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Validation of qPCR assays targeting broiler performance
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Executive Summary

Gut microbiota plays an important role in animal health, performance and nutrition. We have previously shown that diet related changes in gut microbiota are linked to broiler performance (CRC1 03-3a; Torok et al., 2008). The greatest determinant of resident gut microbiota is the host's diet. Although knowledge of the ideal gut microbiota is still incomplete, it is apparent that a variety of diets can equally support optimal bird performance and maintain a healthy gut microbial balance. Despite variations in the gut microbial composition of birds receiving differing dietary treatments we have been able to identify bacterial species or taxonomically related groups of bacteria represented by eight operational taxonomic units (OTU) which were common and related to differences in broiler performance across three Australian feeding trials (CRC1 06-25). Extensive bacterial 16S rRNA sequence information representing these eight potential performance-related OTU was generated. The eight OTU identified may represent 26 different bacterial species or phylotypes. Ten of these phylotypes were identifiable to the species level. Quantitative PCR (qPCR) assays were developed to five of these potential performance related phylotypes which represented two of the eight performance related OTU identified. The five specific qPCR assays developed detected organisms related to *Lactobacillus salivarius, Lactobacillus crispatus, Lactobacillus aviarius, Gallibacterium anatis* and *Escherichia coli*.

In this study the five developed specific assays, as well as a generic qPCR assay detecting total bacterial numbers, were used to screen banks of chicken gut DNA generated from four broiler feeding trials which had shown significant performance differences. These trials included a barley versus barley enzyme feeding trial (done at SARDI PPPI as part of CRC1 03-3a), sorghum feeding trial (done at QDPI Alexandra Hills as part of a RIRDC funded project), litter and dietary fibre trial (done at Inghams Leppington facility as part of CRC1 project 06-18) and Inghams Enterprises feeding trial (done at Inghams Leppington facility by Inghams Enterprises). The five qPCR assays were run against the banks of chicken gut DNA to determine their diagnostic potential for broiler performance.

Across the four feeding trials it was found that *L. salivarius, L. crispatus, L. aviarius*, *E. coli* and total bacterial numbers were significantly altered by diet, environment (litter), and/or sex. Furthermore, changes in the numbers of these bacterial species and total bacteria were significantly linked to broiler performance.

Although, diet related changes in the bacterial community composition varied between performance trials, we found that across trials lactobacilli and total bacteria were significantly increased in poorer performing birds as determined by FCR or body weight. Lactobacilli have previously been linked with impaired broiler performance due to their potential bile deconjugating ability, which impairs fat digestion. Furthermore, increase in total bacteria may be a sign of bacterial overgrowth or dysbacteriosis, which could also lead to impaired performance.

E. coli was potentially linked with improved performance in the sorghum feeding (trial 1) and Inghams feeding (trial 3) trials, but was linked with poorer performance in the barley versus barley plus enzyme trial. This discrepancy may be due to differences at the strain level. Strains of *E. coli* are known to be associated with different functions but strain differences can not be determined using a species specific qPCR approach. Although, *E. coli* are considered pathogens of humans and livestock, not all *E. coli* have been shown to be detrimental with some (*E. coli* Nissle) actually used as a probiotic.

L. salivarius, *L. crispatus* and *E. coli* were detected in all trials while *L. aviarius* and *G. anatis* were only infrequently or sporadically detected. *G. anatis* was detected in two of the four feeding trials, where its presence was shown to have no detrimental effect on broiler performance. This organism has been reported to be a pathogen of poultry, although information within the literature is scarce. Our finding that this organism can be widespread in healthy broilers is an indication that it may not

be the absence/presence of an organism which is important but rather the metabolic function of that organism which may be linked to strain differences or metabolic cross talk.

The qPCR assays to *L. salivarius*, *L. crispatus*, *L. aviarius*, *E. coli* and total bacteria have been shown to be altered by dietary and environmental treatments and linked to performance. These assays are specific, quantitative and rapid compared to other culture dependant and independent methods. This qPCR approach has been shown to be a valid approach in screening large numbers of samples and quantitatively determining organism numbers. Our qPCR results have also supported and validated findings for some of the performance related OTU determined by molecular profiling (T-RFLP) and sequencing. The qPCR assays developed could be used to screen future broiler feeding and performance trials, as well as be combined with other industry relevant qPCR assays targeting gut microbial pathogens of poultry and human zoonotic agents.

A number of other potentially culturable broiler performance related organisms (*Faecalibacterium prausnitzii*, *Clostridium lactatifermentans, Ruminococcus torques*, *Bacteroides vulgatus* and *Alistipes finegoldii*) were also identified in CRC1 06-25 (Torok et al., 2011). However, these findings would still require validation by qPCR. This could be a useful approach based on current results for the five micro-organisms already investigated. Validating association of culturable organisms with broiler performance may provide potential for probiotic development to boost broiler performance in the future.

Overall our results are promising in the quest to identify potential performance related gut bacteria in poultry. Internationally, several research groups are pursuing this field of study, although findings are still to reach broader dissemination via scientific publications. This is primarily due to the relatively new nature of this field of study, as well as its complex nature. Indeed, the Poultry CRCII has invested in gaining a broader knowledge of the broiler gut microbial composition through high throughput sequencing and using this information to develop a poultry specific gut bacterial phylogenetic array chip. Our current project has contributed to the latter by providing all sequence information for broiler commensal gut microbiota (including potential performance related bacteria) generated as part of CRCI 06-25 to CRCII project 2.1.5. Such a poultry specific gut bacterial phylogenetic array chip will help identify suits of bacteria potentially linked with broiler performance across numerous trials. However, findings will still require validation and development into a format which could be easily taken up by both industry and research organisations. qPCR assay development to identified organisms could provide this as the infrastructure for this technology is widespread among diagnostic and research laboratories, is specific and quantitative, affordable compared to other platforms, and high throughput. All these are key criteria for industry uptake.

