Composition of Microbiota in Content and Mucus from Cecae of Broiler Chickens as Measured by Fluorescent In Situ Hybridization with Group-Specific, 16S rRNA-Targeted Oligonucleotide Probes¹

X. Y. Zhu* and R. D. Joergert†,²

*Environmental Science and Technology Center, Gas Technology Institute, 1700 South Mount Prospect Road, Des Plaines, Illinois 60018-1804; and †Department of Animal and Food Sciences, University of Delaware, 018 Townsend Hall, Newark, Delaware 19717-1303

ABSTRACT Six group-specific 16S rRNA-targeted oligonucleotide probes were used to investigate the composition of the microbiota of cecal content and mucus from broiler chickens. Together, the probes hybridized to as many as 94.7% of the bacteria detectable with the universal probe Bact338 in the content of the cecum of 2-d-old chicks. Fewer bacteria gave signals with these probes as the birds aged, and coverage was as low as 76% for the bacteria in cecal content of a 6-wk-old chicken. In the cecal content of 2-d-old chicks, approximately 56, 34, and 3% of the bacteria detectable with the universal probe reacted with the probes Enter1432 (enterics), Lacto722 (Lactobacillus/Streptococcus/Enterococcus), and Bif164 (bifidobacteria), respectively. Probes Clept1240 (Clostridium leptum subgroup), Erec482 (Clostridium coccoides-Eubacterium rectale), and Bacto1080 (Bacteroides groups) did not produce signals. In cecal content from 1-wk-old chicks, all six probes gave signals, and in samples from 6-wk-old birds approximately 3, 9, 6, 32, 22, and 8% of the bacteria detectable with the universal probe hybridized with the probes Enter1432, Lacto722, Bif164, Clept1240, Erec482, and Bacto1080, respectively. At this age, the six probes detected the phylogenetic groups in similar proportions in the microbiota of cecal content and cecal mucus. The exception was the enterics probe because more bacteria from the mucus fraction than from cecal content gave signals with this probe (13.4 vs. 4.4%, \( P < 0.001 \)).

(Key words: broiler chicken, cecum, microbiota, in situ hybridization, 16S rRNA)

INTRODUCTION

Studies on the composition of the intestinal microbiota of chickens date back to 1901 (Rahner, 1901). Additional studies were carried out in the 1940s (Shapiro and Sarles, 1949), but not until the 1970s were comprehensive surveys attempted by culturing as many of the intestinal bacteria as possible (Barnes et al., 1972; Salanitro et al., 1974; Mead and Adams, 1975; Barnes, 1979). These studies were technically difficult and extremely time consuming because strict anaerobic conditions had to be maintained, and numerous biochemical differentiation tests had to be carried out. Using such methods, only an estimated 10 to 60% of the bacteria in the cecum could be cultivated (Barnes et al., 1972; Salanitro et al., 1974; Barnes, 1979). A more comprehensive overview of the intestinal microbiota of the chicken would not only be of interest to basic microbiological ecology but might also be of practical importance.

For example, further development of effective competitive exclusion products might be aided by a better understanding of the intestinal microbiota.

To circumvent some of the problems associated with culture-based surveys, culture-independent molecular approaches have been used to study the composition of the cecal microbiota of chickens (Gong et al., 2002; Zhu et al., 2002). These PCR-based approaches are powerful tools to provide an overview of the microbial diversity present in a particular sample, but they can also be biased by cellular rDNA copy number (Farrelly et al., 1995; Wang et al., 1997), DNA extraction methods (More et al., 1994; von Wintzingerode et al., 1997), primer selection (Rainey et al., 1994; Suzuki and Giovannoni, 1996), PCR amplification (Kyesenbach et al., 1992; Morrison and Gannon, 1994; Speksnijder et al., 2001), and cloning strategy and efficiency (Rainey et al., 1994; Chandler et al., 1997). Such biases were also observed in the study of the cecal microbiota of chickens (Zhu et al., 2002). For example, temporal thermal gradient gel electrophoresis (TTGE) analysis of partial 16S rRNA gene sequences generated with primer

Abbreviation Key: FISH = fluorescent in situ hybridization.
pair 968F-GC/1401R (Nubel et al., 1996) from DNA extracted from the cecal mucus of 18 broiler chickens revealed bacteria from only three phylogenetic groups; on the other hand, random cloning of 16S rRNA gene sequences generated with primer pair 63F/1387R-AC (Marchesi et al., 1998) revealed the presence of 16S rRNA gene sequences representing 17 subdivisions or groups of bacteria.

