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Real-Time Quantitative PCR Measurement of Ileal *Lactobacillus salivarius* Populations from Broiler Chickens To Determine the Influence of Farming Practices▽†

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A real-time quantitative PCR assay targeting a 16S-23S intergenic spacer region sequence was devised to measure the sizes of populations of *Lactobacillus salivarius* present in ileal digesta collected from broiler chickens. This species has been associated with deconjugation of bile salts in the small bowel and reduced broiler productivity. The assay was tested as a means of monitoring the sizes of *L. salivarius* populations from broilers fed diets with different compositions, maintained at different stocking densities, or given the antimicrobial drugs bacitracin and monensin in the feed. Stocking densities did not influence the numbers of *L. salivarius* cells in the ileum. A diet containing meat and bone meal reduced the size of the *L. salivarius* population relative to that of chickens given the control diet, as did administration of bacitracin and monensin in the feed. These changes in the target bacterial population were associated with improved broiler weight gain.

Lactobacilli are common inhabitants of the crop and ileum of broiler chickens, whereas obligate anaerobes predominate in the cecum (5, 7, 14, 32). The bacterial species *Lactobacillus salivarius*, which is a resident of the ileum of the birds, has recently been associated with bile salt deconjugation and growth depression in broilers maintained under standard farming practices (8). It has been proposed that the metabolic activity of *L. salivarius* in the broiler small bowel reduces concentrations of conjugated bile salts (taurocholate and taurochenodeoxycholate), impairing lipid absorption and leading to reduced weight gain (8). This is analogous to "contaminated small bowel syndrome" in humans (6, 29), in which overgrowth of bacteria that produce bile salt hydrolase results in steatorrhea and weight loss due to impaired digestion and absorption of dietary lipid. Antibiotic therapy results in reduction of symptoms (6).

Antimicrobial drugs are added to broiler feeds as prophylaxis against infectious diseases and also to suppress bacterial populations whose presence affects the growth rate and the efficiency of nutrient acquisition (feed conversion) of the birds (4). The addition of antimicrobial drugs to animal feeds has been discouraged by regulatory measures in Europe, and consumer pressure will likely lead to voluntary withdrawal of the drugs from use in other countries (26, 27). Alternative means of maintaining current levels of broiler productivity are therefore required. Guban et al. (8) reported that the administration of antimicrobial drugs did not affect the total number of bacteria in the ileal digesta but, in addition to improving broiler performance, reduced the size of *L. salivarius* populations. They found that a representative isolate of *L. salivarius* deconjugated bile salts in pure culture in the laboratory and that it did the same thing in the ileal contents of chickens previously maintained in a protective *Lactobacillus*-free environment (8). These observations linked bile salt deconjugation in the ileum by *L. salivarius* and decreased weight gain of broilers. This outcome identified *L. salivarius* as a suitable bacterial target for development of methods that allow adequate growth of broilers without administration of antimicrobial drugs.

We developed a real-time quantitative PCR assay to monitor the size of *L. salivarius* populations in the ileal contents of broilers raised under different farming conditions, including feed with or without antimicrobials and altered dietary composition. Given that stocking density is a potential stress factor and since stress is known to influence the composition of the gut microbiota, including *Lactobacillus* populations (24), stocking density was also investigated. Production data (weight gain and feed conversion efficiency) were recorded for each treatment group. Additionally, the sizes of *L. salivarius* populations in birds on different poultry farms were compared to demonstrate the utility of the method for monitoring the bacteriology of commercial flocks.

