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Project 09-21:

Identification of genes associated with mucin production and quality in healthy chickens and those with necrotic enteritis

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Executive Summary

- Proliferation of *Clostridium perfringens* in the intestines of chickens often (but not always) results in clinical or sub-clinical necrotic enteritis and reduced production and health of the birds
- Mucins play a major role in protecting the intestinal epithelium from infection
- On the other hand, *C. perfringens* is mucinogenic
- The relative roles of different mucins in gut pathology following bacterial challenge are unclear, so a method of quantifying changes in mucin and mucin-related profiles is required
- This preliminary study was designed to establish a molecular approach to quantify the expression of mucin and mucin-related genes, using gut samples from a previously-conducted necrotic enteritis challenge trial
- A method for quantifying mucin gene expression was established using a suite of reference genes to normalise expression data. This method was then used to quantify the expression of 12 candidate genes involved in mucin, cytokine or growth factor biosynthesis (IL18, KGF, TLR4, MUC13, MUC2, MUC5ac, TFF2, TNFa, MUC1, MUC4, MUC5b and MUC16)
- Expression of MUC2 and MUC13 was depressed by challenge with *C.perfringens*. Antibiotic treatment prevented a *C. perfringens* induced decrease in MUC2 expression but did not affect MUC13. No other genes were significantly altered in expression**.**
- This preliminary study has paved the way for more extensive studies of the impact of nutrients and other pre- and pro-biotics for the treatment of necrotic enteritis in broiler chickens raised without antibiotics.

Introduction

A healthy gut environment is paramount to the health and productivity of broiler chickens. As the industry moves away from antibiotic use in broiler diets the challenge will be to maintain a healthy gut. Central to gut health is the mucus layer, which is the first line of defence encountered by gut bacteria. Mucins play important roles in host protection and the regulation of microbial colonisation as they provide binding sites and a nutrient source for commensal and pathogenic bacteria. Indeed, important links between gut bacteria and intestinal mucin composition and distribution have been identified (Forder 2008; PhD thesis). The types and quantities of mucin present can impact on intestinal health and in turn bird performance; however, little is known as to what comprises a beneficial mucus layer and how diet can influence mucin dynamics. As part of CRC Mark 2, our research group aims to study the relationships between nutrition, the intestinal environment and bird health and performance. An integral part of understanding intestinal health is knowledge of mucin dynamics. Therefore we aim to develop a series of quantitative real-time PCR-based assays to quantify the expression of a subset of genes known to be pivotal to mucin function. In the long-term these assays will incorporate genes encoding the mucin core peptides (muc genes), genes involved in determining mucin glycoprotein structure (including transferase genes), genes which regulate goblet cell development and mucin exocytosis (including growth factors and cytokines) and genes involved in microbe-host signalling (toll-like receptors). Initially, we have chosen a small subset of genes that we feel are of particular importance to these processes; i) **muc2**, a secreted core mucin peptide, known to be regulated by dietary factors, ii) **muc 5ac**, another secreted mucin, iii) **muc 13**, a membrane-bound mucin, iv) **KGF**, a growth factor involved in goblet cell differentiation and proliferation, v) **TNF-α**, a stimulator

of mucin release, vi) **IL-18**, known to negatively regulate muc2, and vii) **TFF2** a known stimulator of goblet cell migration and a major constituent of the mucus layer.

Samples collected from a previous necrotic enteritis (NE) challenge experiment performed during CRC Project 05-2 (Drs Hughes and Geier) provided the ideal opportunity to develop these assays for future CRC projects within our research team. This experiment included groups that were unchallenged and challenged (untreated and antibiotic treated). The birds treated with antibiotic (ZnB/monensin) were protected from NE whilst the challenged/untreated group displayed gross intestinal lesions and histological damage. We envisaged that the use of these tissues would also provide further mechanistic information as to how antibiotics can prevent NE development. Further characterisation of ZnB-mediated protective mechanisms may lead to the development of non-antibiotic alternatives that have a similar effect.

Objectives

- To develop a series of real-time PCR based assays to study mucin-related genes in the intestine of broiler chickens
- To determine how necrotic enteritis challenge influences expression of these genes
- To evaluate how treatment with antibiotics, organic acids and probiotics influences expression of these genes

Methodology

Tissues were derived from a necrotic enteritis challenge experiment performed as a part of Australian Poultry CRC Project 05-2[1]. Twelve hundred male Cobb 500 broiler chickens (Baiada Hatchery, Kootingal, NSW, Australia) were raised in floor pens in a temperature-controlled room at the University of New England (Armidale, NSW, Australia). All procedures were approved by the Animal Ethics Committee of the University of New England.

