15, propionic acid 30†	5	–	–	–	n.t.	–	–
	24	–	–	–	n.t.	+ + +	+ + +

* Growth: ±, faintly turbid; +, just turbid; + + +, very turbid; n.t., not tested.

† *Salmonella haardt* and *Salmonella agona* were slightly more resistant showing weak growth at pH 6·5 in 5 h.

whilst acetic, propionic and butyric acids continued increasing more slowly, the greatest increase occurring with the *n*-butyric acid. Lactic acid was found at the end of the first week at about 8 μmol/g but it was not found, i.e. was < 5 μmol/g, from 2 weeks onwards. Succinic acid was present at 20–33 μmol/g at 7 days after which the level declined and from 3–7 weeks averaged about 7 μmol/g.

In order to determine whether or not salmonellas would be able to multiply under these conditions, and thus become established in the caeca, tests were carried out in VL broth adjusted to various pH values between pH 5·5 and 6·5 containing the average concentrations of VFA's in the caeca at 3, 7 and 14 days. Further tests were made in the average concentration found between 21 and 49 days. Details of the proportions of acetic, propionic and *n*-butyric acids are shown in Table 4. The numbers of *Salmonella typhimurium* 3698/60 present were determined after incubation at 37 °C for 6, 24 and 48 h and compared with that obtained in the control broth at the same pH but without VFA's. The results are shown in Table 4. It can be seen that the estimated 3 day concentration of about 41 μmol/ml of volatile fatty acids would have been sufficient to prevent salmonella multiplication provided that the pH value was as low as 5·5. At higher pH values salmonella growth was delayed during the first 6 h but reached that of the control in 24 h when the pH value was 6·2 or higher. The same trend was observed with the higher concentrations of volatile fatty acids found in the older chicks. If the pH was in the region of 5·5, there was complete inhibition of growth for 48 h but no real evidence of bactericidal activity. If the pH was above 6·0 then inhibition occurred during the first 6 h.

Table 3. *The* in vivo *anti-salmonella activity* (log_{10} *orgs/g caecal material*) *of* B. hypermegas *and* Bifidobacterium sp. *inoculated into chicks at* 0 *days*

(At 1 day challenged with 10^7 *S. typhimurium*. Chicks killed at 8 days and caeca analysed.)

	Treatment of chicks		
	Control, VLhlf broth	*B. hypermegas* culture in VLhlf broth	*Bifidobacterium sp.* culture in VLhlf broth
S. typhimurium	7·75*, 7·58, 6·23, 8·61 } 7·54	6·62, 7·30, 7·78, 7·43 } 7·28	6·49, 7·88, 6·73, 6·58 } 6·92
Coli-aerogenes bacteria	9·49, 9·57, 8·59, 8·91 } 9·14	8·57, 9·26, 8·97, 8·38 } 8·79	8·62, 9·36, 8·91, 8·49 } 8·84
B. hypermegas	Not tested	8·18, 7·48, 7·53, 7·23 } 7·60	Not tested
Bifidobacterium sp.	Not tested	Not tested	8·79, 9·58, 9·57, 8·82 } 9·19

N.B. One caecum from each bird was also tested individually and all found positive for salmonellas.

* Each count is a bulk sample from 5 birds.

The in vitro *sensitivity of some of the caecal anaerobes to certain antimicrobial compounds*

Antimicrobial compounds used either by Rantala (1974), Nurmi & Rantala (1974) or Smith & Tucker (1975) were tested for their activity against those anaerobes indicated in Table 1 known to produce significant quantities of propionic or butyric acids or high concentrations of acetic acid. All of the organisms tested, except the very strict anaerobes, were grown on BGPhlf agar plates generally containing 1, 10 or 100 μg/ml of the inhibitor (see Methods). The results given in Table 5 indicate that a high proportion of the organisms were sensitive to the additives at 100 μg/ml. All of the strains grew in the presence of 500 μg/ml sulphaquinoxaline whilst all the strains were inhibited by 100 μg/ml furazolidone.