The percentage distribution of 16S rRNA gene sequences generated by PCR from DNA extracted from mixed environmental samples cannot be used to infer the quantitative distribution of species in the microbial community from which the DNA was extracted (Fuhrmann et al., 1993; Farrelly et al., 1995) or to predict the contribution of these species to the activity of the whole microbial community (Sghir et al., 2000). Therefore, fluorescent in situ hybridization (FISH) has been used as an alternative means to quantify the abundance and determine the distribution of bacterial groups in natural communities (Amann et al., 1995; Harmsen et al., 1997, 2000a). Although this approach also has certain biases caused by differential rRNA content and probe penetration, it has been demonstrated to be useful for detection of individual cells in mixed microbiota (Amann et al., 1995; Langendijk et al., 1995; Licht et al., 1996; Harmsen et al., 1997). The FISH technique produced results similar to those obtained using conventional culture techniques for the detection and enumeration of bifidobacteria in fecal material (Langendijk et al., 1995). Harmsen et al. (2000a) confirmed the usefulness of FISH by comparing it to conventional culture-based detection of groups of bacteria in human fecal samples.

In the present study, we describe the whole-cell FISH using a set of 16S rRNA-targeted, group-specific, oligonucleotide probes for the analysis of the microbiota composition in cecal content and cecal mucus of broiler chickens. The two cecal locations were targeted because it was reasonable to believe that they might harbor different microbiota and because further knowledge regarding the composition and distribution of cecal microbiota might enhance our understanding of competitive exclusion processes.

**MATERIALS AND METHODS**

**Sources of Cecal Samples**

Two sets of experiments on cecal samples were carried out for this study. For the first set, 6-wk-old broilers were obtained from a commercial farm. The birds were killed by cervical dislocation, and ceca were removed, placed in sterile plastic bags, and immediately immersed in ice for transport to the laboratory.

For the second set of experiments, birds of different ages were obtained from the University of Delaware Farm where a flock of 2,000 chickens was raised under conditions closely approximating those encountered in commercial broiler operations (e.g., bird density, feed, and litter). The broilers were not exposed to competitive exclusion preparations as newly hatched chicks. For the first 3 wk posthatch, the chicks were fed commercial starter feed containing 90 g monensin per ton, and thereafter they were given commercial feed free of monensin and antibiotics. The birds were killed by cervical dislocation. The ceca were collected aseptically from 2-d-old to 6-wk-old broilers and placed in sterile petri dishes for immediate processing in the laboratory.

**Preparation of Cecal Samples**

Three types of samples (tissue-associated cecal mucus, tissue-free mucus, and cecal content) were prepared for in situ hybridization studies. The tissue-associated cecal mucus samples were obtained as 5 mm long segments cut from the cecum halfway between the cecal tonsil and the tip. The cecal segments were mounted onto Tissue-Tek Cryomolds with Tissue-Tek OCT compound, and immediately frozen in liquid nitrogen. Twenty-micrometer cryostat sections were cut using a Leica CM3050 S Versatile Cryostat, then mounted onto Superfrost/Plus slides, and stored at −70°C until fixation.

Tissue-free mucus samples were obtained by opening the cecal segments longitudinally and washing them with PBS until all visible digesta were removed. The washed tissues were cut into 1 cm long pieces with a razor blade, and surface-associated mucus was separated from the tissues by treatment with 10 mL of PBS containing 250 mg of the reducing agent N-acetyl-L-cysteine (pH 7.2) (Cooper and Narendranathan, 1986) at 37°C for 30 min with shaking at 400 rpm. After removal of the cecal tissues, the liberated mucus fraction was pelleted by centrifugation at 13,000 × g for 5 min.

Cecal content samples were prepared by mixing 0.5 g of digesta collected from the middle portion of the ceca with 4.5 mL of sterile-filtered PBS (pH 7.4). The mixture was vortexed with a dozen glass beads (2.5 mm in diameter) for three min, and centrifuged at 700 × g for 1 min to remove debris.

All cecal samples were fixed with freshly prepared paraformaldehyde/PBS [final concentration 3% (wt/vol)] overnight at 4°C. After two washes with PBS, tissue sections were stored at −70°C until FISH analysis. The cecal content and tissue-free mucus samples were resuspended in 50% ethanol/PBS and stored at −20°C.