All experimental diets were based on a standard commercial starter diet with no added antibiotics or coccidiostats (Ridley Agriproducts, Tamworth, NSW, Australia) and met or exceeded National Research Council guidelines (NRC 1994). The six experimental groups were; an unchallenged control, an *Eimeria* spp./*C. perfringens* challenged control, and *Eimeria* spp./*C. perfringens* challenged groups treated with; antimicrobials (45 ppm zinc bacitracin and 100 ppm monensin), organic acids (OA; proprietary blend[2]; 2 kg/ton), probiotic *L. johnsonii* (10^9 cfu/mL in PBS) and probiotic sham (PBS alone; n = 25 birds/pen, n = 8 pens/treatment). The *L. johnsonii* strain was isolated from the gastrointestinal tract of broiler chickens (Olnood et al. 2007). *L. johnsonii* and sham-treated birds were orally administered 0.5 mL of solution using a crop needle on day 1, and 1 mL on days 3, 7 and 12.

Necrotic enteritis challenge procedure

The necrotic enteritis challenge was performed as described previously (Kocher et al. 2004; Mikkelsen et al. 2009). From placement until day 7, birds were fed starter diets with their

[1] CRC Project 05-2: An integrated approach to understanding gut function and gut health in chickens. Project Leader RJ Hughes. Final Report is available for full experimental details.
[2] Proprietary organic acid blend contained formic, acetic, propionic, sorbic, caprylic and capric acid.

corresponding dietary additive included. Between days 8-15 inclusive (prior to *C. perfringens* inoculation), birds were fed a 50% (w/w) fish meal-based diet (with corresponding dietary additives included). After day 15, original diets were returned.

Eimeria inoculation

On day 9, all birds (except unchallenged controls) were given a suspension of 2,500 oocysts of *Eimeria acervulina*, *E. maxima* and *E. tenella* (Bioproperties Pty Ltd, Glenorie, NSW, Australia) in 1 mL PBS. Unchallenged control birds received sterile PBS in place of *Eimeria*.

Clostridium perfringens inoculation

A primary poultry isolate of *C. perfringens* type A was obtained from CSIRO Australian Animal Health Laboratory (Geelong, Vic, Australia). On day 15, birds in challenged groups were individually inoculated with 1 mL of *C. perfringens* suspended in thioglycollate broth (USP alternative, Oxoid, Hampshire, UK) at a concentration of 3.5×10^8 cfu/mL. Unchallenged control birds received 1 mL of sterile thioglycollate broth.

Tissue collection

On day 18, from 72 birds (n = 12 birds/treatment), a 1 cm segment from the mid-point of the jejunum was removed, rinsed in PBS and collected in a 5 mL tube containing 2 mL of RNAlater for gene expression analyses[3].

Isolation and quantification of total RNA from chicken intestine samples

Total RNA was isolated with the RNeasy mini kit (Qiagen, Germany). Intestine samples were removed from RNAlater (Ambion, USA), patted dry on Kimwipe tissues (Kimberly-Clark, Australia) and 50-100mg of each sample homogenised with an Ultra-Turrax (T25; IKA, Germany) in 2mls of Trizol reagent (Invitrogen, USA). The upper aqueous phase of Trizol was recovered from the homogenised samples as per the manufacturer's instructions. The aqueous phase was then mixed with an equivalent volume of 70% ethanol, loaded onto RNeasy mini columns and centrifuged at 10000rpm for 1 minute (Mikro200; Hettich, Germany). Following this centrifugation step, RNeasy columns were processed according to the manufacturer's instructions. An on-column RNase-free DNase treatment step (Qiagen, Germany) was included and the RNA was eluted in 50ul of EB buffer (Qiagen, Germany). The concentration and purity of total RNA was determined using UV spectrophotometry (Nanodrop 1000, Thermo Scientific, USA). The integrity of all RNA samples was confirmed via agarose gel electrophoresis.

Design and testing of real-time PCR assays targeting chicken genes

The Ensembl chicken genome browser (*Gallus gallus*; Build 56 September 2009) was used to design real-time PCR assays for all the genes examined in this study (Table 1). Keyword searches were performed using human reference sequence (Refseq) gene names, to identify the corresponding chicken cDNA and genomic DNA sequences. Exon-intron boundaries were manually marked on the chicken cDNA sequences and suitable pairs of exon-intron spanning real-time PCR primers were selected with the Universal Probe Library (UPL) design software

[3] For CRC Project 05-2, segments of duodenum, jejunum, ileum and caecum were collected for various analyses including; intestinal lesion scoring, *C. perfringens* enumeration, microbial profiling, assessment of *Lactobacillus* populations, intestinal histology and mucin staining. Performance data are also available.

