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Application of advanced DNA-based tools for monitoring and managing the gut microflora of poultry.

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Executive Summary

Gut microbiology and its role in animal health has become increasingly important, particularly now that the use of antibiotics in animal feeds to promote growth has been banned in the European Union and is being questioned by consumers in other countries. To investigate the composition and ecology of the poultry gut we have developed a high throughput high-resolution microbial profiling method capable of a throughput of 200 samples per day. This molecular technique is based on terminal restriction fragment length polymorphism (T-RFLP) analysis of the bacterial ribosomal gene region and allows a "snapshot" of the complex bacterial population to be produced at any particular time. Furthermore, a database for cataloguing and validating the large quantity of data points generated from the analysis has also been developed. This has been critical in managing data and enabling further statistical analysis to be done. Using T-RFLP in conjunction with multivariate statistical analyses, it has been possible to investigate changes in gut microflora as a result of dietary modification, litter material composition, environmental factors and age. More importantly, this technology has enabled changes in gut microflora composition to be correlated to improved performance for the first time and allowed identification of bacteria linked to these improvements in performance. Improvement in performance may be due to the presence of beneficial and/or absence of detrimental bacterial species.

The use of microbiological culturing techniques, in combination with T-RFLP, has been useful in identifying members of the microbial communities characterised in this project. However, the identities of the majority of bacteria revealed by T-RFLP have remained unknown. Recent advances in molecular techniques will make identification of T-RFLP profiles of interest easier and not reliant on bacterial culturing or generation of clone libraries. This will enable bacterial sequence information of interest to be determined, which may be used to develop specific tests for gut bacteria associated with poultry production traits. From there, it may be possible to develop dietary strategies to induce desirable changes in the gut microflora for enhancement of growth and productivity of commercial chicken flocks.

Diet associated changes in gut microbial community composition were investigated along the length of the gut and found to be detectable only within the ileum and caeca. Bacterial species identified as contributing to diet induced changes, generally contributed less than 5% to the community composition. This indicates that several bacterial species are responsible for the overall differences and not just a select few. Dietary supplementation with antibiotic growth promotants (AGPs) has been shown to alter the overall gut microflora of poultry. However, this needs to be investigated in more detail if alternatives to AGPs are to be evaluated. Supplementation of the poultry diet with several prebiotic products did result in a shift in the overall ileal bacterial population compared with non-

supplemented control groups. However, none of the prebiotics tested resulted in a shift in the ileal bacterial microflora similar to that produced by AGP supplementation (zinc bacitracin).

Gut microflora are affected by factors other than diet. Litter materials have also been shown to affect gut microflora development, with re-used litter having the most significant impact on caecal gut microflora development when compared with other non re-used litter materials. This effect was most apparent at two weeks of age, as compared with four weeks of age. The re-used litter may be acting as a microbiological seed, speeding up mature colonisation of the gut microflora. However, this may also have hygiene and/or food safety implications if undesirable/detrimental bacterial were present in the litter. Differences in caecal microbial communities were also observed between birds raised on some of the non- reused litter materials regardless of age (rice hulls versus shredded paper). However, linkages between changes in gut microflora associated with litter material and bird performance were not investigated further due to lack of opportunity.

Environment and age also impact on the gut microflora of poultry. For example, differences in gut microflora between poultry flocks were related to commercial shed environment. However, regardless of any shed-to-shed differences, the pattern of gut microflora development remained the same. The gut microflora was continually changing and developing in the first two weeks post hatch. It then appeared to reach a mature gut microflora at three week of age with no further changes detected until six weeks of age, when a final shift in gut microbial communities composition was observed. These changes may partially reflect management practices, but strongly indicated that gut microflora development in the first two weeks of life is important and may affect life long performance.

In conclusion, we have established a microbial profiling technique for investigating the chicken intestinal microflora based on high-throughput, high resolution fingerprinting of bacterial ribosomal gene regions. In conjunction with the development of a database for validating and managing the vast output of data from the analysis and multivariate statistical methods, this has allowed us to monitor changes in the intestinal microbial community and conduct comparative studies investigating effects of dietary manipulations, influence of litter material, environment and age on chicken gut bacterial populations. Furthermore, we have been able to link changes in poultry performance with changes in gut microflora composition and identify T-RFLP profiles associated with improved performance. From here it will be possible to identify bacterial species associated with production traits.

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Introduction

Gut microbiology and its role in animal health has become increasingly important, particularly now that the use of antibiotics in animal feeds to promote growth has been banned in the European Union and is being questioned by consumers in other countries. Gastrointestinal microorganisms have a highly significant impact on uptake and utilisation of energy (Choct *et al*., 1996) and other nutrients (Smits *et al*., 1997; Steenfeldt *et al*., 1995), and on the response of poultry to anti-nutritional factors (such as non-starch polysaccharides), pre- and pro-biotic feed additives and feed enzymes (Bedford and Apajalahti, 2001). Microorganisms can also directly interact with the lining of the gastrointestinal tract (Van Leeuwen *et al*., 2004), which may alter the physiology of the tract and immunological status of the bird (Klasing *et al*., 1999). Earlier studies have predominantly used culture-dependent approaches for identifying the composition of the poultry gut microflora (Barnes, 1979, Mead, 1989). However, a large number of bacteria remain unidentified due to lack of knowledge of appropriate culturing conditions. Furthermore, culturing and biochemical techniques have resulted in the misclassification of some bacteria (Tellez, 2006). Alternatively, DNA techniques have the advantages of being rapid, relatively inexpensive and capable of monitoring gene regions of complex populations.

Currently the techniques of choice for microbial community analysis in many disciplines are denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) (Muyzer, 1999). These techniques amplify the 16S subunit of bacterial ribosomal DNA by PCR (polymerase chain reaction), and then separate the amplicons on a denaturing gel to visualise fragment size differences in the ribosomal DNA. However, these techniques are not conducive to high throughput analysis, and differences in amplified ribosomal DNA are not large enough to discriminate between many organisms. Other relevant issues with DGGE/TGGE are that it may not give a true representation of the actual microbial community present and that the choice of PCR primers can affect the outcome. Reproducibility and analysis based on presence/absence/position of bands are also concerns. To this end, there has been emphasis placed on the need to standardise conditions across research groups and the need for good analytical methodologies.

An alternate technique for bacterial community analysis is terminal restriction fragment length polymorphism (T-RFLP) (Osborn *et al*., 2000). This technique also amplifies the 16S subunit of the bacterial ribosomal DNA present in biological samples; however, all bacterial sequences amplified are labelled with a fluorescent dye. The amplified and labelled bacterial sequences are cut with sequence specific enzymes. The resulting fragments are separated according to size and detected by fluorescence emission from the incorporated dye by a DNA sequencing machine. Results are converted to graphical profiles where peaks can represent strains or taxonomically related groups of bacteria. These can be easily compared between samples to identify changes in bacterial community composition. The T-RFLP

technique is high resolution and high throughput, when combined with a high throughput DNA extraction technique uniquely developed at SARDI for soil organisms. This extraction technique has been modified for chicken gut samples, providing high quantities of PCR-ready DNA, and capable of a throughput of 200 samples per day

Objectives

A principal objective of the Poultry CRC is "sustainable production of chicken meat without reliance on antibiotics". Two key industry outcomes to be addressed within this particular project were:-

- Thorough understanding of the key factors influencing digestive function and gut microflora of broiler chickens, maintaining efficient production without the use of antibiotics.
- Controlled microbial colonisation of the gut of newly hatched chickens to maintain a healthy gut microflora throughout the productive life of the bird.

The aims of this project are:

(a) Establish microbial profiling techniques for chicken intestinal microflora based on high-throughput, high resolution fingerprinting of bacterial ribosomal gene regions, allowing us to monitor changes in the intestinal microbial community

(b) Define what constitutes an optimum intestinal microflora in an "elite" chicken

(c) Utilise this technology and benchmark to conduct comparative studies of the effects of dietary manipulations on changes in the "good" and "bad" bacterial population in the gut of chickens.

Methodology

The profiling technique developed for investigating poultry gut microflora was T-RFLP (Marsh, 1999). Like DGGE/TGGE, this technique also relies on PCR amplification of the 16S subunit of the bacterial ribosomal DNA present in biological samples. Two primers (short segments of DNA or oligonucleotides complementary to and flanking the target DNA region) are required to initiate the copying of each DNA strand via PCR. In this way the entire bacterial population in any given gut sample is amplified using a universal primer pair targeting conserved regions within the 16S rDNA gene. All bacterial sequences amplified are terminally labelled with a fluorescent dye attached to one of the primers. The amplified and labelled bacterial sequences are then cut with sequence specific restriction enzymes. The restriction enzyme cuts the amplified DNA wherever its unique recognition sequence (usually four to six base pairs) is present. The resulting fragments are separated according to size by electrophoresis and detected by fluorescence emission from the incorporated dye by a DNA sequencing machine. Only the terminal fragments with the incorporated fluorescent dye attached to the primer are identified. Results are converted to graphical profiles where peaks can represent

taxonomically related groups and/or strains of bacteria. These can be easily compared between samples to identify changes in bacterial community composition (Fig. A). Peaks in Fig 1 represent different bacterial species or taxonomically related groups of bacteria found in the chicken ileum. Numbers above peaks indicate peak position or size of terminal restriction fragments (T-RFs).

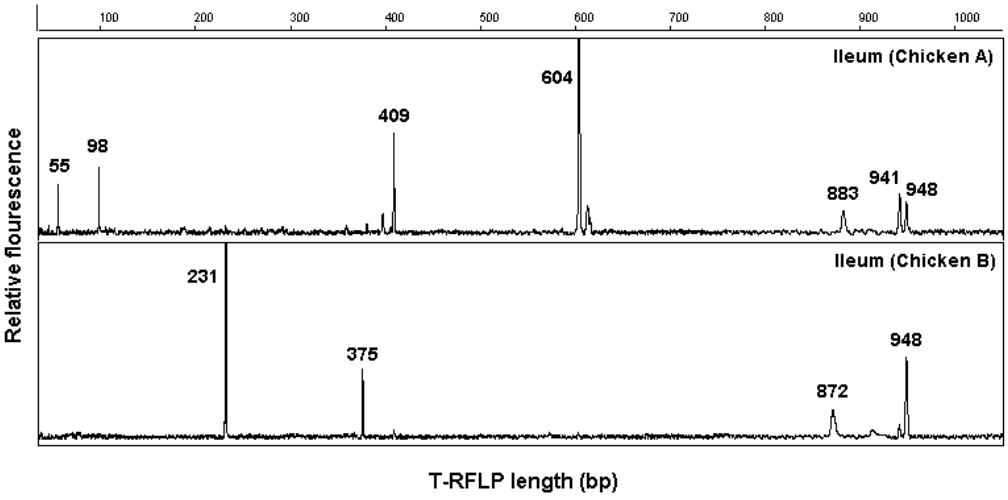

Figure A: Comparison of T-RFLP profiles of the 16S subunit of the bacterial ribosomal DNA gene amplified from DNA extracted from the ileum of two chickens.

The development of T-RFLP methodology will be discussed in greater detail in Chapter 1.

Chapter 1: Development of T-RFLP methodology for chicken gut microbial profiling

Introduction

The T-RFLP technique has both high sensitivity and throughput making it ideal for comparative analysis. This technique is capable of providing a "snap-shot" of the complex bacterial population at any particular time. However, several factors can affect the sensitivity of T-RFLP and the true representation of the bacterial population including: effectiveness of DNA extraction method; PCR primer choice; and restriction enzyme choice (Osborn *et al*., 2000). The optimisation of T-RFLP for chicken gut microbial profiling is presented below.

Selection of bacterial 16S ribosomal DNA primers

A disadvantage of PCR based techniques for amplifying the 16S rDNA gene is primer related bias (Zhu *et al.*, 2002). That is, "universal" primers may not amplify all bacterial species or may preferentially amplify some bacterial species to others. It cannot be guaranteed that a particular primer pair will amplify all bacterial sequences, as primer design is based on currently available bacterial 16S rDNA sequence information, accessible through public genome databases. The amount of 16S rRNA gene sequence information available has grown dramatically over the past three years. In March 2005 there were 128,376 sequences available in the Ribosomal Database Project II (RDP II) bacterial small sub-unit (SSU) 16S rDNA database (http://rdp.cme.msu.edu). This has almost tripled, with a reported 335,830 sequences available in March 2007. Informed decisions on the best primer pairs to use in T-RFLP were made from searching literature and subsequently testing primer prevalence against known 16S rDNA sequences *in silico* using the Microbial Community Analysis III (MiCA III) tools (http://mica.ibest.uidaho.edu). Table 1.1 shows promising 16S rDNA universal primer pairs identified within the literature.

Table 1.1: Universal 16S rDNA bacterial primers

Primer name	Primer sequence	Source
27F	5´ AGA GTT TGA TC(A/C) TGG CTC AG 3´	Lane, 1991
63F	5´ CAG GCC TAA (C/T)AC ATG CAA GTC 3´	Marchesi *et al*., 1998
907R	5´ CCG TCA ATT CCT TT(A/G) AGT TT 3´	Muyzer *et al*., 1995
1492R	5´ GG(C/T) TAC CTT GTT ACG ACT T 3´	Lane, 1991

From *in silico* prediction of the primer prevalence to 16S rDNA sequences (Table 1.2) it was indicated that the 63F/907R primer pair would detect the greatest number of bacterial 16S rDNA sequences,

while the 27F/1492R primer pair would detect the least number of bacterial 16S rDNA sequences. *In vitro* amplification with all four combinations of primer pairs was tested to determine specificity and sensitivity.

Table 1.2: *In silico* primer sequence prevalence to bacterial sequences deposited within the RDP II bacterial SSU 16S rRNA database using MiCA III interface.

Forward primer	Forward matches	Reverse primer	Reverse matches	Combined matches
27F	32,224*	907R	100,696	20,261
27F	32,341	1492R	31,319	12,732
63F	101,491	907R	100,719	66,488
63F	101,492	1492R	31,293	22,392

*Allowing at most 5 mismatches within 15 bases from the 5´ end of the primer

In vitro it was found that PCR primer pair 63F/1492R did not result in amplification of the expected ca.1.4 kb amplicon from the 16S rDNA of bacteria present in DNA extracted from poultry gut samples. This was not due to inhibition of the PCR reaction, as the same DNA templates gave positive reactions with other combinations of 16S rDNA primers. Primer pairs 63F/907, 27F/907R and 27F/1492R all produced expected products of ca. 800 bp, 900 bp and 1.5 kb, respectively. Amount of amplification product generated using each of the three primer pairs from identical DNA template was comparable, although amplicon concentration produced with 63F/907R was consistently the greatest and amplicon concentration produced with 27F/1492R was consistently the lowest. However, primer pair 63F/907R was not developed further for T-RFLP as it was extremely sensitive to exogenous bacterial contamination in the PCR.

All PCR work was done in a designated UV cabinet void of nucleic acids and using designated nucleic acid free pipettes and sterile filter tips. Sources of bacterial contamination were detected in standard primer stocks, which had not been HPLC purified. The DNA polymerase used in PCR amplification was also found to be a source of bacterial DNA contamination. This is not surprising as this enzyme is itself produced by bacteria. This was minimised by sourcing DNA polymerase from manufacturers claiming to have a low bacterial DNA load, such as AmpliTaq LD (Applied Biosystems, Scoresby, Australia).

Primer pairs 27F/1493R and 27F/907R were further evaluated for the T-RFLP procedure to determine sensitivity. T-RFLP was done on the same DNA extract with primer pair 27/F907R (Fig 1.2A) or 27F/1492R (Fig 1.2B). The fluorescent label enabling detection of T-RFs was attached to the 27F primer. If both primer pairs were equal in their sensitivity for detecting bacteria within the population

then the two profiles would be identical. Although, both profiles do show the same dominant peaks at 96, 213 and 287, the profile generated with 27F/907R is more complex, as shown by the greater number of T-RF peaks. These correlate to a greater diversity of bacteria being detected in the sample. Therefore, Primer pair 27F/907R was chosen for T-RFLP analysis.

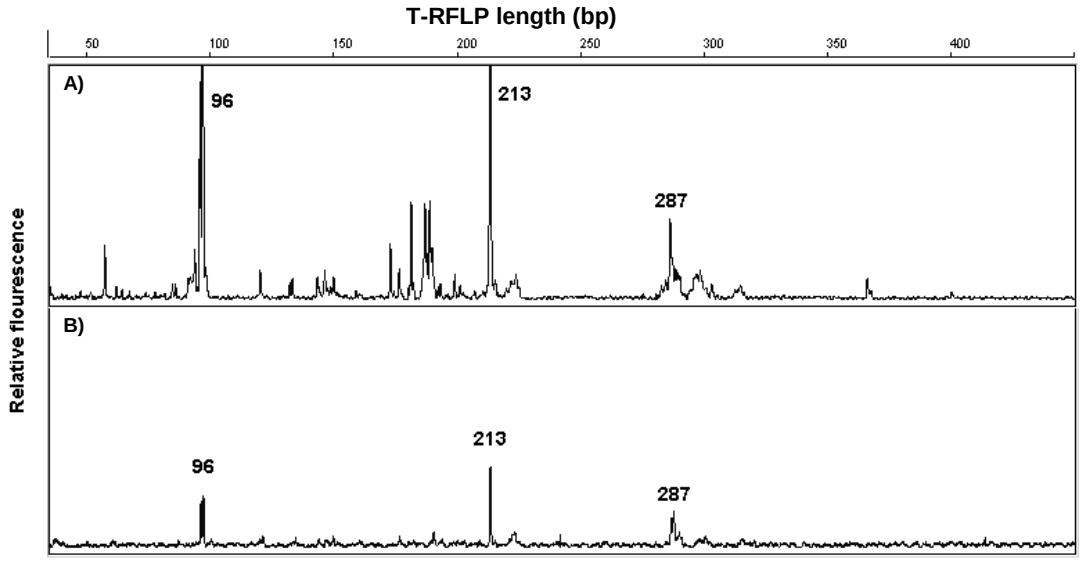

Figure 1.2: Comparison of primer pairs for detecting differences in poultry gut bacterial communities by T-RFLP. A) primer pair 27F/907R. B) primer pair 27F/1492R.