Understanding the dynamics of the gut microbial community, or microbial balance, is necessary to establish or develop strategies to improve 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. Nutritional strategies to manage the composition of the intestinal microbiota and thus detrimental or beneficial outcomes will have practical value in the future.

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Introduction

Feed constitutes the major cost of raising broiler chickens and, as a result, the most common measures of bird performance have been linked to weight gain and feed efficiency. Broiler performance is closely linked to the genetics, diet, age and rearing environment of the bird. Bird genetics has contributed significantly to the vast improvements observed in weight gain and feed efficiency in meat chickens over the last 50 years, although a small proportion of these improvements have been attributed to nutrition and other management practices. The genetic changes associated with improved weight gain and feed efficiency have also resulted in changes to the gut physiology and gut microbial community composition of birds (Lumpkins et al., 2010) and there appears to be a clear link between bird performance and gut microbiota composition. The gut microbiota have significant impacts on their host such as influencing the chick's gastrointestinal development, biochemistry, immunology, gene expression, physiology, and non-specific resistance to infection. Collectively the microbial genome (microbiome) has a coding capacity that vastly exceeds that of the host's genome and encoding biochemical pathways that the host has not evolved.

Several studies have been undertaken to investigate the influence of dietary changes on gut microbial community structure using microbiological culturing techniques, molecular (culture-independent) techniques and indirect measurement of bacterial metabolic products like short chain fatty acids (SCFA). However, understanding how these many diet induced changes in gut bacterial community composition relate to important metabolic changes and ultimately to broiler health and performance is less clear.

Three broiler feeding trials were investigated in order to identify gut bacteria consistently linked with improvements in bird performance as measured by feed efficiency (CRC1 06-25; Torok et al., 2011). Trials were done in various geographic locations and varied in diet composition, broiler breed and bird age. Gut microbial communities were investigated using microbial profiling. Eight common performance linked operational taxonomic units (OTU) were identified within both the ilea (180, 492 and 564-566) and caeca (140-142, 218-220, 284-286, 312 and 482) across trials. OTU 564-566 was associated with lower performance, while OTU 140-142, 482 and 492, were associated with improved performance. Targeted cloning and sequencing from these eight OTU revealed that they represented 26 bacterial species or phylotypes which phylogenetically clustered into seven groups related to *Lactobacillus* spp., *Ruminococcaceae*, Clostridiales, Gammaproteobacteria, Bacteroidales, Clostridiales/*Lachnospiraceae* and unclassified bacteria/Clostridia. Some of the potential performance related phylotypes showed high sequence identity with classified bacteria (*Lactobacillus salivarius, Lactobacillus aviarius*, *Lactobacillus crispatus*, *Faecalibacterium prausnitzii*, *Escherichia coli*, *Gallibacterium anatis*, *Clostridium lactatifermentans, Ruminococcus torques*, *Bacteroides vulgatus* and *Alistipes finegoldii*), many of which have various reported functions (Table 1).

Where performance linked bacteria were identifiable to the phyla level they belong predominantly to the Firmicutes and Bacteroidetes. Although many of our potential performance related bacteria were unclassified, they did show high sequence similarity with those identified from studies investigating the relationship between the gut microbiome and host metabolic phenotype, innate immunity and gut microbiota, gut microbiota in various host species including poultry, and the role of gut microbiota in gut health.

Although five of the potential performance related phylotypes identified could only be classified as unknown bacteria, the remaining 21 phylotypes could be classified to class, order, family, genera or even species level. Twelve of the phylotypes identified belonged to the order Clostridiales and represented sequences obtained from the caeca. The chicken caecum and its mucosal tissue are dominated by clostridia-related species (Bjerrum et al., 2006; Gong et al., 2007). Clostridia related bacteria within the gut have been shown to produce SCFA, with clostridium cluster IX predominantly producing propionate and clusters IV (*Clostridium leptum* group) and XIVa

(*Clostridium coccoides* group) primarily producing butyrate (Van den Abbeele et al., 2010). The *C. coccoides* group is likely to be important in colonization resistance, acting as a barrier against invasion by other potentially pathogenic microbes (Van den Abbeele et al., 2010).

Quantitative PCR (qPCR) assays were developed to five of these bacterial species (*L. salivarius, L. aviarius, L. crispatus, G. anatis* and *E. coli*) in CRC1 06-25. Although microbial profiling and sequencing have indentified many potential performance related bacteria, quantitative assays targeting specific bacteria are required to validate findings.

Table 1: Summary of potential performance related OTU identified in CRC1 06-25 which could be assigned a bacterial species identity. The reported functions of these gut inhabiting bacteria have been summarized from (Torok et al., 2011).

OTU	Closest bacterial identity	Potential function
564-566	*Lactobacillus salivarius* (100%) *Lactobacillus aviarius* (99%) *Lactobacillus crispatus* (100%)	• Some lactobacilli are reported to have bile deconjugating activity leading to growth depression in broilers • Lactobacilli communities are altered by in-feed antimicrobials
942	*Gallibacterium anatis* (99%)	• May cause serious systemic infection affecting multiple organs in poultry • Mechanism of pathogenesis unknown
	Escherichia coli (99%)	• Potential pathogen in livestock & humans • Some strains reported to produce colitis in mice • *E. coli* Nissle widely used as probiotic in humans & livestock
218-220	*Alistipes finegoldii* (91%)	• Anaerobic, non-spore forming non-motile Gram- positive bacteria • Isolated from human faeces • No putative function reported
	Bacteroides vulgatus (99%)	• Accounts for 10 - 12% colonic bacterial population in humans • Inverse relationship between *B. vulgatus* & salmonella in chicken gut • Shown to ameliorate *E. coli* induced colitis development in mice
284-286	*Faecalibacterium prausnitzii* (93%)	• *Clostridium leptum* group (cluster IV) • Dominant in the caeca of chicken • Produce SCFA (butyrate, formate & lactate) • Butyric acid shown to protect against pathogens in poultry
482	*Ruminococcus torques* (94%)	• Belongs to *Clostridium coccoides* (XIVa) group which has been shown to be important in colonization resistance, acting as a barrier against invasion by other potentially pathogenic microbes • Known to degrade gastrointestinal mucin
	Clostridium lactatifermentans (95%)	• Belong to *Clostridium cluster XIVb* which have been shown to produce acetate, propionate, butyrate & isovalerate. • In combination with *L. crispatus* has been shown to inhibit *Salmonella enterica Enteriditis in vitro*.