Effect of incorporating nitrovin at 10 *and* 100 *mg/kg in the feed*

In order to determine the effect of nitrovin as a feed additive on the caecal flora, the pH and concentrations of VFA's and other acids in the caeca of chicks, two separate experiments were carried out using 10 and 100 mg/kg nitrovin (for details see methods).

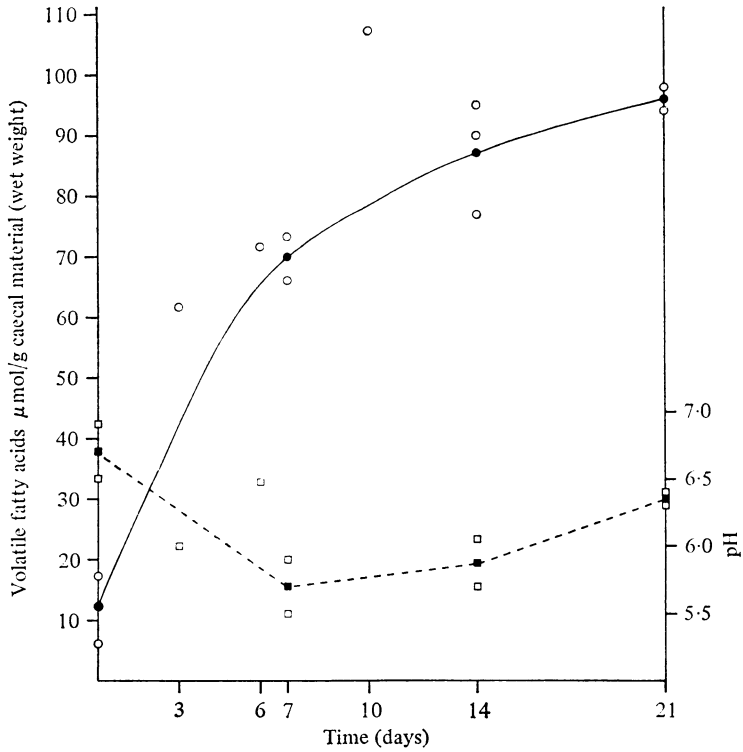

Fig. 1. The pH value and volatile fatty acid (VFA) concentration in the caeca of chicks, 0–21 days. Data from three experiments. ⊙, VFA concentration; ●—●, average VFA's; ⊡, pH value; ■--■, average pH.

Table 4. *The effect of volatile fatty acid (VFA) concentration and pH on the multiplication of* S. typhimurium *in VL broth*

Age of bird (days)	VFA's* (μmol/ml)	pH	Log_{10}/ml *S. typhimurium* 0 h	6 h	24 h	48 h
3	0	5·5, 6·5†	4·45	7·64	8·32	7·42
	41	6·5	4·45	5·22	8·42	8·39
	(A35, P1, n-B5)	6·2	4·45	5·62	8·32	8·19
		5·9	4·45	4·75	7·87	8·30
		5·7	4·45	4·40	6·38	8·10
		5·5	4·45	4·36	4·28	4·18
7	0	5·65, 6·4	4·34	7·20	8·08	6·79
	70	6·4	4·34	5·04	8·20	8·15
	(A55, P4, n-B11)	5·65	4·34	3·99	3·99	3·67
14	0	5·5, 6·45	4·34	7·58	8·15	7·53
	86	6·45	4·34	6·08	8·49	8·36
	(A61, P5, n-B20)	5·5	4·34	4·26	4·15	4·00
21–49 (average)	0	6·0, 6·5	4·43	7·90	8·48	8·28
	101	6·5	4·43	4·91	8·15	8·56
	(A75, P6, n-B20)	6·0	4·43	4·43	6·83	8·30

* Concentrations of VFAs similar to those found in the caeca of chicks at the age given in table. A, Acetic; P, propionic; n-B, *n*-butyric acid.

† Control samples at the two pH values were not significantly different.

