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Quantitative molecular assays for evaluating changes in broiler gut microbiota linked with diet and performance

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Abstract. Changes in the levels of specific gut bacteria have been linked to improved broiler feed efficiency. Quantitative polymerase chain reaction (qPCR) assays were developed to five potential performance-related bacteria (*Lactobacillus salivarius*, *L. crispatus*, *L. aviarius*, *Gallibacterium anatis* and *Escherichia coli*) and generic eubacteria. These were used to screen broiler gut samples from four geographically diverse Australian feeding trials showing significant treatment-related differences in feed efficiency. It was our aim to validate the association of particular bacteria with broiler feed efficiency across a broad range of environmental and dietary conditions, and hence to evaluate their predictive potential for monitoring broiler performance. Across trials *L. salivarius*, *L. crispatus*, *L. aviarius*, *E. coli* and total eubacterial numbers were significantly altered by diet, environment (litter), and/or sex of birds. Furthermore, changes in the numbers of these gut bacteria were significantly linked to broiler performance. Lactobacilli and total eubacteria were significantly decreased in birds that were more feed efficient. *E. coli* was not consistently linked with either improved or decreased performance and these discrepancies may be due to differences at the strain level which were not detectable using our assays. *G. anatis* was detected only in two of the four trials and found not to be significantly linked with broiler performance. These qPCR assays have been useful in either validating or disproving previous reported findings for the association of specific gut bacteria with broiler feed efficiency. This qPCR format can be easily expanded to include other organisms and used as a quantitative screening tool in evaluating dietary additives for improved broiler production.

Additional keywords: feed conversion efficiency, microbial diversity, poultry, poultry nutrition.

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Introduction

Gut microbiota play an important role in animal health, performance and nutrition (Gordon and Pesti 1971; Hammons *et al*. 2010). Although knowledge of the ideal gut microbiota is still incomplete, it is apparent that a variety of diets can equally support optimal broiler performance and maintain a healthy gut microbial balance (Torok *et al*. 2011*b*). Several factors can influence the host's gut microbiota, including age, genetics, rearing environment and stress, although the greatest determinant by far is the host's diet (Lu *et al*. 2003; Burkholder *et al*. 2008; Torok *et al*. 2009, 2011*b*; Lumpkins *et al*. 2010). All these factors also have the potential to influence the animal's performance as measured by growth rate, feed efficiency or productivity, hence indicating a close relationship between gut microbiota and animal performance (Torok *et al*. 2011*b*).

Numerous studies have been undertaken to determine the influence of dietary changes on the structure of the gut microbial community, by using microbiological culturing techniques, culture-independent molecular techniques and indirect measurement of bacterial metabolic products (Bjerrum *et al*. 2006; Choct *et al*. 1999; Hubener *et al*. 2002; Zhu *et al*. 2002; Yin *et al*. 2010). However, understanding how diet-induced changes in the composition of the gut bacterial community relate to important metabolic changes, and ultimately to broiler health and performance, is less clear. Diet-related changes in gut microbiota do not always translate into altered broiler performance (Gunal *et al*. 2006; Pedroso *et al*. 2006; Geier *et al*. 2009), so caution needs to be exercised in identifying gut bacteria linked with broiler performance from limited animal trials. As such, tools need to be developed that can quantitatively validate findings across numerous performance trials, as many molecular techniques (denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism, pyrotag sequencing and phlyogenetic microarrays) used to investigate microbial community structure are at best semiquantitative (Amend *et al*. 2010; Zhou *et al*. 2011; Fraher *et al*. 2012).

We have previously shown that diet-related changes in gut microbiota are linked to broiler performance as measured by feed efficiency, and have identified a common suite of bacterial groups associated with significant changes in performance across various feeding trials (Torok *et al*. 2011*b*). Using microbial profiling, we were able to identify eight operational taxonomic units (OTU) related to differences in broiler performance across three

Australian feeding trials. Targeted cloning and sequencing of these eight OTUs revealed that they could represent 26 different bacterial species or phylotypes. In the present study, we developed quantitative polymerase chain reaction (qPCR) assays to the following two of these performance-related OTUs: OTU 492, which was associated with improved broiler performance and potentially represented *Escherichia coli* or *Gallibacterium anatis*, and OTU 564–566, which was associated with decreased broiler performance and potentially represented *Lactobacillus crispatus*, *L. salivarius* or *L. aviarius*. It was our aim to use these assays, along with a generic eubacterial qPCR assay, to screen banks of chicken gut DNA generated from four broiler feeding trials showing significant performance differences, hence determining the diagnostic potential of these quantitative assays in determining broiler performance.