(https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). In general, amplicon sizes were kept below 100bp, primers spanned exon-intron boundaries that were greater than 500bp in length and 3 real-time PCR primer pairs were synthesised (Invitrogen, USA) for most of the target and reference genes (Table 2).

The PCR oligonucleotides were tested in two stages using a pool of chicken intestine cDNA. In the initial phase of testing, all the assays were tested to determine which genes were expressed in chicken intestine and to prioritise assays for further testing based on their respective sensitivity and specificity. In the second phase of primer testing, the best assay for each gene from phase one testing was assessed against a 2-fold dilution series of pooled chicken intestine cDNA, to determine reaction efficiency and confirm their specificity across a broader dynamic range.

Both stages of the PCR oligonucleotide testing process were performed with a SYTO9-based PCR reagent (200nM dNTPs, 1.33nM SYTO9 and 1 x ROX passive reference dye (Invitrogen, USA), 3.5mM MgCl2, 1 x AmpliTaq Gold buffer and 0.2µ AmpliTaq Gold DNA polymerase (Applied Biosystems, USA)). The concentration of the forward and reverse oligonucleotides was 200nM and the following cycling parameters were performed on a RotorGene3000 real-time PCR machine (Qiagen, Germany): 9º5C for 10 minutes for 1 cycle, followed by 95ºC for 15 seconds, 60ºC for 20 seconds and 72ºC for 15 seconds for 40 cycles. The acquisition of real-time PCR data occurred at 72ºC.

A melt curve profile was generated at the end of each real-time PCR run to check the specificity of the different primer pairs. At the completion of the first phase of primer testing, a 5µl aliquot of each assay was subjected to agarose gel electrophoresis and fractionated against 150ng of pUC19/HpaII DNA markers (Geneworks, Adelaide) to determine the approximate size of the PCR amplicons.

Table 1: Human reference sequence (Refseq) gene names were used to search the Ensembl chicken genome browser to identify chicken homologues. Real-time PCR primers were then designed against chicken cDNA and genomic DNA sequences identified from these searches.

Human Refseq Gene Symbol	Gene Name
KGF	Keratinocyte growth factor
TLR4	Toll-like receptor 4
IL18	Interleukin 18
TFF2	Trefoil factor 2
TNFa	Tumour necrosis factor alpha
MUC1	Mucin 1
MUC2	Mucin 2
MUC4	Mucin 4
MUC5ac	Mucin 5ac
MUC5b	Mucin 5b
MUC13	Mucin 13
MUC16	Mucin 16
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
POLR2l	RNA Polymerase II polypeptide L
UCHL3	Ubiquitin carboxyl-terminal esterase L3
RPL19	Ribosomal protein L19
B2M	Beta-2 microglobulin
EEF2	Eukaryotic translation elongation factor 2
TBP	TATA-binding protein

Table 2: **(**A) Forward and reverse primers that were used to measure gene expression levels in chicken intestine cDNA samples with real-time PCR. (B) Additional forward and reverse primers that were tested on a pooled sample of chicken intestine cDNA but were not used in the real-time PCR component of this study.

(A)

Forward primer	Sequence (5' - 3')	Reverse primer	Sequence (5' - 3')
cKGF_F1	GGATTGATAAGCGAGGCAAA	KGF_R1	CCACTCCTTTGATTGCCACT
cIL18_F13	TGTGTGTGCAGTACGGCTTAG	cIL18_R13	CTTACAAAAGGCATCGCATTC
cTFF2_F157	GCTGTAGCCCTCATCAGCTC	cTFF2_R157	CTGGCAGCTATTTTGCACTG
cTNFa_F49	GAGCGTTGACTTGGCTGTC	cTNFa_R49	AAGCAACAACCAGCTATGCAC
cMUC2_F93	ATGCGATGTTAACACAGGACTC	cMUC2_R93	GTGGAGCACAGCAGACTTTG
cMUC5ac_F38	ACCCACAACACCGTTTCACT	cMUC5ac_R38	TGATGTCGTTGGTGATGATGT
cMUC13_F133	GCATTCCTCAAGCAGAGGTG	cMUC13_R133	CTCAGGCTGCCGTGATATTT
cGAPDH_F39	TGTGACTTCAATGGTGACAGC	cGAPDH_3_R39	GCTATATCCAAACTCATTGTCATACC
cPOLR2l_F74	GGCGGAGTATACGGAAGGA	cPOLR2l_R74	ACGTGGGCTAGGAGCATTC
cUCHL3_F143	GCAGTGCTTCTTCTCTTTCCA	cUCHL3_R143	TTGACATCTTGTCCCTTAGCTTT
cRPL19_F30	ACAAAGCTCGCAAGAAGCTC	cRPL19_R30	AGCCTCCTTGGTTTTGGAC
cB2M_F39	CAGCAGCGGTTCCACCTA	cB2M_R39	CTTCAAACCGTGCTCATCC
cEEF2_F133	CGTGAACGTCATCATCTCCA	cEEF2_R133	CTGTACCAAGCACAGGATCG
cTBP_F72	TCAGCAGCTATGAGCCAGAA	cTBP_R72	CTGCTCGAACTTTAGCACCA