Table 5. *The* in vitro *sensitivity of the chicken caecal anaerobes to certain feed additives*

Anaerobe	No. of strains tested	Resistance (R) or sensitivity (S) to conc. (μg/ml)																	
		Nitrovin			Flavomycin			Tylosin			Bacitracin			Virginiamycin			Tetracycline		
		1	10	100	1	10	100	1	10	100	1	10	100	1	10	100	1	10	100
B. vulgatus	1	R	R	S	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
B. hypermegas	3	R	R	R (2)* S (1)	R	R	R	R	R	R	R	R	S (2) R (1)	R	R	R	R	S	S
F. plauti	1	S	S	S	S	S	S	R	S	S	R	R	R	R	R	S	R	R	S
F. sp. NE1/74	1	R	R	S	R	R	R	S	S	S	S	S	S	S	S	S	R	R	S
F. sp. NE3/82	1	R	S	S	R	R	S	R	R	S	R	R	S	R	S	S	R	R	R
F. sp. NE3/49	2	S	S	S	R	R	R	R	R (1) S (1)	S	S	S	S	R	S	S	S	S	S
Coprococcus sp. NE1/97	1	R	R	R	R	S	S	R	S	S	R	S	S	R	S	S	R	R	S
Bifidobacterium sp.	5	R	R	S	R	S (3) R (2)	S	S (3) R (2)	S (3) R (2)	S (4) R (1)	S	S	S	S (3) R (2)	S	S	R	S (4) R (1)	S (4) R (1)
Cl. symbiosum	2	R	R	S	R	R	R	R	R	S	S	S	S	R	R	S	R	R	S
Cl. malenominatum	1	R	R	S	R	R	R	R	S	S	R	S	S	R	S	S	R	R	S
Tested in broth (Hungate technique)																			
Gemmiger formicilis	1	R	S	S	S	S	S	S	S	S	S	S	S		n.t.		R	R	S
'Budding' bacteria NE3/265	1	R	S	S	S	S	S	S	S	S	S	S	S		n.t.		R	S	S
'Budding' bacteria NE3/209	1	R	R	R	R	R	R	R	S	S	R	R	R		n.t.		R	S	S
'Budding' bacteria NE3/217	1	R	S	S	R	R	S	R	S	S	R	S	S		n.t.		R	S	S

All of the strains grew in the presence of 500μg/ml sulphaquinoxaline. All the strains were inhibited by 100μg/ml furazolidone.
n.t., Not tested.
* () Nos. of strains giving the reaction indicated.

Table 6. *Volatile fatty acids, lactic and succinic acids and pH in the caeca of chicks* 0–49 *days, fed on the control diet and diet* + 10 *mg/kg nitrovin*

Diet	Age (days)	pH	μmol/g caecal material (wet weight)						
			Volatile fatty acids					Other acids	
			Acetic	Propionic	Butyric	Valeric*	Total VFAs	Lactic	Succinic
Control	7	5·9	52	7·4	7·0	trace	66	n.t.	20
Nitrovin		5·5	43	1·4	3·9		48	14	16
Control	14	5·7	55	3·1	18	1·1	77	<5	12
Nitrovin		5·4	65	2·7	12	0·3	80	<5	17
Control	Average	6·44	74·2	4·7	19·6	2·0	100·5	<5	8·6
	21–49	(6·0–6·8)†	(63–82)	(3·6–5·4)	(14–24)	(1–2·5)	(94·1–110·5)		(2–19)
Nitrovin		5·74	75·4	4·5	20·6	1·7	102·2	<5	12·0
		(5·4–6·4)	(71–84)	(3·4–6·2)	(15–26)	(1·0–2·1)	(93–118·2)		(6–18)

n.t. Not tested.
* Valeric acid includes iso and *n*–valeric acids.
† ( )Indicates the range of results obtained.