Materials and methods

Broiler feeding trials

Four independent broiler feeding trials demonstrating significant differences in bird performance as measured by feed efficiency (apparent metabolisable energy; AME or feed conversion ratio; FCR) were investigated.

In Trial I, gut samples were collected from two groups of 24 broiler chickens (12 males and 12 females) that were each fed a barley-based control diet or barley-based diet supplemented with non-starch polysaccharide (NSP)-degrading feed enzyme product (Torok *et al*. 2008). Previously, AME had been shown to be significantly higher for chickens fed the barley-plus-enzyme diet than for chickens fed the control barley diet. No effect of bird gender on AME, or interaction between diet and sex, was detected (Torok *et al*. 2008).

In Trial II, gut samples were collected from 48 male broiler chickens that had been fed one of the following four diets: wheat control supplemented with xylanase; sorghum B; commercial sorghum; and commercial sorghum supplemented with phytase (n = 12 birds/treatment) (Torok *et al*. 2011*b*). It had been previously shown that liveweight gain was significantly ($P < 0.05$) decreased for birds on the wheat control diet supplemented with xylanase as compared with those on the sorghum B, commercial sorghum and commercial sorghum supplemented with phytase diets (Perez-Maldonado and Rodrigues 2009). Furthermore, feed conversion efficiency was significantly ($P < 0.05$) decreased for birds at 0–42 days on the wheat control diet as compared with those on any of the sorghum-based diets (Perez-Maldonado and Rodrigues 2009).

In Trial III, gut samples were collected from 96 broiler chickens that had been raised on one of the following four treatments: paper litter and low-fibre diet; wood litter and low-fibre diet; paper litter and high-fibre diet; and wood litter and high-fibre diet (n = 24 birds/treatment; 12 males and 12 females) (Torok *et al*. 2011*b*). It had been shown that litter type in combination with diet composition significantly ($P < 0.05$) influenced FCR (Torok *et al*. 2011*b*). Birds were more feed efficient on a low-fibre diet if housed on wood litter than if housed on paper litter. Sex of birds also significantly ($P < 0.001$) influenced FCR, with males having a better feed efficiency than females. The bodyweight of male chickens was also significantly ($P < 0.001$) greater than that of the female chickens. Litter type in combination with sex was found to significantly ($P < 0.05$) alter bodyweight, with females growing slower on paper litter than on the wood litter (Torok *et al*. 2011*b*).

In Trial IV, gut samples were collected from 96 broiler chickens that had been fed one of four dietary treatments A, B, F and G (n = 24 birds/treatment; 12 males and 12 females) (Torok *et al*. 2011*b*). Although diet composition varied, Treatments A and B were predominantly sorghum based, while Treatments F and G were predominantly wheat based. Significant differences in FCR among dietary treatments had been previously reported for these birds (Torok *et al*. 2011*b*). Birds on the Treatment G and F diets had significantly improved feed efficiency compared with birds on Treatment A and B diets. Birds fed the Treatment G diet had the most significantly improved feed efficiency of birds on any of the diets investigated. Sex of birds also significantly ($P < 0.001$) influenced FCR, with males having a better feed efficiency than females.

Nucleic acid extraction

Total nucleic acid was extracted from the digesta and associated tissue from the ilea and caeca of individual chickens as previously described (Torok *et al*. 2011*b*, 2008). Total nucleic acid was extracted from ~0.5 g of freeze-dried material.

