The effects of genetics, age and rearing environment on AvBD gene expression and gut anti-microbial activities in three chicken lines.

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Abstract:

The defensins have been shown to be an important component of the innate immune system across many species from plants to man. To date a total of 14 beta defensins have been identified within the chicken genome and anti-microbial activity of an array of these peptides against pathogens has been demonstrated. The innate immune system has been shown to be important in chickens during the first week of life when exposure to pathogens in the environment occurs and the adaptive immune system is not fully developed. The research presented in this thesis attempts to investigate the effects of bird age (aged 0, 7, 14 and 35 days), genetics and rearing environment on components of the innate immune system. A farm trial was performed using three lines of Aviagen birds (lines X, Y and Z) and two distinct rearing environments (low and high hygiene farms).

To determine which of the 14 avian beta defensins (AvBD) to investigate, a panel of potential single nucleotide polymorphisms (SNPs) within the AvBD locus was submitted as part of an Aviagen Ltd genomics initiative. Frequencies of the polymorphisms across the three lines of birds were determined. From these data, gene expression of AvBDs1, 4 and 10 were fully investigated using end-point PCR and then quantitative real-time PCR (qRT-PCR). A pronounced finding from the qRT-PCR was the marked intra and inter-group variation in gene expression levels, which lead to few statistical significant differences. All three genes were shown to be expressed across a panel of ten tissues analysed, but distinct patterns were also seen. Significantly AvBD1 gene expression in the duodenum indicated that of the 7 day old birds the line X birds reared in the low hygiene environment had the highest level of gene expression. In relation to AvBD4 gene expression, the highest level was observed in the spleen of the 0 day old birds, but overall environment did not appear to affect AvBD4 gene expression of the tissues examined. High levels of AvBD10 gene expression were observed in bird kidney and testicle tissue, but again environment in the case of the former tissue did not appear to statistically affect gene expression levels, the YH 7 day old bird testicle samples did have statistically significant higher expression levels compared to the other groups.

The SNP analysis revealed three non-synonymous polymorphisms within the AvBD1 mature peptide locus. The three lines of birds had quite different patterns of these polymorphisms and so three different forms of the peptide along with a single form of the AvBD10 peptide were synthesised using a bacterial hyper-expression system. Peptide levels were quantified using an ELISA and subsequently tested in a bacterial time-kill assay using *Salmonella enterica* serovar Typhimurium phoP, *Staphylococcus aureus* and two strains of *Enterococcus faecalis*. All recombinant peptides showed anti-microbial activity against the bacteria tested. The exception was a clinical isolate of *E. faecalis* which showed resistance to the killing activities of recombinant AvBD10 peptide. Duodenal gut protein extracts were also tested using the same bacterial assay and marked differences in the anti-microbial activities of these samples were seen. The samples taken from the day 0 birds were found to have significant anti-microbial activity compared to those of the older birds. LC/MS identified differences in the proteomes of the respective gut extracts.

These data support that bird genetics, age and the environment have an effect on AvBD gene expression and gut anti-microbial activity. These differences are not uniform for all genes and groups of birds, but clear patterns were observed.

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Abbreviations:

A	adenine
Ab	antibody
AMA	anti-microbial assay
AMP	anti-microbial peptide
AvBD	avian beta defensin
Bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CFU	colony forming units
ChEST	chicken expressed sequence tag
cLEAP-2	chicken liver expressed anti-microbial peptide 2
Cys	cysteine
Da	dalton
DNA	deoxyibose nucelic acid
dNTP	deoxyribonucleotide triphosphate
ds	double stranded
EDTA	ethylenediaminetetracetate
ER	endoplasmic reticulum
F	forward
g	grams
G	guanine
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GALT	gut associated lymphoid tissue
GIT	gastro-intestinal tract
GST	glutathioneS-transferase

h	hour
HBD	human beta defensin
HH	high hygiene
His	histidine
HIV	human immunodeficiency virus
HNP	human neutrophil peptide
HKG	house keeping gene
HPLC	high pressure liquid chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kb	kilobase
kDa	kilodalton
L	liter
LB	Luria Bertani
LCMS	liquid chromatography mass spectrometry
LH	low hygiene
LM	low molecular
LPS	lipopolysaccharide
M	moles
mA	milliamp
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
MCS	multiple cloning site
mg	milligram
min	minute
ml	milliliter
mM	milimolar
MOPS	3-(N-morpholino)propanesulphonic acid
mRNA	messenger RNA
MRSA	methicillin resistant *Staphylococcus aureus*
NCBI	National Centre for Biotechnology Information

nM	nanometers
OD	optical density
PAMP	pathogen associated molecular pattern
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PRR	pathogen recognition receptor
PSI	pounds per square inch
PVDF	polyvinyl
qRT-PCR	quantitative real-time PCR
QTL	quantitative trait loci
R	reverse
rec	recombinant
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcription
SD	standard deviation
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
sec	seconds
SEM	standard error of the mean
SNP	single nucleotide polymorphism
T	thymine
TBE	Tris-borate-EDTA
TEMED	*N'*,*N'*,*N'*,*N'*-tetramethylethylenediamide
Tm	annealing temperature
Tris	tris(hydorxymethyl)methylamine
UK	United Kingdom
UV	ultra violet
V	volts

v/v	volume to volume
w/v	weight for volume

Chapter 1: Introduction

1.1: An overview of innate immunity

Innate immunity is an organisms first line of defense against attack from pathogens and it forms the non-specific fast response unit of the immune system. It is often thought of as a primitive form of the immune system since it is found in the simplest of organisms, for example molluscs and crustaceans (Hancock and Lehrer 1998) and provides protection against bacteria, viruses and parasites that challenge the host.

The innate response is triggered in response to highly conserved motifs such as lipopolysaccharide, peptidoglycan and double-stranded RNA which are collectively known as pathogen-associated molecular patterns (PAMPs). These PAMPs are recognised by pathogen recognition receptors (PRR) in the host. The PRR and PAMPs are highly conserved across species and different PRRs react with specific PAMPs (Akira, Uematsu et al. 2006). The innate immune system minimises the pathogenic challenge to the adaptive or acquired immune system, which is slower to be activated although is more specific in its response. There are several components of the innate immune system including;

- Physical anatomical barriers e.g. skin, nasal passages and integument
- Physical processes e.g. peristalsis and mucocilliary clearance in the upper respiratory tract
- Chemical barriers e.g. gastric acid and mucin

- Phagocytes and macrophages (monocytes in circulation)
- Anti-microbial substances e.g. hydrogen peroxide and nitric acid
- Secretion of proteins and peptides;
    - Microbial binding lectins and pentraxins
    - Anti-microbial proteins and peptides
    - Complement
    - Interferons and other cytokines

The innate immune system is particularly important for the respiratory, gastro-intestinal and reproductive systems, all of which are typified by their epithelial nature. Equally, all of these systems are constantly challenged by pathogens in the surrounding environment.

1.1.1: Innate immunity of the gastro-intestinal (GI) system

The gastro-intestinal system is under a constant threat from opportunistic pathogens found in feed and drinking water. The innate immune system is closely regulated in the healthy individual so that gut homeostasis can occur and responses are only generated against non-self organisms when appropriate. For this to be achieved, a degree of self-tolerance must exist so that the host is not damaged. The major cellular components of the mammalian GI innate system include goblet cells that produce mucin, Paneth cells that produce anti-microbial peptides and dendritic cells that present luminal antigens to the epithelium. Other barriers that form part of this system include the effects of gut peristalsis and locally secreted products such as gastric acid.

1.2: The role and effects of the commensal gut flora

The intestinal gut flora has been shown to influence the normal structural and functional development of the mucosal immune system and a fine level of homeostasis and tolerance of these bacteria exists. Bacterial colonisation occurs straight after birth or in the case of birds, hatch, and is influenced by environment, genetics, age, diet and administration of antibiotics (Hopkins, Sharp et al. 2001; Vaahtovuo, Toivanen et al. 2003). Germ-free reared animals emphasise the importance of this bacterial population by showing increased susceptibility to infection (O'Hara and Shanahan 2006), although, this has been shown to be a reversible phenomena following reconstitution with bacteria (Umesaki, Okada et al. 1995). It has also been shown in the rabbit that there is under-development of gut-associated lymphoid tissue in germ-free reared individuals (Rhee, Sethupathi et al. 2004). The interactions between the microflora and the gut mucosa are extensive and complicated, and often only recognised when there is a disruption in the symbiotic relationship. Clinical diseases such as Crohns disease, ulcerative colitis and inflammatory bowel disease have been associated with a disruption in this population of bacteria (Gophna, Sommerfeld et al. 2006; Willing, Halfvarson et al. 2009). However, identification of such populations can be difficult due to an inability to culture these bacteria out of the host, although new approaches such as metagenomic sequencing are facilitating the identification of human and animal microbiomes (Kuczynski, Costello et al.2010; Nelson, Weinstock et al.2010).

In the chicken the microbiota has been shown to establish within 24 h of hatch but density, and diversity, increase in the more distal regions of the guts. The use of probiotic bacteria in feed can affect this microflora and can be of benefit to the host (Forsythe and Bienenstock 2010). Such benefits include weight gain and reduced mortality through a decrease in the capacity of enteric pathogens to attach and colonise the chicken intestine (Brisbin, Gong et al. 2008). The full role and function of the commensal population is still being investigated, but work published so far suggests that it forms an important component in maintaining a healthy gut environment and affects local immunity by a series of complex interactions (Tsuji, Suzuki et al. 2008). It is highly feasible that the AMPs help establish and maintain the relationship between the host and the commensal gut bacteria population (Salzman, Hung et al.2010).

Bacteria such as *Salmonella* and *Campylobacter* are capable of residing in the chicken intestine without giving rise to clinical symptoms in the birds; however as such they form a major reservoir of these pathogens, which through the consumption of contaminated poultry products can potentially cause food poisoning. Approximately 65% of poultry meat products were found to harbour *Campylobacter* species and 6% *Salmonella* species according to figures from the Food Standards Agency (www.food.gov.uk- 10.03.10). While high, these figures actually reflect a marked reduction in the case of *Salmonella* carriage over the last 20 years, and are evidence of the success of vaccination and tighter biosecurity measures within the poultry industry.

1.3: Overview of anti-microbial peptides and functionality in vertebrates

Anti-microbial peptides (AMPs) are a highly conserved component of the innate immune system (Ganz and Lehrer 1995), and their functions have been shown to be extensive. These peptides are synthesised by a variety of cells and tissue types including epithelia, neutrophils and macrophages (Hancock and Lehrer 1998), and are described as being positively charged, amphipathic and less than 100 amino acids in size (Huttner and Bevins 1999). Their presence has been identified in as diverse organisms as plants, insects and animals. The former two genera lack adaptive immune systems and so the presence of AMPs emphasises their origin as a simple and conserved component of the immune system.

Reported functions of AMPs have focused on anti-microbial activities against bacteria, yeasts, and fungi, but in addition there are reported effects on tumour growth, wound healing, angiogenesis and immune-modulation (Lai and Gallo 2009). Non-immune speculated functions include transporting metal ions (Krause, Neitz et al. 2000) and in the development and protection of sperm (Com, Bourgeon et al. 2003).

Three different models have been proposed to describe the anti-microbial actions of AMPs. These include (i) Barrel-stave pore creation where the peptides integrate into the cell membrane of the target organism and create a pore channel, causing cell death (Oren and Shai 1998), (ii) the Toroidal pore model where the peptides are proposed to aggregate and induce the lipid monolayer to bend and become damaged (Brogden 2005)

and (iii) the Carpet model where the peptides aggregate and cover the target membrane leading to membrane disruption in a detergent like manner (Shai 1995). Cell selectivity of AMPs is particularly important so that the host isn't damaged by their activity.

The principle theory for AMP cell selectivity is based on the electrostatic charge of a cells surface, the cationic peptides being strongly attracted to the very negative charge of the of target cells such as bacteria (Pouny, Rapaport et al. 1992; Matsuzaki 2009). Other postulated contributing factors include the AMP dimerization and the lipid content of the target cell membrane, but a full explanation of cell selectivity of these peptides has to date not yet been elucidated.

Several classes of AMPs have been identified in vertebrates by researchers and can be classified into the following three groups (i) peptides with an α-helical conformation e.g. Magainin, cathelicidins, Temporin A, (ii) cyclic and open-ended cyclic peptides with pairs of cysteine residues e.g. defensins (iii) β-hairpin-like anti-microbial peptides with one and two disulphide bonds e.g. Hepcidin and Protegrin-1 as classified by Bulet, Stocklin et al. (2004).

Many groups of AMPs are hypothesised to contain three specific domains known as the signal peptide, pro-piece and mature peptide, collectively described as a pre-propetide (Boman 2003). It is the mature peptide that is reported to have anti-microbial activity and the other two regions direct the peptide to get to its target location. Enzymatic cleavage at appropriate cellular locations ensures that the correct destination is reached but that self-damage is limited (Figure 1.1).

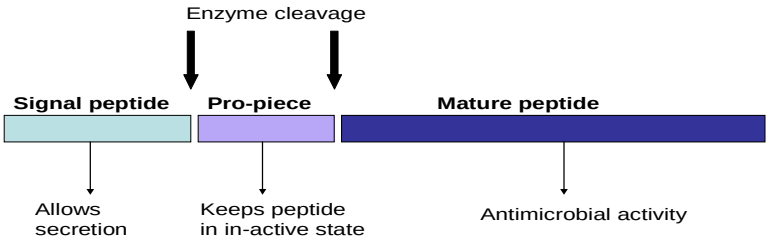


Figure 1.1: Model of an AMP and the three domains.

1.3.1: Defensins

The defensins are generally 5-6kDa (18-45 amino acids) in size, amphipathic, cationic and have six cysteine residues which form three disulphide bonds (Selsted and Ouellette 2005). There are three subgroups; alpha, beta and theta, distinguished by differences between the location of the disulphide bonds and therefore tertiary protein structure (Table 1.1). The absence of α and θ defensin in evolutionary older vertebrates such as birds and fish suggests that evolution from β-defensins by duplication and diversification may have occurred over time (Semple, Rolfe et al. 2003). The first mammalian defensin to be identified was in the trachea of a cow (tracheal anti-microbial peptide or TAP) and this then lead to the discovery of further peptides, based on recognised motifs, initially in the cow and then in other species (Diamond, Zasloff et al. 1991).

	Structure	Size (kDa)	Residues	Cys pairings	Source
α	β-sheet dimer	3.5-4	29-35	1-6, 2-4, 3-5	Human, rabbit, rat, guinea, pig and mouse
β	β-sheet dimer	4-6	38-42	1-5, 2-4, 3-6	Human, bovine, turkey, ostrich, chicken, ovine, pig and king penguin
θ	Cyclic	2	18	1-4,2-5, 3-6	Rhesus monkey

Table 1.1: Classification of the three recognised forms of defensins (adapted from Ganz 2004).

In addition to direct anti-microbial activities, additional defensin functions and properties have been reported. Immunomodulatory properties and the promotion of the adaptive immune system by selective recruitment of monocytes (human neutrophil peptides 1 and 2, HNP 1 and 2) (Territo, Ganz et al. 1989), T lymphocytes (HNP 1 and 2) (Chertov, Michiel et al. 1996), immature dendritic cells (human beta defensins) (Yang, Chertov et al. 1999) and mast cells (Niyonsaba, Iwabuchi et al. 2002) by chemotaxis to sites of inflammation are the immune-related functions of the defensins (human cathelicidin LL-37). In addition, the release of histamine from peritoneal mast cells (Befus, Mowat et al. 1999) and enhancement of macrophage phagocytosis (rabbit anti-microbial peptides) (Fleischmann, Selsted et al. 1985) have also been shown to be stimulated by the presence

of defensins. The reported immune functions other than direct anti-microbial activity emphasise the role that these peptides have in linking the innate and adaptive immune responses, potentially leading to a more favorable outcome in the host that is under attack from pathogens.

1.3.1.1: Alpha defensins

Alpha defensins (known as cryptidins in mice) are synthesised by Paneth cells, specialised epithelial cells within the crypts of Lieberkuhn of the small intestine (ileum), as well as immune cells e.g. neutrophils (Ganz, Selsted et al. 1985). These peptides were first identified in 1966, in rabbit macrophages (Zeya, Spitznagel et al. 1966), but later identified in a variety of species including rat, human, guinea pig, mice, pig, macaque and hamster cells and reviewed by Raj and Dentino (2002). Paneth cells in humans constitutively secrete α defensins (Ouellette 2004), typically human defensin 5 and 6 (HD5 and 6) but the peptides can be upregulated in response to cholinergic stimulation, and bacterial antigen exposure, as demonstrated using *in situ* hybridisation (Ayabe, Satchell et al. 2000). Mice however have been shown to express a greater number of alpha defensins, more than 20 (Ouellette, Hsieh et al. 1994) and the patterns of expression have been shown to be unique to the region of small intestine. Recent research following completion of the horse genome has resulted in the identification thirtyeight equine intestinal α-defensins with at least 20 of this encoding functional peptides, again identification was via a bioinformatics approach (Bruhn, Paul et al. 2009).

Transgenic mice synthesising HD5 exhibited a dramatic increase in resistance to an intestinal infection with *Salmonella typhimurium* (Salzman, Ghosh et al. 2003), indicating the anti-microbial and immune related effects of this defensin in supporting the host. Conditions including Barretts oesophagus, Crohns disease, gastritis and ulcerative colitis can lead to Paneth cell metaplasia leading to subsequent disruptions in AMP production and excessive inflammation (Cunliffe 2003).

1.3.1.2: Beta defensins

Beta defensins have been mainly detected in epithelial surfaces, thus forming an important part of the epithelial barrier, as well as production in some immune-related cells. The peptides have been shown to be more widely distributed amongst vertebrates than α-defensins as they also occur in birds (Bulet, Stocklin et al. 2004). Structurally these defensins have a triple-stranded β-pleated sheet structure (Thouzeau, Le Maho et al. 2003), and disulphide bonds as indicated in Table 1.1. In humans four β-defensins have been well characterised and these include HBD1, 2, 3 and 4. Local constitutive expression of human beta defensin 1 (HBD1), has been shown to be within the picogram to nanogram per milliliter range (Bowdish, Davidson et al. 2006), indicating the low, but constant, local concentrations in the host. In contrast the same study determined that HBD2 expression is induced under the influence of both infection and inflammation. At the level of the skin, HBD2 expression has been noted to be reduced in patients suffering from atopic dermatitis where as concentrations are increased in psoriatic skin sufferers. Interestingly in the former group, atopics, a higher recurrent rate of skin infection, or

pyoderma cases, is reported compared to the psoriatic cases (Ong, Ohtake et al. 2002). This finding suggests, potentially, that HBD 2 has an effect on the bacteria population in these two skin conditions. Conversely an upregulation of the AMP canine β-defensin 1 has been shown to occur in canine cases of atopic dermatitis (van Damme, Willemse et al. 2009). Different mechanisms in disease manifestation may be involved but the complex involvement of AMPs in clinical cases may not be a simple mechanism or may be species specific. Other clinical reports suggest the importance of AMPs in cases such as cystic fibrosis where there is impairment in defensin gene expression with a subsequent susceptibility of the individual to recurrent bacterial infections (Laube, Yim et al. 2006).

To date there are no reports on the effect of gene copy number of any of the AvBDs and disease susceptibility where as in man an altered number of human β-defensin copy number has been linked with Crohns disease susceptibility (Hollox 2008).

1.3.1.3: Theta defensins

The θ-defensins (retrocyclins) are cyclic peptides and to date have been characterised in the neutrophils of non-human primates (Tang, Yuan et al. 1999). They are the smallest of the three groups of defensins (18 residues) (Bulet, Stocklin et al. 2004) and characterised by the presence of six cysteine residues and cyclic conformation. Activity against HIV type 1 has been determined (Venkataraman, Cole et al. 2009) but interestingly it was determined that the mode of inhibition of viral replication was different to that due to α

and β human defensins (Seidel, Ye et al.2010). Evolutionary adaptations to both α and β defensins resulting in θ defensins has been hypothesized (Ganz and Lehrer 1995).

1.3.2: Cathelicidins

Cathelicidins are synthesised by epithelial cells and neutrophils and are typified by a highly conserved N-terminal domain known as the cathelin domain. However, it is the C terminal domain, released following protease cleavage, that has the anti-microbial and immunomodulatory properties (Figure 1.2).

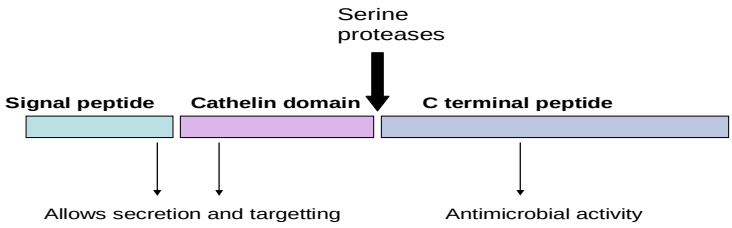


Figure 1.2: Model of a cathelicidin and the three domains.

Cathelicidins have been extensively studied and characterised in mammals but have been shown to be not as widely distributed in non-mammals compared to other groups of AMPs. Cathelicidins show remarkable structural variability unlike the defensins with the conserved cysteine residues. These peptides have been shown to generally be constitutively expressed my myeloid progenitor cells, but can be induced following bacterial stimulation (Chromek, Slamova et al. 2006). Enhanced expression of the human cathelicidin hCAP-18 derivative, LL-37, in transgenic mice lead to increased

resistance to cutaneous bacterial infections, showing the protective effects of this AMP (Nizet, Ohtake et al. 2001). Cathelicidin gene up-regulation has also been shown in pigs following stimulation with lipopolysaccharide, interleukin 16, retinoic acid and *Salmonella* infection (Wu, Zhang et al. 2000), again supporting an immune function of this group of AMP. Other reported functions of cathelicidins include re-epithelialisation, angiogenesis and vasculogenesis (Nizet and Gallo 2003).

1.4: Developments with the chicken genome

In 2006, the second updated version of the chicken genome which had initially become available in 2004 (Wallis, Aerts et al. 2004) was released allowing and improving the accuracy of bioinformatic searches. As part of this facility, data bases of single nucleotide polymorphisms (SNPs) became available allowing searches of regions of genomic DNA to determine the occurrence, and to some degree reported frequencies, of SNPs.

1.5: Anti-microbial peptides in birds

1.5.1: Beta defensins

Apart from avian β defensins 1 and 2 identified through peptide purification of heterophils (Evans, Beach et al. 1994; Kannan, Liyanage et al. 2009), most of the chicken AMPs were identified via a bioinformatics approach using the avian genome information (Lynn, Higgs et al. 2004; Xiao, Hughes et al. 2004).

Only the β-defensin sub-group of defensins has been found to occur in chickens. The probable absence of Paneth cells, a source of gut α-defensins, in the small intestines of birds (van Dijk, Veldhuizen et al. 2008) potentially being a contributory factor to this finding. To date 14 β-defensins have been identified within the genome of *Gallus gallus* (Lynn, Higgs et al. 2007) and these genes have been found to be clustered on a region of approximately 86kb on chromosome 3q3.5-q3.7 in the chicken (Xiao, Hughes et al. 2004). Initially there was some confusion over the numbering system of these β-defensins, two numbering systems were in use. In 2007 a standardised nomenclature system was introduced by the research community and the chicken β-defensins were renamed Avian beta defensins (AvBD) instead of 'Gallinacins' and a uniform coding system was implemented (Lynn, Higgs et al. 2007). This system has been used throughout the text.

As regards molecular architecture, the AvBDs are generally encoded by four exons; the first exon corresponds to the 5'UTR, the second exon encodes the signal peptide and the propiece, and the majority of the mature peptide is encoded by the third and fourth exons (van Dijk, Veldhuizen et al. 2008).

It has also recently been reported that an anti-microbial peptide member of a new avian defensin family, ovodefensins, has been identified within egg white (Gong, Wilson et al.2010). This peptide has been named 'Gallin' and like the other defensins has six cysteine residues although the cysteine spacing differs from the conventional AvBDs. Three forms of this peptide been identified and although anti-microbial activity against

Escherichia coli has been shown, its full role and function within the egg is yet to be determined.

To date only the chicken and the zebra finch genomes are completed and readily available. Recently a cluster of 22 AvBD analogues have been discovered in the Zebra finch, and as for the chicken in a cluster on chromosome 3. These were discovered following bioinformatic searches and support the evidence of the two species sharing a common ancestor (Hellgren and Ekblom). Beta defensin genes have also been shown to exist within the duck (Ma, Liao et al. 2009; Soman, Arathy et al. 2009), penguin (Thouzeau, Le Maho et al. 2003), ostrich (Sugiarto and Yu 2007) and turkey (Brockus, Jackwood et al. 1998).

1.5.2: Functionality of AvBD peptides

Of the 14 identified AvBDs, only eight have been investigated for antimicrobial activities and these include AvBDs 1, 2, 4, 5, 6, 8, 9 and 13. It is difficult to compare data from such studies as the type of assay, incubation medium, incubation time and measurement of peptide concentration are different.

AvBD1 was purified from chicken heterophils and shown to have activity against an array of pathogens including; *Candida albicans, Salmonella enteriditis, Salmonella Typhimurium, Campylobacter jejuni, Escherichia coli, Pasteurella multocida, Bordetella avium, Listeria monocytogenes, Staphylococcus aureus* and *Mycoplasma gallisepticum* at concentrations of peptide in the range 0.4-3.4µM (Evans, Beach et al. 1994; Harwig, Swiderek et al. 1994). In contrast, AvBD2 that was also purified from chicken

heterophils was found to have anti-microbial activity against a narrower spectrum of pathogens and these included; *Escherichia coli* and *Listeria monocytogenes* (Evans, Beach et al. 1994; Harwig, Swiderek et al. 1994).

Recombinant AvBDs 4, 5 and 6 have also been shown to have anti-microbial activity against *Salmonella enterica* serovar Typhimurium phoP, a genetically engineered strain with increased susceptibility to AMPs, *Salmonella enterica* serovar Typhimurium SL1344 and *Salmonella enteriditis* (Milona, Townes et al. 2007) and *Escherichia coli* and *Streptococcus suis* CAB strain, at concentrations of 3-6µg/ml (Ma, Liu et al. 2008). Different forms of recombinant AvBD8, custom synthesised, with varying overall charges have also been shown to have anti-microbial activity against *Salmonella enterica* serovar Typhimurium phoP, *Escherichia coli* and *Listeria monocytogenes*, and moreover these data showed that increasing the charge was associated with increased anti-microbial activity (Higgs, Lynn et al. 2007). Recombinant AvBD9, synthesised in and purified from kidney transfected cells, was shown to have bactericidal activity against *Campylobacter jejuni*, *Salmonella enterica* serovar Typhimurium, *Clostridium perfringens* and *Escherichia coli*, concentrations between 8 and 128µg/ml (van Dijk, Veldhuizen et al. 2007). AvBD13, prepared through custom synthesis, has been shown to demonstrate marked anti-microbial activity against; *Salmonella enterica* serovar typhimurium phoP, *Listeria monocytogenes* (500µg/ml) and reduced activity against *Escherichia coli* (Higgs, Lynn et al. 2005).

1.5.3: Tissue-specific gene expression

Over the years several groups have investigated tissue AvBD gene expression, primarily via end-point PCR. Different breeds and lines of birds have been studied and extensive AvBD gene expression panels have been created. A collation of all of this research has been presented in a review article by van Dijk, Veldhuizen et al. (2008). Importantly differences in gene expression are reported indicating the probable influence of bird genetics and age on such data. A simple summary of tissue expression patterns is shown in Table 1.2. Discrepancies are obvious when tissues such as the 'intestines' are analysed, as the data is not specific enough to specify the exact region of the gut that has been examined. Studies carried out quantifying the α-defensin expression along the gastro-intestinal tract of mice showed that there were regional differences and such a finding could be hypothesised to exist in the chicken (Karlsson, Putsep et al. 2008).

AvBD: Tissue:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Tongue			√		√								√	
Oesophagus			√						√					
Crop									√					
Proventriculus									√				√	
Gizzard									√					
Small intestine	√	√		√		√	√		√				√	
Large intestine	√	√	√	√		√	√		√			√		
Caeca	√	√											√	
Colon														
Cloaca	√	√		√						√				
Pancreas	√	√				√			√				√	
Liver		√		√		√		√	√	√			√	
Gall bladder	√	√		√		√	√	√	√	√			√	
Trachea		√	√	√	√	√	√		√				√	
Lung	√	√		√	√	√	√		√	√			√	
Air sacs			√										√	
Kidneys			√	√		√			√	√	√	√	√	
Testis	√	√		√	√	√	√		√	√		√		
Vas deferens									√	√				
Ovary			√						√	√		√	√	
Oviduct										√	√	√	√	
Infundibulum	√	√	√							√				√
Uterus	√	√	√							√	√	√		
Vagina	√	√	√										√	
Egg yolk										√				
Skin			√						√	√	√			
Eggshell			√								√	√		
Eggshell membrane			√								√	√		
Thymus									√		√			
Spleen		√							√				√	
Bursa	√	√	√	√	√	√	√		√	√		√	√	
Heart												√		
Skeletal muscle									√					
Brain	√	√	√	√	√	√	√		√	√				
Bone marrow	√	√	√	√	√	√	√		√					
Leukocytes	√	√				√	√							

Table 1.2: Summary of AvBD 1-14 gene expression across tissues, modified from van Dijk, Veldhuizen et al. (2008), Ebers, Zhang et al. (2009) and Mageed et al. (2009). Those boxes with √ indicates that gene expression was observed.

1.5.4: Inducibility of gene expression

Several studies have investigated potential inducibility of AvBD gene expression both *in vivo* using challenged birds and *in vitro* using specific cell lines. Such challenges have included infection with *Salmonella* species (Sadeyen, Trotereau et al. 2006; Yoshimura, Ohashi et al. 2006; Milona, Townes et al. 2007; Akbari, Haghighi et al. 2008; Ebers, Zhang et al. 2009; Meade, Narciandi et al. 2009), *Haemophilus paragallinarum* (Zhao, Nguyen et al. 2001), and lipopolysaccharide (Yoshimura, Ohashi et al. 2006; Subedi, Isobe et al. 2007; Mageed, Isobe et al. 2008). Overall the findings, in these numerous studies, showed that the specific AvBD levels of gene expression increased following challenge. The full effect of an increase in gene transcripts and presumably peptide levels, has yet to be fully elucidated, the lack of functional antibodies delaying such investigations. However, if these results are linked with the reported anti-microbial properties of the peptides, it suggests AMPs serve as a protective response generated by the host to fend off pathogens and aid microbe clearance.

1.5.5: Single nucleotide polymorphisms (SNPs) associated with defensins

Single nucleotide polymorphisms have been shown to occur at a rate of 13.2 polymorphisms per kilobase for the 3.25kb region encoding the AvBDs 2, 3, 4,5 and 7 (Hasenstein, Zhang et al. 2006). All, bar one of the polymorphisms located in the exon of AvBD5, were found within the non-coding regions of the genome. The same study linked some of the identified SNPs with disease susceptibility to *Salmonella enteriditis* and bacteria colonisation, and the concept that the polymorphisms may have an effect on

genes at other locations was proposed. Interestingly intronic polymorphisms in HBD1 have also been shown to affect disease susceptibility to a range of diseases including HIV (Ricci, Malacrida et al. 2009), *Candida albicans* carriage (Jurevic, Bai et al. 2003) and airway colonisation with *Pseudomonas aeruginosa* (Tesse, Cardinale et al. 2008).

1.5.6: Other AMPs reported in the chicken

A) Cathelicidins

Cathelicidin genes have been shown to be expressed by chickens and these have been named Fowlicidins 1-3 (Xiao, Cai et al. 2006; Bommineni, Dai et al. 2007), chicken myeloid anti-microbial peptide 27 (van Dijk, Veldhuizen et al. 2005) and cathelicidin-B1 (Goitsuka, Chen et al. 2007). Of these only the three Fowlicidins have been reported to have anti-microbial activity against a broad spectrum of gram positive and negative bacteria at reported concentrations in the range 0.4-2.0µM (Xiao, Cai et al. 2006).

B) Liver-expressed anti-microbial peptides (LEAP)

Chicken LEAP 2 peptide has been identified in the chicken and shown to be induced following challenge with *Salmonella* species (Townes, Michailidis et al. 2004). The recombinant peptide has anti-microbial activity (Townes, Michailidis et al. 2004), and the mechanism of anti-microbial activity has been shown to be via membrane permeabilisation of the bacteria (Townes, Michailidis et al. 2009).

1.5.7: Other roles and functions of chicken anti-microbial peptides

Recently it has been shown that the presence of the gene encoding AvBD1 can have an adjuvant effect when fused with infectious bursal disease virus VP2 gene (Zhang, Yang et al. 2010). The presence of the AvBD1 gene facilitates an enhanced antibody response in birds inoculated with the engineered DNA vaccine. DNA vaccines form a large area of research, especially in those diseases where current vaccines are not as efficacious as desired. The scope for use of the AvBD peptides commercially in this way indicates a very exciting path of study. Other functions of AvBDs can also be hypothesised based on the findings of high levels of gene expression in tissues not linked directly with immune-function or in direct contact with pathogens on the epithelial surface. For example very high levels of AvBD10 gene expression have been reported in the testicle and kidneys (Lynn, Higgs et al. 2004).

1.6: Chicken broiler industry

1.6.1: Overview of production systems

The UK's broiler industry developed extremely quickly following the introduction of broilers from USA in the 1950s, previous to this chicken production had been on a much smaller scale. In 2008 an estimated 800 million chickens were slaughtered to supply the current demands of meat with a calculated average of 25kg consumption of chicken meat per person each year (http://www.ukagriculture.com/livestock/broiler_chickens.cfm - accessed- 19.3.10). The majority of broilers are reared intensively, with slaughter at

approximately 43 days of age weighing close to 2.2kg, in an attempt to maximize production while maintaining reduced rearing costs. Smaller scale production systems, organic, free-range and back-yard rearing practices have become increasingly popular over the past few years.

1.6.2: Management and welfare problems

The major welfare considerations for intensively reared birds include conditions such as foot-pad dermatitis, poor leg strength and breast skin damage. The causes are probably multifactorial but stocking densities and environmental rearing conditions, such as quality of bedding, have been implicated as factors that have an impact on bird welfare (Allain, Mirabito et al. 2009; Buijs, Keeling et al. 2009). The reported incidence of foot-pad dermatitis is very variable with one study showing an average incidence of 14.8% but with a range 9.6-98%, although bird genetics have been shown to be linked to this condition (Pagazaurtundua and Warriss 2006). The birds themselves can be a major factor in bedding quality by the water content of their faeces, which is potentially directing the selection and breeding of birds with perceived good gut health. Bird diet has also been linked to hock burn sores, with an increasing percentage of dietary wheat reducing the incidence of such lesions (Haslam, Knowles et al. 2007).

The breeding of broilers has primarily focused on traits such as efficient feed conversion, growth, muscle percentage, reproductive excellence and a range of general fitness traits such as cardiovascular strength, skeletal form and general survival. With the exception of bird selection to Mareks disease susceptibility, a focus on breeding specifically for a

superior immune system has been neglected, perhaps influenced by the use of prophylactic in-feed antibiotics and absence of effective selection tools.

1.6.3: Disease threats to broilers

As with any animals that are reared intensively indoors, major risks to health need to be considered for the whole population rather than the individual. Strict measures of biosecurity need to implemented to reduce the chance of spread of disease within a flock be the pathogen exotic or endemic. In recent years there has been a series of disease outbreaks in all types of livestock. At the time of commencing this study in 2006, there had been a recent outbreak of avian influenza (H5N1) in the UK (Brown 2010), and identification of further strains of avian influenza occurred up until 2008. The risk that such a disease poses to the poultry industry can not be understated, both on welfare grounds and economically. An outbreak of Newcastle disease also occurred in Scotland in 2006 with the most recent report of disease occurring in Israel earlier this year

(http://www.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/newcastle/recentoutbreaks.htm -accessed 20.3.10). There is therefore an increasing pressure to select birds with good perceived immunity for future breeding programs as well as protecting the stock from common problems such as necrotic enteritis and coccidiosis at the level of the gastro-intestinal system. It is the common endemic health problems, which are the priority of Aviagen Ltd. The full role and importance of the innate immune system can only be speculated.

1.7: Summary of aims of this study

This study was a BBSRC industrial case studentship with Aviagen Ltd. The aims of the study are given in a chronological order as presented within the thesis.

Aim 1 addressed in Chapter 3:

- To perform a farm-based field trial using three pure lines of birds (aged 0, 7, 14 and 35 days) on two distinct farming environments; high and low hygiene, the latter being similar to commercial broiler farms. Parameters such as diet, ambient temperature and humidity and bacteria count of bedding samples were taken into consideration and bird weights were measured to determine the influence of the environment on development and growth.

Aim 2 addressed in Chapter 4:

- To investigate the presence of single nucleotide polymorphisms within the three chosen lines of birds using three different sequencing methods (individual bird cDNA, small group genomic DNA and large group genomic DNA analyses, the latter forming part of a large SNP study by Aviagen Ltd), thus allowing the frequency of the polymorphisms to be determined. The SNP searches were confined to AvBD1, 4 and 10 genes.

Aim 3 addressed in Chapter 5:

- To investigate gene expression levels of AvBD1, 4 and 10 gene expression levels across a panel of tissues taken from the groups of trial birds, initially using the technique of end-point PCR and then quantitative PCR, with the aim of defining expression patterns within and without the bird lines and linking these findings with the SNPs.

Aim 4 addressed in Chapter 6:

- To synthesis recombinant AvBD1 and 10 peptides, and using a time-kill assay to investigate the anti-microbial activities of these AMPs against a panel of bacteria.

Aim 5 addressed in Chapter 7:

- To determine and compare the anti-microbial activities of duodenal gut scrapes taken from birds in the trial and to determine what, if any, differences can be attributed to specific gut proteins and or peptides identified through analyses of their gut proteomes.

Chapter 2: Materials and methods

2.1: Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Bacteriological media and agar were from Oxoid (Basingstoke, Hampshire, UK).

2.2: Molecular Analyses

Figure 2.1 shows an overview of the molecular analyses performed following RNA extraction.

2.2.1: RNA extraction from avian tissues

Following cervical dislocation of each bird, tissue samples from each of the ten organs were taken and placed in a microfuge tube containing 1ml of RNAlater solution (Ambion, Applied Biosystems, UK). The ten tissues were thymus, liver, kidney, testicle, lung, spleen, duodenum, bursa of Fabricius, caecum and caecal tonsil. The tubes were stored at room temperature for 24 h prior to removal of the RNAlater solution. Samples were then stored at -80°C until required. Tissue samples were initially homogenized at room temperature in 1ml of TRIzol reagent (Invitrogen Paisley, UK) with a rotor-stator style homogenizer (Tissue Rupter, Qiagen, Crawley, UK) ensuring that the probe was

thoroughly cleaned between samples (two washes were performed using 10% ethanol and 0.1M PBS, pH7.4).

Instructions for RNA extraction were followed as per manufacturer. Briefly tissue samples were homogenized in 1ml of TRIzol, incubated at room temperature for 5 min followed by the addition of 200µl of isopropanol and gentle mixing with a further 3 min incubation at room temperature. The samples were centrifuged for 15 min at 12,000g and 4°C, the clear supernatant removed and mixed with an equal volume of 70% ethanol. This mixture was added to a purification column (PureLink™RNA mini kit, Invitrogen, Paisley, UK), washed three times with two different wash buffers before elution with 30µl molecular grade water. To aid preservation of RNA, 1µl of RNAse inhibitor (RNAsin, Promega, Southampton, UK), was added to all samples. Samples were stored at -80°C.

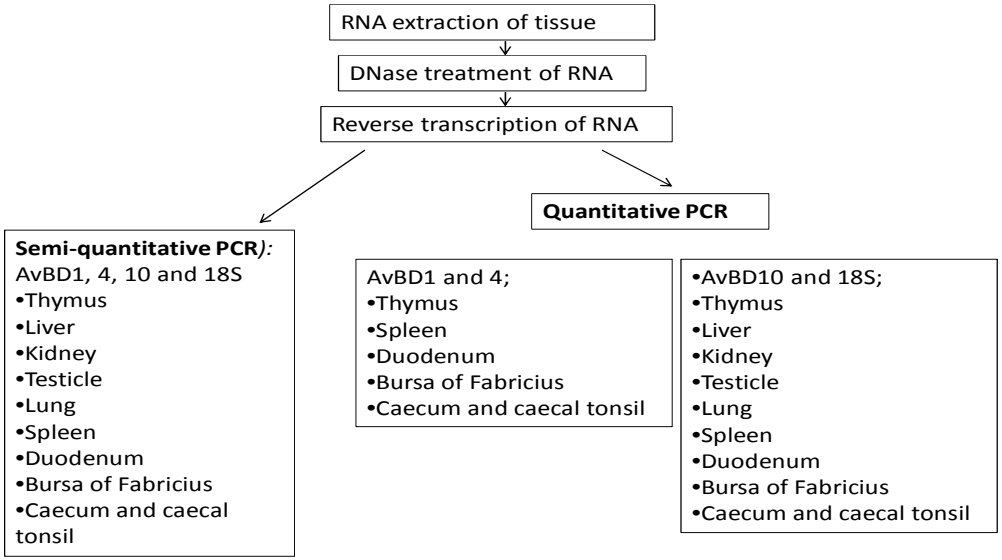


Figure 2.1: Overview of molecular analysis of bird tissues.

2.2.2: Quantification of RNA

RNA concentration was measured using a NanoDrop (NanoDrop®,ND-1000). Measurements were made using the nucleic acid program and involved applying 1.5µl of RNA to the pedestal and measuring of the total nucleic acid concentration at wavelengths of 260nm and 280nm, respectively.

2.2.3: Quality of RNA

As an additional test to confirm RNA quality, a selection of RNA samples were electrophoresed on a modified agarose gel. A 1% (w/v) TBE (54g Tris, 27.5g boric acid, 20ml 0.5M EDTA per 1L de-ionised water) agarose gel was prepared using the wide tooth comb and allowed to set. Working in a fume cupboard 5µl of molecular grade water and 14µl of formamide were added to a microfuge tube containing 5µl of RNA, gently mixed via tapping and incubated at 45°C for 5 min. To this 4.3µl of formaldehyde, 2.8µl of 10xMOPS, 1µl of ethidium bromide and 4µl of agarose gel loading buffer were added. Again the tube contents were gently mixed and incubated at 65°C for 5 min. TBE buffer was added to the tank and the total sample volume was carefully loaded into the gel wells and electrophoresed at 60V for approximately 1 h. The gel was carefully removed from the tank and visualized under UV trans-illuminator and images recorded (302nm, AlphaImager 1200 gel documentation and analysis system, Flowgen, Nottingham, UK).

2.2.4: DNase treatment of RNA

Following quantification of RNA, all samples were treated with DNAse (DNAse kit, Promega, Southampton, UK) to remove any genomic DNA contamination. Approximately 4.5µg of RNA was mixed with 2µl of DNase buffer (400mM Tris-HCL, pH 8.0, 100mM $MgSO_4$, 10mM $CaCl_2$) and 4.5µl of DNase. To this molecular grade water was added so that a total reaction volume of 20µl was made. For those samples which had a poor RNA yield, water was omitted from the sample and instead a larger volume of RNA was added. The tube was briefly vortexed, incubated at 37°C for 30 min and the reaction terminated by the addition of 4µl of DNA stop solution (20mM EDTA, pH8). The reaction was heat treated at 65°C for a further 10 min. Samples were used for reverse transcription or stored at -20°C.

2.2.5: Reverse transcription

Reverse transcription (RT) reactions were set up in microfuge tubes as shown in Table 2.1. The final volume was 20µl.

Reagent	**Volume (µl)**	**Source**
DNase treated RNA (0.94µg)	5	
10xPCR buffer	2	Applied Biosystems, UK
Magnesium chloride [25mM]	1.6	Applied Biosystems, UK
Molecular grade water	1.4	
dNTPs (dATP, dGTP, dCTP, dTTP)[10mM of mixture]	8	Bioline, UK
muLV reverse transcriptase	0.5	Applied Biosystems, UK
RNAsin	0.5	Promega, UK
oligoDTs[15mM]	1	Promega, UK

Table 2.1: Reagents used for reverse transcription reactions.

Following mixing and centrifugation, samples were placed in a thermocycler (Hybaid PCR express, Sprint ™, Hybaid, Middlesex, UK) and treated at 42°C for 1 h, 95°C for 5 min and 5°C for 5 min. All cDNA was stored at 4°C prior to PCR amplification.

2.2.6: Design of primers for RT-PCR

Primers for cDNA amplifications were designed using NCBI sequences of each targeted gene and designed to amplify across at least two exonic regions. Primers were purchased from VH Bio Ltd (Newcastle-Upon-Tyne, UK), supplied in a lyophilised form and re-suspended in water to give a stock solution of 100µM. Working solutions of 10µM (de-ionised H_2O) were prepared from the stock solutions. The 18S primer pair was a commercial product purchased from Ambion (Applied Biosystems, Warrington, UK). The primers and their properties are listed in Table 2.2.

Gene	RefSeq accession no.	Primers (5'to 3')	Product size (base pairs)	Tm °C
AvBD1	NM_204993	F:GGATCGTGTACCTGCTCCTC R: GCAAAAGGAATATGGGGCTGA	144	60
AvBD4	NM_001001610	F:ATCGTGCTCCTCTTTGTGGCAGTTCA R:CTACAACCATCTACAGCAAGAATACT	280	60
AvBD10	NM_001001609	F:GACCCACTTTTCCCTGACACCG R: GCAATGGCCCAGTGAAAGC	166	57
18S		F and R: Universal 18S primers –sequence not given (Ambion)	344	57

Table 2.2: Primers and annealing temperatures used for RT-PCR. Tm indicates the optimal annealing temperature for cDNA amplification.

2.2.7: Polymerase chain reaction (PCR)

PCR reactions were set up in a total volume of 20.25µl as shown in Table 2.3.

Reagent	**Volume (µl)**	**Source**
10X PCR buffer	2	Bioline, UK
Magnesium chloride [50mM]	1	Biloine, UK
Taq DNA polymerase	0.25	Bioline, UK
Forward primer [10µM]	2	VHBio, UK
Reverse primer [10µM]	2	VHBio, UK
Molecular grade water	9	
dNTPs (dATP, dGTP, dCTP, dTTP) [10mM of mixture]	2	Bioline, UK
cDNA from reverse transcription	2	

Table 2.3: Reagents used for PCR reaction in end-point RT-PCR.

Initially an optimal annealing temperature was determined for each set of primers using a 15°C gradient program on the thermocycler; this range was set from 50 to 65°C. The following PCR cycles were applied to the samples; 95°C for 5min and 30 cycles of; 95°C for 30 s, 57-60°C (dependent on primer set) for 30s and 72°C for 30s, and a final elongation stage at 72°C for 12 min. Samples were stored at 4°C prior to being analysed via gel electrophoresis. All reactions were performed in Hybaid PCR express PX2™ PCR machine (Hybaid, Middlesex, UK).

2.2.8: Agarose gel electrophoresis

All PCR products were electrophoresed on 1.5% w/v TBE-agarose gels. The molecular grade agarose was dissolved in 1 X TBE buffer (0.089M Tris-borate, 2mM EDTA, pH8.3) and 5µg/ml ethidium bromide was added prior to pouring into a horizontal electrophoresis tank. When the gel had set it was submerged in TBE buffer and the samples were prepared. To 8µl of PCR product, 2µl of gel loading dye was added (50mM Tris, pH8.5, 5mM EDTA, 50% glycerol (v/v) and 0.1% bromophenol blue (w/v)). The end lanes of each gel were loaded with 4µl of appropriate DNA ladder (Hyperladder 1 or 4, Bioline, UK). Electrophoresis was performed at a voltage of 70V for 45 min. All gels were visualized under UV and an image recorded (BioRad, Hemel Hempstead, UK).

2.2.9: Purification of cDNA

2.2.9.1: Gel extraction kit

Purification of PCR products from gel fragments was performed using a Qiagen gel extraction kit (Qiagen, Crawley, UK) according to manufacturers' instructions. Briefly the gel slice was placed in a microfuge tube and for every gel volume, three volumes of Buffer QG were added. The tube was incubated at 50°C for 10 min with regular vortexing to ensure that the gel had dissolved. One gel volume of isopropanol was added to the sample and this added to a QIAquick column. The column was washed with Buffer PE and the DNA eluted with 30µl Buffer EB (10mM Tris-HCL, pH8.5). DNA samples were purified and either sent for automated nucleotide sequencing (Genevision, Newcastle-Upon-Tyne, UK) or used for cloning.

2.2.9.2: PCR product purification kit

PCR products were purified using a QIAquick PCR purification kit (Qiagen, Crawley, UK). Essentially, five volumes of buffer PB were added to one volume of PCR sample and mixed. DNA was bound to a QIAquick column, washed with buffer PE and the DNA eluted using 30µl of buffer EB. Eluted DNA samples were used directly.

2.2.10: DNA extraction

2.2.10.1: From avian tissue

Genomic DNA was extracted from one tissue sample per bird using the TRIzol (Invitrogen) protocol. This involved the addition of 0.3ml 100% ethanol to the non aqueous remainder of the TRIzol tissue mixture and mixing via inversion. The samples were left for 3 min at room temperature and centrifuged at 2000g for 5 min at 4°C. The phenol-ethanol supernatant was removed and the remaining pellet washed twice in a solution containing 0.1M sodium citrate in 10% ethanol, 1ml being used each time. For each wash, the pellet was left for 30 min, with periodic mixing, before being centrifuged at 2000g for 5 min at 4°C. The pellet was suspended in 1.5ml of 75% ethanol for 20 minutes with periodic mixing and then centrifuged as before. The pellet was allowed to air dry for 15 min in an open tube. Samples were dissolved in 50µl 8mM NaOH and stored at -20°C.

2.2.10.2: From pooled blood samples

Extraction and quantification of genomic DNA from pooled blood samples was performed by Aviagen Ltd. In brief the procedure involved taking equal volumes of settled blood from 20 chickens of the same line. DNA was extracted using a Gentra purification column (Gentra Systems Inc, U.S.A) and the pooled DNA diluted to a target concentration of 100µg/ml using a spectrophotometer (Nanovue, G.E Healthcare, UK).

This technique ensured that each bird in the pool contributed the same amount of DNA. A total of five pooled samples were available for lines X and Y (100 birds) and four samples for line Z (80 birds). SNP analysis was then performed.

2.2.11: PCR for SNP detection in genomic DNA

Initially the pooled genomic DNA samples were diluted to determine the optimum concentration for use in the PCR reactions. PCR reactions were performed in a total volume of 120µl (scaled up from Table 2.3), and a 1:2 dilution of DNA to molecular grade water was found to give the optimum results. Primers were designed to include the potential SNP. Products were electrophoresed on 1% (w/v) TBE-agarose gels, the cDNA bands excised under UV light and the DNA purified (2.2.9.1). Five microliters of the purified sample was electrophoresed on a 1% (w/v) TBE-agarose gel to ensure that product was still present and the remaining sample along with the appropriate forward and reverse primers were sent for automated sequencing (Genevision, Newcastle-Upon-Tyne, UK). Only AvBD1 and 10 were investigated for SNPs in the genomic DNA. The primer sequences are shown for all three genes in Chapter 5. Table 2.4 shows the primer sequences for both the forward and reverse primers, and the predicted size of the PCR product.

Gene	Forward primer (5’ to 3’):	Reverse primer (5’ to 3’):	Size of product (bp)
AvBD1	GGATCGTGTACCTGCTCCTC	GACATCAGGGAATGGGTTCTGTG	960
AvBD4	CTGCAACGTTTTGGCAGCAATAC	AGAGGAGGCTCTGGGTTGGAG	248
AvBD10	GACCCACTTTTCCCTGACACCG	GCAATGGCCCAGTGAAAGC	1,880

Table 2.4: Primers used for RT-PCR of genomic DNA to investigate the presence of SNPs.

2.2.12: Quantitative real-time PCR (qRT-PCR)

2.2.12.1: Overview of technique

To compare gene expression between different lines, ages and groups of chicken, qRT-PCR was performed. Fluorescent reporter molecules were utilised to quantify the amount of amplified PCR product and a Roche Lightcycler 480 (Roche, Basel, Switzerland) was used throughout this study.

Gene expression levels for AvBD1, 4, 10 and 18S were determined using the Sybr green system. The primers used for the AvBD genes were the same as those used for RT-PCR

(Table 2.2), but were HPLC purified as recommended by Roche. A different set of 18S primers were designed as those purchased from Ambion amplified too large PCR product for the analyses.

2.2.12.2: House-keeping genes (HKG)

An appropriate HKG was used to correct for variability in the amount of genetic material between samples, principally caused by changes in the efficiency of mRNA isolation and reverse transcription. 18S was used as the HKG for this study and a recombinant plasmid was made (pBlue-TOPO®, Invitrogen, Paisley, UK) following amplification of tissue cDNA with the Universal 18S primers (Ambion). Following automated sequencing of this plasmid using T7 primers, a set of 18S primers were designed which resulted in a smaller 18S cDNA product suitable for qRT-PCR.

The 18S primers used for qRT-PCR are given in Table 2.5 and the optimal annealing temperature was 57°C.

Gene	**Forward primer (5' to 3'):**	**Reverse primer (5' to 3'):**	**Size of product (bp)**
18S	GTGGTGCATGGCCGTTCTTAGTT	GTCCCTCTAAGAAGTTGGACGCC	126

Table 2.5: 18S Primers used for q RT-PCR.

2.2.12.3: qRT-PCR reactions

PCR reactions were set up as shown in Table 2.6 in a total reaction volume of 10µl.

Reagent	**Volume (µl)**
Sybr green master mix (Roche)	5
Molecular grade water	1.5
Diluted clone/RT product	2.5
Forward primer [10µM]	0.5
Reverse primer [10µM]	0.5

Table 2.6: qRT-PCR reagents and volumes.

2.2.12.4: PCR amplification programme

The following programme was used for PCR amplification on the Roche 480 Lightcycler (Table 2.7). The same programme was used throughout the analyses but the annealing temperatures were changed according to the primers being used.