The effect of 10 *mg/kg nitrovin in the feed*

Two groups of chicks were reared from 0–49 days on (1) a normal starter ration (without feed additives) and (2) the same diet containing 10 mg/kg nitrovin. Samples were taken at 7, 14, 21, 28, 31, 42 and 49 days to determine pH and the concentration of VFA's and other acids present. The results at 7 and 14 days are shown in Table 6 together with the average results for 21, 28, 31 and 49 days. The pH was lower in the nitrovin fed chicks throughout the experiment. Except for the 7-day chicks when the VFA's were lower, little difference was found between the two groups of birds.

A detailed analysis of the microbial flora was made at 31 days. No differences were found between the control and nitrovin fed birds in the numbers of lactobacilli ($1{\cdot}1 \times 10^9$/g and $1{\cdot}2 \times 10^9$/g) or the *coli–aerogenes* bacteria ($9{\cdot}1 \times 10^7$/g and $7{\cdot}5 \times 10^7$/g) whilst with the faecal streptococci the only observed difference was a failure to isolate *Strep. faecalis* from the nitrovin-fed birds. The anaerobic flora was analysed by the Hungate technique (see Methods), ten replicate roll tubes being used at each dilution. The viable counts were similar in both groups ($2{\cdot}9 \times 10^{10}$/g and $2{\cdot}0 \times 10^{10}$/g). All of the colonies from the highest dilution showing growth were tested. In all, 83 colonies were examined from the control birds and 69 from the treated birds, the tests including microscopical examination, Gram reaction and analysis of fermentation products (Barnes & Impey, 1974). Apart from a higher proportion of Gram-positive rods in chains and filaments in the nitrovin-treated birds, no major differences were found. Typical Gram-negative non-sporing anaerobes were present in both groups together with many of the other types listed in Table 1.

The effect of 100 *mg/kg nitrovin in the feed*

The above experiment was repeated with chicks reared on diets containing 100 mg/kg nitrovin. The pH and analysis of VFA's of chicks from 0–84 days is

Table 7. *The pH, volatile fatty acids and other acids in the caeca of chicks* 0–84 *days fed on the control diet and diet* +100 *mg/kg nitrovin*

			Volatile fatty acids μmol/g caecal material, (wet weight)					Other acids	
Diet	Age (days)	pH	Acetic	Propionic	Butyric	Valeric*	Total VFAs†	Lactic	Succinic
Control	0	6·5	16	0·2	1·0		17·2	n.t.	n.t.
Control	7	5·5	58	1·1	14	0·4	73	4	33
Nitrovin		5·7	43	3·0	7	0·5	53	2	16
Control	14	6·1	65	7·6	16	1·4	90	<5	14
Nitrovin		5·4	63	3·5	12	1·0	80	<5	8
Control	21	6·3	71	6·4	19	2·0	98	<3	8
Nitrovin		5·3	69	2·9	12	1·0	85	<3	21
Control	28	6·4	70	8·3	20	2·1	101	<3	6
Nitrovin		5·3	73	3·7	13	1·4	91	<4	33
Control	35	6·1	93	9·6	22	1·9	126	<4	7
Nitrovin		5·5	82	4·8	18	1·5	106	<3	14
Control	49	6·1	66	8·0	21	1·4	95	<3	8
Nitrovin		5·5	72	4·3	17	1·4	94	<4	18
Control	63	6·7	65	6·9	16	0·5	90	n.t.	n.t.
Nitrovin		5·6	60	4·0	16	0·2	82	n.t.	n.t.
Control	84	6·0	102	9·7	32	3·6	147	n.t.	n.t.
Nitrovin		5·9	86	7·3	20	3·1	116	n.t.	n.t.
	Average								
Control	21–49	6·2	75	8·2	20·5	1·9	105·6	<5	7·2
Nitrovin		5·4	74	3·9	15	1·4	94·3	<5	21·5

n.t. Not tested.

* Valeric acid includes iso and *n*-valeric acids.