(B)

Forward primer	Sequence (5' - 3')	Reverse primer	Sequence (5' - 3')
cKGF_F59	GGGACGAGAGAAGCAAACAA	cKGF_R59	CCTTTGATTGCCACTATTCCA
cTLR4_F67	GCAGTTTCTGGATCTTTCAAGG	cTLR4_R67	AAGCCATGGAAGGCTGCTA
cUBIQ_F84	GAAAGAGTCCACCCTGCATC	cUBIQ_R84	GCCAGTCAGGGTCTTCACA
cMUC5b_F80	TGATGTCAATCCTCCACGAA	cMUC5b_R80	TGGATCACAGACACACTCCTG
cIL18_F151	GAAAACCTCTGCCTCTATTTTGAA	cIL18_R151	GATAGTTTTATCCTTACAAAAGGCATC
cPOLR2l_F12	CAAGTGGGAAGCCTATCTCG	cPOLR2l_R12	TTCAAACCAAGGGCATCC
cMUC4_F61	CCAGGCCACCAACTTCAT	cMUC4_R61	TCCCCAAGGTCCACTCAAC
cMUC5b_F76	CCCTACTTCAAGCCGTTCTG	cMUC5b_R76	GGAGGAACTGGAGAATGCAG
cTNFa_F3	GACTTGGCTGTCGTGTGGT	cTNFa_R3	CGCAAAAGGGAATTAAGCAA
cUCHL3_F115	GGAAACCAGCGATGATTCTT	cUCHL3_R115	CAGTGCGATTGCATTAAATCTT
cMUC4_F10	CACCAGGGACACCCACTTA	cMUC4_R10	GCTCCAAATGGGTACAGTGAC
cIL18_F161	AACAGATCAGGAGGTGAAATCTG	cIL18_R161	TGAATGCAACAGGCATCC
cTNFa_F168	TTTGTGCCTGTTTATTTCAGTCA	cTNFa_R168	ATGAAGGTGGTGCAGATGG
cMUC1_F78	CTTCCCCTGGATTTTGCTC	cMUC1_R78	CCACCTTGTGGTTTCCAGTT
cMUC4_F100	GAAGAGAAAGGCAGCACTCAA	cMUC4_R100	TGGTGACTTGTTCTGTCCTGA
cMUC13_F84	TGAAGTTCAGGAAGCAGTAAGGA	cMUC13_R84	ACATGGATAAACAGCACATTGG
cRPL19_F142	AGACAAAGCTCGCAAGAAGC	cRPL19_R142	TTCGAGAGGGTCTTGATGATTT
cMUC1_F128	AGTTCTGTTTTTGCTACCACATTTAC	cMUC1_R128	TCTCCTCAGTCACATTCTCAATTT
cMUC5ac_F55	GCAAGAGGTGTCTGCATTGA	cMUC5ac_R55	CCAGGTGCATAGTAGGTTTCATT
cMUC13_F1	GACCGTCATCGTGAAAGTTG	cMUC13_R1	GTTGCGTTTTCCGTGAAGAT
cTFF2_F114	TAGCCCTCATCAGCTCAACA	cTFF2_R114	CAGTCTGCTGCTGAGATTCCT
cTBP_F142	TTCCTGGCTTAATCTACAGAATGAT	cTBP_R142	CTGCTCGAACTTTAGCACCA
cMUC1_F32	TTGGTGATCAAACTGAAGTAACAGTA	cMUC1_R32	ATGCTTGATCTCTGGGAAGC
cMUC16_F125	AAGGAGAGCCTCTATGTCAACG	cMUC16_R125	TGTGAAATGGCTGGTTGTTG
cTFF2_F84	CAGAAGGAAAACCACCATCAA	cTFF2_R84	AAGCAGCACCCAGCTCTC
cEEF2_F100	TCCTGTCACAGCCCAGAAGTA	cEEF2_R100	CCCTTGGGATCACAGTTCTTA
cMUC2_F32	TCAGTAACTTCAGGCTCCACAGT	cMUC2_R32	TGGAGACTCTGATGGAGTTGG
cMUC5ac_F72	TGTGGTTGCTATGAGAATGGA	cMUC5ac_R72	TTGCCATGGTTTGTGCAT
cMUC16_F32	CTTCAAATACAACGGGACTATGG	cMUC16_R32	TTTTGTTGGTTGCTGCTTCA
cTLR4_F56	ACTCTTGGGGTGCTGCTG	cTLR4_R56	GTGCATCTGAAAGCTGTGCT
cGAPDH_F23	CCGCAGGCAGTCCATTAT	cGAPDH_5_R23	GGGTGGGGATGGAGGATA
cTBP_F129	CACCAGCAGTTCAGCAGCTA	cTBP_R129	CTGCTCGAACTTTAGCACCA
cMUC2_F20	GGCATTGGGAGTGTGATTG	cMUC2_R20	CCATCAAACGTCATATAATGTGG
cMUC5b_F53	CAACACTGTTTATGTGCTCGAAG	cMUC5b_R53	TTGTACTGGCTGCAGAGATCA
cMUC16_F154	CGTCAGAGCAACCTGCAA	cMUC16_R154	TCAACCACTATGGATCCAACC