Program	Temperature/°C	Time (min:sec)	Number of cycles
Preincubation	95	10.00	1
Amplification	95	00:10	30
	57/60 (annealing)	00:30	
	72	00:01	
Melting curve	95	00:05	
	70	00:01	
	97		
Cooling	40	00:10	1

Table 2.7: qRT-PCR programme used for qRT-PCR. Two different annealing temperatures were used dependent on the gene being analysed (57 or 60°C).

Standard curves form an integral part of qRT-PCR, allowing comparisons of PCR efficiency and determination of the relative concentrations of cDNA in the products. Individual standard curves were performed for each gene by carrying out serial dilutions of each cloned PCR product. Further details are provided in Chapter 5.

All samples were loaded onto white real-time 96 well plates (Roche) for PCR amplification.

2.3: Bacterial strains

A number of different bacterial strains were used throughout this study.

E.coli strains were used within this study for (i) synthesis of plasmid stocks;

- DH5α

And (ii) hyperexpression of recombinant peptides;

- Origami B (DE3):: plySs (Novagen, Darmstaft, Germany) (Studier, Rosenberg et al. 1990)

The *Salmonella* strains used for the time-kill assays were;

- *Salmonella enterica* serovar Typhimurium PhoP (a kind gift from Dr S. Miller, Boston, USA (Townes, Michailidis et al. 2004)
- *Salmonella enterica* serovar Typhimurium 1344 (a kind gift from Dr A. Khan, Newcastle University, UK)
- *Salmonella enteriditis* (a kind gift from Dr A. Khan, Newcastle University, UK).

Three clinical isolates;

- *Staphylococcus aureus* (from infected joint of broiler post mortem)
- *Enterococcus faecalis* - isolate 1 (from guts of post mortem of bird A)

- *Enterococcus faecalis* - isolate 2 (from guts of post mortem of bird B)

were obtained from the Aviagen veterinary laboratory and taken from birds at post-mortem, unfortunately no gram negative bacteria were supplied.

2.3.1: Growth media and agar

2.3.1.1: Luria-Bertani (LB) broth

This contained Bacto-tryptone (10g), sodium chloride (10g), Bacto-yeast (5g) in 1L of de-ionised water. The pH was adjusted to 7.4 using 1M sodium hydroxide. The broth was sterilized at 121°C for 20min at 15PSI pressure.

2.3.1.2: LB agar plates and slopes

Two percent (w/v) agar plates were prepared using LB broth and agar (Sigma), and sterilisation was performed as above. Approximately 20ml of autoclaved agar was poured into each petri dish, allowed to set and dried in an oven at 37°C. Where required, antibiotics were added to the cooling agar, concentration given in Table 2.8.

Antibiotic	Working concentration (mg/ml)
Ampicillin	0.05
Chloramphenicol	0.03
Tetracycline	0.01
Kanamycin	0.03

Table 2.8: Working concentrations of antibiotics in LB agar and LB broth.

For the agar slopes, sterilised agar was prepared as for the petri dishes, cooled before being aliquoted into 25ml universals and allowed to set at an angle of 45° at room temperature. Slopes were inoculated as for petri dishes.

2.3.1.3: Blood agar plates

Modified blood agar plates were prepared by dissolving 40g of blood agar powder (Sigma) in1L of de-ionised water and sterilizing as for LB agar. Agar plates were prepared poured as previously, allowed to set and used in the time-kill assays for all bacterial strains. All plates were stored at 4°C prior to use and dried on the day of use.

2.3.1.4: *Salmonella* and clinical isolate agar plates

Prior to each time-kill assay a fresh inoculum of bacteria was taken from each of the frozen glycerol stocks, streaked onto a blood agar plate and incubated at 37°C for 18 hours.

2.3.1.5: *Escherichia coli* plates

A loopful of Origami B (DE3):: PLysS and DH5α glycerol stock was streaked and cultured on LB agar plates containing chloramphenicol, tetracycline and kanamycin (Table 2.8). DH5α was cultured on agar only. Plates were incubated at 37°C for 18 hours and subsequently stored at 4°C.

2.3.1.6: Storage of bacterial strains

Glycerol stocks of all bacterial strains were prepared by inoculating 10ml of LB broth containing the appropriate antibiotics, with a loopful of bacteria and incubating aerobically at 37°C for approximately 16 h. An aliquot of this culture (0.5ml) was placed into a 1.5ml cryovial containing 0.5ml 50% (v/v) glycerol and stored at -80°C.

2.3.2: Competent cells

A loopful of Origami B (DE3):: plySs was used to inoculate 10ml of LB broth containing chloramphenicol 0.03mg/ml, tetracycline 0.01mg/ml and kanamycin 0.03mg/ml and grown overnight at 37°C in an orbital shaker. To prepare competent cells 1ml of this

culture was added into 100ml of LB broth without antibiotics and the culture grown to OD_{600nm} of 0.3. Centrifugation of the culture was performed at 4°C for 10 min at 3000g in a Mistral 3000i, MSE centrifuge. The bacteria pellet was suspended in 4ml chilled 0.1M $MgCl_2$. Centrifugation was repeated as for the previous step, the supernatant discarded and the pellet re-suspended in 4ml of 0.1M $CaCl_2$. The competent cells were incubated on ice for 2h prior to use.

Competent DH5α cells were prepared as described for Origami B (DE3):: plySs except no antibiotics were added to the overnight culture.

2.3.3: Bacterial growth curves

Growth curves were performed for all bacterial strains prior to the time-kill assays. A loopful of bacteria was taken from a sub-master blood agar plate and added to 0.5ml 0.1M PBS, pH7.4, thoroughly mixed and added to 20ml LB broth, containing 25mM glucose in a 500ml duran bottle. The bacteria were grown in an orbital shaker set at 200rpm and 37°C for 3 h. A second sterile duran bottle containing 20ml LB and 25mM glucose was inoculated with 200µl of the first culture and the bacteria cultured under the same conditions. In this case OD_{600nm} of the sample was measured every 30 min using a spectrophotometer (Amersham Biosciences, Ultraspec 43 000 pro, High Wycombe, UK). This process was repeated to 8h of growth.

2.4: Cloning of cDNAs

2.4.1: 18S for qRT-PCR

2.4.1.1: Plasmid vector

The pBlue TOPO vector from Invitrogen (Paisely, UK) was used for cloning (plasmid map in Appendix I).

2.4.1.2: Gene insert

The cDNA template was generated through PCR using nucleic acid prepared from a line Y bird liver and the Universal 18S primers (total volume of 120µl used). This sample was electrophoresed on a 1% TBE-agarose gel (large comb used, 60µl loaded into each well). Gel extraction and purification were performed (2.2.9.1) prior to ligation of the cDNA into the pBLUE TOPO vector.

2.4.1.3: Ligation of insert into vector

One microliter of pBlue TOPO vector, 1µl salt solution (provided in pBlue TOPO cloning kit, Invitrogen, Paisley, UK) and 4µl purified PCR product were combined in a 2ml microfuge tube and incubated at room temperature for 5 min before being placed on ice. Transformation into competent DHα cells was performed (2.4.6) so that large

quantities of plasmid could be made and then isolated from the culture for future experiments.

2.4.2: AvBD cDNA clones for hyperexpression

2.4.2.1: Plasmid vector

The cDNAs for AvBD1 and AvBD10 were each cloned into the plasmid vector pRSETA (Invitrogen, Paisley, UK). The vector plasmid map is shown in Appendix I. This vector contains the bacteriophage T7 promoter which is activated by a small amount of T7 RNA polymerase. The latter is coded for by T7 gene 1, which is within the bacterial strain used (Origami B (DE3):: plySs) for hyperexpression. T7 gene 1 activation is controlled by lacUV5 promoter which is switched on by the addition of isopropyl-bet-D-thyogalactopyronoside (IPTG). By using this form of chemical induction with IPTG, the AvBD gene can be activated and peptide produced.

2.4.2.2: Expressed Sequence Tagged (EST) clones

EST clones (Table 2.6) were purchased from Ark-Genomics, Roslin Institute, UK.

Gene	EST clone
AvBD1	ChEST679b6
AvBD4	ChEST203022
AvBD10	ChEST1015e22

Table 2.9: Chicken EST clones purchased from Ark-Genomics (Roslin Institute, UK). The vector used was pBluescriptII KS+.

2.4.3: Polymerase chain reaction

AvBD cDNAs were amplified from EST clones (diluted 1:100 in de-ionised water) or from actual bird samples (neat DNA) encoding known combinations of the SNPs.

Primers were designed to include the restriction enzyme recognition sites BamHI (GGATCC) on the forward primer and EcoR1 (GAATTC) on the reverse primer (Table 2.10). The region in italics marks the restriction enzyme-specific site. The blue region marks a short sequence added to aid stabilisation of the primer at the restriction enzyme site.

Gene	Forward primer (5' to 3') BamH1	Reverse primer (5' to 3') EcoR1	Product size (bp)
AvBD1	CGC***GGATCC***CGGAAGGAAGTCAGATTGT	CGA***GAATTC***TCAGCCCCATATTCTTTT	123
AvBD10	CGC***GGATCC***CGACCCACTTTTCCCTG	CGA***GAATTC***TTACTGCGCCGGAATCTTGGC	141

Table 2.10: Primers with restriction sites used for cloning into pRSETA. Blue region is to stabilize the primer at the restriction enzyme site.

Optimal annealing temperatures were determined as for other primers, (60°C and 57°C respectively for AvBD1 and 10). PCR conditions are given in 2.2.7.

2.4.4: DNA restriction

The PCR products and the pRSETA vector were restricted with BamHI and EcoRI enzymes (Fermentas, UK). The reactions were scaled as appropriate using the ratio 1:1:2:16 of DNA (1µg/µl): restriction enzyme: buffer (10x): sterile water. Following a 3h incubation and purification (QIAquick PCR purification kit, Qiagen, Crawley, the samples were electrophoresed on 1% agarose-TBE gels, excised from the gels and the

DNA purified (QIAquick gel extraction kit, Qiagen, Crawley, UK). The restricted PCR product and plasmid were eluted into 30 µl Buffer EB.

2.4.5: DNA ligation

The DNA was ligated into the vector using a rapid ligation kit (Fermentas, UK). The samples were combined as in Table 2.11 (including a control with no gene insert) and kept at room temperature for 2 h prior to transformation into competent cells (Origami B (DE3) :: pLysS). The buffer (5X rapid ligation buffer) and enzyme (T4 DNA ligase) were supplied in the kit.

		Volume (µl)		
PCR product	**Vector**	**Buffer**	**Enzyme**	**Water**
10	2	4	1	3
5	1	4	1	9
0	1	4	1	14

Table 2.11: Components of the plasmid ligation.

2.4.6: Transformation of bacteria

A volume of 200µl of freshly prepared competent cells was used for each transformation and to this 5µl of the ligated mix or plasmid DNA was added. Gentle mixing was

performed and the sample placed on ice for 10 min. The sample was heat-shocked at 42°C for 2 min, placed back onto ice for a further 10 min and 100µl of LB broth added. The tube and contents were incubated at 37°C for a further 1 h with gentle shaking in a Gallenkamp orbital shaker (Sanyo Gallenkamp Plc, Leicester, UK). Volumes of 50-100µl were plated onto LB agar containing the appropriate antibiotic and incubated overnight at 37°C. For the hyperexpression experiments where Origami B (DE3):: plySs and pRSETA were used, the plates contained chloramphenicol and ampicillin diluted to 0.03mg/ml and 0.05mg/ml working concentrations respectively, and for those where DH5α bacteria were used, ampicillin alone was added.

2.4.7: Extraction of plasmid DNA from bacterial cells

The bacterial colony (DH5α) containing the required plasmid (pRSETA or PBlue TOPO) was cultured overnight in 10ml LB broth containing ampicillin (0.05mg/ml) at 37°C in an orbital shaker. Approximately 2ml of culture was pelleted by centrifugation at 13000g for one min (Biofuge 13 centrifuge, Heraeus, Seratech). The supernatant was discarded and the plasmid DNA extracted using a plasmid purification kit (Fast plasmid mini-prep, Eppendorf, Cambridge, UK) according to manufacturers instructions. The DNA was eluted in 50µl of molecular grade H_20.

2.5: *In vitro* synthesis of recombinant AvBD peptides

2.5.1: Induction of hyperexpression

Recombinant colonies from the agar plates were used to inoculate 10ml of LB broth containing chloramphenicol (0.03mg/ml) and ampicillin (0.05mg/ml) and incubated at 37°C with gentle shaking for approximately 1.5 h (slight turbidity). A total of 100 universal tubes each containing 10ml LB broth were used (total volume 1L). Expression was induced by the addition of 10µl of 1M isopropyl-beta-D-thiogalactopyranoside (IPTG) to each tube and followed by a further 3h incubation. This method was modified so that a 1L volume of LB containing appropriate antibiotics was inoculated with an overnight culture established from a single transformed colony. This was shaken for 1.5h and induced with 1ml of 1M IPTG and cultured for a further 3h prior to harvesting the bacteria. This latter method used fewer universal tubes and proved more time efficient with similar end results i.e. concentrations of recombinant peptide.

Cells were harvested by centrifugation for 15 min at 4°C and 15 000g in a Biofuge 13 (Heraeus, Seratech). The pelleted cells were stored at -20°C overnight before being re-suspended in 10ml 0.1M PBS, pH7.4 and lysed by sonication for 1 min. After centrifugation the supernatant was removed and retained. Both the pellet and the supernatant were analysed via SDS-PAGE. To the pellet 40µl of SDS-PAGE buffer (5ml of 10%(w/v) Sodium Dodecyl Sulphate, 50% (v/v) 0.25M Tris pH 6.8, 25% (v/v)

50% Glycerol solution and 25% 2-β Mercaptoethanol + 20ml 0.1% Bromophenol Blue dye) was added and 30µl of the soluble fraction was mixed with 15µl of the same buffer. Both samples were boiled for 10 min before being subjected to SDS-PAGE analysis as described in 2.6.1.

2.5.2: Purification of recombinant peptides

Talon resin (2ml, Clontech, Hampshire, UK) was suspended in a column (15ml volume), 2.5ml of the 'soluble extract' was added, left to drain through the resin and the flow-through retained. The resin was washed twice with 1x 0.1M PBS pH7.4 and the flow-through liquid retained. Elution of the protein was performed by the addition of 2ml of 10mM imidazole (X2). This elution was repeated using increasing concentrations of imidazole, (100mM, 250mM and 500mM, 2ml X2). All of the flow-throughs were retained and analysed by Western analysis. A PD-10 de-salting column (G.E. Healthcare, UK) was used to remove any impurities.

2.6: Identification of recombinant peptide

2.6.1: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE)

Protein separation was achieved by the use of vertical 12.5-15% (w/v) bis-acrylamide gels. A low molecular weight pre-stained protein ladder (20µl, range 14-66kbases) was used to confirm protein size. Gels were prepared using the reagents shown in Table 2.12.

A) 15% gel

Resolving Gel			**Stacking Gel**		
Reagent	**Volume**	**Source**	**Reagent**	**Volume**	**Source**
De-ionised water	1.1ml		De-ionised water	1.5ml	
40% bis-acrylamide	3.6ml	Invitrogen	40% bis-acrylamide	0.4ml	Invitrogen
Resolving buffer(0.75M Tris/SDS (pH8.8))	4.7ml		Stacking buffer (0.25M Tris/SDS (pH 6.8)	1.9ml	Sigma
10% (w/v) NH_4 persulphate	90µl		10% (w/v) NH_4 persulphate	60µl	Sigma
TEMED	30µl		TEMED	20µl	Sigma

Table 2.12: Components of 15% SDS-PAGE gel.

B) 12.5% gel

The components for the gel were as shown in Table 2.12 except that the resolving gel contained 3ml of 40% acrylamide and 0.9 ml de-ionised water.

Gels were submerged in a vertical gel tank containing 1x SDS PAGE electrophoresis buffer (3% (w/v) Tris, 14% w/v Glycine and 1% Sodium Dodecyl Sulphate, pH8.3). Samples were prepared by the addition of SDS PAGE loading buffer (5ml of 10%(w/v) Sodium Dodecyl Sulphate, 50% (v/v) 0.25M Tris pH 6.8, 25% (v/v) 50% Glycerol solution and 25% 2-β Mercaptoethanol + 20ml 0.1% Bromophenol Blue dye) in a 2:1 ratio (sample: running buffer). The samples were boiled for 10 min before a sample volume of 20µl was loaded into each well of the stacking gel. An electric current of 30mA was applied for approximately 40 min and then the gel removed and stained with either Coomassie stain, colloidal blue or silver stain. Bands were visualised by exposure to a light source (Bio-Rad, Hemel Hempstead, UK) and photographed (Kodak EasyShare digital camera).

2.6.1.1: Coomassie stain

Electrophoresed gels were placed in a holding vessel and covered with Coomassie stain (4g Coomassie G-250 in 40% (v/v) methanol and 10% (v/v) acetic acid) for 7 h. De-staining (40% (v/v) methanol and 10% (v/v) acetic acid) was performed for at least 1 h or until contrast was optimal.

2.6.1.2: Colloidal blue stain

A colloidal blue staining kit was obtained from Invitrogen (Novex colloidal blue staining kit) and used as per manufacturers instructions. Initially 15% SDS-page gels were used but it was determined that better protein staining was achieved with 12.5% gels.

2.6.1.3: Silver stain

A kit called SilverSNAP® stain kit II was obtained (Thermo Scientific, USA) and staining performed as per manufacturers instructions.

2.6.2: Western blot analysis

An anti-His tag antibody was used for western blot analysis of the His-tag recombinant AvBD fusion protein. Initially samples were subjected to SDS-PAGE (2.6.1), and following electrophoresis the gel was equilibrated for 10 min in transfer buffer (2.5mM Tris base, 150mM glycine in 10% methanol). Three sheets of 3mm Whatmann paper were soaked in transfer buffer and loaded onto the bottom electrode of the semi-dry protein transfer blotter (Biorad, Hemel Hempstead, UK). A section of Hybond PVDF membrane (Thermo Scientific, USA) was activated in 10% methanol for 2 min, washed in de-ionised water and soaked in transfer buffer prior to use. The membrane was laid on top of the moist filter paper. The SDS-PAGE gel was placed on top of the activated membrane and a further three layers of soaked Whatmann filter paper placed on top. All air bubbles were removed and electroblotting conducted at 10V for 30 min.

The membrane was blocked in 5% (w/v) Marvel, 0.1% Tween in 0.1M PBS, pH7.4 overnight at room temperature. Two brief washes of the membrane were performed using 0.1% Tween in 0.1M PBS, pH7.4. A solution of 0.5% milk protein 0.1M PBS, pH7.4, 0.1% Tween was prepared (40ml) and 0.375µl of Mouse anti-His tag horseradish peroxidase conjugated antibody added (working dilution of antibody was 1:12,000). The membrane and antibody solution were incubated at 37°C for 1h.

The membrane was washed for 10 min (X3) in 0.1% Tween in 0.1M PBS, pH7.4. The membrane was treated with 5ml of enzyme chemiluminescent reagent (ECL Western blotting detection reagents, GE Healthcare Life Sciences, UK) for 5 min in the dark. This stain consisted of a 1:40 dilution of Buffer B (Stock acradin solution in dioxin and ethanol) in Buffer A (ECL plus substrate containing Tris buffer). The membrane was dried, placed in an x-ray film cassette with x-ray film (Kodak, Hemel Hempstead, UK), left for between 10 and 60 sec and developed using an automated developer (Konica, SRX-101A).

2.6.3: Enzyme-linked immunoabsorbant assay (ELISA) to determine concentration of recombinant peptide

An ELISA was established to quantify the levels of hyperexpressed peptide, exploiting the six polyhistidine tag. A His-tag labeled protein of known size (53kDa) and a starting concentration of 60µg/ml was used to establish a standard curve on a Maxisorb® (Nunc,

Denmark) 96 well plate. A total volume of 100µl of sample was loaded into each well. Hyperexpressed peptide and control empty samples were loaded onto the plate at neat, 1:2 and 1:4 dilutions using 0.1M PBS, pH7.4 as the diluent. The plate was covered and stored at 4°C overnight.

The wells were washed five times using 200µl 0.1M PBS, pH7.4 and 0.1% Tween, with the plates shaken to remove the liquid in between washes. All wells were blocked for 2h using 100µl of 1% (w/v) bovine serum albumin (BSA) in 0.1M PBS, pH7.4, 0.1% Tween at room temperature. A further five washes were repeated as previous. Mouse anti-His tag horseradish peroxidase conjugated antibody (as used for Western blot analysis) was diluted to a concentration of 1 in 12,000 in 0.1% BSA 0.1M PBS, pH7.4 0.1% Tween and 100µl of this solution added to each well. The plate was incubated at 37°C for 1h. A further five washes were performed. Aliquots of 100µl of 2,2,'-azino-bis(3-ethylbenzthiazoline) (horseradish peroxidase substrate, which had been allowed to reach room temperature) were added to each well and the plate left to develop at room temperature for approximately 20 min. A stop solution of 1% SDS was added to all wells prior to the plate being read at 405nm in a plate reader (Bio-Rad, Hemel Hempstead, UK). A standard curve was plotted and from this, the concentration of the hyperexpressed peptides determined.

2.7: Antimicrobial activity of recombinant peptides

2.7.1: Bradford assay

To determine the protein concentrations of the hyperexpressed peptides and those samples extracted from the gut mucosal scrapes, the Bradford assay was employed (Bradford 1976). Initially a protein standard curve was constructed using bovine serum albumin (BSA at a starting concentration of 0.1mg/ml). The range of protein concentrations was 0-100µg/ml. Test samples were diluted 1:2, 1:5, 1:10, 1:50 and 1:100 (sample:H_2O) to a total volume of 50µl. Bradford reagent (BioRad, Hemel Hempstead, UK) was added to each well (1:5 dilution in H_2O water, volume of 150µl) and the 96 well plate analysed 5min later at an absorbance of 595nm in a plate reader (Bio-Rad, Hemel Hempstead, UK). The amount of protein in the test samples was calculated from the standard curve.

2.7.2: Time-kill assays

A loopful of bacteria (*Salmonella* strain or clinical isolate) was taken from a fresh sub-master plate, used to inoculate 0.5ml 0.1M PBS, pH 7.4 and the suspension vortexed to avoid flocculation. This mixture was added to a duran bottle containing 20ml LB and 25mM glucose and cultured at 37°C and 200rpm for 3 h, 200µl of culture was removed and added to a new sterile duran bottle containing 20ml LB and 25mM glucose. The

bacteria were cultured as previous. A working culture of 10^6 CFU/ml was prepared by diluting the bacteria 1:100 in 0.1M PBS, pH7.4.

Ninety microlitres of the diluted bacterial suspension was added to 10µl of control or test substance (recombinant peptide or gut scrape). Following mixing, 10µl was removed and serially diluted in 0.1M PBS, pH7.4 (10^{-1} to 10^{-4}). A 10µl sample from each of these four dilutions was spread on a blood agar plate, which had been divided into quarters (the quadrants were labeled with sample identity and dilution). The remaining assay mixture (bacteria and test samples) was incubated at 37°C for a further 2 h before the serial dilutions and plating was repeated. The plates were incubated at 37°C for 18 h and the colonies counted. All test and control samples were run in triplicate. Percentage survival of the bacteria was determined as described by Townes, Michailidis et al. (2004).

2.8: Gut mucosal scrapes

2.8.1: Sampling of gut mucosa

The duodenal loop from every bird included in this study was excised, cut longitudinally to expose the mucosal surface the gut contents removed by washing in 0.1M PBS, pH7.4, the mucosal layer of the duodenum collected by scraping with a microscope slide, placed in aluminium foil and labeled. The samples were snap-frozen in liquid nitrogen and stored at -80°C.

2.8.2: Protein extraction of gut mucosal scrapes

Each mucosal scrape was weighed prior to protein extraction. The sample was placed in a 15ml Falcon tube, 1ml of 10% acetic acid added and the sample homogenised using an electric homogeniser (Qiagen Tissue Ruptor as used for tissue, 2.2.1). The probe was cleaned between samples using alcohol and 0.1M PBS, pH7.4. The samples were transferred to microfuge tubes and centrifuged at room temperature, 13000g for 5 min. For each sample the supernatant was removed and placed in a clean microfuge tube. The lid of the tube was removed and the sample lyophilised in a heat block set at 56°C for 36 h or until all liquid had been removed. The residue was reconstituted with 1ml of 0.1M PBS, pH7.4 and vortexed. The day 0 bird gut samples were re-constituted in a smaller volume (150µl) due to the low starting weights of these samples. Total protein concentration was measured using either the Bradford assay or NanoDrop.

2.8.3: Liquid chromatography mass spectrometry (LC/MS)

Acetone precipitation of protein from the gut scrapes was performed before LC/MS analysis. Briefly, to a microfuge tube containing one volume of reconstituted gut scrape in 0.1M PBS, pH7.4, four volumes of ice cold acetone were added, the contents mixed well and stored at -20°C overnight. The sample was centrifuged at 15000g, 4°C for 15 min, the supernatant removed and the tube inverted onto tissue paper to remove any liquid. The sample was lyophilised in a fume-cupboard for 4h.

Samples were stored on ice and delivered to NEPAF Proteome Analysis facility (Cels Business Services Ltd, Newcastle-Upon-Tyne, UK) for LC/MS analysis.

2.8.4: Thin layer chromatography

A pencil line approximately 1cm from the perimeter of a piece of siliconised aluminium plate (Merck, Germany). On the left side of the line 0.5µl of glucose solution (0.1M) was spotted and the plate dried with a hairdryer; 0.5µl of maltose solution (0.1M) was added to the same spot and dried; finally 0.5µl of a tri-saccharide, maltotriose (0.1M) was added and dried. A series of marks were placed at 1cm intervals across the width of the plate. On each mark a 2µl sample of gut extract was spotted, dried and a further two 2µl of the same sample applied in the same place. The plate was placed in hexane solvent for 3 h in a fume-cupboard while the samples migrated proximally. The plate was removed from the solvent and briefly covered in developer (3:1:1, propanol:ethyl:water), wrapped in aluminium foil, incubated at 37°C for approximately 10 min and photographed (Kodak, Easy Share digital camera, UK).

Chapter 3: Farm field trial and analysis of environmental parameters

3.1: Aviagen Ltd

This study was performed in collaboration with Aviagen Ltd (www.aviagen.com). The Aviagen corporation has several commercial interests of which, the chicken broiler industry forms a large component. To maintain competiveness, approximately 10% of turnover per annum is invested into broiler breeding with genetic improvement of commercial lines, and subsequent ability to offer producers appropriate broiler chicks, forming the focus of the research aims for Aviagen Ltd.

The company views the broiler industry as a pyramid, shown in Figure 3.1. Breeding patterns are tightly regulated so that specific qualities are selected from birds at the top of the pyramid and subsequently transferred to the commercial broilers. This then results in the ultimate production of large quantities of chickens which have the previously selected beneficial traits. The diagram also highlights the fact that there is a four year lag in the selection of genetic traits in great-great grandparent stock being transferred to the commercial broilers. This time-delay for trait transfer emphasises the commitment both

financially and with time that is needed for development of the broiler birds.

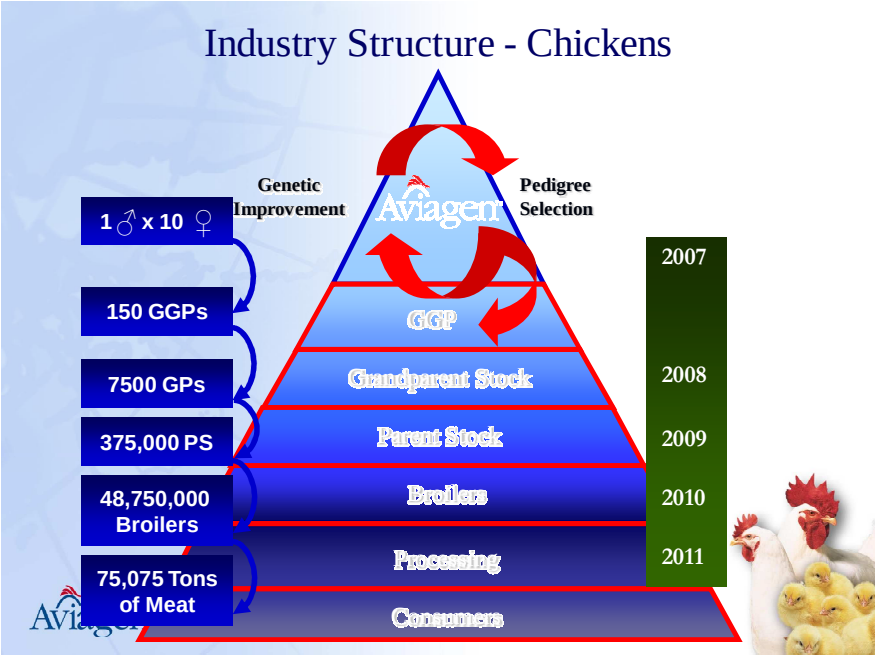


Figure 3.1: The pyramidal structure of the poultry industry (Courtesy of K Laughlin, Aviagen).

(GGP=great-grandparent

GP=grandparent

PS=parent stock)

Within the pedigree selection at the top of the pyramid there are both commercial and developmental lines (the chickens used in this study were commercial lines). Within the selective breeding programme birds are selected using multiple trait measurements to assess their welfare and survival, reproductive, broiler and processing characteristics. As part of this, birds are assessed in different environments to ensure that selected birds have the genetic capability to perform well across a spectrum of commercial farming conditions.

As the pedigree birds need to be kept in high-hygiene facilities, information from how their siblings perform (sib-testing) in the low-hygiene commercial environments is used to aid the selection process.

The weighting of traits is different for each breeding line depending on the intended use of the end broiler cross. For example, more effort is placed on meat yield traits in lines used to make crosses targeted at producers that focus on portioning their broilers.

This investigation encompasses several facets of the research goals for Aviagen Ltd, namely determining genetic potential of different lines via the investigation of different genetic markers.

3.2: Chicken lines

Three pedigree lines of chicken were used throughout this study coded as lines X, Y and Z. These pure lines have their origins in the Plymouth Rock and Cornish lines. They are closed populations that have undergone multiple generations of selection using genetic evaluations based on multiple-trait best linear unbiased prediction analysis. A significant proportion of the worlds' broilers are a hybrid of these three lines (Andreescu, Avendano et al. 2007).

The three lines of birds used within this study were chosen for several reasons, the main one being that they have been shown to have very different gut healths: line X having poor gut health, Y having an intermediate level of gut health and Z having the best gut health. Such problems associated with line X birds include the production of wet litter, which can lead to poor feed conversion ratios and therefore suboptimal bird growth rates as well as foot pad lesions (Meluzzi, Fabbri et al. 2008; personal communication with Dr Barry Thorp, former Director of Veterinary Services, Aviagen Ltd). In addition to the differences in gut health, the three lines are also used for broiler breeding programs

within the pedigree farms and differ in characteristics such as growth potential, yield and reproductive performance.

The initial plan of the study was to rear all three lines of birds within the two distinctive environments (low hygiene LH and high hygiene HH) but unfortunately this proved impossible. Due to the high bio-security controls placed on the high hygiene farm and the outbreak of Avian influenza during the time of the trial in the UK, it was advised to minimise the number of visits made to this farm, and unlike lines X and Y which were reared in tandem using the same barns, line Z bird rearing dates did not fit in with the dates of sampling. It was for this reason that, disappointingly, only lines X and Y were sampled in the high hygiene farm compared to all three lines in the low hygiene farm.

3.2.1: Outline of farm trial

Details of the trial which was performed during Autumn 2007 are shown in the flow-chart (Figure 3.2). A total of 180 birds, all male, were included and the study was performed with the following aims; (the aim which is highlighted in bold is included within this chapter). The other aims are covered in the stated chapter numbers.

- **To define differences in the rearing conditions on the two farms at time points throughout the performance of the trial.**
- To identify potential single nucleotide polymorphisms (SNPs) within the AvBD locus, focusing on AvBD1, 4 and 10 and to determine whether there are any line differences with regard to SNP occurrence (Chapter 4),

- To determine AvBD1, 4 and 10 gene expression data across a panel of 10 tissues for three lines of chickens (rationale for gene selection given in Chapter 5) and to:
  1) explore whether gene expression differs across the different lines of birds,
  2) investigate if there are any age-related patterns in gene expression,
  3) ascertain whether the environmental conditions that the birds are reared in affect gene expression in any of the tissues.
- To determine whether age, line and environmental rearing conditions influenced the antimicrobial effects of duodenal gut scrapes in bacterial time-kill assays and to investigate the contents of duodenal gut scrapes of the different bird lines (Chapter 7).
- Chapter 6 was focused on producing and proving antimicrobial activity of two of the AvBDs and not directly relevant to the farm trial per se.

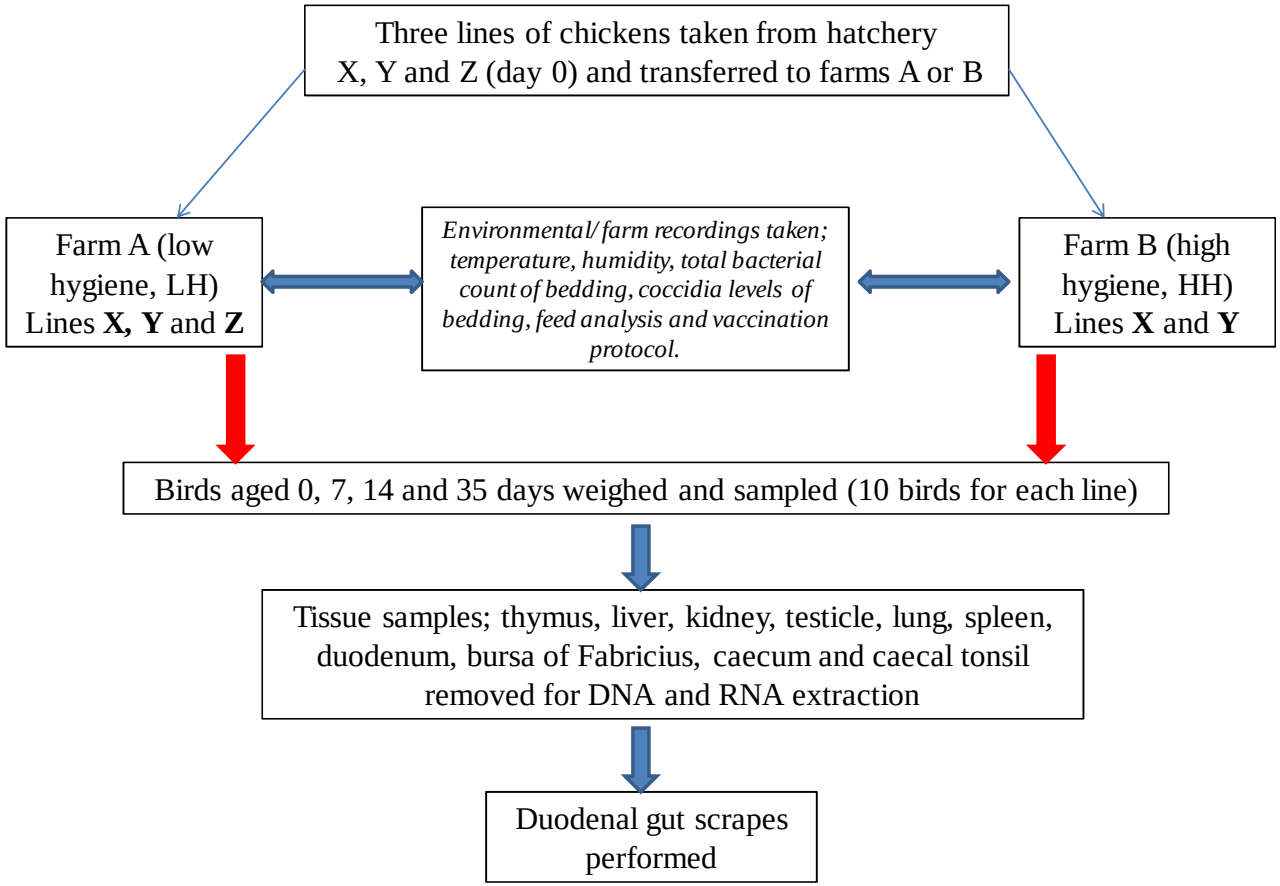


Figure 3.2: Flow-chart of trial performed at two Aviagen farms (low hygiene, LH and high hygiene, HH), Autumn 2007.

3.2.2: Ages of birds

The ages of the birds investigated within this trial were 0 (taken directly from hatchery to the laboratory, all birds were reared at the same hatchery in the same manner and were siblings or half siblings), 7, 14 and 35 days, respectively. The focus for this study was on early life because a previous study (Bar-Shira and Friedman 2006), had shown that the innate immune system is vital to the chick during the first week of life, particularly in the gastro-intestinal system where rapid development and bacterial colonisation occurs at this time. Sampling at day 0 also provided base measurements and allowed any bird line differences to be noted prior to the birds being reared in either of the two distinct environments. By sampling birds at day 0 and 7, any changes associated with both gene expression and gut mucosal scrape activity occurring within the first week of life i.e. before the adaptive immune system is fully functioning, could be identified. The day 14 bird samples not only allowed the generation of data mid-trial, but by day 14 the adaptive immune system is active, as suggested by the presence of lymphocyte colonisation in bird guts (Bar-Shira, Sklan et al. 2003), allowing a good data comparison point. By day 35 the chickens adaptive immune system including secondary lymphoid tissues (with the exception of the bursa of Fabricius) is fully developed (Rezaian and Hamedi 2007) but as most broilers are slaughtered at approximately day 42, this time point provided information on the mature bird.

3.3: Environmental conditions

3.3.1: Farms

The trial was carried out at two Aviagen Ltd farms in Scotland; farm A (low hygiene, LH) is classed as a sib testing farm and is where conditions are similar to those of a commercial broiler unit in the UK. Farm B (high hygiene, HH) has tight bio-security controls and is where breeding of those birds at the top of the poultry pyramid occurs (Figure 3.1). The main differences between the two farms were;

- **Hygiene**: When cleaning barns between new batches of chicks farm A allowed mixing of some of the older bedding with fresh bedding for each new batch of birds (ratio not provided, white wood shavings were used on both farms) while farm B adopted a complete disinfection process between batches of birds.
- **Temperature and humidity**: Environmental controls were less tightly controlled in farm A compared to farm B.
- **Diets**: Different diets were given to the birds on the two farms. Farm A birds were fed a maize-based diet while farm B birds were fed a wheat-based diet.
- **Vaccination protocols**: Different vaccination regimes were used on the two farms and these are listed in Appendix II.
- **Bio-security controls**: Farm A had less tight controls while Farm B had tight controls for example at Farm B a longer interval since contact with chickens was required for visitors and also greater security measures against wildlife were implemented e.g. Farm B was more geographically isolated.

- **Estimated mortality**: Farm A had an estimated mortality rate of 3-5% over a 12 month monitoring period compared to Farm B which had estimated levels of 2-3% (personal communication with Dr Kellie Watson, Aviagen Ltd- no age group breakdown data was available).
- **Stocking density**: Birds were housed in groups of 15 of which, 10 were taken at random for sampling and inclusion in the trial. The stocking densities on the two farms were the same for the period that the trial was performed and this was 34kg/m^2 .

3.3.2: Bedding analysis

On the day of bird sampling (7, 14 and 35 days respectively), a representative bedding sample was taken from each pen that the birds had been reared in (taken from a total of five sites within the pen). Approximately 20 gram bedding samples (shavings combined with faecal matter), were analysed by Poultry Health Service Ltd, Thirsk, UK for the measurement of a) colony forming (cfu) bacteria per gram of sample and also b) coccidia oocyst count per gram (modified McMaster technique was used). No sample was obtained for the day 0 birds as they had arrived directly from the hatchery.

3.3.2.1: Bacterial counts

The total bacterial counts (cfu) for the single bedding samples taken throughout the trial are shown in Figure 3.3. A single sample was submitted for analysis but this involved

sampling from five locations within the pen. No 0 day samples were available as birds had been taken straight from the hatchery to the laboratory.

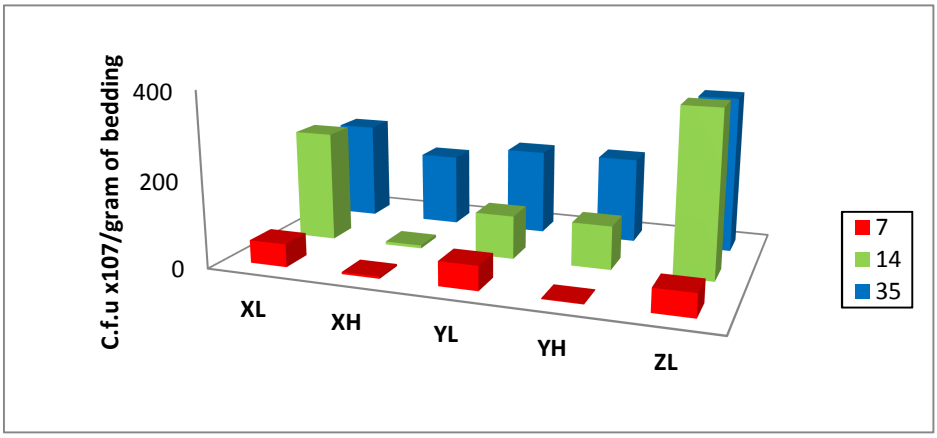


Figure 3.3: Total bacterial counts per gram of bedding (cfu) for a single bedding sample taken at days 7, 14 and 35, respectively for the three lines of birds and two different environments. Lines X, Y and Z and environmental rearing conditions are stated on the x axis; L is low hygiene and H is high hygiene environment.

Only a single sample of bedding was taken at each time point and therefore only trends in the data points can be commented upon. However, differences in the bacterial bedding counts between the two farms were evident at day 7 (red bars in Figure 3.3); in fact the bacterial counts were ten fold greater in the LH samples. By day 14 there was greater variability in the results with a much greater number of bacteria in the XL bedding when compared to XH (approximately three fold difference). The data for day 35 showed that the differences between the two environmental conditions were minimal.

3.3.2.2: Coccidia levels

Coccidia levels were investigated to determine whether there were any differences in the levels of challenge that the birds on the two farms were exposed to during the period of the trial. Coccidiosis is recognised as a major parasitic disease of poultry and is caused by a group of protozoan known as *Eimeria* species. Infection seriously impairs growth and feed utilisation and enforced restrictions on the use of prophylactic anti-coccidia drugs has lead to increased reliance on vaccination (Butler, Spika et al. 1998; Dalloul and Lillehoj 2006). Coccidia oocysts can be shed by normal healthy birds and increased shedding is often observed post vaccination. Both the small intestine and caecum can be colonised depending on the species of *Eimeria*. The same make of vaccine and route of administration was given to birds on both farms but the low hygiene birds received treatment at day 0 while those reared on the high hygiene farm were treated at 5 days of age. The full vaccination protocol is presented in Appendix II.

Coccidia counts obtained from the single bed samples (same sample as for total bacterial counts, n=1 for each group) are shown in Table 3.1. It was observed that for the day 7 birds the parasite challenge was highest in the LH environment compared to the HH farm (range 300-700 oocysts per gram LH bedding compared to <100 in HH material). No distinction between coccidia counts in the samples from the two environments could be seen at day 14. However, at day 35 a difference between the levels on the two farms was evident with the low hygiene samples having higher parasite levels compared to the HH farm (400-5,900 oocysts/g in LH compared to 100 or less in HH group).

Line and environment	7 days	14 days	35 days
XL	700	<100	2,200
YL	300	100	5,900
ZL	700	500	400
XH	<100	<100	100
YH	<100	1,000	<100

Table 3.1: Coccidia levels per gram of bedding for the three lines of chickens at day 7, 14 and 35, respectively. L is low hygiene and H is high hygiene farm.

3.3.3: Environmental temperature and humidity

3.3.3.1: Temperature

A) Pedigree (HH) farm:

In the pedigree farm the barn temperature was tightly regulated. Both lines X and Y were housed within the same barn for each age of sampling. Manual temperature recording was carried out daily by the farm manager and a maximum and minimum ambient temperature recorded for each 24 hour period. Weekly averages were calculated and are presented in Figure 3.4 and Table 3.2.

Figure 3.4 and Table 3.2 show the data for average temperatures over the five week time course of the trial. A gradual decline in ambient temperature from weeks 1 through to 5,

with a drop from 32.2°C to 20.3°C (12°C) was recorded. This controlled decline in temperature is common practice in the rearing of chickens. Fluctuations in temperature during the 24 hour period for weeks 1 and 2 (that is data for birds 0 to 7 days and then 7 to 14 days, respectively) were a maximum of 0.6°C. Greater fluctuations between maximum and minimum temperatures were seen in the subsequent weeks of the trial.

B) Commercial (LH) farm:

Readings for both ambient temperature and humidity were taken every four minutes throughout the 35 day trial using a data logger device (Tinytag ™, model TGP-4500, Gemini Data Logger (UK) Ltd) placed in the middle of the pens of trial birds. Temperature and humidity averages as well as ranges were calculated for each day of the trial using the 359 readings taken over a 24 h period.

The mean ambient temperature data is presented in Figure 3.4 and similar to the results of the high hygiene environment, a gradual decrease was observed over the five weeks of the trial. At the start of the trial the weekly average temperatures for the two farms were comparable but as the trial progressed the gap increased. By week five the difference in the average temperatures for the two farms had increased to 4.9°C with birds raised in LH subjected to higher temperatures.

Table 3.3 presents the data obtained for the LH environment based on the Tinytag™ data from the farms. The average ranges of temperatures over a 24 h period in this environment were greater than those for the HH farm (range difference from 0.6-1.8°C in

the HH farm compared to 1.0-2.4°C range on the LH farm) indicative of greater fluctuations in the barn temperature on the LH farm.

The two temperature profiles were set by Aviagen and are used for the rearing of birds on the pedigree and sib testing standard conditions.

Figure 3.4:

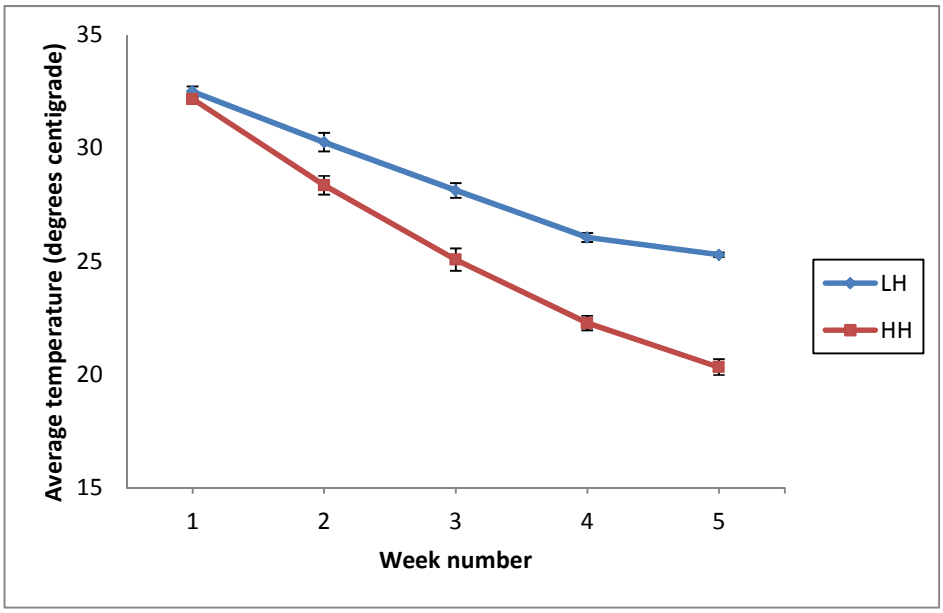


Table 3.2: HH

Week	Average temperature/°C	Std error	Average fluctuation between maximum and minimum temperatures/°C	SEM
1	32.2	0.06	0.6	0.05
2	28.4	0.41	0.6	0.26
3	25.1	0.49	1.0	0.12
4	22.3	0.32	1.6	0.5
5	20.3	0.35	1.8	0.55

Table 3.3: LH

Week	Average temperature/°C	Std error	Average fluctuation between maximum and minimum temperatures/°C	SEM
1	32.5	0.22	1.4	0.16
2	30.3	0.41	2.0	0.23
3	28.1	0.32	1.8	0.21
4	26.0	0.20	1.9	0.14
5	25.2	0.10	2.4	0.27

Figure 3.4, Tables 3.2 and 3.3: Average ambient temperatures and calculated fluctuations for the high and low hygiene farms (mean ± SEM).

3.3.3.2 :Relative humidity

A) Pedigree (HH) farm:

Humidity on the high hygiene farm was determined through a hygrometer. The humidity readings were taken at the same time each day and a weekly average was calculated. Figure 3.5 and Table 3.4 show the weekly changes in mean ambient humidity (±SEM) and show a steady increase from weeks 1 to 5, from 39.9% (±0.94) at the beginning of the trial to 63.7% (±3.01) by day 35.

The range in percentage humidity throughout each seven days was most variable in weeks 3 and 5, respectively. Differences in maximum and minimum readings were 14% and 26% respectively as shown in Table 3.4.

B) Commercial (LH) farm

The Tinytag ™ device was also used for collecting the data for the ambient humidity readings. Figure 3.5 and Table 3.5 shows the weekly average ambient humidity over the five week trial. With the exception of week 1 where, mean humidity ±SEM, was 39.9% ±0.94 in the HH farm compared to 43% ±1.13 on the LH farm, all of the other average readings were lower in the LH environment when compared to the HH farm. In the LH environment, and at the beginning of the trial, the relative humidity was 43% and the peak reading was seen at week 4 when the average reading was 53.8%, an increase of 10.8%. The range of readings within each week of the trial was between 12 and 20% in the LH farm compared to 5 and 26% in the HH farm. However, when the average ranges in readings (difference between maximum and minimum for each 7 days), for each week

of the trial were calculated, the average for HH was 11.8% compared to 16.3% in the commercial farm, indicating greater variability in humidity in the LH farm across the five weeks of the trial.

Figure 3.5:

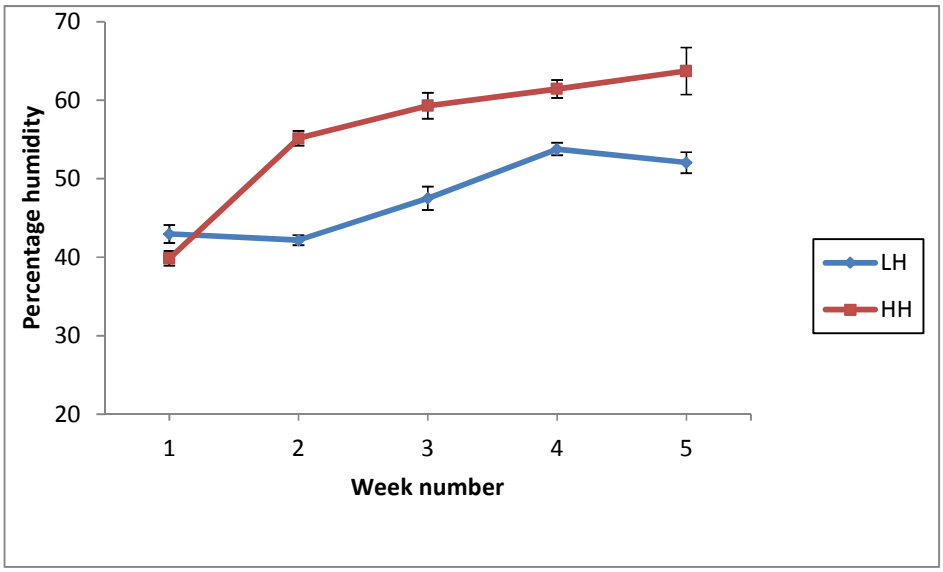


Table 3.4: HH

Week	Average relative humidity/%	Std error	Average fluctuation between maximum and minimum humidity/ %
1	39.9	0.94	5
2	55.1	0.94	5
3	59.3	1.66	14
4	61.4	1.15	9
5	63.7	3.01	26

Table 3.5: LH

Week	Average relative humidity/%	Std error	Average fluctuation between maximum and minimum humidity/ %
1	43.0	1.13	12.31
2	42.2	0.64	12.91
3	47.5	1.49	17.47
4	53.8	0.78	17.76
5	52.0	1.34	20.81

Figure 3.5, Tables 3.4 and 3.5: Average relative humidity and calculated fluctuations for the high and low hygiene farm (mean ± SEM).

3.4: Bird weights

Each bird included within the trial was weighed at the time of tissue sampling. This was performed so that average growth rates (measured by weight gain) for the three different lines of birds could be determined and comparisons between the two farms could be made. Figure 3.6 shows average bird weights for the population of birds included in this study from 0 to 35 days, respectively. As the birds aged, their weight increased and significant differences between the average weights of the birds reared in the two environments were observed.