**FISH**

For FISH analysis, 10 μL of the mucus fraction or of a 1:250 dilution of a content sample was pipetted onto a Superfrost/Plus slide and air-dried. Slides were dehydrated in an ethanol series (50, 80, and 96%, 3 min each). For each sample, a series of identical slides was prepared.
### TABLE 1. Oligonucleotide sequences, target sites, references, formamide concentrations in the hybridization buffer and sodium chloride concentrations in the washing buffer for specific in situ hybridization with FAM-labeled probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Position</th>
<th>Sequence (5′ to 3′)</th>
<th>Target (reference)</th>
<th>Formamide (%)</th>
<th>NaCl (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto 1080</td>
<td>1080–1097</td>
<td>GCCTTAAAGCCGACACCT</td>
<td>Bacteroides group (Dore et al., 1998)</td>
<td>15</td>
<td>353</td>
</tr>
<tr>
<td>Bif164</td>
<td>164–181</td>
<td>CATCCGCAATTCACC</td>
<td>Bifidobacterium group (Harmsen et al., 1997)</td>
<td>20</td>
<td>250</td>
</tr>
<tr>
<td>Clept1240</td>
<td>1240–1257</td>
<td>GTTTTACCGCCAGTCC</td>
<td>Clostridium leptum subgroup (Sghir et al., 1998)</td>
<td>10</td>
<td>500</td>
</tr>
<tr>
<td>Enter1432</td>
<td>1432–1446</td>
<td>CTGGTACCGCAAGACT</td>
<td>enteric group (Sghir et al., 1998)</td>
<td>0</td>
<td>1,000</td>
</tr>
<tr>
<td>Erec482</td>
<td>482–500</td>
<td>GCCGTTGACACGTAATCC</td>
<td>Clostridium acetobutylicum</td>
<td>15</td>
<td>353</td>
</tr>
<tr>
<td>Esco513</td>
<td>513–526</td>
<td>GCCGTTGACACGTAATCC</td>
<td>Escherichia coli (Amann et al., 1990)</td>
<td>25</td>
<td>176</td>
</tr>
<tr>
<td>Lacto722</td>
<td>722–746</td>
<td>YACCCGCTAAGACATGTTCCACT</td>
<td>Lactobacillus, Streptococcus, and Enterococcus groups (Sghir et al., 1998)</td>
<td>25</td>
<td>176</td>
</tr>
<tr>
<td>Lactob338</td>
<td>338–355</td>
<td>GCCGTTGACACGTAATCC</td>
<td>Lactococcus (Brosius et al., 1981)</td>
<td>20</td>
<td>250</td>
</tr>
<tr>
<td>Erec482</td>
<td>482–500</td>
<td>GCCGTTGACACGTAATCC</td>
<td>Eubacterium rectale group (Franks et al., 1998)</td>
<td>15</td>
<td>353</td>
</tr>
<tr>
<td>Clept1240</td>
<td>1240–1257</td>
<td>GTTTTACCGCCAGTCC</td>
<td>Clostridium coccoides group (Harmsen et al., 1997)</td>
<td>10</td>
<td>500</td>
</tr>
<tr>
<td>Bif164</td>
<td>164–181</td>
<td>CATCCGCAATTCACC</td>
<td>Bifidobacterium group (Harmsen et al., 1997)</td>
<td>20</td>
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<td>250</td>
</tr>
</tbody>
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1Position in the 16S rRNA of *Escherichia coli* (Brosius et al., 1981).

to allow determination of optimal hybridization conditions essentially as described by Manz et al. (1992). The oligonucleotides used for this study (Table 1) were selected from the literature, synthesized and labeled with FAM.8 Hybridizations were carried out in 50 μL of hybridization buffer (0.9 M NaCl; 20 mM Tris/HCl, pH 7.2; 0.01% SDS) containing the FAM-labeled probes at 20 ng/μL (content and tissue samples) or 50 ng/μL (mucus sample). Hybridization stringency was optimized for each probe by varying the formamide concentration at 5% increments. All slides were covered with probe-clip press-seal incubation chambers,9 and incubated overnight (16 h) in a humidified Slide Moat Incubator10 at 46 °C. After hybridization, slides were washed at 48 °C for 20 min with prewarmed washing buffer (20 mM Tris/HCl, pH 7.2; 0.01% SDS; 5 mM EDTA) containing NaCl at concentrations that maintained the stringency conditions set by the formamide concentrations in the hybridization buffer. The slides were rinsed gently in distilled water, air-dried, and mounted with SlowFade Light Antifade reagent.11