Objectives

This short-term sub-project will finalise work undertaken in Australian Poultry CRC (CRC1) projects 03-3a and 06-25.

The aims of the short-term sub-project were to:

(a) validate five qPCR assay developed in CRC1 06-25 to determine if they are related to broiler performance
(b) transfer broiler performance related gut bacterial sequence information to Poultry CRC Sub-Project 2.1.5 Geier *Identification of microbial and gut-related factors driving bird performance*
(c) provide sequence information to genome databases
(d) publish findings to date

Methodology

Background

Five qPCR assays targeting two performance related OTU were designed based on 16S rRNA sequence information (CRC1 06-25). All five qPCR assays have been optimised and shown to be specific to the target organism. The assays developed target *L. salivarius*, *L. aviarius*, *L. crispatus*, *G. anatis* and *E. coli.* A sixth qPCR assay was also developed to quantify total bacteria and was used as a control assay.

The six qPCR assays will be validated against samples (DNA) obtained from four broiler performance trials which have shown significant performance differences. These include a barley versus barley enzyme feeding trial (done at SARDI PPPI as part of CRC1 03-3a), Sorghum feeding trial (done at QDPI Alexandra Hills as part of a RIRDC funded project led by R Perez-Maldonado), litter and dietary fibre trial (done at Inghams Leppington facility as part of CRC1 06-18 and led by L Mikkelsen) and Inghams Enterprises feeding trial (done at Inghams Leppington facility and led by R MacAlpine and K Balding).

Trials

Barley versus barley enzyme trial

Broiler gut DNA was obtained from 24 chickens (12 males and 12 females) each fed a barley-based control diet or barley-based diet supplemented with NSP-degrading feed enzyme product (Torok et al., 2008).

Sorghum feeding trial (trial 1)

Broiler gut DNA was obtained from male birds on each of four dietary regimes: wheat control supplemented with xylanase; sorghum B; commercial sorghum; and commercial sorghum supplemented with phytase (n=12 birds/treatment) (Torok et al., 2011).

Litter and dietary fibre trial (trial 2)

Broiler gut DNA was obtained from both male and female birds on each of the 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).

Inghams' feeding trial (trial 3)

Broiler gut DNA was obtained from both male and female birds on each of four dietary treatments A, B, F and G (n=24 birds/treatment; 12 males and 12 females) (Torok et al., 2011).

Quantitative Polymerase Chain Reaction (qPCR)

All qPCR assays were based on SYBR green detection, a relatively simple qPCR technology platform. SYBR green is a dye which binds double stranded DNA (dsDNA), providing a fluorescent signal that reflects the amount of dsDNA product generated during PCR. Sensitivity of our qPCR assays were tested against dilutions of known amounts of target (plasmid standards) as previously described (Final report CRC 06-25). Specificity of our qPCR assays were determined by analysing dissociation or melt curves. Primers used for each assay are shown in Table 2 and assay efficiency shown in Table 3.

qPCR was performed with 50 nM each primer and 2 x Power SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer's recommendations. Reactions were run on a 7900HT real-time PCR system (Applied Biosystems).

Table 2: Primers used in real-time PCR bacterial assays.

Assay	Forward primer	Reverse primer
G. anatis[a]	GallibacF2b 5′ AACGGTAACGGGTTGAAAGC 3′	GallibacR2 5′ CCTTTCATCTCTCGATTCTATGC 3′
E. coli[a]	EcoliF1 5′ CATAATGTCGCAAGACCAAAGAG 3′	EcoliR1 5′ GGTAACGTCAATGAGCAAAGGT 3′
L. salivarius[a]	LsalivF1 5′ GATCGCATGATCCTTAGATGAA 3′	LsalivR1 5′ GCCGATCAACCTCTCAGTTC 3′
L. avarius[a]	LaviarF2 5′ TGACCGCATGGTCATTATGTA 3′	LaviarR2 5′ CAACTCGGCTACGTATCATCAC 3′
L. crispatus[a]	LcrispF1 5′ GCGAGCGGAACTAACAGATTT 3′	LcrispR1 5′ TGATCATGCGATCTGCTTTCT 3′
Bacteria[b]	EubactF1 5′ CCTACGGGAGGCAGCAG 3′	EubactR1 5′ ATTACCGCGGCTGCTGGC 3′

[a] PCR primers developed as part of CRC1 06-25
[b] (Zozaya-Hinchliffe et al., 2010)

Table 3: Determination of real-time PCR efficiencies from plasmid standards. Ct cycles versus plasmid copy number (log10) were plotted to calculate the slope. The corresponding real-time PCR efficiencies were calculated according to the equation: E (%) = $(10^{[-1/slope]} -1) \times 100$.

Assay	Slope	y-intercept	R^2	Efficiency (%)	Amplicon length (bp)
G. anatis	-3.502	31.34	0.999	93%	137
E. coli	-3.782	34.27	1	84%	312
L. 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
Bacteria	-3.493	32.44	0.996	93%	195

Statistical analysis

All statistical analysis was done by Mr Chris Dyson (Statistician, SARDI Sustainable Systems). Data was analysed by ANOVA with significant differences ($P<0.05$) determined between treatment means by the least square difference (LSD) method in Genstat software package. The bacterial copy numbers were logged (ln(x)) before analysis by ANOVA. The covariate ln(x) Bacteria was used to standardise gross bacterial levels.