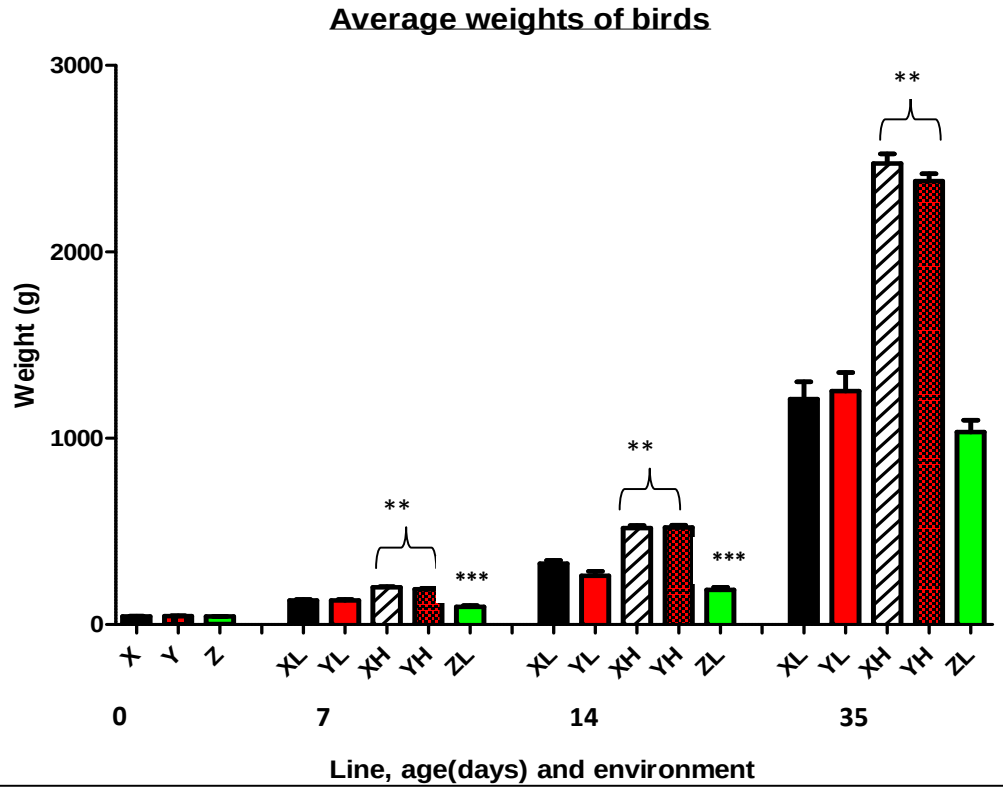


Figure 3.6: Average weight of birds at days 0, 7, 14 and 35 reared in the two different environments (L= low hygiene, H=high hygiene, ± SEM). Significant differences are indicated by *, ** indicates $p<0.05$ when the HH lines are compared to the LH data and *** indicates $p<0.001$ when the ZL group is compared to XH and YH data, n=10 for all groups of birds.

There were no significant differences in the average weights of the three lines of birds at day 0 with the mean weights (±SEM) being; line X 44.2g (±1.4), line Y 42.3g (±1.3) and line Z 41.6g (±0.9). The average weights for the birds at 7 days were (mean±SEM) XL 129.1g (±6.0), YL 130.1g (±5.9), ZL 96.2g (±5.5), XH 199.4g (±4.2) and YH 190.6g (±4.9). These data showed that the two lines of birds reared in the high hygiene environment were consistently heavier than those reared in the LH environment with an average difference of 70.3g for line X and 60.5g for line Y birds at 7 days of age.

The average bird weights for the day 14 time-point were (mean±SEM) XL 327.7g (±16.8), YL 261.9g (±23.6), ZL 186.3g (±12.8), XH 517.7g (±14.9) and YH 521.2g (±11.9). The difference in average weights between birds of the same line, but reared on the two farms was more apparent than at day 7. At day 14 the XL birds were 190g lighter on average than XH and 259.3g separated the average bird weights of line Y chickens. These data were statistically significant, $p<0.05$ for lines X and Y and $p<0.001$ for line Z birds, weights from the HH and LH.

The effects of the two rearing environments on average bird weight were greatest at day 35 (Figure 3.6). The average weight of the five lines of birds were (±SEM); XL 1210g (±92.3), YL 1253g (±99.1), ZL 1033g (±64.8), XH 2475g (±50.0) and YH 2379g (±40.0). In fact, birds reared on the pedigree farm were twice as heavy as those reared in the commercial environment (for both lines X and Y).

In conclusion, line X and Y birds of similar ages had similar weights when reared in the same environment. In contrast, line Z birds weighed less than the other lines at all time

points. As anticipated, those birds reared in the HH environment weighed more than those reared on the LH farm, and this was apparent from day 7, the difference in feed being so great between the two farms.

3.5: Nutrition

The diets that the birds were fed in the two hygiene environments differed in the following ways;

1. Cereal source - HH birds were fed wheat-based diet (high nutritional value) with added enzymes while LH chickens were fed a maize based diet (lower nutritional value, 100% maize due to concurrent food trial on farm).
2. Quality/size of crumble - HH flocks received a good quality crumble/pellet (3mm in size) while LH birds were fed a poor quality crumble, produced from 3mm pellets. Essentially the LH feed was very powdery in texture where as the HH feed was well formed.

No further information on the diet composition of the two feed types was available due to commercial interests.

3.7: Discussion

The trial pivotal to this study was performed at Aviagen farms in Scotland in Autumn 2007, and provided all the samples for subsequent analyses. However, due to increased biosecurity associated with bird flu in the UK, the line Z birds were not sampled in the HH rearing conditions. This was unfortunate, yet unavoidable, and so only the data generated from line X and Y birds could be directly compared with regard to rearing environment. The trial ran very smoothly and there were no major problems in sampling, which was due to the involvement and commitment of a team of enthusiastic individuals working at Aviagen. All the samples were collected over a period of five visits to the Aviagen farms i.e. more than one age group was sampled on the same day and the high hygiene farm was always visited first.

There were marked differences between the rearing conditions on the LH and HH farms that the birds were reared in. The main differences included the bacterial counts of bedding samples with the LH environment having higher levels (11-35 fold higher at day 7 compared to HH however, only single samples were taken); the presence of higher levels of coccidia oocysts in the LH bedding samples (300-700 in LH compared to less than 100 in HH samples at day 7); less tightly controlled ambient temperature and humidity on the LH farm and the use of two different feeds on the two farms (wheat-based diet on HH and maize-based diet on LH). Marked differences in average bird weights at day 7, 14 and 35 were also noted when the LH birds were compared to those reared on the HH farm with those reared in the HH environment being heavier.

The birds used in the trial from each of the three lines were genetically related to each other due to breeding practices, in fact direct siblings or half brothers, and so any differences in results obtained for a specific line between the two rearing environments could be attributed to the farm conditions.

A large difference in environmental bacterial challenge, based on the bedding sample results, between the two farms was seen at day 7 (11 fold difference between XL and XH pens and 35 fold for the two line Y bird pens). This result was anticipated due to the practice of mixing old bedding from previous batches of birds with new shavings on the low hygiene farm. This was carried out as it is common practice on commercial farms (North and Latin America) and is believed to help the stabilisation of a commensal bacteria population within the gastro-intestinal system of the chicks in the first week of life. With regard to bacterial counts, only trends can be discussed due to the fact that only a single bedding sample was analysed, but these results provided an indicator of the environmental conditions on the two farms during the time of the trial. The moisture content was also different between the samples from LH and HH pens. The samples were posted to the laboratory for analysis so the bacterial numbers may well have altered during this transit period. However, the sample counts were all relative to each other and so this would not be expected to influence the pattern in results. Analysis of further samples and at several additional time-points would have provided validation of such results. Moreover the addition of in- house sample analysis so that processing could be performed immediately would have supported these observations. The data presented was

for total bacterial counts and does not give any indication of the nature of these microbes and whether they are potentially pathogenic or commensal populations.

As well as the total bacterial counts for the 7 day LH bedding samples being higher than those in the HH group, a similar pattern was observed with regard to coccidia oocyst counts with the LH group presenting with higher levels relative to the HH group. The nature of the bedding, mixing old with new litter, and bio-security controls of the two farms were the most likely reasons for these observed differences. Interestingly, it has been shown that the mixing of bedding from previous batches of birds has no effect on the acquired immunity against *Eimeria maxima*, one of the species which can infest the large intestine (Chapman and Rayavarapu 2007). This therefore suggests that this method of rearing has no benefit in the prevention of coccidiosis. Following discussion with the parasitology team at Poultry Health Services, it was concluded that all of the samples analysed had low levels of coccidia oocysts and that such levels were not a threat to the birds in the trial. Much higher levels need to occur, thousands of oocysts per gram, to pose as a potential health threat. As discussed previously, both sets of birds received oral vaccination against coccidia at day 0 (LH) and day 5 (HH) respectively, which is particularly important following the ban of prophylactic coccidiostats, known to have antimicrobial properties. In addition such treatments are now also less effective due to the developing resistances of *Eimeria* species to such drugs (Vermeulen, Schaap et al. 2001).

The control of ambient temperature and humidity was different on the two farms. The HH farm was tightly regulated with computerised heating and cooling systems and the

specialized building materials had been used for the HH barns. The daily fluctuations in both temperature and humidity were reduced on the HH compared to the LH farm. Both farms started at average temperatures of 32°C for the first week, but over the course of the trial this was reduced to 20.3°C on the HH and 25.2°C on the LH farms. The initial high temperature was essential for rearing of the young stock due to low body weight to surface area.

The average temperatures in the low hygiene conditions were less tightly regulated and therefore more variable compared to those observed on the high hygiene farm. This finding is emphasised when the fluctuations in weekly temperatures presented in Tables 3.2 and 3.3 are compared i.e. the HH farm ranged 0.6-1.8°C and the LH ranged of 1.4-2.4°C. The greatest control of temperature was seen in the high hygiene environment during the first two weeks of the trial however, this tightness of regulation was not observed on the commercial farm. The first week is the time when broilers are most susceptible to health problems due to the immature adaptive immune system, lack of prior exposure to pathogens and reliance on the non-specific innate immune system (Bar-Shira and Friedman 2006) and therefore is perhaps the most important period for the controlled regulation of environmental conditions. Major fluctuations in ambient temperature may increase disease susceptibility due to the added external stress on the birds. In fact, this has been shown with the development of ascites (Bendheim, Berman et al. 1992; Buys, Scheele et al. 1999), such variations were not observed within this trial. In addition, fluctuations in temperature may have possible effects on both feed and water intake with a potential change in bird growth rates. This could be an important economic

consideration and may well be a factor which has contributed to the differences observed between the LH and HH birds.

Work published on the ambient temperature for rearing chickens focuses on high temperatures (greater than 28°C for maturing birds, 4 weeks and older). However, birds of this age were not subjected to such high temperatures during this trial. Interestingly, marked physiological responses were only seen in older birds when they were reared in temperatures greater than 30°C (Donkoh 1989). In this trial the temperature fluctuations over a 24 hour period on the LH farm were greater than those observed on the HH farm, but remained within a narrow range, the largest fluctuation in readings being 2.4°C. This fluctuation in temperature can be considered small for the time that the trial was performed, Autumn, when outside ambient temperatures were particularly variable. It was not determined whether there were any physiological effects due to such small temperature changes within the population of birds studied. Most published work focuses on very marked temperature fluctuations, for example 20°C differences (Yahav, Straschnow et al. 1997), with a clear focus on commercial units in hotter climates. Such variations in temperature do not currently occur within the UK, although with changing climates this may change.

Whereas the ambient temperatures across the five weeks of the trial were higher in the LH group, the relative humidity was higher, on average, in the HH group. It was observed in both groups that as the temperature fell, the humidity increased (Figures 3.4 and 3.5). Several factors contribute to relative humidity including; external weather

conditions, water content of bedding in the barn, stocking densities of birds and the airflow system installed in the barn. The data presented in Tables 3.4 and 3.5 show that with regard to relative humidity, there was tighter control during the first two weeks of this parameter within the HH farm relative to the LH farm. As for published work on ambient humidity there is a particular focus on extreme fluctuations, for example levels which are experienced within the tropics where high levels are often seen in conjunction with high temperatures. However, it has been reported that for chickens aged 5-8 weeks, a maximum body weight and feed intake was recorded at relative humidities within the range 60-65% (Yahav 2000), as was achieved on the HH unit in weeks 4 and 5 of this trial.

The line differences were interesting especially in relation to line Z, which is slower to grow and gain weight from day 0 to 14 relative to the other two lines. This finding is interesting considering that line Z birds have the best gut health out of the three bird lines. A fine balance between gut immunity, function and nutrient absorbing efficiency may be responsible for such an observation. In view of this, it would have been particularly useful to have sampled line Z birds on the HH farm so that full comparisons could have been made between the lines.

The differences in nutrition between the two farms perhaps contributes to the differences observed more so than the environmental differences. The feeding of birds reared on the HH farm the maize based diet and vice versa would allow the full effects to be investigated. The diets may also be the responsible factor for the gut immunity of these

two groups of birds. To date no work has been published investigating defensin expression and birds fed different diets. However, Sherman, Chapnik et al. (2006) showed that both albumin and amino acids affected human beta-defensin 1 expression following exposure in a human colon cell line. Whether similar affects *in vivo* are seen remains to be determined.

This chapter has explained how the trial was performed and identified the differences between the two rearing environments which were used throughout this study. The subsequent chapter investigates the differences between line X, Y and Z birds at the genetic level, focusing via the identification of SNPs on the genes encoding three of the AvBDs.

Chapter 4: Chicken genomics

4.1: Introduction

The three lines of birds used in this study X, Y and Z, revealed by linkage disequilibrium, to be phylogenetically distinct (Andreescu, Avendano et al. 2007), form part of the breeding stocks for Aviagen broilers and were selected for study with the potential of being used in future breeding programmes.

Commercial breeding programmes, over the years, have focused on establishing rapid bird growth rates. Breeding for disease resistance has generally been hard to perform due to poor selection markers, especially when the immune system is considered. The introduction of National and International regulations limiting the use of antibiotics in livestock, including poultry, means that knowledge of indigenous defense systems is essential if a healthy poultry industry is to be maintained. Thus the study of traits which can help protect birds against specific diseases including those caused by bacterial, viral and fungal vectors are becoming increasingly important. The main defense against disease is going to be largely influenced by supply of stock and feed free from pathogens, good husbandry and appropriate biosecurity.

During their first seven days of life chicks rely on their innate defenses. Thus this facet of the immune system is of particular interest to the poultry industry as the greatest bird mortality occurs in birds of less than seven days of age (personal communication with Dr Barry Thorp, former Director of Veterinary Services, Aviagen Ltd). An important

component of the chicken innate immune response is the synthesis of anti-microbials including lysozyme and the defensins. The avian β-defensins (AvBDs) in particular have been shown to have potent broad spectrum anti-microbial properties (van Dijk, Veldhuizen et al. 2008) and as a consequence are proposed to function in the protection of birds from disease.

This part of the study focused on the DNA locus encoding the AvBD genes and aimed to explore whether there were any genetic differences between this locus in the Aviagen bird lines X, Y and Z. To facilitate this a panel of SNPs, determined via bioinformatic analyses, was submitted for investigation as part of a large SNP study commissioned by Aviagen Ltd and performed by Illumina (a global company that develops innovative array based solutions for DNA, RNA and protein analysis, San Diego, Califironia, USA). The aim of this chapter was,

- **To identify potential single nucleotide polymorphisms (SNPs) within the AvBD locus of Aviagen bird lines X, Y and Z, and to investigate and compare SNP frequencies in selected AvBD genes.**

4.2: SNPs in the Illumina study

In 2006 a project was initiated by Aviagen, which involved the analysis of panels of SNPs across ten different lines of chicken. The first panel, performed in 2006, contained a total of 6,144 SNPs; the second panel contained 12,046 (2007) and it is this latter data-set, which was used within this study. The reasons for investigating these SNP panels was to identify genetic markers linked to specific traits which are otherwise difficult to

measure, and incorporate such findings into the genetic selection of birds with subsequent appropriate breeding programmes. All of the initial sequencing for SNPs in the first two panels was performed by Illumina. A minimum of 200 birds from each of ten selected lines, including lines X, Y and Z, were analysed for the presence of the SNPs.

4.2.1: SNPs within the AvBD locus

As part of this AvBD project, a list of 44 potential SNPs located within the chicken AvBD locus on chromosome 3 were identified and submitted to Illumina for investigation. This list of SNPs was constructed using the following techniques:

- Use of Basic Local Alignment Search Tool (BLAST) of mRNA sequences of AvBD against the chicken genome (NCBI, www.ncbi/nlm.nih.gov/Blast.cgi - 10.4.07 and Ensembl, www.ensembl.org/Multi/blastview - 10.4.07) to identify any novel SNPs.
- Searching the SNP database (Ensembl) for reported polymorphisms via the entry of gene accession numbers and manually scrolling the region of the chromosome 3 for other SNPs within the vicinity. A limited amount of data was available on these sites. Much of the work had been carried out on Silkie, and layer chickens, which are genetically different to broilers and so it was not known whether such SNPs would exist within bird lines X, Y and Z. There was also little available data on actual frequencies of the SNP allele forms within the published populations and so such sites were used only as a starting point for SNP identification.

Of the 44 potential SNPs, only 15 were identified as actual polymorphisms within the three lines of Aviagen chickens and these are listed in Table 4.1. The frequency given is that of allele A, i.e. whichever allele has the DNA base T. The location, gene code and, if available, the recognised SNP code are given for each polymorphism detected within the Illumina study. SNPs were identified in AvBD1, 3, 4, 6, 8, 9 and 10 genes respectively.

						Frequency		
Code	**AvBD**	**Gene code**	**SNP code**	**SNP**	**Location**	**Line X**	**Line Y**	**Line Z**
1	1	ENSGALG00000022815	Rs15457749	G/T	Non synonymous within mature peptide	0.87	0.02	0.55
2	1	ENSGALG00000022815	Rs15457745	C/T	Intronic	0.87	0.22	0.72
3	3			C/T	Exon 1 region	0.87	0.23	0.89
4	4	ENSGALG00000019843	Rs16341536	T/C	5' UTR region	0.99	1	0.58
5	6	ENSGALG00000016668	Rs13526000	T/G	Intronic	0.49	0	0.28
6	6	ENSGALG00000016668	Rs16341514	G/T	Intronic	0.17	1	0.28
7	8	ENSGALG00000019844	Rs15457650	G/T	Intronic	0.08	0	0.26
8	8	ENSGALG00000019844	Rs15457653	T/C	Intronic	0.58	0.21	0.72
9	8	ENSGALG00000019844		C/T	Intronic	1	1	0.82
10	9	ENSGALG00000019845	Rs3137928	T/C	3'UTR region	0.08	0.14	0.61
11	9	ENSGALG00000019845	Rs14411786	C/T	Intronic	0.97	0.86	0.65
12	9	ENSGALG00000019845		T/C	Intronic	0.99	0.86	0.36
13	10	ENSGALG00000016667	Rs15457607	T/C	Intronic	0.88	0.98	0.45
14	10	ENSGALG00000016667	Rs14411785	T/C	5'UTR	0.94	0.86	0.65
15	10	ENSGALG00000016667		T/C	Intronic	0.85	0.98	0.44

Table 4.1: List of 15 SNPs from Illumina study which were successfully identified in Aviagen lines X, Y and Z. Frequency is for the presence of the T base in allele designated A.

4.2.2: Allele frequency from Illumina results

For those SNPs present within lines X, Y and Z, a single frequency value was given and this value (p component) represents the occurrence of the T containing allele within the 200 birds (values shown in Table 4.1). Allele frequency within that population of birds was determined using the equation, $p^2 + 2pq+q^2=1$ where p=frequency of T allele and q=frequency of the non T containing allele (G or C). Values are given as percentages of the tested population (where n=200). The frequency of the T allele is given irrespective of whether it is on the forward or reverse strand so that there can be uniformity.

For example, if a SNP was reported with the result of 0.7 this would be the value of p (frequency of T base in allele A), and therefore q would have a value of 0.3 (since p+q must equal 1). As this SNP is a T/G base change the following calculations can be performed to determine the frequency (as a percentage) within this population of the four genotypes (TT, GT, TG and GG);

p^2x100=49% TT

(2pq) x100=21% TG/GT

q^2=9% GG

4.3: SNPs in AvBD1, 4 and 10 loci

With the eventual aim of investigating the effects, if any, of the SNPs on chicken innate defenses, this study focused on three avian β-defensin genes - AvBDs1, 4 and 10. The AvBD1 gene was selected following the identification of an exonic non-synonymous SNP within the region coding the mature peptide, and the finding that different allelic forms occurred within the three lines of chickens. The rationale for the inclusion of the AvBD4 gene was that the encoded peptide had been shown to be anti-microbial (Milona, Townes et al. 2007) and the AvBD4 SNP was located in the 5'UTR with possible effects on gene expression. The AvBD10 gene was investigated as again a SNP was identified in the 5'UTR with possible effects on gene expression and there was little information in the literature about this gene or its encoded peptide. AvBD10, has been shown to be highly expressed within the kidney, liver, reproductive system as well as the gastro-intestinal tract (Lynn, Higgs et al. 2004), which was viewed as an unusual panel of tissues regarding possible anti-microbial functions and properties.

4.3.1: Novel SNP identification within AvBD1 and 10 in individual birds

In addition to the SNPs identified via the Illumina panel, studies involving birds from each of the three lines were performed to determine whether there were any novel SNPs within the coding regions of AvBD1 and 10, respectively. To achieve this, PCR amplification of cDNA from individual birds was performed and the products sequenced. For SNP identification DNA from ten birds from each of the three lines was sequenced and the DNA chromatograms analysed for the presence of SNPs.

4.3.2: Analysis of sequencing for pooled genomic DNA

To improve accuracy when determining SNP allele frequency within the three lines of birds, pooled genomic DNA as well as individual bird cDNA was used. Each pool contained DNA from 20 birds and six pools were available for lines X and Y, and five for line Z. A published technique was used to estimate SNP allele frequency from DNA chromatograms of pooled DNA samples (Ye, McLeod et al. 2006). Briefly this involved examining the chromatogram at the location of the SNP and the height of the peaks was used to calculate the frequencies of the bases relative to the population investigated. The equation used to calculate the frequency of a SNP allele e.g. 'T' within the population is shown in Figure 4.1.

(x/x+y) X 100 = estimated frequency (%)

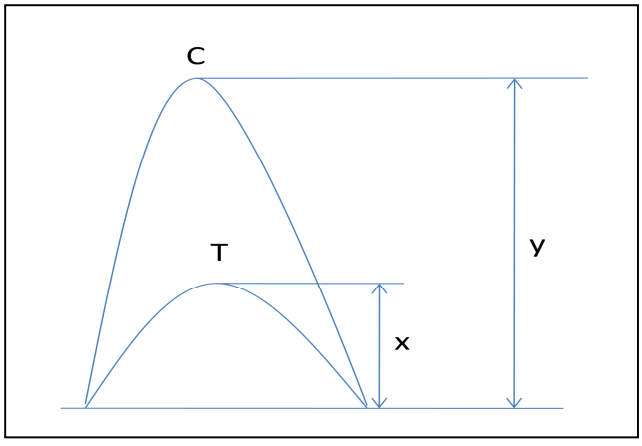


Figure 4.1: Estimation of allele frequency taken from two peaks (C and T bases) on a chromatogram reading obtained following DNA sequencing. Measurement of x and y were used to determine approximate base frequency.

Poor sequencing results can affect the accuracy of this technique and so it can only be performed on good quality sequencing data.

A selection of, but not all sequencing chromatograms are included as examples within this chapter. These illustrate the base peaks and show how the subsequent calculations were performed to determine the frequencies of the bases within the given chicken populations.

4.4: SNPs within AvBD1 locus

This section reports the data for the SNPs detected within the AvBD1 gene locus. The genomic sequence for this region is shown in Figure 4.2. Identified SNPs are indicated as well as the primers used for the PCR amplification reactions. Two additional SNPs in the mature peptide coding region of AvBD1 were identified following sequencing of the individual birds. One of these SNPs, rs15457747, is a recognized SNP, which was overlooked in the initial SNP searches that were performed thus unfortunately there is no Illumina frequency data for this polymorphism. The second SNP highlighted in green in Figure 4.2 and designated SNP 'VB1' is novel and previously unreported. All three SNPs are located in exon 2 of the gene, are non-synonymous and thus change the amino acid sequence of the peptide.

Figure 4.2: The genomic sequence AvBD1 with the primers used for sequencing underlined and the four SNPs marked as coloured highlighted boxes.
The exonic regions are bold and highlighted in a peach colour. The same forward primer was used for both forms of sequencing and is underlined within exon 1. The reverse primer used for individual bird sequencing (cDNA) is in green and underlined. The reverse primer used for the genomic DNA sequencing is in black and also underlined. The ATG start codon is in red but the remainder of the sequence encoding AvBD1 peptide is in black and bold. The sequence encoding the mature peptide of AvBD1 spans exons 2 and 3 and has the stop codon TGA which is underlined in black.

SNPs Green= SNP VB1
Purple= rs15457749
Blue= rs15457747
Yellow= rs15457745

```
3781 TGTGTGGCCTTGGTTTCTCCCCTCTGTAGCCCTGTGAAAACCCGGGACAGACGTAAACCA 3840   Exon 1
3841 TGCGGATCGTGTACCTGCTCCTCCCCTTCATCCTCCTCCTGGCCCAGGGTGCTGCAGGTG 3900
3901 AGGTGTGAGTTCTGTGGGGTTCTCCATATCCCAGGAGGTGGCTTGTCAGGGATGGGTAAC 3960
3961 GACTAGGAGGGCTCTGATCAGTTGGTTCAGGAGGGAGGGAAGATTTAGGTTGGATATCAG 4020
4021 GGGGAAGTTCTTTACAGAGAGAGAGGTGAGGTGCTGGAACAGCTGCCCAGAGAGGCTGTG 4080
4081 GATGCCCCGTCCATCCCTGGAGGTGTTCAAGGCCAGGTTGGATGGGGCCCTGGGCAGCCT 4140
4141 GGGCTGGTATTAAATGGGGAGGTTGGTGGCCCTGCCTGTGGTGGGTGGGTTGGAGCTTCA 4200
4201 TGATCCTTGGGGTCCCTTCCAACCCAACCATTCTGTGATTCTGTGGTTTGGATGAGTGGC 4260
4261 TGGGCTTTTGGGTTTGGTGCTTTGTGCGCGTGTTAGACTGAGATCCATGGGACAGCCACT 4320
4321 CTAGAACCACACACAGCTTTTACAGGTATCCTACACTCATTTTCTTTTGGTCTGTGCAGG 4380
4381 ATCCTCCCAGGCTCTAGGAAGGAAGTCAGATTGTTTTCGAAAGAATGGCTTCTGTGCATT 4440   Exon 2
4441 TCTGAAGTGCCCTTACCTCACTCTCATCAGTGGGAAATGCTCAAGATTTCACCTCTGCTG 4500
4501 CAAAAGGTAAGCTTTGGAATTAGGGATGAAATTGGATCTGCTACCACGATGGCAGAAATA 4560
4561 GCTGTTGTTGTGTTTGATCCCCAAACCTAGCTACTGGCTTTGGGCTATATATGATCCAGG 4620
4621 GCAGGGGCTTGGGGAGGAAAGGAGAAGGTGCTAGGACCGGTCCTTTAAAGGAACTGGAGG 4680
4681 AACCCCAGATCAGACGCTGGCCTCCCCATTGCCCTCAGTTACACGGGGCTGCCTGGCTTG 4740
4741 CTGGTTTCACAAATGCTTCCCCAGTTGGTGCAGAGTGGAGACTCTCCCCTGGGTAGTGTG 4800
4801 AGGCACAGAACCCATTCCCTGATGTCTCTGCAAAACCTTGGAAACCAAGCTGAAACCAAG 4860
4861 CTGTCTGCTATGCAGGCTGCTTACTACCTGCATTGAGATTAGTGTCAATGTGTCAGTGTT 4920
4921 ATCCAGGAGAAGTGATGCATACTGAGAGACAGAAAAAGGAGAATAAAAAGAGGTGACCTC 4980
4981 ACAGAGTGTTTTCTTCCTGCAGAATATGGGGCTGAAGAGCCAGACATCCCAAGCAGGACA 5040   Exon 3
5041 TCACCCTGGCTTCTCGCTTCTGGAAACTTCCCCCATTGACCTCTCCCCTTCCCACCTCTG 5100
5101 CAGTCTCCCATGGTGTGAGCGTGGCAGTAGAAGTTGGAGACATCCCACCATGGGCCTGCA 5160
5161 GTTGTTTGGCCAGTTGCTGCTTTTCCCTGCTGAATAAAGGTGTGCAGTTTAGCATTGCAG 5220
5221 CTGGTGGGGAGTGTGAGTGTGTCCTTGTGCCAGTGGGTGCCCAGGGATGCTCTTCCCCAG 5280
```

A fourth SNP was also detected within the intronic region between exons 2 and 3.

Table 4.2 lists details of the four AvBD1 SNPs that were identified within the three lines of chickens and indicates the location of the polymorphism within the codon (highlighted in green). The amino acid sequences are given for the two forms of the polymorphism that can exist at each of these locations.

SNP	**SNP code**	**Genotype**	**Location/type**	**Codon (T form)**	**Codon (non T form)**
1	Rs15457749	G/T (reverse strand)	Non-synonymous coding	TAC Y residue Tyrosine	TCC S residue Serine
2	Rs15457745	C/T	Intronic		
3	Rs15457747	C/T	Non-synonymous coding	TAC Y residue Tyrosine	CAC H residue Histidine
4	VB1	C/T (reverse strand)	Non-synonymous coding	AAT N residue Asparagine	AGT S residue Serine

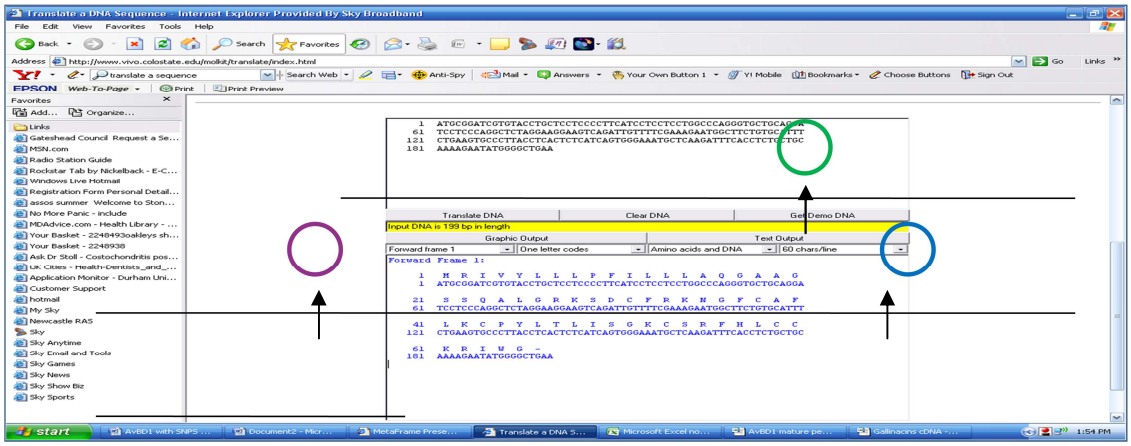
Table 4.2: Four SNPs identified in the AvBD1 locus. The location of the SNP is highlighted in each of the codons and the single letter amino acid coding system has been used.

The cDNA sequence and corresponding amino acid sequence of AvBD1 is shown in Figure 4.3. The locations of the three SNPs are marked with arrows and the affected amino acids with circles.

Figure 4.3: Translated sequence for cDNA of AvBD1. The mature peptide is underlined in black. The three non-synonymous coding SNPs are identified by rings around the corresponding amino acid and the polymorphism is marked by an arrow.

The SNP combination indicated by this cDNA sequence is NYH (seen in line X birds); the other combinations of amino acid were SSY and N/SYY (for line Y and Z birds, respectively).

SNPs: Green= novel SNP VB1
Purple=rs15457749
Blue =rs15457747



Each of the four SNPs in the AvBD1 gene locus will be considered in detail. Initially the Illumina data will be discussed and then the data, where available, for the individual bird and pooled DNA sequences will be addressed.

4.4.1: SNP rs15457749

This SNP is purple-coded in Figures 4.2 & 4.3 and Table 4.2.

A) Frequency from Illumina data

The frequency data obtained from Illumina for the SNP rs15457749 calculated as percentages of the total population is presented in Table 4.3. All four allele genotypes are given with anticipated frequencies calculated as percentages.

Line	**TT**	**TG**	**GT**	**GG**
X	75.7	11.3	11.3	1.7
Y	0.04	1.98	1.98	96
Z	30.25	24.75	24.75	20.25

Table 4.3: Percentage frequencies of four different genotypes for SNP rs15457749 within AvBD1 locus.

These data show that line X birds were most likely to have a T base at this SNP, 76% TT homozygote frequency, and therefore as shown in Table 4.2, the TAC codon is predicted to be the most abundant within these birds. This combination of bases would code for a tyrosine (Y) residue. Line Y birds had a higher incidence of G bases, 96% predicted

frequency of GG genotype, and as can be seen in Table 4.2 this would encode a serine (S) residue. Line Z birds showed a mix of all four genotypes.

B) Results from individual bird cDNA sequencing

Of the ten cDNAs from line X birds that were sequenced, all of them showed a single T base peak on the chromatograms, a tyrosine amino acid. An example of one of the chromatograms is shown in Figure 4.4A with the three coding SNPs circled and the base peaks marked with an arrow. SNP rs15457749 is located at base position 107. It can be seen that only one base peak is seen at each of these three locations.

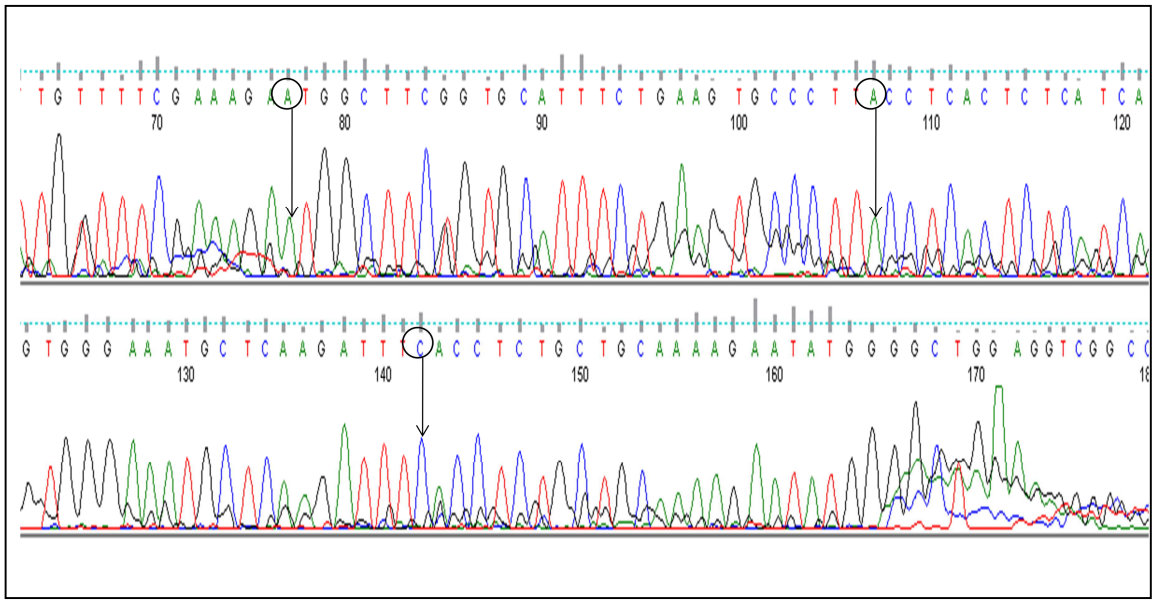


Figure 4.4A: Region of sequenced PCR product from an individual line X bird showing the location of the three non-synonymous SNPs in AvBD1. The grey bars above the base letter indicate the strength of the signal output. This sequence was the result from the forward primer amplification. SNP rs15457749 is located at position 107 on the chromatogram and is an A base. The different bases are colour-coded, A-green, T-red, G-black and C-blue.

An additional example of the single peak observed for this SNP within the group of ten line X birds is shown in Figure 4.4B. This amplified section of DNA chromatogram from a second bird sample clearly shows a single peak indicating an A base. This supports this X line bird having the same SNP as for the bird represented in Figure 4.4A.

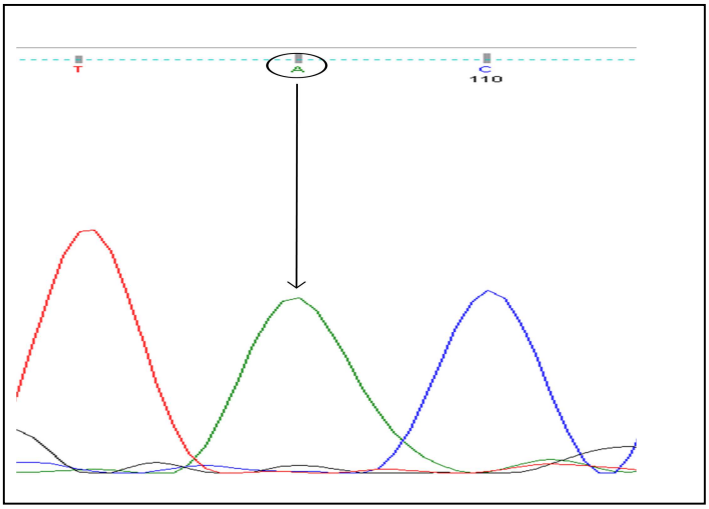


Figure 4.4B: Single peak representing an A base in one of the sequence outputs for a line X bird. SNP rs15457749 is marked with an arrow.

In line Y birds, eight of ten bird chromatograms showed a distinct single peak indicating a G base (G base on reverse strand and so a C base on the forward strand), i.e. a serine residue is encoded. An example of a chromatogram from a line Y bird is shown in Figure 4.5A. The three non synonymous SNPs are marked and SNP rs15457749 is located at position 101.

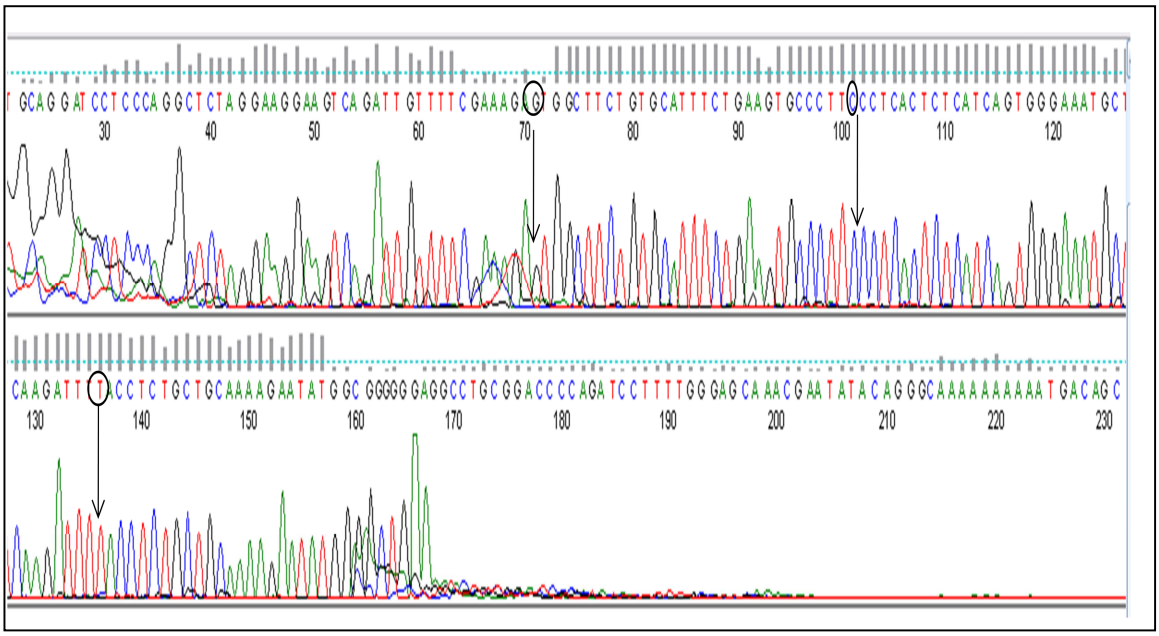


Figure 4.5A: Region of sequenced PCR product from an individual line Y bird showing the location of the three non-synonymous SNPs in AvBD1. The grey bars above the base letter indicate the strength of the signal output. This sequence was the result from the forward primer amplification. SNP rs15457749 is located at position 101 on the chromatogram and is a C base. The different bases are colour-coded, A-green, T-red, G-black and C-blue.

Figure 4.5B shows an additional example of the SNP rs15457749 in a line Y bird and shows the presence of a C nucleotide.

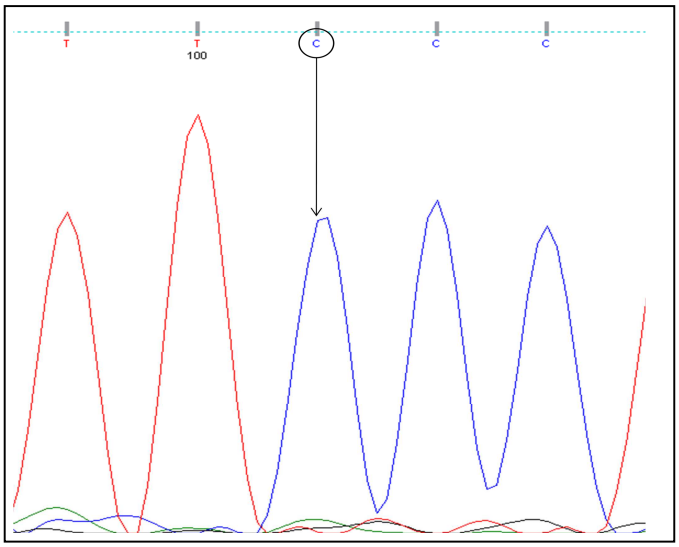


Figure 4.5B: Single peak representing a C base in one of the sequence outputs for a line Y bird. SNP rs15457749 is marked with an arrow. Colour coding as for Figure 4.4A.

One of the ten line Y birds had a definite T base at this point in the chromatogram while the remaining bird had two peaks in a 1:3 ratio of G:T (Figure 4.5C). This bird was likely to be a heterozygote (note the frequencies from Illumina are given on the reverse strands and therefore the SNP is G or T). When the frequency of the two alleles was calculated, there was a 74% occurrence of the C form and 26% for the A form (x/(x+y) x 100 or y/(x+y) x 100, respectively), supporting 'C' being the most frequent base in this group of line Y birds.

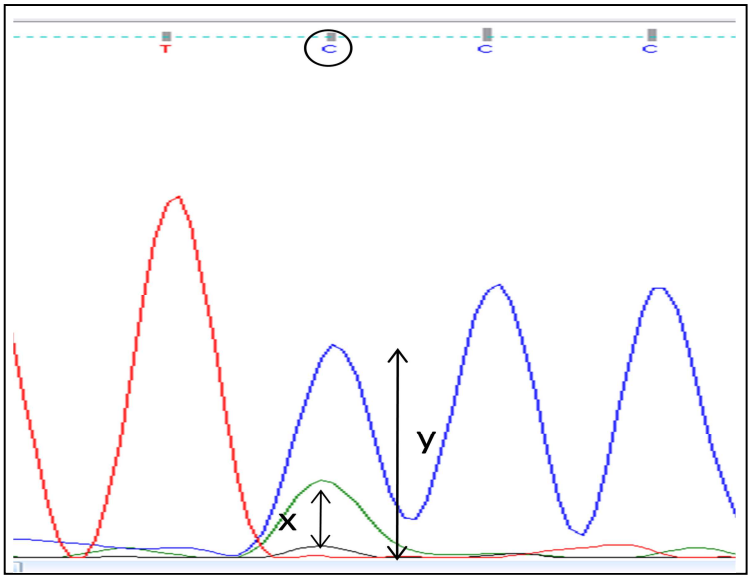


Figure 4.5C: DNA sequence of a line Y bird showing two base peaks at the location of SNP rs15457749. This chromatogram is from the forward sequence. Blue peak-C base, green peak-A base.

The third line of chickens (Z) had a very mixed predicted genotype. This mixed genotype was also suggested in the Illumina data. Figure 4.6A shows an example of a DNA sequence obtained for one of the line Z birds. SNP rs15457749 is located at position 105 and shows the presence of both C and A peaks. It has been assigned a C base as this peak is slightly larger in amplitude compared to the A base peak.

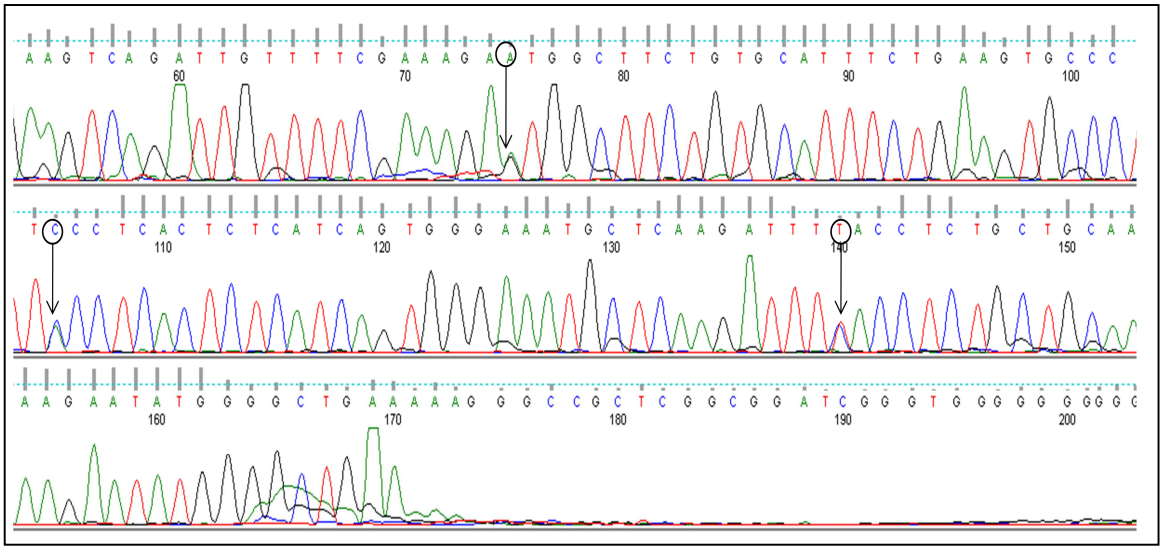


Figure 4.6A: Region of sequenced PCR product from an individual line Z bird showing the location of the three non-synonymous SNPs in AvBD1. The grey bars above the base letter indicate the strength of the signal output. This sequence was the result from the forward primer amplification. SNP rs15457749 is located at position 105 on the chromatogram and is classified as a C base although a peak representing an A base also occurs at this point. The different bases are colour-coded, A-green, T-red, G-black and C-blue.

Two peaks were present for seven out of the ten birds, indicating a mixture of T and G bases within this group. An amplified section of a chromatogram from one of these birds is shown in Figure 4.6B. Three birds out of the ten had a distinct single peak indicating a T base (tyrosine encoded, shown in Figure 4.6C), and only one had the G base (serine shown in Figure 4.6D).

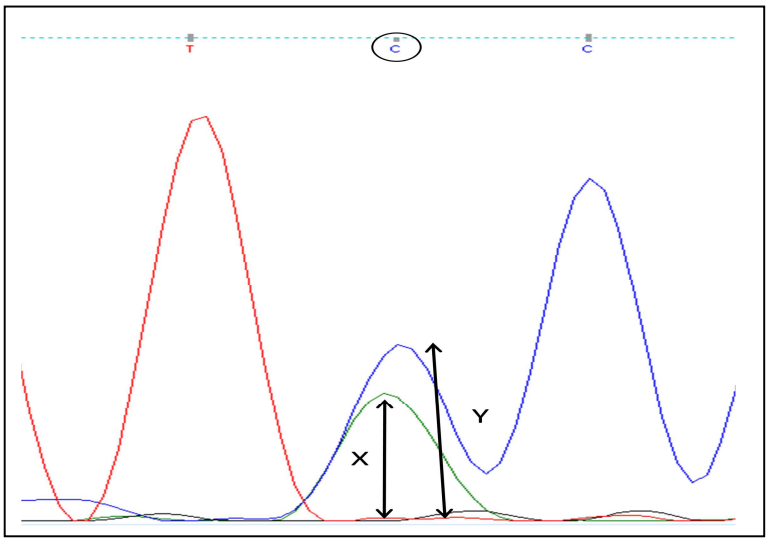


Figure 4.6B: DNA sequence of a line Z bird showing two base peaks at the location of SNP rs15457749. Blue peak- C base and green peak- A base.

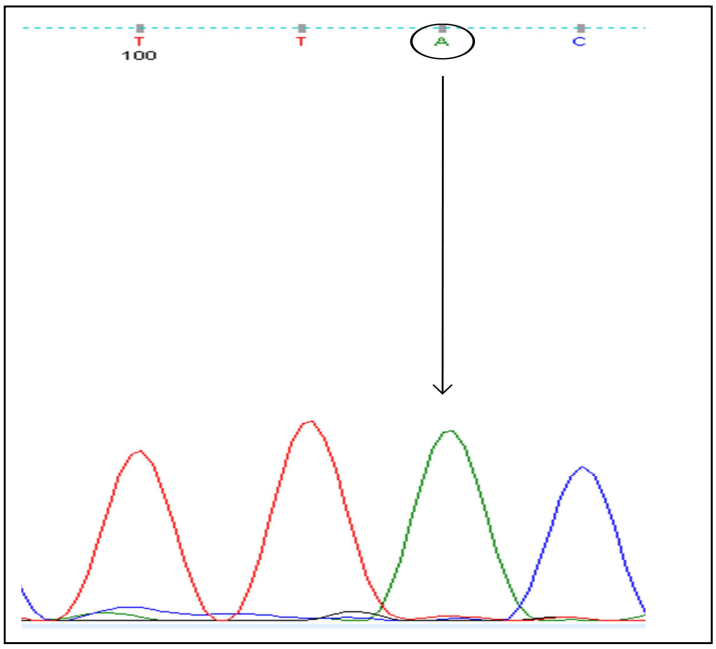


Figure 4.6C: DNA sequence of a line Z bird showing a single base peak at the location of SNP rs15457749. Green peak- A base.

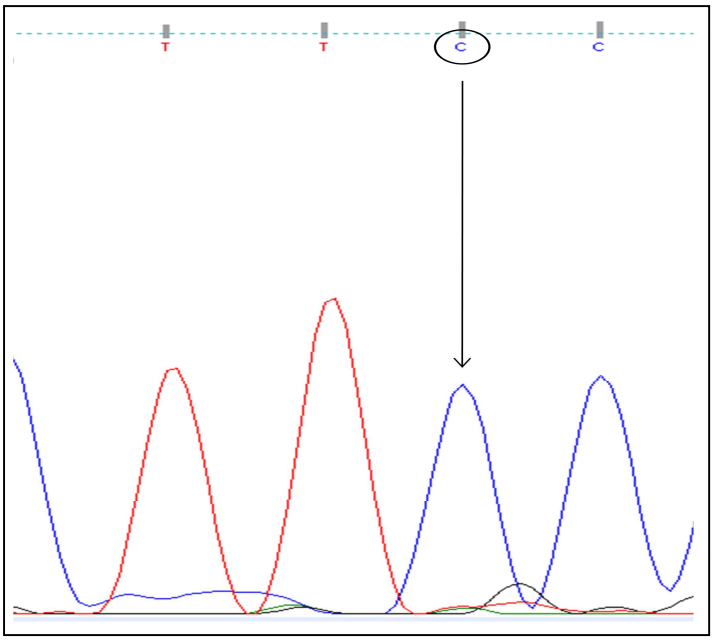


Figure 4.6D: DNA sequence of a line Z bird showing a single base peak at the location of SNP rs15457749. Blue peak- C base.

C) Results from pooled DNA samples

In addition to individual bird cDNA sequences, samples of pooled genomic DNA were also investigated. Each of these pooled samples contained equal amounts of DNA from 20 birds from each of the lines. Six samples were available for both lines X and Y (120 bird DNA samples) and five samples of pooled line Z bird DNA (100 bird DNA samples). PCR amplification and sequencing of these genomic samples was performed. As for the sequencing of the individual birds, the ratios of multiple peaks were calculated so that the frequency of the two bases in the population could be determined. The results are shown in Table 4.4. Sequencing results of the line X birds indicated that all of the birds tested were homozygotes (TT, tyrosine encoded). The G base was most common within the population of 120 line Y birds, mean frequency 95.7% (±4.3SEM), resulting in

a serine. Only five pools of DNA were available for line Z, but as for line Y, the G base was most common, mean frequency of 61.1% (±1.1SEM), and again a serine was encoded.

Line of birds	T base frequency/%	G base frequency/%	Standard error
X (n=120)	100	0	0
Y (n=120)	4.3	95.7	4.3
Z (n=100)	38.9	61.1	1.1

Table 4.4: Calculated percentage frequencies of SNP bases for rs15457749 in the pooled genomic DNA samples.

The presence of a double peak at the location of SNP rs15457749 is indicated in Figure 4.7.

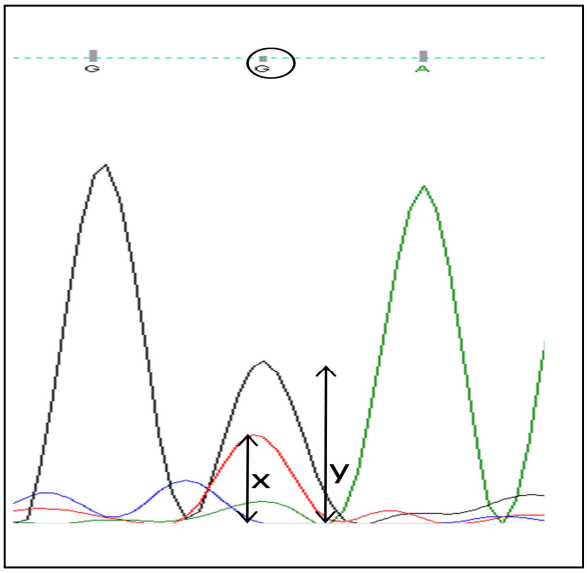


Figure 4.7: DNA sequence of a pooled genomic DNA sample of line Z birds showing two base peaks at the location of SNP rs15457749. This chromatogram is from the reverse sequence. Black peak- G base, red peak- T base.

The results obtained from Illumina, individual bird sequencing and pooled DNA sequences were comparable and reinforced the use of the Illumina data. Overall these analyses showed that AvBD1 gene of the line X birds most commonly encoded a tyrosine residue at the location of SNP rs15457749 compared to lines Y and Z where a serine amino acid was more likely to be encoded.

4.4.2: SNP rs15457745

This intronic SNP is encoded in yellow in Figure 4.2 and Table 4.2.

A) Frequency from Illumina data

The calculated frequencies for SNP rs15457745 for the four different genotypes based on the Illumina results are presented in Table 4.5.