Optimisation of bacterial DNA extraction from chicken gut samples

DNA was extracted from freeze-dried chicken gut samples containing digesta by the SARDI proprietary extraction method. This method was developed for extraction of DNA from soil samples and is high throughput allowing up to 200 samples to be processed per day. However, the standard soil method was found not to be optimal for extracting DNA from chicken gut samples. A modification was made to the initial steps of the method, which optimised the method while still retaining its high throughput nature. The modification made was that freeze-dried gut samples were incubated at 70^{o}C in extraction buffer for an hour prior to commencement of the SARDI extraction method. The extraction buffer was also modified to contain 1.5M $NaCl_2$, 1.3 M guanidine thiocyanate, 30 mM Tris HCl pH 7, 65 mM phosphate buffer pH 8.0, 3.4% (w/v) N-lauroylsarcosine, 1.7% (w/v) polyvinylpolypyrrolidone. Fig 1.3 shows the effect extraction method has on the sensitivity of T-RFLP.

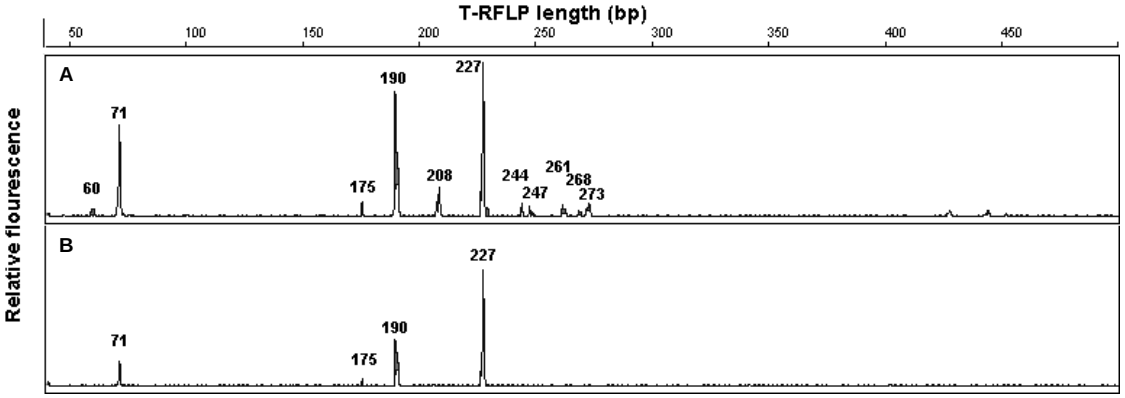

Figure 1.3: The effect of extraction method on obtaining a representative profile of the gut bacterial community from adjacent gut sections from the same bird. A) modified SARDI extraction method for chicken gut samples. B) SARDI commercial soil extraction method.

Selection of restriction enzymes

Choice of restriction enzyme affects the resolving power of the T-RFLP technique. Restriction enzymes with a four base pair recognition sequence will have a higher resolving power than six base pair cutting restriction enzyme, as they cut more frequently. Furthermore, some restriction enzymes may discriminate between species of a taxonomic group better than others. This can be demonstrated *in silico* using MiCA III (Table 1.3) and *in vitro* (Fig 1.4). The greater the numbers of unique T-RFs (or peaks) are produced by a particular restriction enzyme, the higher the resolving power of the T-RFLP technique.

Table 1.3: *In silico* resolving power of various restriction enzymes for discriminating between bacterial species or taxonomically related groups of bacteria amplified with primer pair 27F/907R (MiCA III).

Restriction enzyme	Unique T-RFs	Restriction enzyme (cont)	Unique T-RFs (cont)
*NIa*III	165	*Mse*I	645
*Hpy*CH4V	170	*Hpy*CH4IV	670
*Dde*I	374	*Hpy*188I	723
*Bam*HI	413	*Tsp*509I	727
*Eco*RI	426	*Dpn*I	728
*Bst*UI	445	*Sau*3AI	729
*Sau*961	455	*Bfa*I	772
*Stu*I	481	*Hha*I (*Cfo*I)	772
*Hae*III	511	*Hin*P1I	774
*Taq*I	520	*Hpy*188III	776
*Alu*I	560	*Scr*FI	779
*Msp*I	582	*Rsa*I	780
*Hinf*I	590		

From the *in silico* analysis of the resolving power of restriction enzymes (Table 1.3) it could be predicted that *Rsa*I would have a better resolving power than *Msp*I. However, *in vitro* it was found that *Msp*I (Fig 1.4B) had a higher resolving power than *Rsa*I (Fig 1.4D) for chicken gut microflora. The apparent inconsistency can be explained by the fact that the *in silico* analysis is based on all available bacterial sequences in public databases, which include bacteria from environmental sources (i.e. thermal springs, marshes, landfill, etc.) not representative of chicken gut microflora.

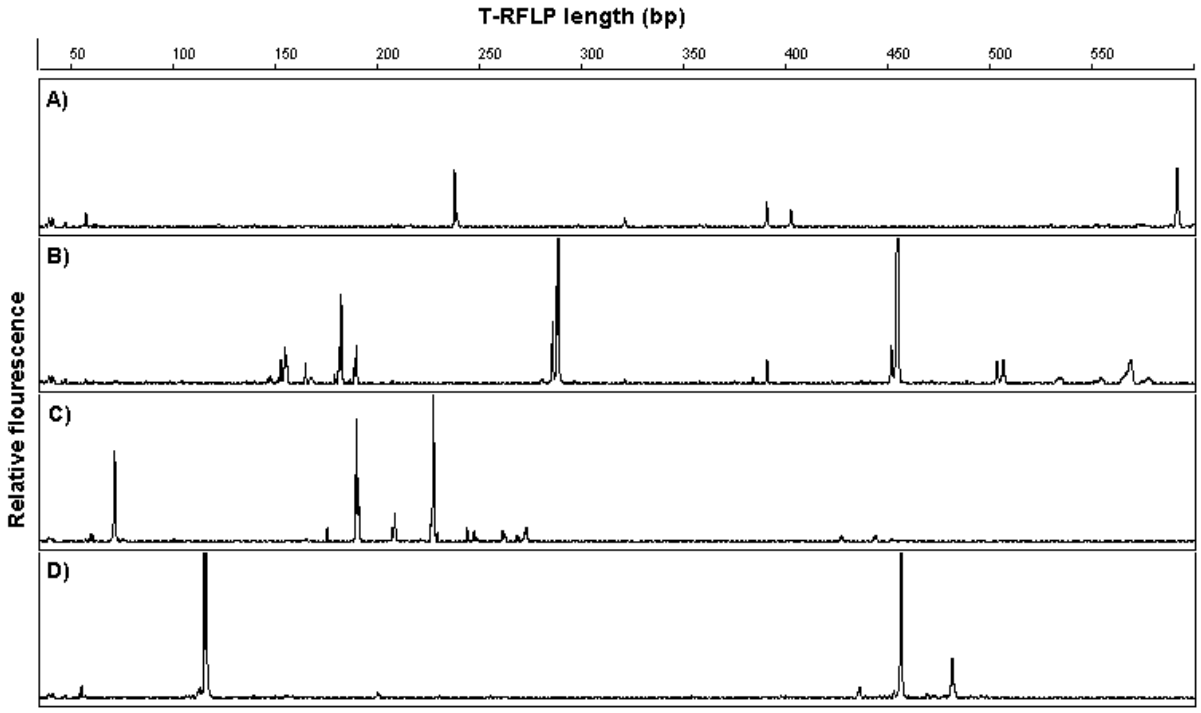

Figure 1.4: Different resolving power of four restriction enzymes for differentiating between bacterial gut populations from the ileum of a healthy chicken. The restriction enzymes compared were A) *Cfo*I. B) *Msp*I C) *Alu*I D) *Rsa*I.

From the *in vitro* analysis it was found that the resolving power of *Msp*1 and *Alu*I was higher than for *Cfo*1 and *Rsa*1 for distinguishing bacterial populations from the ileum of health chickens (Fig 1.4) as indicated by the greater number of T-RFs produced.

Sensitivity of detection

Seven bacterial species isolated from the ilea of healthy chickens were used to determine sensitivity of detection for the T-RFLP technique. The bacterial species were identified by generation of genome sequence information from the 16S rDNA gene and found to be *Bifidobacterium pseudolongum*, *Clostridium perfringens, Lactobacilli crispatus, L. reuterii, L. salivarius, L johnsonii,* and an uncharacterised *Lactobacilli* species. Pure cultures of each bacterial species were grown to a concentration of 10^8 cell/ml and serially diluted 10 fold to a final concentration 10^1 cells/ml. A mixture

of all seven bacterial species (10^8cells/ml each) was also made and diluted to a final concentration of $7x10^1$ cells/ml. T-RFLP was done on the individual and mixed bacterial cultures to determine level of sensitivity. The level of sensitivity was found to be 10^5-10^6 cell/ml of original sample or 10^3-10^4 cells per PCR reaction. Fig 1.5 shows that all seven bacterial species present within the mixed culture ($7x10^5$ cell/ml) could be identified in the T-RFLP profile. Bacterial species and/or taxonomically related groups of bacterial can be qualitatively detected by T-RFLP, however detection is only semi-quantitative.

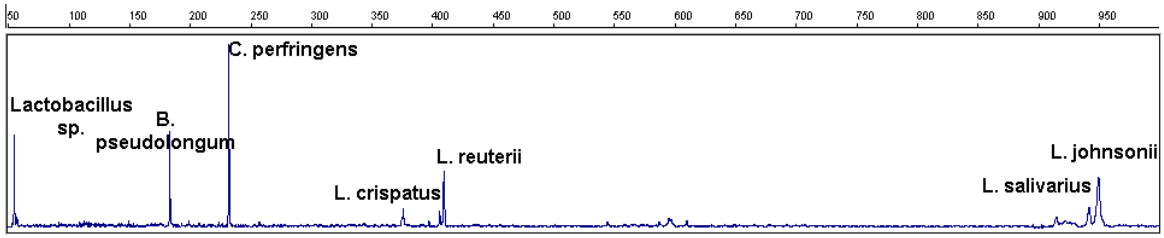

Figure 1.5: T-RFLP profile showing detection of seven bacterial species within a mixed culture. *Cfo*I was the restriction enzyme used in the R-RFLP analysis.

T-RFLP methodology

Total nucleic acid was extracted from chicken gut samples by a modification of a proprietary extraction method developed by the South Australian Research and Development Institute. Bacterial rDNA was amplified with universal 16S bacterial primers 27F (Lane, 1991) and 907R (Muyzer *et al*., 1995). The forward primer was 5′-labelled with 6-carboxyfluorescein (FAM). PCR reactions were done in 50 µl volumes containing 1 × PCR buffer II (Applied Biosystems, Scoresby, Australia), 1.5 mM $MgCl_2$, 200 µM of each dNTP (Invitrogen, Mulgrave, Australia), 0.2 µM of each primer (Sigma Proligo, Lismore, Australia), 1U AmpliTaq DNA polymerase LD (Applied Biosystems, Scoresby, Australia) and 50-100 ng total nucleic acid template. All PCR reactions were done in duplicate and run in a MJ Research PTC-225 Peltier thermal cycler (GeneWorks, Adelaide, Australia) with the following amplification conditions: initial denaturation at 94^{o}C for 5 min followed by 33 cycles of denaturation at 94^{o}C for 45 sec, annealing at 48^{o}C for 45 sec and extension at 72^{o}C for 1 min with a final extension step at 72^{o}C for 7 min. PCR products were quantified by fluorometry with Quant iT™ Pico green (Invitrogen, Mulgrave, Australia) according to manufacturer's instructions, in a Wallac Victor^2 1420 multilabel counter (Perkin Elmer, Life Sciences, Australia). If duplicate PCRs were consistent, they were pooled and products analysed by gel electrophoresis on a 1.5% agarose gel and visualised after staining with ethidium bromide. Approximately 200 ng PCR product was restricted with 2 U *Msp*I (Genesearch, Arundel, Australia) in the recommended enzyme buffer. Digests were done on pseudo-duplicates in a final volume of 15 µl and incubated at 37^{o}C for 4 hrs. Following

restriction, enzyme was inactivated by incubation at 65^{o}C for 15 min. Efficiency of restriction was checked by gel electrophoresis of a single species bacterial positive control on a 2% agarose gel and visualised following ethidium bromide staining. If restriction was complete pseudo-duplicates were pooled. The length of fluorescently-labelled terminal restriction fragments (T-RFs) were determined by comparison with an internal standard (GeneScan 1000 ROX; Applied Biosystems, Australia) following separation by capillary electrophoresis on a ABI 3700 or ABI 3730 automated DNA sequencer (Applied Biosystems, Australia) and data analysed using GeneScan 3.7 or GeneMapper software (Applied Biosystems, Australia).

Chapter 2: Managing and analysing raw T-RFLP data points.

Introduction

T-RFLP is an extremely useful tool for microbial community analysis and has been shown to have higher resolution for detecting less abundant species than other microbial profiling techniques, such as denaturing gradient gel electrophoresis (DGGE) (Moeseneder *et al*., 1999). However, the power of T-RFLP has not been fully utilised when it comes to analysis of resulting data. For example, many T-RFLP studies have predominantly used visual interpretation of graphical T-RFLP profile outputs for identification of presence/absence of particular peaks indicative of bacterial species (Fairchild *et al*., 2005, Gong *et al*., 2002a, Gong *et al*., 2002b, Miyamoto *et al*., 2004). Such studies have typically been based on small numbers of individual samples or even pooled samples per treatment. The high throughput nature of T-RFLP makes it conducive to individual analysis of a large number of samples and therefore, appropriate statistical methods need to be investigated for analysing the vast amount of data which can be generated. Therefore, appropriate statistical methods for analysing T-RFLP are outlined in this chapter.

Development of database for validating raw T-RFLP outputs

Following T-RFLP the size and height of T-RFs were determined by comparison with a known internal sizing standard which had been added to each sample. T-RF data points were determined using either the GeneScan or GeneMapper programs (ABI Biosystems) depending on the model of DNA sequencer used for analysis. Data point outputs required further analysis to validate "true" T-RF's, validate positions of T-RFs, standardise within sample T-RF heights, set thresholds, automate analysis, create outputs for statistical analysis, and catalogue data. To achieve this, an Access (Microsoft Windows) database was developed in conjunction with 2ic Software Pty Ltd. Assumptions used to design queries in the database were modified from Dunbar *et al*. (2001) and Egert *et al*. (2004). The resulting fragments were treated as operational taxonomic units (OTUs), representing particular bacterial species or taxonomically related groups.

Multivariate statistical analysis of operational taxonomic units

OTUs were analysed using multivariate statistical techniques (PRIMER 6 and PERMANOVA+β1, PRIMER-E Ltd., Plymouth, UK). These analyses were used to examine similarities in chicken gut microbial communities, identify OTUs accounting for differences observed in microbial communities,

and examine correlations between the composition of the microbial community and bird performance data.

Bray-Curtis measures of similarity (Bray and Curtis, 1957) were calculated to examine similarities between gut microbial communities of birds from the T-RFLP generated (OTU) data matrices, following standardization and fourth root transformation. The Bray-Curtis similarity co-efficient (Bray and Curtis, 1957) is a reliable measure for biological data on community structure and is not affected by joint absences that are commonly found in microbial data (Clarke, 1993). Two-way crossed analysis of similarity (ANOSIM) (Clarke, 1993) was used to test if gut microbial communities were significantly different between treatments. The *R*-statistic value describes the extent of similarity between each pair in the ANOSIM analysis, with values close to unity indicating that the two groups are entirely separate and a zero value indicating that there is no difference between the groups.

Similarity percentages (SIMPER) (Clarke, 1993) analyses were done to determine which OTUs contributed most to the dissimilarity between treatments. The overall average dissimilarity ($\bar{\delta}$) between gut microbial communities of birds on the two treatments were calculated and the average contribution of the *i*th OTU ($\bar{\delta}_i$) to the overall dissimilarity determined. Average abundance ($\bar{y}$) of important OTUs in each of the groups were determined. OTUs contributing significantly to the dissimilarity between treatments were calculated ($\bar{\delta}_i/SD(\delta_i)>1$). Percent contribution of individual OTUs ($\bar{\delta}_i\%$) and cumulative percent contribution ($\Sigma\ \bar{\delta}_i\%$) to the top 50% of average dissimilarities were also calculated.