Quantitative PCR

Quantitative PCR primers (Table 1) were designed to target organisms using Primer3Plus (Untergasser *et al*. 2007) and tested for specificity *in silico* by using Primer-BLAST (http://blast.ncbi.nlm.nih.gov/, verified 26 April 2013). All qPCR assays were based on SYBR green detection. Sensitivity of qPCR assays was tested against dilutions of known amounts of target (plasmid standards). Sequences of 16S rRNA representative of *L. crispatus* (JF798181), *L. salivarius* (JF798161), *L. aviarius* (JF798223),

Table 1. Primers used in qPCR assays

qPCR assay	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Reference
Gallibacterium anatis	GanatF: AACGGTAACGGGTTGAAAGC	GanatR: CCTTTCATCTCTCGATTCTATGC	This study
Escherichia coli	EcoliF: CATAATGTCGCAAGACCAAAGAG	EcoliR: GGTAACGTCAATGAGCAAAGGT	This study
Lactobacillus salivarius	LsaliF: GATCGCATGATCCTTAGATGAA	LsalivR: GCCGATCAACCTCTCAGTTC	This study
L. aviarius	LaviarF: TGACCGCATGGTCATTATGTA	LaviarR: CAACTCGGCTACGTATCATCAC	This study
L. crispatus	LcrispF: GCGAGCGGAACTAACAGATTT	LcrispR: TGATCATGCGATCTGCTTTCT	This study
Eubacterial	EubactF CCTACGGGAGGCAGCAG	EubactR: ATTACCGCGGCTGCTGGC	(Zozaya-Hinchliffe *et al*. 2010)

G. anatis (JF798066), *E. coli* (JF798201) and eubacteria (JF798192) were cloned into pGEM-T (Promega, Madison, WI, USA), as previously described (Torok *et al*. 2011*a*, 2011*b*). Plasmids were quantified using spectrophotometry (NanoDrop 2000, Thermo Scientific, Wilmington, DE, USA). The number of plasmid copies per microlitre was calculated using the following formula:

$$\text{Plasmid number (copies/}\mu\text{L)} = \text{(plasmid concentration (g/}\mu\text{L)} \times \text{Avagadro's number)/(plasmid size (bp)} \times \text{molar mass)},$$

where Avagadro's number is 6.022×10^{23} molecules/mol and the average molar mass for double-stranded DNA is 660 g/mol.

All plasmids were diluted to 2×10^{10} copies/μL. Standard curves were produced from a 10-fold dilution series, using the following range: 10^6 to 1 copy for specific bacteria and 10^8 to 10^2 copies for general eubacterial detection. The efficiency of each assay was determined using the plasmid standards (Table 2). Specificity of qPCR assays was confirmed visually using gel electrophoresis, as well as by determining the nucleotide sequence of resulting PCR amplicons. Purified PCR amplicons were cloned and recombinant plasmid recovered and sequenced, as previously described (Torok *et al*. 2011*a*). All qPCR amplicons were generated from total nucleic acid extract obtained from chicken gut digesta and associated gut tissue that was representative of a diverse bacterial template. Sequencing was performed from five independent clones per assay. All sequences were ≥99% identical with the target.

All qPCR assays were run in a 384-well format, with master mix and template being dispensed using a Biomek 3000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA). qPCR was performed in a reaction volume of 10 μL, containing 2 μL of total nucleic extract, 50 nM each primer (Table 1; Sigma Aldrich, Castle Hill, NSW, Australia) and 2 × *Power* SYBR Green PCR Master Mix (Applied Biosystems, Mulgrave, Vic, Australia), according to manufacturer's recommendations. All reactions were run on a 7900HT Sequence Detection System (Applied Biosystems), with the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. To determine the specificity of all SYBR green qPCR assays, a dissociation-curve analysis of amplified DNA fragments was performed following PCR in the range of 60−95°C. All data were analysed using the 7900HT v2.3 SDS software (Applied Biosystems). Raw data were analysed for *L. salivarius*, *E. coli*, *G. anatis* and *L. crispatus* assays, by using a cycle threshold (Ct) of 0.2 and a baseline of 3–10. Assays for eubacteria used a Ct of 0.2 and a baseline of 2–10 and assays for *L. aviarius* used a Ct of 0.05 and an automatic baseline. Bacterial copies were calculated using standard curves.