Synthesis of complementary DNA (cDNA) from chicken intestine RNA

The concentration of all chicken intestine RNA samples was normalised to 40ng/µl with the aid of a liquid handling robotics system (EpMotion 5075; Eppendorf, Germany). Complementary DNA (cDNA) was synthesised from 400ng of total RNA using the High Capacity cDNA Synthesis kit (Applied Biosystems, USA). The cDNA synthesis reactions were set up according to the manufacturer's instructions. In addition to the components supplied in the kit, 20 units of RNAse inhibitor (RNaseOUT; Invitrogen, USA) and 500nM of oligodTV primer (5'-TTTTTTTTTTTTTTTV, where V = A,C and G) were included in the cDNA synthesis reactions. The cDNA synthesis reactions were incubated at 39ºC for 2 hours and the reverse transcriptase was subsequently inactivated at 65ºC for 20 minutes. The cDNA stocks were diluted 1:4 with 10 mM Tris pH 8.0 (Ambion, USA) and stored at -80ºC until required.

Real-time PCR assessment of gene expression levels in chicken intestine

The stock cDNA (1:4) was diluted a further 5-fold with 10mM Tris 8.0 immediately prior to use in real-time PCR. For each chicken intestine sample, 10µl of 1:20 diluted cDNA was combined with 30µl of the SYTO9-based PCR reagent (composition described above). Ten microlitres of the cDNA/SYTO9 mixture was transferred in triplicate to a 384 well MicroAmp plate (Applied Biosystem, USA). For each gene assay, the chicken intestine cDNA preparations were examined in conjunction with a 7 point standard curve and a no template control. The standard curve was prepared by pooling a portion of all the 1:4 chicken intestine stock cDNA samples. Standard curves were prepared fresh before each real-time PCR run using 7 consecutive 2-fold dilutions in 10mM Tris pH8.0. The dilution series consisted of 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512 dilutions of pooled cDNA. Real-time PCR measurements were performed on two 384 well machines (7900; Applied Biosystems, USA) using the following cycling parameters: 95ºC/10minutes for 1 cycle, and 95ºC/15seconds, 60ºC/20seconds and 72ºC/40seconds for 40 cycles, with data acquisition occurring at the 72ºC step.

Processing, normalisation and statistical analysis of the real-time PCR data

Real-time PCR data was processed and normalised using in-house computer software, qEXPRESS, which was modelled on the qBase software package (Hellemans et. al. 2007). Tab delimitated text files from each real-time PCR run were exported from the SDS 2.3 software (Applied Biosystems, USA) and imported into qEXPRESS, where the data was processed. In brief, the reaction efficiency of each gene assay was determined from the standard curve and applied to a delta Ct quantification model to calculate relative quantities between samples. The non-normalised relative quantification data were exported from qEXPRESS and imported into the GenEx software package (MultiD, Sweden), where reference gene stability analyses were performed using the NormFinder application (Andersen et. al. 2004). This analysis identified the best pair of reference genes, which were then used to normalise the target gene measurements within qEXPRESS using geometric averaging (Vandesompele et. al. 2002). Statistical analyses of the normalised real-time PCR data were performed with a General Linear Model (GLM) in SAS (v9.1; SAS Institute, USA).

