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Competitive exclusion of salmonellas from the chick caecum using a defined mixture of bacterial isolates from the caecal microflora of an adult bird

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SUMMARY

Colonization of the caeca of newly hatched chicks by *Salmonella typhimurium* was prevented by oral administration of a mixture of cultures comprising 48 different bacterial strains originating from an adult bird known to be free from salmonellas. The treatment conferred protection to the same degree as that obtained previously with a suspension of adult caecal contents or an undefined anaerobic culture from the same source and was demonstrated in four separate laboratory trials.

Examination of the caecal microflora of chicks one day after being given the protective treatment showed that the presence of high levels of lactobacilli and *Bacteroides* spp. which are not found usually at two days of age in chicks produced under commercial conditions was indicative of the successful establishment of an adult-type microflora.

Although the usual method of administering the protective organisms was to dose the chicks directly into the crop, it was also found possible to incorporate the organisms in the drinking water given to the birds at dilutions up to one in five, the maximum tested.

When chicks were given the bacterial mixture via the crop and fed on a diet containing 10 mg kg^{-1} nitrovin and 100 mg kg^{-1} monensin, the bacteroides failed to establish in the caeca and the birds were not protected against salmonella colonization. However, when the bacterial cultures were incorporated in the drinking water and the chicks given the same feed, normal protection was obtained; possible reasons for these observations are discussed.

INTRODUCTION

The greater susceptibility of young chicks to intestinal colonization by food poisoning salmonellas when compared with adult chickens (Milner & Shaffer, 1952) has been attributed to the simpler composition of the gut microflora in the young bird and the relatively slow rate at which the adult flora is acquired under commercial conditions because of the particular emphasis on hygiene and disinfection in hatching and rearing operations (Nurmi & Rantala, 1973).

As a possible means of overcoming the problem, Nurmi & Rantala (1973) and

Rantala & Nurmi (1973) fed chicks a suspension of gut contents or an anaerobic culture of intestinal material from healthy adult birds and, by establishing an adult-type microflora in the caeca, successfully protected the chicks against subsequent challenge with *Salmonella infantis*. More recently, other studies in different parts of the world have amply confirmed the efficacy of this type of treatment against salmonella colonization in both young chicks and turkey poults (Idziak & Caldwell, 1977; Lloyd, Cumming & Kent, 1977; Rigby, Pettit & Robertson, 1977; Snoeyenbos, Weinack & Smyser, 1978, 1979; Soerjadi, Lloyd & Cumming, 1978; Barnes, Impey & Cooper, 1980*a*; Rigby & Pettit, 1980; Dorn & Krabisch, 1981).

With regard to the possible commercial application of the 'protective' treatment, it is clear that the organisms used should not include any avian pathogens or organisms which could create a public health hazard if transmitted to man via the processed carcass. From these points of view, the treatment of commercial flocks with suspensions of gut contents or undefined, mixed cultures of intestinal organisms would appear to be unacceptable.

In attempting to develop a pure-culture treatment, Soerjadi *et al.* (1978) reported that chicks were successfully protected against colonization by *S. typhimurium* when given a pure culture of *Streptococcus faecalis* isolated from an adult bird, whilst Rigby *et al.* (1977) obtained temporary protection of chicks against *S. typhimurium* by using a *Clostridium* sp. However, previous experience in our laboratory (Barnes, Impey & Stevens, 1979; Barnes *et al.* 1980*a*) suggests that not only would a limited number of strains be unlikely to confer complete protection but may even disturb the ecological balance of the gut. When chicks were given pure cultures of lactobacilli alone or in various combinations with *Bacteroides vulgatus*, *Bifidobacterium* spp. and an unidentified anaerobe, no protection against *S. typhimurium* was obtained and in some cases the numbers of salmonellas in the caeca were 10- to 100-fold higher than those in control birds. Subsequently, Barnes, Impey & Cooper (1980*b*) used a mixture of 23 organisms which included representatives of the major bacterial groups present in a saline suspension of caecal contents from an adult bird shown previously to prevent colonization of chicks by *S. typhimurium*. The organisms were used in the proportions in which they had occurred in the original suspension and gave substantial protection when treated chicks were challenged with the salmonella.