† Formic acid was not estimated–but was unlikely to exceed 6 % of the total VFAs (see Methods).

shown in Table 7. It can be seen that, as previously, the concentration of VFA's after 7 days was lower in the nitrovin-fed chicks, 53 μmol/ml and a pH of 5·7, as compared with 73 μmol/ml and a pH of 5·5 in the control chicks. From 14 days onwards the concentration of VFA's (particularly propionic and butyric acids) remained slightly lower in the nitrovin fed chicks but there was also a much lower pH value (average 5·4). The average concentration of VFA's (without valeric) in the nitrovin-fed birds from 21–49 days (Table 7) was tested for the ability to inhibit salmonellas by growing *S. typhimurium* in heart infusion (Difco) broth adjusted to pH 5·4 with and without the addition of the 20·5 μmol/ml succinic acid. Salmonella multiplication was completely inhibited for 48 hr but the organisms were not killed. The addition of the succinic acid had no effect.

Aerobic flora. Analyses were carried out on bulk caecal samples at 7, 14, 21, 28, 35, 49 and 84 days for lactobacilli, enterobacteria and faecal streptococci. The results given in Fig. 2 indicate that the numbers of lactobacilli tended to be slightly higher in the control birds. Both *Strep. faecalis* and *Strep. faecium* types

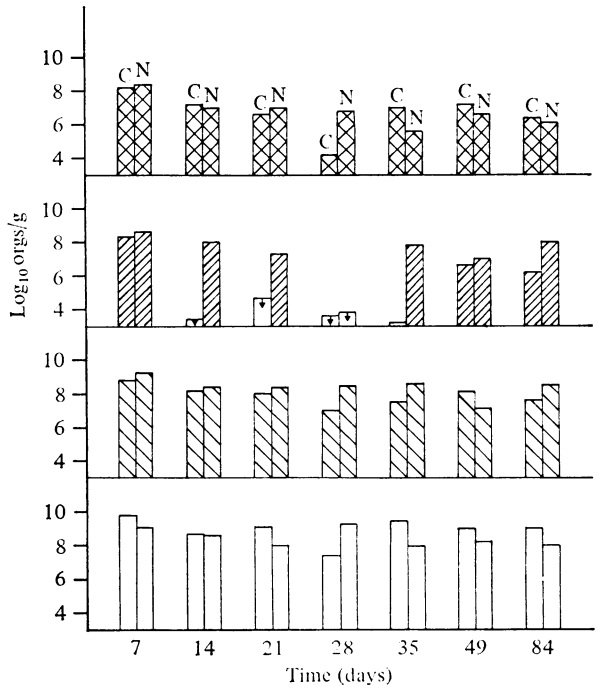

Fig. 2. The effect of 100 mg/kg nitrovin on the aerobic caecal flora of chicks, 7–84 days. C, Control; N, nitrovin 100 mg/kg. ▩, Faecal streptococci; □, Lactobacilli; ▨, Enterobacteria (lactose negative); ▧, *Coli-aerogenes* bacteria; ↓, not found at level indicated.

were isolated from the control birds but *Strep. faecalis* occurred less frequently in the nitrovin-fed birds. Although there was not a great deal of difference in number of the *coli-aerogenes* bacteria in the two groups, lactose negative strains, probably *Proteus* spp., occurred much more frequently in the nitrovin-fed birds.

Anaerobic flora. Caecal samples were analysed at 14, 35, 49 and 84 days. Direct microscopical counts varied between $1{\cdot}2 \times 10^{11}$ and $3{\cdot}3 \times 10^{11}$/g (wet weight) in the control birds and $9{\cdot}0 \times 10^{10}$/g and $6{\cdot}0 \times 10^{11}$/g in the nitrovin-fed birds. The predominant anaerobic flora was determined by making tenfold dilutions of the samples in SM10 broth (see Methods) and analysing the two or three highest dilutions showing any growth. In some cases dilutions were also made in lactate broths and the results were similar.

