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Development of the normal gastrointestinal microflora of specific pathogen-free chickens

By P. J. COLOE,* T. J. BAGUST† and L. IRELAND*

* *Victorian Department of Agriculture, Veterinary Research Institute, Parkville, Victoria, Australia,* † *Commonwealth Scientific and Industrial Research Organization, CSIRO Division of Animal Health, Parkville, Victoria, Australia*

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SUMMARY

The development of the normal intestinal microflora of the small intestine, caecum and large intestine of specific pathogen-free (SPF) chickens, was studied in the period from hatching to 84 days of age.

No bacteria were detected in any of the sites at hatchery (day 1), but by day 3 significant levels of faecal streptococci and coliforms were isolated from all sites. The flora of the small intestine was limited to faecal streptococci and coliforms for the first 40 days and then lactobacilli became established and dominated the flora.

A large variety of facultative and strictly anaerobic organisms colonized the caecum. Many of these species were transient and were only present for a limited period; after 40 days the flora stabilized to consist predominantly of faecal streptococci, *Escherichia coli*, *Bacteroides* spp. and *Lactobacillus* sp.

The flora of the large intestine was composed of organisms also present in the small intestine or the caecum.

These findings differ from previously published studies on conventionally reared chickens in that the number of species isolated and the population levels of organisms are much lower. This probably reflects the absence of continuous environmental challenge to the chickens because of the housing and feeding facilities in which the chickens were maintained.

INTRODUCTION

There has been an upsurge in interest in the role that the normal intestinal flora, both anaerobic and facultative anaerobic, plays in protecting against enteric infections. The presence of a developed gastrointestinal microbial flora has been shown to significantly reduce the susceptibility of chickens to salmonellosis (Barnes, Impey & Stephens, 1979; Schneitz, Senna & Rizzo, 1981) and also limit colonisation of the gut of chickens by pathogenic strains of *Escherichia coli* (Weinack, Sneoyenbos, Smyser and Soerjadi, 1981) and *Campylobacter fetus* subsp. *jejuni* (Soerjadi, Sneoyenbos & Weinack, 1982).

Studies on the development of the normal gastrointestinal microflora of poultry using chickens which are housed under conventional (commercial) conditions are complicated by the profusion of bacterial species encountered and by the potential

for colonization by flora from the mother (Smith, 1965), as well as from the environment. Alternatively, using gnotobiotic chickens (germ-free or holaxenic) cannot accurately reflect the competition for attachment sites occurring among the variety of bacterial species which comprise the normal gastrointestinal microflora. Specific pathogen-free (SPF) chickens would appear to offer distinct advantages for studies on enteric diseases in chickens since the results should not be biased by the absence of competitive flora as is the case for germ-free chickens, nor do they carry the risk of additional infectious agents (i.e. viruses) being present as may occur with holaxenic (conventionally raised) chickens.

In this study we have analysed the types and numbers of bacteria which develop in the small intestine, caecum and large intestine of SPF chickens over a period of 12 weeks. The initial period of 2–6 weeks, when the anaerobic flora of holaxenic chickens is known to be developing (Barnes, Mead, Barum & Harry, 1972) was extended to cover the slower development of intestinal flora expected in chickens raised in SPF isolators.

MATERIALS AND METHODS

SPF chickens and their management

Chickens of the CSIRO-Mini SPF strain were studied. On the day of hatching within an SPF unit, a group of SPF chickens were transferred from the incubator, using a sterile transfer module, into a fibreglass poultry flock isolator (as Cooper & Timms, 1972) with wire-mesh flooring. These chickens were supplied *ad libitum* with commercial chick crumbles (KMM-Barastoc, Melbourne) which had been sealed in plastic bags under vacuum and then gamma-irradiated (2·5 Mrads). Tap water was supplied after acidification to pH 2 following standard practices for SPF poultry. Three chickens were removed from this isolator for bacterial examination on each of 1, 3, 7, 9, 14, 17, 21, 24, 28, 31, 33, 35, 42, 56 and 84 days after hatching.