Results

Barley versus barley enzyme trial

Previously apparent metabolisable energy (AME) had been shown to be significantly higher for chickens fed a barley-plus-enzyme diet (13.87±0.22 SD MJ/kg dry matter) than chickens on the control barley diet (13.47±0.29 SD MJ/kg dry matter). No effect of bird sex on AME and no interaction between diet and sex was detected (Torok et al., 2008).

qPCR quantification of ileal microbiota showed that *L. salivarius* was significantly higher in the ileum of broilers on the barley plus enzyme diet than for birds on the barley control diet (Table 4). However, within the caeca *L. salivarius* was significantly higher in the birds fed the barley control diet than birds on the barley plus enzyme diet. Within the caeca *E. coli* was also significantly increased in birds on the barley control diet. No diet related differences were detected in total bacterial numbers or *L. crispatus* in either gut sections. *G. anatis* and *L. aviarius* were not detected in any of the birds from this experiment. Sex of birds was found to significantly influence total bacterial numbers within the caeca (P=0.013, LDS= 0.834) with males having increased bacterial numbers (14.513) as compared with females (14.148).

Table 4: qPCR quantification of bacteria within the gut of broilers fed a control barley diet and a barley diet supplemented with exogenous NSP enzyme. Values indicate mean of the ln(copy number).

Assay	Diet		Diet	
	Control	Enzyme	P	LSD
Ilea				
Bacteria	10.22	10.47	0.506	0.756
E. coli [492*]	6.48	6.09	0.535	1.234
G. anatis [492*]	-	-	-	-
L. crispatus [564-566*]	5.63	5.93	0.558	1.001
L. salivarius [564-566*]	5.50[a]	6.92[b]	**0.009**	1.048
L aviarius [564-566*]	-	-	-	-
Caeca				
Bacteria	14.274	14.387	0.426	0.2834
E. coli [492*]	10.89[a]	9.45[b]	**0.016**	1.145
G. anatis [492*]	-	-	-	-
L. crispatus [564-566*]	8.54	8.26	0.623	1.150
L. salivarius [564-566*]	10.10[a]	8.42[b]	**0.002**	1.023
L aviarius [564-566*]	-	-	-	-

* indicate candidate OTU represented.

Sorghum feeding trial (trial 1)

It has previously been shown that live weight gain was significantly (P<0.05) decreased for birds on the wheat control diet supplemented with xylanase (2595±22 gm/bird) as compared to those on the sorghum B (2679±18 gm/bird), commercial sorghum (2675±23 gm/bird) and commercial sorghum supplemented with phytase (2674±21 gm/bird) 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 (1.788±0.010 gm/gm) diet as compared to those on the sorghum B (1.721±0.013 gm/gm), commercial sorghum (1.728±0.006 gm/gm) and commercial sorghum supplemented with phytase (1.739±0.004 gm/gm) diets (Perez-Maldonado and Rodrigues, 2009).

The only bacterial community significantly affected by diet was the *E. coli* community within the caeca (Table 5). Better performing birds on the sorghum based diets had significantly higher

counts of *E. coli* than the poorer performing birds on the wheat control diet. *G. anatis* was only infrequently detected in the ilea and caeca of birds in this experiment (10/48) with the same birds testing positive for both gut sections.

Table 5: qPCR quantification of bacteria within the gut of broilers from the sorghum feeding trial (trial 1). Values indicate mean of the ln(copy number).

Assay	Diet				Diet	
	Wheat+xyl	Sorghum B	Commercial Sorghum	Comm Sorg +phyt	P	LSD
Ilea						
Bacteria	9.58	9.89	9.68	9.44	0.724	0.805
E. coli [492*]	8.49	9.03	8.77	8.71	0.770	1.030
G. anatis [492*]	-	-	-	-	-	-
L. crispatus [564-566*]	6.39	5.93	7.04	7.46	0.404	1.93
L. salivarius [564-566*]	5.22	5.66	5.38	6.92	0.077	1.41
L aviarius [564-566*]	7.38	6.45	6.14	7.04	0.056	0.97
Ceaca						
Bacteria	13.258	13.366	13.395	13.245	0.720	0.3214
E. coli [492*]	7.32^{a}	8.68^{b}	8.79^{b}	9.13^{b}	**0.049**	1.350
G. anatis [492*]	-	-	-	-	-	-
L. crispatus [564-566*]	6.89	5.35	6.65	6.90	0.315	1.918
L. salivarius [564-566*]	4.86	6.41	5.40	6.39	0.109	1.497
L aviarius [564-566*]	7.02	6.32	6.38	7.00	0.301	0.962

* indicate candidate OTU represented.

Litter and dietary fibre trial (trial 2)

Litter type in combination with diet composition was shown to have significantly ($P<0.05$) influenced FCR (Torok et al., 2011). Birds were more feed efficient on a low fibre diet if housed on wood litter (1.481±0.018 gm/gm) than if housed on paper litter (1.538±0.023 gm/gm). Sex of birds also significantly ($P<0.001$) influenced FCR at 35 days of age with males having a better feed efficiency (1.482±0.009 gm/gm) than females (1.551±0.010 gm/gm). The body weight of male chickens at 35 days of age (2285±14 gm/bird) was significantly ($P<0.001$) heavier than the female chickens (1993±13 gm/bird). Litter type in combination with sex was found to significantly ($P<0.05$) alter body weight with females growing slower on paper litter (1960±17 gm/bird) than on the wood litter (2026±6 gm/bird) (Torok et al., 2011).

qPCR data from this trial is shown in Table 6. Within both the ileum ($P=0.022$, LSD=0.3534) and caeca ($P=0.020$, LSD=0.1454) total bacterial numbers were significantly increased in birds raised on the wood litter as opposed to paper litter material (Fig 1). A significant sex by diet interaction ($P=0.033$ LSD 0.4998) was also detected for ileal bacterial communities. Females on the high fibre diet had lower numbers of total bacteria than females on the low fibre diet, although no significant differences were observed in males on either of the diets (Fig 2).