Further experience with the bacterial mixture described by Barnes *et al.* (1980*b*) showed it to be less reproducible in protecting chicks than a suspension of caecal contents or an anaerobic culture derived from the suspension (Impey & Mead, unpublished). For this reason, the test mixture has been extended to include virtually all the organisms isolated previously from the original suspension studied by Barnes *et al.* (1980*b*) and from chicks given the suspension as a protective treatment: a total of 48 strains. The present paper describes an evaluation of the larger mixture of organisms in laboratory trials aimed at developing an acceptable treatment for reducing the carriage of salmonellas in commerical flocks and hence the contamination of processed poultry meat.

MATERIALS AND METHODS

Origin and composition of protective bacterial mixture

The analysis of the caecal material was described by Barnes *et al.* (1980*b*). The test organisms originated from a 45-week-old Light Sussex hen which was salmonella-negative and had been obtained from a specific pathogen-free flock kept at the Houghton Poultry Research Station. The strains were isolated either from a 1 : 10 saline suspension of caecal contents or from the caeca of 3-day-old chicks to which 0·5 ml of the suspension had been given orally immediately after hatching. The mixture of 48 strains studied here included the 23 tested by Barnes *et al.* (1980*b*) for their ability to protect chicks against salmonella colonization.

The composition of the 48-organism mixture and the source of each strain are given in Table 1. Strains of *Lactobacillus* spp. were identified as described by Mitsuoka (1969) and methods used to identify the streptococci were those described by Barnes *et al.* (1978). The strains of *Escherichia coli* and *Bacillus coagulans* were identified according to Cowan (1974). The clostridial isolates were characterized by the methods of Mead *et al.* (1979) and, where possible, identified with the scheme of Smith (1970). The non-sporing anaerobes were characterized by the methods of Barnes & Impey (1974) and strains of *Bacteroides hypermegas* identified according to Cato & Barnes (1976) whilst *B. vulgatus* was identified as described by Cato & Johnson (1976).

The anaerobic, Gram-positive, curved rod (F109/33) resembled the type NE1/22 of Barnes *et al.* (1979). The three strains of budding bacteria were similar to the type NE3/235 described by the same workers and the peptostreptococci all belonged to the group 2 of Barnes & Impey (1970). Classification of the anaerobe F109/28 as a *Streptococcus* sp. was based on cell morphology, Gram stain reaction and the presence of lactic acid as the major end-product in cultures of this organism (Barnes *et al.* 1977). The *Bifidobacterium* and *Eubacterium* spp. also were characterized on the basis of cell morphology, Gram stain reaction and the end-products of glucose metabolism but did not resemble any known species.

In relation to possible pathogenicity, the strains of *E. coli* were kindly examined by Dr B. Rowe, Central Public Health Laboratory, London. None was an enteropathogenic serotype; in addition, the strains failed to produce ST or LT enterotoxins and were not enteroinvasive. Dr Barbara M. Lund (Food Research Institute) kindly examined the strains of clostridia for toxicity by intraperitoneal inoculation of mice, with and without prior treatment of culture supernatants with trypsin. All strains gave negative results. The strains of *B. vulgatus* were confirmed by Dr A. T. Willis, Public Health Laboratory, Luton as being distinct from *B. fragilis*.

Preparation of the protective mixture

Table 1 shows the media used to grow each of the 48 strains individually and the amount of culture included in the final mixture to give the required number of organisms, in approximate proportion to their original incidence. In each case, the medium used was that giving the highest number of cells after incubation at 37 °C for 24 h.