Examination procedures

Chickens were killed by cervical dislocation and immediately dissected. Sections of the small intestine, caecum and large intestine were each ligated with string and the sections removed, weighed and immediately placed within an anaerobic chamber. All specimens were placed into the anaerobic chamber within 2 min of sacrificing the chicken.

Anaerobic chamber

An anaerobic chamber similar to that described by Draser (1967) was manufactured at the Veterinary Research Institute. To maintain anaerobiosis, the chamber was first flushed with CO_2 and then twice with an oxygen-free nitrogen/hydrogen (95%/5%) gaseous mixture. One kg of palladium catalyst was incorporated into the chamber and changed weekly. The chamber was flushed every 3 days with the oxygen-free N_2/H_2 mixture. Immediately prior to use each time the atmosphere within the chamber was established at 85% N_2:10% H_2:5% CO_2 by the addition of H_2 and CO_2. Resasurine indicator was used to monitor anaerobiosis.

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Preparation of specimens for culture

Measured weights of intestinal portions were squeezed into a tube containing 9 ml of anaerobic dilution broth (Barnes & Impey, 1970). Serial ten-fold dilutions of each specimen were performed using anaerobic dilution broth and 100 μl aliquots of each dilution were then spread onto specific agar culture media.

Media

The following media were used:

Horse blood agar (HBA)	5% horse blood in Oxoid columbia agar base (1·5% agar).
Sheep blood agar (SBA)	7% sheep blood in Oxoid columbia agar base (1·5% agar).
Firm blood agar	7% sheep blood in Oxoid columbia agar base (4·5% agar).
Vancomycin blood agar	Horse blood (as above) plus 5 μg/ml vancomycin.

Media for selective isolation or differentiation of individual species were prepared as described by Barnes *et al.* (1972).

Storage media

All isolates were stored in chopped meat medium (CMM), freshly prepared as described by Holdeman & Moore (1972).

Incubation conditions

Media used for anaerobic plate culture were held in the anaerobic chamber for at least 24 h prior to use to remove all traces of oxygen. Inoculated plates were loaded within the anaerobic chamber into anaerobic jars (Oxoid, Aust.) equipped with a palladium catalyst. A fresh gas kit (Oxoid Aust.) was added to each jar to maintain anaerobiosis and ensure that the CO_2 level was optimal and the jar was then sealed and removed from the chamber for incubation. This was necessary since incubation facilities were not available inside the anaerobic chamber.

Reading of plates and sub-culturing of isolates was performed within the anaerobic chamber, except for isolates known to be aerobic species.

Isolation and identification of organisms

To determine the numbers of each bacterial species present, individual colony types were identified from all selective and non-selective media at each countable dilution. Selected examples of each species were then sub-cultured onto the medium from which they were isolated, onto HBA and into CMM.

All strict anaerobes were identified biochemically and on the basis of their metabolic products according to the key of Holdeman & Moore (1972). All facultative anaerobes were identified according to Cowan & Steel (1975). Gas–liquid chromatography of metabolic products was performed according to the protocol of Rizzo (1980).

Table 1. *Characteristics of the bacterial flora isolation from the intestinal tract of specific pathogen-free chickens*

Morphological group	Gram	Site of isolation*	O_2 tolerance	Major metabolic products†	Identification
I. Rods	−	Caec. SI	−	S.L.	*Bacteroides* sp.
II. Rods	+	SI Caec.	−	L.S.a	*Lactobacillus* sp.
III. Rods	−	Caec. SI. LI	−	A. ic iv	*Clostridium* sp.
IV. Rods	−	Caec	−	A	*Clostridium* sp.
V. Rods	−	SI. LI.	−	B.A.	*Eubacterium* sp.
VI. Rods	+	Caec. LI	−	P.a.	*Propionibacterium* sp.
VII. Rods	+	SI	+‡	A.P.B.iv.ib.	*Clostridium* sp.
VIII. Rods	+	Caec	−	S.I.	*Eubacterium* sp.
IX. Rods	−	Caec. LI	−	P.S. a.l.	*Bacteroides* sp.
X. Cocci	−	LI	−	A.b.	*Acidaminococcus* sp.
XI. Cocci	−	SI	+	L.S.	Unknown
XII. Coccobacilli	−	Caec	−	B.iB.P.	*Megasphaera* sp.
XIII. Cocci	+	Caec. LI	−	A	*Peptostreptococcus* sp.
XIV. Rods	−	Caec. SI. LI.	+	Nt	*E. coli*
XV. Cocci	+	Caec. SI. LI.	+	Nt	*Streptococcus* sp.
XVI. Rods	−	Caec. SI. LI.	+	Nt	*Proteus* sp.