MATERIALS AND METHODS

Birds, diets, and sampling. Three separate trials examining the effects of different poultry management practices on *L. salivarius* populations were conducted with male Ross broiler chickens kept on wood shavings in an environmentally controlled poultry house from days 1 to 35 after hatching. The birds

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were managed according to the recommendations of the New Zealand animal ethics committee, including normal commercial practices, and were fed nutritionally balanced diets (see Tables S1 and S2 in the supplemental material). In trial 1, the influence of dietary composition was tested by feeding four diets: diets A (maize-soy), B (maize-wheat-soy), C (maize-barley-soy), and D (maize-soy-meat and bone meal) (see Table S1 in the supplemental material). The stocking density was 20 birds/m^2. Trial 2 tested the effect of stocking density (number of birds per unit of area). The birds were fed diet A in this trial. Three stocking densities (16, 21, and 24 birds/m^2) were tested (equivalent to 40, 48, and 56 birds/pen). Trial 3 measured the effect of adding antimicrobial drugs (100 mg/kg monensin [Elancoban] and 100 mg/kg zinc bacitracin [Albac]) to the feed (see Table S2 in the supplemental material). The stocking density was 20 birds/m^2. Treatments were applied to five (trials 1 and 2) or four (trial 3) pens. Fifty birds per pen were used in trials 1 and 3. Performance data (bird weight and amount of feed consumed) were recorded at weekly intervals. In trials 1 and 2, two birds per pen were randomly selected each week for collection of ileal samples. In trial 3, four birds were selected per pen at 7, 14, and 21 days, and two birds were selected at 26 days of age. The birds were killed by cervical dislocation, and digesta samples were obtained from the terminal ileum. Samples were collected in sterile tubes, frozen immediately, and stored at −20°C until analysis. Forty-six digesta samples were also collected (trial 4) from 2- and 4-week-old broilers raised on five commercial poultry farms located in different geographical locations in New Zealand (Auckland, Waikato, New Plymouth, and Christchurch). This was done to test the utility of the method for monitoring *L. salivarius* populations in the ilea of birds in commercial flocks. Standard farming practices were followed on all five commercial farms. The stocking density was 16 birds/m^2, and wheat-based diets supplemented with exogenous xylanase enzyme, in-feed antibiotics, and coccidiostat were used on all of the farms.

Bacterial strains. The bacteria used to validate the *L. salivarius*-specific PCR primers and probe are listed in Table S3 in the supplementary material. These bacteria represent taxonomically and ecologically related gram-positive species known to inhabit the ileum of broilers (5, 11, 14). Clostridia were cultured using cooked meat medium (Difco) and Columbia agar (Difco) supplemented with 5% sheep blood, enterococci were cultured using fastidious anaerobic broth (Difco) and brain heart infusion medium (Difco), and lactobacilli were cultured using lactobacillus MRS medium (Remel). All bacteria were cultured at 37°C under anaerobic conditions.

DNA extraction. Total DNA of pure bacterial cultures and of ileal digesta was extracted as described previously (30). Briefly, 100 mg of ileal digesta was added to a sterile tube containing 300 mg of sterile zirconium beads (diameter, 0.1 mm) and suspended in 1 ml of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4). The suspension was vortexed thoroughly and centrifuged at 10,000 × *g* for 5 min. The supernatant was discarded, and the pellet was washed three times with 1 ml of PBS. After the final wash, the pellet was suspended in 1 ml of PBS. The cells were lysed by physical disruption with a mini-bead beater (Biospec Products, Bartlesville, OK) at 5,000 rpm for 3 min and placed on ice to cool. Subsequently, the sample was centrifuged at 10,000 × *g* for 5 min. A 600-μl portion of the supernatant was extracted sequentially with 600 μl of Tris-EDTA-saturated phenol (pH 6.6 or 7.9) and an equal volume of chloroform-isoamyl alcohol (24:1), and this procedure was repeated three times. The nucleic acids were precipitated with 2 volumes of cold ethanol and 0.1 volume of 3 M sodium acetate and then stored overnight at −20°C. The DNA was collected by centrifugation at 10,000 × *g* for 20 min, and the pellet was dried at 37°C for 1 h. Finally, the DNA was dissolved in 100 μl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