Table 1. *Bacterial strains, culture media, component volumes and numbers of organisms included in the 'protective' mixture*

Reference no.	Organism	Source	Medium	Vol. used (ml)	No. of orgs/ml of mixture
F109/3	*Lactobacillus acidophilus*	S	MRS	10	10^7
6	*L. acidophilus*	S	MRS	10	10^6
5	*L. salivarius*	S	MRS	10	10^7
8	*L. salivarius*	S	MRS	10	10^6
9	*L. salivarius*	S	MRS	10	10^6
27	*L. salivarius*	S	MRS	10	10^7
43	*L. salivarius*	S	MRS	10	10^7
7	*L. fermentum*	S	VLhlf	20	10^6
10	*L. fermentum*	C	VLhlf	20	10^7
12	*L. fermentum*	C	VLhlf	20	10^7
13	*L. fermentum*	C	VLhlf	20	10^7
14	*Streptococcus faecalis* s.s. *liquefaciens*	S	HI	2·0	10^6
15	*Strep. faecium*	S	HI	2·0	10^5
16	*Escherichia coli* type I	S	HI	0·2	10^5
17	*E. coli* type I	S	HI	0·2	10^5
18R	*E. coli* type I	S	HI	0·2	10^5
18S	*E. coli* type I	S	HI	0·2	10^5
19	*E. coli* type I	C	HI	0·2	10^5
20R	*E. coli* type I	C	HI	0·2	10^5
20S	*E. coli* type I	C	HI	0·2	10^5
21	*E. coli* type I	C	HI	0·2	10^5
52	*Bacillus coagulans*	S	VLhlf	0·2	10^4
50	*Clostridium subterminale*	S	BGPhlf	20	10^7
63	*C. subterminale*	S	BGPhlf	20	10^6
53	*C. tertium*	S	BGPhlf	20	10^6
55	*C. cochlearium*	C	BGPhlf	1·0	10^6
60	*C. cochlearium*	C	BGPhlf	1·0	10^6
56	*Clostridium* sp.	C	BGPhlf	1·0	10^5
59	*Clostridium* sp.	C	BGPhlf	1·0	10^6
61	*Clostridium* sp.	S	BGPhlf	1·0	10^3
62	*Clostridium* sp.	S	BGPhlf	1·0	10^3
51	*Clostridium* sp.	S	VLhlf	1·0	10^6
22	*Bacteroides hypermegas*	S	VLhlf	20	10^6
36	*B. hypermegas*	C	VLhlf	20	10^6
37	*B. vulgatus*	C	BGPhlf	20	10^8
38	*B. vulgatus*	C	BGPhlf	20	10^8
2	*Eubacterium* sp.	S	SM10	10	10^5
23	*Eubacterium* sp.	S	VLhlf	20	10^7
25	*Bifidobacterium* sp.	S	SM10	10	10^6
34	*Bifidobacterium* sp.	C	SM10	10	10^6
33	Anaerobic, Gram-positive, curved rod (NEI/22)	C	SM10	10	10^5
26	Anaerobic, budding bacterium (NE3/235)	S	BGPhlf	20	10^5
32	Anaerobic, budding bacterium (NE3/235)	C	VLhlf	20	10^6
39	Anaerobic, budding bacterium (NE3/235)	C	SM10	10	10^5

Table 1 (*cont.*)

Reference no.	Organism	Source	Medium	Vol. used (ml)	No. of orgs/ml of mixture
28	Anaerobic *Streptococcus* sp.	S	BGPhlf	20	10^6
29	*Peptostreptococcus* sp. (Group 2: NE3/225)	S	VLhlf	20	10^7
30	*Peptostreptococcus* sp. (Group 2: NE3/225)	S	VLhlf	20	10^7
31	*Peptostreptococcus* sp. (Group 2: NE3/225)	C	VLhlf	20	10^7

Strain designations given by Barnes *et al.* (1979) in ( ). S, saline suspension of caecal contents from adult bird. C, caecum of chicks given 'protective' suspension (S, above). Details of media given in text.