Table 2. Determination of qPCR assay efficiency

Ct cycles versus plasmid copy number (log10) were plotted to calculate the slope. Real-time PCR efficiencies were calculated according to the equation Efficiency (E; %) = $(10^{[-1/\text{slope}]} - 1) \times 100$

Assay	Slope	*Y*-intercept	R^2	Efficiency (%)	Amplicon length (bp)
Gallibacterium anatis	–3.502	31.34	0.999	93	137
Escherichia coli	–3.782	34.27	1	84	312
Lactobacillus salivarius	–3.506	33.12	0.999	93	130
L. aviarius	–4.744	43.23	0.998	63	117
L. crispatus	–3.390	30.82	1	97	150
Eubacterial	–3.493	32.44	0.996	93	195

Statistical analyses

Data obtained from individual birds were analysed by ANOVA, with significant ($P < 0.05$) differences determined between treatment means by the least significant difference (l.s.d.) method in GENSTAT v14 (VSN International, Hemel Hempstead, UK). The bacterial copy numbers were logged (ln (x)) before analysis by ANOVA. The covariate ln(copy number) eubacteria was used to standardise gross bacterial levels.

Results

Trial I

Quantitative PCR of *L. salivarius* showed that this microorganism was present in significantly higher numbers within the ileum of the improved performing broilers on the barley plus enzyme diet than within the ileum of birds on the barley control diet (Table 3). However, within the caeca, the numbers of *L. salivarius* were significantly higher in the birds fed the barley control diet (Table 4). Within the caeca, the numbers of *E. coli* were also significantly increased in the poorer-performing birds on the barley control diet. No diet-related differences were detected in total eubacterial or *L. crispatus* numbers in either gut sections. *G. anatis* and *L. aviarius* were not detected in any of the birds from this trial. Gender of birds was found to significantly influence total eubacterial numbers within the caeca, with males having increased numbers of eubacteria compared with females (Table 4).

Trial II

The only bacterial species significantly affected by diet was *E. coli* within the caeca (Tables 3, 4). Better-performing birds on the sorghum-based diets had significantly higher numbers of *E. coli* than did the poorer-performing birds on the wheat control diet. *G. anatis* was only infrequently detected within the ilea and caeca of birds within this trial (in 10 of 48 birds), with the same birds testing positive for both gut sections.

Trial III

Total eubacterial, *E. coli* and *L. aviarius* numbers within the ilea (Table 3), as well as, *L. salivarius* numbers within the caeca (Table 4), were significantly increased in birds fed the low-fibre diet. Ileal and caecal eubacterial numbers were also significantly increased in birds raised on wood litter as opposed to paper litter. Male broilers had significantly increased ileal eubacterial and *L. aviarius* numbers (Table 3) and decreased caecal *L. crispatus* numbers (Table 4) compared with female birds. Significant diet by sex and litter by sex interactions were also detected for ileal bacterial communities (Table 3). Females on the high-fibre diet had lower numbers of total eubacteria than did females on the

Table 3. qPCR quantification of bacteria within the ilea of broiler chickens from four feeding trials

See text for explanation of treatments in different trials. Least significant differences (l.s.d.) among treatment means (ln(copy number)) are shown. Within columns, means followed by the same letter for each factor are not significantly different ($P > 0.05$). n.s., not significant. ND, not detected