Line	**TT**	**TC**	**CT**	**CC**
X	75.7	11.3	11.3	1.7
Y	4.8	17.2	17.2	60.8
Z	51.8	20.1	20.1	7.8

Table 4.5: Percentage frequencies of four different genotypes for SNP rs15457745 within AvBD1 locus.

These data indicate that the genotype TT is most common in the line X (75.7% frequency) and Z bird populations (51.8%) where as the CC genotype is predicted to be the most prevalent in line Y birds (60.8%). Thus the T base SNP allele is most prevalent in lines X and Z, while the C base SNP allele is most abundant in line Y birds.

B) Results from pooled DNA samples

The calculated percentages for the sequencing of the pooled genomic DNA samples are presented in Table 4.6. All of the DNA sequences of line X birds showed a single T base at the location of this SNP, supporting the suggestion that these populations of birds were TT homozygotes for this SNP. The C base was most frequent within the line Y birds, mean 78% (±5.5SEM). As for line X birds, the line Z bird sequence data showed that the T base was most frequent with a mean 84.3% (±7.8SEM). This overall frequency pattern was comparable to the Illumina data (Table 4.5).

Line of birds	**T base frequency/%**	**C base frequency/%**	**Standard error**
X (n=120)	100	0	0
Y (n=120)	22	78	5.5
Z (n=100)	84.3	15.7	7.8

Table 4.6: Percentage frequencies of SNP bases for rs15457745 in the pooled genomic DNA samples.

4.4.3: SNP rs15457747

This SNP is coded in blue in Figures 4.2 and 4.3 and Table 4.2.

A) Frequency from Illumina data

No Illumina data was available for this SNP as it was not entered into the panel for analysis.

B) Results from individual bird sequencing

The DNA chromatograms of all ten of the line X birds had a single peak representing a C base at the location of SNP rs15457747. As shown in Table 4.2, a C SNP allele encodes a histidine (H) amino acid. In line Y birds, eight out of ten bird DNA sequences showed the T SNP allele (tyrosine residue). The sequences of the remaining two birds had two peaks at this location with a ratio 3T:1C, making T the predominant SNP in this group of birds. The line Z bird sequences showed a mixed pattern; of the ten bird sequences analysed, three had a single peak representing a C base, one bird sequence showed the presence of a T base and the other six sequences showed two peaks for C and T bases superimposed on each other. These latter observations indicated that the birds were heterozygotes for SNP rs15457747 with both allelic forms being transcribed. An example of the two superimposed peaks is shown in Figure 4.8.

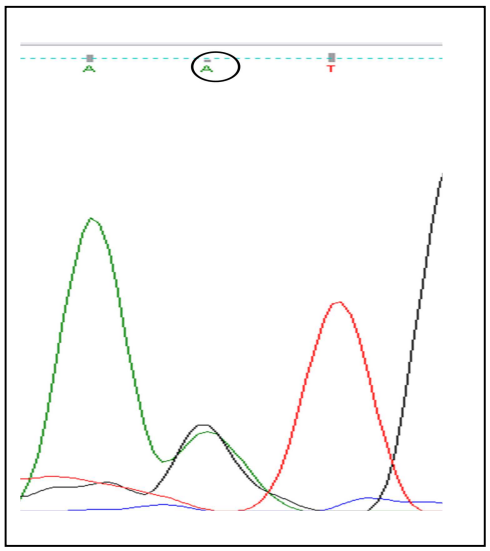


Figure 4.8: DNA sequence of an individual line Z bird showing two base peaks at the location of SNP rs15457747. This chromatogram is from the forward sequence. The green peaks indicates an A base and the black a G base. The two peaks are superimposed on each other indicating an even frequency of these two bases in this individual bird.

C) Results from pooled DNA samples

As there was no data available from the Illumina study for the occurrence of this SNP, it was particularly important to investigate its occurrence using the pooled genomic DNA samples. This would then confirm that the data from the ten individual birds was representative of the larger populations as a whole. The data for the pooled genomic DNA samples is presented in Table 4.7.

Line of birds	**T base frequency/%**	**C base frequency/%**	**Standard error**
X (n=120)	13.8	86.2	3.2
Y (n=120)	89.7	10.3	7.4
Z (n=100)	61.4	38.6	1.6

Table 4.7: Percentage frequencies of SNP bases for rs15457747 in the pooled genomic DNA samples.

Of the 120 line X birds analysed within the pooled DNA samples the C base was the most frequent with a calculated mean 86.2% (±5.5SEM). As C is the most common base within these samples, it is proposed that a histidine residue is most commonly encoded in the mature peptide of AvBD1.

The sequencing of the 120 line Y samples showed that the T base was most commonly observed within this population with the mean percentage of T base 89.7% (±7.4 SEM). This result was comparable to sequencing obtained from the individual birds (eight out of ten, 80%). Sequencing of line Z bird pooled DNA also showed that a T base was most

common with an estimated frequency of 84.3% (±1.6 SEM). The T base is associated with the presence of a Y residue in the mature peptide.

These data support that AvBD1 produced by line X birds contains a H residue at the location of this SNP whereas the other two lines i.e. lines Y and Z would synthesise AvBD1 with a Y at this position.

4.4.4: Novel SNP 'VB1'

This novel SNP, for thesis purposes named as 'VB1', is coded in green in Figures 4.2 and 4.3 and Table 4.2.

A) Frequency from Illumina data

No Illumina data was available for this SNP as it was detected after the large SNP panel had been analysed. In fact the presence of this SNP was detected following the sequencing of individual bird cDNAs.

B) Results from individual bird sequencing

The cDNAs of ten line X birds were sequenced and eight provided good quality DNA sequences. Two sequences failed and the data discussed herein relates to only eight birds. All eight birds showed a single peak on the chromatograms at position 'VB1', indicative of a T base for this SNP (shown in Figure 4.4A at position 107). This base supports an asparagine (N) residue (Table 4.2).

With regard to the line Y birds, seven out of ten birds had a single C peak on their DNA chromatograms at the position of this SNP, which would subsequently lead to a serine (S)

amino acid being encoded. One bird showed a single T base peak. The remaining two birds showed two peaks with a 3G:1A ratio (reverse strand sequence). It can be concluded that the G nucleotide supports the incorporation of a serine amino acid and was therefore most abundant amongst this group of chickens.

Four of the line Z birds for which DNA sequencing was available indicated the presence of a T base for this SNP (asparagine (N) encoded) and only one bird had a single peak indicative of C nucleotide (serine). The remaining five birds had double peaks for G and T bases, which were superimposed over each other, suggesting that both SNP allelic forms were present and transcribed.

C) Results from pooled DNA sample

As previously mentioned, this SNP was not entered into the Illumina panel as it had not been detected at the time of panel submission.

Table 4.8 shows the data for the occurrence of the two SNP forms within the bird populations included within the pooled DNA samples. As with the previous three SNPs in the AvBD1 locus, line X birds had a T base (asparagine, N residue) and no double peaks were seen. The sequence data of the line Y birds showed that the C base was most common with mean 89.3% (±6.6SEM), which equates to a serine residue being encoded, and indicates that the group of ten individual birds, were representative of a larger bird population. However, only four out of the five pooled DNA samples for line Z birds provided adequate sequencing data and so the number of birds represented within this data was reduced to 80. Line Z birds showed an equal occurrence of both SNP alleles

(51.2% C and 48.8% T, ±1.2 SEM) and so approximately half encoded an asparagine amino acid and the other half a serine amino acid, again consistent with individual bird cDNA sequencing data.

Line of birds	T base frequency/%	C base frequency/%	Standard error
X (n=120)	100	0	0
Y (n=120)	10.7	89.3	6.6
Z (n=80)	48.8	51.2	1.2

Table 4.8: Percentage frequencies of SNP bases for the novel SNP in the pooled genomic DNA samples.

Summary of AvBD1 SNPs:

Three SNPs were detected within the peptide coding sequence of the AvBD1 gene examined in the three Aviagen chicken lines. All three SNPs were non-synonymous directing the insertion of different amino acids into the mature AvBD1 peptide.

The three lines of birds showed distinctive combinations of the three SNPs and these are shown in Table 4.9. Line X birds most commonly had the amino acid combination NYH, whereas line Y had SSY, and line Z had N/S YY residues. The potential effects of these different amino acids on the anti-microbial properties of the mature peptides are presented, and discussed in Chapter 6.

Line	**SNP and amino acid base**		
	SNP VB1	**Rs15457749**	**Rs15457747**
X	T Asparagine **N**	T Tyrosine **Y**	C Histidine **H**
Y	C Serine **S**	G Serine **S**	T Tyrosine **Y**
Z	C/T Asparagine/serine **N/S**	T Tyrosine **Y**	T Tyrosine **Y**

Table 4.9: The most frequently estimated alleles for the three non-synonymous SNPs located in the mature peptide of AvBD1 in the three lines of chicken. The single letter code for the appropriate amino acid is given.

A fourth SNP, rs15457745 was found to occur between exons 2 and 3 of the AvBD1 gene (Figure 4.2), and was entered into the Illumina panel. As this SNP was located within an intron it was not amplified in the cDNA of the individual birds, and thus was only detected in the chromatograms of the pooled genomic DNA samples. With regard to this SNP, line X birds predominantly had a T base at this site (98.3-100%) as did line Z (84.3-92.2%), but line Y birds had a higher frequency of the C base (78-95.2%). All percentages were based on Illumina and pooled sample data.

4.5: SNPs within AvBD4 locus

Of the panel of 49 SNPs that were submitted as part of the Illumina study, two were identified in the AvBD4 locus but only one SNP was found in the DNA of the three lines of birds used in this study. This SNP was located in the 5' untranslated region (5' UTR) of the gene.

4.5.1: SNP rs16341536

Figure 4.9 shows the exonic regions of AvBD4 marked against the genomic DNA, the location of the single SNP, highlighted in yellow, and the primers used for the PCR amplification reactions.

I) Frequency from Illumina data

The previous studies relating to AvBD1 had shown that the Illumina data was very comparable to that obtained through the sequencing of individual and pooled bird DNA samples. For this reason it was deemed adequate to only consider Illumina data to ascertain SNP frequencies for the polymorphism rs16341536 in the three Aviagen lines. No individual or pooled bird sequencing was performed.

The calculated frequencies of the four genotypes of SNP rs16341536 are presented in Table 4.10.

Figure 4.9: The genomic sequence for AvBD4 with the primers used for sequencing underlined and the one SNP marked as a coloured highlighted box (yellow).

The exonic regions are highlighted in peach but the AvBD4 gene is in bold ink. The SNP within the 5'UTR region is high-lighted and within a region recognised as exonic but does not code for any of AvBD4 peptide (or other peptides). The ATG start sequence encoding the signal peptide is in red but the remainder of the peptide is in black and bold and spans exons 1, 2 and 3. The forward and reverse primers used for measuring gene expression are in green and underlined (F in exon 1 and R in exon 2 and 3). The letter N marks regions where the genomic sequence is still unknown. The two transcription factor binding sites for Thing1-E47 are underlined in black and in red ink respectively.
SNP - yellow =rs16341536

```
110276382 CATGACAGAAACCTTGAGCAAGTCGTTGTGGTCTAACGTTGCACAGGCAGAGGTTCCAAG 110276323
110276322 TTCATTTTGCAGGCAGCTCAGCGCATTTCTTGAGGTGATGCTGCAACGTTTTGGCAGCAA 110276263
110276262 TACCTTGGTCCCTCACAGGAGGACGCTAACAGAGAAGATCTGCTGAACGGCGCCTCGACT 110276203
110276202 CTCTCCAGAACTGCAGACATCTCCTGATTGGCCCCAGGAAGGCTGTGAAGAGCAAGCATC 110276143
110276142 CTTCCCAGCCTCCAGAAGCCACGTAGAGCCACGCTCTGCTCCTCCATCTTAATGCGTTTG 110276083
110276082 GGTGGCTGACGCTGATCTGCAGGACTACTCCAACCCAGAGCCTCCTCTTTGGTGGTAATG 110276023
110276022 ACCCAAATCTTCTCATTATTCAGCCTCTCTTCTACAGCTCTGTACTTCTGAAAATGGCTG 110275963
110275962 GAGATTTCAGATCTTCCATGGATAGCTGCTTTACTCTGTCGTAACCTTAACTGTGCAGTG 110275903
110275902 ACTCAGTTCATCAAGCATTAGTTTAGGCAAATATTGATTGGAAAGAAGAAATGTTTGTTG 110275843
110275842 GCTGCAGAAGGAGATCTAGGAGGAAATTTGTCTTGGAAATAGTTCTGGAATTGAACAAAA 110275783
110275782 AAGCGGTGGTTGGACTAGATGACCTTCATGGTCTTTCCAACCTTAATGATTCCTTCTCTA 110275723
110275722 CTCTTGCTTTAGAAAGGTTTGAGAACGTGCTTCGTGTTGCCTTGCATGAATGTAGTAAGA 110275663
110275662 ATTGTCATGTTCTGGTTTCTGAATTAACAACTTCCACAGAGCAGGTTCAATTTATTGCAG 110275603
110275602 TGTATTGCAACATGTCAAGCAATTTAGGATAAACGTCTACTTAATTAACATGGTAATTAA 110275543
110275542 AAGTTGGATTTAAAATGGCATAGTTCTGCACAGAAGCTTCTGCCATGATCTTACGTGTGG 110275483
110275482 TCCAGGGAACCCTTAAGACAAAGCACATGATTGTGAAGAAAGTGTATTCTATATGATTCT 110275423
110275422 CAATGATAATTTCTGTCCTTCACTCCTCAGCCCACTGTGTCTGTAGGTGGACAACATCTC 110275363
110275362 AGTGTCGTTTCTCTGCAGTGACAGGATTTCCCAGTCTGCCTTCTGCCATGAAAATCCTTT 110275303
110275302 GCTTTTTCATCGTGCTCCTCTTTGTGGCAGTTCATGGAGCTGTGGGTAAGGAGTAAGTGA 110275243
110275242 AAGCGTGAGGCTGTATACAAGCCGTATGATATTGGTGTCTCATAAAGGTCTTCTGTCCCC 110275183
110275182 TTTGGGAGGTGGCCCAGTTTGGATATTAGTAAAAATTCTCATAAGGAGCAGTGCTGCAGT 110275123
110275122 GGCACAGCTGCCCAGGGGGGCGGTGGGGTCACCATCCCTGGAGGTGTTCAATGTGGAGAT 110275063
110275062 GTGGCGCTGAGGGACATAGTGGGCAGTGGGCACGGTGGGGGTGGGGTTGGACTTGGGGAT 110275003
110275002 CTTAGAGGTCTAATTGCAACCTGAGTGATTCTATGATTCCATGAATAGGGTGGTAAGTTC 110274943
110274942 CTCCAGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 110274883
110274882 >N<NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 110274343
110274342 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTCGATTCTATGATTCCATGAATA 110274283
110274282 GGGTGGTAAGTGTCCTCCAGGTGATTATGGATGGGAAAAGACTGTGACGGATTGAGAAAG 110274223
110274222 AGAAGGGAGAAGTGGGAGAAATATCGTATCTGCAACAGTCTCCCTTTTTCTTTTCTTTCT 110274163
110274162 TTTTTTCAATTTTTCTTTTCTCTTTTTTAAATACTGCAGGCTTTTCCCGTTCTCCAAGAT 110274103
110274102 ATCACATGCAATGTGGATATCGCGGGACCTTCTGCACCCCTGGGAAATGCCCTCATGGGA 110274043
110274042 ATGCTTACCTGGGGCTATGCCGTCCCAAGTATTCTTGCTGTAGATGGTAAGATTAAGACT 110273983
110273982 TGACTATGGCTAAACTGACTTCCCAGATTTTAAGTTCTATATGGTGGGATTTTCCCCTTC 110273923
110273922 AACTTAGGTGTGAAAACCCTGTACTCTTCTTTCTTTTGCATAGGTTGTAGTGTGAATAAT 110273863
110273862 TGCACAGGATCTCCAGAAGTCTGGAAATGGTCTCTTCTTGCATTGTTGGATTTGGGGAAC 110273803
110273802 CATTGTACCGGTCATTCTTTTAACAAATTTTTGCCATTCTTTTAATAAAAGCAATATCTG 110273743
110273742 GGGAGATTGTATGGCAGCCATGTGTGTTTTTGTTATTAATTTCATTAGAAATAGTATTAA 110273683
110273682 TCTTCAGAAGTTTCAACAAGATAAGACTTCTGAAATCAGATTCAGGTATGTTTAGTTTAC 110273623
110273622 CAAACTAAACTGTTTCCCCTTTCATGCTGCTGGTACCAGCATGTGCTCCACCCAGACCTG 110273563
110273562 CTAGGAGCCACTGTCCTGATCTCAGCTGGAGTCA                          110273529
```

Exon 1

Exon 2

Exon 3

Line	TT	TC	CT	CC
X	98	1	1	0.01
Y	100	0	0	0
Z	33.6	24.4	24.4	17.6

Table 4.10: Percentage frequencies of four different genotypes for SNP rs16341536 within AvBD4 locus.

For bird lines X and Y nearly all (98-100%) of the 200 birds were homozygotes for the TT combination. In contrast line Z birds showed different calculated frequencies for all four genotype combinations, 33.6% TT, 48.8% TC/CT and 17.6% CC, respectively.

II) Potential transcription factor binding site (TFBS)

As the SNP rs16341536 is located in the 5'UTR region of the AvBD4 gene, it was investigated whether there was a recognized TFBS at this location, and if so whether its functionality was dependent on whether a T or C base was present. This was achieved by pasting a section of the genomic DNA sequence (coded 110276143-110276262 as shown in Figure 4.9) and its two SNP forms into a specific website (CONSITE (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite) to identify specific TFBS sequences.

A potential TFBS, Thing1-E47 (TCTCCAGAAC), was identified in the T allele form of the SNP and on the reverse strand (Figures 4.9 and 4.10). When the C form was analysed the TFBS was not identified. No other TFBS could be identified at this location, although a second potential Thing1-E47 site was found adjacent to the first (Figure 4.10). To date this TFBS it is not currently documented within *Gallus gallus*.

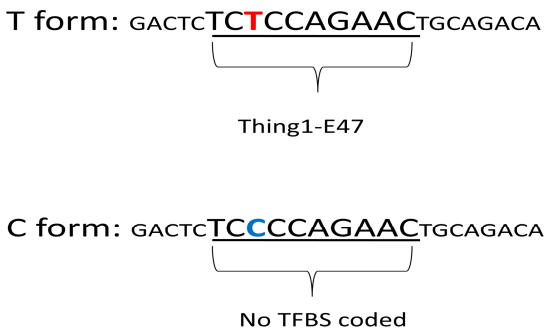


Figure 4.10: The T and C forms of SNP rs16341536. When the T base is present a potential TFBS is encoded (Thing1-E47). The TFBS is underlined and the SNP is marked in colour. NB. This is on the reverse strand of the genomic DNA.

Summary of AvBD4 SNP:

Only the Illumina data was available for SNP rs16341536. Alleles containing a T base at this location were most common in bird lines X and Y, respectively. This SNP is located in the 5'UTR of the gene and encodes a potential TFBS, Thing-E47. Line Z has a mixed genotype population but again the T form was the most common (34%).

4.6: SNPs within AvBD10 locus

Of the seven potential SNPs in the AvBD10 locus entered into the Illumina panel, three SNPs were identified in the Aviagen lines included in this study (Table 4.1). These SNPs are marked on the AvBD10 genomic sequence (Figure 4.11) and their details are presented in Table 4.11. None of the three SNPs identified code for the actual AvBD10 peptide, one was located intronically, one was located upstream of the ATG start codon and the third was found downstream of the polyA tail of the gene coding sequence.

Of the three confirmed SNPs, only SNP rs15457607, high-lighted in blue in Figure 4.11, was detected by PCR using the primers shown. A PCR amplification product 1883 bp in size was predicted when the genomic DNA was used as the PCR template, and indeed a product of this size was obtained (Figure 4.12). However, all sequencing of such products failed (returned as N, unknown bases). The Illumina data had been shown to be highly comparable to both individual and pooled DNA sequencing SNP data and so in this case amplification and sequencing of individual and pooled DNA was not pursued. Therefore only the Illumina data was used in the analyses of this SNP.

Figure 4.11: The genomic sequence for AvBD10 with the primers used for sequencing underlined and the three SNPs marked as coloured (yellow, blue and purple) highlighted boxes.

The exonic regions are highlighted in a peach colour. The start ATG sequence is in red but the remainder of the peptide is in black and bold (peptide spans exons 1, 2 and 3). The same forward and reverse primers were used for both forms of sequencing and are in black and underlined (F in exon 2 and R in exon 3).

SNPs Yellow=rs14411785
Blue= rs15457607
Purple=novel SNP

```
110227725 CGCGATATGGGGGATAACCAATTGCTTGACAAAAGGTTGGGTGCAGTTACTTATTGACAG 110227784
110227785 GGCACTCAGCCGTGAAACTTTCACAGTCCCACAACCCCTATAAATGCCAGGCGCCTTCCC 110227844
110227845 TTGCCTCTTCCTCAAACAAACGTCATCCTCCTTCGGTCTTCGAGGAATTGGGGCACGCAG 110227904
110227905 TCCACAACTGAGCCATGAAGATCCTCTGCCTGCTCTTCGCTGTTCTCCTCTTCCTCTTCC 110227964
110227965 AGGCTGCTCCAGGTGAGATACAACGTAAATCCAACCACTGAAAGGAGAGGCAAGCACAAG 110228024
110228025 TGTGTGCCGAGGAGGAAAAGGAGGCTGTGGCCAACTGATAGGCAATATACGATGCTGGTG 110228084
110228085 CTGTGCACTGGATTAGCCCAAGAACATCTGTTCTCAGGCAGACTCTATTGAAAAAAAATC 110228144
110228145 AGCAGCATGTTATTTATTGTATATTCATTTTGTATTGTCTATTTATCCTTCACTGTTTAA 110228204
110228205 AACTAAAAAGTGAGATTTTAACGCCTTCCTCTCCCCACAGGCTCAGCAGACCCACTTTTC 110228264
110228265 CCTGACACCGTGGCATGCAGGACTCAGGGGAATTTCTGCCGTGCTGGGGCATGCCCCCCC 110228324
110228325 ACCTTCACCATCTCTGGGCAGTGCCATGGGGGGCTGTTAAACTGCTGTGCCAAGTAAGTG 110228384
110228385 CTGCGGGGCGGAAGGGAGAGCATTTTCTTTTTGCCATCTGCCTGCTGCACCTCTGTGCGG 110228444
110228445 GATGCACACATCTGTGGGGGGGGTCTTGCAGGGCAGCTATTTGTGAGGAATATGTATGGA 110228504
110228505 GGGTGAACATAAATATACTGTATAGAATCATGGAATCATAGAAACACCACGGTTGGCAAA 110228564
110228565 GACCTTTAAGGTCATCCAGTCCAACCATCCACCTACCACCATTATTTCCCCACTAAACCA 110228624
110228625 TGTCCCTCTGTGCAATATCTAAACACTTATTGAACACCCACACGGATGGCGACCCCACCA 110228684
110228685 CCTCCCTGGGCATCCTGTGCCACTATATTGCCACCCTTTCTGAGGAGAAATATTTCCTAA 110228744
110228745 TATCCAACCTGACCCTCCCCTGGAGCAACTTGAGGCCATTCCCTCTTGTCCTGTTGGTAG 110228804
110228805 TTTTGCTGGAGAAGTGGCCGTGTTAAAAGAGGTAATTAATTTTGATGGAAGCTGTGTACA 110228864
110228865 AGAGCAAAAGTTCAAGAGGGCCAACATTTCCCAGAATTCAGCATTTTCTTCTTGAATTTG 110228924
110228925 AATTTAGATGGGAAAAAAACTCAAAGGAACTTACTACAAAAATATCTCACAGTATTTTTA 110228984
110228985 CCCGTTAGAGGGAAAAATGTGTCCTCCATTCCTCTGAACTTTTCCTTTCTCAAAAGACCT 110229044
110229045 AGATTTCAATAAGGAAAACATCACTTCTTCCTCCCATAGCTGTGCATCTCTTAGGTGTGA 110229104
110229105 ATCTGTGTGTGCATGAAGACAATTGCATTTCTATTGCACTTTTGACCTTGGAGACCAAGT 110229164
110229165 AGTAGCAAGACTTTTTCTTTCCTTAAGGATTTAAACACACATTTGAAGAGTGCACTTCAG 110229224
110229225 CTTAGGATCAAGCATACTTCTTAGTATACATTTACTTGCTTAGAAGCTGTCAGACTGTGG 110229284
110229285 TTTCAAGAAACCCAAGCCACACCAATATTAGGACACGTTCACTGCCGTGATATCAATGAC 110229344
110229345 GTTAAGGAAACATCCGAGAAGAACTGGGGCTGGGGAGTGAATGAGACCTCCTGAGGTTTT 110229404
110229405 TTTCAAGTACAATTTTCTGCAGAAATACTCCTTCTGAGTAGAATGTCCCATGCATGATAT 110229464
110229465 CAGTGGATGCTAATTGTTCAGGATTCAATCTCTTCTTATTGAGATGCTCTGTTTTCAGCT 110229524
110229525 GGCTTGATTCACTACGGTAGAAGCTGAAACCAATAAGGTATTCTGATGTACAATATCTTC 110229584
110229585 TTTTTCCAACTGAGAATTCAGTCTACTTGGAAGGAAATTGGACACAAAGGATTTGATTCC 110229644
110229645 ATAAACAAAGCTGTGGTGCTTAGTGGCATTTGAGGTGCCTTGGATTATCTAAACCACATT 110229704
110229705 TATAGGTCTGGCTGATATGGACCTATGGGAAAACAATGCTGGAGACCAAGCAGACACCTA 110229764
110229765 CTCCAGGGCTCTTCAAAAGGCACTCAACTTGTGTGTTTAAATCATGAAACAAAATCCCTA 110229824
110229825 GTCTTTATTTTGAGCAGAGCTGAATCAGTAACTTACTACCAGTGGTAACAGAACTGGTTT 110229884
110229885 GCCAGTGAGGACAGTTCATGTAGGCTGGTTGTGTGCAGACCTACACACCCAGCGCTCTTA 110229944
110229945 GGGACCCTTCTTTGCAGGCATCCACGTGCAGGTTTTTTACAAACTGCCTCTCAAACTCAA 110230004
110230005 CATATATGTTTATTCTTAATGGTAATTAGCAGTAATTGACCGAGCTTCATCCCACTTCAG 110230064
110230065 CAAAGTAATGAGGTTCTTTCTATCCTTACAGGATTCCGGCGCAGTAAGGAAAGGCTTTCA 110230124
110230125 CTGGGCCATTGCCCTTGTACCAGCAACATTTCCACTGCCTTGAGGAGCTGATTGTGATGG 110230184
110230185 ATTCCTCCTCTGTCTAGACCCTCTGCAAATGAGCATTCAAAGAAGCTTCCAGAGGGCACG 110230244
110230245 TCCTGTTAGCACACTGCTTCTGGAGATCCAGGTTTTCCTTGCTGAAGTCACTCCTTTTTT 110230304
110230305 TTTTGCTTTTAATTAATACACAAGCCACTTTGGCACCTTGGCCACCTATTCCAGCCTACC 110230364
110230365 TCACGGGATCTTTGGATGCTCAGTCACTTIGGGTTCATGCAGCTCTTGGAGCATATGAAG 110230424
110230425 CAATACATACGTTATTATTTTATTCACACACCCAAATAAATGTCTTCTTAAACCAAAAAC 110230484
```

Exon 1

Exon 2

Exon 3

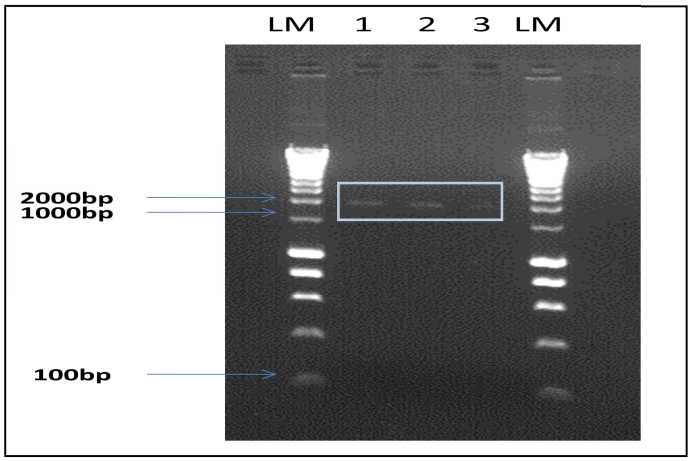


Figure 4.12: Amplification of genomic DNA using the AvBD10 primers (shown in Figure 4.11). Lanes 1-3 show a single band approximately of 1,880 bp (in box). LM = low molecular weight marker (no negative control).

SNP	**SNP code**	**Genotype**	**Location/type**
1	Rs15457607	T/C	Intronic
2	Rs14411785	T/G	Upstream of ATG start codon
3	Novel	T/C	Downstream of PolyA tail

Table 4.11: Details of the three SNPs identified in AvBD10

4.6.1: SNP rs15457607

This SNP is coded in blue in Figure 4.11 and Table 4.11.

A) Frequency from Illumina data

SNP rs15457607 is located between exons 2 and 3 of AvBD10 (Figure 4.11). The calculated frequencies of the four genotypes for SNP rs15457607 based on the Illumina results are given in Table 4.12. No individual or pooled sequencing data is available for this SNP.

Line	TT	TC	CT	CC
X	77.4	10.6	10.6	1.4
Y	96	2	2	0
Z	20.25	24.75	24.75	30.25

Table 4.12: Percentage frequencies of four different genotypes for SNP rs15457607 within AvBD10 locus.

In both lines X and Y, the TT genotype was the most abundant, 77.4% and 96% respectively. The 200 line Z birds that were analysed showed a mixture of the four genotypes which equates to approximately 50% having a T base and the remainder having a C base at the location of this SNP.

4.6.2: SNP rs14411785

This SNP is coded in yellow in Figure 4.11 and Table 4.11.

I) Frequency from Illumina data

This SNP is located upstream of the AvBD10 ATG start codon (at base 110227785 in Figure 4.11). The predicted frequencies of the four genotypes were calculated from the Illumina study for the three lines of birds and shown in Table 4.13.

Line	TT	TG	GT	GG
X	88.4	5.6	5.6	0.4
Y	74	12	12	2
Z	42.3	22.7	22.7	12.3

Table 4.13: Percentage frequencies of four different genotypes for SNP rs14411785 within AvBD10 locus.

As for SNP rs15457607, bird lines X and Y showed similar results. For these two lines, the TT genotype was the predominant form within the populations, 88.4% and 74% respectively. The calculated predicted frequencies for line Z again showed mixed genotypes across the 200 birds analysed, but the TT form at 42.3%, was the most common pattern of the four.

II) Potential transcription factor binding sites (TFBS)

SNP rs14411785 is located upstream of the start codon (Figure 4.11) and as discussed previously this is a region containing TFBS and involved in gene regulation. The two forms of the genomic sequence were analysed using the TFBS web-based package to determine the presence of any such sites

(http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite). No potential TFBS sites were located at this location for either of the two forms of the genomic sequences.

4.6.3: Novel SNP 'VB2'

This SNP is coded in purple in Figure 4.11 and Table 4.11.

A) Frequency from Illumina data

The predicted frequencies of the novel polymorphism across lines X, Y and Z are presented in Table 4.14.

Line	TT	TC	CT	CC
X	72.2	12.8	12.8	2.2
Y	96	2	2	0
Z	19.4	24.6	24.6	31.4

Table 4.14: Percentage frequencies of four different genotypes for the novel SNP within AvBD10 locus.

As for the previous two SNPs, the TT genotype predominated in bird lines X and Y. The results for bird line Z again showed mixed frequencies across the four genotypes.

Summary of the three AvBD10 SNPs:

Three SNPs were detected within the AvBD10 locus. None of these SNPs were located within the coding region of the gene but were found to be located upstream of the ATG start codon, within an intron and downstream of the PolyA tail. For all three SNPs, bird

lines X and Y showed similar distributions, with the TT genotype being most predominant. Line Z birds showed mixed populations across all four genotypes for each of the three recognised SNPs.

4.7: Discussion

The focus of this chapter was the investigation of single nucleotide polymorphisms (SNPs) within the avian β-defensin locus of three Aviagen bird lines - X, Y and Z. Investigation of SNPs as genetic markers for particular traits including disease susceptibility and resistance is becoming increasingly popular across many livestock species including chickens. Indirectly immune system traits will have been investigated as part of general bird fitness selection, but not in isolation. Moreover the presence of specific SNPs associated with disease susceptibility can help identify individuals and/or specific groups, in the case of poultry, particular lines, at risk. In addition where strong correlations between SNPs and disease are established in domestic livestock, controlled breeding programmes can be implemented to select for or 'breed out' targeted genes.

Chicken breeding over the last few decades has focused on traits such as muscle deposition and rate of growth. Historically, immune system-related traits have not featured in the selection process. Investigations into the presence of single nucleotide polymorphisms (SNPs) either as a form of quantitative trait loci (QTL) or related to performance with an immunity focus are now increasing in number (Ye, Avendano et al. 2006; Biscarini, Bovenhuis et al. 2009). These are being driven by increasing concerns regarding endemic flock health problems but also food security caused by recent disease outbreaks including Avian Influenza, the banning of antibiotics as growth promoters, and the ever-emerging antibiotic resistant strains of bacteria. The Aviagen lines selected for

this study form three of the pure lines used for commercial broiler breeding programmes and it is for this reason they were selected SNP analyses.

In humans the defensins are known to be important components of the innate immune system with reported anti-microbial, chemotactic and immunomodulatory effects (Lai and Gallo 2009). In poultry there are 14 AvBD genes and although their tissue expression patterns have been well reported their actual roles in defense are less well explored. In addition there are few reports of the effects of SNPs on AvBD gene expression and peptide function. As part of a large genomics initiative by Aviagen Ltd I created a panel of 49 SNPs in the AvBD locus to investigate potential differences in occurrence of the polymorphisms between lines, X, Y and Z birds. Fifteen SNPs were found to exist in the three lines of birds (Table 4.1). SNP frequency at a rate of 13.2 per kilobase within the AvBD locus has been reported, and with the defensin locus being in the region of 86kb in size, approximately 1135 SNPs would be predicted across the whole region (Hasenstein, Zhang et al. 2006). The reasons for the observed >70 fold difference in numbers is difficult to explain. It may reflect the original number of SNPs included in the Illumina study or it may reflect the fact that defensin functions are fundamental to the host and as such the genes are not readily susceptible to mutation. Of the SNPs identified via the Illumina study, one SNP rs15457749, was located in the exonic region of the AvBD1 gene and was non-synonymous. This AvBD gene was therefore selected for further evaluation as it was hypothesised that the different lines of birds could potentially synthesise peptides with different amino acids. As AvBDs are anti-microbial agents, functionally this could have subsequent downstream effects,

including those involving bird health and disease resistance. The selection of AvBD1 was further supported by the identification of a further two non-synonymous SNPs in AvBD1 following sequencing of cDNA from individual birds. This finding of three such 'exonic located' SNPs was very interesting as defensins have been shown to be evolutionary conserved (Radhakrishnan, Fares et al. 2007). In addition a fourth SNP was located in the intronic region of AvBD1.

With regard to the location of SNPs and defensins, most of the published work focuses on those located within introns. In particular Lamont's group has focused on intronic SNPs within the AvBD11, 12 and 13 genes. These investigators, using birds with different SNP combinations, identified different caecal bacterial yields following infection of the birds with *Salmonella* species. These authors suggested that the intronic SNPs may well be acting as linkage markers with another functional polymorphism associated with bacterial colonisation of the gut (Hasenstein and Lamont 2007).

A single SNP was located in the 5'UTR region of AvBD4 (Table 4.1). This gene had been shown to encode a peptide with anti-microbial activity against *Salmonella* spp (Milona, Townes et al. 2007), and was therefore selected for further analysis. Three SNPs were identified in the AvBD10 locus, and because there is little in the literature relating to this gene, apart from its unusual tissue expression panel, this gene was also selected for further analysis. The three lines of birds had very distinct frequencies of the SNPs, in particular with regard to the three non-synonymous SNPs located within the AvBD1 mature peptide encoding region and the subsequent alteration in amino acids. Four

combinations of SNPs were found to exist within this encoding region in the bird populations that were analysed and these therefore lead to four combinations of amino acids at these three sites. Line X birds were found to encode the amino acids NYH, line Y birds predominately encoded SSY residues and the line Z population of birds either NYY or SYY amino acids at the three respective sites of the SNPs in the mature peptide of AvBD1.

In this study the Illumina data was compared with sequencing data from individual and pooled bird DNA samples. The Illumina and the pooled genomic DNA SNP frequency were comparable across the three lines, which reinforced the use of the technique described by Ye, McLeod et al. (2006) of using pooled DNA samples to determine SNP frequency within a population. Benefits of this technique include the analysis of larger populations with reduced sequencing being performed, thus allowing a better representation of what is happening in the population as a whole, rather than in an individual or small selected sample numbers, in a cost and time effective manner. Essentially the Illumina data provides a global picture of different genotypes while in contrast the individual sample data provides only a local impression i.e. at the level of mRNA.

It must be noted that a disadvantage of the individual bird sequencing was that the use of cDNA as the template meant that only exonic SNPs could be detected. As observed from the Illumina data, heterozygotes for some of the SNPs e.g. rs15457745 are well represented across the populations of chickens, in particular within the line Z birds. Thus

when individual bird sequence outputs are considered, there is a risk that the presence of any heterozygote birds will be masked if only one of the alleles has been transcribed to mRNA. Interestingly, where double peaks were observed in the DNA sequencing chromatograms they were often not of equal amplitude suggesting that there were higher levels of one transcript relative to the other. This perhaps suggests that one gene allele is 'switched on' and being transcribed more than the other.

An area of study which perhaps warrants further investigation is gene copy number of AvBD1 within the three groups of birds, in particular with reference to the frequencies of the SNPs if multiple alleles exist. Differences in gene copy number in the three lines of birds could also have had an effect on the expression data considered later in Chapter 5. This may prove an interesting area when differences in reported gut health between the lines of birds is considered as there have studies linking human defensin copy number and specific disease susceptibilities (Hollox 2008).

Apart from those SNPs identified in AvBD1, none of the other SNPs were located in the peptide coding region of the genes. The relative absence of non-synonymous SNPs suggests that the genes that encode the AvBD peptides are highly conserved and this is of potential benefit to the chicken population. A link to the importance of the innate immune system as a first line of defense, and the roles that AvBDs contribute to this, could be the reason for the relative absence of non-synonymous SNPs. The presence of non-synonymous SNPs within the mature active peptide of AvBD1 is therefore an exciting finding.

Studies in other species have linked defensin polymorphisms with changes in disease susceptibility and one example is the Human Beta-Defensin 1 (HBD1) gene and airway colonization with *Pseudomonas aeruginosa* (Tesse, Cardinale et al. 2008). The authors reported that the presence of two SNPs in the HBD1 non coding region leads to an increased susceptibility to bacterial colonisation in those individuals homozygous for these two SNPs. A SNP associated with HBD1 has also been linked with the oral carriage of *Candida albicans* (Jurevic, Bai et al. 2003). A protective role of two HBD1 SNPs one, of which was the same SNP as described in the *Candida* study, has been reported in children born to HIV mothers (Ricci, Malacrida et al. 2009). All three of these HBD1 studies, linked to disease susceptibility, have included SNPs located within non-coding regions, it is therefore possible that the intron located SNPs identified in this avian study may be linked to similar properties.

In 2007 Andreescu, Avendano et al. published a study, which involved broiler lines X, Y and Z and used the presence of SNPs to determine how closely certain pure lines of birds were related. The resultant trees suggest that relative to all of the other lines and to themselves, lines X, Y and Z are not closely linked within the study parameters selected. However, the results presented within this thesis show line patterns for some of the SNPs. For example for the three SNPs that are discussed in the AvBD10 locus, similar SNP patterns existed between lines X and Z, in that they have, statistically, the same SNP allele frequencies. In contrast, this pattern is not reproducible when the SNP frequencies in AvBD1 and 4 are considered, as distinct differences can be seen between the three lines of birds.

When the Illumina data for the three Aviagen lines and that from the pooled bird DNA samples were compared, the data was unchanged. This finding indicates that despite the two year gap between the analysis of these two populations, 2007 for Illumina and 2009 for the pooled DNA samples, the SNPs were still present at relatively the same frequencies. This strongly suggests that the SNPs have been selected for over the two year breeding period. An example of a non-coding SNP, located in the 5'UTR, and that may effect gene expression per se is rs16341536, found in AvBD4. A potential TFBS called 'Thing 1-E47' (Figure 4.10) was shown to be encoded by one of the SNP forms. However, this site was identified using a human TFBS database, as none of the websites that are readily available to investigate potential TFBS have data specifically for *Gallus gallus*. Bird lines X and Y were shown to have SNP frequencies close to 100% for the TT genotype, the form which would encode the 'Thing 1-E47' site. However according to the Illumina data only half of the line Z birds, would encode this TFBS. Whether this TFBS is functional in the avian is not known but this TFBS has been shown to be over-represented in sites of co-expressed genes (Huang et al 2005). Interestingly the AvBD genes are expressed at the same time, and within the same tissues, and so could be described as being 'co-expressed'.

In summary novel SNPs and unique SNP allele patterns were determined for the three lines of chickens. A total of four SNPs within the AvBD1 locus, one within AvBD4 and three within the region encoding AvBD10 were detected and characterised within the population of chickens used in this study. The detection of three non-synonymous SNPs within the gene encoding the mature peptide of AvBD1 proved to be an exciting finding.

Chapter 5: Avian Beta Defensin Gene Expression

5.1: Introduction

Defensins are considered to be important components of the innate immune system, being particularly important at the epithelial barrier, a site where the host is exposed to and vulnerable from pathogenic attack. Thus avian β-defensins (AvBDs) may play important roles in relation to the resistance and or susceptibility of birds to disease. This is not only important with respect to bird health but also in relation to food safety and therefore public health.

This part of the study investigated AvBD gene expression in the tissues of birds raised in the two distinctive rearing environments described in Chapter 3, i.e. LH and HH. The expression of three AvBDs genes were studied; AvBD1, AvBD4 and AvBD10, with these gene selections based on factors including the presence and location of SNPs within the gene loci and previously reported tissue expression panels. An emphasis was also placed on the gastro-intestinal system as both AvBD1 and 4 have previously been shown to be expressed in gut tissue. The third gene investigated was AvBD10 and selected due to its unusual tissue expression panel, kidney, testicle and liver, and unreported anti-microbial activity to date.

The use of three distinct lines of breeding birds i.e. X, Y and Z, and sampling at different ages (0, 7, 14 and 35 days of age), also allowed the influence of such parameters on gene expression to be ascertained and any patterns to be observed.

A panel of ten tissues were extracted from each of the birds (one tissue sample per bird); thymus, liver, kidney, testicle, lung, spleen, duodenum, bursa of Fabricius, caecum and caecal tonsil. An emphasis was placed on tissues with an immunological role as well as the gastro-intestinal tract. The latter was particularly important so that a direct comparison between defensin gene expression and gut anti-microbial activities (Chapter 6) could be made.

The aims of the work presented in this chapter were;

- **To explore using end-point PCR and quantitative real time PCR AvBD1,4 and 10 gene expression in panels of tissues taken from three lines of chickens, X,Y and Z, raised in the low (challenged) and high hygiene conditions and at 0, 7 and 35 days of age.**

5.2: Tissue extracted RNA quality

RNA was extracted from the bird tissue samples using TRIzol Plus (Invitrogen). The RNA quality of the samples was assessed by gel electrophoresis to ensure that the RNA had not undergone degradation following extraction. Figure 5.1 shows the electrophoresis pattern for two of the RNA tissue samples analysed as described in Chapter 2. Three distinct bands can be seen showing the 5S, 18S and 28S ribosomal RNA (rRNA) components of total RNA. The good RNA quality of these samples is indicated by the sharp 18 and 28S bands.

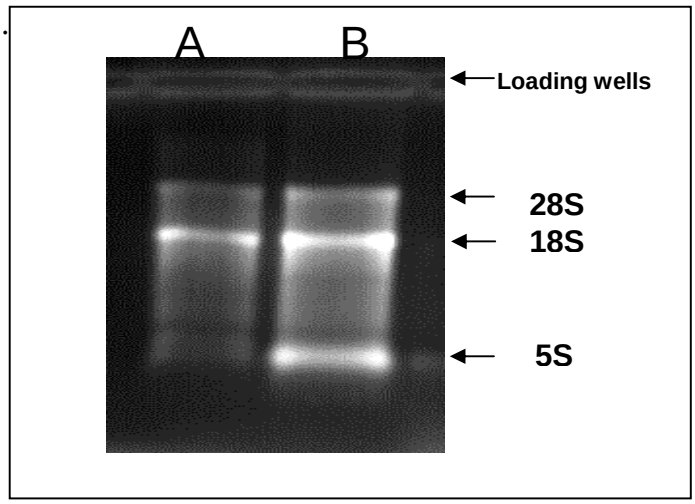


Figure 5.1: RNA gel of A (thymus) and B (liver) from a 7 day old bird. Ribosomal units are marked, 28S, 18S and 5S.

As described in Chapter 2, RNA quality and quantity were also measured using the NanoDrop and these readings supported the visual data from the RNA gels. Table 5.1 shows examples of the readings taken from the NanoDrop to measure the quality and quantity of RNA for a 7 day old line Y bird, which had been reared in the HH environment. The calculated ratio reflects the amounts of 18S and 28S in the total RNA. There should, theoretically, be twice the amount of rRNA for the 28S component compared to 18S and so the closer this ratio is to 2 the better, since deviations away from this value suggest sample contamination or poor extraction.

Bird and tissue	[RNA] mg/ml	Ratio 260nm:280nm
Thymus	1.274	2.00
Liver	2.287	2.00
Kidney	0.414	1.98
Testicle	0.170	1.89
Lung	0.102	2.00

Table 5.1: Examples of RNA quantification following extraction of total RNA from tissue samples from a line Y bird using TRIzol (Invitrogen) as measured by the NanoDrop.

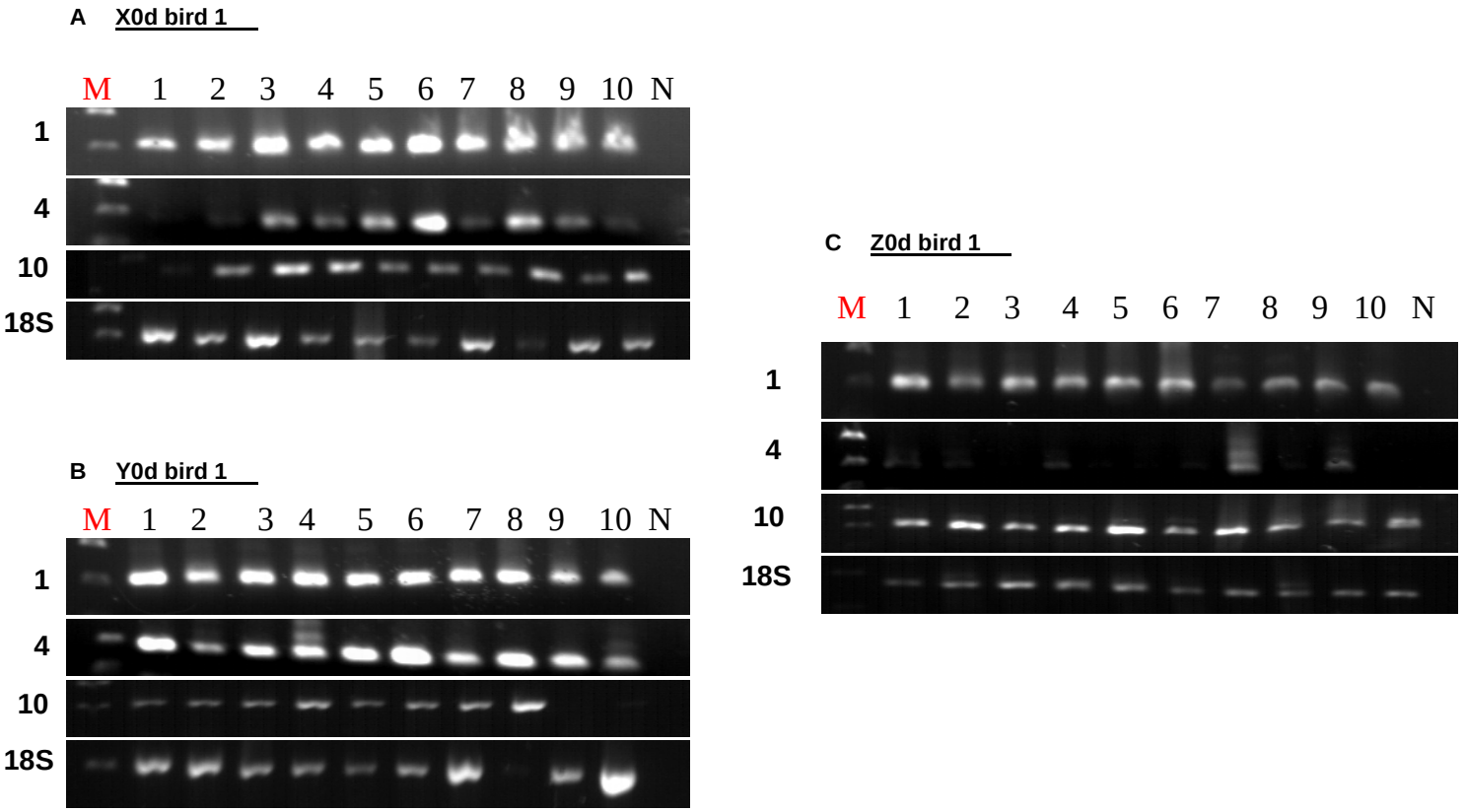
The table above high-lights that there was variability in the total RNA concentrations of the different tissue samples. RNA concentrations ranged from 0.016 to 5 mg/ml. The lower concentrations of total RNA were extracted from those tissues which were smaller in size or difficult to homogenize e.g. the testicles were very small in birds aged below 35 days. Despite low RNA yields from some tissue samples, no problems were encountered with the down-stream application and processing i.e. expression analysis.

5.3: Tissue gene expression panels

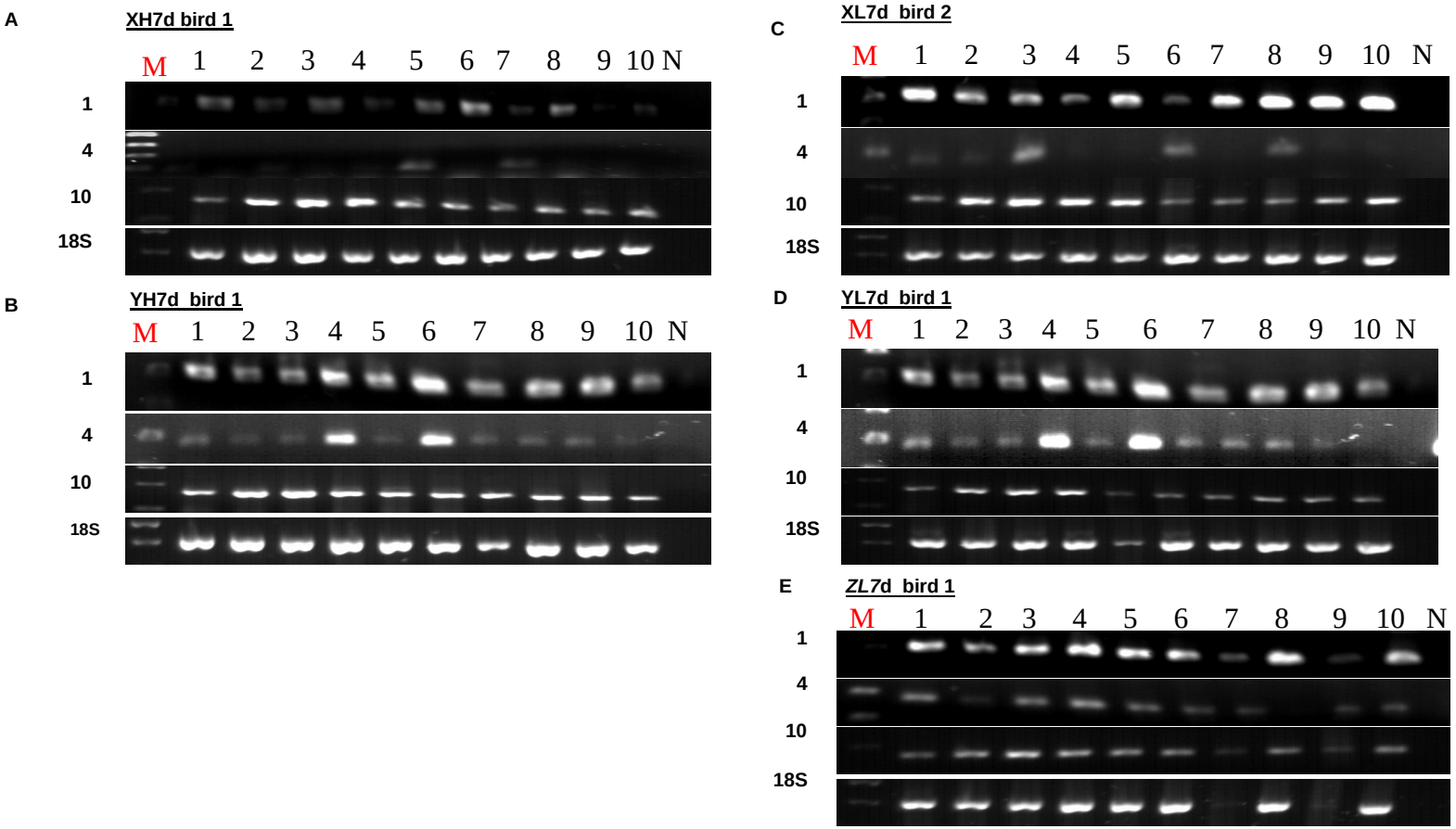
End-point RT-PCR using gene expression primers designed for the detection of AvBD1, 4, 10 and 18S (house-keeping gene) was performed on the extracted RNA from the bird tissue samples. These primers are shown marked against the genomic DNA sequences in Figures 4.2, 4.9 and 4.11, respectively and allowed gene expression patterns to be

determined in the bird tissue samples. As shown in the trial plan in Figure 3.2, ten tissue samples were taken from each bird. Initially end-point PCR was performed on nucleic acid material from two birds from each age, and line, so that gene expression patterns could be visualised and to rationalise which tissues should be further investigated using quantitative real time PCR (qRT-PCR).