L. crispatus numbers were significantly altered in both the ileum and caeca (Fig 3). Within the ilea a significant litter x sex interaction ($P=0.025$, LSD=0.956) was detected with males raised on the wood litter having significantly lower *L. crispatus* counts than either males raised on paper litter or females raised on wood litter but not females raised on paper litter (Fig. 3a). Within the caeca *L. crispatus* numbers were significantly altered by sex ($P=0.033$, LSD= 0.826) with males having lower *L. crispatus* numbers than female birds (Fig. 3b).

Within the ileum *E. coli* numbers significantly differed between diets ($P=0.006$, LSD=0.765) with the high fibre diet having less *E. coli* than the low fibre diet (Fig. 4). No significant differences in *E. coli* numbers were detected within the caeca.

Table 6: Treatment means for bacteria determined by qPCR quantification within the gut of broilers from the litter and dietary fibre trial (trial 2). Values indicate means of the ln(copy number).

Assay	High fibre				Low fibre			
	Paper		Wood		Paper		Wood	
	Female	Male	Female	Male	Female	Male	Female	Male
Ilea								
Bacteria	9.996	10.903	10.594	11.192	10.909	10.969	11.372	11.274
E. coli [492*]	8.43	8.31	8.89	8.41	10.10	9.30	9.24	9.70
G. anatis [492*]	-	-	-	-	-	-	-	-
L. crispatus [564-566*]	6.26	6.49	6.97	5.76	6.51	7.34	6.80	6.01
L. salivarius [564-566*]	9.50	8.94	9.32	8.49	9.24	9.71	9.67	9.17
L. aviarius [564-566*]	-	-	-	-	-	-	-	-
Caeca								
Bacteria	13.715	13.712	13.823	14.009	13.845	13.774	13.805	14.103
E. coli [492*]	9.06	9.70	8.80	8.79	8.92	9.74	9.41	8.53
G. anatis [492*]	-	-	-	-	-	-	-	-
L. crispatus [564-566*]	6.79	7.01	7.56	5.94	7.64	7.10	8.36	6.61
L. salivarius [564-566*]	7.66	8.11	8.76	8.08	8.98	8.92	9.41	8.68
L. aviarius [564-566*]	-	-	-	-	-	-	-	-

* indicate candidate OTU represented.

Within the caeca *L. salivarius* was significantly influenced by diet (P=0.006, LSD=0.604) with birds on the high fibre diet having lower numbers of *L. salivarius* than birds on the low fibre diet (Fig. 5). No significant difference in *L. salivarius* numbers were detected within the ileum.

No *G. anatis* was detected in the either the ileum or caeca of broilers in this performance trial. *L aviarius* was only infrequently detected within the ilea (34/96) and caeca (15/96).

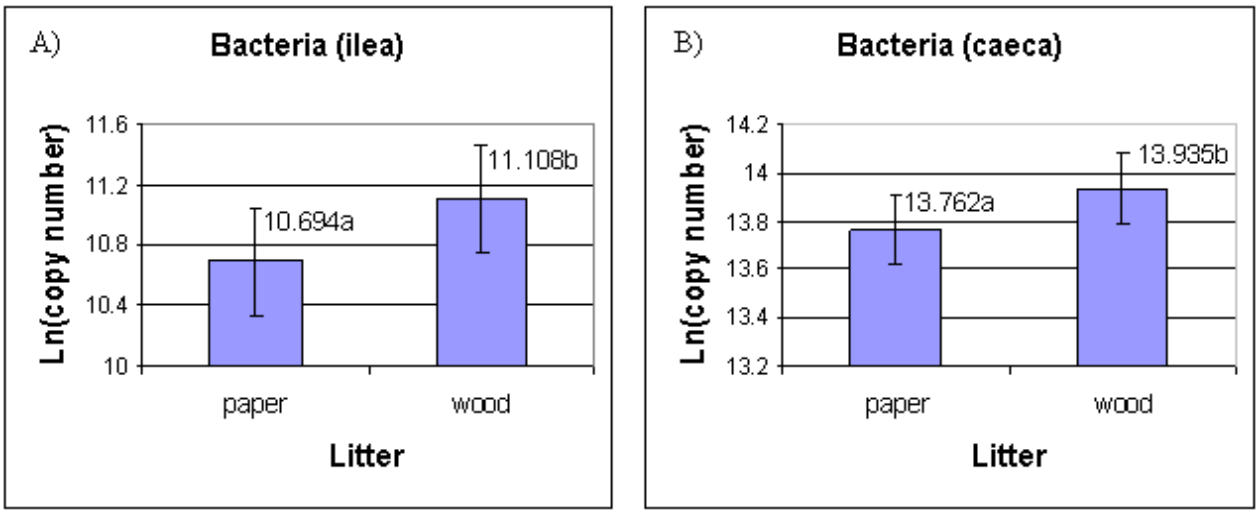

Figure 1: qPCR quantification of total bacteria within the gut of broilers raised on different litter materials. A) Ileal bacterial community. B) Caecal bacterial community. Treatment means are indicated. Treatment means with a different subscript are significantly different. Error bars represent LSD values.

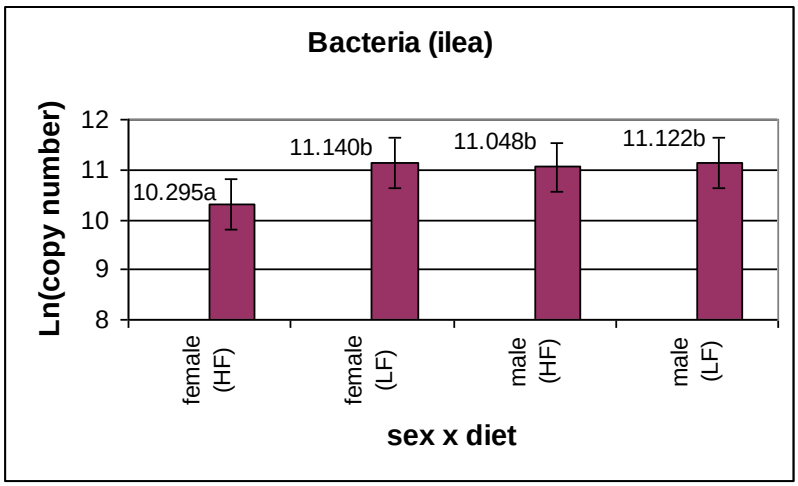

Figure 2: qPCR quantification of total bacteria within the gut of female and male broilers raised on different diets. Treatment means with a different subscript are significantly different. Error bars represent LSD values.