Development of primers and probe. Sequences (obtained by direct sequencing using fluorescent dye terminator technology or retrieved from the NCBI database) of the 16S-23S intergenic spacer region of *L. salivarius* and taxonomically and ecologically related bacteria were aligned using CLUSTAL-W (http://www.ebi.ac.uk/clustalw/) and were examined for regions unique to *L. salivarius*. PCR primers and a probe specific to *L. salivarius* were then designed using Primer Express v1.5 (Applied Biosystems) by following the manufacturer's instructions. The forward primer For-Sal-3 (5′-GTCGTAACAAGGTAGCCGTAGGA-3′) and the reverse primer Rev-Sal-1 (5′-TAAACAAAGTATTCGATAAATGTACAGGTT-3′) were used to amplify a 97-bp product. The minor groove binder (MGB) probe SalivarISRDes-4 (5′-CGGCTGGATCACC-3′) was labeled with the fluorescent reporter dye 6-carboxyfluorescein at the 5′ end, and there was a nonfluorescent quencher at the 3′ end. Primers were synthesized by Sigma-Proligo (Australia), and the MGB probe was synthesized by Applied Biosystems. The specificities of candidate primer-probe sets were evaluated by performing BLASTN (1) homology searches for short, nearly exact matches against the NCBI database and subsequently by performing SYBR green and fluorogenic 5′ nuclease PCR assays. The concentrations of the forward and reverse primers were 9 and 3 μM, respectively, for the SYBR green PCR assays, and the concentrations of the forward primer, reverse primer and MGB probe were 3, 9, and 1.75 μM, respectively, for the fluorogenic 5′ nuclease PCR assays.

SYBR green PCR assay conditions. Amplification reaction mixtures (total volume, 25 μl) contained 5 μl of reference strain template DNA (1 ng/μl) (see Table S3 in the supplemental material), 12.5 μl of 2× SYBR green PCR master mixture (Applied Biosystems), 2.5 μl of each primer, and sterile ultraPURE distilled water (GibcoBRL). For the no-template control, sterile ultraPURE distilled water (GibcoBRL) was used instead of the template DNA. Tests were performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems). Before amplification, the reaction mixture was heated to 50°C for 2 min, followed by 95°C for 10 min. Subsequently, standard thermal cycling conditions consisting of 40 cycles of 95°C for 15 s and 60°C for 1 min were used. To determine the specificity of the SYBR green PCR assay, a dissociation curve analysis or a melting analysis of the DNA fragments was performed according to the manufacturer's instructions.

Fluorogenic 5′ nuclease PCR assay conditions. Amplification reaction mixtures contained 5 μl of either a 1-ng/μl reference strain template DNA solution (see Table S3 in the supplemental material) or gut digesta template DNA, 12.5 μl of 2× Taqman universal PCR master mixture (Applied Biosystems), and 2.5 μl of primers and probe. Each sample was analyzed once, with 10 replicates per treatment. For the no-template control, sterile ultraPURE distilled water (GibcoBRL) was used instead of the template DNA. Four calibration standards (0.001, 0.1, 1, and 100 ng/μl) of *L. salivarius* subsp. *salivarius* ATCC 11741 DNA were used in each run. The thermal cycler conditions were the same as those used in the SYBR green PCR assays.

Determination of specificity and the limit of detection. The specificity of the primers was first determined by a SYBR green PCR assay using purified template DNA from reference strains of *L. salivarius* and other bacteria (see Table S3 in the supplemental material) as described above. The analysis was subsequently repeated with the MGB probe in a fluorogenic 5′ nuclease PCR assay. The limit of detection for the assay was determined with concentrations of purified DNA of *L. salivarius* subsp. *salivarius* ATCC 11741 ranging from 0.001 to 100 ng in duplicate. Concentrations were determined using a combination of GenQuant II (Pharmacia Biotech) and a low-molecular-mass DNA ladder (Life Technologies) comparison in agarose gels.

Estimation of the *L. salivarius* genome size. Two methods were used to estimate genome size: pulsed-field gel electrophoresis (PFGE) and calculation of the average genome size for strains originating from chickens using the method of Byun et al. (2). The PFGE method used was adapted from the methods of Liu et al. (13) and Tynkkynen et al. (28). DNA in plugs was digested using I-Ceu I (New England Biolabs).