The results given in Table 8 indicate that the main difference was the presence of Gram-negative non-sporing anaerobes which were identified as *Bacteroides vulgatus* in all of the control samples whilst they were completely absent from the highest dilutions in the nitrovin-fed birds. A tiny Gram-variable rod was also found in the control samples at 2, 5 and 7 weeks but not in the nitrovin fed birds. The predominant organisms in the nitrovin-fed birds were clostridia, consisting of several types normally found in chicken caeca (differentiated morphologically and by their fermentation products), peptostreptococci and several unidentified *Eubacterium* spp. Direct microscopical examination of the caecal material con-

Table 8. *The predominant anaerobic flora in the caeca of chicks fed on a control diet and a diet containing* 100 *mg/kg nitrovin*

		Control diet		Diet + nitrovin 100 mg/kg	
Age (days)	Highest dilutions showing growth	Gram-negative non-sporing anaerobes (Bacteroidaceae)	Other organisms present	Gram-negative non-sporing anaerobes (Bacteroidaceae)	Other organisms present
14	10^{-9}	+	Clostridia, tiny Gram ± rods	−	Clostridia, Other Gram +ve rods*, Gram +ve coccobacilli
	10^{-10}	+	Gram +ve coccobacilli	No growth	
35	10^{-9}	+	Cocci in chains, Gram +ve filaments	−	Clostridia, cocci in chains, Gram +ve rods
	10^{-10}	+	Tiny Gram ± rods	−	Peptostreptococci, Gram +ve filaments
	10^{-11}	+	−	No growth	
49	10^{-10}	+	Clostridia, tiny Gram ± rods	−	Gram +ve rods (several types)
	10^{-11}	+	Cocci in chains	No growth	
84	10^{-10}	+	Lactobacilli, Gram +ve coccobacilli	Not tested	
	10^{-11}	No growth		−	Clostridia, Gram +ve rods, cocci in chains
	10^{-12}	No growth		−	Clostridia, Gram +ve rods

* Gram-positive non-sporing anaerobic rods (*Eubacterium* spp.).

firmed the absence of Gram-negative rods from the nitrovin-fed birds. The general impression was that there were fewer types of organisms in these samples as compared with the control birds where many of the types found in the nitrovin treated birds were also present but in proportionately fewer numbers. Attempts were made to determine the number of bifidobacteria by isolating on China Blue agar (see Methods). Recognition is difficult because several other types of organisms are also isolated on this medium. Evidence indicated that the bifidobacteria were possibly 20–100 fold fewer in the nitrovin-fed birds.

The absence of lactic acid from the caecal material in both groups of chickens after about 14 days was surprising considering the high numbers of lactobacilli present. A number of the anaerobes, including some of the clostridia isolated were shown to utilize lactic acid as an energy source when tested by the methods of Barnes & Impey (1974). In particular, all the *Bacteroides vulgatus* strains were found to utilize lactic acid with the production of propionic acid when grown in a lactate medium supplemented with chicken faecal extract. This was contrary to

the findings of Cato & Johnson (1976) for this species but tests with the type strain *B. vulgatus* ATCC 8482 confirmed that lactate was utilized when it was grown under comparable conditions. The *B. vulgatus* strains isolated from the highest dilutions of the control samples were tested to determine whether they had any anti-salmonella activity (see Methods). In addition to tests being made on VLhlf agar and BGPhlf agar, tests were also carried out on a lactate agar, the lactate replacing the glucose in VLhlf agar. No anti-salmonella activity was detected with any of the strains tested. The possible relevance of these findings in the ecology of the caecum is being further studied.

DISCUSSION

The results in this paper go some way towards explaining the behaviour of the food poisoning salmonellas in the caeca of the young chick. The fact that it is much easier to infect a chick during the first few days of life may be due to the low concentrations of volatile fatty acids (VFA's) present which are insufficient to prevent salmonella multiplication. The high pH during the first few days after hatching renders the VFA's even less inhibitory. The concentrations of the VFA's will vary considerably according to the numbers and types of anaerobes present which the chick must acquire from the general environment. A further source of acetic acid in the caeca could be from the decomposition of uric acid as Mead & Adams (1975) showed that organisms, including faecal streptococci, which were capable of utilizing uric acid were present in high numbers immediately after hatching.