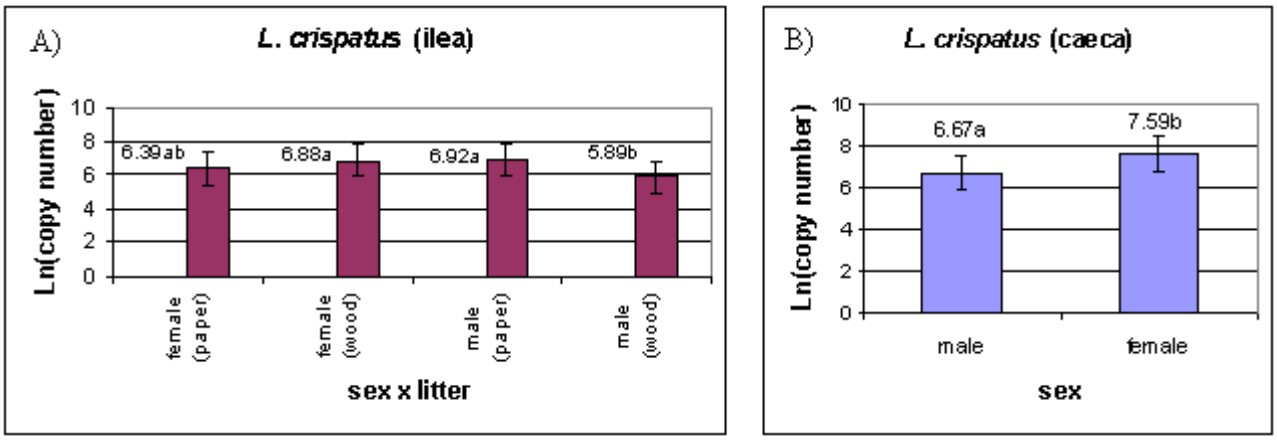

Figure 3: qPCR quantification of *L. crispatus*. A) ileal *L. crispatus* communities associated with sex and litter material. B) Caecal *L. crispatus* communities influenced by bird sex. Treatment means with a different subscript are significantly different. Error bars represent LSD values.

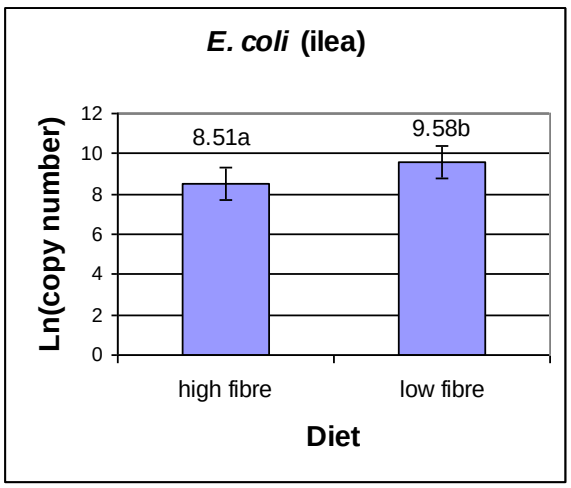

Figure 4: qPCR quantification of *E. coli* within the ileum of broilers fed different diets. Treatment means are indicated. Treatment means with a different subscript are significantly different. Error bars represent LSD values.

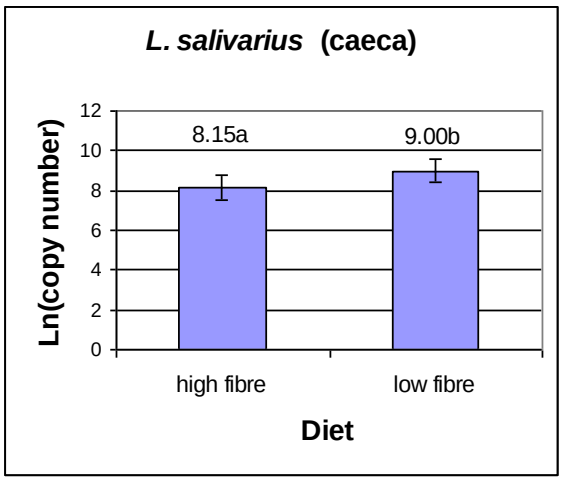

Figure 5: qPCR quantification of *L. salivarius* within the caeca of broilers fed different diets. Treatment means are indicated. Treatment means with a different subscript are significantly different. Error bars represent LSD values.

Inghams' feeding trial (trial 3)

Significant differences in FCR among dietary treatments have been previously shown for the Inhgams' feeding trial (trial 3) (Torok et al., 2011). Birds on the commercial G (1.501±0.050 gm/gm) and commercial F (1.554±0.068 gm/gm) diets had significantly improved feed efficiency as compared with birds on commercial A (1.583±0.052 gm/gm) and commercial B (1.603±0.061 gm/gm) diets. Birds fed the commercial G diet had the most significantly improved feed efficiency of birds on any of the diets investigated. Sex of bird also significantly (P<0.001) influenced FCR with males having a better feed efficiency (1.465±0.008 gm/gm) than females (1.666±0.009 gm/gm).

Table 7: qPCR quantification of bacterial within the gut of broilers from the Inghams' feeding trial (trial 3). Values indicate means of the ln(copy number).