RESULTS AND DISCUSSION

**In Situ Hybridizations on Samples from 6-Wk-Old Chickens**

Optimal hybridization conditions for the probes are listed in Table 1 along with the formamide concentrations used for the hybridization step and the salt concentration used in the wash buffer. In preliminary experiments with cecal samples, no hybridization signals could be obtained with the Strc493 probe regardless of the hybridization conditions used. Under stringency conditions of 20% formamide, the probe gave signals with cells from a *Lactococcus lactis* subsp. *lactis* ATCC11454 laboratory culture cells, and these signals were strongest with cells treated with lysozyme (1 mg/mL) for 20 min. In contrast, no signals were observed in cecal samples treated with lysozyme in the same manner. Possibly, bacteria targeted by the Strc493 probe were not present in high enough numbers for detection in the sample volume deposited on the slides. It is also possible that these bacteria require hybridization protocols different from those tried. This probe was therefore omitted from the quantitative experiments. The probes Bact338, Arch915, Bif164, and Erec482 have previously been used in FISH experiments (Amann et al., 1990; Langendijk et al., 1995; Franks et al., 1998). The specificity of the remaining probes had previously been established using dot blot hybridization against 46 reference species (Sghir et al., 2000) and was assumed to apply also for whole-cell hybridization under optimized conditions (Amann et al., 1990; Franks et al., 1998).

At the outset of the studies, attempts were made to use cross sections of the cecum for the analyses. Although different bacteria groups could be detected in the tissue-associated mucus samples, it was not possible to obtain a count. The bacteria usually were packed densely in the

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8MWG Biotech, High Point, NC.
9Sigma Chemical Co., St. Louis, MO.
10Boekel Scientific, Feasterville, PA.
11Molecular Probes, Inc., Eugene, OR.
12Carl Zeiss Inc., Thornwood, NY.
13Carl Zeiss Inc., Thornwood, NY.
invaginations of the cecal tissue (Figure 1, panels A and B), and it was frequently impossible to distinguish individual cells. The bacteria were distributed unevenly across the tissue sections, and a large number of tissue sections would have to be done to ensure that any non-homogenous distribution of mucus-associated bacteria species could be documented. Therefore, only cecal content and samples consisting of mucus released from the cecal wall were used for the bacteria quantification. Examples of fluorescent images obtained with cecal content and mucus but that the number of bacteria with signals from the enteric probe was higher in the mucus than the content samples (13.4 ± 0.65% vs. 4.4 ± 0.75%, P < 0.001). The presence of a relatively high number of bacteria with signals from the enteric probe in the mucus fraction might be due to a combination of specific characteristics of these bacteria including the capability of utilizing oxygen transported to the cecal tissues through the blood supply.

Some variability among the cecal microbiota of individual broilers was noticed in our study, especially in the digesta sample. Bacteria hybridizing with the C. leptum group probe made up between 19 and 32% of the total bacteria in content samples (13.4 ± 0.65% vs. 4.4 ± 0.75%, P < 0.001). The finding that many of the bacteria in cecal content and mucus belong to the same phylogenetic groups is consistent with results from the earlier PCR-based studies of the cecal bacteria populations (Zhu et al., 2002). Gong et al. (2002) compared terminal restriction fragment length polymorphism profiles generated from the bacteria associated with cecal and mucosa from chickens and obtained relatively similar profiles with only a few peaks that were different between the two samples. Our earlier study (Zhu et al., 2002) also detected the presence of enterics bacteria in most of the cecal mucus samples examined, and in some of these samples the majority of 16S rRNA gene sequences obtained were from enteric bacteria. Cecal samples analyzed by Gong et al. (2002) also contained sequences from E. coli. The current FISH-based study indicated that enterics were present in cecal content and mucus but that the number of bacteria with signals from the enteric probe was higher in the mucus than the content samples (13.4 ± 0.65% vs. 4.4 ± 0.75%, P < 0.001). The presence of a relatively high number of bacteria with signals from the enteric probe in the mucus fraction might be due to a combination of specific characteristics of these bacteria including the capability of utilizing oxygen transported to the cecal tissues through the blood supply.

For each content and mucus sample, at least three microscopic fields were enumerated. The average counts of cells in content samples stained with SYTO13 and of cells hybridizing to the Bact338 probe in each field were 218 ± 53. The relative abundance of cells hybridizing with a specific probe was normalized to the total cell count determined with the Bact338 probe. The percentage of cells hybridizing with each probe is illustrated in Figure 2. Together, the six probes hybridized to 78.4 ± 0.95% and 76.9 ± 1% of the bacteria hybridizing with the universal probe in cecal content and mucus samples, respectively.