Results

A total of 72 samples were processed, but 13 samples were removed from the final analysis due to either degraded RNA or RNA that had been purified in a manner not consistent with the other samples. Therefore, the study is based on 59 samples and high quality data were obtained for 14 out of 15 genes.

A key aspect of a good gene expression study is the ability to normalise the data to a stable set of reference genes. A total of 7 reference genes (Table 1) were included in this study, 6 of which were quite stably expressed (Fig. 1). TATA-binding protein (TBP) was the most stably expressed and Eukaryotic translation elongation factor 2 (EEF2) the least stably expressed. This analysis identified TBP and GADPH as the best two genes for data normalisation so these reference genes were used to normalise the data.

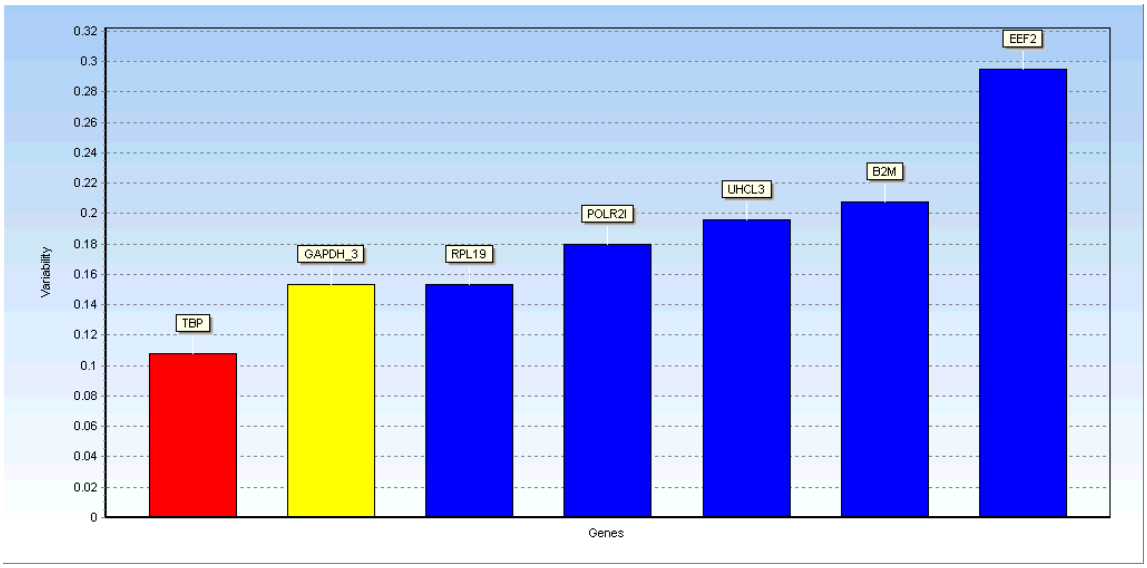

Figure 1. Variability in the expression of reference genes. TBP and GAPDH were the most stably-expressed genes.

The MUC2 and MUC13 genes were differentially expressed between the treatment groups (Fig 2 and 3). MUC2 expression was significantly greater in unchallenged controls and challenged birds fed antimicrobials compared to challenged controls and challenged birds treated with organic acids, probiotics or sham. MUC13 expression was significantly greater in unchallenged controls compared to birds in all challenged groups.