Assay	Diet				Diet	
	A	B	F	G	P	LSD
Ilea						
Bacteria	10.335^{a}	10.233^{a}	9.194^{b}	10.695^{a}	**<0.001**	0.494
E. coli ^{492*}	5.28^{a}	4.04^{b}	3.62^{b}	6.56^{c}	**0.039**	1.210
G. anatis ^{492*}	3.58	3.93	4.41	5.24	0.055	1.349
L. crispatus $^{564-566*}$	7.33^{a}	7.39^{a}	6.34^{b}	5.43^{c}	**<0.001**	0.791
L. salivarius $^{564-566*}$	6.91^{a}	6.60^{a}	6.62^{a}	5.58^{b}	**0.035**	0.965
L aviarius $^{564-566*}$	5.61^{a}	5.46^{a}	4.54^{b}	6.02^{a}	**0.016**	0.851
Caeca						
Bacteria	14.281^{a}	14.245^{a}	13.683^{b}	14.005^{c}	**<0.001**	0.2108
E. coli ^{492*}	8.05^{a}	7.42^{a}	10.32^{b}	9.54^{b}	**<0.001**	0.926
G. anatis ^{492*}	5.59	4.24	5.07	5.43	0.153	1.342
L. crispatus $^{564-566*}$	7.65	7.83	7.14	7.39	0.685	1.034
L. salivarius $^{564-566*}$	8.42	7.78	7.97	8.49	0.357	0.746
L aviarius $^{564-566*}$	6.26^{a}	5.33^{ab}	4.11^{b}	5.73^{a}	**0.031**	1.329

* indicate candidate OTU represented.

Diet significantly influenced most of the bacterial species investigated (Table 7). Total bacteria numbers were significantly decreased within the ileum of improved performing birds on diet F as well as being significantly decreased within the caeca of improved performing birds on diets F and G (Table 7). *E. coli* was significantly increased within the ileum of the best performing birds on diet G as well as being increasing within the caeca of the better performing birds on diets F and G. *L*

crispatus, *L. salivarius* and *L. aviarius* were all significantly decreased within the ileum of better performing birds on diets F/G, G and F respectively. *L. aviarius* was also significantly decreased within the caeca of improved performing birds on diet F.

Sex also had a significant influence on *E. coli* (P<0.001, LSD=0.610) and *L. crispatus* (P=0.024, LSD 0.681) communities within the caeca with males having higher numbers of *E. coli* (9.37) and *L. crispatus* (7.90) than females (8.30 and 7.11, respectively).

It is interesting to note that *G. anatis* was detected in the ileum and caeca of most birds within the trial without being linked to adverse performance in these birds. Indeed all birds in this trial performed well, regardless of diet, by industry standards.

Transfer broiler gut bacterial sequences to Poultry CRC 2.1.5 & submission to genome database

Two hundred and sixty eight 16S rRNA sequences (accession numbers HQ704902-HQ705169) were generated from the guts of broiler chicks aged 3-17 days post-hatch, which had been fed a commercial starter diet either containing no antimicrobials or supplemented with one of three antimicrobials (zinc bacitracin, avilamycin or flavophospholipol) (CRCI 6-25). These sequences represent the commensal gut microbiota, age and antimicrobial related changes.

Six hundred and twenty seven 16S rRNA sequences (accession numbers JF797629-JF798255) were generated from the guts of broilers from three Australian feeding trials (CRCI 06-25). These sequences represent commensal chicken gut bacteria including bacteria potentially related with broiler performance.

All the above sequences have been transfered to CRC 2.1.5 for potential use in the development of a poultry gut chip. All sequences have also been submitted to NCBI database (accession numbers HQ704902-HQ705169 and JF797629-JF798255).

Scientific publication

Two scientific publications have been produced:

Torok, V. A., G. E. Allison, N. J. Percy, K. Ophel-Keller, and R. J. Hughes. 2011. Influence of in-feed antimicrobials on broiler commensal post-hatch gut microbiota development and performance. Appl. Environ. Microbiol. **77(10):**3380-3390.

Torok, V. A., R. J. Hughes, L. L. Mikkelsen, R. Perez-Maldonado, K. Balding, R. MacAlpine, N. J. Percy, and K. Ophel-Keller. 2011. Identification and characterization of potential performance related gut microbiota in broiler chickens across various feeding trials. Appl. Environ. Microbiol. Published ahead of print doi:10.1128/AEM.00165-11

Discussion of Results

Although knowledge of the ideal gut microbiota is still incomplete, it is apparent that a variety of diets can equally support optimal bird performance and maintain a healthy gut microbial balance. Identifying which gut microbiota are common and related to performance is the challenge.

In this study, five developed specific qPCR assays (*L. salivarius, L. crispatus, L. aviarius, G. anatis* and *E. coli*), as well as a generic qPCR assay detecting total bacterial numbers, were used to screen banks of chicken gut DNA generated from four broiler feeding trials which had shown significant performance differences.

In the barley versus barley enzyme trial only *E. coli* and *L. salivarius* were found to be influenced by dietary treatment. Both *E. coli* and *L. salivarius* were increased within the caeca of the poorer performing birds on the barley diet, however, *L. salivarius* was decreased within the ilea of the poorer performing birds on the barely plus enzyme supplemented diet. Although sex was not found to influence performance within this trial it was observed that male birds had significantly increased number of total bacteria within the caeca as compared with females.

Within the sorghum feeding trial (trial 1) only *E. coli* was influenced by diet and was significantly increased within the caeca of improved performing birds on all the sorghum diets.

In the litter/dietary fibre trial (trial 2), litter, diet and sex were found to influence total bacteria, *E. coli*, *L. crispatus* and *L. salivarius,* however, linkages with performance were difficult to establish because of the multiple factors within the trial. The only clear linkage that could be drawn was that the heavier male birds had significantly decreased *L. crispatus* numbers within the caeca. Birds on high fibre diets had significantly lower numbers of *L. salivarius* and *E. coli*. Total bacterial numbers were also significantly increased for birds on the wood litter as opposed to the paper litter.

Within the Inghams' feeding trial (trial 3), total bacteria and lactobacilli were significantly decreased within the gut of improved performing birds, while *E. coli* was significantly increased within the gut of improved performing birds.