Bacteria fluorescing with the Clept1240 probe (C. leptum subgroup) and bacteria with hybridization signals from the Erec482 probe (C. coccoides-E. rectale group) each accounted for more than 20% of the total bacteria in content and mucus samples (Figure 2). Bacteria with signals from the Lactobacillus/Streptococcus/Enterococcus, Bacteroides, and Bifidobacterium probes were also present in similar proportions in the content and mucus samples. The finding that many of the bacteria in cecal content and mucus belong to the same phylogenetic groups is consistent with results from the earlier PCR-based studies of the cecal bacteria populations (Zhu et al., 2002). Gong et al. (2002) compared terminal restriction fragment length polymorphism profiles generated from the bacteria associated with cecal and mucosa from chickens and obtained relatively similar profiles with only a few peaks that were different between the two samples. Our earlier study (Zhu et al., 2002) also detected the presence of enterics bacteria in most of the cecal mucus samples examined, and in some of these samples the majority of 16S rRNA gene sequences obtained were from enteric bacteria. Cecal samples analyzed by Gong et al. (2002) also contained sequences from E. coli. The current FISH-based study indicated that enterics were present in cecal content and mucus but that the number of bacteria with signals from the enteric probe was higher in the mucus than the content samples (13.4 ± 0.65% vs. 4.4 ± 0.75%, P < 0.001). The presence of a relatively high number of bacteria with signals from the enteric probe in the mucus fraction might be due to a combination of specific characteristics of these bacteria including the capability of utilizing oxygen transported to the cecal tissues through the blood supply.

Some variability among the cecal microbiota of individual broilers was noticed in our study, especially in the digesta sample. Bacteria hybridizing with the C. leptum group probe made up between 19 and 32% of the total number of bacteria detected, and the percentage of bacteria reacting with the C. coccoides-E. rectale group probe varied between 23 and 30%. This finding is consistent with previous work on chicken cecal flora that observed appreciable variation in the composition of the cecal microflora of different chickens within a flock and even, at various times, within the same individual (Salani et al., 1974). In clone libraries of 16S rRNA sequences obtained from the bacteria of the cecal mucosa of nine different chickens, sequences from the C. leptum group accounted for between less than 10 and more than 40% of all the sequences, and sequences belonging to the C. coccoides group were present in the range of 0 to over
30%. Sequences belonging to the enterics and relatives were found in the range of 2% to more than 90% (Zhu et al., 2002).

In Situ Hybridization of Cecal Samples from Chickens of Different Ages

Earlier studies (Barnes et al., 1972; Mead and Adams, 1975) have demonstrated significant changes in the intestinal populations with age. Temporal thermal gradient gel electrophoresis analyses of 16S rRNA gene sequences obtained from cecal samples from chickens of different ages also indicated population changes (Zhu et al., 2002). The current FISH analysis of cecal content samples from two broiler chickens killed at five different ages (2 d, or 1, 2, 4, or 6 wk) demonstrated that at 2 d of age, only three probes (Enter1432, Lacto772, and Bif164) gave fluorescent signals, and the bacteria detected with these probes accounted for more than 90% of all bacteria detected (Table 2). This finding is consistent with an earlier report by Lev and Briggs (1956), who found that the bacterial population in the ceca can reach $10^{10}$/g within a few hours after hatching, but usually comprise only a few types, including enterococci, coliforms, and clostridia. Approximately 56 and 34% of the bacteria detectable with the universal probe in cecal content of 2-d-old chicks gave signals with the enteric and the Lactobacillus/Streptococcus/Enterococcus probe, respectively. At 1 wk of age, cecal content contained about 6% bacteria reactive with the enterics probe and between 26 and 29% bacteria with signals from the Lactobacillus/Streptococcus/Enterococcus probe. The corresponding values for cecal content from broilers 6 wk of age were approximately 3 and 9%. The decrease in the percentage of bacteria reactive with the enteric probe mirrored culture-based observations on a decrease in enteric bacteria with age. This decrease has been attributed to competition for nutrients and adhesion sites once anaerobic bacteria become established, the production of antibacterial compounds from lactic acid bacteria (Lactobacillus and Lactococcus spp.), or volatile fatty acids from Bifidobacteria spp. and Bacteroides spp. (Bonhoff et al., 1964; Gibson and Wang, 1994). Mead and Adams (1975) reported that the levels of coliforms and enterococci declined significantly when the anaerobes begin to predominate. A low percentage of enteric bacteria in cecal content of adult chickens has also been observed in other studies (Barnes, 1979; Netherwood et al., 1999). Culture-based studies (Smith, 1965; Mead and Adams, 1975) had shown that fecal coli-aerogenes bacteria predominated in the ceca of chicks for the first few days after hatching and that lactobacilli became established more slowly.