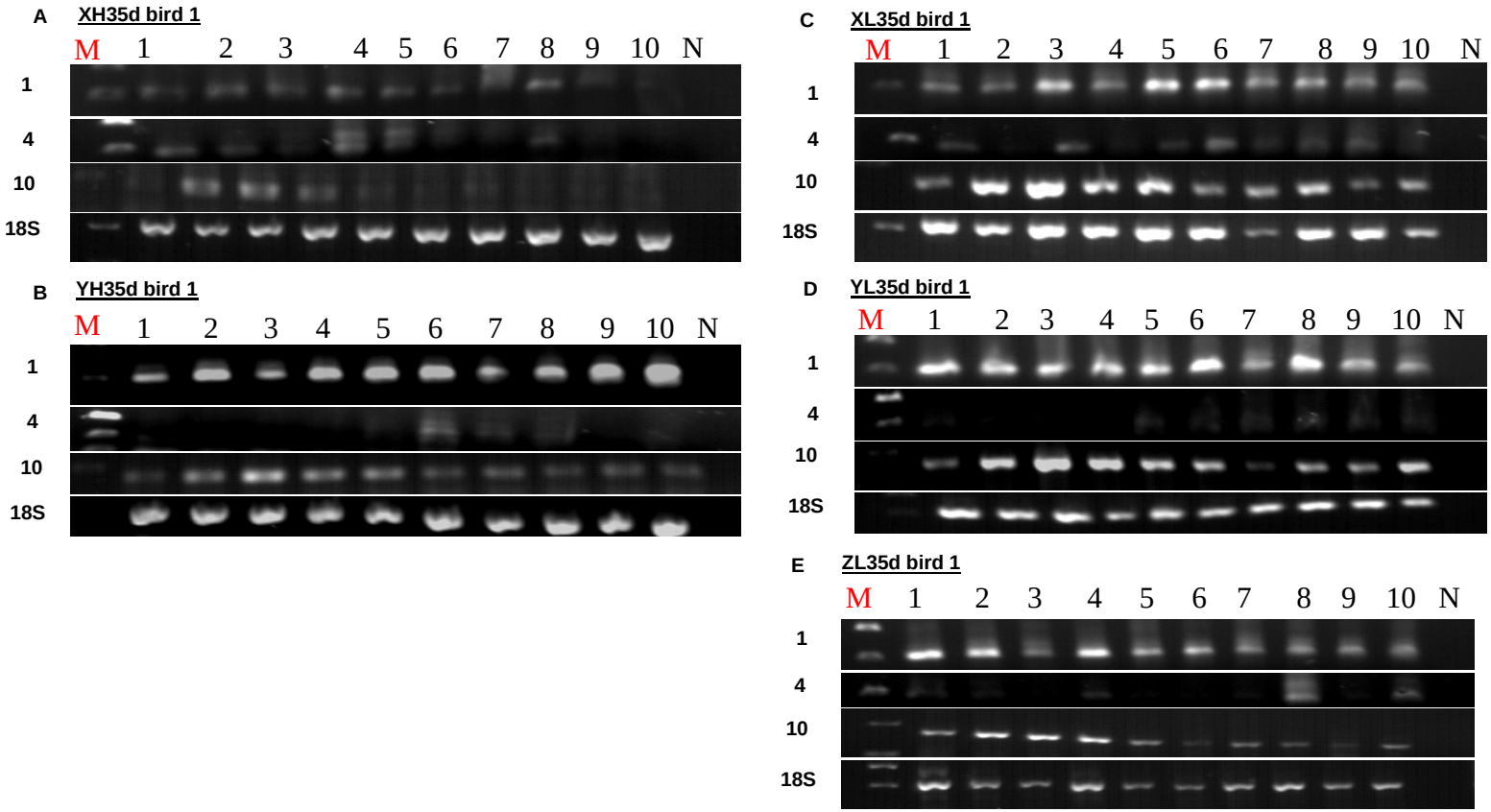
The end-point PCR products for one of the two birds from each age group are shown in Figures 5.2, 5.3 and 5.4. The ten tissues included in these panels were; thymus, liver, kidney, testicle, lung, spleen, duodenum, bursa of Fabricius, caecum and caecal tonsil (labeled 1-10, respectively).



Figures 5.2A-C: Tissue panels of gene expression of AvBD1, 4, 10 and the house-keeping gene 18S for three birds, one from each of the three groups at day 0 (M=marker, 1=thymus, 2=liver, 3=kidney, 4=testicle, 5=lung, 6=spleen, 7=duodenum, 8=Bursa of Fabricius, 9=caecum and 10=caecal tonsil, N= negative control). Panels are taken from individual agarose gels, hence contrast is slightly different between row of panels. 1µg of RNA was used and 30 cycles of amplification were performed.



Figures 5.3A-E: Tissue panels of gene expression of AvBD1, 4, 10 and the house-keeping gene 18S for five birds, one from each of the five groups at day 7 (M=marker, 1=thymus, 2=liver, 3=kidney, 4=testicle, 5=lung, 6=spleen, 7=duodenum, 8=Bursa of Fabricius, 9=caecum and 10=caecal tonsil, N= negative control). Panels are taken from individual agarose gels, hence contrast is slightly different between the rows of panels. 1µg of RNA was added and 30 cycles of amplification were performed.



Figures 5.4A-E: Tissue panels of gene expression of AvBD1, 4, 10 and the house-keeping gene 18S for five birds, one from each of the five groups at day 35 (M=marker, 1=thymus, 2=liver, 3=kidney, 4=testicle, 5=lung, 6=spleen, 7=duodenum, 8=Bursa of Fabricius, 9=caecum and 10=caecal tonsil, N= negative control). Panels were taken from individual agarose gels, hence contrast is slightly different between the rows of panels. 1µg of RNA was added and 30 cycles of amplification were performed.

5.3.1: AvBD1, 4, 10 and 18S expression panels; day 0, 7 and 35 birds

One microgram of RNA was used and thirty cycles were performed for all of the PCR amplifications. Only trends in expression can be commented upon when the tissue panels using end-point RT-PCR were compared, because of the unknown effects of the level of ethidium staining and gel UV exposure.

Data from one of the day 0 birds from each of the lines X, Y and Z are represented in Figure 5.2A-C. The gene expression panels from the second bird tissues was, in all cases, comparable.

AvBD1 gene expression was observed across the panel of ten tissues for all three day 0 birds. In contrast, AvBD4 gene expression appeared more variable across the three day 0 birds. For example in the line X, day 0 bird, the thymus, liver and duodenum produced weaker bands compared to the other tissues. Tissue expression in the line Z, day 0 birds was weak apart from the testicle (lane 4), the duodenum (lane 7) and caecum (lane 9). The line Y, day 0 bird showed strong banding across all tissues following analysis of AvBD4 expression. AvBD10 expression was observed across all of the day 0 bird tissue samples except for caecum and caecal tonsil in the line Y bird panel.

Figure 5.3A-E represents the five groups of day 7 birds. Variability in banding was noted in the XH 7 day bird AvBD1 tissue panel, in comparison the XL bird panel revealed weaker cDNA bands. Differences were also observed between these two birds in relation to AvBD4 expression that was only observed in the lung and duodenum of the XL bird

compared to thymus, liver, kidney, spleen and bursa of the XH bird. In contrast the expression panels of AvBD10 were similar.

Both of the line Y birds showed similar expression across all ten tissues and three genes. Of particular note however were the strong bands for both the testicle (lane 4) and spleen (lane 6) for the AvBD4 panel. AvBD4 expression was not detected in the line ZL caecal tonsil sample. Similar banding for the AvBD1 and 10 panels was observed between the day 0 and 7 line Z bird tissues. Interestingly, more tissues showed AvBD4 gene expression at day 7 than day 0 for the line Z birds.

The gene expression panels for the day 35 birds were different to those for both the day 0 and 7 birds (Figures 5.4A-E). All five birds showed AvBD1 across all ten tissues. AvBD4 gene expression was generally weak for all birds, with both YL and YH having no banding in the thymus, liver, kidney and testicle. AvBD10 gene expression was observed in all the bird tissues analysed. The XH bird had particularly weak banding across all tissues and for all three genes.

The overall observed patterns from Figure 5.2-5.4 were; (i) that AvBD1 gene expression appeared consistent and comparable across most of the bird samples with the exception of the 7 and 35 day XH birds, (ii) AvBD4 gene expression bands were stronger in the line Y birds at days 0 and 7, but all birds showed panels suggestive of low levels of expression at day 35, (iii) greatest variability in bands was observed with AvBD4 gene expression panels and (iv) AvBD10 bands appeared most intense and uniform across the bird tissue samples analysed.

5.4: Quantitative real-time PCR (qRT-PCR)

QRT-PCR was performed so that the gene expression levels of the AvBDs could be quantified. Analyses allowed patterns of gene expression between tissues, bird lines, ages of birds and rearing environments to be compared.

5.4.1: Amplification curve of PCR products in qRT-PCR

An example of an amplification curve relating to serial dilutions of the 18S plasmid is shown in Figure 5.5. The level of fluorescence (Sybr green) is indicated on the y axis and the number of amplification cycles is indicated along the x axis. The more dilute, i.e. the less starting PCR template present, the higher the number of cycles needed to achieve the crossing point (CP) value, that is the point at which the fluorescence of the Sybr green reaches a threshold level that is constant for all reactions. The dilutions are colour-coded with the most concentrated plasmid curves on the left side and the most dilute on the right.

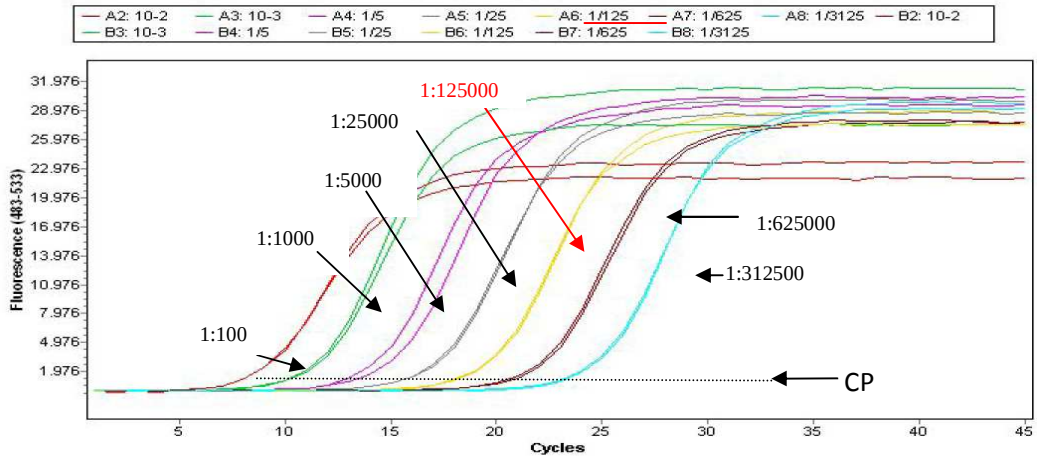


Figure 5.5: Amplification curves of diluted 18S plasmid. The most concentrated plasmid is on the left side of the graph (1:100) and the most dilute (1:312500) on the right hand side. The standard selected for PCR of the tissue samples is marked above with a red arrow (1:125000 of original stock) and the sample identity is underlined in red in the key above the graph. Each sample was performed in duplicate and so there are two curves for each dilution. A hypothetical level of fluorescence has been marked with a dashed line which corresponds to the determined CP value.

5.4.2: Standard curves

A standard curve was automatically plotted for each series of samples analysed (Lightcycler 480 Roche), and an example of one of the standard curves for 18S is presented in Figure 5.6. The dilutions of the 18S plasmid, high-lighted in Figure 5.5, are marked on this graph. To allow relative concentrations of product to be calculated, each dilution used within the standard curve was assigned an arbitrary number relative to copy number. The most dilute sample was assigned the value 1, the next point on the curve was given the value 5 (as 1:5 dilution was used), the subsequent value was assigned the value of 25 (1:25 dilution) and so on along the curve.

One dilution from the standard curve was applied to a sample well in all subsequent PCR reactions to ensure that the amplification had worked efficiently, and so that concentration calculations could be made. The dilution marked with a red arrow was used as the standard (1:125000 dilution of the original plasmid prep or 1/125 of the 10^{-3} dilution of the stock) with a CP of approximately 17.1. This standard was chosen because the CP value falls in the middle of the results obtained for a selection of tissues (range listed for tissue CP values was 15.20-17.79).

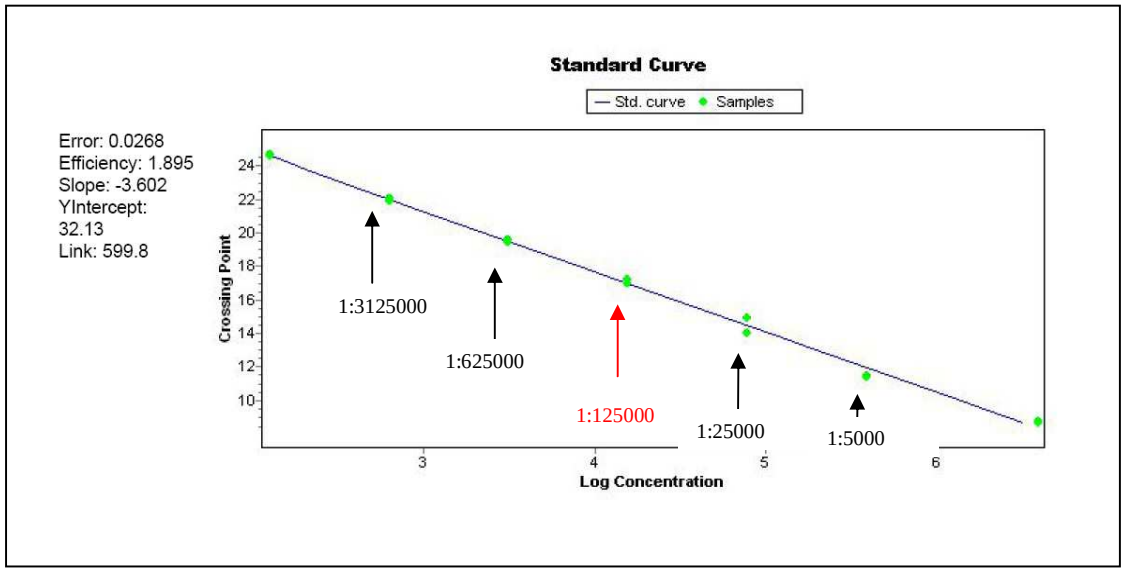


Figure 5.6: Standard curve for serial dilutions of 18S plasmid against calculated CP values. The arrows mark the dilution of the samples from the original plasmid mini-prep. The red sample was the standard used on subsequent PCR plates that allowed standardization of the tissue sample.

5.4.3: Calculation of relative concentrations of gene expression within tissues

Crossing point (CP) values were used to quantify gene expression within a tissue sample. The relative concentration of PCR product was calculated using the standard curve established using the serial dilutions of the appropriate plasmid. The use of arbitrary units (AU) for the plasmid dilutions allowed relative concentrations of a PCR product to be calculated for each of the tissue samples. Table 5.2 shows examples of the CP values for 18S gene expression relating to a selection of tissue samples from a 7 day line Y bird which was reared in the HH environment.

Bird and tissue	**Crossing point value**	**Relative concentration**
Thymus	15.2	4.40E4
Liver	15.16	4.51E4
Kidney	17.79	8.39E3
Standard (1:125000)	17.1	1.44E4

Table 5.2: Examples of data for tissue samples quantifying 18S levels showing CP values and relative concentrations calculated relative to an established plasmid standard curve. The standard sample from this curve is also shown to confirm the efficiency of the analysis.

5.4.4: Melt curves

Each PCR product had a distinctive melting temperature based on the size of product produced. Sybr green is non-specific in its binding to double-stranded DNA PCR products and these include non-specific products such as primer dimers, which can affect subsequent quantification. Analysis of the melting temperature ensures that only one product seen as a single peak on the melt curve is taken into consideration with regard to the calculated CP value.

Primer dimers are smaller in length than the true specific PCR product and therefore have a lower melting temperature relative to the specific PCR product. If primer dimers were present as well as the real PCR product, two peaks would be visualized on the melt curve.

Each of the four genes, AvBD1, 4, 10 and 18S resulted in products of different sizes (123bp, 164bp, 141bp and 126bp, respectively), and so each had a unique melting temperature. Figure 5.7 shows an example of a melt curve for the PCR product following the use of the specific primers for 18S. Only one peak was observed and a melting temperature of approximately 85.3°C was determined for this product. If primer dimers were present a second peak to the left of this would have been observed.

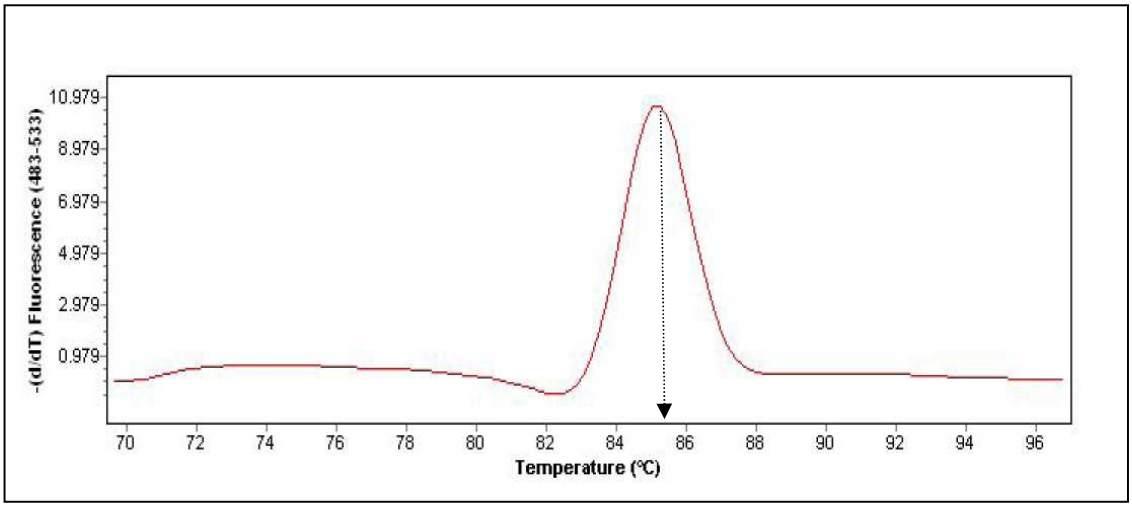


Figure 5.7: Melt curve for 18S PCR product. Only one peak is visible. The template for this PCR amplification was 18S plasmid at a dilution of 1:125000. The melting temperature was approximately 85.3°C

5.4.5: Normalisation of AvBD gene expression

The AvBD gene expression values were normalized to 18S for each particular tissue analysed. The data allowed trends in expression of the three AvBD genes to be determined. However, as different plasmid concentrations were used for the three genes in establishing standard curves, the numbers generated cannot be directly compared between the three AvBDs.

5.5: Gene expression

A total of 1800 different tissue samples were extracted from birds included in the farm trial and because of time constraints the numbers of tissues analysed via this technique had to be limited. The end-point PCR studies, although subjective, indicated that regardless of age, or environment, most tissues expressed the AvBD1, 4 and 10 genes. It

was of particular interest however, that the lymphoid organs including the thymus, spleen and bursa of Fabricius, more commonly associated with adaptive rather than innate immunity, also expressed these genes. The roles of the AvBDs within these primary and secondary lymphoid organs is not known (in humans or birds) and so these tissues were selected for further study. In addition, the duodenal tissue was selected to complement parallel studies investigating the gut anti-microbial activities of the birds reared in the different environments.

Thus AvBD1, 4 and 10 expression levels were measured in the thymus, spleen, duodenum and bursa of Fabricius tissues excised from birds aged 0, 7 and 35 days reared in the two (LH and HH) environments.

In addition caecum and caecal tonsil samples were analysed from two birds from each of the groups. AvBD10 gene expression was measured only in the testicle, kidney, liver and lung tissues from all groups of birds. Table 5.3 summarises the tissues and genes investigated.

	T	**Sp**	**Si**	**BF**	**L**	**K**	**Te**	**Lu**	**C**	**CT**
AvBD1	+	+	+	+	-	-	-	-	+	+
AvBD4	+	+	+	+	-	-	-	-	+	+
AvBD10	+	+	+	+	+	+	+	+	+	+
18S	+	+	+	+	+	+	+	+	+	+

Table 5.3: Summary of genes and tissues analysed by qRT-PCR. Code: T=thymus, Sp=spleen, Si=small intestine, BF=bursa of Fabricius, L=liver, K=kidney, Te=testicle, Lu=lung, C=caecum and CT=caecal tonsil (n=5 for day 0 and 35 birds; n=10 for day 7 birds; n=2 for caecum and caecal tonsil analyses).

Each sample was analysed in duplicate and any anomalous results were repeated on a separate occasion. Summaries of all of the data for each of the three genes, normalised to 18S, across the panels of tissues are presented in Tables 5.4, 5.5 and 5.6.

	X0	Y0	Z0	XL7	YL7	ZL7	XH7	YH7	XL35	YL35	ZL35	XH35	YH35
T	34(12)	54(42)	237(182)	24(9)	5(12)	20(12)	0.3(0.1)	1.4(0.6)	0.6(0.4)	2(2)	3(2)	6(6)	0.9(0.7)
Sp	219(85)	524(306)	545(291)	87(46)	34(19)	4(2)	4(2)	3(2)	16(9)	3(0.9)	6(6)	25(9)	5(4)
Si	72(53)	10(4)	14(7)	151(77)	2(0.4)	31(17)	3(2)	1(0.4)	1(0.4)	0.9(0.5)	3(2)	4(2)	3(2)
BF	18(10)	15(6)	28(10)	11(5)	10(9)	11(14)	0.7(0.3)	5(2)	3(13)	3(2)	2(9)	3(17)	4(3)
L													
K													
Te													
Lu													
C	4	428	3	2	18	2	2	2	40	5	9	3	0.7
CT	12	37	6	8	0.1	0.8	12	0.4	3	11	12	3	1

Table 5.4: AvBD1 gene expression (average AU ± SEM). Code: T=thymus, Sp=spleen, Si=small intestine, BF=bursa of Fabricius, L=liver, K=kidney, Te=testicle, Lu=lung, C=caecum and CT=caecal tonsil. N=5 for day 0 and 35 birds and n=10 for day 7 birds but n=2 for caecum and caecal tonsil.

	X0	Y0	Z0	XL7	YL7	ZL7	XH7	YH7	XL 35	YL 35	ZL 35	XH 35	YH 35
T	44(8)	594(322)	49(14)	25(11)	4(1)	17(4)	6(4)	5(3)	3(1)	9(6)	13(7)	6(6)	3(1)
Sp	1715(908)	2299(694)	3897(2289)	743(559)	18(6)	34(24)	190(117)	1254(53)	96(37)	13(7)	82(62)	10(3)	16(7)
Si	35(16)	19(6)	12(8)	36(17)	2(0.7)	7(4)	12(6)	13(7)	12(6)	0.6(0.4)	7(3)	5(2)	6(3)
BF	50(14)	103(52)	60(34)	9(5)	15(9)	25(14)	15(6)	29(14)	8(13)	1(0.5)	10(9)	2(5)	12(5)
L													
K													
Te													
Lu													
C	6	57	11	16	24	3	4041	109	8	7		7	4
CT	6	109	9	103	1	1	328	16	185	45		10	3

Table 5.5: AvBD4 gene expression (average AU ± SEM). Code: T=thymus, Sp=spleen, Si=small intestine, BF=bursa of Fabricius, L=liver, K=kidney, Te=testicle, Lu=lung, C=caecum and CT=caecal tonsil. N=5 for day 0 and 35 birds and n=10 for day 7 birds but n=2 for caecum and caecal tonsil.

	X0	Y0	Z0	XL7	YL7	ZL7	XH7	YH7	XL35	YL35	ZL35	XH35	YH35
T	2(0.5)	4(2)	3(0.5)	13(7)	9(3)	43(21)	12(6)	336(281)	0.3(0.3)	16(16)	1(0.8)	11(9)	0.9(0.3)
Sp	288(359)	13(3)	97(94)	132(78)	9(3)	15(7)	49(51)	10(4)	21(15)	2(2)	3(3)	30(7)	8(2)
Si	26(23)	9(5)	31(29)	55(21)	20(5)	384(353)	39(7)	44(31)	19(15)	2(1)	44(31)	148(134)	12(4)
BF	84(50)	5(3)	168(159)	3(5)	8(9)	7(14)	24(14)	103(73)	2(13)	12(5)	9(9)	16(17)	17(6)
L	205(147)	777(524)	2041 (1509)	247(78)	567(168)	1135 (423)	5202 (2882)	10003 (302)	902(582)	591(181)	287(79)	3276 (2013)	817(383)
K	4157 (2526)	12227 (7571)	45221 (27866)	6836 (3653)	15067 (6583)	68347 (27544)	23287 (9532)	43848 33273	132193 (41029)	31907 (14765)	10962 (5240)	99288 (56550)	33556 (14266)
Te	791(747)	423(389)	13398 (12885)	2385 (1398)	1033 (539)	2967 (1464)	8754 (6322)	146935 (93551)	1244 (566)	2428 (1099)	257(152)	440(80)	1889 (1570)
Lu	2(1)	18(16)	465(302)	190(100)	133(98)	457(270)	109(55)	324(219)	279(263)	117(44)	44(18)	37(14)	26(12)
C	0.6	43	11	11	71	7	9	17	17	22	22	3	4
CT	3	35	10	2	2	10	13	17	3	88	11	12	0.3

Table 5.6: AvBD10 gene expression (average AU ± SEM). Code: T=thymus, Sp=spleen, Si=small intestine, BF=bursa of Fabricius, L=liver, K=kidney, Te=testicle, Lu=lung, C=caecum and CT=caecal tonsil. N=5 for day 0 and 35 birds and n=10 for day 7 birds but n=2 for all groups for both caecum and caecal tonsil.

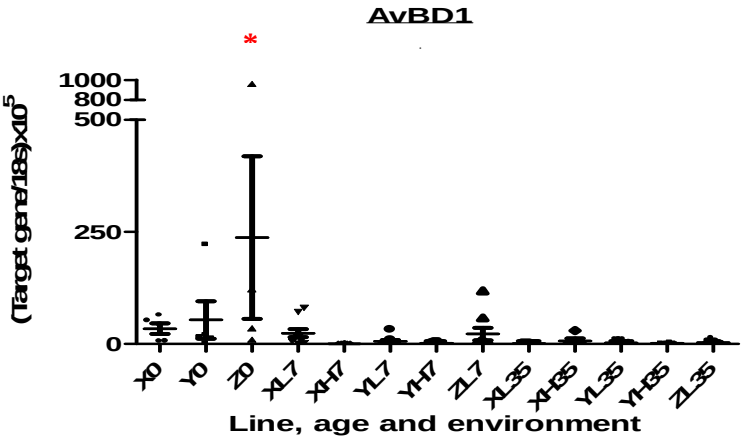
5.5.1: Thymus

Individual AvBD gene expression values for the bird thymic tissues are shown in Figures 5.8A-C. Marked intra-group variation in AvBD expression was noted e.g. AvBD1 gene expression values in the line Z, day 0 group (Figure 5.8A) ranged 100 fold from 9.4 AU to 960.7 AU. Such large variations were not unique and in fact were common within many of the data sets presented within this chapter.

Thymic AvBD1 gene expression was highest in all lines of birds at day 0 with mean (±SEM) AU expression levels of 34.0 (±12.0) for line X, 53.5 (±42.0) line Y and 237.3 (±181.8) line Z. As indicated in Figure 5.8A, gene expression appeared lower in the older birds, with the mean values ranging from 0.3-24 for day 7 birds and 0.6-6.4 for day 35 birds.

As regards trends, overall both line X and Y birds showed similar patterns of AvBD1 expression. However, the only statistical difference between groups of birds was that between line Z, day 0 birds (mean 237.3) and all other groups aged 7 and 35 days respectively ($p<0.05$). Figure 5.9 provides the AvBD1 gene expression profiles specifically for line X birds. Although there were no statistically significant differences in gene expression between line X birds reared in the two different environments, the mean AvBD1 expression was raised in the line X, day 7 birds raised in the LH environment, suggestive of an environmental effect on gene expression.

A



B

C

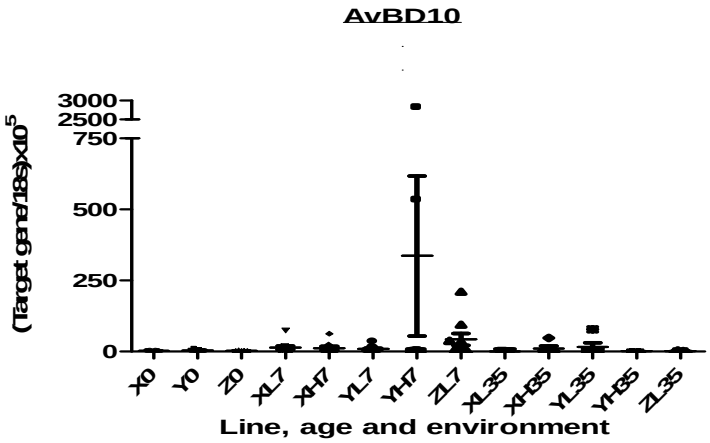


Figure 5.8A, B &C: AvBD1, 4 and 10 gene expression in the thymic tissue across all lines, ages and environments (±SEM). L= low hygiene, H=high hygiene and numbers depict age of birds in days. * indicates $p<0.05$, Z 0d birds relative to all other day 7 and 35 groups (AvBD1) and Y 0d relative to other groups except YH7day (AvBD4).

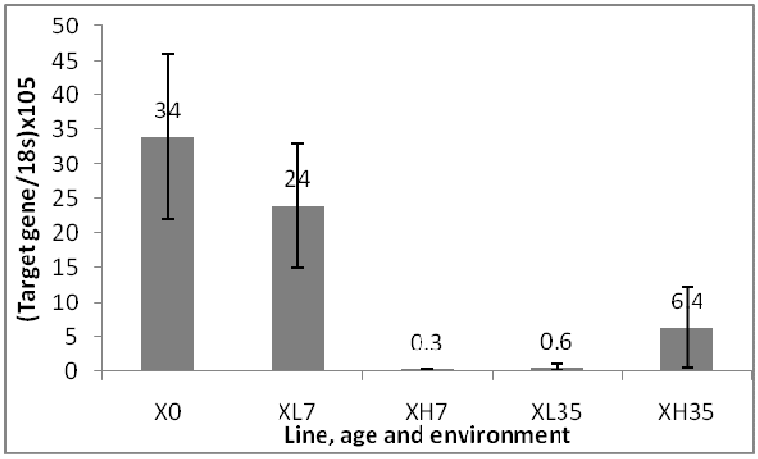


Figure 5.9: Mean AvBD1 gene expression in the thymus of line X birds (±SEM). (y axis should read $18Sx10^5$).

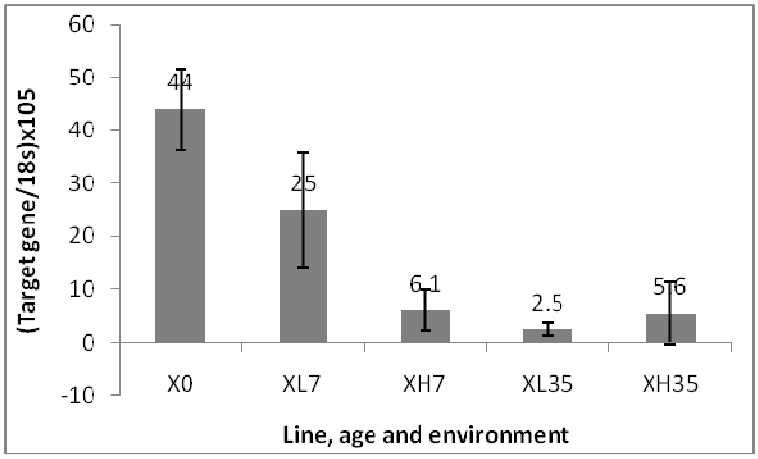


Figure 5.10: Mean AvBD4 gene expression in the thymus in line X birds (±SEM). (y axis should read $18Sx10^5$).

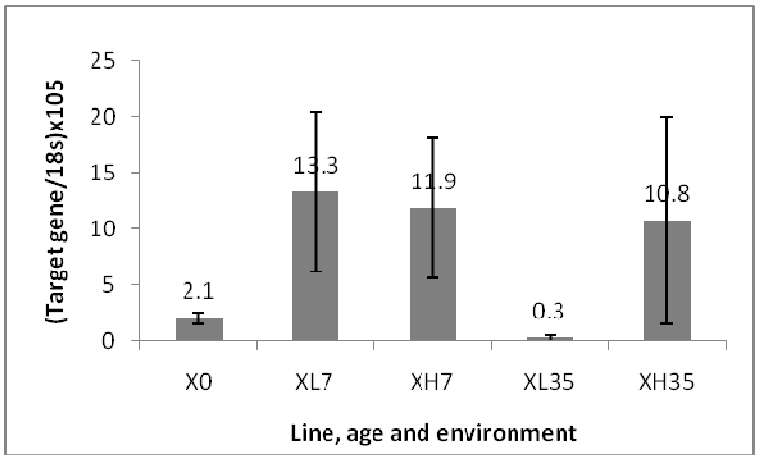


Figure 5.11: Mean AvBD10 gene expression in the thymus of line X birds (±SEM). (y axis should read $18Sx10^5$).

Line Y, day 0 birds showed the highest mean (± SEM) AU expression of AvBD4, 594 (±321.5) compared to line X 44 (±7.6) and line Z 49 (±13.8) birds. However as shown in Figure 5.8B two birds within the line Y day 0 group had markedly elevated, but very reproducible, expression levels. Line Y, day 0 birds had significantly higher expression levels of AvBD4 when compared to all other groups and ages of birds ($p<0.001$).

A similar trend in AvBD4 gene expression as for AvBD1 was observed (Table 5.5 and Figure 5.8B). Again highest expression was observed in the day 0 group and this reduced with age. Figure 5.10 shows the data specifically for the line X birds, although again there were no statistically significant differences in gene expression between line X birds reared in the two different environments, the mean AvBD4 expression was raised in the line X, day 7 birds raised in the LH environment, suggestive of an environmental effect on gene expression.

Figure 5.8C shows AvBD10 gene expression and in contrast with the other two genes, expression was not maximal at day 0. Expression between the five groups of chickens was similar, with the exception of YH day 7 birds, which had a mean (±SEM) AU of 336.0 (±281) compared to other day 7 birds; 13.3 (±7.1), 11.9 (±6.2), 8.9 (±3.2) and 43 (±20.5) (XL, XH, YL and ZL, respectively). However, there were no significant differences between any of the groups. Figure 5.11 shows the mean expression data for the line X birds and a different pattern, in comparison with the AvBD1 and 4 data is evident. These data suggest that neither age nor environment affects line X AvBD10 expression.

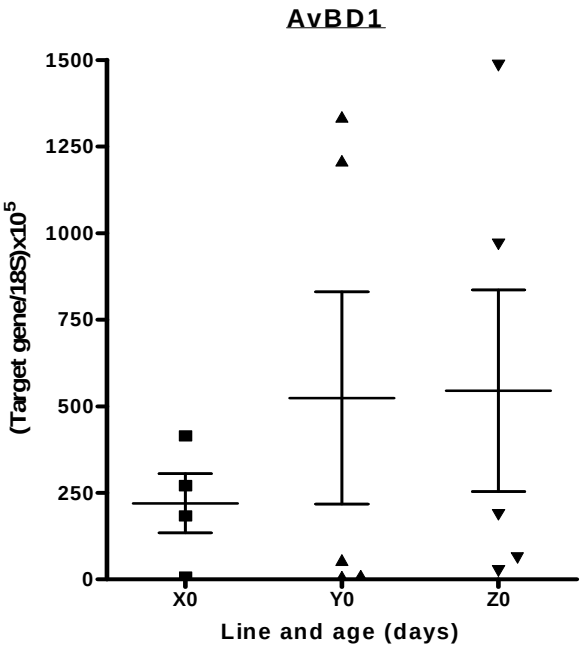
5.5.2: Spleen

The mean splenic AvBD expression values are presented in Tables 5.4-5.6. Figure 5.12 i-ii shows the individual bird AvBD1 splenic expression data; the data is presented for day 0 birds (Figure 5.12i) and day 7 and day 35 birds (Figure 5.12ii), and high-lights the large differences in gene expression between the birds themselves and in relation to age. The data was divided into two graphs due to the higher relative gene expression in the day 0 bird samples compared to the older birds.

Expression levels of all three genes showed similar patterns to those observed in the thymus with the highest levels recorded at day 0. With regard to AvBD1, the day 0 birds of lines Z and Y expressed the highest mean levels and line X birds the lowest (544.9 ±291 SEM, 523.7 ±306.4SEM and 219.3 ±85.4 SEM, respectively for lines Z, Y and X), however, these data were characterized by marked intra-group variability. Statistically the day 0, line Z birds showed significantly higher AvBD1 gene expression than all other day 7 and 35 groups of birds ($p<0.05$); line Y day 0 bird expression was significantly higher than all day 7 and 35 groups relating to lines Y and Z ($p<0.05$).

The splenic expression of AvBD1 is plotted for the different lines and ages of birds in Figure 5.13A-C. These data indicate that overall, AvBD1 splenic gene expression fell with age, and apart from a suggestion in the day 7 line X birds, was not affected by the rearing environment.

i



ii

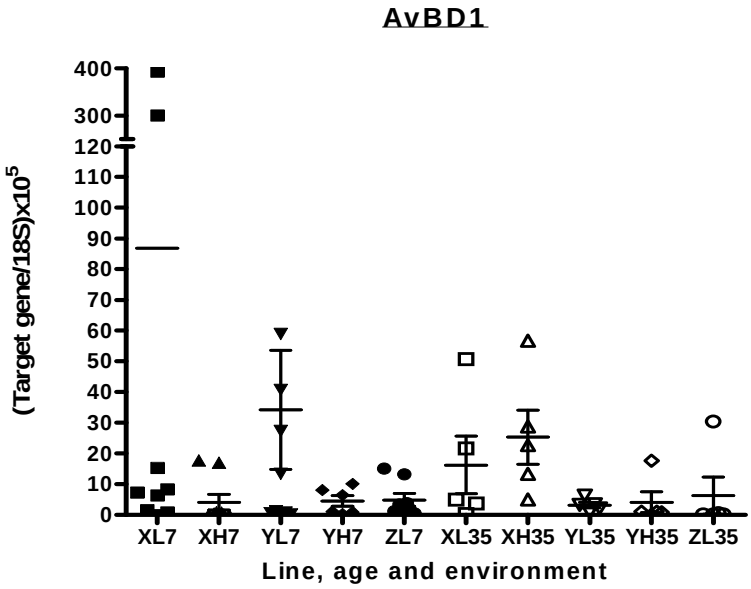
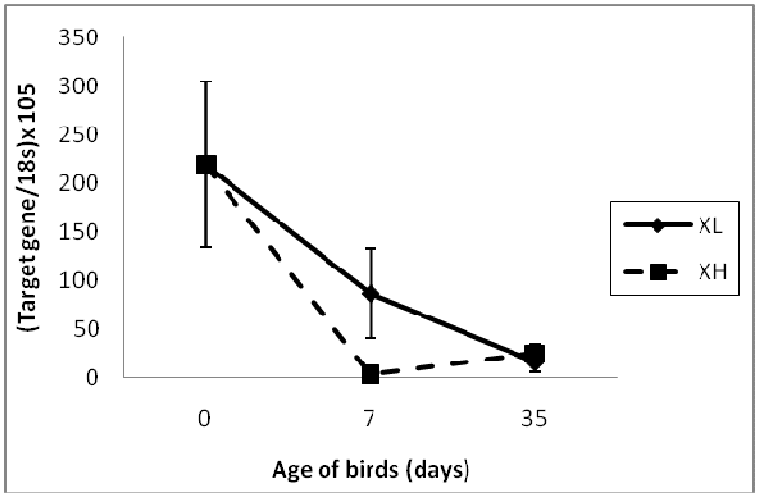
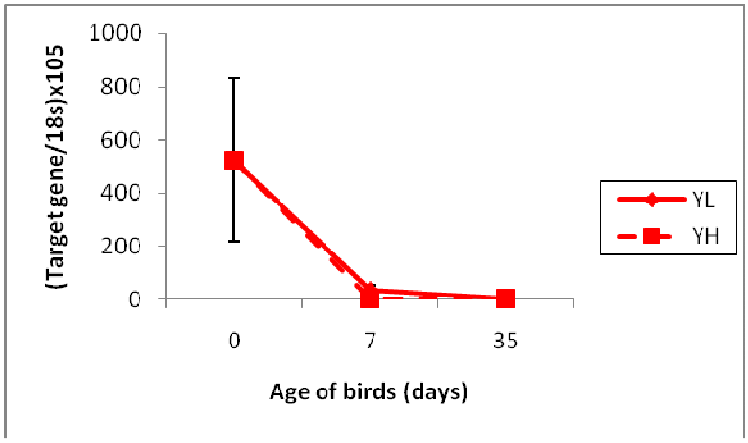


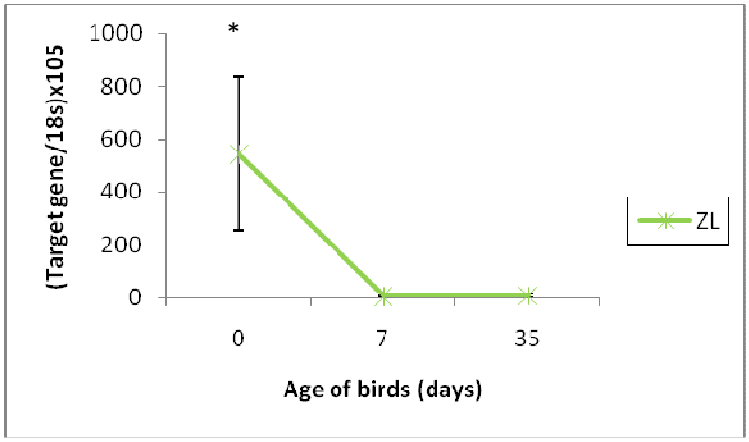
Figure 5.12i and ii: Splenic AvBD1 gene expression across all lines ages and environments respectively. L=low hygiene, H=high hygiene and numbers depict the age of birds in days. (i) day 0 birds (ii) day 7 and day 35 birds

A



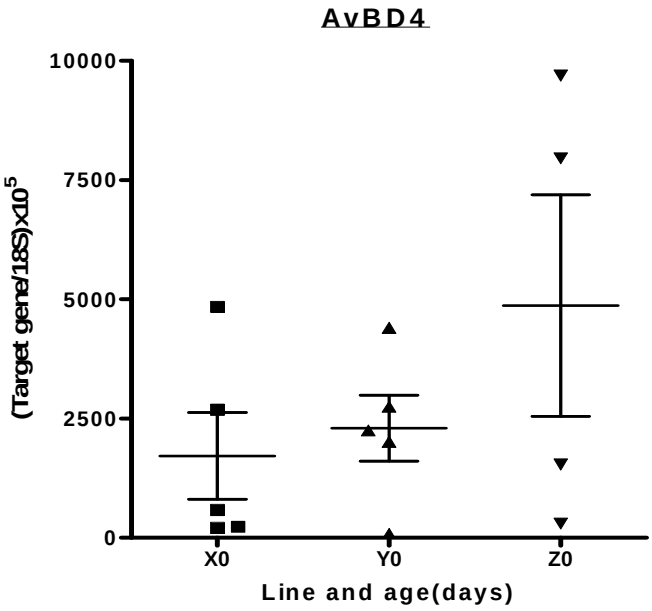


C



Figures 5.13A-C: AvBD1 gene expression in the spleen of different groups of chickens at different ages. A: line X birds, B: line Y birds and C: line Z birds (±SEM). L indicates low hygiene environment and H for the high hygiene conditions (* $p<0.05$, significantly higher levels of expression compared to all other groups except Y 0 day birds). (y axis should read $18Sx10^5$).

i



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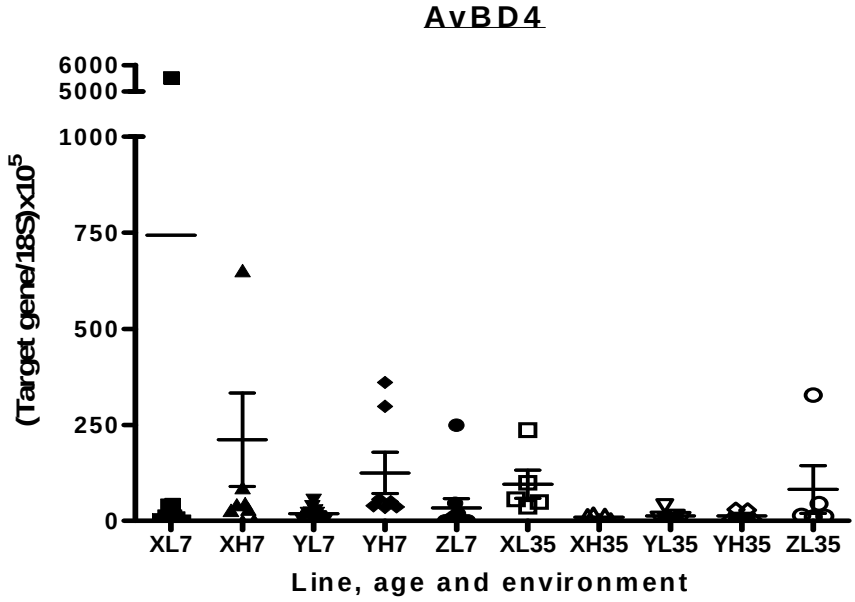


Figure 5.14 i and ii: Splenic AvBD4 gene expression across all lines ages and environments respectively. L=low hygiene, H=high hygiene and numbers depict the age of birds in days. (i) day 0 birds (ii), day 7 and 35 birds

The AvBD4 gene expression data is presented in Figure 5.14i and ii. These data are presented separately as the day 0 birds had much higher levels of expression than the day 7 and 35 birds (as for AvBD1). AvBD4 expression was highest in line Z day 0 birds (mean ±SEM AU) 3,896.6 (±2,289) compared to 2,298.8 (±693.7) and 1,715.4 (±1,715.4) for lines Y and X respectively. In fact, line Z day 0 birds had significantly higher levels of gene expression than all other groups of birds except line Y day 0 ($p<0.05$) (Figure 5.13C). This observation was similar to that found for this group of birds and AvBD1 gene expression. Interestingly, the line Z day 0 bird with the highest level of AvBD1 mRNA also had a very high level of AvBD4 expression. The rearing environment appeared to have no effects on splenic AvBD4 expression although these data (Figure 5.14ii) were skewed by a number of outliers.

The splenic expression levels of AvBD10 are shown in Figure 5.15. These data are characterized by marked intra-group variation, especially at days 0 and 7, respectively. However, statistically neither age nor rearing environment affected splenic AvBD10 expression.

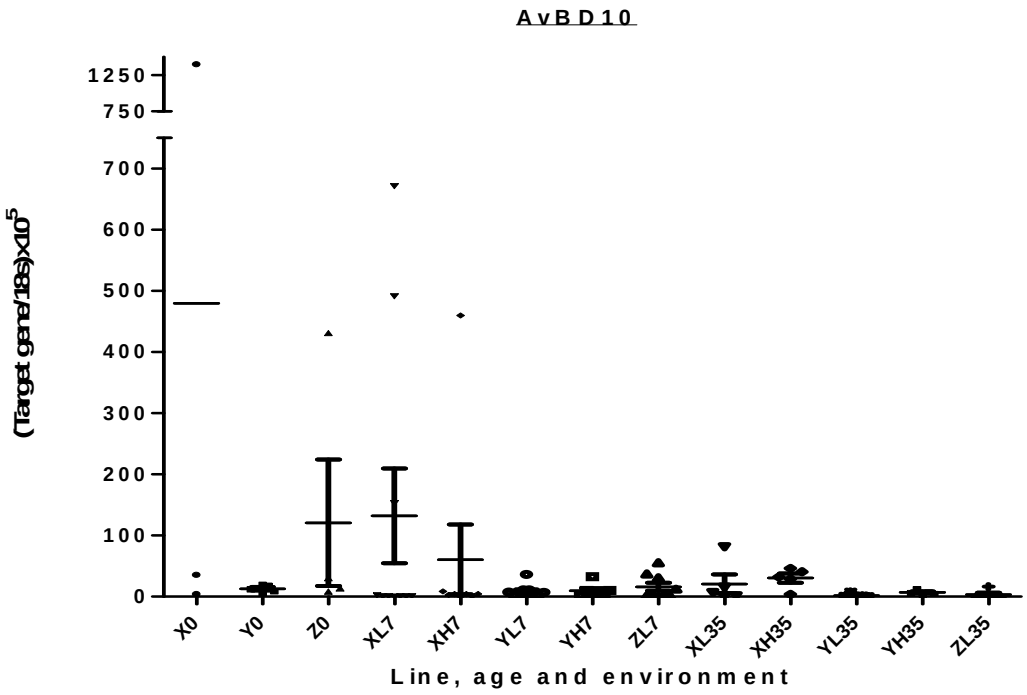


Figure 5.15: Splenic AvBD10 gene expression across all lines, ages and environments respectively. L=low hygiene, H=high hygiene and numbers depict the age of birds in days. (y axis should read 18S). (Data in table format in Table 5.7).

X0	Y0	Z0	XL7	XH7	YL7	YH7	ZL7	XL35	XH35	YL35	YH35	ZL35
3.98	20.41	430.80	0.47	4.17	6.78	9.86	3.51	81.38	46.30	0.48	4.03	16.49
35.92	18.01	13.36	0.96	8.77	10.91	2.77	0.34	0.04	3.72	7.75	7.88	0.39
1398.99	5.41	30.42	490.80	1.15	7.03	2.18	1.24	1.67	28.80	0.03	6.19	0.03
	9.22	8.47	152.68	2.54	7.31	7.30	37.37	7.01	32.05	0.09	12.45	0.02
	12.30		0.53	459.56	36.54	2.10	55.38	15.55	40.85	1.14	4.68	0.03
			671.15	4.06	0.20	32.68	0.91					
			0.84	0.04	0.81	10.31	12.52					
			1.84	4.66	6.98		3.24					
			0.07		0.40		30.86					
			0.44		10.84							

Table 5.7: Splenic AvBD10 gene expression across all lines, ages and environments respectively. Data as plotted in Figure 5.15. Units calculated as ((Target gene/18S) x 10^5). L= low hygiene and H =high hygiene.

5.5.3: Small intestine (duodenum)

Figure 5.16A-C shows the expression levels of the three AvBD genes in the duodenal tissue samples of the day 0, 7 and 35 birds respectively. Again the expression data was characterised by large intra-group variation and no definite trends between age and gene expression were identified.

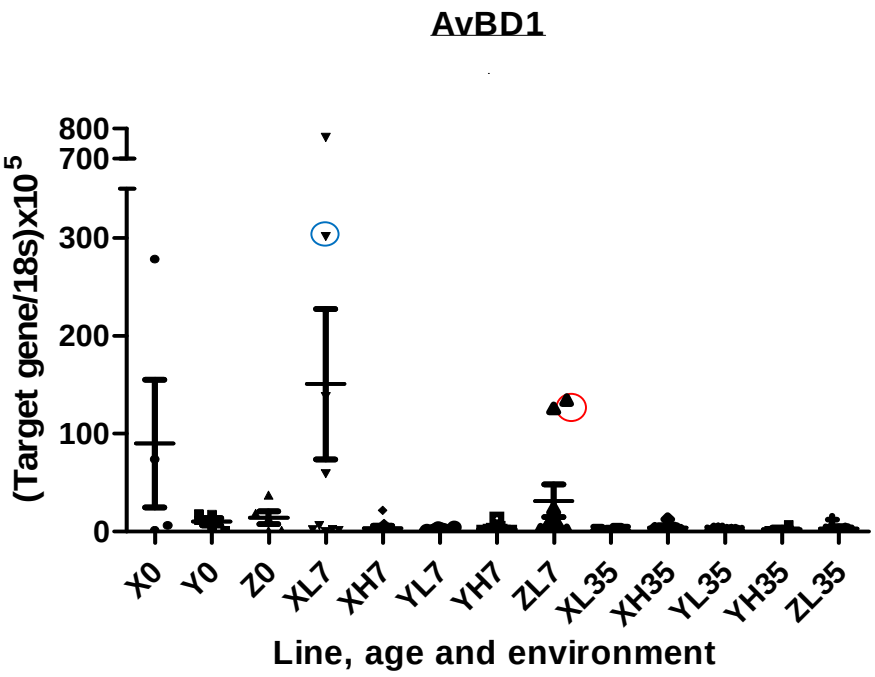
Figure 5.16A shows that the majority of the bird samples analysed had low levels of AvBD1 gene expression and this was consistent with the end-point PCR results (Figures 5.2-5.4). Interestingly, two bird samples within the XL 7 day-old group were found to have high levels of both AvBD1 and 4 gene expression (blue ring in Figure 5.16A and B).

AvBD1 expression was shown to be significantly higher ($p<0.05$) in XL 7 day-old birds compared to XH, YL and YH 7 day-old birds (Figure 5.17). In fact, these data indicated a 50 fold difference between XL, mean 150.7 (±76.8 SEM) and XH birds, mean 3.3 (± 2.4 SEM), which suggested that environment was affecting AvBD1 gene expression in such birds.