Trial	Attribute	*n*	Eubacteria	OTU 492		OTU 564–566		
				Escherichia coli	*Gallibacterium anatis*	*Lactobacillus crispatus*	*L. salivarius*	*L. aviarius*
Trial I								
					Diet			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	1.05	ND
	Barley control	48	10.22	6.48		5.63	5.50a	
	Barley enzyme	48	10.47	6.09		5.93	6.92b	
					Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	n.s.	ND
	Female	48	10.14	6.31		5.66	6.32	
	Male	48	10.56	6.26		5.90	6.11	
					Diet × Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	n.s.	ND
Trial II								
					Diet			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Wheat+xyl	12	9.58	8.49	2.11	6.39	5.22	7.38
	Sorg. B	12	9.89	9.03	0.44	5.93	5.66	6.45
	Sorg. comm	12	9.68	8.77	2.03	7.04	5.38	6.14
	Sorg. comm+phyt	12	9.44	8.71	1.08	7.46	6.92	7.04
Trial III								
					Diet			
	l.s.d. ($P < 0.05$)		0.35	0.77	ND	n.s.	n.s.	1.30
	High fibre (HF)	48	10.67a	8.51a		6.37	9.06	1.07a
	Low fibre (LF)	48	11.13b	9.58b		6.66	9.45	2.57b
					Litter			
	l.s.d. ($P < 0.05$)		0.35	n.s.	ND	n.s.	n.s.	n.s.
	Paper	48	10.69a	9.04		6.65	9.35	1.86
	Wood	48	11.11b	9.06		6.38	9.16	1.78
					Sex			
	l.s.d. ($P < 0.05$)		0.35	n.s.	ND	n.s.	n.s.	1.30
	Female	48	10.72a	9.17		6.63	9.43	1.00a
	Male	48	11.09b	8.93		6.40	9.08	2.64b
					Diet × Sex			
	l.s.d. ($P < 0.05$)		0.50	n.s.	ND	n.s.	n.s.	2.10
	HF female	24	10.30a	8.66		6.61	9.41	0.68a
	LF female	24	11.14b	9.67		6.65	9.46	1.32a
	HF male	24	11.05b	8.36		6.13	8.72	1.45a
	LF male	24	11.12b	9.50		6.68	9.44	3.82b
					Litter × Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	0.96	n.s.	n.s.
	Paper female	24	10.45	9.27		6.39ab	9.37	0.93
	Wood female	24	10.98	9.06		6.88b	9.50	1.08
	Paper male	24	10.94	8.80		6.92b	9.32	2.79
	Wood male	24	11.23	9.05		5.89a	8.83	2.48
					Diet × Litter			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	n.s.	n.s.
					Diet × Litter × Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	n.s.	n.s.
Trial IV								
					Diet			
	l.s.d. ($P < 0.05$)		0.49	1.71	n.s.	0.79	0.97	0.85
	A	24	10.34b	4.28b	3.58	7.33c	6.91b	5.61b
	B	24	10.23b	2.34a	3.93	7.39c	6.60b	5.46b

Continued next page

Table 3. (*continued*)

Trial	Attribute	*n*	Eubacteria	OTU 492		OTU 564–566		
				Escherichia coli	*Gallibacterium anatis*	*Lactobacillus crispatus*	*L. salivarius*	*L. aviarius*
	F	24	9.19a	2.46a	4.41	6.34b	6.62b	4.54a
	G	24	10.70b	5.98b	5.24	5.43a	5.58a	6.02b
					Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Female	48	10.02	3.91	4.59	6.46	6.22	5.56
	Male	48	10.21	3.63	3.99	6.80	6.63	5.26
					Diet × Sex			
	l.s.d. ($P < 0.05$)		n.s.	2.33	n.s.	1.08	1.32	n.s.
	A female	12	10.29	5.25cd	3.67	6.54bc	5.78ab	5.51
	A male	12	10.38	3.31abc	3.48	8.12d	8.04c	5.72
	B female	12	10.27	2.73ab	4.69	7.64d	6.61ab	5.43
	B male	12	10.20	1.95a	3.16	7.15cd	6.58ab	5.49
	F female	12	8.85	3.12abc	5.08	6.29abc	7.08bc	4.73
	F male	12	9.54	1.80a	3.74	6.40abc	6.17ab	4.35
	G female	12	10.66	4.53bc	4.91	5.35a	5.42a	6.57
	G male	12	10.73	7.43d	5.58	5.51ab	5.74a	5.48

low-fibre diet, while males on the high-fibre diet had lower numbers of *L. aviarius* than did males on the low-fibre diet. Furthermore, males raised on wood litter had significantly lower *L. crispatus* numbers than did males raised on paper litter (Table 3). Within this trial, *L. aviarius* was only infrequently detected within the ilea (in 34 of 96 birds) and caeca (in 15 of 96 birds), while no *G. anatis* was detected in either the ileum or caeca of any of these chickens.