* Caec, Caecum; LI, large intestine, SI, small intestine.

† Aa, Acetic acid; Bb, butyric acid; iBb, Isobutyric acid; iC, Isocaproic acid; Iv, isovaleric acid; P. propionic acid. Capital letters designate major peaks. Small letters designate minor peaks.

‡ Microaerophilic only.

RESULTS

Isolation of bacteria from the chicken intestinal tract

A variety of facultative anaerobic and strictly anaerobic bacteria were isolated and identified during the course of the experiment and several species were consistently isolated from consecutive samples (Table 1).

Comparison of media for isolation of intestinal flora

To compare the efficacy of HBA for the isolation of fastidious anaerobes, specimens were cultured onto HBA and onto the caecal-extract-agar of Barnes & Impey (1970). The results found at day 21 are shown because it included the largest range of bacterial species isolated at any of the sampling periods. The relative numbers of the organisms and the time they took to grow on the media are shown in Table 2. Horse-blood agar was found to be adequate for isolation of the anaerobic flora for these SPF chickens. The use of caecal extract agar enabled the more rapid isolation of *Eubacterium* sp. and *Peptostreptococcus* spp. and in slightly higher numbers, but did not appear to increase the number of species that could be isolated.

Bacterial colonization of the chicken intestine

No bacteria were detectable at hatching (day 1), but by day 3, significant levels (10^8/g) of faecal streptococci and coliforms were present, and by day 7 these were accompanied by *Proteus* sp. at levels in excess of 10^7/g. These groups were the only

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Table 2. *Comparison of the use of horse blood agar and caecal extract agar for the isolation of strict anaerobes from the chicken caecum*

	Anaerobic horse blood agar		Caecal extract agar	
Species	No./g	Incubation time for observable growth (days)	No./g	Incubation time for observable growth (days)
Clostridium sp.	$1{\cdot}6 \times 10^8$	2	$2{\cdot}4 \times 10^7$	2
Clostridium sp.	$2{\cdot}6 \times 10^8$	2	$4{\cdot}9 \times 10^8$	2
Eubacterium sp.	$4{\cdot}2 \times 10^8$	2	5×10^8	2
Peptostreptococcus	$1{\cdot}4 \times 10^6$	5	$6{\cdot}9 \times 10^7$	2
Eubacterium spp.	$1{\cdot}6 \times 10^7$	5	$4{\cdot}9 \times 10^9$	2
Bacteroides sp.	$6{\cdot}8 \times 10^8$	2	$1{\cdot}3 \times 10^8$	2