A dot plot showing AvBD4 duodenal gene expression data is presented in Figure 5.16B. The expression range (AU) was between 0.11 and 165.9 for individual birds. No statistically significant differences were identified between any of the groups, however one of the birds which was found to have high expression levels of AvBD1 also had high levels of AvBD4 (blue ring in Figures 5.16A and B, 771.0AU for AvBD1 and 165.9 AU for AvBD4). One bird in the ZL 7 day group showed a similar pattern, but in this case

also had a very high expression level of AvBD10 (marked with red rings in Figures 5.16B and C with 134.5AU, 43.2 AU and 3554.9 AU for AvBD1, 4 and 10 expression respectively).

A



B

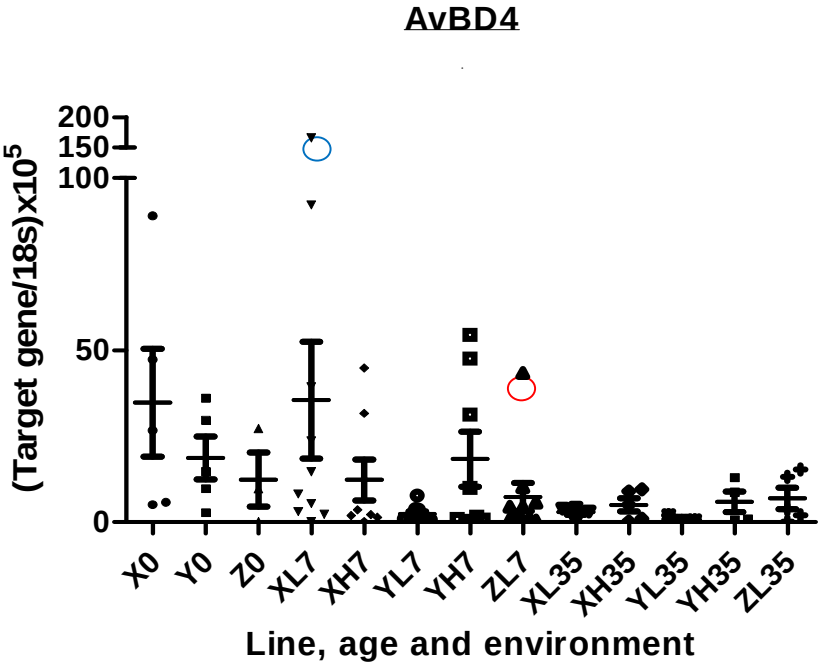


Figure 5.16A-B: Duodenal AvBD1 and 4 gene expression respectively across all lines, ages and environments. L= low hygiene, H=high hygiene and numbers depict age of birds in days. The blue and red circles indicate the same bird within that specific group across the different genes. (y axis should read 18S). (Data in table form Tables 5.8A-B).

X0	Y0	Z0	XL7	XH7	YL7	YH7	ZL7	XL35	XH35	YL35	YH35	ZL35
3.72	17.67	1.05	3.86	73.80	19.44	67.93	3554.85	3.35	43.68	5.44	7.98	18.19
118.80	22.68	2.91	133.56	39.14	2.33	0.10	5.05	2.48	9.40	0.05	11.13	165.91
3.65	1.47	2.15	0.42	57.23	53.70	0.11	13.82	64.89	684.88	0.42	23.37	7.38
2.27	0.22	117.56	19.82	46.43	15.29	4.78	3.89	4.09	2.17	0.72	3.97	6.23
1.65	0.70		0.38	19.92	32.72	3.54	25.11		0.71	2.70		20.90
			30.73	27.00	13.33	3.67	6.23					
			5.32	28.37	3.28	2.07	4.55					
			182.52	8.35	36.88	268.47	15.57					
			126.10	70.06	16.78	20.63	46.95					
			47.31	14.37	8.79		163.72					

Table 5.8C: Duodenal AvBD4 gene expression across all lines, ages and environments respectively. Data as plotted in Figure 5.16C. Units calculated as ((Target gene/18S) x 10^5). L= low hygiene and H =high hygiene.

X0	Y0	Z0	XL7	XH7	YL7	YH7	ZL7	XL35	XH35	YL35	YH35	ZL35
5.71	36.05	0.21	23.54	31.65	2.71	47.47	43.25	3.88	8.96	0.37	1.01	13.18
88.95	9.79	9.66	8.22	1.38	1.33	0.69	0.43	0.56	5.07	0.56	0.73	0.11
47.19	2.76	27.17	0.19	3.62	3.81	1.51	0.43	4.17	9.58	2.01	12.98	2.04
5.08	14.77		39.24	44.74	0.91	0.34	1.99	3.12	0.99		8.77	3.77
26.61	29.66		3.10	0.21	7.72	31.21	0.80		0.28			15.35
			2.34	2.17	1.09	0.94	1.27					
			5.40	12.43	3.59	9.98	4.41					
			165.93	1.93	0.94	54.37	5.78					
			14.69		0.69		10.17					
			92.07		0.54		4.42					

Table 5.8B: Duodenal AvBD4 gene expression across all lines, ages and environments respectively. Data as plotted in Figure 5.16B. Units calculated as ((Target gene/18S) x 10^5). L= low hygiene and H =high hygiene.

X0	Y0	Z0	XL7	XH7	YL7	YH7	ZL7	XL35	XH35	YL35	YH35	ZL35
6.33	18.78	0.78	59.34	0.17	3.54	0.70	134.49	1.96	12.85	0.88	0.85	12.10
74.07	1.96	1.37	1.61	0.19	0.81	0.00	1.05	0.16	3.02	2.72	0.42	0.11
278.11	0.53	18.60	0.16	21.80	2.10	0.34	8.91	0.90	3.21	0.17	7.50	1.47
1.13	17.93	13.39	138.08	0.90	0.84	0.96	5.42	0.81	0.18	0.68	2.03	0.40
	11.77	37.14	1.96	0.22	4.28	0.10	1.87		0.71	0.28		2.42
			6.39	0.63	2.09	3.91	2.60					
			2.70	0.11	0.19	0.23	10.31					
			771.02	0.57	0.49	2.00	23.66					
			301.54	8.89	1.73	13.59	125.73					
			223.80		0.80		0.42					

Table 5.8A: Duodenal AvBD1 gene expression across all lines, ages and environments respectively. Data as plotted in Figure 5.16A. Units calculated as ((Target gene/18S) x 10^5). L= low hygiene and H =high hygiene.

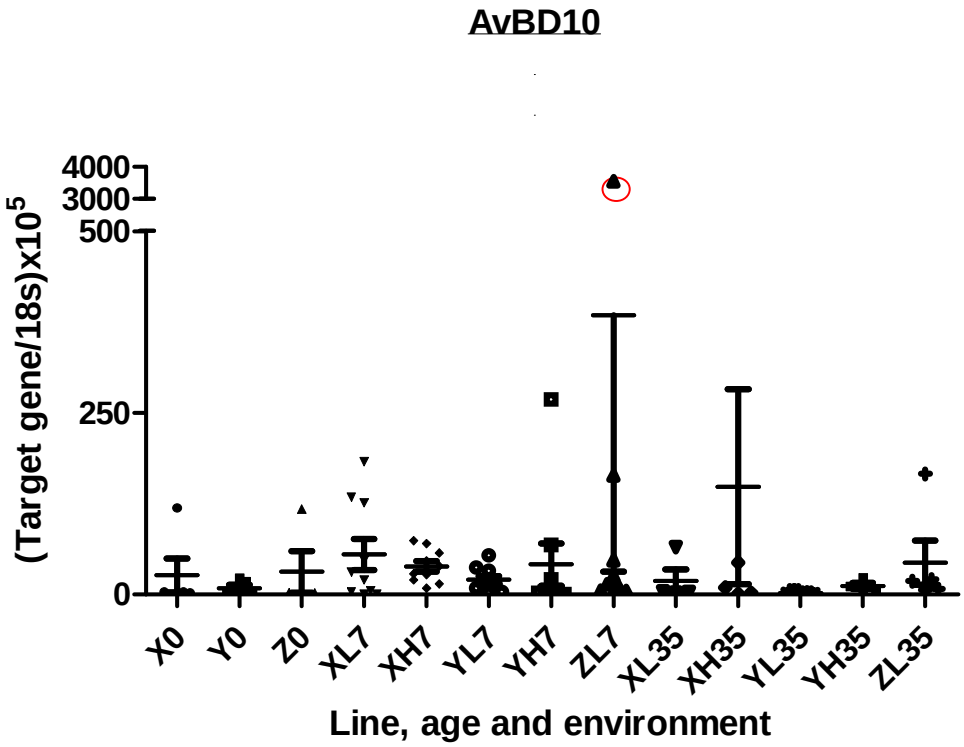


Figure 5.16C: Duodenal AvBD10 gene expression across all lines, ages and environments. L= low hygiene, H=high hygiene and numbers depict age of birds in days. The red circle indicate the same bird within that specific group across the different genes. (y axis should read 18S). (Data in table form Table 5.8C).

Duodenal expression of AvBD10 across all the groups was low when compared to other tissues such as the testicle and kidney, and ranged between 1.9 (±1 SEM) AU and 384.0 (±352.7 SEM) AU. The latter figure was however skewed by one bird which had a very high expression level (ZL 7 day-old group, data point with red circle in Figure 5.16C).

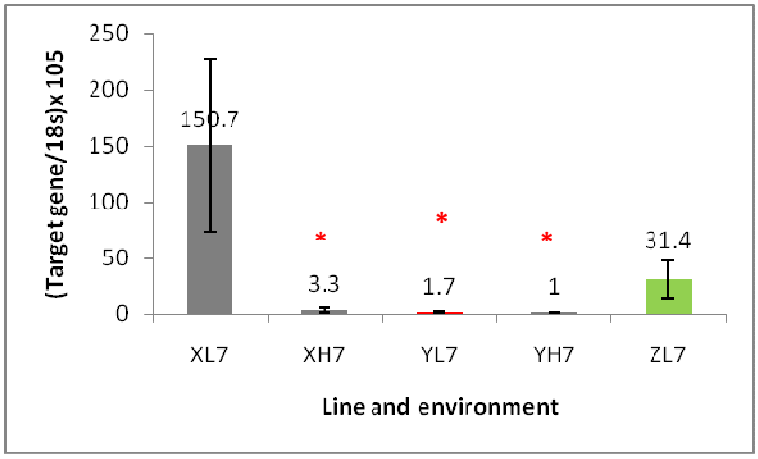


Figure 5.17: Mean AvBD1 gene expression levels for day 7 birds in the duodenum. Red asterisk indicates significant difference to XL 7day group ($p<0.05$). (y axis should read $18Sx10^5$).

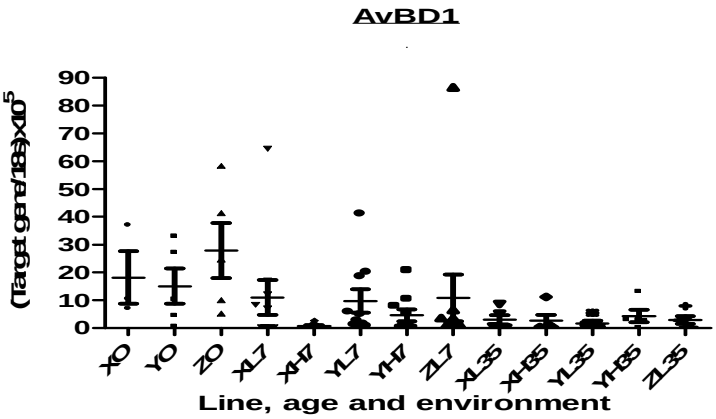
5.5.4: Bursa of Fabricius

The data for AvBD gene expression in the bursa of Fabricius samples is presented in Figure 5.18A-C. Again there was variability in AvBD1 expression levels between birds in each of the groups. The highest mean level of gene expression was observed in day 0 birds and expression appeared to fall with increasing age. The overall range of means for all groups fell within the range (AU±SEM) 0.7 (±0.3) and 27.9 (±9.9), indicative of relatively low levels of expression of AvBD1 in this tissue across all bird groups.

Similar to the trend observed for AvBD1, AvBD4 gene expression was also highest in day 0 birds (Figure 5.18B). Line Y 0 day-old birds had the highest mean level of expression of AvBD4 102 (±52SEM) AU, but there were no statistically significant differences between this group and any of the other groups. AvBD4 gene expression across all lines was lowest at day 35, again suggesting a fall in expression with increasing age.

No significant statistical differences were detected between groups with regard to AvBD10 expression. Both lines X and Z showed highest mean expression levels (±SEM) AU at day 0 (83.8 ±49.8 and 167.6± 158.6), but line Y showed peak levels in the YH 7 day group (103.0AU±73.4). With regard to the latter group, two of the birds had very high levels of expression, one of which was the bird found to have increased gene expression of all three AvBDs. It can therefore be concluded that the bursa of Fabricius expressed all three AvBD genes, but apart from a few birds as exceptions, expression levels were relatively low compared to other tissues. Age but not rearing environment affected gene expression.

A



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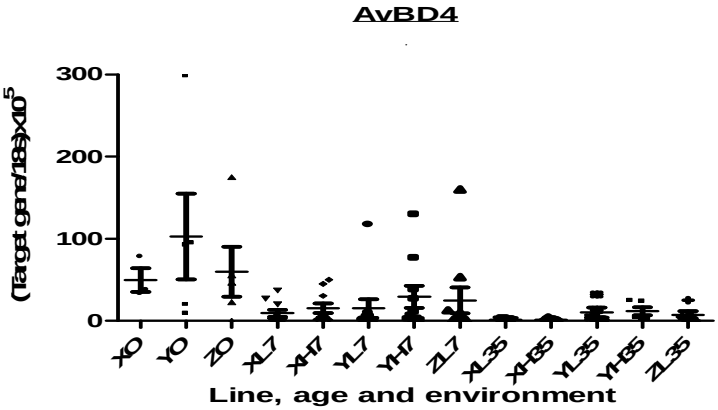

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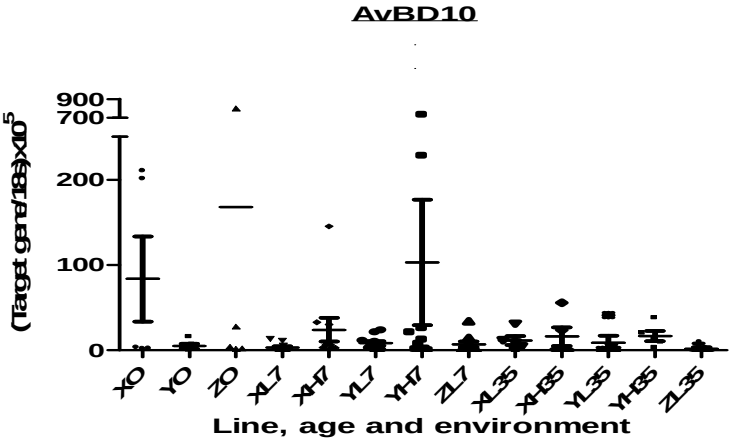


Figure 5.18A-C: AvBD1, 4 and 10 gene expression across all bird lines, ages and environments in the bursa of Fabricius. L= low hygiene, H=high hygiene and numbers depict age of birds in days. (y axis should read (Target gene/18S x 10^5)).

5.5.5: Caecum and caecal tonsil

Neither the caecum nor the caecal tonsil were included in the initial qRT-PCR panel for analysis of AvBD1, 4 and 10 expression because the end-point PCR data showed cDNA bands of similar intensities for those AvBD genes analysed across all groups (Figures 5.2-5.4). However, due to the location of these tissues within the gastro-intestinal tract and the lymphoid function of the caecal tonsil it was decided to analyse the tissues from two birds, from each of the groups, to provide insight into the actual gene expression levels. The premise was that these data would provide an interesting comparison to the data obtained for the small intestine, and indicate whether AvBD gene expression is regionally regulated in the GI tract. The data for these birds is shown in Tables 5.9 (caecum) and 5.10 (caecal tonsil).

The sample sizes were small (n=2) and characterized by intra-group variation, thus it is difficult to report on possible trends. However caecal expression levels of AvBD1 across all groups were generally low, exceptions being the line Y 0 day-old bird 2 (855.3AU), the YL 7day-old bird 2 (35.7AU) and XL35 day-old bird 1 (78.7AU). All other bird values fell within the range 0.5-16.5AU. For those samples analysed the AvBD4 expression levels were variable in the caecal tissues with levels ranging from 0.1 to 7730.1. The results suggested that both XH 7 day-old birds had higher levels of expression compared to other groups (351.6 and 7730.1AU respectively). AvBD10 gene expression levels were reduced when compared to other tissues such as the kidney and testicle. As for the other two genes, no definite age related patterns were noted.

However, from Table 5.9 it was noted that the line Y 0 day-old bird 1 had relatively high expression levels of all three AvBDs and similar patterns were observed for YL 7 day-old bird 2 and the XL 35 day-old bird 1 (although the latter is missing AvBD4 expression data).

Bird identity (line, age and number)	AvBD1	AvBD4	AvBD10
X 0day bird 1	2.7	4.7	0.2
X 0day bird 2	5.6	7.9	1.0
Y 0day bird 1	855.3	110.8	84.7
Y 0day bird 2	0.9	3.5	0.7
Z 0day bird 1	3.2	8.4	19.2
Z 0day bird 2	2.3	13.1	3.6
XL 7day bird 1	0.1	0.1	0.2
XL 7day bird 2	3.4	32.2	21.6
XH 7day bird 1	0.8	351.6	1.0
XH 7day bird 2	2.3	7730.1	16.4
YL 7 day bird 1	0.9	3.7	3.8
YL 7 day bird 2	35.7	43.6	138.7
YH 7 day bird 1	2.7	44.1	5.5
YH 7 day bird 2	1.0	174.0	27.6
ZL 7 day bird 1	2.7	4.0	7.1
ZL 7 day bird 2	0.5	2.3	6.8
XL 35 day bird 1	1.0	15.8	1.3
XL 35 day bird 2	78.7	NA	33.2
XH 35 day bird 1	3.8	6.8	3.7
XH 35 day bird 2	1.7	NA	1.5
YL 35 day bird 1	0.7	7.3	1.3
YL 35 day bird 2	8.7	NA	43.1
YH 35 day bird 1	1.2	3.8	5.9
YH 35 day bird 2	1.7	NA	1.5
ZL 35 day bird 1	16.5	NA	14.3
ZL 35 day bird 2	0.8	NA	30.2

Table 5.9: AvBD 1, 4 and 10 gene expression in the caecum (AU). L is low hygiene and H high hygiene. NA indicates samples not analysed.

Again only two birds from each of the groups were analysed for AvBD1, 4 and 10 expression in the caecal tonsil. The data is presented in Table 5.10.

Again it is difficult to comment on trends with such small sample sizes. However the line Y 0 day-old bird 1, XL 7day-old bird 2, XH 7 day-old bird 2, XL35 day old bird 1 and YL 35 day bird 1 all showed higher expression of the AvBD genes, particularly AvBD4. Some of these birds e.g. XH 7 day-old bird 2 and Y 0 day-old bird 1 also revealed high levels of AvBD gene expression in the caecum suggesting a genetic link.

Bird identity (line, age and number)	AvBD1	AvBD4	AvBD10
X 0day bird 1	0.2	0.9	2.9
X 0day bird 2	23.9	11.9	3.8
Y 0day bird 1	73.9	218.1	70.1
Y 0day bird 2	0.5	0.2	0.6
Z 0day bird 1	9.4	16.4	16.9
Z 0day bird 2	1.9	0.9	3.9
XL 7day bird 1	2.0	5.4	0.2
XL 7day bird 2	14.6	201.1	3.6
XH 7day bird 1	0.1	4.2	10.9
XH 7day bird 2	23.5	651.5	15.4
YL 7 day bird 1	0.1	0.6	3.4
YL 7 day bird 2	0.2	1.8	1.2
YH 7 day bird 1	0.2	23.3	2.1
YH 7 day bird 2	0.5	9.4	30.9
ZL 7 day bird 1	1.3	2.1	16.7
ZL 7 day bird 2	0.2	0.5	2.2
XL 35 day bird 1	6.2	184.8	6.3
XL 35 day bird 2	0.4	NA	0.1
XH 35 day bird 1	1.3	9.5	2.6
XH 35 day bird 2	4.4	NA	21.9
YL 35 day bird 1	10.0	45.3	129.7
YL 35 day bird 2	12.0	NA	46.7
YH 35 day bird 1	1.0	2.5	0.4
YH 35 day bird 2	NA	NA	0.1
ZL 35 day bird 1	24.1	NA	11.0
ZL 35 day bird 2	0.6	NA	9.9

Table 5.10: AvBD 1, 4 and 10 expression in the caecal tonsil (AU). L is low hygiene and H high hygiene. NA indicates samples not analysed.

5.5.6: Lung

Due to the size of the study and sample numbers, qRT-PCR analyses of all three AvBD genes was only performed on four tissues i.e. thymus, spleen, bursa of Fabricius and duodenum. However, the AvBD10 gene was the first to be analysed via qRT-PCR and all ten tissue samples, including the lung, kidney, testicle and liver were analysed. The lung expression values for the individual birds is shown in Figure 5.19 and again the data is characterized by the marked spread within bird groups, particularly the 7 day-old bird groups. No significant statistical differences between any of the lines and or ages were identified. The intra-group variation in AvBD10 gene expression makes it difficult to comment on potential trends, although expression appeared more variable in the birds of the day 7 groups, and expression appeared reduced in all of the day 35 groups when compared to the day 7 data. However, no clear distinctions between the data from the two rearing farms could be made.

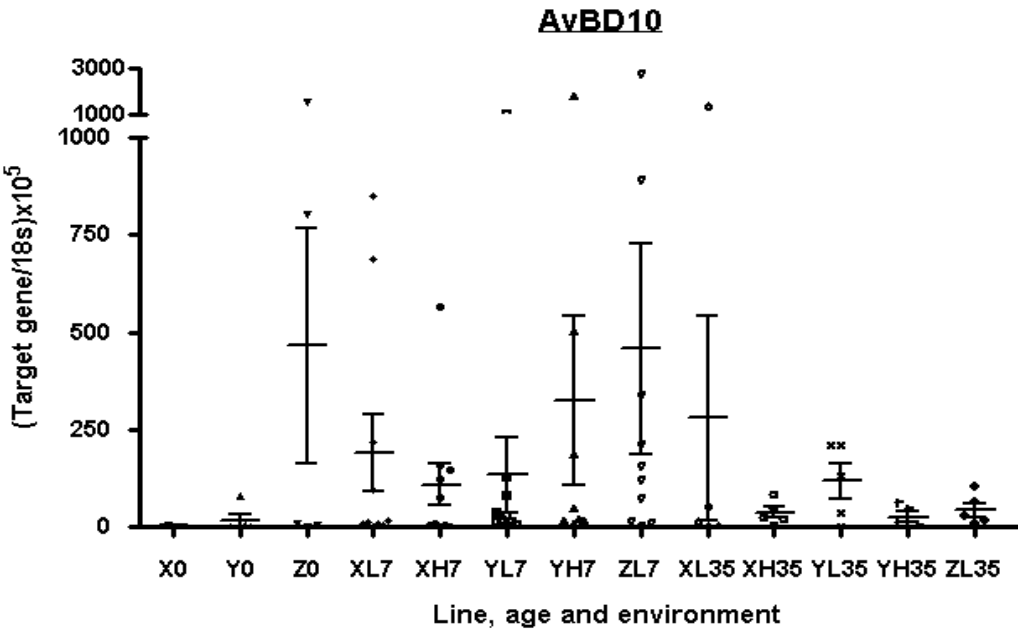


Figure 5.19: Lung tissue expression of AvBD10 across all groups of chickens. (±SEM). (y axis should read 18S).

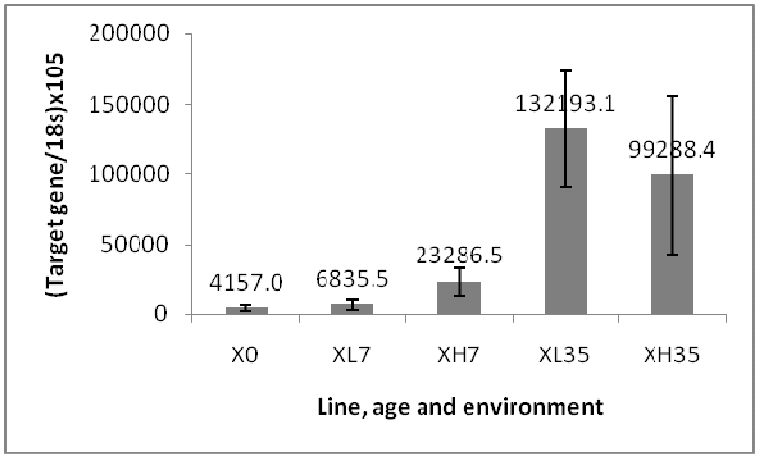
5.5.7: Kidney

Very high levels of gene expression were detected within kidney tissues relative to the other tissues analysed (Table 5.6 and Figure 5.20A-C)

Data relating to AvBD10 gene expression within the kidneys of line X birds is shown in Figure 5.20. Peak expression was shown to occur in the day 35 bird samples (132,193.1(± 41,028.6 SEM) and 99,288.4 (±56,550.4 SEM) respectively, for lines XL and XH), but there was no statistically significant difference between these two groups. Line Y birds showed the highest mean expression level in the YH 7 day group (43,848.4), however there was marked intra-group variation. The line X groups showed higher expression

levels than the line Y groups. In contrast to these birds, line Z birds showed peak gene expression at day 7 (68,346.7 ±27,544.0 SEM) and the lowest mean expression level was found to occur in the day 35 birds (Figure 5.20C).

A



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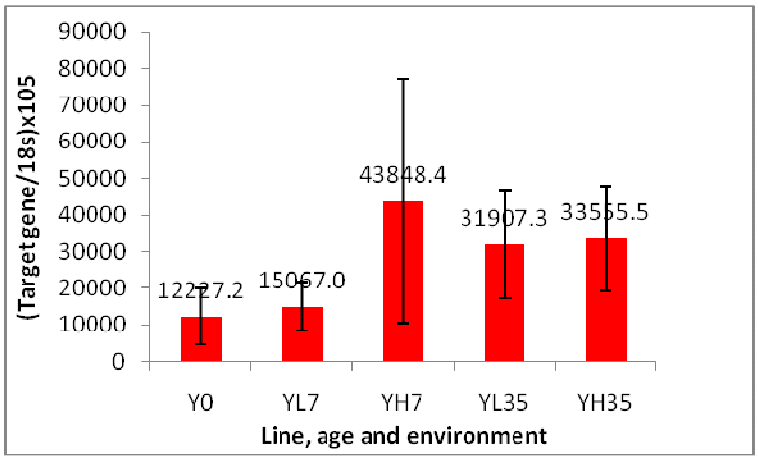

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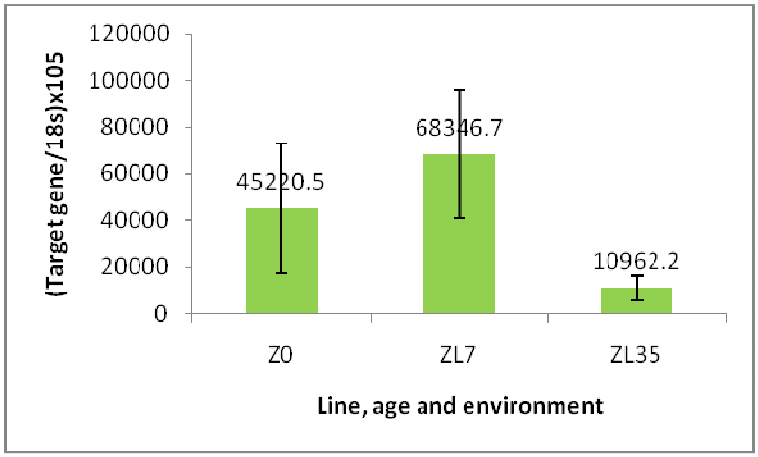

Figure 5.20A-C: Mean AvBD10 expression in the kidney of the different groups of birds (±SEM). A: line X, B: line Y and C: line Z. (y axis should read 18Sx10 ^{5}).

5.5.8: Testicle

High levels of testicular AvBD10 gene expression were detected across all groups of birds compared to the other tissues analysed (Table 5.6). However, again large intra-group variation was observed (Figure 5.21).

For both lines X and Y, the highest expression levels were noted in the 7 day birds reared in the high hygiene environment with mean (±SEM) levels of 8,753.8 (±6,322.1) and 146,934.4 (±93,351.3)AU, respectively. The average fold difference in expression for the 7 day birds was four fold between groups XH and XL, and 142 fold for YH compared to YL. However, bird number 6 in YH 7 day group had an extremely high level of gene expression (699,588) and when this bird was omitted from the data set the mean expression average level fell to 54,825.5 (±17,959 SEM) (Figure 5.21). For the statistical analyses this value was omitted as it was such an outlying value (>2SD) relative to the other data.

Within the YH 7 day group there appeared to be two distinct sub-groups, one with very high levels of expression (red ring, n=4) and the other with lower levels (blue ring, n=2), although it is accepted that the numbers are small. When statistical analysis was performed the YH 7day group had significantly higher expression levels of AvBD10 compared to all of the other bird groups ($p<0.05$). Generally, expression levels fell in the day 35 group of birds with the exception of the YL 35 birds.

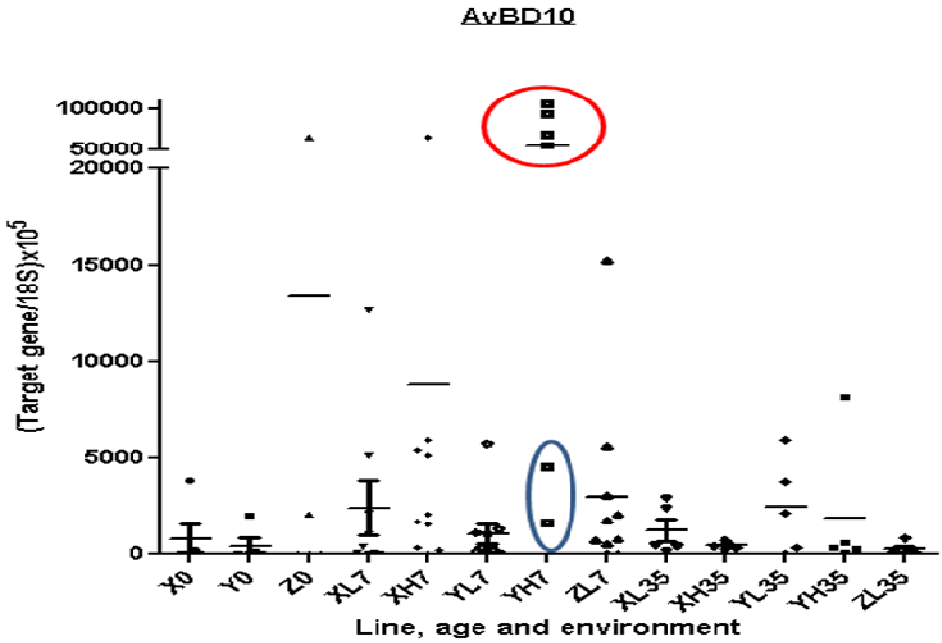

Figure 5.21: AvBD10 expression patterns in the testicle of all groups of chicken (mean ±SEM, for those groups where SEM is very large only mean is given). The red and blue rings mark the two distinct groups of expression within the YH 7 day birds.

5.5.9: Liver

Moderate levels of AvBD10 gene expression were noted in the liver tissues of the line X, Y and Z chickens compared to other tissues analysed (Table 5.6 and Figure 5.22A-C).

Very different patterns in gene expression level were found to occur across the three lines of birds as illustrated in Figure 5.22. The line Y 0 day birds showed a higher mean level of gene expression (777AU) compared to line X birds (147AU), but these data were characterised by their degree of intra-group variability. Interestingly line X birds reared on the HH farm showed the highest mean expression levels for both the 7 and 35 day old

birds, 5,202.3(±2,882.3SEM) and 3,276.2(±2,013SEM) respectively, compared to the same birds reared on the LH farm. This suggested the rearing environment was affecting gene expression. Similar to line X, the highest mean level of expression was observed in the YH 7 day group (1,002.5AU). In fact both of the line Y high hygiene groups at day 7 and 35 showed higher average expression levels than the low hygiene birds but these findings were not as pronounced when compared to the line X data.

In contrast to both lines X and Y, the line Z birds were observed to have the highest mean AvBD10 gene expression at day 0, 2,040.5(±1,509.3SEM). Mean levels decreased as the birds aged (1,135.1 and 287 respectively for day 7 and 35), a pattern similar to that was observed for AvBD10 in the spleen.

Although trends were evident, no statistical significant differences could be observed between the groups of birds, again due to large degree of variation between birds in the same group.

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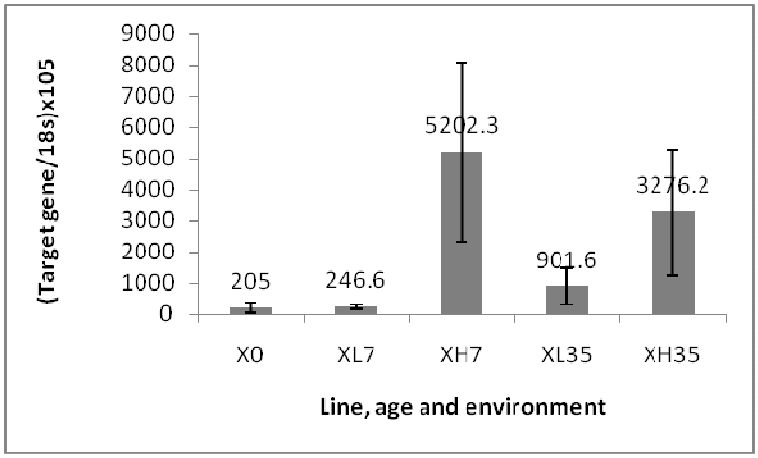

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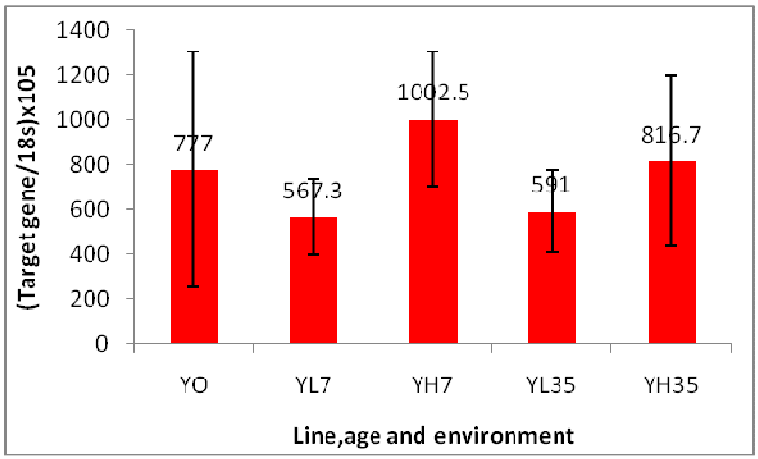

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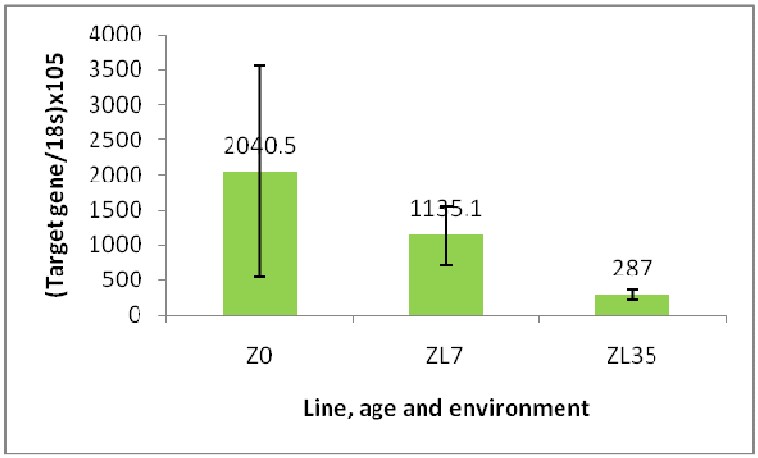


Figure 5.22A-C: Mean AvBD10 gene expression in all groups of chickens in the liver. A: line X, B: line Y and C: line Z (±SEM). (y axis should read 18S x 10^5).

5.6: Discussion

To investigate the potential effects of bird rearing environment, genetics on the tissue levels of AvBD, a molecular approach was employed. This was driven due to lack of specific antibodies against AvBDs, and therefore the inability to measure actual protein levels within tissues. The trial included a total of 180 birds, and from each of these birds a total of ten tissue samples were extracted providing 1,800 tissue samples. For analyses it was decided to focus on the day 0, 7 and 35 samples, the former based on the importance of the innate immune system in young birds, and the latter age group acting as a sensible end-point for the trial as commercial broilers are usually slaughtered around day 42.

Initially gene expression was investigated using end-point PCR analyses. This allowed the selection of specific tissues for quantification of AvBD expression by real-time PCR analysis. Time and financial constraints made it impossible to analyse all 1,800 samples via qRT-PCR, especially considering that samples were run in duplicate. Tissue and sample numbers had to be limited but not compromised so that sample sizes were sufficiently large enough to allow statistical analysis.

Expression levels of AvBD1, 4 and 10 were measured in the thymus, spleen, duodenum and bursa of Fabricius. As stated in the results, these tissues were chosen based on the end-point PCR data but the initial focus was to select and analyse tissues which had an immunological role. The selection of the duodenum allowed AvBD expression patterns to be analysed in conjunction with the anti-microbial activities of gut mucosal scrapes taken

from the same regions and this is discussed further in Chapter 7. Only AvBD10 levels were measured in the liver, kidney, testicle and lung, because prior to obtaining all of the SNP data (Chapter 4), AvBD10 was the gene of focus for the study. High levels of this gene had also been shown to be expressed in the liver, kidney and testicle in the literature (Xiao, Hughes et al. 2004; Higgs, Lynn et al. 2005) but the effects of age, bird genetics and rearing environment had not been previously reported. In addition, these three tissues are not directly linked with innate defense. It could be argued that all ten tissues should have been analysed for the expression of all three genes, and in fact the analyses of additional AvBD genes was also considered based on the SNP data shown in Chapter 4 e.g. AvBDs 3, 6 and 9. However, the study had to be focused due to time and finite resources. Throughout this chapter a significant, but recurrent finding, was the intra-group variability; such variation had not been anticipated. This combined with the sample sizes lead to large standard errors within the groups with the effect of reducing the chance of establishing statistically significant differences. Increasing the number of birds in each group would have improved the statistical power, allowing potential trends to be statistically proven, future studies need to address this.

The presence of the AvBD SNPs outlined in Chapter 4, may help to explain the observed differences in AvBD expression between birds and lines, perhaps becoming more apparent when the birds were reared in more challenging conditions. For example focusing on AvBD1 where three non-synonymous SNPs specific to line X birds were detected, the levels in the duodenal tissues of birds from the XL 7 day group were higher than the other groups of birds. The birds within each group were fed the same diets and

reared in the same conditions and so the observed data must be due to individual and/or local factors. It can only be hypothesised that the high expression levels seen in some birds correlated with high levels of specific protein production, but of particular interest was those birds that expressed high levels of more than one AvBD gene, indicating that either co-expression occurs or suggesting that the bird was under some form of challenge to that specific tissue. Whether expression of high levels of the gene is beneficial to a particular bird needs to be determined with further studies.

The house-keeping gene used in the qRT-PCR and data normalisation, was 18S. 18S is ubiquitously expressed within all tissue types and so high levels in qRT-PCR were detected, particularly in very active tissues such as the liver. In hindsight, the use of a less highly expressed house-keeping gene with expression levels closer to those observed for some of the AvBD genes would have been more appropriate. However, the selection of such a gene would have made the analysis of AvBD10 in tissues, such as the kidney and testicle, challenging since such high levels were observed in these tissues. Quantification using both β actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the real time assays was attempted but were not pursued due to lack of working plasmid standard and primers. The use of an array of house-keeping genes is becoming increasingly popular in studies utilising qRT-PCR, with subsequent selection of the appropriate gene for normalization. Much of the published work on chicken anti-microbial peptide expression is based on semi-quantitative analysis, and most of the reported literature used β- actin as the house-keeping gene (Wu, Zhang et al. 2000; Zhao, Nguyen et al. 2001; Yoshimura, Ohashi et al. 2006; Subedi, Isobe et al. 2007; Akbari,

Haghighi et al. 2008; Mageed, Isobe et al. 2008). Beta actin has also been used as the house-keeping gene in two quantitative real-time PCR studies (Yoshimura, Ohashi et al. 2006; Meade, Narciandi et al. 2009). The latter study chose β- actin as it was reported to be the most stable out of a panel containing both GAPDH and lactate dehydrogenase A (LDHA), but like 18S it is also highly expressed.

Anti-microbial peptides have been speculated to help link the innate and adaptive immune systems by having immuno-modulatory effects via their chemokine properties (Bowdish, Davidson et al. 2006). This function could be considered particularly important within the lymphoid tissues of the body where there are large populations of cells of lymphoid origin. The thymus is a primary lymphoid tissue and is the site of T cell development and cellular immunity (Cooper, Raymond et al. 1966). To date only AvBD9 (Ma, Liu et al. 2008) and AvBD11 have been reported to be expressed in this tissue (Xiao, Hughes et al. 2004) but in this chapter expression of AvBD1, 4 and 10 was also shown. Both AvBD1 and 4 showed the highest expression levels within the thymus in the day 0 bird samples and this may well correlate with either the development of this tissue, or in preparation for life outside the shell and challenges of the local environment. In contrast AvBD10 levels were shown to peak in birds aged 7 days, but the physiological and/or immunological reasons for this are not known. Higher levels of AvBD1 gene expression in the thymus were observed in the XL 7day birds compared to the XH 7 day group (mean AU of 24.0 and 0.3 respectively). The difference could, potentially, be related to the bacterial challenge of the low hygiene rearing environment (Figure 3.3) but the full effects of the different diets probably is a significant factor.

This study for reasons explained previously, focused on AvBD1, 4 and 10. Expression of AvBD1 and 4 had previously, using a chicken 44K agilent microarray been shown to occur in the spleen (Li, Chiang et al. 2008). The same authors also identified AvBD2 and 9 expression within the spleen, and both AvBD11 (Higgs, Lynn et al. 2005) and AvBD13 (Xiao, Hughes et al. 2004) have been shown to be expressed at this site. With respect to this study a similar pattern of AvBD gene expression was observed in the splenic tissue as found in the thymus. The spleen was chosen because it is a secondary lymphoid tissue with functions including the synthesis of antibodies within the white pulp and removal of antibody-coated bacteria and red blood cells, the latter two being particularly important in a successful adaptive immune response. The importance of the innate immune response within the first week of life has been supported by work carried out by Bar-Shira and Friedman (2006), and the levels of the AvBD gene expression in the day 0 birds support this. As the birds age, the immune system develops and they become more capable of developing a successful adaptive immune response with both cell mediated and humoral responses functioning. It could therefore be speculated that less emphasis is placed on the innate non-specific response with increasing age, and this could explain the reduced AvBD gene expression patterns in the day 35 group in the spleen.

The primary role of the bursa of Fabricius is to generate a humoural immune response and so it forms part of the adaptive immune response. However, maturation of the lymphoid cell population within the bursa is a slower process than that which occurs in the thymus (Peterson and Good 1965), and this explains the delay in antibody generation in young stock and perhaps places a greater emphasis on the innate immune system in

early life. For such reasons it was be proposed that the AvBD peptides may have an important role in this tissue and it was selected for further study. Moreover, the bursa anatomically is located close to the cloaca and is therefore exposed to potential pathogens in the gastro-intestinal and reproductive tracts. This supports the bursa being a site of marked AvBD expression, especially in the birds that were reared in the more challenging (LH) environment.

As observed in the thymus and spleen, both AvBD1 and 4 expression levels were highest in the day 0 birds in the bursal tissue consistent, potentially, with a protective role. Of note was just how low these levels were compared to other tissues (Table 5.3.and 5.4), although the bursal levels may just reflect the poor development of this organ in the young birds. Interestingly all AvBDs with the exception of 8 and 11 (van Dijk, Veldhuizen et al. 2008), have been reported in the literature to be expressed in the bursa of Fabricius, again supportive of their importance in this lymphoid organ.

Alpha defensins, located in Paneth cells of mammalian GI tract, have not been shown to exist in chickens. The duodenum was selected for AvBD expression analyses due to ease of locating this part of the GI tract at the time of sampling, thus allowing sampling reproducibility and to be able to compare the expression data with the anti-microbial activities of gut mucosal scrapes taken from the same section of tissue. Part of the rationale for selection of the three lines of birds was based on perceived gut health of the birds with the line X birds having the poorest health (wet litter) and the line Z having fewer gut-related issues.

Unlike the thymus and the spleen where peak levels of AvBD1 expression were observed in the day 0 groups, the highest level of AvBD1 in the duodenum was in the XL 7 day group (Table 5.4). The mean was significantly higher than the other day 7 groups (except ZL birds). This is an interesting finding because 1) the level was higher than the XH group and so the environment may have played a role and 2) it is line X birds which have the reported poorer gut health. In the gut tissues it is therefore feasible that both genetics and environmental rearing conditions have an effect on AvBD1 gene expression. While no significant statistical differences were found between XL and ZL day 7 birds means 150.7 and 31.4 AU, respectively (Figure 5.16), these data show a definite trend.

It is also possible that the SNPs which were detected in the AvBD1 gene (Chapter 4) may have affected gene expression levels. For example the presence of SNPs specific to line X birds may have had an effect on AvBD1 gene expression levels due to potential alterations in peptide function for example the synthesis of a peptide with reduced anti-microbial activity may result in increased mRNA expression levels. Differences in anti-microbial properties of the three forms of the mature peptide are considered in Chapter 6.

Each region of the small intestine; duodenum, jejunum and ileum, has distinct features in its structure and physiology, and supports very different commensal bacteria populations. The data presented in this Chapter shows that AvBD1, 4 and 10 were expressed in the duodenal tissues in day 0 and 7 birds. Other groups have shown expression within the small intestine of AvBD1, AvBD2 (Lynn, Higgs et al. 2004), AvBD4 (Milona, Townes et al. 2007), AvBD6 (Ma, Liu et al. 2008), AvBD9 (van Dijk, Veldhuizen et al. 2007),

AvBD10 (Ma, Liu et al. 2008), AvBD11 (Higgs, Lynn et al. 2005) and AvBD13 (same as AvBD11 group). Milona, Townes et al. (2007) also showed that there was significant up-regulation of duodenal AvBD4 expression following oral challenge with *Salmonella* species supporting the link between potential anti-microbial effects and host defense following infection.

Karlsson et al (2008), showed that in mice different anti-microbial peptides were secreted in the different regions of the small intestine and that the levels were also influenced by animal age. The same authors also determined that there were differences in expression levels between lines of mice, again suggesting that there is a genetic component influencing expression, as was also observed in this study with the three lines of chickens. Interestingly work in transgenic mice supports a role for defensins in selecting gut commensal populations (Salzman, Hung et al. 2010). Thus sectioning the chicken gut to include the duodenum, ileum, jejunum and caecum, and analyzing AvBD expression patterns and bacterial populations would therefore be of significant interest. Interestingly although only trends can be suggested due to the small number of actual values available and their variability, the gut expression data reported in this study suggests a reduction in duodenal AvBD expression with increasing bird age, and an increase in caecal expression, particularly in those birds raised in the LH environment.

A study involving the feeding of swine AMPs to 0-49 day-old chickens has also been performed in an attempt to enhance their gut immunity. Birds included in this study were shown to have enhanced intestinal mucosal immune parameters including increased intra-

epithelial lymphocyte, mast cell and goblet cell numbers, indicating the local effects of such peptides (Wang, Ma et al. 2009). Other reported effects of the swine peptides includes an increased ability of the treated chickens to absorb nutrients (Bao, She et al. 2009), with subsequent superior growth rates. Administration of AMPs from rabbits to chickens also showed improved mucosal parameters including villus height increase and increased numbers of intra-epithelial lymphocytes (Liu, She et al. 2008). Whether up-regulation of endogenous AMPs would have a similar effect is not known as it is still speculative as to whether high endogenous levels of AvBD expression are of actual benefit or detriment to the host.

The caecal tonsils are located at the base of the two caecal sacs and can be considered as modified Peyers patches and act as secondary lymphoid tissues. M cells, characteristic of Peyers' patches, have been detected only in birds aged over two months (Kato, Hashimoto et al. 1992) and since the maximum age of birds in this study was 35 days, there is a strong possibility that these birds do not have M cells within their epithelial layer. Absence of such cells and under-development of this tissue may be correlated with the AvBD expression data and explain the relatively low levels observed in the birds analysed. Akbari, Haghighi et al. (2008), assessed AvBD expression (AvBD1, 2, 4 and 6) within the caecal tonsil following challenge with *Salmonella enterica* serovar Typhimurium with and without prior administration of probiotics. Their conclusion was that the probiotics dampened down the AvBD expression levels following challenge with *Salmonella*. Microbial colonisation of the gut with flora from the environment might also have a dampening effect on AvBD expression and so lead to the older birds having lower

expression levels. The data presented in Table 5.8 does not support this theory, although it is acknowledged that this data is compromised by the very small sample number. This also raises the question again as to whether high levels of AvBD expression are of benefit or detriment to the host.

As expected in a tissue continuously exposed to potential pathogens via aerosols a number of different AvBDs have been shown to be expressed in the lungs of chickens including AvBD1, 2, 4, 6, 7, 9, 10 and 13 (Xiao, Hughes et al. 2004; Higgs, Lynn et al. 2005). In this study for reasons discussed previously the lung tissue was only investigated for AvBD10 expression. Peak AvBD10 expression within the lungs was observed in the day 7 birds, but interestingly expression was relatively low compared to kidney and testicle, tissues arguably not as exposed to pathogens. In fact the AvBD 10 gene expression levels observed in the bird kidney samples were very high; the XL 35 day group had a mean expression level of 132193.1 AU, which was nearly a thousand fold higher than some of the other tissues which were analysed. In the literature AvBD3, 6, 9, 10, 11, 12 and 13 have been shown to be expressed within kidney tissue (Xiao, Hughes et al. 2004; Higgs, Lynn et al. 2005). These data indicate that the kidney is an active site for AvBD gene expression, but whether the defensins function as only anti-microbial agents or in some other roles is not known.

As for the kidney, high levels of AvBD10 gene expression were observed within bird testicles. Both the kidney and testicle are in close proximity to each other in the chicken

and embryologically they are derived from the same cells, which may potentially explain these findings.

Of particular note is the result that line YH 7 day birds expressed very high testicular AvBD 10 levels compared to the other groups of birds (Figure 5.21) with the presence of two distinct sub-groups. None of the birds within this study were sexually mature (normally 18-24 weeks of age) and so this proves an exciting finding, and raises the question as to why line Y birds in the high hygiene environment were expressing such high levels of this gene within a tissue that is not fully developed or active. One explanation is that the Vas deferens opens into the cloaca and since the cloaca has multiple functions, and is not perceived as a sterile environment, there is a theoretical risk of bacterial colonization within this duct and an ascending infection to the testicles. Interestingly other AvBDs have been shown to be highly expressed within the testicle and these include AvBD, 2, and 6 while low to moderate expression levels of 5, 9 and 12 have also been reported (Xiao, Hughes et al. 2004; Higgs, Lynn et al. 2005). Most of these studies involved male birds which had not reached sexual maturity and so had developing testes. Neither this study nor the other studies specify the section of the testicle responsible for AvBD expression. However Com, Bourgeon et al. (2003), investigated anti-microbial gene expression in the reproductive tract of rats, mice and man, and focused on distinct areas of the male reproductive system. Resident macrophages, Leydig cells and seminiferous tubules were found to express these genes with subsequent protection of sperm post-ejaculation. These data support the possibility of a non immune function of AvBD10 within this tissue as well as the kidney.

The final tissue for discussion is the liver and previously moderate to high levels of AvBD 8, 9, 10 and 13 gene expression have been reported (Xiao, Hughes et al. 2004; Higgs, Lynn et al. 2005) as well weaker levels of AvBD2, 4 and 6. In this study only AvBD10 expression levels were quantified. Both lines X and Y birds reared in the HH environment at day 7 showed higher mean expression levels of AvBD10 in the liver compared to the same lines reared on the commercial farm with line X showing the highest levels. This is difficult to explain as HH environment would be expected to provide greatest microbial challenge. It is possible that the two different diets affected expression, as the liver is in close proximity to the gastro-intestinal system and is linked to the hepatic portal vein from the gut. However, the possibility of a non immune function of AvBD10 within the liver cannot be excluded. In conclusion, AvBD gene expression patterns and trends were found in the bird tissues analysed in this study. However, the data was characterized by large intra-group variability making definite conclusions as to whether the environmental factors, diet or bird genetics together, or individually, were responsible for such observations difficult. Increased numbers of analyses per group studies are required to increase the statistical power to allow such conclusions to be made. However the results presented within this chapter may lead to further studies allowing for example, protein ELISAs to determine peptide levels and immuno-histochemistry to determine cell types responsible for AvBD gene expression.

Chapter 6: Expression and functionality of recombinant AvBD1 and 10 peptides

6.1: Introduction

Based on the presence of single nucleotide polymorphisms it was determined to focus on the study of three AvBD genes, 1, 4 and 10. As outlined in Chapter 4, no coding SNPs were detected in either of the AvBD 4 or 10 genes but three non-synonymous coding SNPs were detected in the gene encoding the mature peptide of AvBD1. Each of the three lines of birds were shown to have different frequencies of these polymorphisms, which suggested the production of AvBD1 peptides with different combinations of amino acids and, potentially, different antimicrobial activities. AvBD4 had previously been shown to have anti-microbial properties (Milona, Townes et al. 2007), but to date no reports exist demonstrating the anti-microbial properties of chicken AvBD10. It was thus decided to produce recombinant AvBD1, specifically, the three different variants, and AvBD10, to determine their anti-microbial properties and establish whether one form of the mature peptide was more effective than the others.