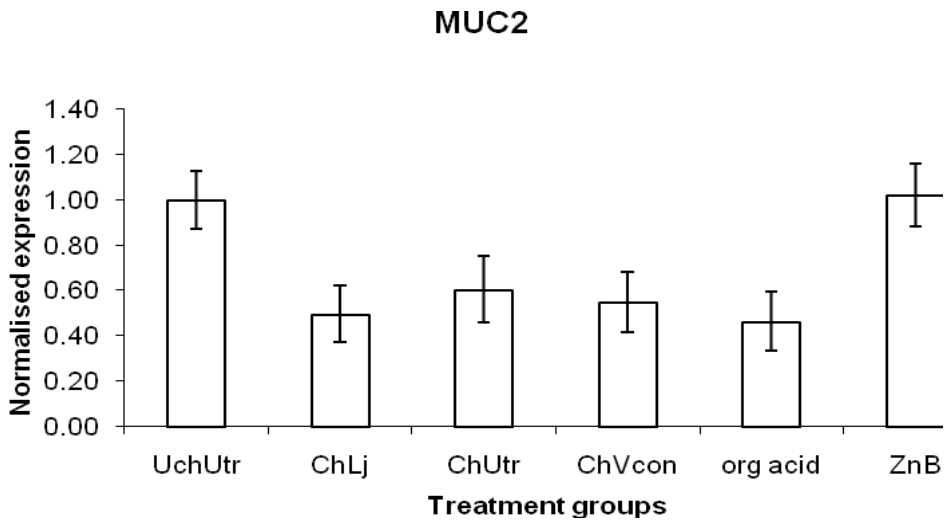

Figure 2. Expression of MUC2 was depressed in all challenged birds with the exception of those treated with antibiotic (ZnB/monensin)

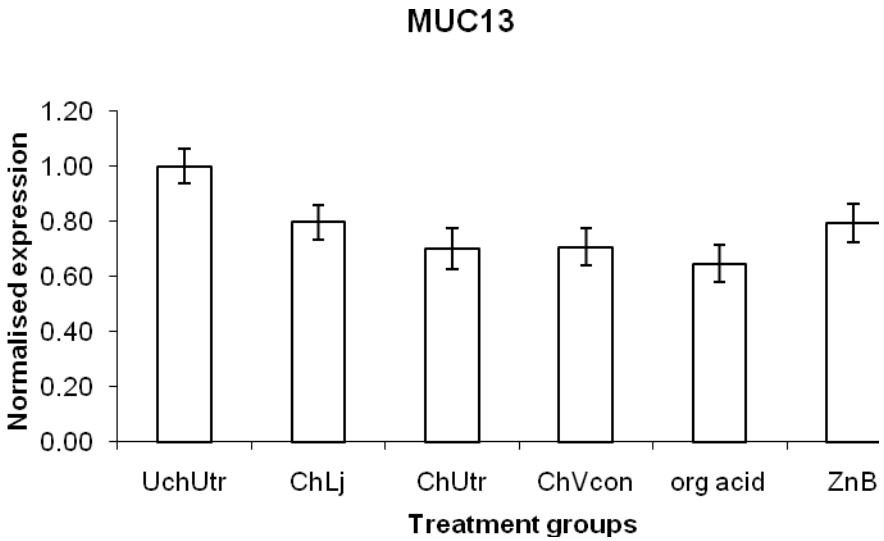

Figure 3. Expression of MUC13 was depressed in all challenged birds

The expression of other target genes (IL18, KGF, MUC5ac, TFF2 and TNFalpha) was not statistically different between the treatment groups (data not shown).

Another aspect of this project was the identification of members of the mucin gene family which are expressed in chicken intestine, or more specifically, the particular region of the intestine which was sampled in this study. We were unable to detect mRNA for the following genes: MUC1, MUC4, MUC5b and MUC16. Three separate assays were designed to each mucin gene so it is likely that none of these genes are expressed in the intestine.

Discussion

The objective of this study was to establish methods for quantifying changes in the expression of a suite of genes associated with mucin production in the intestines of chickens. The opportunity was taken to utilise tissues derived from a previous experiment in which birds were challenged with bacteria and provided a range of potential 'protective' agents.

Normalisation of expression data obtained using qPCR assumes that the expression of the reference gene is uniform and independent of treatment. The most stably expressed reference genes were TBP and GAPDH, so these were subsequently used to normalise the expression data for all other genes, and will be the standard reference genes for future studies of intestinal gene expression in chickens in our laboratory.

Changes in the expression of the suite of mucins revealed significant differences between mucin types. Several of the mucins MUC1, MUC4, MUC5b and MUC16 were not detectable in this region of the intestines at all, regardless of challenge and enteritis status. Previous studies have shown significant decreases in the expression of cell surface mucins following infection (Linden et al. 2008). Expression of MUC2 and MUC13 was significantly depressed following infection by *C. perfringens*. *L. johnsonii* and the organic acid blend could not prevent this decline in mucin production, but the conventional antibiotic (ZnB/monensin) prevented the decline in MUC2 but not MUC13.

We conclude that the methods developed in this study produce sensible, reproducible and accurate quantitative data on the expression of mucin and other gut-related genes potentially involved in protection of the chicken from invasion and pathogenesis by infective organisms such as *C. perfringens*.

Implications

Differential expression of mucin and related genes during infection by enteric organisms, suggests that targeted approaches to altering the mucin profile of the intestines (eg nutritionally, using prebiotics and probiotics) may be possible.