The failure of *in vivo* experiments with *Bacteroides hypermegas* or *Bifidobacterium* sp. demonstrated that it was insufficient to try and establish a single VFA-producing anaerobe without at the same time establishing those organisms which will contribute towards lowering the pH of the caecum. The role of the lactobacilli which are not usually found in large numbers in the caeca until about the 2nd or 3rd day (Mead & Adams, 1975) remains to be investigated. Thus when Rantala & Nurmi (1973) prevent infection by *Salmonella infantis* by administering an anaerobic VL culture of organisms from the caeca of the adult bird they are probably speeding up the establishment of a mixture of organisms which will both lower the pH and produce the VFA's.

For the first 21 days after hatching there was a steady increase in the concentration of acetic, propionic and butyric acids in the caeca which corresponds with the gradual establishment of the caecal flora (Barnes *et al.* 1972). From 21 days onwards the concentrations and pH fluctuated (cf. controls in Table 7) but the average over 21–49 days determined in two groups of chicks reared at different times was remarkably similar (cf. the control groups in Table 6 and 7). In chicks after the first 7 days the problem of the salmonella carrier rate appears to be one of the gradual elimination of the salmonellas which are now in a less favourable environment. In an optimal growth medium, it was shown that in the presence of the concentrations of VFA's found from 7 days onwards (Table 4) if the pH was about 5·6 salmonella multiplication would be inhibited, whilst between

pH 6·0 and 6·5 it would be considerably delayed. However, none of the mixtures tested were actually bactericidal for the organisms.

Whilst some of the effects of the feed additives can be explained others cannot. The elimination of the Gram-negative anaerobes as a major component of the flora when 100 mg/kg nitrovin was incorporated in the diet did not lead to a major change in the VFA concentration (Table 7) and the pH was lower in the nitrovin fed chicks. The importance of the Gram-negative anaerobes in relation to the elimination of the salmonellas may therefore lie in additional properties such as the production of unidentified anti-microbial compounds or competition for limiting energy sources. The significance of lactic acid shown to be utilized by *Bacteroides vulgatus* as well as other species including *B. hypermegas* has still to be determined. In the above experiment the method of analysis was such that only the Gram-negative anaerobes such as *B. vulgatus*, which outnumbered all other organisms, were isolated but previous studies have shown the enormous diversity of types in the caeca at numbers $> 10^9$/g. Direct microscopical examination indicated that the Gram-negative anaerobes were not present in the chicks reared on the diets containing 100 mg/kg nitrovin and possibly all of the species are needed in the control of the salmonellas.

The lactose-utilizing *coli-aerogenes* bacteria are as susceptible to the VFA's *in vitro* as the salmonellas although they survive and form part of the normal flora of the caeca (Fig. 2). It was shown by Bohnhoff *et al.* (1964*b*) that when the Gram-negative anaerobes in mice were destroyed by streptomycin the VFA's were reduced to a low level and the numbers of coliforms increased considerably together with the salmonellas. The actual numbers of *coli-aerogenes* bacteria may be controlled by the VFA's in the caeca of chicks but it is possible that they can survive and multiply more readily than the salmonellas as they can utilize other energy sources, particularly any lactose present which is not utilized by the chick. In this respect it was interesting to note the higher numbers of *Proteus* spp. in the nitrovin-fed birds. These organisms like the salmonellas cannot utilize lactose.

Although in this complex environment the factors controlling the salmonellas are not understood, evidence suggests that if an antibacterial compound is given to a chick in sufficient concentration to destroy the major components of the normal anaerobic flora then the chick will be particularly susceptible to re-infection by salmonellas. In this respect the failure of all the anaerobes tested *in vitro* to grow in the presence of 100 μg/ml furazolidone may explain the problems encountered by Rantala & Nurmi (1974) with this compound. The use of nitrovin at 10 mg/kg both *in vitro* and *in vivo* had far less effect on the caecal flora than 100 mg/kg nitrovin and is in agreement with the findings of Smith & Tucker (1975) concerning differences in the salmonella carrier rate when the feed contained 10 mg/kg rather than 100 mg/kg nitrovin.

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