Previous studies determining anti-microbial properties of the AvBD have involved using either synthetic peptides or relied upon hyper-expression systems that utilise a vector system such as bacteria (Ma, Liu et al. 2008) or specific cell lines (van Dijk, Veldhuizen et al. 2007). The use of insect hyper-expression systems (Wonderling, Powell et al. 2002) and synthetic peptide synthesis are other methods of protein production (Higgs, Lynn et

al. 2005). Previously within the research group hyper-expression of the chicken AMPs cLEAP-2, AvBD4, 5 and 6 had been performed using a bacterial system (Townes, Michailidis et al. 2004; Milona, Townes et al. 2007). Other anti-microbial peptides that have been produced in bacterial systems include mouse cryptidins (Satchell, Sheynis et al. 2003), human neutrophil peptide 1 (HNP-1) (Piers, Brown et al. 1993), human beta defensins 26 and 27 (Huang, Leong et al. 2009), human beta defensin 3 (Chandrababu, Ho et al. 2009), human beta defensin 2 (Vargues, Morrison et al. 2009) and duck AvBD9 and 10 (Ma, Liao et al. 2009). The advantage of using a bacterial production system is that it allows mutated peptides to be sythesised in a cost effective manner. Bacterial production systems can also lead to high yields of peptide, but ensuring correct folding of the peptides can be problematic, although this is true irrespective of the method utilized for synthesis.

AvBD1 has been shown to have anti-microbial effects at concentrations within the range 0.4-3.4µM against several classes of bacteria that include, *Escherichia coli*, *Salmonella enteriditis*, *Salmonella typhimurium*, *Campylobacter jejuni*, *Bordetella avium*, *Listeria monocytogenes* and *Staphylococcus aureus* (Evans, Beach et al. 1994; Harwig, Swiderek et al. 1994; Evans, Beach et al. 1995; Ma, Liao et al. 2009). The four combinations of the three non-synonymous SNPs that were detected within the birds analysed were predicted to code the following amino acid combinations; NYH, SSY, NYY and SYY (Table 4.9). To investigate the potential effects of such mutations on peptide functionality the three different forms of AvBD1 were synthesised, the amounts quantified and their functionality determined using anti-microbial time-kill assays. In

addition, AvBD10 was also synthesized and the anti-microbial properties of the recombinant peptide analysed.

The aims for this chapter were;

- **To hyper-express and purify the mature peptides AvBD1 and 10,**
- **To establish an appropriate enzyme-linked immunosorbant assay (ELISA) to quantify peptide levels,**
- **To determine functionality and potency of the recombinant peptides in bacterial time-kill assays with *Salmonella enterica* serovar Typhimurium phoP and clinical isolates.**

6.2: Engineering of AvBD 1 and 10 cDNA clones

6.2.1: Gene inserts

Specific primers, as described in Chapter 2, with restriction sites engineered at the 5' ends allowed AvBD cDNAs to be amplified from a template that was either a pre-purchased cDNA AvBD clone or a cDNA amplified from an individual bird. The restriction sites (EcoRI and BamHI) were chosen based on compatibility with those within the chosen expression vector (pRSETA, Invitrogen, Paisely, UK). A cDNA clone, ChEST 1015e22 (ARK genomics, Edinburgh) was used to amplify the DNA encoding the mature peptide of AvBD10.

However, no PCR product could be amplified from the AvBD1 clone (ChEST 679b6), suggesting a problem with the clone. The AvBD1 primers were therefore used to amplify cDNAs from actual bird tissue samples. Figure 6.1 shows restriction enzyme treated AvBD1 and 10 PCR products prior to ligation into the pRSETA vector.

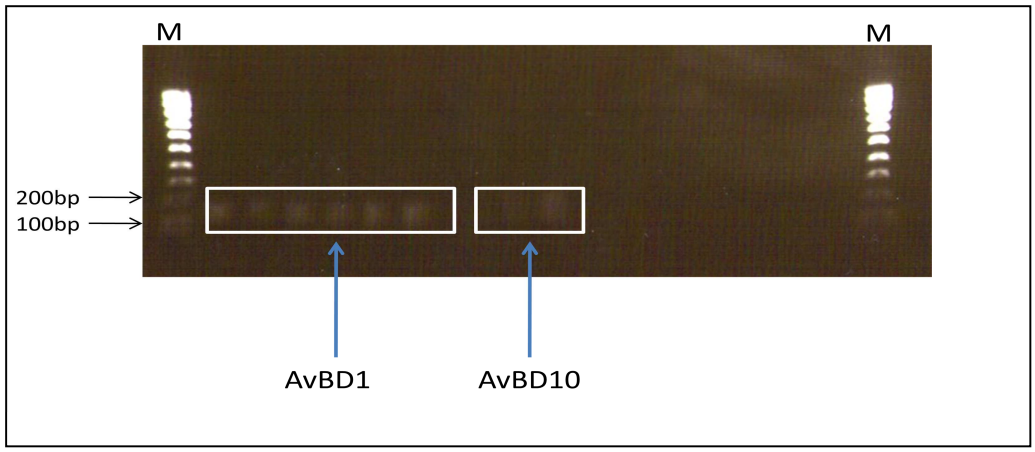


Figure 6.1: Gel electrophoresis of amplified cDNA gene inserts for both AvBD1 and AvBD10 prior to ligation. AvBD1 PCR product was 144bp and AvBD10 166bp.

The three non-synonymous SNPs located within the mature peptide of AvBD1 were predicted to encode six different amino acid combinations; however, only four combinations were detected in the populations analysed (Chapter 4). The three most common combinations of SNPs were amplified, the cDNAs cloned and the encoded peptides expressed. The predicted amino acid sequences of the mature peptides for the chosen gene inserts are shown in Figure 6.2. The green hi-lighted amino acid contains the novel, newly identified polymorphism, SNP 'VB1', within its codon, the purple amino acid incorporates SNP rs15457749 within its codon, the blue marks the third non-synonymous polymorphism, and is associated with SNP rs15457747.

AvBD10: DPLFPDTVA**C**RTQGNF**C**RAGA**C**PPTFTISGQ**C**HGGLLN**CC**AKIPAQ

AvBD1 **A**:GRKSD**C**FRKNGF**C**AFLK**C**PYLTLISGK**C**SRFHL**CC**KRIWG

AvBD1 **B**:GRKSD**C**FRKNGF**C**AFLK**C**PYLTLISGK**C**SRFYL**CC**KRIWG

AvBD1 **C**:GRKSD**C**FRKSGF**C**AFLK**C**PSLTLISGK**C**SRFYL**CC**KRIWG

Figure 6.2: The amino acid sequences of the mature peptide of AvBD10 and the three encoded AvBD1 (A-C) peptides. The cysteine residues are in bold and underlined. The amino acids resulting from the presence of the SNPs are hi-lighted.

6.2.2: Predicted peptide properties

The encoded amino acid sequences of the AvBD1 mature peptides are given in Figure 6.2 with the three different forms of AvBD1 referred to as A, B and C. Predicted properties of these four peptides are presented in Table 6.1 and determined using bioinformatic programmes (www.bioinformatics.org/sms/prot_mw.ntml and http://aps.unmc.edu/AP/prediction/prediction_main.php for the molecular weight and hydrophobic ratio and charge respectively, data accessed 10.8.2009).

Recombinant peptide	**Molecular weight (kDa)**	**Total hydrophobic ratio (%)**	**Net charge**
AvBD10	4.78	43	+2
AvBD1 A	4.64	45	+9
AvBD1 B	4.67	45	+8
AvBD1 C	4.57	45	+8

Table 6.1: Predicted properties of the four mature AvBD peptides

6.2.3: pRSETA cloning vector

The plasmid vector used throughout for the hyper-expression of the AvBDs was pRSETA (plasmid map in Appendix I). This vector encodes an N-terminal six poly-histidine tag that facilitates purification and identification of the recombinant protein. All recombinant DNA constructs were verified by sequencing to ensure that the cDNA sequence was correct and that it was in frame with the T7 promoter prior to transformation and peptide hyper-expression. Examples of sequenced plasmid constructs are shown in Figure 6.3.

Example 1- AvBD1 pET T7 promoter

CTTTAAGAAGGAGATATACAT***ATG***CGGGGTTCTCATCATCATCATCATCATGGTCTGGT
TCCGCGTGGATCC**GGAAGGAAGTCAGATTGTTTTCGAAAGAGTGGCTTCTGTGCAT**
TTCTGAAGTGCCCTTCCCTCACTCTCATCAGTGGGAAATGCTCAAGATTTTACCTC
TGCTGCAAAAGAATATGGGGCTGAGAATTCGAAGCTTGATCCGGCTGCTAACAAAG
CCCGAAAGGAAGCTGAGTTGGCTGCTG

Example 2- AvBD1 pET T7 promoter

GAGATATACAT***ATG***CGGGGTTCTCATCATCATCATCATCATGGTCTGGTTCCGCGTGGA
TCC**GGAAGGAAGTCAGATTGTTTTCGAAAGAGTGGCTTCTGTGCATTTCTGAAGT**
GCCCTTCCCTCACTCTCATCAGTGGGAAATGCTCAAGATTTTACCTCTGCTGCAAA
AGAATATGGGGCTGAGAATTCGAAGCTTGATCCGGCTGCTAACAAAGCCCGAAAGGA
A

Figure 6.3: Two examples of sequencing from AvBD1 construct plasmids showing the DNA encoding the mature peptide in bold ink, the location of the SNPs in yellow and the start ATG codon in red. Both inserts are in frame with the T7 promoter (both are forward sequences from the use of the T7 promoter primer).

6.2.4: Preparation of competent cells for hyper-expression studies

The recombinant AvBD pRSETA clones were each transformed into competent Origami B (DE3) :: pLysS (Chapter 2). This strain of bacteria had been previously used for AMP expression by co-workers (Townes, Michailidis et al. 2004; Milona, Townes et al. 2007). The production of T7 RNA polymerase by Origami B is counteracted by the presence of the plasmid pLysS. This plasmid functions by expressing low levels of T7 lysozyme that acts as a natural inhibitor of T7 RNA polymerase, thus preventing activation of the T7 promoter in un-induced cells. Following IPTG induction, expression of T7 RNA polymerase increases substantially, counteracting inhibition by T7 lysozyme with the subsequent expression of the target gene (Studier 1991). An additional property of Origami B:: pLysS is that its cytoplasm provides a higher oxidizing environment which facilitates the folding of protein by increasing di-sulphide bond formation (Xu, Lewis et al. 2008).

The procedures and conditions for hyper-expression of the AvBD1 and 10 peptides are described in Chapter 2. Following hyper-expression, total protein was measured and the soluble fraction analysed by SDS-PAGE to confirm peptide synthesis.

6.3: Measurement of total protein levels by Bradford and NanoDrop Analysis

Bradford analysis of total protein, as described in Chapter 2, was performed on the cell-free extracts following hyper-expression. Only the cell-free extracts (soluble peptide)

were analysed as these were the samples used in the anti-microbial assays. However, problems were encountered in that as the recombinant peptide was diluted, the calculated protein concentration increased. This finding suggested that there were substances within the cell-free extracts that interfered with the activity of the Bradford reagent and thus resulted in erroneous results. To address this, the NanoDrop was used to measure total protein. Examples of the concentrations obtained following hyper-expression of all four peptides are given in Table 6.2.

Sample	**mg/ml**
Empty vector	25.0
AvBD1 A	6.5
AvBD1 B	6.0
AvBD1 C	30.6
AvBD10	13.7

Table 6.2: Examples of mean total protein concentrations of cell-lysates as determined by the NanoDrop method (read at OD_{280}nm, n=3).

6.4: Recombinant AvBD10 peptide

6.4.1: SDS-PAGE gel analysis of recombinant AvBD10 peptide

The cell-free extracts were subjected to SDS-PAGE and the gels Coomasie stained. The hyper-expressed peptide was visualised generally as a diffuse band (Figure 6.4). A band

(circled) can be seen in the cell extract lane and is smaller than 14kDa in size. This band was presumed to be recombinant AvBD10. The mature peptide has a calculated weight of 4.75kDa but as the His-tag was still attached the full molecular weight was calculated to be 5.68kDa.

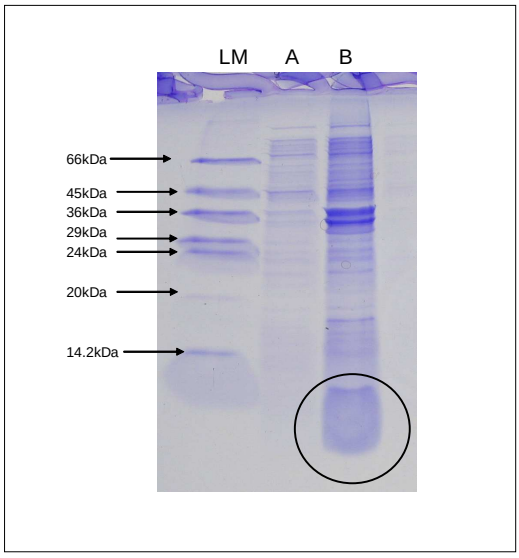


Figure 6.4: Coomassie blue-stained 12.5% SDS-PAGE gel of recombinant AvBD10 (200µg of protein was loaded). Lane A contains the cell free extract. Lane B contains the cell pellet. LM is the low molecular marker with the molecular weights of the proteins marked on the left side of the figure. Presumed AvBD10 peptide is circled.

6.4.2: Western analysis of recombinant AvBD10

It was difficult to prove that the band visualised in Figure 6.4 was recombinant AvBD10 peptide and so Western analysis was performed. This analysis relied upon the presence of the poly-histidine tag attached to the AvBD mature peptide and the use of an anti-His tag monoclonal horse-radish peroxidase conjugated antibody (purchased from Sigma).

Western analyses confirmed that peptide of the correct size, indicative of recAvBD10, had been synthesised. Figure 6.5 shows an example of a Western analysis of hyper-expressed AvBD10 and the bands indicate His-tag labeled AvBD10 peptide. The band in lane A relates to peptide produced from 200ml of culture, lane B to 600ml of culture and lane C to one litre of culture. Poor transfer onto the membrane has occurred in lane B.

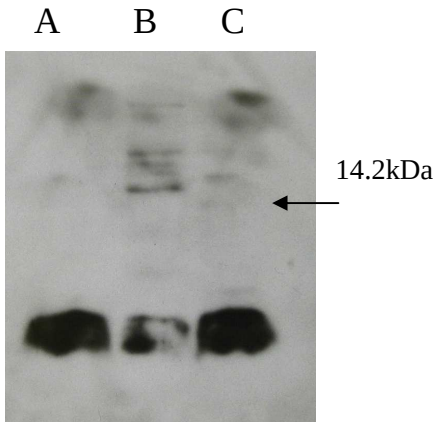


Figure 6.5: Western analysis of AvBD10 peptide. A-C indicate total volume culture (200µg protein added to each well).

For each hyper-expression experiment a control was set up using bacteria transformed with pRSETA vector without a gene insert. No protein bands were visualised following Western analysis of this sample (Figure 6.8C, lane 2).

6.4.3: Enzyme-linked immunosorbant assay (ELISA)

Only the total protein concentration of the hyper-expressed samples had been measured, thus it was not known what percentage of this, the recombinant peptide constituted. To

determine the actual amount of peptide, a direct capture ELISA method based on the presence of the His-tag was established.

Initially a standard curve using a His-tag labeled protein, 53kDa in size, but of known concentration was constructed, within the protein range of 0.025µg/ml to 60µg/ml. Figure 6.6 shows an example of the standard curve generated. As the curve plateaued at concentrations greater than 1.7µg/ml, test samples had to be diluted so that their concentration fell below this point.

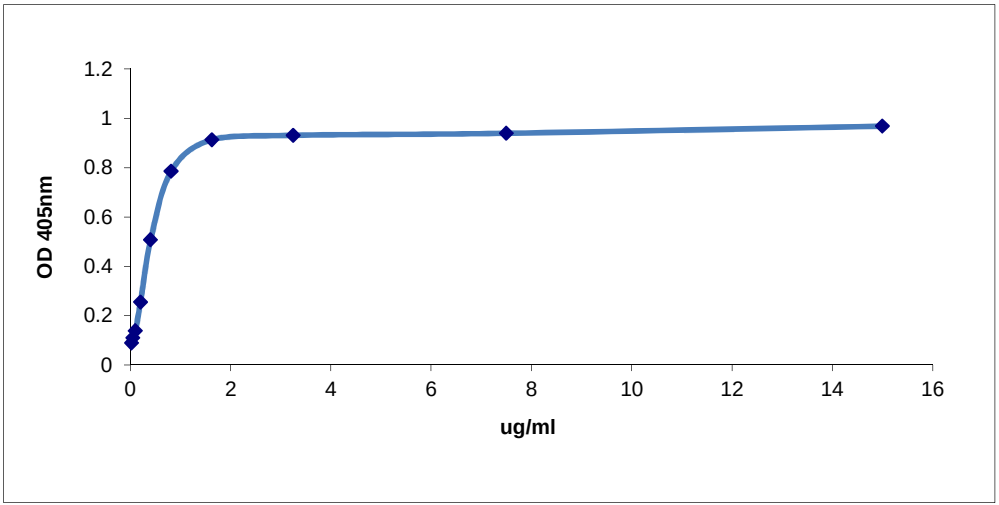


Figure 6.6: Standard curve generated for His-tag labeled proteins. The first six points are shown in Figure 6.7. After a peptide concentration greater than 1.7µg/ml the curve can be shown to plateau out and the points no longer follow the same pattern.

A standard curve was therefore produced focusing on the points below 1.7µg/ml and an example of such a standard curve is presented in Figure 6.7 (NB a narrower range of 0-0.8µg/ml is represented in this curve).

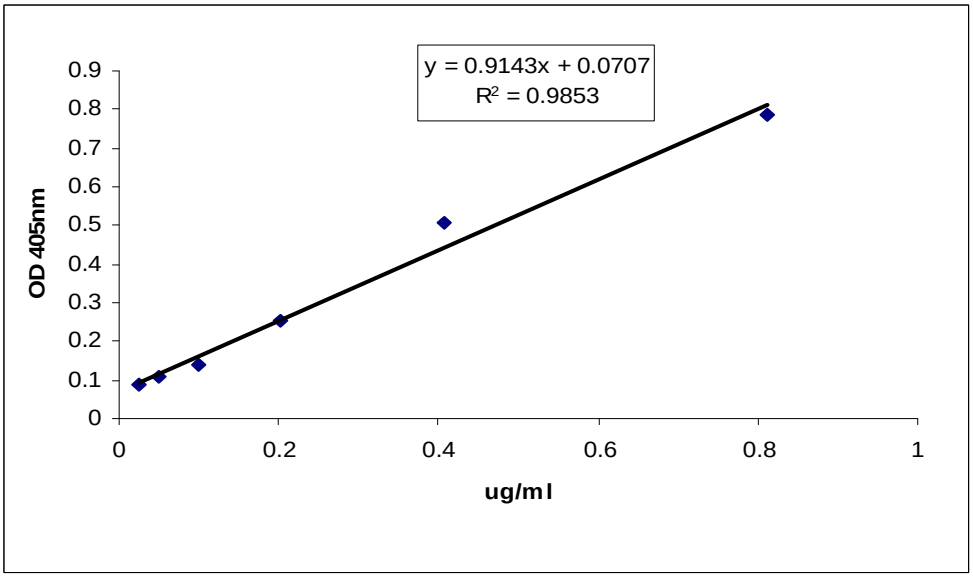


Figure 6.7: Standard curve generated for His-tag labeled protein (53kDa in size).

The equation of best fit (Figure 6.7) was used to calculate the concentrations of the recombinant peptides based on the individual optical density readings and dilution factors. Molar concentrations of peptide were calculated using the predicted molecular mass of the peptide.

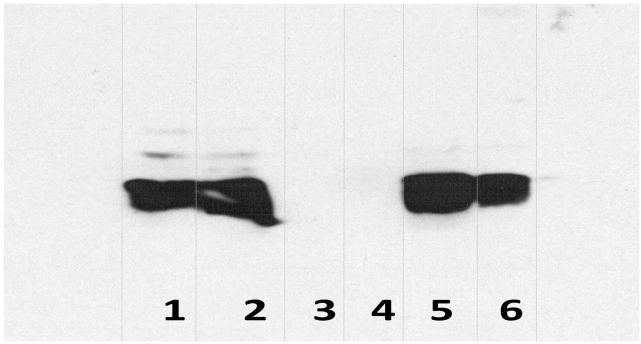
6.4.4: Purification of recombinant AvBD10

Once synthesis of the recombinant peptide had been confirmed by Western analyses, attempts were made to purify the peptide from the soluble fraction using a talon resin (Clontech, Hampshire, UK). This technique, described in Chapter 2, uses the principles of immobilized metal affinity chromatography (IMAC) (Porath, Carlsson et al. 1975), to bind recombinant peptides with poly-histidine tags to the cobalt-based talon resin. Bound proteins are then eluted from the column by the addition of imidazole. Cell-free extract

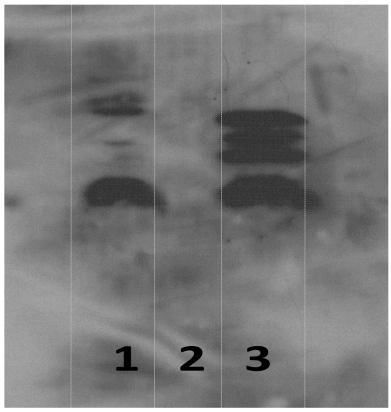
(200µg/ml total protein, 2.6nM recombinant AvBD10 peptide) was applied to the talon column, washed and recombinant AvBD peptide eluted with increasing concentrations of imidazole. An example of the Western analyses following electrophoresis of these fractions is shown in Figure 6.8A. No His-tag labeled protein was detected in the flow-through sample (lane 3), indicating that all of the recombinant protein had presumably bound to the talon resin. Both the 100mM and 200mM imidazole elution samples contained His-tag labeled protein as shown in lanes 5 and 6 respectively, although not the 10mM elution fraction (lane 4). No staining of bands on the corresponding SDS-PAGE gels was visualised, presumably due to low concentrations of peptide. Figure 6.8B, lane 3 also shows that elution of the peptide occurred when a higher concentration of imidazole (500mM) was used, suggesting that the recombinant protein had a high affinity for the talon column.

Following confirmation that the recombinant peptide had been eluted into imidazole, the sample was passed through a PD-10 desalting column (G.E. Healthcare, UK) to remove the imidazole with subsequent final elution into 0.1M PBS, pH7.4. The use of this column had the effect of diluting the sample into a larger final volume (3.5ml, from a total start volume of 2.5ml). To compensate for this dilution effect, the final sample was placed into a concentrating column (Vivaspin, 3-50kDa proteins, VWR USA). It was determined from Western analyses that significant amounts of peptide freely passed through the concentrator membrane and were lost.

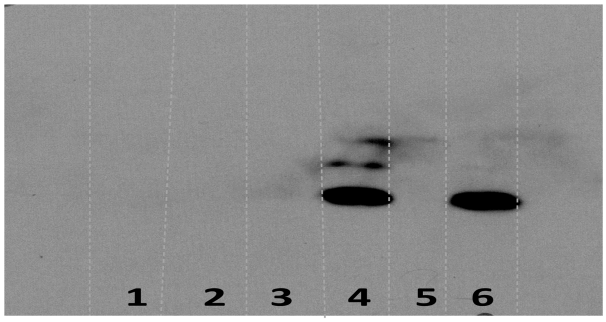
A



B



C



Figures 6.8A, B and C: Western analysis of His-tag labeled recombinant AvBD10 with HRP-conjugated anti-His tag antibody.

A: lane 1:=recAvBD10 from 1L LB broth, 2=recAvBD10 from 1L LB broth, 3=flow through from talon resin, 4= 10mM imidazole elution, 5=100mM imidazole and 6=200mM imidazole elutions.

B: lane 1= 100mM imidazole elution 2=unloaded lane and 3= 500mM imidazole elution.

C: lane 1-low molecular weight marker, 2= empty pRSETA, 3= empty lane, 4= recAvBD10 from 1L LB broth (flask), 5=empty lane and 6= purified AvBD10 (elution from PD10 column).

6.4.5: Problems encountered with purification of recombinant AvBD10

Hyper-expression and purification were attempted on numerous occasions but despite the appearance of recombinant protein in Figure 6.8C, purification of AvBD10 proved inconsistent. Essentially difficulties were encountered while de-salting the samples. Thus obtaining reasonable levels of pure peptide proved very difficult.

6.4.6: MALDI-TOF of purified AvBD10

To confirm the identity of the hyper-expressed protein, MALDI-TOF was used. A sample of pure material was submitted for analysis at Pinnacle Proteomics (Newcastle University). Unfortunately analyses proved inconclusive, with insufficient protein given as the reason for this failure.

6.5: Recombinant AvBD1 peptides

6.5.1: SDS-PAGE gel analysis of recombinant AvBD1 peptide

Figure 6.9 shows a colloidal blue-stained SDS-PAGE gel containing the products from hyper-expression of each of the three peptide forms of AvBD1 (A-C).

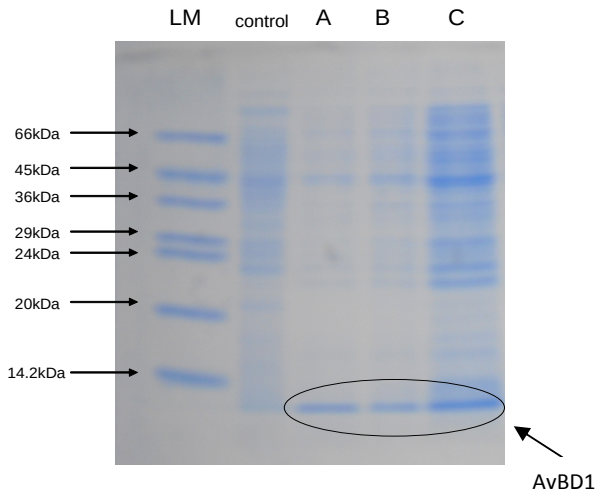


Figure 6.9: Colloidal blue-stained SDS-PAGE gel of recombinant AvBD1 peptides. Lanes A-C, A: AvBD1-NYH, B: AvBD1-NYY and C: AvBD1-SSY. The control lane contained the products from hyper-expression of pRSETA vector (no gene insert). All lanes were loaded with cell free extract. Approximately l00µg of total protein was added to wells A and B and 500µg to lane C. LM is the low molecular marker. Presumed AvBD1 peptide is circled below the 14.2kDa marker.

Lanes A to C in Figure 6.9 reveal distinctive bands (shown in circle) below the 14.2kDa molecular weight marker, presumed to be the AvBD1 peptides. Their predicted peptide masses were 5.57kDa, 5.6kDa and 5.5kDa, respectively for forms A, B and C, including the His-tag. The identification of bands was aided by the absence of a comparable band in the control lane. The multiple bands in all of the lanes reflect the samples being cell free extracts.

6.5.2: Western analysis of recombinant AvBD1

Western analysis to actually identify the AvBD1 peptides was performed as described for AvBD10. Figure 6.10 shows detection of the three forms of AvBD1 (Figure 6.9 is the corresponding SDS-PAGE gel). Approximately 100µg of total protein was loaded onto wells A and B and 500µg to well C (as for the SDS-PAGE analysis).

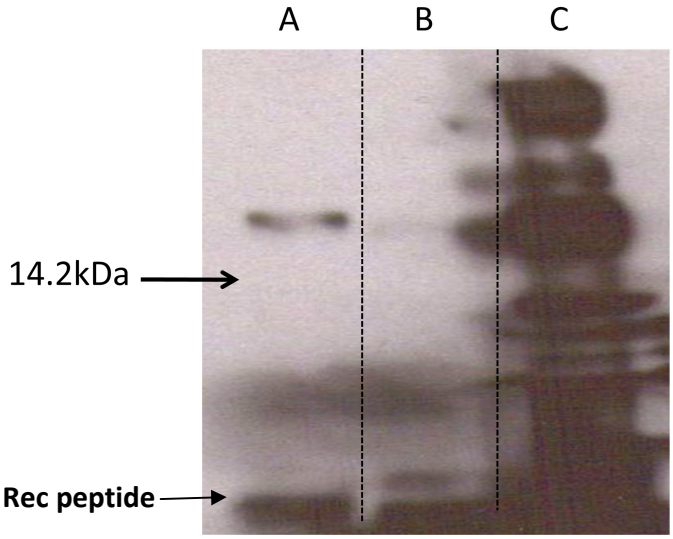


Figure 6.10: Western analysis of His-tag labeled recombinant AvBD1 peptides with HRP-conjugated anti-His tag antibody. Lanes A- AvBD1-NYH, B- AvBD1-NYY and C-AvBD1-SSY as for Figure 6.9. Multiple banding can be seen in lane C and overspill into the right lane occurred. Approximately 100µg of total protein was added to lanes A and B and 500µg to lane C.

Due to the problems encountered previously, neither protein purification nor MALDI-TOF of the recombinant AvBD1 peptides were attempted. However, following confirmation of peptide synthesis via Western analysis, the established ELISA was used to quantify the level of recombinant peptide in the cell free extracts.

6.6: Bacterial time-kill assays

Following identification and quantification of the AvBD peptides, their anti-microbial activities were determined against different strains of bacteria using a time-kill assay as used by (Townes, Michailidis et al. 2004; Milona, Townes et al. 2007).

6.6.1: Bacterial strains

A) *Salmonella enterica* serovar Typhimurium phoP

Salmonella enterica serovar Typhimurium phoP is used routinely as a sensitive tool to detect, gauge and compare the anti-microbial activity of AMPs. This strain of bacteria contains a mutated form of the gene regulon phoP which leads to increased sensitivity to the actions of AMPs (Miller, Loomis et al. 1993).

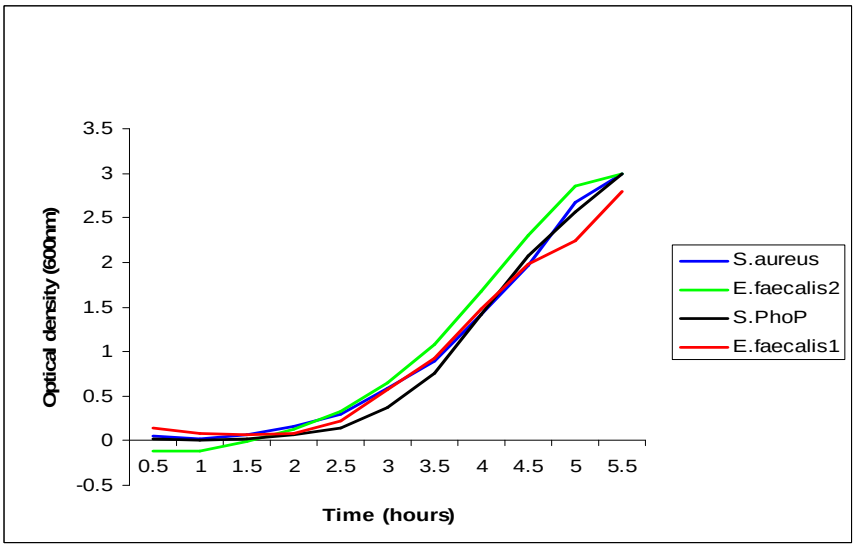


Figure 6.11: The bacteria growth curves for the strains used in the time-kill assays. All strains reached mid-exponential growth at approximately 3 hours.

Time-kill assays were performed using known amounts of the recombinant peptide and for each experiment, controls were used. These controls were from a hyper-expression experiment using bacteria transformed with empty pRSETA vector and contained an equal amount of total protein to the recombinant samples.

B) Clinical isolates:

In addition to *Salmonella enterica* Typhimurium phoP the killing activities of the recombinant peptides were tested against a panel of bacteria. These included three clinical isolates, *Staphylococcus aureus* and *Enterococcus faecalis* (two strains), isolated and identified from post-mortem cases from the Aviagen farms. The time-kill assay relies on using bacteria, which are in mid-exponential growth and it was necessary to

perform growth curves of the clinical isolates to determine whether any modifications to the assay, established using *Salmonella* strains needed to be made. The procedures performed to determine the growth rates of the bacteria were carried out as described in Chapter 2. The growth curves for all the bacteria are shown in Figure 6.11

6.6.2: Results of the bacterial time-kill assays

In all of the time-kill assays, all analyses were performed in triplicate and the assays were performed on at least three different occasions. The recombinant AvBD10 peptide was tested for anti-microbial activity against all three of the clinical isolates as well as *Salmonella enterica* serovar Typhimurium phoP. The three forms of recombinant AvBD1 were only tested against *Salmonella enterica* serovar Typhimurium phoP as an indicator as to whether a change in the three amino acids affected anti-microbial activity.

6.6.3: Determination of recAvBD10 anti-microbial activity

A) Crude cell-lysate containing recombinant AvBD10

To normalise the anti-microbial assay data, identical amounts of total protein from the AvBD recombinant and control cell-lysate extracts were analysed. The total amount of recombinant peptide based on the ELISA results was then calculated from this and the molar concentration determined. This resulted in all samples analysed, be they control or recombinant AvBD10 peptide, having the same concentration of total protein. Following the first hyper-expression a total of 40ng of total protein was used

in the anti-microbial assays and this corresponded to an AvBD10 concentration of 2.6nM.

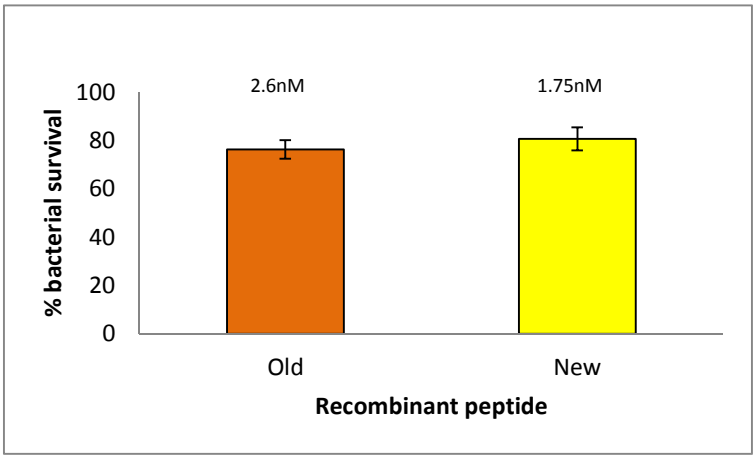


Figure 6.12: Percentage bacterial survival of *S.phoP* in time-kill assays using different batches of recombinant peptides (n=6 for Old, 2.5nM batch and n=5 for New, 1.75nM batch experiments).

The results obtained for the time-kill assays using the four strains of bacteria, *Staphylococcus aureus* (one strain), *Enterococcus faecalis* (two strains) and *Salmonella enterica* serovar Typhimurium phoP, are shown in Figure 6.13 and the peptide was used at a concentration of 2.6nM throughout these experiments.

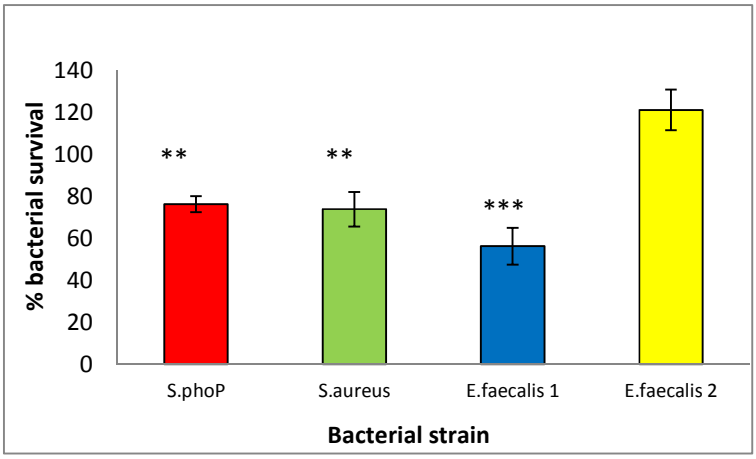


Figure 6.13: Bacterial time-kill assay results using 2.6nM of recAvBD10. Results have been normalized to pRSETA cell-lysate. ** indicates $p<0.01$ and ***$p<0.001$ relative to *Enterococcus faecalis* strain 2 (n=9, n=12, n=15 and n=18 for strains left to right, respectively).

The average percentage survival for the *Salmonella enterica* serovar Typhimurium phoP was 76.3% (±3.9SEM, n=6) and a similar result was obtained for *Staphylococcus aureus* (73.9% ±8.3SEM, n=4). Anti-microbial activity was also observed against *E. faecalis* strain 1 with a mean bacteria survival of 56.3% (±8.8SEM, n=3). In contrast the second strain of *Enterococcus faecalis* showed no susceptibility to the effects of the recombinant peptide with mean survival 121.3% (±9.7SEM, n=5). The percentage survival of *Salmonella enterica* serovar Typhimurium phoP, *Staphylococcus aureus* and *Enterococcus faecalis* 1 were significantly lower ($p<0.01$) than that of *Enterococcus faecalis* 2, and so it was determined that recAvBD10 at a concentration of 2.6nM had anti-microbial effects against three of the strains of bacteria tested.

(i) *Enterococcus faecalis* 2:

All three of the clinical isolates were supplied by Aviagen Ltd and arrived as bacterial culture slopes. Good microbiological practice had been adhered to when preparing glycerol stocks from the original slopes and single colonies had been used throughout. However, an interesting finding in relation to the *Enterococcus faecalis* 2 isolate was the presence of what appeared as a mixed population of colonies characterized by their different sizes. This mixture of colony sizes was consistent and observed when counting the colonies following the anti-microbial assays. In most cases the large colonies predominated but small colonies, which made counting difficult were present in all four serial dilutions (Figure 6.14).

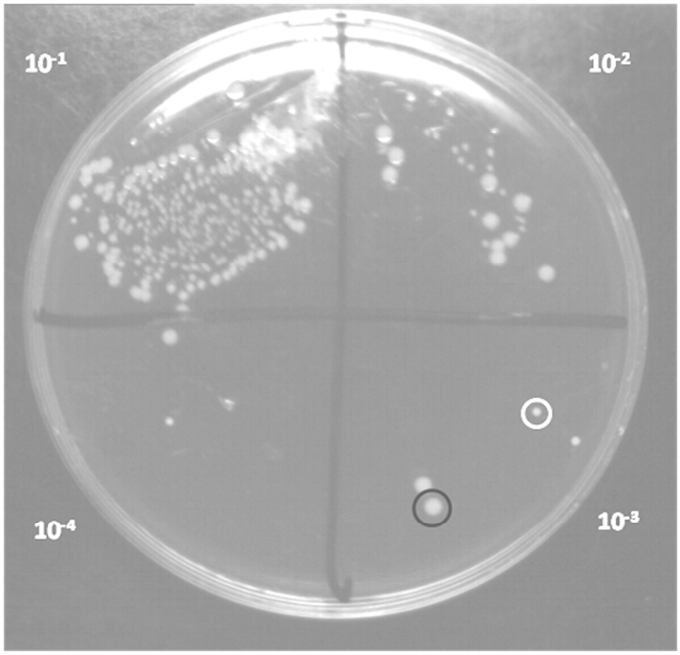


Figure 6.14: Blood agar plate from time 0 of an anti-microbial assay with *Enterococcus faecalis* 2 and recAvBD10. Each quarter contains a sample of bacterial dilution (10^{-1} to 10^{-4}). The white circle indicates one of the small colonies and the black circle the large sized colonies.

B) Purified recombinant AvBD10

It was determined by ELISA that the concentration of pure peptide produced was 0.35µg/ml. Time-kill assays using *Salmonella enterica* serovar Typhimurium phoP were performed using neat sample and also 1/10 and 1/100 dilutions (protein diluted into 0.1M PBS, pH7.4).

The time-kill assay was repeated on three different occasions but the results were inconsistent (Table 6.2, the bacterial percentage survival data is normalised to the PBS

control). When 10µl of 500mM imidazole was added to the time-kill assay, anti-microbial effects were seen with 45.8% bacterial survival i.e. 54.2% bacteria killing. Potential carry-over and failure to remove all of the imidazole in the PD 10 column could explain the result on day1 (0% survival for neat and 1/10 samples) or it could be that the peptide is new and very effective. The reasons for the inconsistent data presented in Table 6.3 has not been identified but of note is that there were three days between experiment 1 and 2 and it is possible that the peptide had become degraded or altered (data presented in chronological order). It appears that the 6nM concentration in experiment 2 was anomalous when compared to the other concentration, and also experiment 3.

Experiment	**Neat** ***60nM***	**1 in 10** ***6nM***	**1 in 100** ***0.6nM***
1	0%	0%	84.5%
2	100%	0%	87.13%
3	103%	93.3%	115%

Table 6.3: Percentage bacteria survival using different concentrations of purified recAvBD10 on three different occasions (*Salmonella enteriditis* serovar typhimurium phoP).

6.6.4: Determination of recombinant mutant AvBD1 anti-microbial activity

Chapter 3 showed that the three lines of chicken included in this study were characterised by three non- synonymous SNPs within the gene encoding the mature peptide of AvBD1. To determine whether there were any effects on the peptide anti-microbial activity, based on the presence of different amino acids, the three different protein forms were synthesised, concentrations measured by ELISA (anti-His tag antibody) and tested in the anti-microbial time-kill assays. The three forms of recAvBD1 were described as A-C with the corresponding amino acid combinations based on SNP presence as given below.

A: AvBD1- NYH

B: AvBD1- NYY

C: AvBD1- SSY

As described for the recAvBD10 experiments, each assay had a total of 40ng of total protein added to the test samples. All three peptides were tested within the same assays, performed at the same time, to reduce the number of variables and therefore allow normalisation to the same control, pRSETA cell lysate data.

The concentrations of the three forms of AvBD1 were different as determined by ELISA. A much higher concentration of recAvBD1-NYH was synthesised (360nM) compared to the other peptides (70nM, 36nM and 1.75nM for AvBD1-NYY, AvBD1-SSY and AvBD10 respectively). This result in itself was interesting considering the same volumes

of inoculant and broth were used for the hyper-expression experiments. It could therefore be suggested that as significantly higher amounts of the AvBD1- NYH (A form) were synthesised, this peptide had less deleterious effects on the bacteria used to hyper-express it, allowing increased amounts to be made. Approximately twice as much of AvBD1-NYY was produced compared to AvBD1- SSY; again this may have been related to the potential toxic effects of leaking peptide on the bacterial host.

The difference in peptide concentration meant that the data for each of the peptides against *Salmonella enterica* serovar Typhimurium phoP could not be compared directly (Table 6.4). Had purification of the peptides been achieved then this would have been possible and allowed direct comparison of the potency of each peptide against the different bacterial strains.

Code	**Amino acids**	**Concentration (nM)**	**Percentage bacteria survival (%)**	**± Standard error of mean**
A	AvBD1-NYH	360	37.5	6.8
B	AvBD1-NYY	70	58.5	8.5
C	AvBD1-SSY	36	79.8	3.8

Table 6.4: Mean percentage bacteria survival following treatment with recAvBD1 A-C (±SEM, n=5).

However Table 6.4 shows that all of the recombinant peptides at the concentrations used had anti-microbial activity against *Salmonella enterica* serovar Typhimurium phoP. It also shows that the concentration of peptides used in the assays differed by 2 to 10 fold. While the most potent peptide, AvBD1 A, was also the most concentrated peptide, 360nM (0.36µM) and the data suggests that the more concentrated the recombinant peptide the greater the bacteria kill, a linear response cannot be assumed. In conclusion these data suggest that the three forms of AvBD1 all showed anti-microbial activity against *Salmonella enterica* serovar Typhimurium phoP with potentially different killing potencies.

6.7: Discussion

None of the AvBDs are currently commercially available and so the aims of this chapter were to (i) synthesise three of the mutant forms of the AvBD1 mature peptide as identified in Chapter 4, and determine anti-microbial activities of these proteins and (ii) synthesise AvBD10 recombinant peptide and confirm whether this peptide had anti-microbial activity. Peptide synthesis was performed utilising a bacterial hyper-expression system, which had previously been used for other peptides within the research group. Alternative expression systems that were considered included a) the use of yeast cells and b) insect expression systems and c) tissue culture techniques (AvBD9 has been synthesized using the latter method (van Dijk, Veldhuizen et al. 2007)). The purchase of synthetic peptide was also discussed but this option was cost prohibitive. The system used was reasonably successful but only resulted in nanomolar concentrations of peptide being produced. It was also a time consuming process principally because of the methods needed for successful identification of low levels of peptide i.e. Western analysis and ELISA. The small sizes of the peptides, their hydrophobicity and cationic properties also contributed to the difficulties in peptide production, identification and later the purification process.

Anti-microbial activity of crude recAvBD10 peptide was observed against *Salmonella enterica* serovar Typhimurium phoP, and the clinical isolates *Staphylococcus aureus* and *Enterococcus faecalis* 1. To date no published data has shown successful determination of the anti-microbial effects of chicken AvBD10. However, recently, a study has been

published on the identification of an AMP described as AvBD10 in the duck and this protein showed 85% homology to the chicken form and was shown to have anti-microbial activity against *Escherichia coli* (Ma, Liao et al. 2009).

Purification of AvBD10 was attempted but problems were encountered. The major problem was the initial low amounts of peptide purified. These low amounts may have been due to the majority of synthesised recombinant peptide being insoluble and trapped within the bacterial pellet. As only the cell-lysate was utilized, then much of the protein may have been inadvertently discarded. As stated earlier, the AMPs are hydrophobic and cationic, and losses due to the peptides sticking to the sides of tubes may also have occurred. Alternative techniques for de-salting the peptides following elution from the talon column, such as dialysis, and the use of different systems for concentrating such small peptides (<10KDa) should have been considered further, and may have improved the yields. However due to the unsuccessful purification of recAvBD10, the work became focused on the crude bacterial extracts and with the appropriate controls the anti-microbial assay data indicated AvBD10 to have bacterial killing properties. In the literature several groups have hyper-expressed AvBD genes and then tested the anti-microbial potency of these peptides. van Dijk, Veldhuizen et al. (2007), successfully hyper-expressed AvBD9 using a His-tag as a fusion protein and used human embryonic kidney cells as the expression system. The authors purified the peptide and cleaved off the His-tag prior to use but unfortunately the mode, by which, the protein concentration was determined was not presented. The reported concentration of this peptide was 32µg/ml or 32mg/litre, much higher than the concentration achieved in this study; in fact

the AvBD1 A form had the highest level of hyper-expression (2µg/ml). Ma, Liu et al. (2008), also reported the successful hyper-expression of AvBD6 and a fusion protein of AvBD6-AvBD10 using an *Escherichia coli* system. Instead of a poly-histidine tag, they used a glutathione S-transferase (GST) fusion system. The peptide oncentration was determined by Bradford's method as 1mg/ml or astonishingly 1g/litre. Duck AvBD9 and 10 have also been hyper-expressed as fusion proteins with GST in an *Escherichia coli* system with protein quantification determined by SDS-PAGE and again the Bradford's method (Ma, Liao et al. 2009). This study by the same research group did not report the final concentration of peptide, but the experiments were performed at concentrations of 1mg/ml suggesting high levels of peptide had been expressed. It can therefore be deduced from the literature that other groups have hyper-expressed more concentrated levels of recombinant peptide than those present in this chapter with the use of GST rather than a His-tag more commonly being reported. However, anti-microbial activity was still determined even at the low concentrations (nanomolar) analysed in this chapter. Anti-microbial effects of AvBDs have been reported in the literature at various concentrations; AvBD1 0.4-3.4µM (Evans, Beach et al. 1995), AvBD2 1.9-3.7µM (Evans, Beach et al. 1994) and AvBD13 57-114µM (Higgs, Lynn et al. 2005). However, it must be noted that the anti-microbial assays used in each of the studies were not standardised.

All three forms of AvBD1 were shown to be anti-microbial, despite containing different amino acids in the mature peptide. The alteration in amino acids could potentially have effects on factors such as hydrophobicity, weight and charge (Table 6.2) and therefore

alter functionality of the peptides. Spheniscin 2, a β defensin identified in the stomach of penguins has been shown using 2 dimensional nuclear magnetic resonance and molecular modeling to consist of three standard β sheets which are stabilized by three disulphide bridges. The presence of an α helix within the region of the N terminal was also shown to exist (Landon, Thouzeau et al. 2004). The avian β defensins are likely to have a similar structure to Spheniscin 2 based on the presence of conserved motifs and patterns.

It has been reported that defensin anti-microbial activity is dependent on charge of the peptide and not its 3D structure i.e. the more cationic a peptide is the greater the anti-microbial effects (Wu, Hoover et al. 2003). Interestingly both the B and C forms of AvBD1 showed similar percentages of bacterial killing when the data, assuming a linear relationship, is extrapolated, to reflect the effects of the addition of 10nM of recombinant peptide. It could therefore be suggested that the A peptide form is the least anti-microbial and this would also support the theory that more of this was produced because it had less deleterious effects on the expression system. However, this result is in direct conflict with that predicted, as when Table 6.2 is examined it can be seen that AvBD1 form A is the most positively charged peptide of the three peptides.

AvBD1 B form has the amino acid pattern NYY while AvBD1 C has the pattern SSY both of which contain a Y at position 32 of the peptide sequence. However the presence of a histidine (H) at position 32, as seen with AvBD1 A, is unique and it is possible that this affects the anti-microbial properties of the peptide. It is possible therefore that the tyrosine side-chain affects 1) the hydrophobicity of the mature peptide and thus its ability

to penetrate membranes and/or 2) the folding of the protein. Production of pure peptide and crystallography would help determine any structural differences between the three forms of AvBD1, which in turn may explain the differences in their anti-microbial activities. The use of model prediction programs could also be useful in determining potential differences between the three forms.

The absence of any non-synonymous SNPs within the mature AvBD10 suggest that the mature peptide is evolutionary conserved. This finding may confirm the physiological as well as immunological importance of the AvBD10 peptide in the chicken population. The conservation of SNPs in mammals has been shown to give a subsequent advantage to the individual (Hughes and Bumstead 1999). The presence of three SNPs in the mature AvBD1 peptide of AvBD1 across three pure lines with possible differences in anti-microbial activity adds to this theory.

To enable quantification of the hyper-expressed peptides a direct capture ELISA utilising the presence of the His-tag was established. The ELISA was performed using only a single type of antibody, which had been shown to have a strong affinity to the His-tag even at low concentrations (1:12000). It could be argued that using a secondary labeled antibody against a primary antibody may have provided a more accurate assay. Potential inaccuracies with the ELISA may have occurred if the recombinant peptide had not bound to the wells as efficiently as the standard protein and, if due to poor binding, the peptide had inadvertently been washed off following the numerous plate washes. Low levels of recombinant peptide were measured using the ELISA assays and this may have

been simply due to low expression levels or poor peptide isolation. However, the results obtained from the ELISAs were reproducible and comparable, and gave insight into the relative concentrations of recombinant peptides that were synthesised.

No attempt was made to cleave the His-tag from the AvBD peptide. The main reason was that the amounts of peptide expressed were low and the effect of adding an additional purification system would, potentially, have reduced the final peptide concentration. Tobacco etch virus derived (TEV) proteolysis would have been the system of choice for cleavage of the His-tag and this method has been used successfully in the production of AvBD9 (van Dijk, Veldhuizen et al. 2007). In addition, the anti-microbial activities of His-tag recombinant peptide fusion proteins have been shown not to be significantly altered by the presence of the His-tag (Yenugu, Hamil et al. 2003).

The results presented in Figure 6.13 showed that the four strains of bacteria used within the time-kill assays had different sensitivities to recAvBD10. Approximately 25% of both *Salmonella enterica* serovar Typhimurium phoP and *Staphylococcus aureus* were killed following the addition of the peptide (concentration of 3nM) and *Enterococcus faecalis* 1 showed the greatest susceptibility (44% killing) to recAvBD 10. These data are interesting in relation to the ever-growing concern regarding Methicillin-resistant *Staphylococcus aureus* (MRSA), with animals acting as both carriers and vectors. Indeed there are reported findings of the presence of an MRSA strain within the broiler population (Persoons, Van Hoorebeke et al. 2009). The *Staphylococcus aureus* strain used in this study was taken from an infected bird joint. Infections with this bacterium in

chickens have been associated with femoral head necrosis and bubblefoot, both having low mortality rates but high morbidity and major welfare implications (McNamee and Smyth 2000).

Enterococcus faecalis infection is generally via the oral or aerosol route and infection can lead to a series of diseases including septicemia, endocarditis, osteomyelitis and encephalomyelitis (Chadfield, Christensen et al. 2004). It is most commonly a problem in young birds, which for some reason are unable to mount a successful immune response to low-grade infection. The bacterial isolates used in these studies were sent from the Aviagen laboratory and no further typing had been performed.

Unlike *Enterococcus faecalis* 1, the *Enterococcus faecalis* 2 strain was not susceptible to the potential anti-microbial effects of recAvBD10 at a concentration of 2.6nM. A sample of *Enterococcus faecalis* 2 was sent to the Veterinary Laboratory Agency, Bury St Edmunds, for sub-typing but this was unsuccessful. However both of the *Enterococcus faecalis* strains had been taken from different post-mortems and so it is feasible that they were different sub-types. The identification of small colonies following the use of *Enterococcus faecalis* 2 in the assays may explain the resistance that this strain showed to recAvBD10 killing. It has been shown that small colony variants of clinical isolates have a reduced susceptibility to antibiotics and have the ability to cause latent or recurrent infections in the host by having altered phenotypic and pathogenic traits (Proctor, von Eiff et al. 2006). The colonies may have virulence factors, which gives

them a superior phenotype that may be an advantage when exposed to AMPs. This needs further investigation.

The resistance of *Enterococcus faecalis* strains to antibiotics is affected by the growth medium, temperature and general conditions under which they are cultured (Jackson, Fedorka-Cray et al. 2005). However, the culturing and preparation of the bacterial stocks was kept constant following the arrival of the bacterial slopes in the laboratory so the sensitivity of the two strains to the AvBD is probably not linked to their growth conditions. It has also been shown that some strains of *Enterococcus faecalis* evade the effects of antibiotics via the production of a slime factor (59.7% of isolated strains in a reported study by (Ciftci, Findik et al. 2009)). Slime production was not obvious, but further characterisation of the two strains and individual colonies would confirm whether slime was involved.