Unconstrained ordinations were done to graphically illustrate relationships between treatments using non-metric multidimensional scaling (nMDS) (Shepard, 1962, Kruskal, 1964) and principal coordinate analysis (PCO) (Gower, 1966). nMDS ordinations attempt to place all samples in an arbitrary two-dimensional space such that their relative distances apart match the corresponding pair-wise similarities. Hence, the closer two samples are in the ordination the more similar are their overall gut bacterial communities. “Stress” values (Kruskal’s formula 1) reflect difficulty involved in compressing the sample relationship into the 2-D ordination. PCO ordinations also show the relationship between samples. However, nMDS use the ranks of similarities, whereas PCO use the actual similarity measures from the underlying Bray-Curtis similarity matrix.

Subsets of OTUs found to best represent results from nMDS ordinations on the full set of OTU data were also determined using the BVSTEP procedure (Clarke and Warwick, 1998) on random selection of starting variables. Matches of the nMDS ordination produced from the subset of OTUs to the full set of OTUs were determined by Spearman rank correlation (ρ) of elements from the two underlying

Bray-Curtis similarity matrices. A good correlation between underlying similarity matrices is determined when $\rho \geq 0.95$. Both SIMPER and BVSTEP identify differences in community composition, but in slightly different ways. SIMPER identifies individual species (OTUs) contributing to the overall dissimilarity between treatments, whereas BVSTEP identifies sets of species (OTUs) summarising the overall pattern differences in microbial community composition (Clarke and Warwick, 2001).

Constrained canonical analysis of principal coordinates (CAP) biplots (Anderson and Willis, 2003) were constructed to investigate the relationship between OTUs associated with diet and bird performance measures, such as AME. The *a priori* hypothesis that gut microbial communities were different between diets was tested in CAP by obtaining a P value using permutation procedures (999 permutations) on the canonical test statistic (squared canonical correlation, δ_1^2). The number of PCO axes (m) was chosen to achieve the maximum proportion of correct allocations (% of Trace (G)) of samples to diet. Pearson's correlation (R) was calculated between the first canonical axis (CAP1) and AME.

Discussion

Several previous T-RFLP studies have used cluster analysis to depict grouping of related samples (Dunbar *et al*., 2000, Gomez *et al*., 2004, Kuske *et al*., 2002, Lan *et al*., 2004, Moeseneder *et al*., 1999, Perez-Jimenez *et al*., 2005). However, a disadvantage of this method is that it groups samples into discrete clusters, and does not display their inter-relations on a continual scale (Clarke and Warwick, 2001). Other studies have used principal component analysis (PCA) to examine community structure resulting from T-RFLP data (Kuske *et al*., 2002, Park *et al.*, 2006, Wang *et al.*, 2004). However, PCA analysis is not appropriate where data contain many "zeros" or where observations (species) exceed total number of samples (Clarke and Warwick, 2001), as is usually the case for T-RFLP data.

We have shown that T-RFLP in conjunction with several multivariate statistical techniques, such as, unconstrained (nMDS and PCO) and constrained (CAP) ordinations, statistical tests of the hypothesis (ANOSIM) and characterisation of species responsible for the pattern differences (SIMPER and BVSTEP), are all useful tools for investigating the composition of the poultry gut microbial community.

Chapter 3: Effect of diet on gut microflora

Introduction

This study investigated the use of T-RFLP, in conjunction with multivariate statistical methods, for examining changes in gut microbial communities in response to addition of a non-starch polysaccharide (NSP) degrading enzyme product to a barley-based diet. The dietary regime was chosen to maximise differences between diets, rather than to emulate commercial practice in Australia. Changes in gut microflora composition were then related to bird performance. NSP degrading additives are widely used in commercial poultry production (Choct, 2006), however, the manner by which they benefit their host is not completely understood (Bedford, 2000). This is the first published study, which directly links diet-induced changes in gut microflora with subsequent improvement in poultry performance, as measured by energy metabolism.

Materials and methods

Chickens

All experimental work with animals was done at the Pig and Poultry Production Institute (PPPI) Poultry Research Unit, Roseworthy Campus, University of Adelaide with appropriate animal ethics approval. Cobb 500 broiler chickens were raised from hatch in two rearing pens in a controlled temperature room. Male and female chickens were reared separately. All birds were given commercial starter crumbles (Steg 600 starter, Ridley Agriproducts, Australia). At age day 13, a total of 48 chickens were placed, in single-sex pairs, in 24 metabolism cages located in a controlled-temperature room kept at 25-27^{o}C, and continued to receive starter crumbles. Birds had free access to feed and water prior to and during the experimental period. At age day 15, chickens were transferred into individual metabolism cages for the start of the 7-day apparent metabolisable energy (AME) experiment. Barley-based diets, with and without addition of a non-starch polysaccharide degrading feed enzyme product (Avizyme 1100 at 1 kg/tonne inclusion rate), were given to 24 chickens each (12 male and 12 female). Avizyme 1100 (Feedworks, Australia) contains endo 1,3(4)-β- glucanase, 1,4-β-xylanase and protease activities (according to the manufacturer). Each diet contained (per kg) 800 g barley, 155 g casein, 20 g dicalcium phosphate, 11 g limestone, 7 g DL-methionine, 5 g mineral and vitamin premix, 3 g salt, and 2 g choline chloride (60%). Both diets were cold-pressed to form pellets approximately 6 mm in diameter and 6 mm in length.

Measurement of AME

AME values of barley-based diets with and without feed enzyme product were determined in a classical energy balance study involving measurements of total feed intake and total excreta output

and subsequent measurement of gross energy values of feed and excreta by bomb calorimetry (Choct *et al*., 1996). Diets were fed for seven days. The first three days enabled chickens to adapt to the feeds. During the following four days, all excreta were collected daily and dried at 85^{o}C, then pooled for each chicken. Moisture content of excreta voided over a 24 h period, and feed intake during the adaptation and collection phases of the study were measured. Birds were weighed at the start and end of the 7-day period. Dry matter (DM) contents of samples of pelleted and milled feeds were measured. Gross energy values of dried excreta and pelleted feeds were measured with a Parr isoperibol bomb calorimeter (Parr Instrument Company, Moline, Illinois). AME of grain was calculated by subtracting from the total energy intake the energy contribution of casein, which is assumed to be 20.1 MJ/kg dry matter (Annison *et al*., 1994).

Intestinal sample collection

At age day 22, following completion of the metabolism study, all chickens were killed by intravenous injection of pentobarbitone sodium 325 mg/ml at a dose rate of 0.5 ml/kg live weight (Lethobarb, Virbac Australia Pty Ltd, Australia). The gastrointestinal tracts from the base of the gizzard down to the rectum were dissected and sections approximately 3 cm long (including digesta) were cut from the mid regions of the duodenum, jejunum, ileum and caeca. Samples were snap-frozen by immersion in liquid nitrogen and stored at -20^{o}C prior to freeze-drying. Dissecting instruments were cleaned with 70% ethanol following use on each bird.

T-RFLP and multivariate statistical analysis of OTU's

T-RFLP methodology is described in Chapter 1 and statistical analysis of T-RFLP generated data points is stated in Chapter 2.

Univariate statistical analysis

Univariate analysis of variance (ANOVA) was used to determine effects of block, diet and sex (fixed factors) on bird performance, as measured by AME, using the GLM model (Base SAS® software, SAS Institute). Equal numbers of birds (n=24) received either the barley control diet or barley diet supplemented with enzyme. Each dietary group contained equal numbers of males and female chickens (n=12).

Results

Univariate analysis of classical growth/performance data showed that chickens fed a barley plus enzyme diet had a significantly higher (ANOVA, $F_{1,11}$=46.47 P<0.0001) AME (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). There was no effect of bird sex on AME (P>0.05), and no interaction between diet and sex was detected (P>0.05).

Multivariate statistical analysis showed that the composition of the gut bacterial community was significantly different between gut sections and diet. The global *R*-value for differences between gut sections across diets was 0.440 (P<0.001) and for differences between diets across all gut sections was 0.186 (P<0.001). Bacterial community composition was significantly different between all gut sections, except between the duodenum and jejunum, regardless of diet (Table 3.1). This is graphically shown in the nMDS ordination as separation into three groups; ileum, caeca and duodenum-jejunum combined (Fig 3.1A). However, when the samples are identified by diet (Fig 3.1B) the influence of diet on gut microbial community composition becomes apparent, particularly within the caeca. It should be noted that the stress value for these 2-D ordinations is moderately high (0.24), indicating that it is not a good representation of the overall gut bacterial community differences. Stress is known to increase with reducing dimensionality and increasing quantity of data (Clarke and Warwick, 2001).

Table 3.1: Two-way ANOSIM of gut microbial communities associated with gut sections and diet. The *R* statistic (**bold**) and significance level (*italics*) are shown between gut sections across both the barley control and barley plus enzyme diets.

	Duodenum	Jejunum	Ileum	Caeca
Duodenum		**0.033**	**0.371**	**0.541**
Jejunum	*0.066*		**0.405**	**0.625**
Ileum	*0.001*	*0.001*		**0.631**
Caeca	*0.001*	*0.001*	*0.001*	

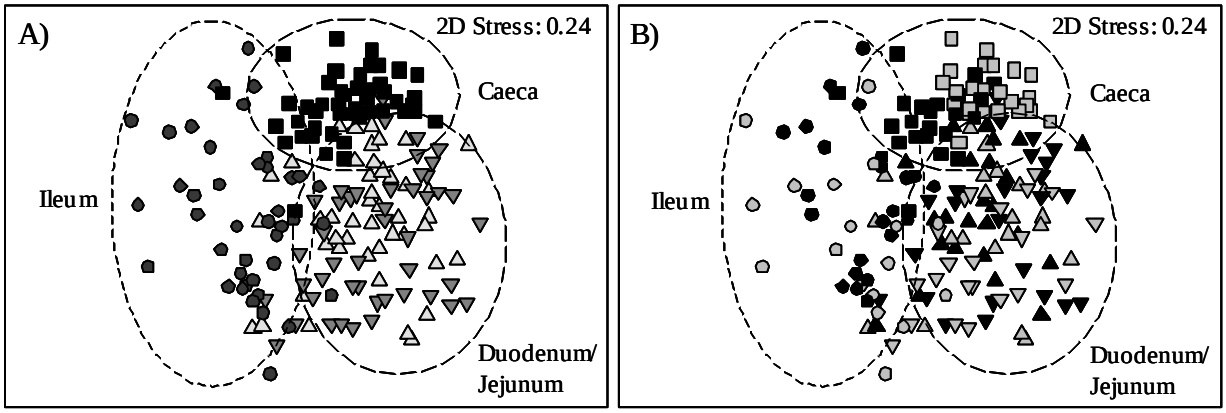

Figure 3.1: nMDS ordination of gut microbial communities identified by gut section and diet. A) gut microbial communities from the duodenum (▲), jejunum (▼), ileum (●) and caeca (■) from all birds (n=48) regardless of diet. B) The same nMDS as in A) however, samples are identified by both gut section and diet. Gut microbial communities from the four gut sections of birds on the barley control diet are identified as duodenum (▲), jejunum (▼), ileum (●) and caeca (■), while those of birds on the barely diet supplemented with enzyme are identified as duodenum (▲), jejunum (▼), ileum (●) and caeca (■). The ordination is based on Bray-Curtis similarities calculated from 4^{th}-root transformed OTU abundances (147 OTUs).

The effects of diet and sex were further investigated for each of the four gut sections. Significant differences in bacterial community composition associated with diet were only detected within the ileum and caeca (Table 3.2). No significant diet-associated differences in bacterial community composition were detected within the duodenum or jejunum. Furthermore, no significant differences were found in gut bacterial community composition between male and female birds (Table 3.2).

Table 3.2: Two-way ANOSIM of gut microbial communities associated with sex and diet for each of the four gut sections investigated. The global *R* statistic (**bold**) and significance level (*italics*) are shown for each of the factors within each gut section.

	Duodenum	Jejunum	Ileum	Caeca
Sex	**0.038**, *0.178*	**0.057**, *0.117*	**0.052**, *0.141*	**0.008**, *0.394*
Diet	**0.007**, *0.402*	**0.061**, *0.090*	**0.197**, *0.003*	**0.47**, *0.001*

The difference in ileum bacterial community composition associated with diet is shown in Fig 3.2A. The relationship is represented in two-dimensional space, which has been reduced from a much higher-dimensional species (OTUs) space. This implies that many OTUs must be interchangeable in the way they characterise the samples, and that an analysis of a small subset of the total number of OTUs may give a similar result to that of the full set. Hence, a subset of 17 OTUs was identified by the BVSTEP procedure, which showed a good correlation (ρ=0.95) with the relationship generated from the full set of 91 OTUs. The nMDS shows a close match between the wider community pattern based on all 91 identified OTUs (Fig 3.2A) and the pattern based on the subset of 17 OTUs (Fig 3.2B).

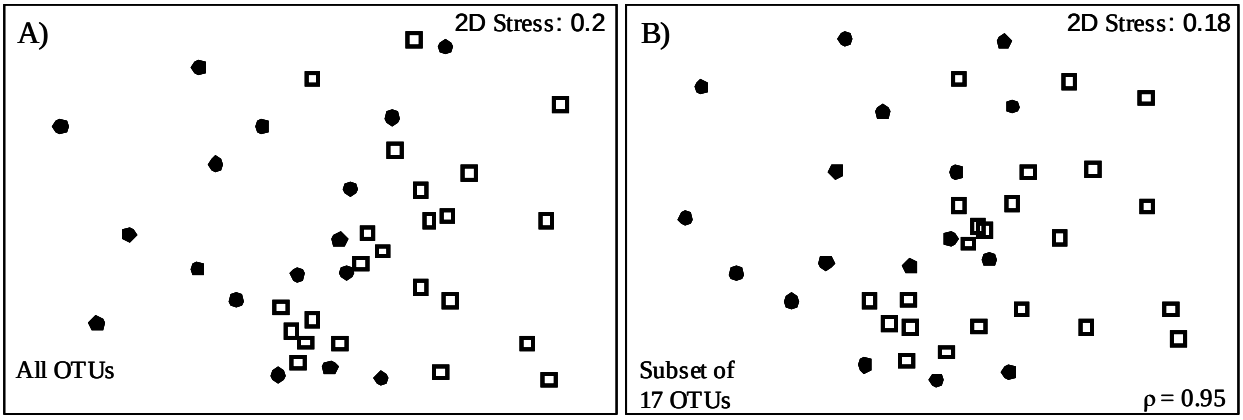

Figure 3.2: nMDS ordination of gut microbial communities from the ileum of birds fed either a barley control (●) or barley supplemented with enzyme (□) diet showing the extent to which A) the overall community pattern generated from the full set of 91 OTUs, is reproducible by B) a smaller subset of 17 OTUs identified by the BVSTEP procedure. The matching coefficient (ρ) to A) is shown in B). The subset of 17 OTUs identified was: 70; 96; 148; 182; 184; 190; 214; 224; 286; 288; 290; 300; 480; 522; 580; 590; and 880.

The difference in caecal bacterial community composition associated with diet is shown in Fig 3.3A. A subset of 29 OTUs was identified by the BVSTEP procedure, which showed a good correlation (ρ=0.95) with the relationship observed from the full set of 111 OTUs. Figure 3.3B shows the nMDS generated from the subset of 29 OTUs that is similar to the nMDS produced from the full set of OTUs (Fig 3.3A). Of the subset of 17 and 29 diet associated OTUs identified within the ileum and caeca, respectively, nine OTUs (70, 96, 184, 214, 286, 290, 300, 580, 590) were common to both gut sections. The remaining eight (148, 182, 190, 224, 228, 480, 522, 880) and twenty (92, 94, 146, 158, 188, 198, 212, 216, 222, 486, 488, 490, 496, 526, 528, 530, 536, 538, 546, 548) diet associated OTUs identified were unique to the ileum and caeca, respectively.

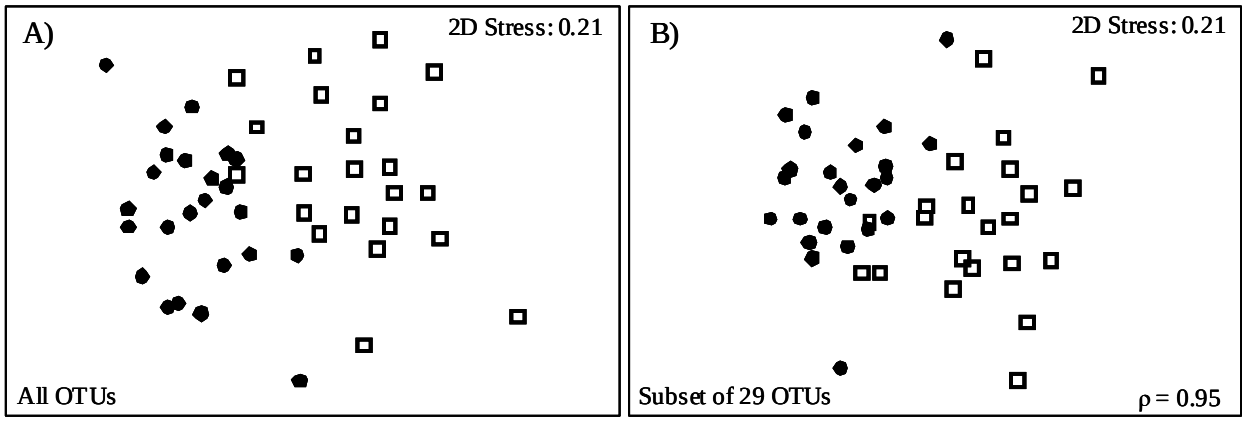

Figure 3.3: nMDS ordination of gut microbial communities from the caeca of birds fed either a barley control (●) or barley supplemented with enzyme (□) diet showing the extent to which A) the overall community pattern identified from the full set of 111 species, is reproducible by B) a smaller subset of 29 species generated by the BVSTEP procedure. The matching coefficient (ρ) to A) is shown in B). The subset of 29 OTUs identified were: 70; 92; 94; 96; 146; 158; 184; 188; 198; 212; 214; 216; 222; 286; 290; 300; 486; 488; 490; 496; 526; 528; 530; 536; 538; 546; 548; 580; and 590.