Of the five specific and one general total bacterial assay investigated in this study all but *G. anatis* were identified as being significantly influenced by dietary and/or environmental treatments, and linked with broiler performance. *L. salivarius*, *L. crispatus* and *E. coli* were detected in all trials, while *L. aviarius* and *G. anatis* were only infrequently or sporadically detected. Birds in two of the experiment trials were totally devoid of *G. anatis* while in the Inghams' feeding trial (trial 3) the organism was present in most birds without having a detrimental effect on performance as measured by FCR. *G. anatis* has been reported to cause serious systemic infection affecting multiple organ systems although the mechanism of pathogenesis remains unclear (Bojesen et al., 2003). *Gallibacterium* spp. have been found to be present in the upper respiratory tract as well as the lower genital tract of chicken (Bojesen et al., 2003).

Although diet related changes in the bacterial composition investigated in this report varied between performance trials, the common feature noted was that lactobacilli and total bacteria were significantly decreased in improved performing birds as determined by FCR or body weight. Some lactobacilli have been linked with impaired broiler performance due to their bile deconjugating activities (Knarreborg et al., 2002). Furthermore, an increase in total bacteria may be a sign of bacterial overgrowth or dysbacteriosis also leading to impaired performance. *E. coli* was potentially linked with improved performance in both the sorghum and Inghams' feeding trials but seemed linked with impaired performance in the barley vs barley plus enzyme trial. This discrepancy may be due to differences at the strain level and hence potential functional differences which can not be determined using our broader species specific qPCR approach. It should be noted that not all *E. coli* are pathogenic or detrimental, with some strains (*E. coli* Nissle) being used as a probiotic (Barth et al., 2009).

The fact that total bacterial numbers were shown to be significantly altered and linked with broiler performance indicates that there are other bacterial species which were not investigated in this study driving these shifts. The assays validated in this study represent only five of the 26 potential performance related bacterial phylotypes previously identified (CRC 6-25; Torok et al., 2011). Furthermore, these five bacterial species also only represent two of the eight performance related OTU identified. Hence, there are many candidate organisms which have not yet been validated for their association with broiler performance. We already possess the 16S rRNA genome sequences information which would allow future development of quantitative assays for some of these other identified and culturable organisms *(Faecalibacterium prausnitzii*, *Clostridium lactatifermentans, Ruminococcus torques*, *Bacteroides vulgatus* and *Alistipes finegoldii*). Considering the success of the qPCR assays investigated in this study *(L. salivarius, L. crispatus, L. aviarius, E. coli* and total bacteria) to monitor diet and performance related changes, future development of new qPCR assays to encompass other candidate organisms may be warranted. It is promising that so many 16S rRNA sequences showed similarity with classified bacteria (10/26; Table 1). If they could be validated as being performance related, this may lead to the development of poultry specific probiotics.

Overall our results are promising in the quest to identify potential performance related gut bacteria in poultry. Internationally, several research groups are pursuing this field of study, although findings are still to reach broader dissemination via scientific publications. This is primarily due to the relatively new nature of this field of study, as well as its complex nature. Indeed, the Poultry CRCII has invested in gaining a broader knowledge of the broiler gut microbial composition through high throughput sequencing and using this information to develop a poultry specific gut bacterial phylogenetic array chip. Our current project has also contributed to the latter by providing all sequence information for broiler commensal gut microbiota (including potential performance related bacteria) generated from CRCI 06-25 to CRCII project 2.1.5. Such a chip poultry specific gut bacterial phylogenetic array will help identify suites of bacteria potentially linked with broiler performance across numerous tails. However, findings will still require validation and development into a format which could be easily taken up by both industry and research organisations. qPCR assay development to organisms of interest could provide this as the infrastructure for this technology is widespread among diagnostic and research laboratories, is specific and quantitative, affordable (compared to other platforms), and high throughput. All these are key criteria for industry uptake.

Understanding the dynamics of the gut microbial community, or microbial balance, is necessary to establish or develop strategies to improve 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. Nutritional strategies to manage the composition of the intestinal microbiota and thus detrimental or beneficial outcomes will have practical value in the future.

Implications

- A variety of diets can equally support optimal bird performance and maintain a healthy gut microbial balance.

- qPCR assays have potential for investigating shifts in gut microbiota in poultry driven by dietary and environmental factors and linked to broiler performance.

- The developed qPCR assays (*L. salivarius, L. crispatus, L. aviarius, G. anatis*, *E. coli* and total bacteria) could be combined with other qPCR assays to organisms of interest to the poultry industry (i.e. livestock and zoonotic pathogens) and run as a commercial screen.

- Developed qPCR assays can be used to monitor strategies to improve feed efficiency and feed formulation for optimal gut health.

- qPCR is more specific and less labour intensive than microbiological cultivation techniques.

- Potential pathogenic bacteria related to *Gallibacterium anatis* can inhabit the gut without causing disease or impaired performance.

- Care needs to be taken not to directly link changes in microbial community structure with changes in microbial function. For example, not all strains of a particular bacterial species have the same function. Alternatively, several bacterial species (belonging to different genera) have been reported to have the same function.

- The 16S rRNA bacterial sequence information generated in CRC 6-25 could allow further quantitative assays to be developed which will confirm which of these phylotypes are truly performance related.

- Developed qPCR assays can be used as a quick and inexpensive screen to investigate changes in gut inhabiting bacteria.

Recommendations

- Run qPCR assays on independent performance trials to confirm findings.

- Development of further qPCR assays to other candidate performance related bacteria (*Faecalibacterium prausnitzii*, *Clostridium lactatifermentans, Ruminococcus torques*, *Bacteroides vulgatus* and *Alistipes finegoldii*) to validate findings of Torok et al., 2011.

- Further work to be undertaken in an attempt to identify gut microbiota linked with broiler performance which could lead to manipulating the gut microbiota with various dietary interventions to lift animal performance.

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