Calculation of bacterial cell numbers. To estimate the amounts of DNA in broiler digesta samples, the threshold cycle (C_T) values were first converted into amounts of DNA using the calibration standards. The C_T values for the calibration standards were regressed onto the $\log_{10}$ DNA amount, allowing a different equation for each run. For these regressions, the 0.001-ng/μl calibration standards were excluded, as some of these had C_T values of 40 (i.e., no detectable response). The regressions generated a very close fit to the data (R^2 = 98.7 over all runs). Using the results of the regressions, the C_T values for each sample (omitting C_T values of 40) were converted into estimated amounts of DNA in ng/μl. Samples with a C_T value of 40 were defined as samples that contained no DNA. To convert amounts of DNA to ng/mg of digesta, the amount of digesta in each assay well had to be determined. Since DNA from an average 3 mg of gut digesta was represented in each well of the reaction plate (see above) and the calibration standards were measured in ng of genomic DNA/μl, the estimated DNA levels from the main statistical analysis described below were expressed in ng of DNA per 3 mg/5 μl (= 0.6 mg of gut digesta/μl of digesta DNA). These estimates were converted to estimated ng of DNA/mg of digesta. To convert estimated amounts of DNA to numbers of bacterial cells (estimated number of cells per mg digesta), knowledge of the size of the genome was necessary. Since this information was not known for *L. salivarius* at the time, an estimate of genome size was calculated using PFGE analyses, as well as by calculating the average genome size for strains originating in chickens using the method of Byun et al. (2) (see above), which gave an average value of 1,950,666.7 bp. This value was converted into mass per genome using Avogadro's number (Na) (6.02214 × 10^{23} bp/mol) and an average molecular mass of a base pair (M) of 609.5 g/mol as follows: mass of DNA in ng = $10^9 \times (1{,}950{,}666.7 \times M)/\text{Na}$. The digesta estimates in ng DNA/mg were converted to estimated numbers of *L. salivarius* cells per mg of digesta by dividing the estimated DNA mass by this genome mass.

Statistical analysis. (i) Performance data. Weight gain, feed intake per bird, and feed conversion efficiencies were calculated for each pen from the raw bird weights and feed weights. The data were adjusted for bird mortality (natural

TABLE 1. Populations of *L. salivarius* in ileal digesta of broilers fed different diets[a]

Age of birds (wk)	Mean $\log_{10}$ no. of lactobacilli/mg of digesta (95% confidence limits)			
	Diet A	Diet B	Diet C	Diet D
3	6.4 (6.1–6.7)	6.1 (5.8–6.5)	4.9 (4.5–5.3)	5.0 (4.5–5.5)
4	6.0 (5.6–6.4)	5.6 (5.3–5.9)	4.7 (4.3–5.1)	3.0 (2.7–3.3)
5	6.0 (5.7–6.4)	4.3 (3.9–4.6)	5.7 (5.2–6.2)	4.1 (3.5–4.6)

[a] Diet A, maize and soy; diet B, maize, wheat, and soy; diet C, maize, barley, and soy; diet D, maize, soy, meat, and bone meal.

TABLE 3. Effect of antimicrobial drugs in feed on the numbers of *L. salivarius* cells in the ilea of broilers

Age of broilers (days)	Mean $\log_{10}$ no. of lactobacilli/mg of digesta (95% confidence limits) with:	
	No drugs	Bacitracin and monensin
7	5.1 (4.3–6.0)	3.0 (2.6–3.4)
14	5.3 (4.8–5.8)	3.5 (3.1–4.0)
21	6.1 (5.8–6.4)	6.1 (5.7–6.5)
26	6.3 (6.0–6.7)	6.0 (5.6–6.3)

mortality and birds removed for sampling). The calculated data were analyzed by analysis of variance.