Trial IV

Diet had a significant influence on most of the bacterial species investigated. Total eubacterial numbers were significantly decreased within both the ileum (Table 3) and caeca (Table 4) of the better-performing birds fed Treatment F, and Treatment F or G diets, respectively. Diet also had a significant influence on *E. coli* numbers. Within the ilea, *E. coli* was significantly influenced by diet but was not clearly linked to performance, while within the caeca, *E. coli* was significantly increased in the better-performing birds fed Treatments F and G (Table 4). Gender also had a significant influence on *E. coli* communities within the caeca, with males having higher numbers than females (Table 4). A significant diet by sex interaction for ileal *E. coli* was detected, with females fed Treatment A diet having higher numbers of *E. coli* than those fed Treatment B diet. Furthermore, significant differences between the sexes were noted only for birds fed the Treatment G diet, with males having higher *E. coli* numbers than females.

Unlike for other trials, *G. anatis* was detected in the ileum and caeca of most birds in the present trial, but numbers were not significantly influenced by diet nor linked to performance.

Lactobacilli were significantly influenced by both diet and sex. *L. aviarius*, *L. crispatus* and *L. salivarius* were significantly reduced within the ilea of better-performing birds on Treatment F and or G diets (Table 3). *L. salivarius* and *L. crispatus* were also significantly increased in the caeca of males (Table 4). Within the ilea, a significant diet by sex interaction was observed for both *L. salivarius* and *L. crispatus*. The most feed-efficient male and female birds fed Treatment G diet had significantly lower numbers of *L. crispatus* than the same-sex birds on the lower-performing diets (Treatments A and B). Furthermore, *L. salivarius* and *L. crispatus* differed significantly between the sexes only for the less feed-efficient birds fed Treatment A diet, with males having higher ileal numbers of these species than females (Table 3).

Discussion

Of the 26 bacterial species or phylotypes previously identified as potentially representing eight performance-related OTUs, only six phylotypes could be identified to the species (>97% nucleotide-sequence identity) (Torok *et al.* 2011*b*). In the present study, we developed qPCR assays to five of these known phylotypes (*L. salivarius*, *L. crispatus*, *L. aviarius*, *E. coli* and *G. anatis*), so as to determine whether they are consistently linked to broiler performance from a variety of feeding trials differing in feed composition, environment, broiler breed and bird age. qPCR results supported previous findings that OTU 564–566 (*L. salivarius*, *L. crispatus*, *L. aviarius*) is associated with decreased broiler performance. However, qPCR results for OTU 492 (*E. coli* and *G. anatis*), previously thought to be associated with improved performance, showed discrepancies across trials. These findings are promising in identifying broiler gut bacteria associated with performance, but also highlighted the need to validate research findings across various performance trials with specific quantitative tools such as qPCR.

Across four broiler feeding trials, each being linked to significant changes in performance as determined by feed efficiency, it was found that *L. salivarius*, *L. crispatus*, *L. aviarius*, *E. coli* and total eubacterial numbers were significantly altered by dietary treatment. Furthermore, changes in the numbers of these bacteria could be linked to broiler performance. Although diet-related changes in the composition of bacterial communities varied among feeding trials, we found that across trials, the numbers of lactobacilli and total eubacteria were significantly increased in less feed-

Table 4. qPCR quantification of bacteria within the caeca of broiler chickens from four feeding trials

See text for explanation of treatments in different trials. Least significant differences (l.s.d.) among treatment means (ln(copy number)) are shown. Within columns, means followed by the same letter for each factor are not significantly different ($P > 0.05$). n.s., not significant. ND, not detected