Similarities in gut bacterial communities between birds on the same diet were calculated with SIMPER. Within the ileum, gut bacterial communities were 28% similar for birds on the barley control diet and 34% similar for birds on the barley diet supplemented with enzyme. Within the caeca, similarity in gut bacterial community composition was higher and found to be 48% and 42% for birds on the barley control diet and barley diet supplemented with enzyme, respectively. Dissimilarity in bacterial communities associated with diet were 73% in the ileum and 66% in the caeca. OTUs contributing to the top 50% of dissimilarity in bacterial community composition between diets were identified within the ileum (Table 3.3) and caeca (Table 3.4). Nine OTUs within the ileum, and twenty-one OTUs within the caeca, were identified as good discriminators between diets. Six of these diet-associated OTUs (286, 214, 590, 580, 96, and 224) were common to both the ileum and caeca.

Table 3.3: OTU contribution to the dissimilarity in ileal microbial communities associated with diet. Average abundance of important OTUs in ileal microbial communities of birds fed either a barley diet (B) or barley plus enzyme diet (BE) are shown. OTUs are listed in order of their contribution ($\overline{\delta}_i$) to the average dissimilarity $\overline{\delta}$ (=72.86%) between diets. Percent contribution of individual OTUs and cumulative percent contribution to the top 50% of average dissimilarities are shown. OTUs identified as being good discriminators between diet are marked with an asterix *

OTU	Av. abundance		$\overline{\delta}_i$	$\overline{\delta}_i$/SD(δ_i)	Individual contribution %	Cumulative contribution %
	BE diet	B diet				
522	0.15	1.24	3.69	0.98	5.06	5.06
190*	1.47	1.49	3.44	1.21	4.72	9.78
288*	1.14	0.72	3.01	1.18	4.14	13.92
286*	1.23	0.50	2.98	1.26	4.09	18.01
182*	0.65	0.83	2.68	1.02	3.68	21.69
214*	0.86	0.96	2.55	1.20	3.50	25.19
590*	0.95	0.69	2.51	1.09	3.45	28.64
290	0.73	0.29	2.44	0.72	3.35	31.99
580*	1.32	1.04	2.40	1.08	3.29	35.28
96*	0.65	0.62	2.20	1.06	3.01	38.29
224*	0.80	0.27	2.14	1.19	2.94	41.24
880	0.68	0.00	1.86	0.98	2.55	43.79
186	0.40	0.37	1.65	0.83	2.27	46.05
184	0.38	0.26	1.65	0.66	2.26	48.32
300	0.50	0.24	1.53	0.92	2.10	50.42

Table 3.4: OTU contribution to the dissimilarity in caecal microbial communities associated with diet. Average abundance of important OTUs in caecal microbial communities of birds fed either a barley diet (B) or barley plus enzyme (BE) diet are shown. OTUs are listed in order of their contribution ($\bar{\delta}_i$) to the average dissimilarity $\bar{\delta}$ (=65.64%) between diets. Percent contribution of individual OTUs and cumulative percent contribution to the top 50% of average dissimilarities are shown. OTUs identified as being good discriminators between diet are marked with an asterix *

OTU	Av. abundance		$\bar{\delta}_i$	$\bar{\delta}_i$/SD(δ_i)	Individual contribution %	Cumulative contribution %
	BE diet	B diet				
70*	1.51	0.04	2.64	2.56	4.03	4.03
96*	1.15	2.36	2.43	1.52	3.70	7.73
224*	1.24	0.42	1.88	1.32	2.86	10.59
212*	0.58	1.08	1.71	1.17	2.61	13.20
286*	1.47	0.69	1.68	1.28	2.56	15.76
548*	0.46	1.13	1.62	1.30	2.47	18.23
222*	0.76	1.02	1.56	1.27	2.38	20.61
188*	1.21	0.61	1.54	1.26	2.35	22.96
496*	0.79	0.60	1.49	1.16	2.26	25.22
94*	0.31	0.79	1.43	1.03	2.18	27.40
214*	0.74	1.18	1.37	1.19	2.09	29.49
198*	0.80	0.51	1.34	1.14	2.05	31.54
92	0.12	0.72	1.33	0.97	2.03	33.56
522*	0.05	0.72	1.27	1.07	1.94	35.50
158*	0.20	0.78	1.27	1.34	1.93	37.43
146*	0.76	0.26	1.22	1.20	1.86	39.29
580*	0.55	0.64	1.19	1.10	1.82	41.10
300*	0.52	0.50	1.17	1.02	1.78	42.88
590*	0.58	0.46	1.15	1.02	1.75	44.63
318*	0.97	0.60	1.11	1.09	1.69	46.32
530*	0.59	0.35	1.09	1.05	1.65	47.97
290*	0.60	0.29	1.08	1.02	1.65	49.62

PCO was done on the subset of 17 OTUs identified within the ileum (Fig 3.4) and 29 OTUs identified within the caeca (Fig 3.5). The PCOs have been overlayed with the subset of OTUs identified by the BVSTEP procedure, indicating which OTUs are most strongly associated with a particular diet. These PCO ordinations confirm the separation in gut bacterial communities associated with diet. Table 3.5 summarises common diet associated OTUs identified within the ileum and caeca by both the SIMPER and BVSTEP procedures. Within the ileum, nine diet associated OTUs were confirmed by both the SIMPER and BVSTEP analyses with five of these having a strong association with the barley diet supplemented with enzyme and one OTU having a strong association with the barley control diet (Table 3.5). OTU 522 and 480 also had a strong association with the barley control diet as illustrated by the PCO ordination (Fig 3.4). However, these were not identified as good discriminators between diets (from the SIMPER analysis), even though OTU 522 was identified as contributing to the top 50% of dissimilarity between dietary treatments. Within the caeca, 18 diet-associated OTUs were confirmed by both the SIMPER and BVSTEP analyses (Table 3.5). Seven of these OTUs were shown to have strong associations with the barley diet supplements with enzyme, and six OTUs had strong associations with the barley control diet (Table 3.5). Most of the diet-associated OTUs identified were specific to either the ileum or caeca. However, OTUs 286 and 590 were common to both the ileum and caeca and associated with the barley plus enzyme treatment group.

Table 3.5: Common diet-associated OTUs identified within the ileum and caeca by both the SIMPER and BVSTEP procedures and identification of those OTUs showing strong association with a particular diet.

	SIMPER/BVSTEP OTUs	Strong diet association OTUs	
Gut section		Barley plus enzyme	Barley
Ileum	96, 182, 190, 214, 224, 286, 288,580, 590	224, 286, 288, 580 590	182
Caeca	70, 92, 94, 96, 146, 158, 188, 212, 214, 222, 286, 290, 300, 496, 530, 548, 580, 590.	70, 146, 188, 286, 290, 530, 590	92, 96, 212, 214, 158, 548

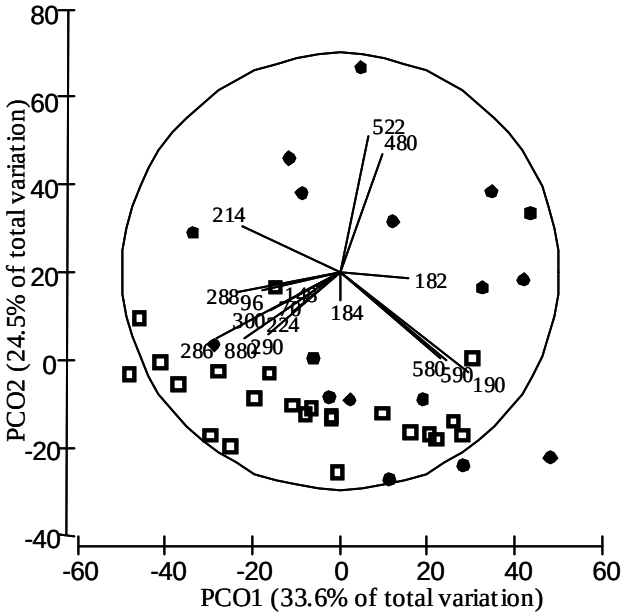

Figure 3.4: PCO ordination of ileal microbial communities from birds fed either a barley control (●) or barley supplemented with enzyme (□) diet. Overlaid onto the PCO are vectors of the subset of 17 OTUs identified by the BVSTEP procedure indicating association of OTUs with particular diets.

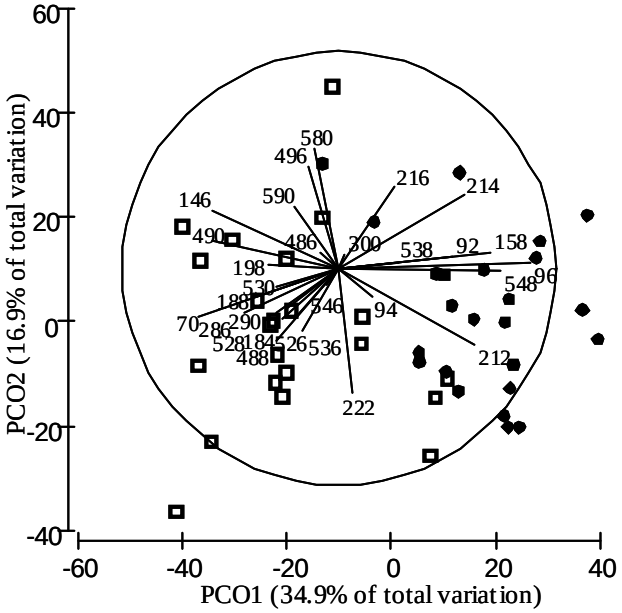

Figure 3.5: PCO ordination of caecal microbial communities from birds fed either a barley control (●) or barley supplemented with enzyme (□) diet. Overlaid onto the PCO are vectors of the subset of 29 OTUs identified by the BVSTEP procedure indicating association of OTUs with particular diets.

A constrained CAP analysis done on gut bacterial communities from either the ileum or caeca produced biplots from the first canonical analysis of principal coordinates axis (CAP1) against bird performance as measured by AME. A good correlation was seen between ileal bacterial community composition and AME (R=0.56) (Fig 3.6A) and between caecal bacterial community composition and AME (R=0.55) (Fig 3.6B).

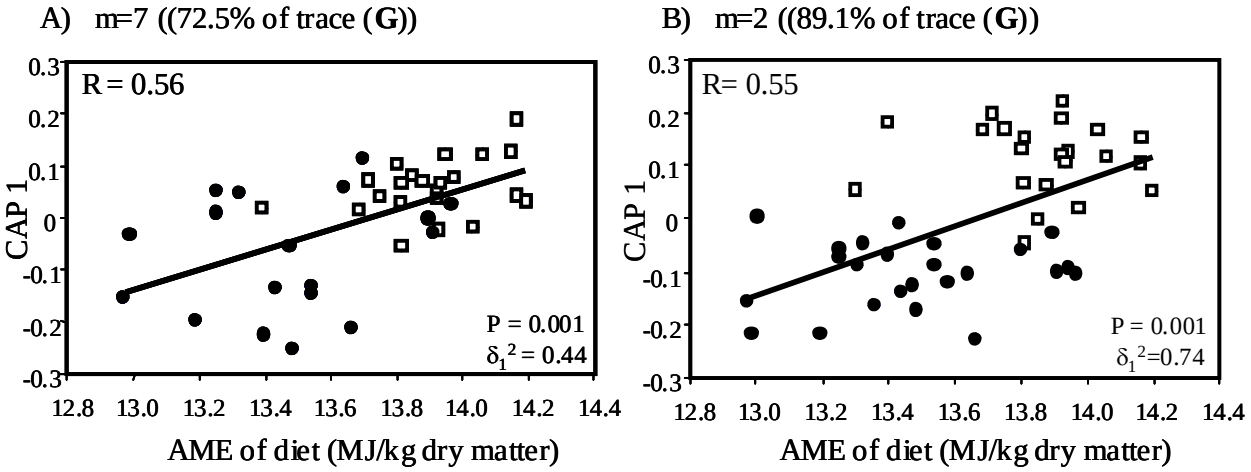

Figure 3.6: CAP of diet associated gut microbial communities related to AME. CAP versus AME biplot for A) the ileum and B) the caeca. CAP analysis was based on Bray-Curtis similarities calculated from fourth-root transformed species abundances. m achieves the maximum proportion of correct allocations (% of Trace (G)) of samples to diet. □ = birds on the barley plus enzyme diet, ● = birds on the barley control diet.

Discussion

We have demonstrated positive correlation of gut microbial communities and bird performance for the first time. We have identified several indicator bacterial species contributing to the diet-induced differences in the overall gut microbial community. The presence of specific beneficial bacterial species and/or the absence of specific detrimental bacterial species may be partially responsible for the improved AME in these chickens.

Classical growth/performance analysis showed that chickens fed the barley plus enzyme diet had a significantly higher AME than chickens fed the control barley diet as expected. Improvements in performance of birds on barley-based diets due to supplementation with NSP degrading enzyme (β-glucanase) have been widely reported (Almirall and Esteve-Garcia, 1994, Brenes *et al*., 1993, Jamroz *et al*., 2001a, Jamroz *et al*., 2001b).

Supplementation of wheat-based diets with the exogenous NSP degrading enzyme, xylanase, has been shown to alter broiler gut microflora by lowering counts of Enterobacteria, total gram-positive cocci (Vahjen *et al.*, 1998) and *Clostridium perfringens* (Choct *et al*., 2006). The current study however, shows that overall gut bacterial community composition is altered by exogenous enzyme supplementation of a barley-based diet. It is also the first report that directly correlates diet-associated changes in gut microbial community with improved performance.

The identity of some of the diet associated OTUs can be predicted from *in silico* PCR amplification and restriction of 16S rDNA gene sequences found in a public database (http://mica.ibest.uidaho.edu/). Hence, barley plus enzyme diet associated OTUs 580 and 188 identified within the ileum and caeca, respectively, may represent various Lactobacilli species. Furthermore, the barley control diet associated OTUs 182 and 522 identified within the ileum may represent Lactobacilli and *Clostridium perfringens* (or other Clostridium species), respectively.

Lactobacilli (Firmicutes) are present throughout the gastrointestinal tract of poultry (Guan *et al*., 2003, Knarreborg *et al*., 2002a, Lan *et al*., 2002). Lactobacilli have various biochemical properties, including production of antibacterial compounds (De Angelis *et al.*, 2006, Stern *et al*., 2006), β-glucanase (Jonsson and Hemmingsson, 1991), and bile salt hydrolase compounds (Knarreborg *et al.*, 2002b). Lactobacilli with β-glucanase activity have been identified in faeces of piglets and linked to presence of β-glucans in the diet (Jonsson and Hemmingsson, 1991). Other bacteria containing 1,3-1,4-β-endoglucanase activities (*Bacteroides ovatus*, *B. uniformis*, *B. capillosus*, *Clostridium perfringens* and *Streptoccus bovis*) have recently been isolated from poultry (Beckmann *et al*., 2006). β-glucanase activity attributed to these bacterial species may explain the association of Lactobacilli (OTU 182) and *Clostridium perfringens* (OTU 522) with the barley control diet. Alternatively, the incidence of *C. perfringens* has been shown to decrease in poultry fed a wheat-based diet supplement with xylanase (Choct *et al*., 2006).

Diet-associated differences in the composition of the gut microbial community were only detected within the ileum and caeca. Diet has previously been shown to modify overall caecal microflora composition (Apajalahti *et al.*, 2001). We have shown that many bacterial species, not just a select few, are responsible for the overall difference in gut bacterial community composition associated with diet. Single OTUs identified as being good discriminators between diets (SIMPER) generally contributed 1-5% to the overall dissimilarity. Both SIMPER and BVSTEP have enabled potential OTUs (bacterial species) associated with a barley-based diet, either with or without supplementation of exogenous enzyme, to be identified. Most of the diet-associated OTUs identified were unique to either the ileum or caeca, however a few were common to both gut sections.

Chapter 4: Effect of prebiotics on gut microflora development

Introduction

Microbial profiling was done on ileal digesta samples from two experiments investigating effects of prebiotics in chickens. Both experiments were done by Mr Janak Vidanarachchi at the University of New England, Armidale as part of his PhD candidature and were integral parts of the Australian Poultry CRC funded project 03-04 (Control over life-long productivity by dietary manipulation immediately post hatch). The two experiments were identified as: UNE experiment 1- commercial products and plant extract as prebiotics in broiler chickens; and UNE experiment 3- native plant extracts as prebiotics in broiler chickens.