(ii) Real-time quantitative PCR data. An analysis of the estimated DNA amounts was carried out separately for each of the four trials, using the method of Murray et al. (16). Briefly, since the estimated amount of DNA is an indirect estimate of the number of whole-genome molecules (i.e., cells) of the target bacterium in a sample, the DNA estimates have many of the properties of count data. Thus, methods appropriate for count data were used to analyze these data. A mixed modeling approach was used to allow assessment of random factors such as differences between pens and between real-time PCR runs. The models were fitted using a hierarchical generalized linear model (HGLM) (12), which is a mixed model extension of the common Poisson generalized linear model with a logarithmic link (15) for count data. The HGLM also allows the variability in the data at the lowest level (dispersion) to be modeled according to other factors in the data. Random effects for pens and real-time PCR runs were not found to be important, so they were not included in the final analyses. However, the dispersion did vary substantially between treatments in many cases, so adjustments for this were included. Comparisons between treatments and sample times for each trial were made within the HGLM analysis, using contrasts. Confidence limits for the estimated mean amount of DNA for each treatment were calculated using the estimated standard errors for the estimates on the logarithmic scale and then back-transformed. These data and the estimated mean DNA amounts were then converted into approximate numbers of *L. salivarius* cells as described above. All analyses were carried out with GenStat (release 9, parts 1 to 3, 2006; GenStat Committee; R. W. Payne, VSN International, Oxford, United Kingdom). A P level of 0.05 was used throughout the analyses to determine significance.

RESULTS

Primer and probe design and specificity. In SYBR green PCR assays, average C_T values of 18.40 and 39.92 were obtained for the *L. salivarius* strains and negative controls, respectively, validating the specificity of the assay for *L. salivarius*. The dissociation curve analysis revealed a single melting peak at 78°C corresponding to the melting temperature of the specific amplified product. No other products from the negative controls were detected, indicating a lack of interference from nontarget bacteria, further indicating the specificity of the signal. The analysis was subsequently repeated with the primers and MGB probe using a fluorogenic 5′ nuclease PCR assay. Average C_T values of 18.79 and 39.98 were obtained for the *L. salivarius* strains and negative controls, respectively. No amplification (C_T, 40) was observed for negative controls that did not contain DNA template. DNA amounts equivalent to less than one *L. salivarius* cell per mg of digesta could be detected.

Impact of dietary composition on the number of *L. salivarius* cells in the ileum and on production data (trial 1). *L. salivarius* populations were larger in the ileum of broilers fed diet A throughout the production cycle than in the ileum of birds fed diet D ($P < 0.05$) (Table 1). The *L. salivarius* populations were about 100 times smaller in birds fed diet D than in birds fed diet A. Diet D-fed broilers showed the greatest weight gain ($P < 0.05$), but feed conversion was not significantly improved ($P = 0.11$) (see Table S4 in the supplemental material).

Impact of stocking density on the number of *L. salivarius* cells in the ileum and on production data (trial 2). Stocking density did not significantly affect the numbers of *L. salivarius* cells in ileal samples ($P > 0.05$) (Table 2). The highest stocking density (24 birds/m^2) resulted in decreased efficiency of feed conversion ($P = 0.02$) (see Table S5 in the supplemental material).

Impact of antimicrobials on the number of *L. salivarius* cells in the ileum and on production data (trial 3). Antimicrobials (bacitracin and monensin) administered in the feed reduced the size of the *L. salivarius* population in the ileum for the first 14 days of treatment ($P < 0.05$) (Table 3), after which the numbers of bacterial cells were similar to those in untreated birds ($P > 0.05$). Weight gain and feed conversion were improved as a result of antimicrobial drug administration; however, the differences were not significant ($P > 0.05$) (see Table S6 in the supplemental material).

***L. salivarius* populations in the ileal digesta of broilers on commercial poultry farms (trial 4).** The *L. salivarius* populations in the ilea of broilers were similar for all farms ($P > 0.05$), except that the number of bacteria was lower in ileal samples collected from birds that were 2 weeks old on farm D ($P < 0.05$) (Table 4).

TABLE 2. Effect of stocking density on numbers of *L. salivarius* cells in the ileum of broilers

Age of broilers (wk)	Mean $\log_{10}$ no. of lactobacilli/mg of digesta (95% confidence limits)		
	16 birds/m^2	20 birds/m^2	24 birds/m^2
3	5.0 (4.4–5.6)	4.3 (3.8–4.8)	4.1 (3.5–4.6)
4	4.3 (3.8–4.8)	4.5 (4.0–5.1)	4.5 (3.9–5.1)
5	4.7 (4.1–5.1)	5.4 (4.8–6.0)	4.0 (4.1–5.1)