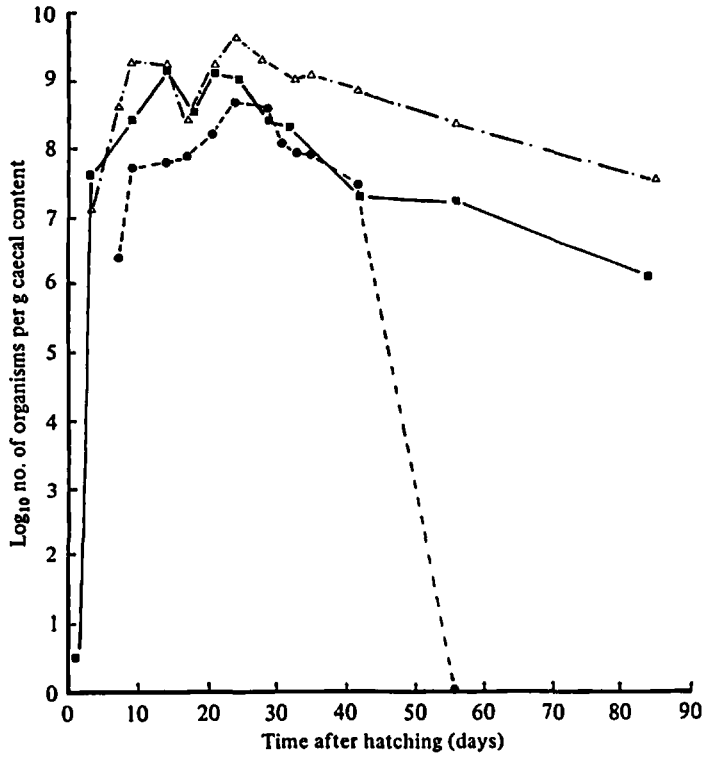

Fig. 1. Development of the facultative anaerobic microflora of the chicken caecum with time. △–·–△, *Streptococcus* sp.; ■—■, *E. coli*, ●--●, *Proteus* spp.

facultative anaerobes found to colonise the caecum (Fig. 1). Anaerobes appeared to colonize the caecum more slowly with only small numbers of *Clostridium* spp. present at day 3, *Eubacterium* sp. did not appear before 10 days, *Bacteroides* sp. before 21 days or *Lactobacillus* sp. before 42 days (Fig. 2). Other anaerobes detected (refer Table 1) were transient, being detected only one or twice throughout the experimental period.

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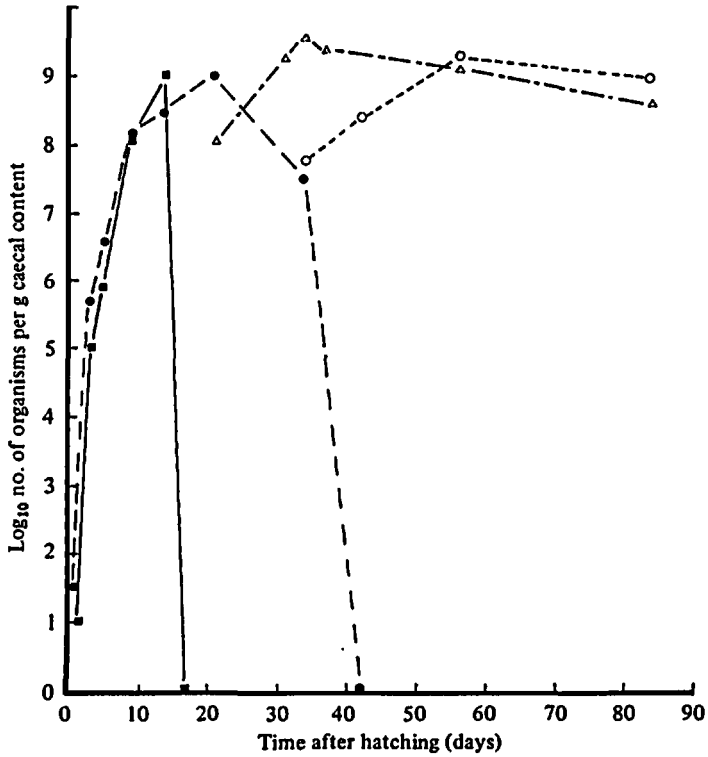

Fig. 2. Development of the predominant species of strictly anaerobic microflora in the chicken caecum with time. △--△, *Bacteroides* sp. (group 1); ■—■, *Clostridium* sp. (group 3); ●--●, *Clostridium* sp. (group 4); ○--○, *Lactobacillus* sp. (group 2).

Distribution of bacterial species within the chicken intestinal tract

(*a*) *Small intestine.* The development of the flora of the small intestine was restricted to only four species. Initially faecal streptococci and coliforms colonized at day 3 at 10^2 and 10^3/g respectively and by day 7 had each reached 10^7/g. These organisms remained at 10^7/g until day 42 when *Lactobacillus* sp. and *Eubacterium* sp. appeared at 10^7 and 10^6/g respectively. With the establishment of the *Lactobacillus* sp. and the *Eubacterium* sp. the numbers of faecal streptococci and coliforms fell to 10^6 and 10^5/g respectively.