The probes for the C. leptum subgroup, C. coccoides-E. rectale group, and Bacteroides group did not produce signals at 2 d of age; however, at 1 wk of age, hybridization signals were observed. The percentage of bacteria reactive with the probe for the C. leptum subgroup increased from about 9% at 1 wk of age to about 32% at 6 wk of age, and the percentage of bacteria with hybridization signals from the probe for the C. coccoides-E. rectale group was highest in the birds killed at 2 wk of age and was lower again in the older birds. The percentage of bacteria hybridizing with the probes for the Bacteroides and Bifidobacterium groups remained relatively stable from the age of one week (3 to 8% and 3 to 6%, respectively).

For the cecal content samples, SYTO 13 DNA staining dye was used to detect and count the prokaryotes present.
The counts obtained with this dye were compared to counts obtained with the Bact338 and Arch915 for domain Bacteria and Archaea, respectively. Cells with strong hybridization signals from the Archaea probe were present at a similar level in birds of different ages and counts ranged from 0.7 to 3.3% of the counts obtained with SYTO 13 (data not shown). The hybridization data thus might be in agreement with those from earlier studies (Miller et al., 1986) that indicated the presence of methanogens in fecal material of adult chickens, but they are in conflict with results from PCR assays (Zhu et al., 2002) that failed to retrieve 16S rRNA gene sequences of Archaea from fecal samples using archaea-specific primer pairs A109F/A934R (Whitehead and Cotta, 1999). Surprisingly, hybridization signals with the Archaea probe were obtained even from samples of very young birds that apparently harbored few if any strict anaerobic bacteria. Further studies using other Archaea probes will be necessary to confirm the presence of Archaea.

The total number of cells detected with the Bact338 probe accounted for approximately 82 to 90% of the cells detected by SYTO 13. A number of technical factors can influence oligonucleotide probe hybridization and lead to differences in counts between a nucleic acid stain and probe hybridization. In addition, it is also possible that Bact338 might miss some bacterial phyla as observed by Daims et al. (1999).

The set of group-specific probes used for the current study was not expected to cover all bacteria detected by Bact338 probe. The probes detected 93% of the bacteria in content from broilers at 2 d (Table 2) presumably since the microbiota consisted of bacteria predominantly from the enteric and Lactobacillus/Streptococcus/Enterococcus groups. Coverage by the six probes decreased with age of the birds to as low as approximately 80%. These results suggest that as the birds age, an increasing number of bacteria that cannot be detected by universal probe Bact338 or the six group-specific probe set colonize the cecum. Therefore, there is a need to expand the spectrum of probes. For example, sequences from bacteria belonging to the Atopobium, Sporomusa, and Pseudomonas groups have been retrieved from cecal bacteria (Zhu et al., 2002), and probes covering these bacteria would be desirable. While this study was underway and after its completion, a number of studies using in situ hybridization with 16S rRNA-derived oligonucleotide probes for intestinal bacteria samples have been carried out (Schwiertz et al., 2000; Harmsen et al., 2000b, 2002; Tannock et al., 2000; Apostolou et al., 2001; Kleessen et al., 2001; Tuohy et al., 2001). Some of the probes used were those employed for this study, but additional probes were also introduced. For example, Tannock et al. (2000) included a probe for Atopobium, Eggertella, and Collinsella spp. in their study of human fecal bacteria.

Future advances in probe development such as the design of probes that are specific perhaps to the species level will allow the location and enumeration of bacterial species directly in cecal samples. In situ hybridization with 16S rRNA-targeted oligonucleotide probes will then provide a direct and quantitative estimate of the microbial composition and its natural variations in the chicken cecum or other intestinal sites without tedious culturing and identification. This approach can then be used to study the impact of various treatments on the avian intestinal microbiota and to obtain data to aid in formulating more effective competitive exclusion culture to prevent the pathogenic bacteria such as Salmonella and Campylobacter from colonizing the chicken cecum.

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