TABLE 4. Comparison of *L. salivarius* populations in the ilea of broilers raised on different poultry farms

Age of broilers (wk)	Mean $\log_{10}$ no. of lactobacilli/mg of digesta (95% confidence limits)				
	Farm A	Farm B	Farm C	Farm D	Farm E
2	4.6 (3.8–5.4)	4.7 (4.1–5.3)	5.3 (5.1–5.4)	2.1 (1.3–3.0)	4.4 (3.8–5.0)
4	4.9 (4.4–5.3)	5.4 (4.9–5.9)	5.0 (4.4–5.7)	5.6 (5.0–6.1)	6.0 (5.4–6.5)

DISCUSSION

Studies aimed at devising poultry management practices that would result in reduced populations of *L. salivarius* in the ileum required a specific assay system. The assay had to be specific for *L. salivarius* and be appropriate for quantification of this bacterium in ileal samples collected in various parts of New Zealand. Real-time PCR has been used to measure populations of *Campylobacter jejuni*, *Clostridium perfringens*, and *Lactobacillus thermotolerans* in the gut or feces of chickens (20, 21, 31). Real-time quantitative PCR provided a suitable assay method for *L. salivarius* because, on the basis of in silico and laboratory evaluations, it was a specific and sensitive assay for this species and, since it was DNA based, could be used with samples that had been frozen for transportation to the analytical laboratory. The 16S-23S intergenic spacer sequence of *L. salivarius* proved to be a suitable region of DNA to target for these real-time assays, as has been reported previously for other *Lactobacillus* species (22, 25). The results obtained with the PCR-based assay cannot be directly compared with plate counts (CFU) because a single bacterial colony can result from multiplication of one or several cells, whereas our assay quantifies the bacteria on the basis of genome copies. Valid, relative comparisons of treatment groups result, however, from application of a standard methodology.

The bacterial community inhabiting the ilea of broilers is likely to affect the efficiency of utilization of food constituents. Lipid absorption occurs maximally in the distal jejunum and proximal ileum of broilers (19). Deconjugation of conjugated bile salts in the ileum by *L. salivarius* could thus affect the efficiency of food conversion because these substances have critical roles in the emulsification and absorption of dietary lipids (10, 23). Young birds are less able to absorb lipids (9, 17), so suboptimal concentrations of conjugated bile salts resulting from bacterial hydrolytic activity could influence weight gain and feed conversion in early stages of the broiler production cycle. Moreover, the "enterogastric reflex" of birds results in reflux of the digesta by antiperistaltic movements at 15- to 20-min intervals (3). Therefore, bacterial activities in the ileum could affect the physiology of the whole small bowel as a result of this retrograde movement of the digesta.

Poultry management aimed at reducing the numbers of *L. salivarius* cells in the ileal digesta would provide a strategy to improve broiler production outcomes. Our research using the real-time quantitative PCR assay showed that the numbers of *L. salivarius* cells were reduced in broilers given antimicrobial drugs during the first part of the production cycle (the initial 2 weeks), as has been described previously (8). A reduction in the numbers of *L. salivarius* cells was associated with improved weight gain and greater efficiency of food conversion, although larger numbers of birds would have to be examined to obtain statistically significant results. Stocking density, although affecting the production data, did not alter the sizes of *L. salivarius* populations. Addition of meat and bone meal to the diet, however, resulted in reduced *L. salivarius* populations in the ileum and heavier birds compared to the standard diet. Protein in meat and bone meal is poorly digested (18), resulting in larger amounts of amino acids reaching the distal bowel, which might, due to the presence of an increased nitrogen source, alter the proportions of different bacterial types inhabiting the ileum. Feed conversion ratios did not differ, but this part of the study clearly demonstrated that a reduction in the numbers of *L. salivarius* cells could be achieved by modification of dietary composition rather than by use of antimicrobial agents. The results of our study demonstrated the utility of real-time quantitative PCR for monitoring *L. salivarius* populations in gut digesta collected from commercial flocks in various disparate geographical locations and provide a basis for monitoring specific modifications of the bacterial community of the broiler ileum in future studies.

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