The medium used for cultivating the lactobacilli was either MRS (de Man, Rogosa & Sharp, 1960) or the VL broth of Barnes *et al.* (1979) supplemented with (per l) haemin, 1 mg; liver extract, 50 ml and chicken faecal extract, 50 ml (VLhlf) as described by Barnes & Impey (1974).

Streptococcus faecalis, *Strep. faecium* and *E. coli* were grown in Difco Heart Infusion medium (HI).

Both the single strain of *Bacillus coagulans* and one strain of *Clostridium* sp. were grown in VLhlf whilst the remaining clostridia were cultivated in the same medium modified by the inclusion of (gl^{-1}): glucose, 1 (instead of 2·5) and Na_2HPO_4, 4 (BGPhlf)

The other anaerobes were grown in either VLhlf, BGPhlf or the SM10 medium of Barnes & Impey (1974).

Just prior to use, individual cultures were mixed in two batches, one comprising only the lactobacilli, the other the remaining organisms. Where required for use without any dilution, the two batches were combined immediately before treatment of the chicks. When dilution was required, the lactobacilli were added after dilution of the other organisms with non-chlorinated tap water. In all cases the pH value of the final mixture, determined electrometrically, was 5·6–5·7.

The protective mixture was given to the birds either directly into the crop (0·5 ml), using a hypodermic syringe fitted with a beaded needle, or as the first available liquid for drinking.

Experimental animals

The chicks were either Shaver Starbro or Ross I, supplied without 'sexing' from commercial hatcheries.

For treatment with the protective bacterial mixture, the birds were housed in brooder cages with wire mesh floors but after challenging with *S. typhimurium* (see below) they were transferred to flexible-wall isolators with wire mesh floors (Plysu Ltd, Milton Keynes), kept under a negative pressure.

Normally, the birds were fed on a standard broiler, heat pelleted, starter ration without any antimicrobial additives. In one experiment, however, the diet contained ($mg\ kg^{-1}$): nitrovin, 10 and monensin, 100. Both food and water were available *ad libitum*.

Methods of salmonella challenge and recovery

The organism used was a nalidixic acid-resistant strain of *S. typhimurium* obtained from Dr H. Williams Smith, Houghton Poultry Research Station.

A culture of the salmonella in HI, incubated at 37 °C for 24 h, was diluted in 0·1 % peptone diluent to give *ca.* 5×10^3 orgs ml. Each chick was given 0·2 ml of the appropriate dilution into the crop, using a hypodermic syringe and beaded needle. The challenge dose was checked by plating on HI medium.

At 7 and 14 days of age, the chicks were killed by dislocating the neck and the caeca removed. Samples of caecal contents were collected from each bird in 9 ml amounts of peptone diluent containing a few glass beads to aid dispersion. After preparing serial tenfold dilutions in peptone diluent, the salmonellas were enumerated by surface plating on Oxoid Brilliant Green Agar (modified) containing 20 μg ml^{-1} nalidixic acid. The plates were incubated at 37 °C at 24 h.

Microbial analysis of chick caecal contents

The media and methods of Barnes *et al.* (1979) were used except that clostridia were isolated as follows. Serial ten-fold dilutions of the sample were prepared in Oxoid Reinforced Clostridial Medium, freshly boiled and cooled to expel oxygen before use. Using appropriate dilutions, one ml amounts were used to inoculate freshly deoxygenated cooked meat medium in triplicate at each dilution. Cultures were incubated at 37 °C for seven days. Then, 2 ml of each culture were transferred to a sterile test-tube, heated in a thermostatically-controlled water bath at 70 °C for 10 min and cooled rapidly. The heated cultures were streaked on VL agar + 5 % horse blood (Barnes & Impey, 1968) and incubated at 37 °C for 3 days under hydrogen + 10 % carbon dioxide. The number of clostridia present in the original sample was calculated by reference to Most Probable Number tables.