Materials and methods

UNE experiment 1: Commercial products and plant extract as prebiotics in broiler chickens

Forty-eight freeze-dried chicken ileal digesta samples were provided by Mr Janak Vidanarachchi for microbial profiling analysis. Six prebiotic dietary treatments were tested and compared to control diets containing either no supplement, or supplemented with antibiotic. Each treatment group contained six replicates.

The dietary treatments tested were: no supplement (negative control); supplementation with 45 ppm zinc bacitracin (positive control); supplementation with 0.5% arabinogalactan product; supplementation with 1% arabinogalactan product; supplementation with 0.5% fructooligosaccharide product; supplementation with 1% fructooligosaccharide product; supplementation with 0.5% rengarenga extract; and supplementation with 1% rengarenga extract.

UNE experiment 3: Native plant extracts as prebiotics in broiler chickens

Forty-eight freeze-dried chicken ileal digesta samples were provided by Mr Janak Vidanarachchi for microbial profiling analysis. Six prebiotic dietary treatments were tested and compared to control diets containing either no supplement, or supplemented with antibiotic. Each treatment group contained six replicates.

The dietary treatments tested were: no supplement (negative control); supplementation with 45 ppm zinc bacitracin (positive control); supplementation with 0.5% cabbage tree extract; supplementation

with 1% cabbage tree extract; supplementation with 0.5% seaweed extract; supplementation with 1% seaweed extract; supplementation with 0.5% acacia extract; and supplementation with 1% acacia extract.

T-RFLP and statistical analysis of OTUs

T-RFLP methodology is detailed in Chapter 1 and statistical analysis of generated data points is detailed in Chapter 2.

Screening of bacterial cultures

Twenty-nine pure bacterial cultures isolated from digesta of birds in UNE experiment 3 were provided by Dr Lene Mikkelsen to aid in identification of observed OTUs from the microbial profiling analysis.

The 16S ribosomal gene of each of the twenty-nine pure bacterial cultures were analysed by restriction fragment length polymorphism (RFLP) analysis to differentiate isolates. Representative bacteria from each of the identified groups were sequenced to determine identity and used as standards in the bacterial profiling analysis. Total nucleic acid was extracted from twenty-nine bacterial cultures by a modified SARDI proprietary method. The bacterial ribosomal DNA was amplified with unlabelled universal 16S bacterial primers, identical in sequence to those used for T-RFLP analysis. The resulting amplicons were restricted with three different specific recognition sequence restriction enzymes (*Msp*I, *Cfo*I and *Rsa*I) and separated by standard gel electrophoresis. Bacterial isolates were grouped according to restriction banding patterns. Representative isolates from each of the restriction banding patterns were cloned and sequenced. Clones were used as standards in T-RFLP. Bacterial isolates were identified by sequence similarity with known organisms in public genome databases using the BLAST interface (http://www.ncbi.nlm.nih.gov/blast/).

Results

UNE experiment 1: Commercial products and plant extract as prebiotics in broiler chickens

There were significant differences in ileal bacterial community composition between dietary treatments (ANOSIM, Global R=0.412, P=0.001). Table 4.1 shows the results of the one-way ANOSIM for differences between ileal bacterial communities associated with the dietary treatments. There was a significant difference (P=0.004) in ileal bacterial community composition of birds receiving no dietary supplementation (negative control) and those supplemented with antibiotics (positive control). This is graphically demonstrated in the nMDS ordination representing the gut bacterial communities from the ilea of birds on the two control diets (Fig 4.1).

Table 4.1: One-way ANOSIM of ileal microbial communities associated with dietary treatments from UNE experiment 1. The *R* statistic (**bold**) and significance level (*italics*) are shown between diets. Ar = arabingalactan product, Fr = fructooligosacharide product, Re = rengarenga extract.

	Negative	Positive	0.5 % Ar	1 % Ar	0.5 % Fr	1 % Fr	0.5 % Re	1 % Re
Negative		*0.004*	*0.002*	*0.002*	*0.017*	*0.019*	*0.006*	*0.004*
Positive	**0.621**		*0.002*	*0.002*	*0.002*	*0.011*	*0.002*	*0.002*
0.5% Ar	**0.821**	**0.833**		*0.004*	*0.063*	*0.002*	*0.039*	*0.024*
1% Ar	**0.899**	**0.796**	**0.535**		*0.002*	*0.002*	*0.004*	*0.043*
0.5% Fr	**0.328**	**0.472**	**0.191**	**0.359**		*0.175*	*0.238*	*0.002*
1% Fr	**0.307**	**0.393**	**0.437**	**0.533**	**0.095**		*0.082*	*0.002*
0.5% Re	**0.339**	**0.376**	**0.164**	**0.376**	**0.083**	**0.148**		*0.035*
1% Re	**0.835**	**0.585**	**0.363**	**0.300**	**0.465**	**0.632**	**0.240**	

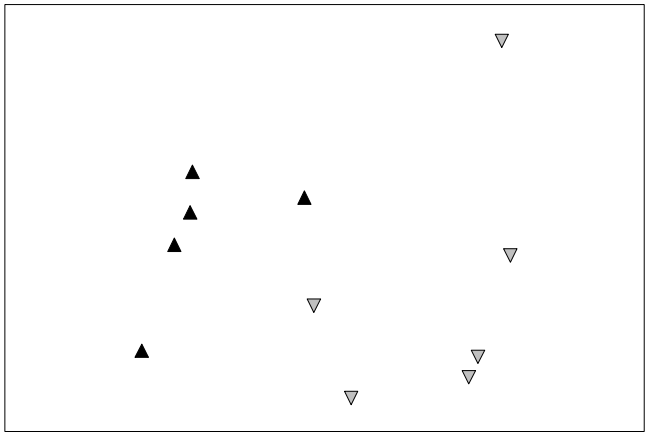

Figure 4.1: 2-D nMDS ordination of OTUs from T-RFLP analysis of bacterial communities from ileal contents of chickens in ▼ positive and ▲ negative treatment groups. Stress value=0.11. Each point in the ordination represents the overall ileal bacterial profile of an individual chicken and its relationship to the ileal bacterial profiles of all other chickens in the comparison. The closer two points are in the ordination the more similar are the overall ileal bacterial profiles of those two chickens.

OTUs contributing to the top 50 % of overall differences observed in ileal bacterial community composition between positive and negative control groups were identified using SIMPER (Table 4.2).

Table 4.2: OTU contribution to the dissimilarity in ileal bacterial communities associated with diet. Average abundance of important OTUs in ileal bacterial communities of birds fed either a negative or positive control diet are shown. OTUs are listed in order of their contribution ($\bar{\delta}_i$) to the average dissimilarity $\bar{\delta}$ (=69.1%) between diets. Percent contribution of individual OTUs and cumulative percent contribution to the top 50% of average dissimilarities are shown. OTUs identified as being good discriminators between diet are marked with an asterix *

OTU	Av. abundance		$\bar{\delta}_i$	$\bar{\delta}_i$/SD(δ_i)	Individual contribution %	Cumulative contribution %
	Negative control	Positive control				
578*	1.78	0.46	4.89	1.58	7.07	7.07
522*	1.28	0.00	3.73	4.31	5.39	12.46
142*	0.00	1.09	3.02	1.79	4.36	16.82
182*	1.40	0.38	3.01	1.60	4.35	21.18
62*	0.26	1.17	2.89	1.43	4.19	25.36
576	0.00	1.12	2.88	0.93	4.16	29.53
590*	1.07	0.50	2.86	1.09	4.14	33.67
152*	1.02	0.00	2.76	1.85	3.99	37.66
574	0.69	0.50	2.29	0.98	3.31	40.97
178*	0.76	0.00	2.04	1.18	2.95	43.92
58*	0.42	0.71	2.04	1.05	2.95	46.87
586	0.37	0.54	1.82	0.81	2.63	49.50
224*	0.64	0.00	1.77	1.17	2.56	52.06

There were significant differences ($P<0.05$) in ileal bacterial community composition between the negative control and: arabinogalactan; fructooligosaccharide; and rengarenga, regardless of inclusion rate (Table 4.1). However, there were also significant differences ($P<0.05$) in ileal bacterial community composition between the positive control and: arabinogalactan; fructooligosaccharide; and rengarenga (Table 4.1).

On comparison of the prebiotic treatments it was found that there were significant differences ($P<0.05$) in ileal bacterial community between: either concentration of arabinogalactan and 1% fructooligosaccharide; 1% arabinogalactan and 0.5% fructooligosacharide; either concentration of fructooligosaccharide and 1% rengarenga; arabinagalactan and rengarenga, regardless of inclusion rate (Table 4.1). However, there were also significant differences in bacterial community composition between differing inclusion rates of a single prebiotic treatment, as found between: 0.5% and 1% arabinogalactan; and 0.5% and 1% rengarenga. There were no significant differences ($P>0.05$) in ileal bacterial community composition between: 0.5% and 1% fructooligosaccharide; 0.5% fructooligosaccharide and 0.5% arabinogalactan; and 0.5% rengarenga and either concentration of fructooligosaccharide (Table 4.1).

UNE experiment 3: Native plant extracts as prebiotics in broiler chickens

There were significant differences in ileal bacterial community composition between dietary treatments (ANOSIM, Global *R*=0.478, P=0.001). Table 4.3 shows the results of the one-way ANOSIM for differences between ileal bacterial communities associated with the dietary treatments. There was a significant difference (P=0.020) in ileal bacterial community composition of birds either receiving no dietary supplementation (negative control) or those supplemented with antibiotics (positive control).

Table 4.3: One-way ANOSIM of ileal microbial communities associated with dietary treatments from UNE experiment 3. The *R* statistic (**bold**) and significance level (*italics*) are shown between diets. CT = cabbage tree extract, SW = seaweed extract, AC = acacia extract.

	Negative	Positive	0.5% CT	1% CT	0.5% SW	1% SW	0.5% AC	1% AC
Negative		*0.020*	*0.182*	*0.020*	*0.002*	*0.032*	*0.435*	*0.188*
Positive	**0.324**		*0.002*	*0.002*	*0.013*	*0.009*	*0.006*	*0.024*
0.5% CT	**0.106**	**0.769**		*0.002*	*0.002*	*0.002*	*0.203*	*0.152*
1% CT	**0.401**	**0.716**	**0.724**		*0.030*	*0.043*	*0.016*	*0.002*
0.5% SW	**0.639**	**0.467**	**0.976**	**0.392**		*0.043*	*0.002*	*0.002*
1% SW	**0.324**	**0.616**	**0.716**	**0.323**	**0.267**		*0.002*	*0.002*
0.5% AC	**0.016**	**0.501**	**0.099**	**0.402**	**0.909**	**0.656**		*0.485*
1% AC	**0.109**	**0.341**	**0.130**	**0.643**	**0.872**	**0.702**	**-0.032**	

The current analysis did not use Bonferroni correction (more stringent significance levels) of ANOSIM results, hence in some cases has led to slightly different interpretation of results from those previously reported. The Bonferroni correction states that if there are *n* pairwise comparisons in total then the test uses a significant level of 0.05/*n*. The correction in theory minimises the risk of making a Type I error (the probability of detecting a difference when there is no genuine difference). However, a too demandingly low Type I error (significance level) will be at the expense of a greater risk of Type II error (the probability of not detecting a difference when one genuinely exists) (Clarke and Warwick, 2001)

OTUs contributing to the top 50 % of overall differences observed in ileal bacterial community composition between positive and negative control groups was identified using SIMPER (Table 4.4).

Table 4.4: OTU contribution to the dissimilarity in ileal bacterial communities associated with diet. Average abundance of important OTUs in ileal bacterial communities of birds fed either a negative or positive control diet are shown. OTUs are listed in order of their contribution ($\bar{\delta}_i$) to the average dissimilarity $\bar{\delta}$ (=74.1%) between diets. Percent contribution of individual OTUs and cumulative percent contribution to the top 50% of average dissimilarities are shown. OTUs identified as being good discriminators between diet are marked with an asterix *

OTU	Av. abundance		$\bar{\delta}_i$	$\bar{\delta}_i$/SD(δ_i)	Individual contribution %	Cumulative contribution %
	Negative control	Positive control				
580*	0.84	1.70	4.08	1.07	5.51	5.51
564*	1.24	0.00	3.66	1.30	4.94	10.45
182*	1.17	1.80	2.98	1.06	4.02	14.47
576	0.26	0.93	2.82	0.80	3.81	18.28
62*	0.46	1.31	2.72	1.44	3.67	21.94
574	1.07	0.00	2.71	0.84	3.65	25.59
190*	0.00	0.92	2.65	1.27	3.58	29.17
184	0.67	0.53	2.48	0.89	3.34	32.52
578	0.84	0.00	2.48	0.64	3.34	35.86
142*	0.00	0.79	2.36	1.27	3.18	39.04
186*	0.20	0.90	2.23	1.20	3.01	42.05
522*	0.87	0.00	2.21	1.32	2.98	45.03
216*	0.88	0.40	2.15	1.15	2.91	47.94
58*	1.10	0.56	2.09	1.20	2.82	50.76

There were significant differences ($P<0.05$) in the ileal bacterial communities of birds on the negative control diet as compared with: 1% cabbage tree extract; and either concentration of seaweed extract. However, there were no significant differences ($P>0.05$) in ileal bacterial community of birds on the negative control diet as compared with: 0.5% cabbage tree extract; and either concentration of acacia extract. Ileal bacterial communities of birds on all prebiotic treatments were significantly different ($P<0.05$) from the positive control (antibiotic supplemented) group (Table 4.3). The ordination in Fig 4.2 visually demonstrates that the ileal microbial communities of chickens on the acacia extract supplemented diet were not different from the negative control group but were different from the positive control group.

There were statistically significant differences ($P<0.05$) in ileal bacterial community composition between seaweed extract and acacia extract, and between seaweed extract and cabbage tree extract, regardless of inclusion rate (Table 4.3). This is graphically shown in Fig 4.3. There were also significant differences in ileal bacterial community composition between 1% cabbage tree extract and either concentration of acacia extract, but no significant differences between 0.5% cabbage tree extract and either percentage of the acacia extracts ($P>0.05$) (Fig 4.3).

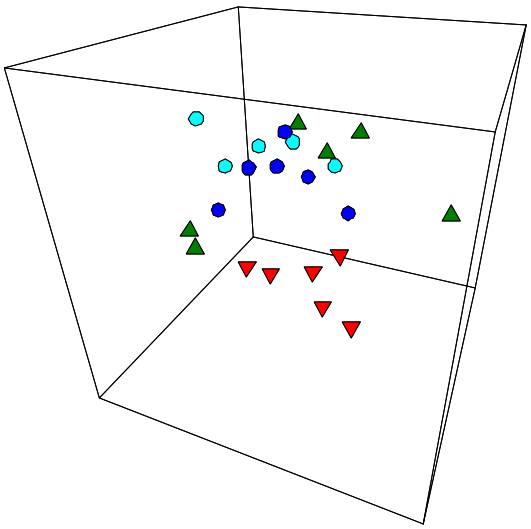

Figure 4.2: 3-D nMDS ordination of OTUs from T-RFLP analysis of bacterial communities from ileal contents of chickens in ▼ positive, ▲ negative, ● 0.5% acacia ● and 1% acacia treatment groups. Stress value=0.12.

There were no significant differences in ileal bacterial community composition between 0.5% and 1% acacia extract (P>0.05). However, there were significant differences in ileal bacterial community between 0.5% and 1% cabbage tree extract and between 0.5% and 1% seaweed extract (Table 4.3).

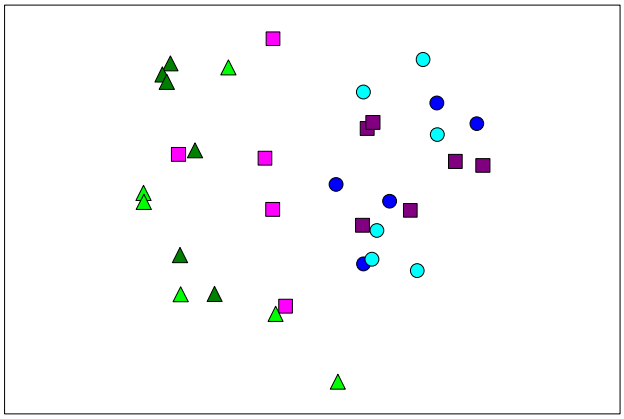

Figure 4.3: 2-D nMDS ordination of OTUs from T-RFLP analysis of bacterial communities from ileal contents of chickens in the ■ 0.5% cabbage tree extract, ■ 1% cabbage tree extract, ▲ 0.5% seaweed extract, ▲ 1% seaweed extract, ● 0.5% acacia extract, and ● 1% acacia extract. Stress value=0.18.