Trial	Attribute	*n*	Eubacteria	OTU 492		OTU 564–566		
				Escherichia coli	*Gallibacterium anatis*	*Lactobacillus crispatus*	*L. salivarius*	*L. aviarius*
Trial I								
					Diet			
	l.s.d. ($P < 0.05$)		n.s.	1.15	ND	n.s.	1.02	ND
	Barley control	48	14.27	10.89b		8.54	10.10b	
	Barley enzyme	48	14.39	9.45a		8.26	8.42a	
					Sex			
	l.s.d. ($P < 0.05$)		0.28	n.s.	ND	n.s.	n.s.	ND
	female	48	14.15a	10.35		8.46	9.60	
	male	48	14.51b	9.99		8.34	8.92	
					Diet × Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	n.s.	ND
Trial II								
					Diet			
	l.s.d. ($P < 0.05$)		n.s.	1.35	n.s.	n.s.	n.s.	n.s.
	Wheat+xyl	12	13.26	7.32a	0.79	6.89	4.86	7.02
	Sorg. B	12	13.37	8.68b	0.00	5.35	6.41	6.32
	Sorg. comm	12	13.40	8.79b	1.37	6.65	5.40	6.38
	Sorg. comm+phyt	12	13.25	9.13b	0.87	6.90	6.39	7.00
Trial III								
					Diet			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	0.60	n.s.
	High fibre	48	13.82	9.09		6.82	8.15a	0.43
	Low fibre	48	13.88	9.15		7.43	9.00b	1.31
					Litter			
	l.s.d. ($P < 0.05$)		0.15	n.s.	ND	n.s.	n.s.	n.s.
	Paper	48	13.76a	9.36		7.13	8.42	0.77
	Wood	48	13.94b	8.88		7.12	8.73	0.96
					Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	0.83	n.s.	n.s.
	Female	48	13.80	9.05		7.59b	8.70	0.47
	Male	48	13.90	9.19		6.67a	8.45	1.27
					Diet × Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	n.s.	n.s.
					Litter × Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	n.s.	n.s.
					Diet × Litter			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	n.s.	n.s.
					Diet × Litter × Sex			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	ND	n.s.	n.s.	n.s.
Trial IV								
					Diet			
	l.s.d. ($P < 0.05$)		0.21	0.93	n.s.	n.s.	n.s.	1.33
	A	24	14.28c	8.05a	5.59	7.65	8.17	6.26b
	B	24	14.25c	7.42a	4.24	7.84	7.83	5.33ab
	F	24	13.68a	10.32b	5.07	7.14	7.88	4.11a
	G	24	14.01b	9.54b	5.43	7.39	8.48	5.73b
					Sex			
	l.s.d. ($P < 0.05$)		n.s.	0.61	n.s.	0.68	0.57	n.s.
	Female	48	14.06	8.30a	4.84	7.11a	7.78a	5.70
	Male	48	14.05	9.37b	5.33	7.90b	8.40b	5.01
					Sex × Diet			
	l.s.d. ($P < 0.05$)		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

efficient birds. Some lactobacilli, including *L. salivarius*, *L. aviarius* and *L. crispatus*, have been previously reported to have bile-deconjugating activity (Knarreborg *et al*. 2002; Majidzadeh Heravi *et al*. 2011). This bacterial activity lowers the detergent properties of bile acids in the emulsification of fat and leads to growth depression in chickens (Knarreborg *et al*. 2002; Harrow *et al*. 2007). Furthermore, increase in the total number of eubacteria in the less feed-efficient chickens may be a sign of bacterial overgrowth, which can lead to impaired gut health (Teirlynck *et al*. 2011) and, hence, impaired performance.

Conflicting results for the association of *E. coli* with performance were obtained across feeding trials. Increased numbers of caecal *E. coli* were linked to improved performance in feeding Trials II and IV, but in feeding Trial I, *E. coli* was linked to poorer performance. Furthermore, within the ilea, significant diet-related changes in *E. coli* numbers were not linked to broiler performance. These discrepancies may be due to differences at the strain level and, hence, functional level of the bacteria. Although, *E. coli* is considered a pathogen of humans and livestock, not all strains of *E. coli* have been shown to be detrimental, with some strains (*E. coli* Nissle) being used as a probiotic in both humans and livestock (Waidmann *et al*. 2003; Barth *et al*. 2009). Any strain differences present within trials could not be differentiated by using our species-specific qPCR approach.