(*b*) *Caecum.* Development of the caecal microflora with time is shown in Figs. 1 and 2. No bacteria were present at hatching (day 1), but by day 3 high concentrations (10^8/g) of faecal streptococci and coliforms (including *E. coli*) had developed and by day 7 there was also greater than 10^7/g of *Proteus* sp. These three groups of bacteria were the only facultative anaerobes that colonized the caecum. The anaerobes were slower in colonizing the caecum and only small numbers of *Clostridium* sp. were established by day 3. Other anaerobes were then detectable at varying intervals, with *Eubacterium* sp. appearing at 10 days, *Bacteroides* sp. at 21 days, and *Lactobacillus* sp. at 42 days after hatching. Each of these species once established were regularly isolated as part of the normal flora.

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(c) *Large intestine.* The composition of the bacterial flora of the large intestine closely resembled that of the caecum, but bacterial population densities were reduced by approximately ten-fold for each species. Only once was an anaerobe recoverable from the large intestine that was not also present in the caecum. The *Eubacterium* sp. (Group V, Table 1) isolated in this instance was, however, also present in the small intestine at that time.

DISCUSSION

Even though the group of birds in this study were maintained in isolators supplied with filtered air, irradiated feed and acidified water, less than 3 days were required for the development of a bacterial flora comprising in excess of four species. By 3–4 weeks the SPF intestinal flora had stabilized to consist of seven species. The results presented in this paper thus differ significantly from the earlier reports by Ochi, Mitsuoka & Sega (1964) and Barnes *et al.* (1972), who reported that lactobacilli are the only organisms present in the small intestine from 2–6 weeks of age. In contrast to their findings, we found that coliforms, faecal streptococci and *Proteus* spp. had colonized the small intestine of SPF chickens within a few days of hatching and persisted for the duration of the experiment, while lactobacilli were not detected in the small intestine until 42 days of age.

Our findings resemble those of Smith (1965), who also reported coliforms and faecal streptococci in the small intestine of chicks aged from 1 to 18 days. He reported lower numbers of coliforms and faecal streptococci than we found and he also reported the presence of *C. perfringens* and *Staphylococcus aureus* in the small intestine, but we could find no evidence of these latter organisms in our study.

Our findings could be thought to reflect some invasion of the small intestine from the caecum post-mortem, but the very short interval occurring before the experimental separation of the segments makes such contamination unlikely. A more plausible explanation of our finding is that the coliforms and faecal streptococci could only colonize the small intestine in the absence of competitive flora such as the *Lactobacillus* spp., and this is further supported by our observation that with the establishment of the lactobacilli, the levels of coliforms and faecal streptococci declined. (See later in Discussion). A further variation from Barnes *et al.* (1972) is noted in total numbers of caecal micro-organisms as 100-fold less (10^9/g) in SPF chickens. This may reflect the lack of constant environmental challenge in SPF isolators together with a lack of microbial by-products that enhance microbial proliferation.

Development of the components of the SPF microflora occurred quite rapidly considering that sources of maternal and environmental challenge were negligible after hatching. Indeed, the limited flora of these chickens would appear to have been initially derived from the shells and contents of their eggs, even though SPF chickens are excluded from any significant exposure to the flora of their dams through such procedures as formalin fumigation of eggs, incubators and isolators prior to occupation by the chickens.

For the purposes of discussion, the caecum may be considered as the segment of the intestinal tract best reflecting the development of the microflora of SPF

chickens. No bacteria were detectable at hatching, but by day 3, greater than 10^7 faecal streptococci and coliforms, 10^4 *Proteus* spp. and 10^5 clostridia/g of caecal content were present. By 7 days of age, all of the bacterial species that were to dominate the flora, excepting *Lactobacillus* spp. and *Bacteroides* sp., had established in the intestinal tract, even though final concentrations relative to the other components of the flora was still stabilizing. Thus, populations of bacteria within the microflora of the caecum appear to undergo significant fluctuations in numbers before a dynamic equilibrium is established between the species.