Screening of bacterial cultures

The 29 pure bacterial cultures isolated from digesta (UNE experiment 3) were screened by PCR amplication of the 16S rDNA followed by RFLP analysis. Of the three restriction enzymes used (*Msp*I, *Cfo*I and *Rsa*I) in RFLP it was found that *Msp*I had the greatest discriminating power between isolates. This resulted in the identification of seven unique RFLP patterns among the 29 bacterial isolates (Fig 4.4). Bacterial species representative of the RFLP patterns were identified by genome sequence information obtained from the rDNA region. Four characterised *Lactobacillus* (*L. crispatus, L. salivarius, L. reuterii* and *L johnsonii*), as well as, one uncharacterised *Lactobacillus* species were identified. In addition *Bifidobacterium pseudolongum* and *Clostridium perfringens* were also identified.

Genome sequence information and observed position of T-RFs from generated clones were used to identify OTUs. Based on this information potential identity of OTUs were: *L. salivarius* (OTU 578/580); *L. johnsonii* (OTU 190); *L. crispatus* (OUT 182); *L. reuterii* (OTU 29); *Lactobacillus* sp. (OTU 590); *C. perfringens* (OTU 522); and *B. pseudolongum* (OTU 74).

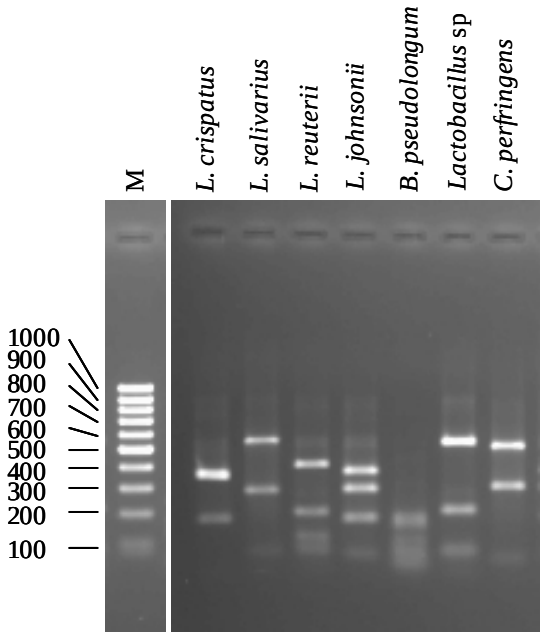

Figure 4.4: Unique RFLP patterns detected from screening 29 pure bacterial cultures isolated from ileal digesta and identification of bacterial species. RFLP analysis was done with *Msp*I. M = 100 bp marker

Discussion

UNE experiment 1: Commercial products and plant extract as prebiotics in broiler chickens

Ileal bacterial community composition was different between the negative control diet (no added supplement) and positive control diet (supplemented with antibiotic). Ten OTUs (taxonomically related bacterial groups and/or species) contributing to the top 50% of dissimilarity in bacteria community composition between these two diets were identified. With the aid of sequence information and cloned standards, generated from bacterial cultures isolated from UNE experiment 3, four of these OTUs may represent *L. salivarius* (578), *L. crispatus* (182), *Lactobacillus* (590) and *C. perfringens* (522). However, it should be noted that these are not the only possibilities and are in no way conclusive. OTUs 578, 182 and 590 were present in both control groups but more prominent in the negative control group, while OTU 522 was only found in the negative control group. Other discriminating OTUs identified between diets were 142, 62, 152, 178, 58 and 224.

Dietary supplementation with all of the prebiotic products (arabinogalactan, fructooligosaccharide or rengarenga) significantly altered ileal bacterial community composition as compared with the negative control group (no added supplement). However, none of the prebiotic products tested resulted in an ileal bacterial community composition similar to that of the antibiotic positive control group.

Ileal bacterial community composition varied between different inclusion rates of the same prebiotic product, suggesting bacterial communities were sensitive to inclusion rate. For example ileal bacterial communities of birds on 0.5% arabinogalactan were different from those on 1% arabinogalactan, as were ileal bacterial communities of bird on 0.5% rengarenga as compared with 1% rengarenga. Only fructoologosaccharide supplementation didn't seem sensitive to inclusion rate as ileal bacterial communities of birds on 0.5% and 1% fructooligosaccharide product were similar.

Prebiotic dietary supplementation altered ileal bacterial community composition between arabinogalactan and fructologosaccharide products, and fructooligosaccharide product and rengarenga extract, but was also sensitive to inclusion rate. Dietary supplementation with arabinogalactan product resulted in a different ileal bacterial community composition as compared with rengarenga extract regardless of inclusion rate.

UNE experiment 3: Native plant extracts as prebiotics in broiler chickens

Ileal bacterial community composition was different between the negative control diet (no added supplement) and positive control diet (supplemented with antibiotic). Ten discriminating OTUs

contributing to the top 50% of the dissimilarity in bacterial community composition between control diets were identified. With the aid of sequence information and cloned standards from available bacterial cultures, four of these OTUs may represent *L. salivarius* (OTU 580), *L. crispatus* (OTU 182), *L. johnsonii* (OTU 190) and *C. perfringens* (OTU 522). OTU 580 and 182 were found in both control treatments but were predominantly associated with the antibiotic positive control group. OTU 190 was only found associated with the positive control group, while OTU 522 was only found associated the negative control group.

Prebiotic dietary supplementation altered ileal bacterial community composition as compared to the negative control group (no added supplement) for all treatments, except for 0.5% cabbage tree extract and acacia extract. Furthermore, ileal bacterial communities of 0.5% cabbage tree extract and acacia extract were also similar. None of the prebiotic treatments resulted in a similar ileal microbial community composition as compared with the antibiotic positive control group.

Ileal bacterial community composition varied between different inclusion rates of the same prebiotic product, suggesting bacterial communities were sensitive to inclusion rate. Ileal bacterial communities of birds on 0.5% cabbage tree extract were different from those on 1% cabbage tree extract, as were ileal bacterial communities of bird on 0.5% seaweed extract as compared with 1% seaweed extract. Only acacia supplementation didn't seem sensitive to inclusion rate as ileal bacterial communities of birds on 0.5% and 1% acacia extract were similar.

Prebiotic dietary supplementation did alter ileal microbial community composition between seaweed extract and acacia extract, and cabbage tree extract and seaweed extract groups, regardless of inclusion rate. Differences observed in ileal bacterial community composition of birds on the cabbages tree versus acacia extract were sensitive to inclusion rate.

Conclusion

Supplementation of the diet with AGPs alters the overall gut microflora of poultry. This confirms findings of others (Engberg *et al*., 2000, Faichild *et al*., 2005, Knarreborg *et al.*, 2002a). Supplementation of the poultry diet with prebiotics (arabinogalactan product, fructooligsacharide product, rengarenga extract, cabbage tree extract, seaweed extract or acacia extract) has been shown to shift the overall ileal bacterial population in most cases from a non-supplemented control group, with the exception of 0.5% cabbage tree extract and acacia extract. None of the prebiotics tested produced a shift in the ileal bacterial microflora similar to that produced by supplementation with the antibiotic zinc bacitracin.

Interpretation of statistical results may vary whether or not Bonferroni correction is used in the pairwise comparisons of ANOSIM. Furthermore, from our experience replicates per treatment should equal at least 12 (n=12) due to between bird variations in gut microflora and the replication in this set of experiments was lower. Hence, caution should be taken not to over interpret results from statistically underpowered experiments.

Chapter 5: Effect of litter material on gut microflora

Introduction

Poultry litter materials commonly used in Australia include shavings and sawdust from soft and hard woods, rice hulls, chopped straw and shredded paper, similar to those described by Grimes *et al*. (2002). In most locations, chicken growers have limited choices of litter materials due to cost and/or availability. It has been suggested that litter quality and composition can affect flock performance (Fries *et al*., 2005), however the direct effect of litter material on poultry gut microflora development and composition has not been investigated.

The microflora from caecal contents of chickens aged two and four weeks old were investigated by T-RFLP to determine if there were differences in the gut microbial community composition of birds raised on seven different litter materials commercially used in Australia.

Materials and methods

Ingham's litter trial

Seven litter materials were tested in one of Ingham's nutrition trials to determine the effects on growth, performance and gizzard development of broiler chickens. The experiment was done at Ingham's Leppington facility in New South Wales by Ms Moreen Ali and Dr Ron MacAlpine.

The litter materials used in the experiment were: rice hulls from New South Wales; softwood sawdust from Victoria; pine shavings from New South Wales; re-used single batch litter from a previous Leppington trial done on pine shavings; hardwood sawdust from Western Australia; shredded paper from Queensland; and chopped straw from South Australia. All birds in the experiment received the same diet.

Caecal contents were taken from 12 birds (n = 6 females, n = 6 males) per treatment at two and four weeks of age. All samples were freeze dried prior to being sent to SARDI for T-RFLP analysis.

T-RFLP and statistical analysis of OTUs

T-RFLP methodology is outlined in Chapter 1 and statistical analysis of generated data points is outlined in Chapter 2.

Results

Multivariate statistical analysis showed that the composition of the caecal bacterial community was significantly different between age groups and litter materials. The global *R*-value for differences between ages across all litter materials was 0.362 (P=0.001) and for differences between litter materials across both ages was 0.121 (P=0.001). The influence of age and sex of chickens on caecal microbial community composition were further investigated for each of the seven litter materials. Sex was found not to have an influence on gut microbial community composition (P>0.05), whereas age was found to have a significant influence on caecal microbial community composition (P<0.05), regardless of litter material used (Table 5.1). The difference in caecal microflora associated with age across all litter treatments is shown in Fig 5.1.

Table 5.1: Two-way ANOSIM of caecal microbial communities associated with sex and age for each of the seven litter materials tested. The global *R* statistic (**bold**) and significance level (*italics*) are shown for each of the factors for individual litter materials. RH = rice hulls, SWS = softwood sawdust, PS = pine shavings, RSBL = re-used single batch litter, HWS = hardwood sawdust, SP = shredded paper, ChSt = chopped straw.

	RH (NSW)	SWS (Vic)	PS (NSW)	RSBL (Lepp)	HWS (WA)	SP (Qld)	ChSt (SA)
Sex	**0.016**, *0.396*	**0.121**, *0.114*	**0.034** *0.374*	**0.119**, *0.101*	**0.165**, *0.051*	**-0.010**, *0.521*	**0.109**, *0.073*
Age	**0.447**, *0.002*	**0.511**, *0.001*	**0.187**, *0.031*	**0.163**, *0.029*	**0.435**, *0.001*	**0.421**, *0.001*	**0.571**, *0.001*

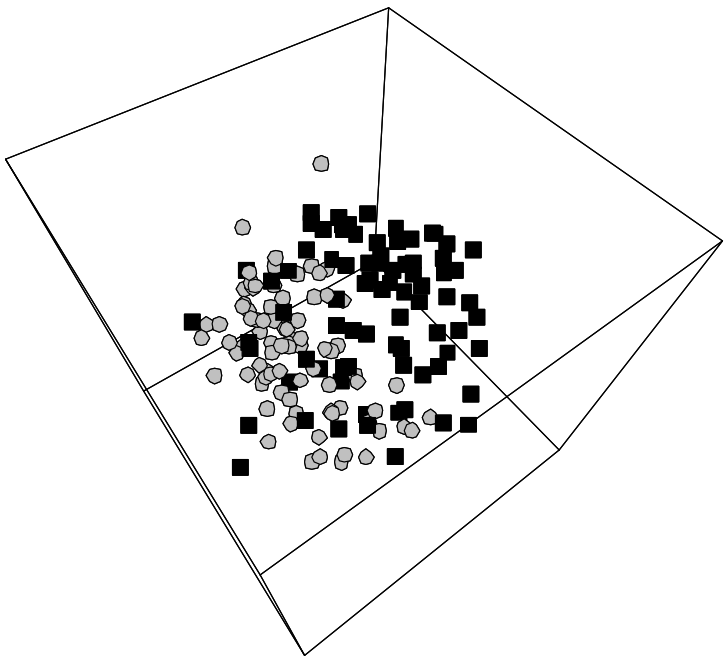

Figure 5.1: 3-D nMDS ordination identifying caecal microbial communities of chickens aged two ■ and four ● weeks across all litter treatments. 3-D Stress = 0.23.

At two weeks of age the caecal microbial communities of chickens raised on the re-used single batch litter was significantly different (P<0.05) from the caecal microbial communities of chickens raised on any of the other six litter material tested (Table 5.2). This is visually demonstrated in the ordination in Fig. 5.2.

Table 5.2: One-way ANOSIM of caecal microbial communities at two weeks of age associated with litter treatments. The *R* statistic (**bold**) and significance level (*italics*) are shown between litter treatments. The global *R*-value for differences between litter treatments was 0.164 at a significance level of 0.001. RH = rice hulls, SWS = softwood sawdust, PS = pine shavings, RSBL = re-used single batch litter, HWS = hardwood sawdust, SP = shredded paper, ChSt = chopped straw.

	RH (NSW)	SWS (Vic)	PS (NSW)	RSBL (Lepp)	HWS (WA)	SP (Qld)	ChSt (SA)
RH (NSW)		*0.010*	*0.371*	*0.001*	*0.009*	*0.043*	*0.256*
SWS (Vic)	**0.200**		*0.558*	*0.001*	*0.015*	*0.511*	*0.055*
PS (NSW)	**0.018**	**-0.021**		*0.001*	*0.067*	*0.092*	*0.395*
RSBL (Lepp)	**0.287**	**0.422**	**0.264**		*0.003*	*0.001*	*0.002*
HWS (WA)	**0.184**	**0.201**	**0.112**	**0.318**		*0.028*	*0.308*
SP (Qld)	**0.112**	**-0.009**	**0.095**	**0.446**	**0.162**		*0.118*
ChSt (SA)	**0.035**	**0.117**	**0.011**	**0.369**	**0.023**	**0.060**	

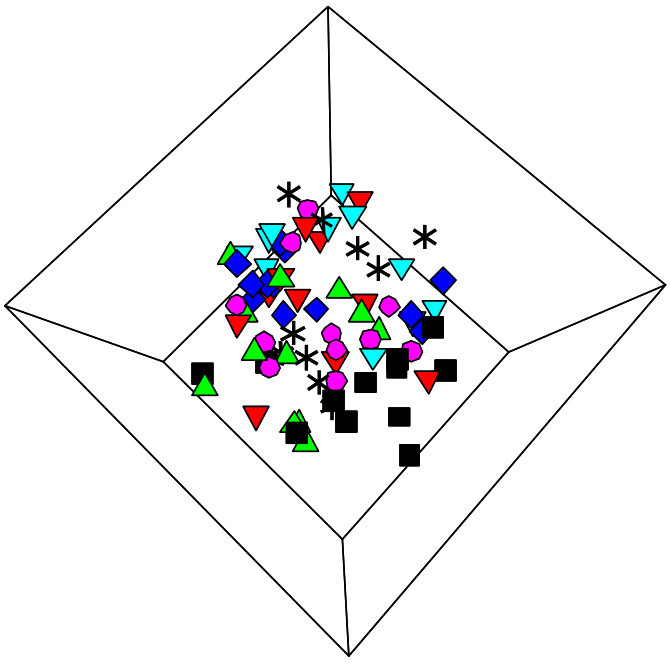

Figure 5.2: 3-D nMDS ordination of caecal microbial communities from two-week-old chickens raised on: ▲ rice Hulls (NSW); ▼ softwood sawdust (Vic); ✱ pine shavings (NSW); ■ re-used single batch litter (Lepp); ● hardwood sawdust (WA); ◆ shredded paper (Qld); ▼ chopped straw (SA). 3-D Stress = 0.22.

Other significant differences in caecal microbial communities at two weeks of age were between chickens raised on rice hulls as compared with softwood sawdust, hardwood sawdust or shredded paper, and between chickens raised on shredded paper versus hardwood sawdust and between chickens raised on softwood sawdust versus hardwood sawdust. No other significant differences in caecal microbial communities were detected between birds raised on any of the other litter treatments at two weeks of age (Table 5.2).

At four weeks of age there were also significant differences in caecal microbial communities between chickens raised on re-used single batch litter as compared with all other litter materials except pine shavings (Table 5.3). Other significant differences in caecal microbial communities at four weeks of age were between birds raised on rice hulls as compared with shredded paper or chopped straw (Fig 5.3). No other significant differences in caecal microbial communities were detected between birds raised on any of the other litter treatments at four weeks of age (Table 5.3).

Table 5.3: One-way ANOSIM of caecal microbial communities at age four weeks associated with litter treatments. The *R* statistic (**bold**) and significance level (*italics*) are shown between litter treatments. The global *R*-value for differences between litter treatments was 0.082 at a significance level of 0.002. RH = rice hulls, SWS = softwood sawdust, PS = pine shavings, RSBL = re-used single batch litter, HWS = hardwood sawdust, SP = shredded paper, ChSt = chopped straw

	RH (NSW)	SWS (Vic)	PS (NSW)	RSBL (Lepp)	HWS (WA)	SP (Qld)	ChSt (SA)
RH (NSW)		*0.791*	*0.659*	*0.018*	*0.365*	*0.011*	*0.003*
SWS (Vic)	**-0.050**		*0.958*	*0.007*	*0.397*	*0.191*	*0.050*
PS (NSW)	**-0.029**	**-0.092**		*0.528*	*0.240*	*0.055*	*0.092*
RSBL (Lepp)	**0.168**	**0.170**	**-0.007**		*0.001*	*0.001*	*0.001*
HWS (WA)	**0.017**	**0.009**	**0.037**	**0.299**		*0.790*	*0.197*
SP (Qld)	**0.131**	**0.054**	**0.101**	**0.375**	**-0.051**		*0.477*
ChSt (SA)	**0.158**	**0.087**	**0.069**	**0.276**	**0.040**	**0.000**	

Similarity in caecal microbial community between chickens raised on the same litter material was overall low, but generally higher for birds at four weeks of age (29-40%) than at two weeks of age (25-32%).