In the case of tests for the presence of *Bacteroides hypermegas* and *B. vulgatus*, it was necessary to carry out microscopical checks and to test for the presence of catalase because neither the ethyl violet-azide agar of Barnes & Goldberg (1962) nor the kanamycin–vancomycin agar of Finegold, Miller & Posnick (1965) is entirely selective for the required organisms.

Tests were carried out for naturally-occurring salmonellas, just prior to challenging birds with the marker strain, using the method of Edel & Kampelmacher (1969).

RESULTS

The main components of the caecal microflora of different batches of newly hatched chicks were determined as soon as the birds arrived from the hatchery. Results are given in Table 2 and include data for all four experiments carried out. At this stage, before the birds had received food or water, neither lactobacilli nor non-sporing anaerobes were present and the microflora comprised mainly lactose-positive coliform bacteria, group D streptococci and lower but more variable numbers of *Clostridium* spp. Pediococci (Barnes *et al.* 1980*b*) were detected in two batches of birds and these appeared on Rogosa medium. Tests carried out for salmonellas on the following day, prior to challenging the birds with the marker strain of *S. typhimurium*, were all negative.

Three separate experiments were carried out to determine the effectiveness of

Table 2. *Caecal microflora of chicks on arrival from the hatchery*

Expt no. ...	1	2	3	4
Hatchery ...	I	II	II	II
Breed ...	Shaver Starbro	Ross I	Ross I	Ross I
Coliform bacteria				
Lactose +	9·56	9·56	9·38	9·26
Lactose −	< 7·49	< 7·77	< 7·26	7·97
Strep. faecalis	9·04	8·11	8·52	9·11
Strep. faecium	8·81	8·04	8·92	8·04
Clostridium spp.	7·74	4·04	5·51	6·74
Pediococcus spp.	< 2·49	7·08	8·59	< 2·49

Figures are $\log_{10}$ organisms/g of a bulk sample from 5–8 birds in each case.

Table 3. *Effect of 'protective' treatment on levels of* S. typhimurium *in the caeca of three different batches of chicks*

	No. of birds positive at each level of salmonella carriage											
Expt no. ...	1				2				3			
Chick group ...	Control		Treated		Control		Treated		Control		Treated	
	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d
Salmonellas/g												
10^7	8	3	1	0	4	0	0	0	7	2	0	0
10^6	0	5	0	1	2	0	0	0	2	6	0	0
10^5	2	1	0	0	1	3	0	1	1	2	1	0
10^4	0	0	0	1	0	5	0	0	0	0	0	0
10^3	0	0	0	0	2	1	0	0	0	0	0	0
10^2	0	0	0	0	0	0	0	2	0	0	0	0
Not found	0	0	9	8	1	1	10	7	0	0	9	10
Total birds sampled	10	*9	10	10	10	10	10	10	10	10	10	10

* One bird died. Infective dose of *S. typhimurium* $3{\cdot}4 \times 10^2$–$3{\cdot}8 \times 10^3$.

the protective treatment in preventing the establishment of *S. typhimurium* when birds were challenged one day later with 10^2–10^3 cells of the marker strain, the birds being dosed directly into the crop. The results obtained with birds of differing breed and origin showed that in all cases a high degree of protection was obtained by comparison with untreated controls (Table 3).

Although the large numbers of *S. typhimurium* present in untreated controls at seven days showed some decline by day 14, in most cases levels remained relatively high, whereas salmonellas were not detected on either occasion in the majority of treated chicks.

On the day after giving chicks the protective treatment but before the salmonella challenge, 20 birds were taken from each group to determine the influence on the caecal microflora of the organisms administered. Tests were made using appropriate selective media. Of the non-sporing anaerobes, only *B. hypermegas* and *B. vulgatus* could be enumerated directly (using EVA and KVA media) but supplementary checks were still necessary (see Methods).