It was apparent that facultative anaerobes, such as the faecal steptococci, the coliforms and the *Proteus* spp., initially rose to very large numbers in all the sites from which they were cultured, and particularly in the caecum. These species reached maximum levels (10^9/g) at about 20 days of age, and then slowly declined in number. It was also around this time (30 days of age) that the *Bacteroides* spp. and the *Lactobacillus* sp. were becoming established in high concentrations. It would seem probable that the development of the latter species would provide competition for food sources and production of volatile fatty acids (Barnes, Impey & Stephens, 1979) so as to result in the coincident decrease in the numbers of facultative anaerobes.

Our findings show that SPF chickens develop a resident flora without any obvious environmental contribution and that this resident flora appeared to stabilize around 6 weeks of age. We have shown (Bagust & Coloe, unpublished data), that soon after hatch SPF chickens are extremely susceptible to *Salmonella typhimurium* infection, but that susceptibility decreases significantly with the development of the normal intestinal flora. Therefore the limited flora that we have identified in these SPF chickens may open the possibility of using combinations of pure bacterial cultures to protect against salmonellosis in chickens rather than the potentially hazardous procedure of supplying faecal material obtained from commercial adult hens as a source of 'normal' flora for chicks (Rantala & Nurmi 1973; Sneoyenbos, Weinack & Smyser, 1978).

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REFERENCES

Barnes, E. M. & Impey, C. S. (1970). The isolation and properties of the predominant anaerobic bacteria in the caeca of chickens and turkeys. *British Poultry Science* **11**, 467–481.

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.

Barnes, E. M., Mead, G. C., Barnum, D. A. & Harry, E. G. (1972). The intestinal flora of the chicken in the period 2–6 weeks of age, with particular reference to the anaerobic bacteria. *British Poultry Science* **13**, 311–326.

Cooper, D. M. & Timms, J. R. (1972). The rearing and maintenance of breeder chickens in isolators: 1. Glassfibre isolators. *Avian Pathology* **1**, 47–57.

Cowan, S. T. & Steel, K. J. (1975). *Manual for the Identification of Medical Bacteria*, 2nd ed. Revised by S. T. Cowan, Cambridge University Press.

Draser, B. (1967). Cultivation of intestinal anaerobes. *Journal of Pathology and Bacteriology* **94**, 417.

HOLDEMAN, L. V. & MOORE, W. E. C. (1972). *Anaerobe Laboratory Manual*, ed. Virginia Polytechnic. Institute and State University Anaerobe Laboratory, Blacksburg, Va.

OCHI, Y., MITSUOKA, T. & SEGA, T. (1964). Untersuchungen über die Darmflora des Huhnes. III. Die Entwicklung der Darmflora von Kuken bis zum Huhn. *Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* (1. Orig.) **193** (5), 80–85.

RANTALA, M. & NURMI, E. (1973). Prevention of the growth of *Salmonella infantis* in chicks by the flora of the alimentary tract of chickens. *British Poultry Science* **14**, 627–630.

RIZZO, A. (1980). Rapid gas chromatographic method for identification of metabolic products of anaerobic bacteria. *Journal of Clinical Microbiology* **11**, 418–421.

SCHNEITZ, C., SENNA, E. & RIZZO, A. (1981). The anaerobically cultured caecal flora of adult fowls that protects chickens from salmonella infections. *Acta path. microbiol. Scand.* S B **89**, 109–116.

SMITH, H. W. (1965). The development of the flora of the alimentary tract in young animals. *Journal of Pathology and Bacteriology* **90**, 495–513.

SNEOYENBOS, G. H., WEINACK, O. M. & SMYSER, C. F. (1978). Protecting chicks and poults from Salmonella by oral administration of 'normal' gut microflora. *Avian Diseases* **22** (2), 273–287.

SOERJADI, A. S., SNEOYENBOS, G. H. & WEINACK, O. M. (1982). Intestinal colonisation and competitive exclusion of *Campylobacter fetus* ssp. *jejuni* in young chicks. *Avian Diseases* **26** (3), 520–524.

WEINACK, O. H., SNEOYENBOS, G. H., SMYSER, C. F. & SOERJADI, A. S. (1981). Competitive exclusion of intestinal colonization of *Escherichia coli* in chicks. *Avian Diseases* **25** (3), 696–705.