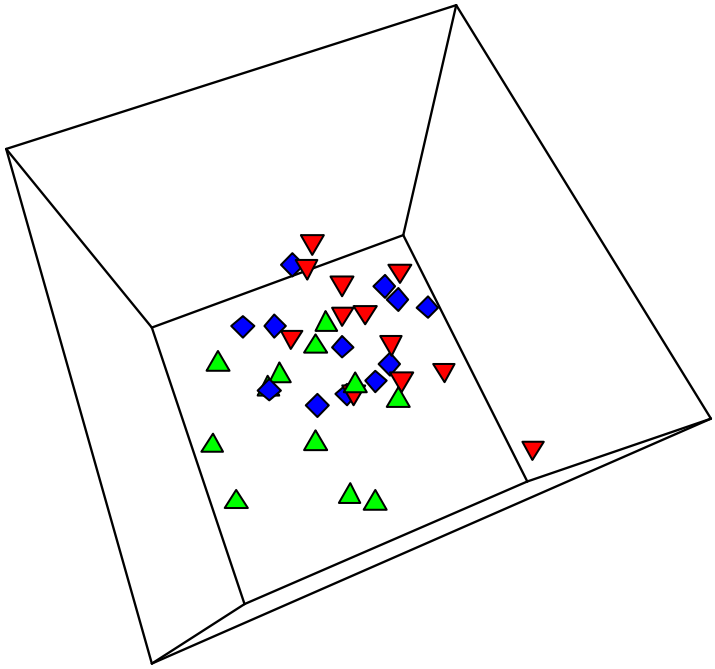

Figure 5.3: 3-D nMDS ordination of caecal microbial communities from four-week-old chickens raised on: ▲ rice hulls (NSW); ◆ shredded paper (Qld); ▼ chopped straw (SA). 3-D Stress = 0.18.

Discussion

Gut microbial profiling was done on the caecal contents of broiler chickens aged two and four weeks which had been raised on one of seven litter materials. The litter materials tested were rice hulls (NSW), softwood sawdust (Vic), pine shavings (NSW), re-used single batch litter (Leppington), hardwood sawdust (WA), shredded paper (Qld), and chopped straw (SA).

Significant differences were detected in the caecal microflora between birds aged two and four weeks, regardless of litter material on which birds had been raised. This indicates that shifts in caecal microflora composition are occurring at this early age. Indeed variability in caecal microflora among chickens raised on the same litter material was higher at two weeks of age than at four weeks of age, suggesting gut microflora is starting to stabilise by four weeks.

It was found that re-used single batch litter changed the gut microbial community composition of two-week-old broiler chickens relative to all other litter types. Caecal microbial communities of birds raised on re-used litter were also different from those raised on other litter materials at four weeks of age, with the exception of pine shavings. At four weeks of age, birds raised on pine shavings did not have a significantly different caecal microbial community from chickens raised on the re-used litter. It

is interesting to note that pine shavings were the source of original litter material used in the re-used litter group. The re-used litter may be acting as a microbiological seed, speeding up mature colonisation of the caecal microflora. However, this may also have hygiene and/or food safety implications if undesirable/detrimental bacteria are present in the litter.

The only other litter treatments for which caecal microbial community composition differed at both two and four weeks of age were rice hulls and shredded paper. In hindsight, samples of litter material for microbial profiling should have been taken prior to placement of birds. This would have allowed identification of bacterial species in the litter material itself, which may be a potential source of inoculum resulting in differences in gut microbial communities of these birds.

Several bacterial species were identified as contributing to the differences in caecal microbial community composition between birds raised on re-used litter and some of the other litter materials (data not shown). However, no one or select few bacterial species accounted for a large proportion of these differences. No one bacterial species contributed more than 4% to the total difference in caecal microbial community composition observed.

Chapter 6: Effect of age and environment on gut microflora development

Introduction

Knowledge about the composition of the poultry gut microflora, microbial ecology of the gastrointestinal tract and factors affecting its development are still limited. The effect of age on broiler chicken gut microflora development was investigated over a six-week period in a commercial production setting. Inter-flock environmental associated differences in gut microflora composition were also investigated within farms, as well as, between farms.

Materials and methods

South Australian poultry producers

Two commercial broiler production farms in South Australia participated in this study. Both farms were from the same poultry company and sourced their chickens from the same commercial hatchery. Chickens were placed on each of the two farms surveyed within 10 days of each other.

Chickens (n=12) were taken from an individual shed per farm at weekly intervals from one week of age to six weeks of age. Chickens (n=12) from a second shed per farm were also taken at two and six weeks of age. Caecal contents were collected from each bird and freeze dried prior to microbial profiling analysis.

T-RFLP and statistical analysis of OTUs

T-RFLP methodology is outlined in Chapter 1 and statistical analysis of generated data points is outlined in Chapter 2.

Results

Environment

Significant differences were found between caecal microflora of chickens aged two and six weeks (two-way ANOSIM; Global R=0.392, P=0.001) regardless of environment. However, there were also significant differences in caecal microflora between chickens reared in the four different poultry sheds (two way ANOSIM; Global R=0.201, P=0.001) across both age groups. Closer investigation of the between-shed variation in caecal microflora on an age basis, showed that at two weeks of age there

were no significant differences (P>0.05) between sheds (1 and 2) on farm A, or shed 2 on farm A and shed 1 on farm B (Table 6.1). There were significant differences between sheds on farms B, shed 2 on farm B and either of the sheds on farm A, and shed 1 on farm A and shed 1 on farm B. However, by six weeks of age there were significant (P<0.05) differences between all four sheds regardless of farm (Table 6.2).

Table 6.1: One-way ANOSIM of caecal microbial communities from chickens housed in different sheds on two farms at two weeks of age. Two sheds per farm (A and B) were investigated. The *R* statistic (**bold**) and significance level (*italics*) are shown between sheds. The global *R*-value for differences between sheds was 0.126 at a significance level of 0.003.

	Farm A Shed 1	Farm A Shed 2	Farm B Shed 1	Farm B Shed 2
Farm A Shed 1		*0.148*	*0.003*	*0.002*
Farm A Shed 2	**0.060**		*0.345*	*0.013*
Farm B Shed 1	**0.189**	**0.017**		*0.013*
Farm B Shed 2	**0.249**	**0.129**	**0.136**	

Table 6.2: One-way ANOSIM of caecal microbial communities from chickens housed in different sheds on two farms at six weeks of age. Two sheds per farm (A and B) were investigated. The *R* statistic (**bold**) and significance level (*italics*) are shown between sheds. The global *R*-value for differences between sheds was 0.276 at a significance level of 0.001.

	Farm A Shed 1	Farm A Shed 2	Farm B Shed 1	Farm B Shed 2
Farm A Shed 1		*0.004*	*0.002*	*0.001*
Farm A Shed 2	**0.256**		*0.008*	*0.001*
Farm B Shed 1	**0.247**	**0.193**		*0.003*
Farm B Shed 2	**0.411**	**0.315**	**0.222**	

Age

The development of caecal microflora was tracked over a six-week period (at weekly intervals) from one week of age in a single shed on each farm (farm A shed 1 and farm B shed 2). Significant differences in caecal microflora were again found between age groups (two-way ANOSIM; Global *R*=0.376, P=0.001) across sheds, and between sheds (two-way ANOSIM; Global *R*=0.363, P=0.001) across all age groups. The overall difference in caecal microflora (regardless of age) as a result of being reared in different sheds is graphically demonstrated in the 3-D nMDS ordination in Fig 6.1. However, if the same samples are identified by age it becomes apparent that the caecal microflora differs between age groups, regardless of rearing environment (Fig 6.2). By investigating caecal microflora changes occurring with age in each of the two sheds individually, significant differences were found between each of the age groups with the exception of between age groups 3 and 4 weeks and 4 and 5 weeks on farm A shed 1 (Table 6.3), and between 3 and 4 weeks, 3 and 5 weeks and 4 and 5 weeks on farm B shed 2 (Table 6.4).

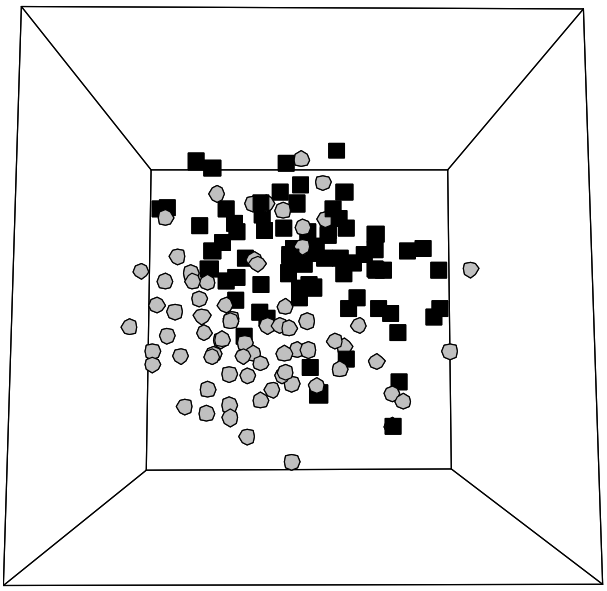

Figure 6.1: 3-D nMDS ordination of caecal microbial communities from chickens ranging in age from 1- 6 weeks reared in a single shed each on two different farms: ■ farm A shed 1; ● farm B shed 2. 3-D Stress = 0.24.

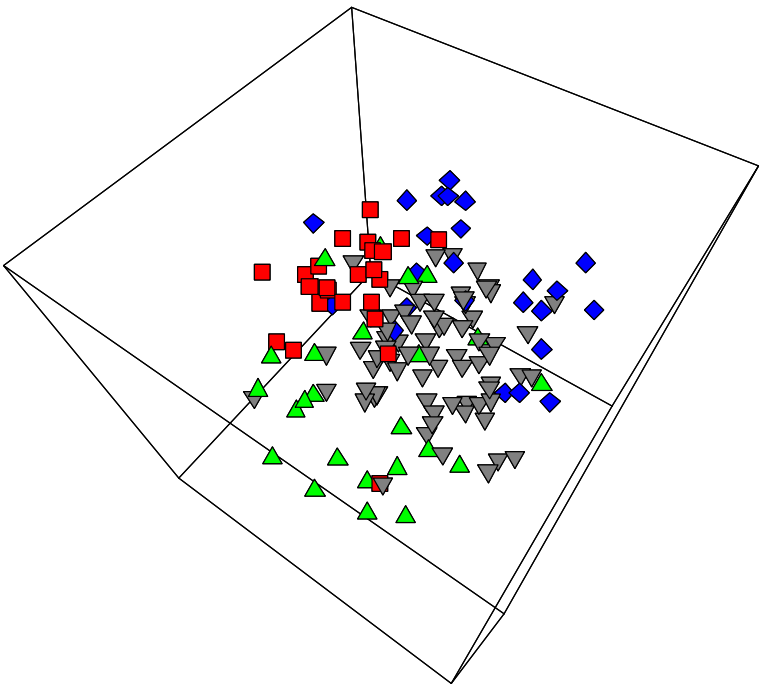

Figure 6.2: 3-D nMDS ordination of caecal microbial communities from chickens ranging in age from 1- 6 weeks reared in a single shed each on two different farms: ▲ one week old; ■ two weeks old; ▼ three to five weeks old; ◆ six weeks old. 3-D Stress = 0.24. The ordination is based on the same samples as in Fig 6.1 but identified by age rather than shed.

Table 6.3: One-way ANOSIM of caecal microbial communities from chickens aged 1- 6 weeks housed in shed 1 on farm A. The *R* statistic (**bold**) and significance level (*italics*) are shown between age groups. The global *R*-value for differences between age groups was 0.409 at a significance level of 0.001.

	1 wk old	2 wks old	3 wks old	4 wks old	5 wks old	6 wks old
1 wk old		*0.001*	*0.001*	*0.001*	*0.001*	*0.001*
2 wks old	**0.475**		*0.001*	*0.001*	*0.001*	*0.001*
3 wks old	**0.452**	**0.534**		*0.584*	*0.001*	*0.001*
4 wks old	**0.332**	**0.468**	**-0.017**		*0.099*	*0.001*
5 wks old	**0.490**	**0.705**	**0.214**	**0.073**		*0.001*
6 wks old	**0.716**	**0.487**	**0.457**	**0.350**	**0.451**	

Table 6.4: One-way ANOSIM of caecal microbial communities from birds aged 1- 6 weeks housed in shed 2 on farm B. The *R* statistic (**bold**) and significance level (*italics*) are shown between age groups. The global *R*-value for differences between age groups was 0.342 at a significance level of 0.001.

	1 wk old	2 wks old	3 wks old	4 wks old	5 wks old	6 wks old
1 wk old		*0.001*	*0.002*	*0.001*	*0.001*	*0.001*
2 wks old	**0.477**		*0.001*	*0.001*	*0.001*	*0.001*
3 wks old	**0.255**	**0.431**		*0.368*	*0.218*	*0.001*
4 wks old	**0.333**	**0.525**	**0.012**		*0.598*	*0.001*
5 wks old	**0.313**	**0.644**	**0.048**	**-0.021**		*0.001*
6 wks old	**0.571**	**0.449**	**0.414**	**0.309**	**0.374**	

Discussion

T-RFLP has been used to monitor shifts in the chicken gut microbial population associated with environment and age. Environment was show to have a large impact on gut microflora development with differences detected between birds raised in different sheds on the same farm, as well as, between sheds on different farms by six weeks of age. It is interesting to note that on one of the two farms surveyed, differences in caecal microflora between sheds were already apparent at two weeks of age. These early differences detected in gut microflora at two weeks of age may indicate differences in hygiene or management practices between farms, and between sheds on the same farm.

Overall, caecal microbial community composition changed significantly within the first 2-3 weeks of age. Caecal microbial communities remained constant between 3-5 weeks of age and then shifted again at 6 weeks of age. The complexity of bacterial profiles also increased with age. This trend was observed on both farms surveyed regardless of overriding differences in caecal microbial communities between sheds. Although previous studies have shown the composition of the gut microflora can vary with age (Knarreborg *et al*., 2002a; Lu *et al*., 2003), this is the first report which follows the succession of gut microflora over a six week period of individual chickens within a commercial

production setting, and is not dependant on the production of bacterial sequence information for identifying bacteria community differences.

Chapter 7: Collection of chicken gut bacterial clones

Introduction

T-RFLP is an extremely useful tool for screening changes in the gut microflora of poultry associated with diet, litter material, age and environment. However, it does not readily allow characterisation of OTUs as particular bacterial species. To this end we have generated bacterial clones of the 16S rDNA gene region analysed in T-RFLP from a range of characterised and uncharacterised bacterial cultures either isolated from chicken gut, poultry rearing environments or reference cultures of bacteria known to inhabit the poultry gut. Genome sequence information from these clones was determined and will become an important resource for predicting OTU identity and developing diagnostic assays for bacteria of interest in the future.

Materials and methods

Characterised pure bacterial cultures

Characterised pure bacterial cultures of *Campylobacter coli*, *C. jejuni*, *Escherichia coli*, *Salmonella enterica* serovar Singapore, *S. enterica* serovar Vichow and *S. enterica* serovar Bovis-morbificans were provided by Dr Pat Blackall and Dr Nalini Chinivasagam (QDPI&F). Characterised pure bacterial cultures of *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterobacter aerogenes*, *Citrobacter fremdii*, *E. coli*, *Proteus mirabilis*, *Salmonella infantis* and *S. sofia* were provided by Dr Peter Groves (Birling Avian Laboratories). DNA from a characterised pure bacterial culture of *Bacillus cereus* was provided by Dr Stephen Barnett (SARDI). DNA from characterised pure bacterial cultures of *Enterococcus faecalis*, *E faeceum*, *Lactobacillus gallinarum*, *L. amylovorus*, *L. crispatus*, *L johnsonii*, *L. gasseri*, *L. salivarius*, *L. aviaries*, *L. fermentum* and *Pediococcus pentosaceus* were provided by Dr Gwen Allison (ANU).

Uncharacterised pure bacterial cultures

One hundred and eleven uncharacterised pure bacterial cultures isolated from the ileum and caeca of chickens aged 14 days from the Inghams litter trial (Chapter 5) and 29 uncharacterised pure bacterial cultures isolated from the ileal and caeca of chickens from the UNE experiment 3 prebiotic trial (Chapter 4) were provided by Dr Lene Mikkelsen (UNE). Bacterial isolates were differentiated by RFLP analysis as described in Chapter 4 (Screening of bacterial cultures). Representative bacteria from each of the identified RFLP groups were cloned and sequenced to determine identity.

RFLP and genome sequence analysis of the 29 uncharacterised pure bacterial cultures from the UNE experiment 3 identified seven different bacterial species (Chapter 4), including four characterised Lactobacilli species (*L. crispatus, L. salivarius, L. reuterii* and *L johnsonii*), as well as, one uncharacterised Lactobacilli species. In addition *Bifidobacterium pseudolongum* and *Clostridium perfringens* were also identified.