Results for bulked samples shown in Table 4 demonstrate marked differences

Table 4. *Caecal microflora of chicks, one day after treatment with the bacterial mixture and prior to the salmonella challenge*

Expt no. ...	1		2		3	
Chick group ...	Control	Treated	Control	Treated	Control	Treated
Lactobacillus spp.	4·43	9·20	< 3·18	8·80	5·48	9·39
Coliform bacteria						
Lactose +	10·00	10·26	9·61	9·69	8·59	8·54
Lactose −	9·18	< 8·63	8·52	< 7·15	< 6·08	< 6·74
Streptococcus faecalis	10·15	9·63	8·98	8·93	8·45	9·45
Strep. faecium	9·04	9·32	8·48	7·70	< 7·08	< 6·74
Clostridium spp.	8·99	8·89	7·41	7·40	6·34	5·99
Total anaerobes* (VLhlf)	10·57	10·43	9·82	10·32	9·11	10·40
Bacteroides hypermegas	NF	9·16	NF	8·53	NF	7·78
B. vulgatus	NF	NT	NF	10·15	NF	10·64

Figures are $\log_{10}$ organisms/g of a bulk sample from 20 birds in each case. NF, not found; NT, not tested. * This includes facultative anaerobes.

between treated and control chicks in relation to the incidence of lactobacilli and the two *Bacteroides* spp. and hence such organisms appear to be useful indicators of the successful establishment of a protective microflora in the caeca.

The fourth experiment combined two objectives. One was concerned with the adaptation of the protective treatment for use as a drinking water supplement because this method of application would appear to be more appropriate for possible commercial use. In parallel, a test was carried out to determine the effect of a commercial starter ration containing 10 mg kg^{-1} nitrovin and 100 mg kg^{-1} monensin, by comparison with the experimental diet which contained no antimicrobial compounds. All birds were challenged with *ca.* $6{\cdot}0 \times 10^3$ *S. typhimurium* and results are presented in Table 5.

First, the protective effect of the bacterial mixture against salmonella colonization was confirmed for chicks dosed directly into the crop and fed on a diet without antimicrobial additives. The experiment also demonstrated successful protection when the mixture was incorporated in the drinking water and diluted up to one in five, even with birds given the nitrovin–monensin diet. By contrast, the latter appeared to prevent protection against salmonella colonization when the protective dose was given via the crop.

Table 6 shows that successful protection of the chicks was again paralleled by the establishment in the caeca of large numbers of lactobacilli, *B. hypermegas* and *B. vulgatus*. In the case of those chicks fed on the nitrovin–monensin diet and given the bacterial mixture via the crop (group C), the lactobacilli appeared to establish but not the bacteroides and hence the birds were not protected against the salmonella challenge. Regardless of the composition of the predominant microflora, the 'total anaerobic' count was comparable for all six groups of chicks.

Table 5. *Influence of feed additives and method of treating chicks to prevent salmonella colonization*

	No. of birds positive at each level of *Salmonella* carriage											
Chick group ...	A		B		C		D		E		F	
Nitrovin/monensin feed ...	−		+		+		+		+		+	
	Method of administering bacteria mixture											
	Via crop (undiluted)		None		Via crop (undiluted)		Via drinking water (undiluted)		Via drinking water (1 : 3)		Via drinking water (1 : 5)	
Salmonella/g	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d
10^8	0	0	2	0	0	0	0	0	0	0	0	0
10^7	0	0	2	4	5	3	1	0	0	0	0	0
10^6	0	0	4	1	2	3	0	1	1	0	0	0
10^5	0	0	2	4	1	3	1	0	0	0	0	1
10^4	0	0	0	1	2	0	0	0	0	0	0	0
10^3	0	0	0	0	0	1	0	0	2	0	2	0
10^2	0	0	0	0	0	0	0	0	1	0	2	0
Not found	10	10	0	0	0	0	8	7	6	9	6	9
Total birds sampled	10	10	10	10	10	10	10	8*	10	9†	10	10

* Two birds died; † one bird died.