- We have successfully developed a series of rt-PCR assays to study gene expression of a number of key genes involved in mucin dynamics, inflammatory pathways and host-microbe interactions
- These assays can be applied to further studies investigating links between i) pathogens and gut health, and ii) diet and gut health; particular focus can be applied to novel gut modulators in an NE setting
- We have demonstrated that NE affects mucin expression (MUC2 and 13) and that antibiotics can prevent NE-induced shifts (MUC2). Further investigation is needed to identify any possible relationships between MUC expression, NE incidence and bird performance

Recommendations

Further research is required to

- apply these assays to future work within our research team to study the impact of diet and bacterial challenge on gut health
- expand the assays (to cover more mucin-related genes) to develop a better understanding of factors driving mucin and its involvement in gut pathology
- understand how diet influences gut health and how we can improve production and disease resistance by dietary manipulation
- apply studies of gene expression, structural analysis, bacterial binding assays and histological analyses to provide a more complete picture of the role of mucins in gut health and disease

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Plain English Compendium Summary

Project Title:	**Identification of genes associated with mucin production and quality in healthy chickens and those with necrotic enteritis**
Project No.:	CRC 09-21
Researcher:	Prof Phil Hynd
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Objectives	• To develop a series of real-time PCR based assays to study mucin-related genes in the intestine of broiler chickens • To determine how necrotic enteritis challenge influences expression of these genes • To evaluate how treatment with antibiotics, organic acids and probiotics influences expression of these genes
Background	A healthy gut environment is paramount to the health and productivity of broiler chickens. As the industry moves away from antibiotic use in broiler diets the challenge will be to maintain a healthy gut. Central to gut health is the mucus layer, which is the first line of defence encountered by gut bacteria. Mucins play important roles in host protection and the regulation of microbial colonisation as they provide binding sites and a nutrient source for commensal and pathogenic bacteria.
Research	Samples of jejunum and ileum were collected from the CRC Project 05-2 necrotic enteritis (NE) challenge experiment. We focussed on samples taken from birds in the unchallenged and challenged control groups and from challenged birds treated with zinc bacitracin (ZnB). RNA from 12 birds from each group was extracted and rt-PCR assays developed. Initially our target genes were muc2, TNF-a, KGF, TLR2, IL-18 and TFF. This work can be extended in the future to cover more target genes. Real-time PCR assays were designed with the aid of the chicken genome sequence. Six target gene (muc2, kgf, TNFa, TLR2, IL18 and TFF) and 4 reference gene (rpl19, ß-actin, ubiq and elf) assays were designed. The latter set of assays was used for the normalisation of the gene expression data. The performance and specificity of the PCR assays were thoroughly tested with a SYBR green reagent and a real-time PCR machine (RotorGene3000). PCR products from assays that performed well were purified and submitted for DNA sequencing, to confirm the identity of all amplified genes. Total RNA was isolated from the jejunum and ileum samples (Project 05-2) and treated with DNAse I to remove traces of contaminating genomic DNA. Complementary DNA (cDNA) was synthesised from 500ng of jejunum and ileum RNA. The mRNA levels of the target and reference genes were measured in triplicate in each cDNA sample. DNA standard curves were used to calculate the PCR efficiency of the gene assays, while inter-run calibrator samples were included to minimise run-to-run variation. The GenEx software package (MultiD, Sweden) was used to select the most stably expressed set of reference genes. The resulting geometric averages was used to normalise the target gene data and undertake statistical assessments.
Outcomes	- We have successfully developed a series of rt-PCR assays to study

	gene expression of a number of key genes involved in mucin dynamics, inflammatory pathways and host-microbe interactions - These assays can be applied to further studies investigating links between i) pathogens and gut health, and ii) diet and gut health; particular focus can be applied to novel gut modulators in an NE setting - We have demonstrated that NE affects mucin expression (MUC2 and 13) and that antibiotics can prevent NE-induced shifts (MUC2). Further investigation is needed to identify any possible relationships between MUC expression, NE incidence and bird performance
Implications	The successful development of these key assays will complement and strengthen future CRC projects which aim to develop novel dietary strategies to improve broiler health and production. A mechanistic understanding of how novel dietary components influence intestinal health (including mucin dynamics) will be important for identifying dietary additives with potential to be used for commercial broiler production. Identification of key genes that can regulate mucin quantity and composition may provide targets for intervention (by dietary or other means) which can promote the establishment of a beneficial gut microbiota and subsequently improve broiler performance
Publications	None to date but work will be submitted for scientific journal publication