Lactobacillus salivarius, *L. crispatus* and *E. coli* were detected in all trials, while *L. aviarius* and *G. anatis* were only infrequently or sporadically detected. Interestingly, in two trials, we were unable to detect *G. anatis*, while in one trial, most birds harboured this organism without exhibiting any detrimental effects on performance. This pattern of colonisation is consistent with previous observations where birds within a flock were either all colonised with *G. anatis* or were free of this organism (Bojesen *et al*. 2003*a*). This 'all or none' colonisation has been shown to be linked to the biosecurity level on the farm. The importance of *Gallibacterium* species as pathogens in poultry is unclear. *Gallibacterium* is part of the commensal microbiota of the upper respiratory and the lower genital tracts of healthy chickens (Bojesen *et al*. 2003*b*). However, *Gallibacterium* isolates have also been recovered in pure culture from a range of pathological lesions in chickens, including septicemia, oophoritis, follicle degeneration, salpingitis with or without peritonitis, peritonitis, enteritis and diseases of the respiratory tract (Bojesen *et al*. 2003*b*). Our finding that this organism can be widespread in healthy broilers is an indication that it may not be the absence or presence of the organism that is important but rather its metabolic function, which again may be linked to differences among strains. Indeed, several *G. anatis* biovars have been detected with clonal differences identified between tracheal and cloacal isolates from within an individual bird (Bojesen *et al*. 2003*b*). Detection of *G. anatis* in healthy chicken may have greater implications for detrimental performance within the layer and breeder industries due to the longer life span of those birds. Our studies on broilers were all performed at or <6 weeks of age, whereas previous investigations were conducted in layers and breeder at 13–63 weeks of age (Bojesen *et al*. 2003*a*).

We found that bird gender also had a significant influence on *L. salivarius*, *L. crispatus*, *L. aviarius*, *E. coli* and total eubacterial numbers within the gut. This is supportive of previous findings in which male and female chickens were found to differ in their ileal bacterial communities (Lumpkins *et al*. 2008). The fact that gender does influence gut microbiota is not surprising, considering that gender also influences performance (feed efficiency and growth rate) (Lumpkins *et al*. 2008). Better understanding of these gender-related differences in gut microbiota may, in future, lead to the development of gender-specific broiler diets to optimise gut microbial balance and performance.

Advances in high-resolution methods for determining the structure of microbial community, such as pyrotag sequencing and gut bacterial phylogenetic microarrays, will help identify suites of bacteria potentially linked to broiler performance. Several studies have already used pyrotag sequencing to investigate the community structure of the chicken gut (Callaway *et al*. 2009; Yin *et al*. 2010; Hume *et al*. 2011; Stanley *et al*. 2012). Although this methodology gives a detailed phylogenetic description of the complexity of the microbial community, quantitative interpretation of results should be performed with some caution (Amend *et al*. 2010; Zhou *et al*. 2011). Furthermore, direct comparison of pyrotag sequencing and phylogentic microarray results have indicated discrepancies in the phylogenetic groups identified due to the different classification schemes used by the two techniques (Claesson *et al*. 2009). If we are to extrapolate research findings from individual broiler feeding or performance trials, tools are needed for rapid and specific identification of organisms across trials. qPCR is ideal and can be developed into a diagnostic format which can be easily adopted by both industry and research organisations because the infrastructure for this technology is widespread, specific, quantitative, affordable and of high throughput, compared with other platforms.

The qPCR assays developed for *L. salivarius*, *L. crispatus*, *L. aviarius*, *E. coli* and total bacteria have been used to show that dietary and environmental treatments alter these bacterial communities and that they are linked to broiler feed efficiency. This qPCR approach has been shown to be a valid approach in screening large numbers of samples for the quantitative determination of organism numbers. Our qPCR results have also validated or disproved earlier findings for the association of specific bacteria with broiler performance. The qPCR assays developed could be used to screen future broiler feeding and performance trials, as well as expanded to include further gut bacteria thought to be linked to broiler performance, poultry health (pathogens) or consumer product safety (zoonotic agents). Understanding the dynamics of the chicken gut microbial community is necessary if we wish to develop further strategies for improved feed efficiency and growth rate, avoid intestinal diseases and proliferation of food-borne pathogens, and identify better feed additives and nutrient levels that influence beneficial microbial communities.

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