Table 6. *Presence of caecal organisms indicating successful establishment of 'protective' microflora in chicks examined one day after treatment*

Chick group ...	A*	B	C	D	E	F
Lactobacillus spp.	9·11	5·04	8·28	7·76	7·87	7·83
Bacteroides hypermegas	8·82	NF	NF	9·41	8·46	9·36
B. vulgatus	10·23	NF	NF	10·08	9·94	10·61
Total anaerobes† (VLhlf)	10·99	10·20	10·04	10·23	10·18	10·53

* The groups correspond to those in Table 5; the figures are $\log_{10}$ organisms/g of a bulk sample of 12 birds in each case; NF, not found. † This includes facultative anaerobes.

DISCUSSION

The primary objective of this study has been to determine whether a defined mixture of caecal bacteria from an adult chicken can be substituted for a suspension of caecal contents or an undefined anaerobic culture from the same source as a means of protecting newly-hatched chicks against caecal colonization by food poisoning salmonellas. The results obtained by treating chicks with a mixture of 48 different organisms showed that the treatment not only conferred protection to the same degree as that demonstrated previously with a suspension of adult caecal contents tested under comparable conditions (Barnes *et al.* 1980*a*) but the protective effect was consistently reproducible. Also, the component organisms did not include types likely to cause any particular hazard to human or animal health.

As yet, no attempt has been made to identify the essential components of the protective mixture or to reduce the number of strains used. Because of the importance of maintaining the ecological balance of the caecal microflora, it could be argued that any disturbance is less likely with a bacterial mixture which includes the main elements of the adult caecal microflora at levels corresponding to their proportionate incidence *in vivo*. Moreover, the inclusion of more than one strain of each type in most cases is thought to increase the chance of establishing at least one of the strains in different batches of birds which are given the protective treatment. Since the initial gut microflora of the young chick varies in composition and changes rapidly during the first few days of life (Mead & Adams, 1975), no account can be taken of the possible presence in the caeca of some of the bacterial types which are also included in the treatment mixture.

For experimental purposes, the method of administering both the protective organisms and subsequently the salmonella by direct dosing into the crop ensured that all birds received a comparable inoculum of the required organisms within a short space of time. However, such a laborious method would not be feasible for protecting large numbers of birds under commercial conditions and therefore particular attention was given to the alternative of adding the protective organisms to the birds' drinking water, as used by Rantala (1974*b*) for administering anaerobic cultures of intestinal material. The results given in Table 5 show that this method was highly successful and presumably facilitated rapid transfer of the organisms to the caeca of the chicks.

With direct dosing and feeeding of the birds on a diet containing nitrovin and monensin, both *B. hypermegas* and *B. vulgatus* failed to establish in the caeca and

no protective effect was observed. By contrast, when chicks were given the protective organisms via the drinking water, there was no interference from the diet. It is conceivable that monensin or the combination of nitrovin, monensin and the low pH conditions of the crop were inimical to survival of the key organisms. Feed containing nitrovin alone at 10 mg kg^{-1} did not interfere with salmonella exclusion when tested by Rantala (1974*a*), using anaerobic cultures of intestinal contents inoculated directly into the crop. Also, incorporation of nitrovin in the diet at 10 mg kg^{-1} was found by Barnes *et al.* (1979) to have no significant effect on the caecal microflora.

A pertinent but so far unresolved question is why a small proportion of the birds used in these experiments remained susceptible to salmonella colonization after being given the protective treatment and, assuming this is due to failure of the necessary organisms to become established in the caeca, why it should occur. However, the fact that such birds continued to shed large numbers of salmonellas into the environment of the isolator and remained in contact with the other birds without infecting them underlines the effectiveness of the protective treatment in the majority of cases.

The experimental isolators used in this study provided suitably controlled conditions for housing the chicks and appropriate containment for the salmonella. Nevertheless, the rate of chick growth is such that birds could not be kept in the isolators for more than about 14 days. In subsequent trials, it will be necessary to test the efficiency of the protective mixture under field conditions where, of course, the birds are reared on litter and kept for much longer periods as well as being subjected to a more natural challenge with different salmonella serotypes.

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