Determination of genome sequence identity of bacterial cultures

The bacterial 16S rDNA was amplified with unlabelled universal 16S bacterial primers, identical in sequence to those used for T-RFLP analysis. Resulting PCR amplicons were cloned and sequenced. Bacterial isolates were identified by sequence similarity with known organisms in public genome databases using the BLAST interface (http://www.ncbi.nlm.nih.gov/blast/).

Results

RFLP analysis with *Msp*I, *Cfo*I and *Rsa*I of the 111 chicken gut bacterial cultures isolated from the Ingham's litter trial identified 11 unique RFLP types. *Msp*I had the highest resolving power for discriminating among isolates. Unique RFLP types identified and their bacterial identity are shown in Fig. 7.1.

A summary of all bacteria identified in this study on the basis of 16S rDNA sequence information and the use of this sequence information for identifying OTUs *in silico* with *Alu*I, *Cfo*I, *Msp*I and *Rsa*I is shown in Table 7.1.

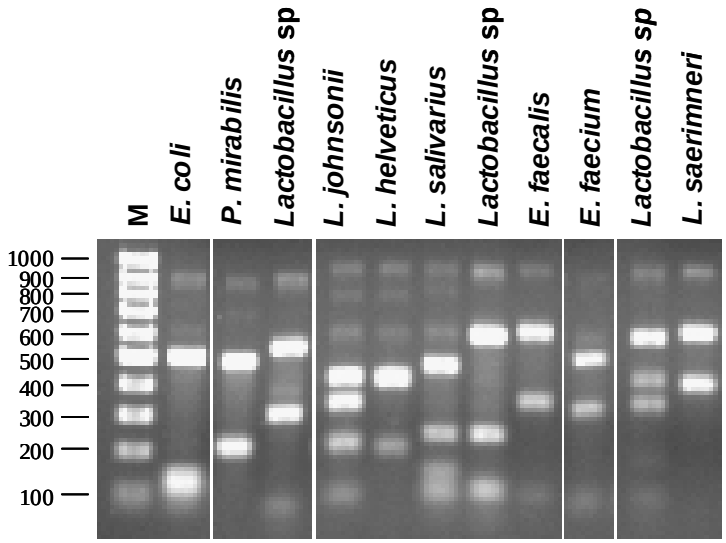

Figure 7.1: Eleven unique RFLP types identified from RFLP analysis of 111 uncharacterised pure bacterial cultures isolated from chicken gut samples in Ingham's litter experiment. Restriction analysis was done with *Msp*I. M = 100bp marker.

Table 7.1: Bacterial 16S rDNA sequence information determined from bacterial cultures analysed. Predicted TRFs are shown from *in silico* restriction with four different restriction enzymes. Bacteria represented were either isolated from poultry guts, poultry inhabited environments or were reference cultures of bacterial species know to inhabit the poultry gut.

Bacterial identity	**Source**	**Predicted OTU**			
		***Alu* I**	***Cfo* I**	***Msp* I**	***Rsa* I**
Bifidobacterium pseudolongum	Chicken gut	74	185	82	627
Staphylococcus aureus	Poultry environment	74	238	155	486
Bacillus cereus	Unknow	74	579	147	488
Clostridium perfringens	Chicken gut	239	233	522	453
Enterococcus faecalis	Reference culture	258	218	566	903
Enterococcus faecalis	Poultry environment	258	218	566	903
E. faecalis	Chicken gut	258	218	566	903
E. faecium	Reference culture	79	218	74	903
E. faecium	Chicken gut	79	218	89	58
Lactobacillus species	Chicken gut	273	61	29	919
Lactobacillus species	Chicken gut	222	267	29	918
Lactobacillus species	Chicken gut	273	267	29	918
Lactobacillus species	Chicken gut	272	60	580	917
L. reuterii	Chicken gut	273	406	29	58
L. gallinarum	Reference culture	209	596	181	907
L. amylovorus	Reference culture	209	596	181	907
L. crispatus	Reference culture	209	596	181	907
L. crispatus	Chicken gut	209	596	181	907
L. helveticus	Chicken gut	209	596	181	907
L. johnsonii	Reference culture	63	948 (uncut)	189	914
L. johnsonii	Reference culture	63	948 (uncut)	189	914
L. johnsonii	Chicken gut	63	948 (uncut)	189	914
L. gasseri	Reference culture	63	948 (uncut)	189	914
L. salivarius	Reference culture	875	939 (uncut)	568	905
L. salivarius	Chicken gut	874	939 (uncut)	576	904
L. aviaries	Reference culture	876	394	569	906
L. saerimneri	Chicken gut	272	61	580	917
L. fermentum	Reference culture	273	406	581	918
Pediococcus pentosaceus	Reference culture	274	608	582	86
Campylobacter coli	Reference culture	67	98	470	453
C. jejuni	Reference culture	67	98	470	453
Citrobacter fremdii	Poultry environment	75	373	496	427
Escherichia coli	Reference culture	75	373	496	427
E. coli	Poultry environment	75	373	496	427
Proteus mirabilis	Poultry environment	75	373	496	427
P.mirabilis	Chicken gut	75	373	496	427
Salmonella infantis	Poultry environment	75	373	496	427
S. sofia	Poultry environment	75	373	496	427
S. typhimurium	Poultry environment	75	373	496	427
S.enterica serovar Singapore	Reference culture	75	373	496	427
S.enterica serovar Vichow	Reference culture	75	373	496	427
S.enterica Bovis-morbificans	Reference culture	75	373	496	427
Enterobacter aerogenes	Poultry environment	74	371	494	425

Discussion

The collection of bacterial cultures has been extremely useful for generating clones, which can be used as standards in T-RFLP for identifying OTUs. Furthermore, the 16S rDNA sequence information has allowed identity of characterised bacterial cultures to be confirmed and the identity of uncharacterised bacterial cultures of chicken gut origin to be determined. However, culturing techniques used to isolate bacteria from chicken gut samples may have been biased towards certain bacterial groups, such as the Lactobacilli. Therefore, the compiled list presented in this chapter is incomplete, possibly biased by culturing techniques, and does not include bacteria that are difficult to culture.

Nevertheless, the sequence information obtained from the 16S rDNA gene region has allowed *in silico* prediction of TRFs with a range of restriction enzymes. Restriction analysis with *Msp*I has shown that many bacteria can be successfully differentiated at the genus level, as well as, at the species level. The latter is particularly true of the Lactobacilli. However, for the genera Salmonella, Proteus, Escherichia and Citrobacter, bacteria could only be differentiated at the family level (Enterobacteriaceae) with restriction enzymes *Alu*I, *Cfo*I, *Msp*I or *Rsa*I. Other restrictions enzymes may successfully differentiate genera within this family and the generated 16S rDNA sequence information can be used to test this *in silico*. Finally, the generated genome sequence information is a valuable resource if diagnostic assays were to be developed for any of these bacteria in the future.

In conclusion the use of traditional culturing techniques, in combination with T-RFLP, has been useful in identifying members of the microbial communities characterised in this project.

Discussion of results

Knowledge about the composition of the poultry gut microflora and microbial ecology of the gastrointestinal tract is still limited. The gastrointestinal microflora have one of the highest cell densities for any ecosystem, and in poultry ranges from 10^7-10^{11} bacteria per gram gut content (Apajalahti *et al*., 2004). A large number of these bacteria have remained unidentified by traditional microbiological techniques due to lack of knowledge of appropriate culturing conditions.

Recent molecular studies targeting the bacterial DNA in poultry gut have yielded more detailed insights into the composition of the microbial community (Amit-Romach *et al.*, 2004, Apajalahti *et al*., 1998, Gong *et al*., 2002a, Gong *et al*., 2002b, Hume *et al*., 2003, Lan *et al*., 2004, Lu *et al*., 2003, Zhu *et al*., 2002, Zhu *et al*., 2003). It has been estimated that the caecal microflora consists of at least 640 species from 140 genera, of which 10% of the identified bacterial 16S rDNA sequences represent previously known bacterial species, and the remaining sequences belong to new species or even new genera (Apajalahti *et al*., 2004). However, most molecular techniques currently used for investigating gut microflora, such as PCR, hybridisation, DGGE/TGGE or G+C% content, are either unable to characterise the entire bacterial community in a single test, or are not conducive to high throughput analysis. Much information on the composition of the gut microflora as a whole has been obtained through the generation of bacterial 16S rDNA clone libraries (Lan *et al*., 2002, Lu *et al*., 2003, Zhu *et al.*, 2002). However, generation of clone libraries is laborious and expensive. Ideally it would be best to screen for significant differences in the overall gut microbial community composition without the need for prior knowledge of the individual bacterial components. If significant differences were detected in the screening process then detailed information on the organisms of interest could be obtained.

In this project we have developed a high throughput high-resolution method for investigating the gut microflora of poultry. This molecular technique is based on T-RFLP analysis of the bacterial ribosomal gene region and allows a “snapshot” of the complex bacterial population to be produced at any particular time. Furthermore, a database for cataloguing and validating the large quantity of data points generated from the analysis has also been developed. This has been critical in managing data and enabling further statistical analysis to be done. In conjunction with multivariate statistical analyses, it has been possible to investigate changes in gut microflora as a result of dietary modification, litter material composition, environmental factors and age. More importantly this technology has enabled changes in gut microflora composition to be correlated to improved performance for the first time and allowed identification of bacteria (OTUs) linked to these improvements in performance. Improvement in performance may be due to the presence of beneficial and/or absence of detrimental bacterial species. Indeed, it has recently been shown that in genetically

predisposed obese mice versus lean mice that the gut microflora differ in relative abundance of the Bacteroidetes and Firmicutes (Turnbaugh *et al.,* 2006) indicating particular bacterial groups have increased capacity for energy harvest.

The use of microbiological culturing techniques, in combination with T-RFLP, has been useful in identifying members of the microbial communities characterised in this project. However, the identities of the majority of bacteria revealed by T-RFLP have remained unknown. Recent advances in molecular techniques will make identification of T-RFLP profiles of interest easier (Widmer *et al.,* 2006) and not reliant on bacterial culturing or generation of clone libraries. This will enable bacterial sequence information of interest to be determined, which may be used to develop specific tests for gut bacteria associated with poultry production traits. From there, it may be possible to develop dietary strategies to induce desirable changes in the gut microflora for enhancement of growth and productivity of commercial chicken flocks, and to design appropriately targeted probiotic products.

Diet associated changes in gut microbial community composition were investigated along the length of the gut and found to be detectable only within the ileum and caeca. Bacterial species identified as contributing to diet induced changes, generally contributed less than 5% to the community composition. This indicates that multiple bacterial species are responsible for the overall differences and not just a select few.

Dietary supplementation with antibiotic growth promotants (AGPs) has been shown to alter the overall gut microflora of poultry. However, this needs to be investigated in more detail if alternatives to AGPs are to be evaluated. Supplementation of the poultry diet with prebiotics (arabinogalactan product, fructooligsacharide product, rengarenga extract, cabbage tree extract and seaweed extract) did shift the overall ileal bacterial population compared with non-supplemented control groups. However, none of the prebiotics tested resulted in a shift in the ileal bacterial microflora similar to that produced by AGP supplementation (zinc bacitracin).

Gut microflora can be affected by more than just diet. Litter materials have also been shown to affect gut microflora development, with re-used litter having the most significant impact on caecal gut microflora development when compared with other non-reused litter materials. This effect was most apparent at 14 days of age as compared with 28 days of age. It is possible that the re-used litter is acting as a microbiological seed, speeding up mature colonisation of the gut microflora. However, this may also have hygiene and/or food safety implications if undesirable/detrimental bacterial were present in the litter. Differences in caecal microbial communities were also noticed between birds raised on some of the non re-used litter materials regardless of age (rice hulls versus shredded paper).

However, linkages between changes in gut microflora associated with litter material and bird performance were not investigated further due to lack of opportunity.

Environment and age also impact on the gut microflora of poultry. For example, differences in gut microflora between poultry flocks were related to commercial shed environment. However, regardless of any shed-to-shed differences, the pattern of gut microflora development remained the same. The gut microflora was continually changing and developing in the first two weeks post hatch. It then appeared to reach a mature gut microflora at three week of age with no further changes detected until six weeks of age, when a final shift in gut microbial communities composition was observed. These changes may partially reflect management practices, but strongly indicated that gut microflora development in the first two weeks of life is critical and may affect life long performance.

In conclusion, we have established a microbial profiling technique for investigating the chicken intestinal microflora based on high-throughput, high resolution fingerprinting of bacterial ribosomal gene regions. In conjunction with the development of a database for validating and managing the vast output of data from the analysis and multivariate statistical methods, this has allowed us to monitor changes in the intestinal microbial community and conduct comparative studies investigating effects of dietary manipulations, influence of litter material, environment and age on chicken gut bacterial populations. Furthermore, we have been able to link changes in poultry performance with changes in gut microflora composition and identify T-RFLP profiles associated with improved performance. From here it will be possible to identify bacterial species associated with production traits. These are the major outcomes of this project and are key in achieving the Australian Poultry CRC objectives of: gaining a thorough understanding of the key factors influencing digestive function and gut microflora of broiler chickens and maintaining efficient production without the use of antibiotics; and controlled microbial colonisation of the gut of newly hatched chickens to maintain a healthy gut microflora throughout the productive life of the birds.

Implications

- A high-throughput, high resolution microbial profiling tool capable of providing a “snapshot” of the complex bacterial population has been developed for investigating the gut microflora of poultry. This technology enables comparative studies to be undertaken for screening differences in gut microbial communities associated with dietary, environmental and developmental changes.
- Gut microflora composition is correlated with performance, as measured by AME. This implies that the specific organisms, or microbial functions attributed to particular organisms, are contributing to improved productivity. Identifying and characterising groups of performance related microbes would provide greater insight into how gut microflora influence performance and how gut microbial communities could be modified by diet to achieve an optimal gut microflora for improved production.
- Prebiotics have been show to modify the gut microflora of poultry, however not in the same way as AGPs. A detailed understanding of how AGPs change the gut microflora and how these are linked to performance enhancement need to be understood if alternatives to AGPs are to be developed and evaluated.
- Rapid post hatch gut microflora development and succession is observed during the first 2-3 weeks of age, after which the gut microflora reaches a mature or stabilised state. This implies that changes in gut microflora immediately post hatch, and dietary modifications targeted at this age group could result in improved performance.
- Litter type plays a role in poultry gut microflora development. This implies that the choice of litter material and whether it is re-used for subsequent batches of chickens are important factors in modulating the gut microflora, hence possibly improving performance.

Recommendations

- Poultry gut microflora composition is correlated with performance. Linkages need to be established with poultry performance trials in order to validate these findings in a commercial setting and to begin to characterise important performance related bacteria. This could lead to the identification of groups of bacteria consistently related with improved production traits.
- A greater understanding of how AGPs work *in vivo* to modulate the gut microflora of poultry and the linkage to growth promotion need to be fully understood in a commercial poultry setting. Information on modes of action of most AGPs against particular bacteria is reported *in vitro* and not *in vivo*. Developing such an understanding will greatly contribute to the reduced reliance on AGPs in the Australian poultry industry by enabling evaluation and development of alternatives, including probiotic products.
- Normal poultry gut microflora development needs to be characterised in the first two weeks post hatch. This has been shown to be an important period in the gut microflora development and succession before which a stable microflora is established. During this early period of gut microflora development it may be possible to modulate the microflora development towards an optimal gut microflora for life long health and performance. Knowledge gained into the transitional phase of gut microflora development from 2-3 weeks may also provide useful insight into the mode of necrotic enteritis development during this period.
- The role of poultry litter in inoculating the gastrointestinal tract needs to be better understood and the impact on overall poultry performance and health determine.
- Further work into the identification and characterisation of gut bacteria associated with production traits is required. Identifying and characterising groups of performance related organisms would provide greater insight into how gut microflora influences performance and how gut microbial communities are modified by diet.

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Appendixes

Appendix A: Abbreviations

AGP	antibiotic growth promotant
AME	apparent metabolisable energy
ANOSIM	analysis of similarity
ANOVA	analysis of variance
ANU	Australian National University
CAP	canonical analysis of principle components
DGGE	denaturing gradient gel electrophoresis
dNTP	deoxynucleotide triphosphates
DM	dry matter
DNA	deoxyribonucleic acid
FAM	6-carboxyfluorescein
G+C%	percent-guanine-plus-cytosine
HPLC	high performance liquid chromatography
MiCA III	microbial community analysis III
nMDS	non-metric multidimensional scaling
NSP	non-starch polysaccharide
OTU	operational taxonomic units
PCA	principal component analysis
PCO	principal coordinate analysis
PCR	polymerase chain reaction
PPPI	Pig and Poultry Production Institute
QDPI&F	Queensland Department of Primary Industries and Fisheries
rDNA	ribosomal deoxyribonucleic acid
SSU	ribosomal small sub-unit
TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism
T-RF	terminal restriction fragment
RDP II	ribosomal database project II
RFLP	restriction fragment length polymorphism
SARDI	South Australian Research and Development Institute
SD	standard deviation
SIMPER	similarity percentages
U	units

UNE	University of New England
UV	ultra violet

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