In conclusion the data presented in this chapter describes the attempts made to investigate the anti-microbial activity of the three forms of recAvBD1 and recAvBD10. A regulated bacterial expression system was employed but while peptides were produced the amounts were too low to allow purification and comparative anti-microbial studies. Peptide production ideally needs to be scaled up so that purification can occur as this would allow peptide killing curves to be constructed as well as further studies investigating protein structure and folding to be performed. The following chapter continues the work of the time-kill assays but the substrates are the small intestinal gut extracts where it is anticipated that AvBDs will function.

Chapter 7: Analysis and anti-microbial activity of duodenal gut mucosal scrapes

7.1: Introduction

One of the major focuses for this study was the gastro-intestinal health of growing birds and the potential roles of AvBD peptides during this period. Differences in perceived gut health between the three lines of birds have already been discussed i.e. line X was characterised by 'poor gut health' with problems such as wet litter being common, while in contrast the line Z birds exhibited a 'superior gut health' with fewer related issues. In Chapter 4 characterisation of the different SNPs within the AvBD loci of the three lines revealed marked differences between the three lines of birds, especially in the context of AvBD1, but whether this impacted on the actual gut AMAs was not known. AvBD1, 4 and 10 gene expression had been shown within the duodenal tissue samples; thus to determine the presence of such peptides as well as confirm the local anti-microbial activity of the duodenal mucosal layer scrapes were taken from all of the birds, and their AMAs analysed and compared.

Gut duodenal mucosal scrapes were collected and the peptides extracted as described previously (Nile, Townes et al. 2006). To determine potential bird line, age and rearing environmental effects on gut AMAs, the samples were analysed using bacterial time-kill assays. Characterisation of the proteome of gut scrapes was performed using SDS-PAGE, MALDI-TOF and LC-MS.

The aims for the work performed and presented within this chapter were;

- **To determine the anti-microbial properties of gut mucosal scrapes collected from individual birds so that the effects of bird line, age and rearing conditions could be compared.**
- **To profile the proteomes of duodenal scrapes and attempt to identify peptides associated with gut innate immunity.**

7.2: Peptide extraction from the bird gut mucosal scrapes

Peptide extraction was performed on the duodenal gut mucosal scrapes as described in Chapter 2. Following extraction all samples were analysed using the NanoDrop to quantitate total protein and the samples diluted as necessary in 0.1M PBS, pH7.4. A working concentration of 4µg/µl was used for the bacterial time-kill assays thus allowing comparison of results. This concentration was selected as this was the lowest sample protein concentration measured following extraction and reconstitution of the proteome.

7.3: *In vitro* anti-microbial activity of gut mucosal scrapes

Individual samples were analysed for anti-microbial activity using bacterial time-kill assays described in Chapter 6. A total of 10µl of the extracted peptides was used in the assays i.e. 40µg of total protein from each of the scrapes in all of the assays, and each sample was run in triplicate.

7.3.1: Bacterial strains

The time-kill assays used were initially established using the mutant strain *Salmonella enterica* serovar Typhimurium phoP (Nile, Townes et al. 2004). As mentioned earlier, this is a laboratory strain engineered to have increased susceptibility to the effects of antimicrobial peptides (Behlau and Miller 1993), and is a good tool to gauge anti-microbial activity. However, wild-types strains, *Salmonella enteriditis* and *Salmonella enterica* serovar Typhimurium SL 1344 were also used in these analyses to corroborate these data.

Reproducibility of this assay relies on the bacterial culture being in mid-exponential phase, and this was determined by comparing the growth curves of all three strains of bacteria (Figure 7.1). All three bacteria showed similar growth rates and the bacterial cells were used in the assay at approximately 2.5 to 3 h post inoculation i.e. mid-exponential growth phase, no modifications to the original assay were therefore made.

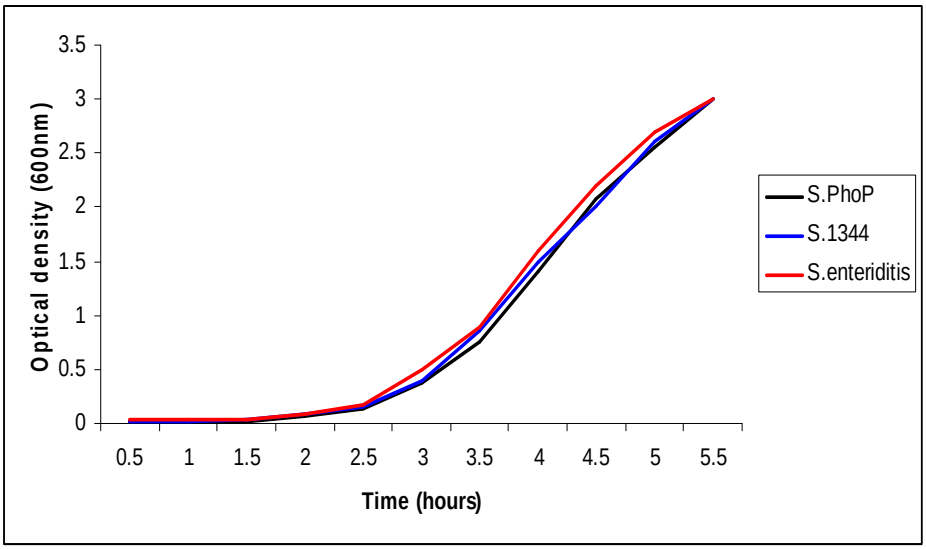


Figure 7.1: Bacterial growth curves for the three strains of *Salmonella* (*S. enterica* Typhimurium phoP, *S. enteriditis* and *S. enterica* serovar Typhimurium SL1344).

7.4: Anti-microbial activity of gut extracts

7.4.1: *Salmonella enterica* serovar Typhimurium phoP - Day 0 bird gut scrapes

Gut extracts from the line X (n=7), Y (n=6) and Z (n=6), 0 day-old birds were analysed for anti-microbial activity. The data for the 0 day old birds is presented in Figure 7.2. All of the data points are below 100%, and so every sample showed anti-microbial activity. However, marked variation in anti-microbial activity was observed between birds in each line e.g. in line X the anti-microbial assays scores ranged from 0 to 58% bacterial survival. Thus, when the data for each group was averaged, there were no statistically significant differences between the groups. The calculated bacterial survival (means and

standard errors), for the three lines were; X 19.3% (±11.3), Y 8.5% (±11.7) and Z 28.6% (±16.6).

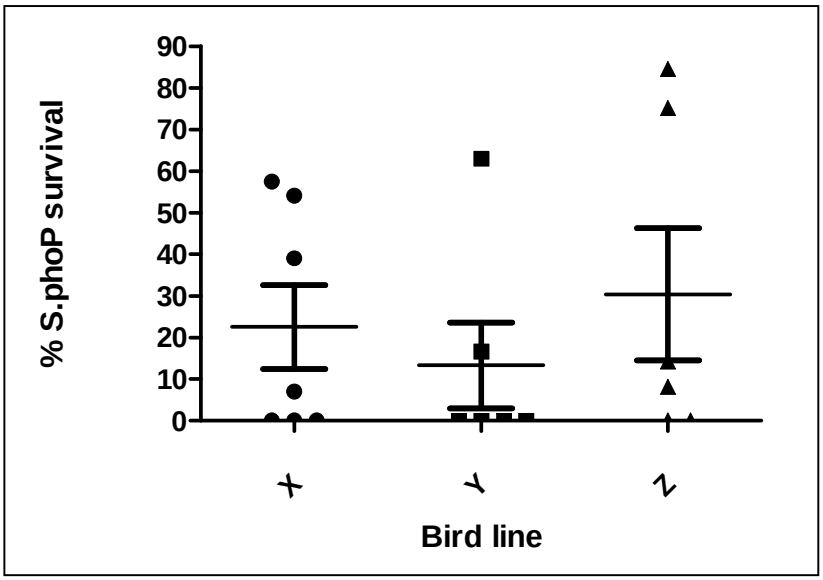


Figure 7.2: Bacterial survival (*S. enterica* serovar Typhimurium phoP) following a time-kill assay using gut extracts from day 0 birds (n=7 for line X and n=6 for both lines Y and Z).

7.4.2: *Salmonella enterica* serovar Typhimurium phoP- Day 7 bird gut scrapes

Figure 7.3 shows the time-kill assay results for the five groups of day 7 birds. The data suggests that there was a large difference between the birds reared on the LH farm compared to those reared in the HH conditions. Again variation in individual values within each of the five groups was noted, with the XH group showing the greatest variability between birds (range 572-1606%). Focusing initially on the LH data, the

average bacterial survival for the three lines of birds (mean ±SEM) were, XL 94.9% (±31.4), YL 89.2% (±19.6), ZL 60.0% (±14.3), providing evidence for gut anti-microbial activity. Of the three lines, Z showed the greatest average killing (40%). However, there were no statistically significant differences between the three populations reared on the LH farm, probably a reflection of the inter group variation.

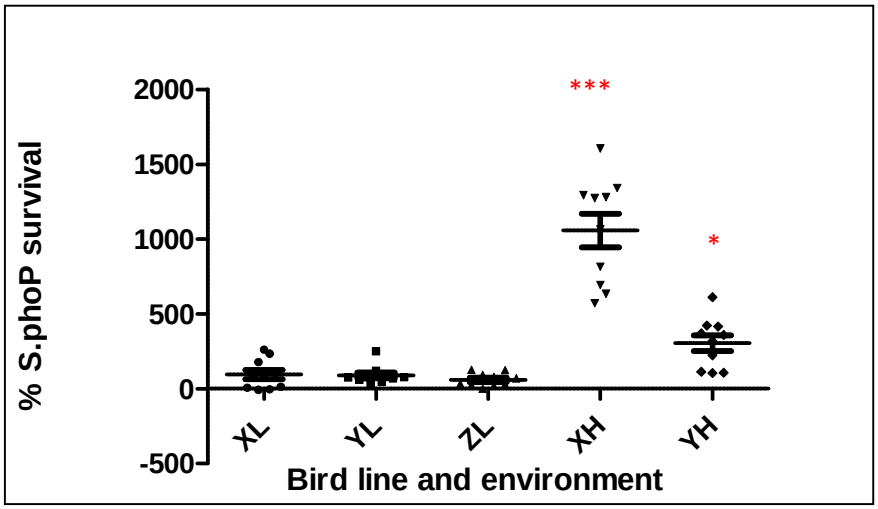


Figure 7.3: Bacterial survival (*S.enterica* serovar Typhimurium *phoP*) following a time-kill assay using gut extracts from day 7 birds (*** indicates p<0.001 and * p<0.05) (n=10 for all groups).

Both of the groups (X and Y) reared in the HH conditions showed increased levels of bacterial survival compared to those reared on the LH farm (XH 1,058.0% ±112.6 and YH 304.8% ±52.7). A percentage survival greater than 100% was recorded when there was greater bacterial growth on the plates at time 2h compared to the PBS control plates. This therefore indicated that the gut protein extract had pro-microbial properties.

Statistically, the XH group gut extract activities were significantly higher than the YH group ($p<0.001$). Comparing the XL and XH mean data showed that the gut extracts of the XH group were significantly less anti-microbial than those of the XL group, in fact the difference was approximately ten-fold (95% compared to 1058%). Similarly the YH group had a higher mean percentage bacteria survival compared to the YL group, although this was not statistically significant. Statistically, the XH group mean was higher when compared to the other four groups of birds ($p<0.001$). The percentage *Salmonella* survival of the line YH bird gut extracts (305%) was significantly higher than the ZL group ($p<0.05$) but not the other three groups.

These data indicated clear and significant differences in the anti-microbial effects of the gut protein extracts. Most notable was the difference that the rearing environment appeared to have on the bacteria survival, with those protein extracts taken from the LH reared birds haD anti-microbial properties yet those from HH kept birds haD pro-microbial effects. In addition, line differences were also identified.

7.4.3: *Salmonella enterica* serovar Typhimurium phoP - Day 14 bird gut scrapes

The time-kill assay data for the five groups of day 14 birds are presented in Figure 7.4. The mean percentages (±SEM) for bacterial survival for the five groups were XL 571% (±42.8), YL 150% (±16.4), ZL 143% (±38.9), XH 272% (±64.5) and YH 217% (±31.9). These data indicated that none of the gut scrapes provided evidence of anti-microbial activity, all were found to be pro-microbial.

When data from groups raised in the LH conditions were compared, line X showed the most pro-microbial effects (571% compared to 150% and 143% for lines Y and Z respectively). This difference was statistically significant ($p<0.001$).

In contrast both XH and YH protein extracts showed similar effects on *S. enterica* serovar Typhimurium phoP survival (272% and 217% ,respectively).

The greatest intra-group variability in bacteria survival was seen in relation to the ZL and XH groups, but the most interesting finding from this data set was the high level of bacteria survival seen in the XL group.

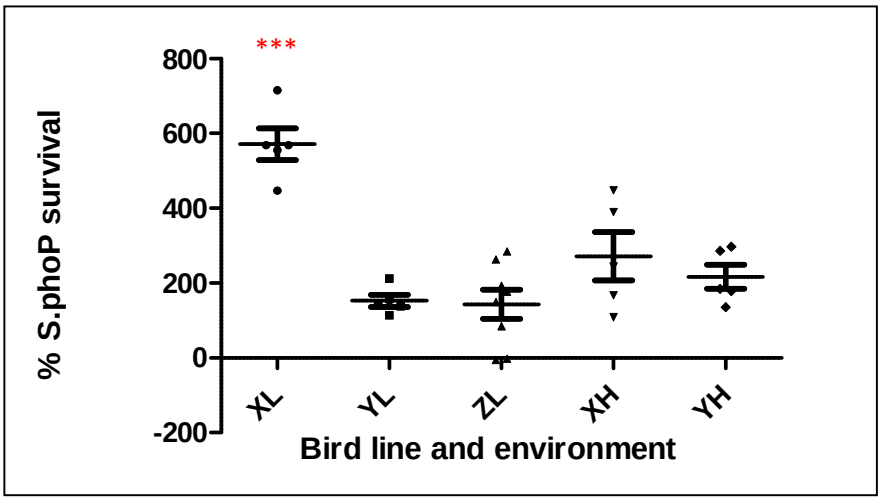


Figure 7.4: Bacterial survival (*S. enterica* serovar Typhimurium *phoP*) following a time-kill assay using gut extracts from day 14 birds (n=5 for all groups, ***=p<0.001).

7.4.4: *Salmonella enterica* serovar Typhimurium phoP - Day 35 bird gut scrapes

The results of the time-kill assays for the gut extracts from the day 35 birds are shown in Figure 7.5. The means (±SEM) for bacterial survival were XL 217% (±33.2), YL 384% (±111.9), ZL 277% (±43.9), XH 314% (±18.9) and YH 315% (±43.1). The YL group showed the greatest intra-group variation but there were no statistically significant differences between any of the five groups.

Interestingly bacterial survival relating to the day 35 gut extracts were comparable to the 14 day results with the exception of XL 14 days (day 35 range of means 217.1-384.3% and day 14 152.8-271.5%).

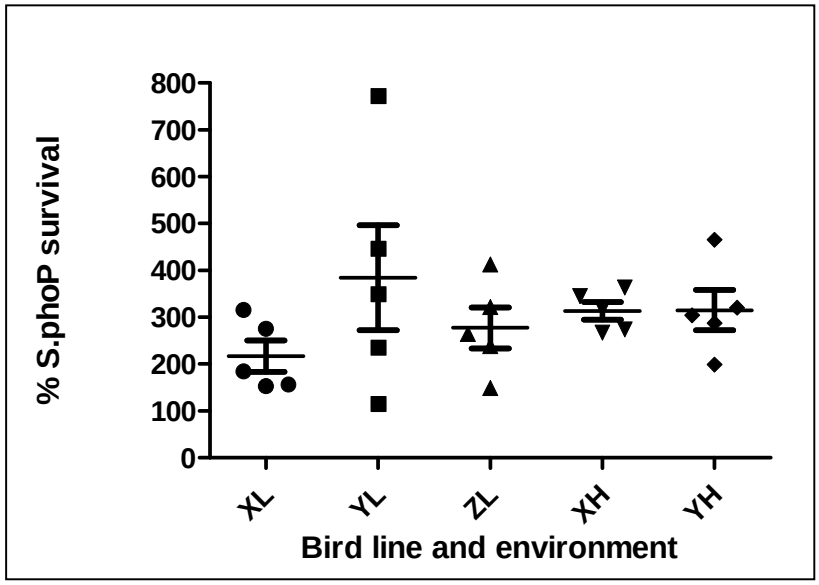


Figure 7.5: Bacterial survival (*S.enterica* serovar Typhimurium *phoP*) following a time-kill assay using gut extracts from day 35 birds (n=5 for all groups).

7.4.5: Conclusions from *Salmonella enterica* serovar Typhimurium phoP time-kill assays

Although the data sets were characterized by intra-group variability the trends and differences between the five groups of birds are most clearly visualised when the data for the different age groups are plotted (Figures 7.6A-D, LH groups and Figure 7.7A-C HH groups).

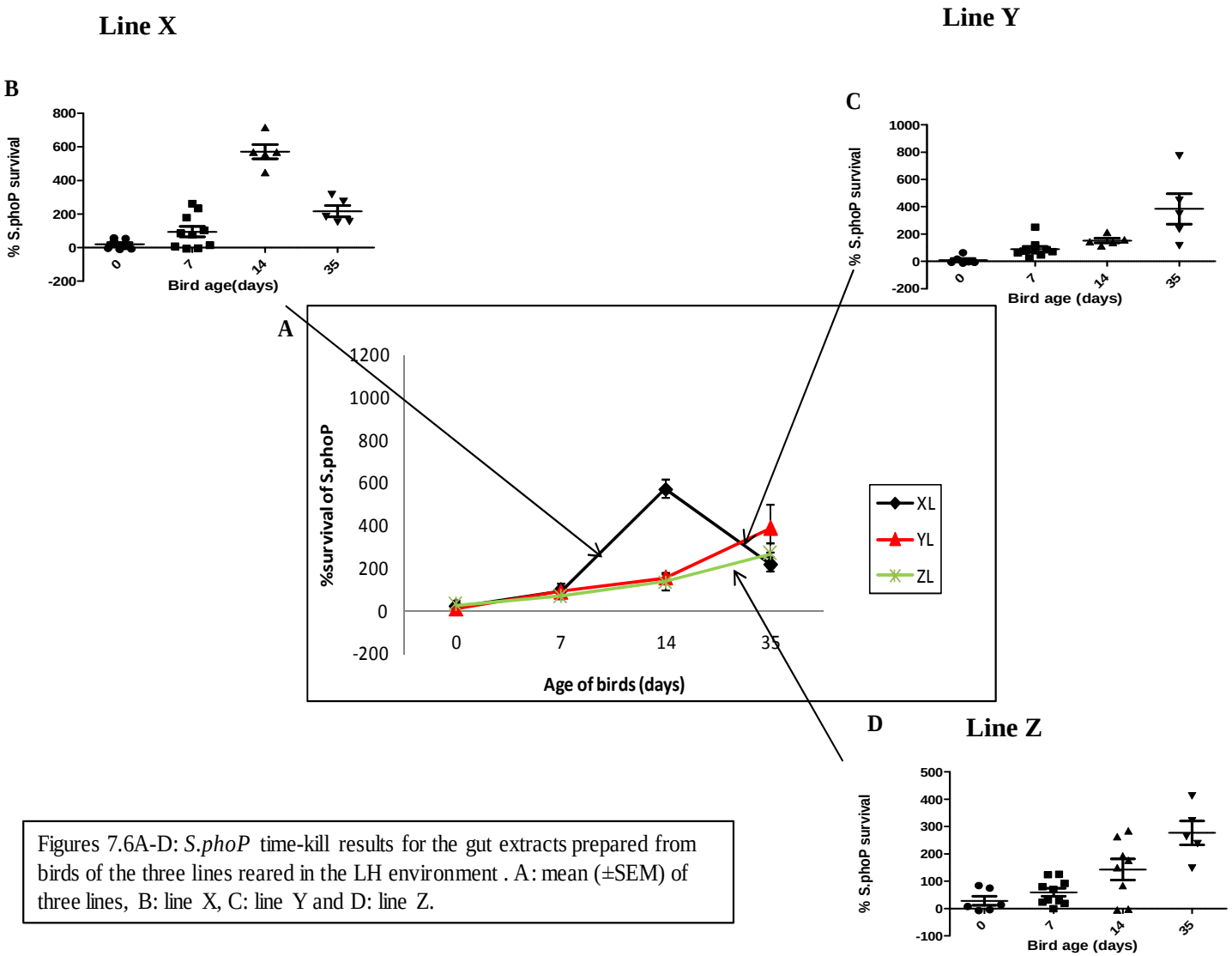
It was noted that all 0 day old bird gut extracts were anti-microbial. However, the anti-microbial effects were less pronounced when the gut extracts from 7 day old birds were analysed, particularly in assays using the samples from HH birds where pro-microbial

effects were observed. The XH extracts were particularly pro-microbial, resulting in a mean bacteria survival percentage of 1,058%. Essentially, both environment and bird line appeared to affect the results.

Of particular note is the peak in bacterial survival seen in the day 14 XL group compared to the XH group, where the highest mean survival of bacteria was seen at day 7. Interestingly lines YL and ZL showed similar results. Generally over time, as the birds aged, the observed trend was a decrease in gut AMA. The exception was the gut extracts of the line X birds. Line X bird gut scrapes produced very different results to the other lines, most notably in relation to the XH 7 day old and XL 14 day old groups. The pro-microbial effects of the XL (mean 571%) group were half those recorded for the XH 7 day group (mean 1,058%), and this was statistically significant ($p < 0.05$).

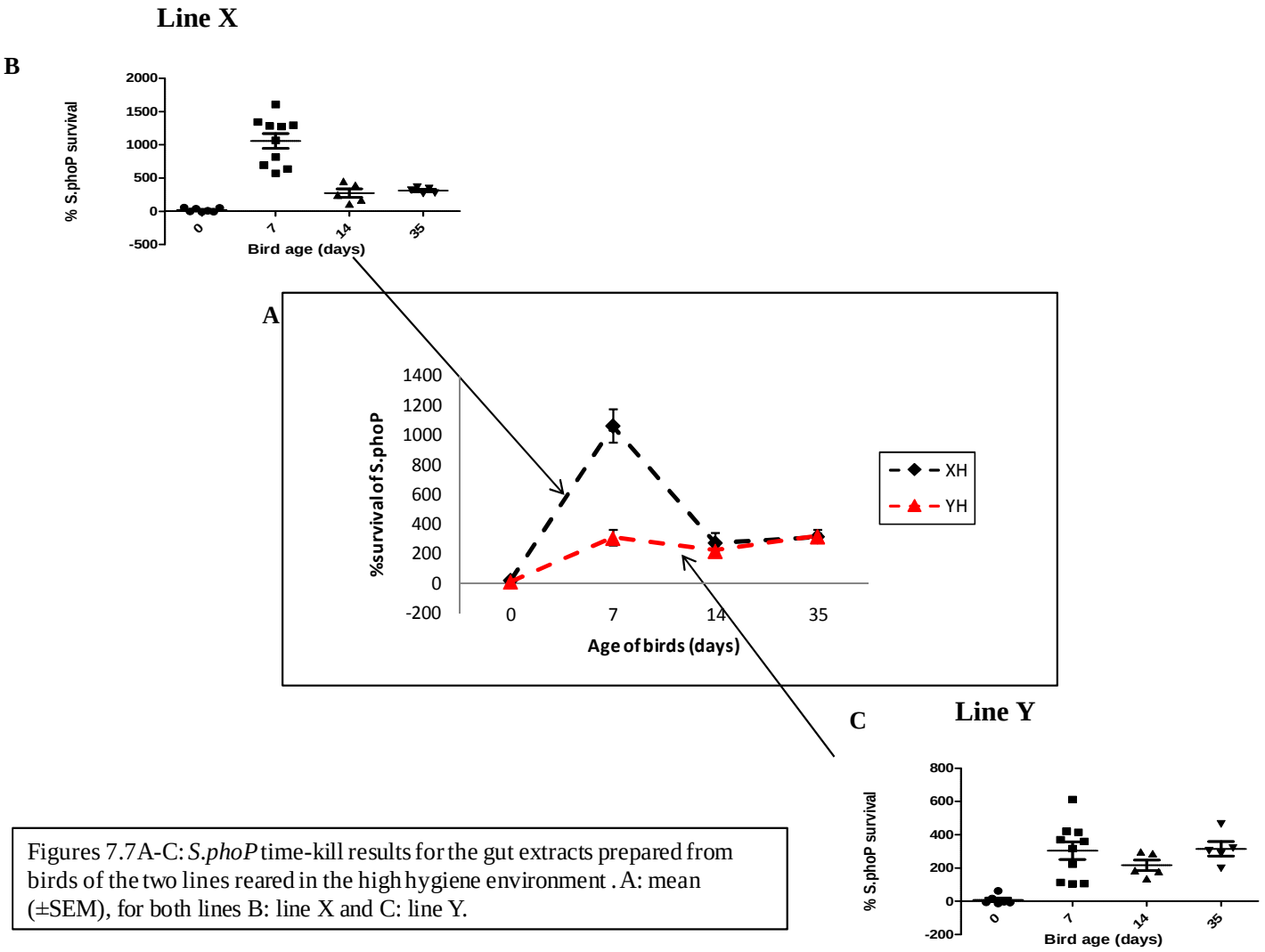
These data were collated using *S. enterica* serovar Typhimurium phoP so to investigate if the data followed a similar pattern for wild-type *Salmonella* strains, the time-kill anti-microbial assays were performed using *Salmonella enteriditis* and *S. enterica* serovar Typhimurium SL 1344. Only the day 7 gut scrapes were analysed in these assays; it was not possible to assay the 0 day old bird gut extracts as there was not enough sample due to the small size of the original scrapes.

Low Hygiene Groups



Figures 7.6A-D: *S.phoP* time-kill results for the gut extracts prepared from birds of the three lines reared in the LH environment . A: mean (±SEM) of three lines, B: line X, C: line Y and D: line Z.

High Hygiene Groups



Figures 7.7A-C: *S.phoP* time-kill results for the gut extracts prepared from birds of the two lines reared in the high hygiene environment . A: mean (±SEM), for both lines B: line X and C: line Y.

7.4.6: *Salmonella enteriditis* and *Salmonella enterica* serovar Typhimurium SL 1344 – Day 7 bird gut scrapes

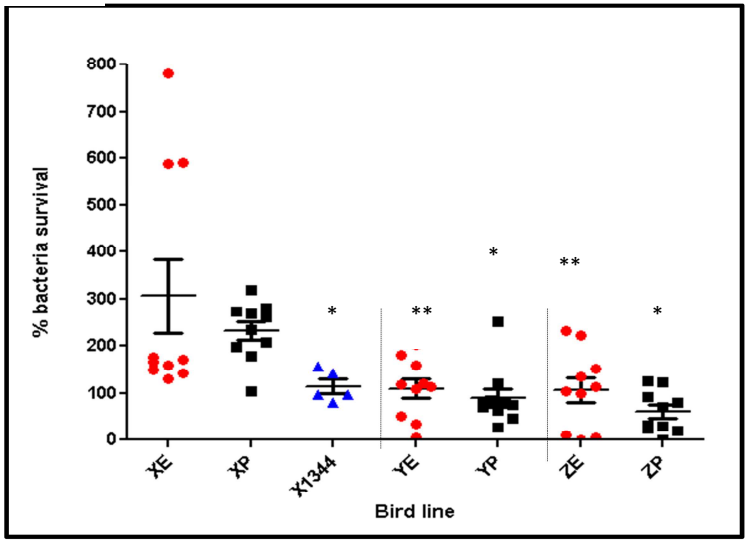
Figure 7.8A shows the anti-microbial assay data for *S. enteriditis*, *S. enterica* serovar Typhimurium phoP and *S. enterica* serovar Typhimurium SL 1344 respectively, focusing on the gut scrapes of birds reared in the LH environment. Only line X bird gut extracts were tested against all three strains of *Salmonella* and mean (±SEM) bacterial survival data was 306% (±77.7), 232% (±19.6) and 115% (±14.8) for *S. enteriditis*, *S. enterica* serovar Typhimurium phoP and *S. enterica* serovar Typhimurium SL 1344 respectively. Pro-microbial effects were observed in relation to all three strains analysed, but these effects related particularly to the engineered *S. enterica* serovar Typhimurium phoP strain. The *S. enteriditis* data was statistically significant compared to that of the *S. enterica* serovar Typhimurium SL 1344 strain ($p<0.05$) but not *S. enterica* serovar Typhimurium phoP.

The results of the time-kill assays using the line Y and Z gut extracts and the *S.enteriditis* indicated the mean bacterial survival to be similar in these two groups (109% ±20.2SEM and 107% ±26.8SEM, respectively). In contrast the line X birds had a higher mean bacteria survival, 306% ±77.6 SEM.

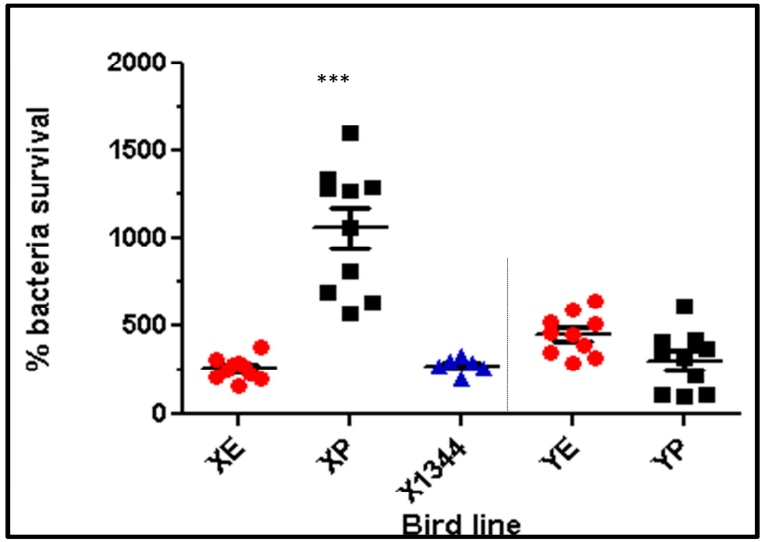
The data for the X and Y bird lines reared on the HH farm is shown in Figure 7.8B. Again only line X gut scrapes were tested against all three strains of *Salmonella*. The mean survival of the two wild-type strains in the assays using the line X group extracts

were very similar to each other and statistically not significantly different to the XL data. However, the *S. enterica* serovar Typhimurium phoP mean data for the line X birds (1058.0 ±112.6 SEM), was significantly higher ($p<0.001$), than those recorded for all of the other groups investigated. The line YH data showed the bird gut extracts to have greater pro-microbial effects for both strains compared to those from the line Y birds reared on the LH farm ($p<0.001$).

A- LH



B- HH



Key:

- *S. enteriditis*
- *S.* Typhimurium SL 1344
- *S. phoP*

Figure 7.8A and B: Time-kill assays for day 7 bird gut scrapes using *Salmonella enteriditis* (E), *S. enterica* serovar Typhimurium SL 1344 (1344) and *S. phoP* (P). A is the LH data and B HH. (statistically significant difference are marked as follows and details given in text * =$p<0.05$, **=$p<0.01$ and= ***$p<0.001$). Scales for two figures are different due to the results obtained for XP high hygiene assays and n=10 for all of the groups of birds.

7.4.7: Conclusions from *S. enteriditis* and *S. enterica* serovar Typhimurium SL 1344 time-kill assays

In summary, when the LH and HH data are compared (Figures 7.8A and B), three significant observations can be made (i) the line XH results for the AMA using *S. enterica* serovar Typhimurium phoP were significantly elevated compared to all other groups i.e. the duodenal gut extracts appeared exceptionally pro-microbial; (ii) the XL data for the *S. enteriditis* assays was significantly higher i.e. pro-microbial, when compared to both the YL and ZL data and (iii) that when the LH and HH groups were compared, the YL *S. enteriditis* survival data was significantly reduced compared to that of YH.

7.5: Thin layer chromatography (TLC) of gut extracts from line X birds

Line X bird gut extracts produced the most interesting data for the time-kill assays in both the LH and HH groups, with marked pro-microbial effects being a major finding. In order to determine whether these extracts contained potential nutrients e.g. sugars, and thus supported bacterial growth in the assay, TLC was performed on a selection of the gut extracts. Figure 7.9 shows an example of a TLC analysis of both 0 day and 7 day old gut extracts.

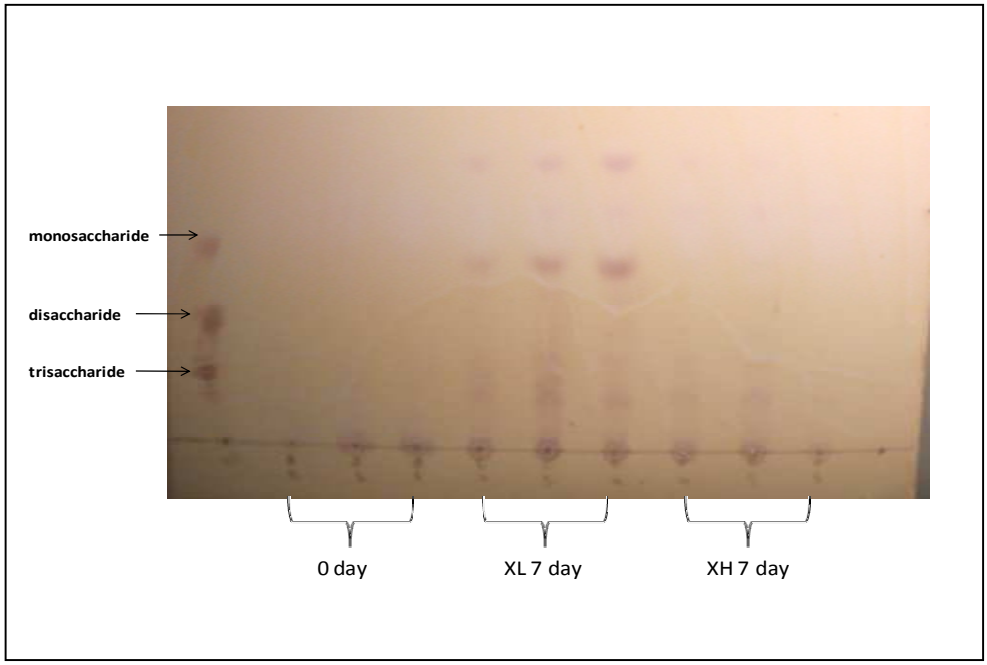


Figure 7.9: TLC of extracted gut scrapes from 0 and 7 day old line X birds. The sugar standards are shown on the left side of the figure. The 0 day old group included three gut extracts taken from birds on the day of hatch, XL is from 7 day old birds reared in the LH environment and XH7 indicates those reared on the HH farm.

However, all the XL 7 day samples produced bands and these appeared to be very similar in all three bird gut extracts. The XH 7 day old samples did produce some faint bands, although not as pronounced as the XL group. The banding patterns did not compare directly with the sugar standards. This could have been due to modifications of the sugars such as the addition of an amino acid or the nature of the sample e.g. the presence of contaminants altering the migration of the sugars. From the chromatogram it appears that the XL samples contained the greatest amounts of sugars. The presence of multiple bands also suggested a mix of sugars within the gut extracts.

7.6: Protein analysis and identification of gut extracts

Clear patterns in gut anti-microbial activity were detected between the different groups of birds. To determine whether differences in the proteomes of these samples were responsible for such observations, attempts to identify the different proteins were made. Initially, SDS-PAGE of the prepared gut extracts from the 0 and 7 day old birds was performed to determine whether there were gross differences in the proteins present (details in Chapter 2). The gels were stained with either colloidal blue or silver stain.

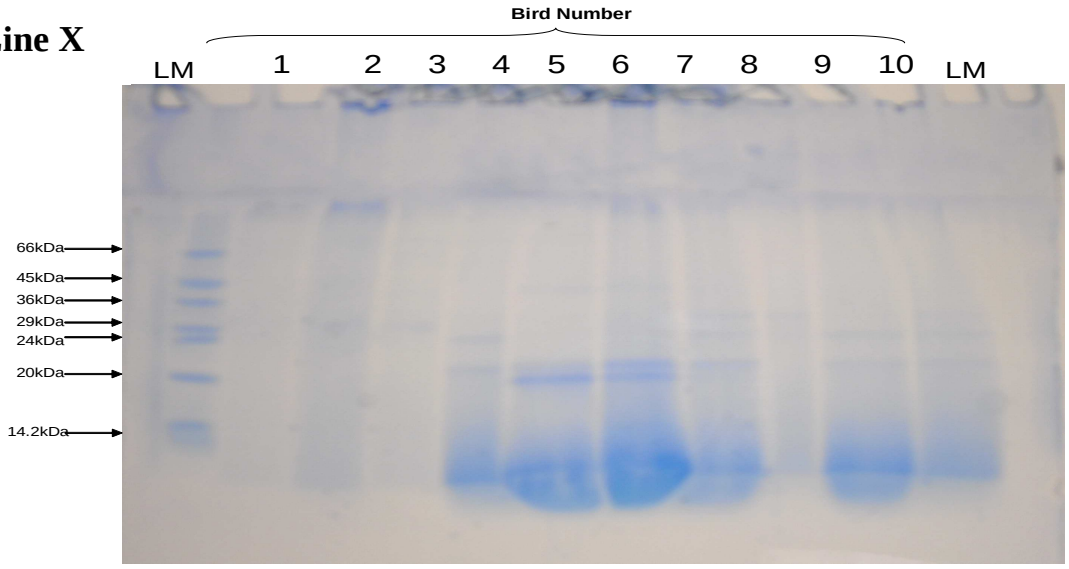
7.6.1: Colloidal Blue-stained SDS-PAGE gels

Examples of SDS-PAGE stained gels are presented in Figures 7.10A and B. Figure 7.10A shows stained proteins from the gut extracts of the ten line X 0 day old birds and B shows the line Y 0 day old bird gut extracts. No obvious differences in the protein staining patterns were observed. The gels show marked staining at the bottom of the gel, suggestive of high concentrations of proteins less than 14.2kDa in size, as indicated by the low molecular weight marker.

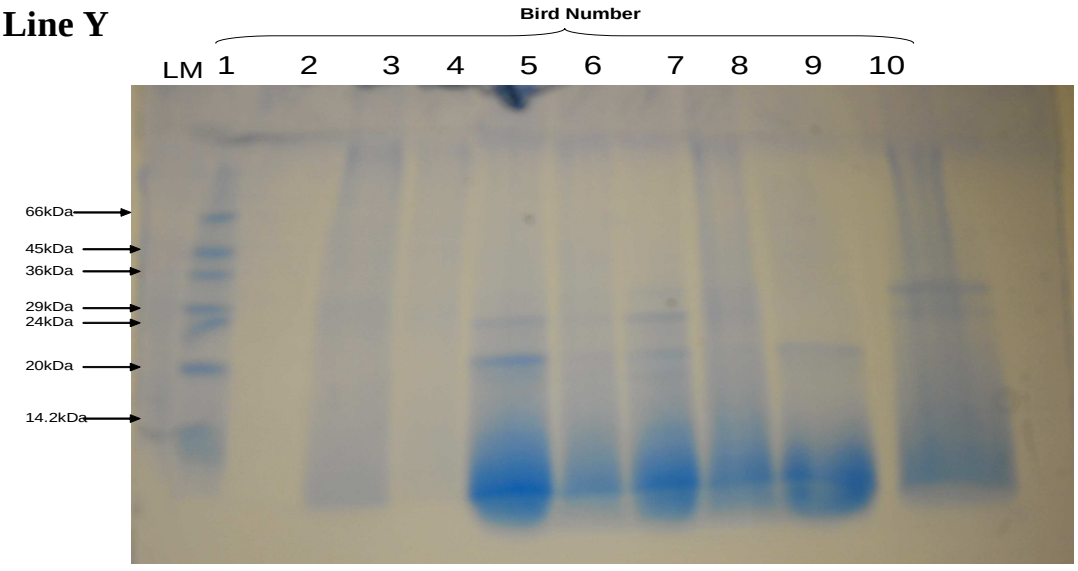
Small cationic peptides have previously been shown to be extracted from chicken gut mucosal scrapes in 10% acetic acid (Nile, Townes et al. 2006) and the observed staining patterns may represent such peptides. Another possible explanation for this region of dense staining could be due to protein degradation, which has occurred during the extraction process. More discreet bands can be seen proximally in some of the individual bird samples with a clear band visible at 20kDa (lanes 4-6 in Figure 7.10A and lane 5 in

Figure 7.10B). The colloidal blue staining system was relatively insensitive, and inconsistent, so reproducibility of the data was a major issue. To address this silver staining of the SDS-PAGE gels was performed as this stain has been shown to increase sensitivity (Kerenyi and Gallyas 1973).

A: Line X



B: Line Y

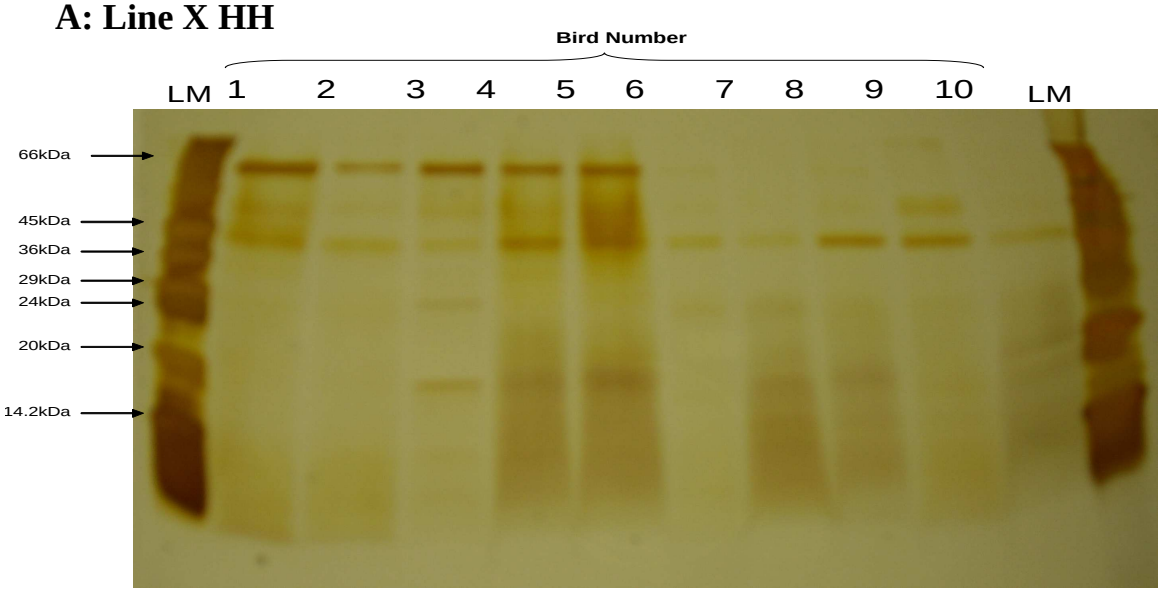


Figures 7.10 A and B: Colloidal blue-stained 12.5% SDS-PAGE gel following electrophoresis of protein extracts from the gut mucosal scrapes of the ten 0 day old line X and Y birds. A: line X and B: line Y. LM is the low molecular weight marker. Approximately 190µg of protein was added to each lane.

7.6.2: Silver-stained gels

Figures 7.11A and B show examples of two silver-stained SDS-PAGE gels of the day 7 bird gut extracts. It was not possible to analyse the day 0 samples in this manner as there was insufficient sample. Figure 7.11A shows the stained proteins from the gut extracts of the ten line X 7 day old birds reared in the HH environment and, B, those reared on the LH farm. Differences in the protein staining are evident when these two gels are compared i.e. there is an increase in the number of stained protein bands in the LH samples compared to the HH samples, with distinct bands occurring at approximately 50, 36, 24 20 and 18kDa in the LH extracts compared to bands at 60, 45 and 18kDa in the HH bird extracts.

A: Line X HH



B: Line X LH

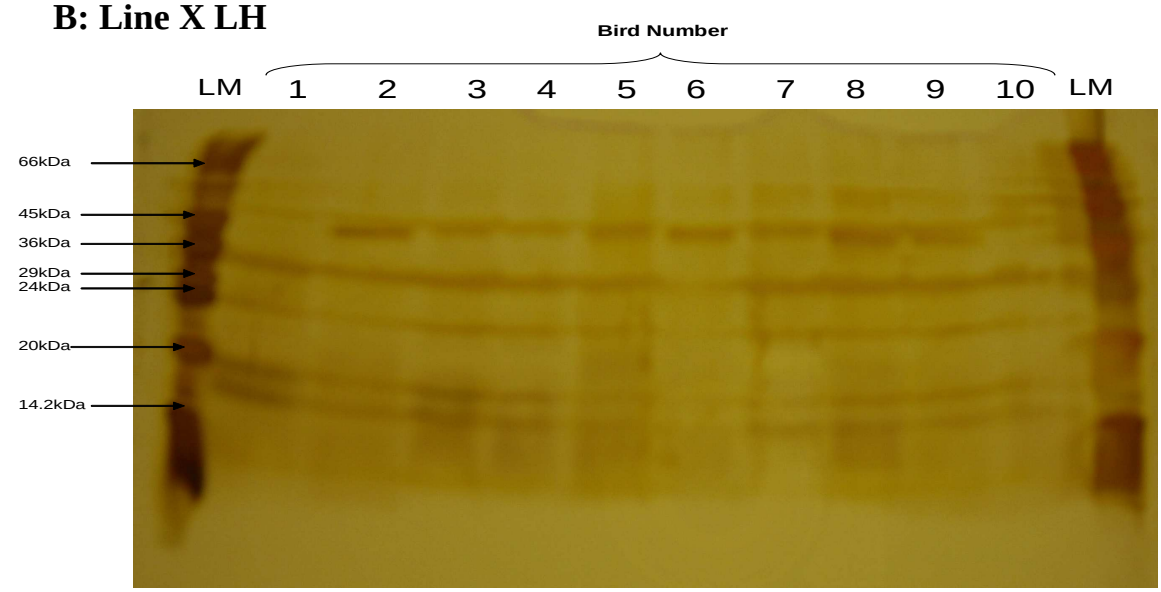


Figure 7.11A and B: Silver-stained 12.5% SDS-PAGE gels following electrophoresis of protein extracts from the gut mucosal scrapes of the ten 7 day old line X birds. A: high hygiene B: low hygiene. LM is the low molecular weight marker. Approximately 100µg of protein was added to each lane.

7.6.3: Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF)

The SDS-PAGE analysis and staining was too insensitive to accurately determine differences in the gut profiles of the birds raised in the different environments. To address this MALDI-TOF was employed to improve the sensitivity and identify actual proteins. A total of four bands from the 7 day bird XH gel were analysed by Pinnacle (Newcastle University). The size of the bands of the four samples relative to the molecular weight marker were; 1) > 66kDa, 2) approximately 45kDa, 3) approximately 20kDa and 4) <14.2kDa (Figure 7.12). Unfortunately no MALDI-TOF data was obtained. It was reported that the protein concentrations of the samples were too low to be analysed by this method.

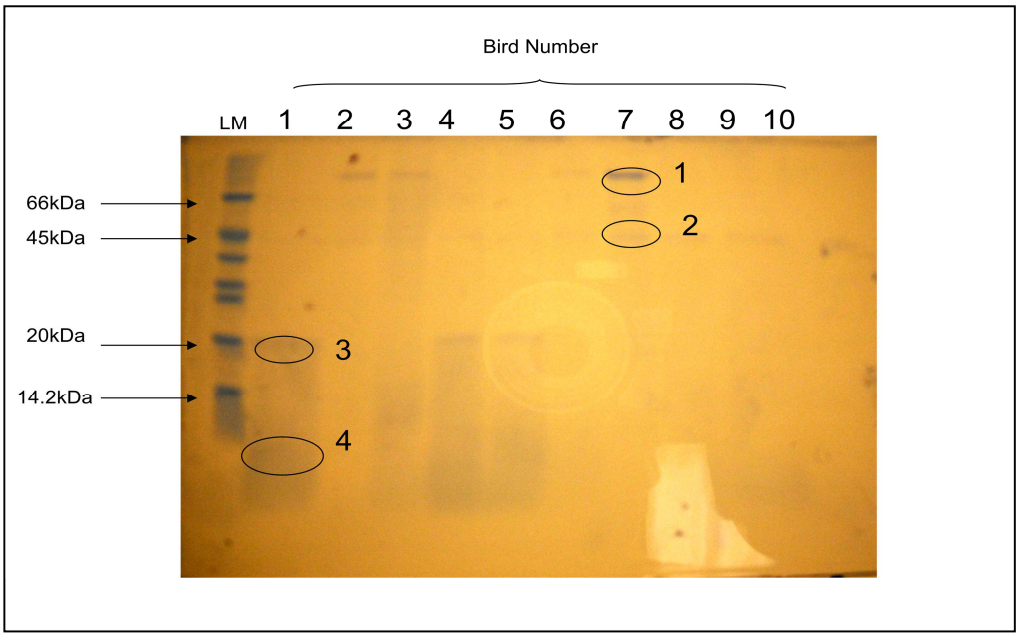


Figure 7.12: Colloidal blue-stained 12.5% SDS-PAGE gel showing protein profiles of gut extract samples from ten XH 7 day old birds, indicating the four bands (1-4) which were extracted and sent for analysis by MALDI-TOF (circled).

7.6.4: Liquid chromatography- mass spectrometry (LC-MS)

The failure of MALDI-TOF analysis to identify any of the proteins in the gut scrapes lead to the use of LC-MS. This technique was chosen following discussion with Dr Achim Treuman of NEPAF (Cels Business Services Ltd, Newcastle-Upon-Tyne) and, if successful, would allow a global analysis of the proteome of each of the gut scrapes analysed. Analysis was limited to two samples as part of a pilot study and it was decided to examine the protein composition of those gut extracts, which had resulted in very different results in the time kill assays. The first sample was from a 7 day old line X bird reared in the HH environment, which had been shown to be very pro-microbial in the *S. enterica* serovar Typhimurium phoP assays. The second sample was from 0 day old line X birds (pooling of three extracts was performed due to the small sample volumes) and each of the selected gut extracts had been shown to have marked anti-microbial effects. Two milligrams of total protein, as measured using the NanoDrop, for each of the two samples was precipitated in acetone as described in Chapter 2, and submitted for LC-MS analysis.

Analysis was performed by Dr A Treuman (NEPAF, Cels Business Services Ltd, Newcastle-Upon-Tyne) using an LTQ XL orbitrap™ mass spectrometer (Thermo Scientific, USA). Data generated for both samples was extensive (Appendix III), and included multi-dimensional mass spectrum readings of peptides of varying size identified in the samples. Dr A Treuman also utilised several bioinformatic programmes to generate the final data and identify potential peptide identification in the samples.

An example of the readings obtained from LC-MS for six of the proteins identified in Sample 2 is presented in Table 7.1.

Identification	Log(i)	ri	Log(e)	pI	Mr	Description
ENSGALP00000034463	6.16	1	-11.0	5.9	18.2	**BCL2-like protein 15**
ENSGALP00000012445	6.33	1	-8.2	6.1	80.3	**TNF receptor-associated protein 1** Source: RefSeq_peptide NP_001006175 IPR009079 4 helix cytokine-like coreIPR003594 ATP bd ATPaseIPR001404 Hsp90
ENSGALP00000011124	6.03	2	-15.5	6.1	55.5	**protective protein for beta-galactosidase** Source: RefSeq_peptide NP_001026662 IPR001563 Peptidase S10IPR002410 Peptidase S33
ENSGALP00000016629	7.61	18	-130.0	8.2	22.3	**Peroxiredoxin-1** (EC 1.11.1.15)(Thioredoxin peroxidase 2)(Thioredoxin-dependent peroxide reductase 2)(Proliferation-associated gene protein)(PAG)(Natural killer cell-enhancing factor A)(NKEF-A) Source: 1.11.1.15 IPR000866 AhpC-TSAIPR013740 RedoxinIPR012336 Thiordxn-like fd
ENSGALP00000018616	6.42	3	-11.2	6.6	69.8	**leukotriene A4 hydrolase** Source: RefSeq_peptide NP_001006234 IPR012777 Leuk A4 hydro aminopeptIPR006025 Pept M Zn BSIPR014782 Peptidase M1 N
ENSGALP00000019853	6.42	3	-10.9	8.8	35.2	**lectin, galactoside-binding, soluble, 3 (galectin 3)** Source: RefSeq_peptide NP_999756 IPR008985 ConA like lec glIPR001079 Galectin bdIPR000694 PRO rich

Table 7.1: Data output for six of the proteins identified in Sample 2.

1) The “identification column” is the code for that specific protein and its full analysis linked to the website www.gpm.org ,
2) “Log (i)” is the log of the sum of the intensities of all spectra contributing to this protein (based on the multi-dimensional mass spectrum, the closer to 8 the better, values closer to 5 are less desirable),
3) “ri” is the number of peptides which contribute to the protein (will depend on size of protein but if many peptides are included then this improves the credibility of the protein identification),
4) “Log(e)” is an indicator of confidence that the peptides identified do contribute to the protein identified (the larger this negative number, the more credible the data, a cut off of -3 has been used throughout),
5) “pI” is the iso-electric point of that protein,
6) “Mr” is the molecular weight of the protein (kDa).
7) “Description” includes protein identification.

7.6.4.1: Overview of the data for Sample 1 (day 7 XH gut extract) and Sample 2 (day 0 line X bird gut extracts)

LC-MS proved successful in identifying peptides in both of the samples submitted. In sample 1 (XH 7 day old bird gut extract) a total of 143 proteins which met the analysis criteria discussed previously were positively identified. A total of 198 samples were identified in sample 2 of which 126 were unique to this sample, Figure 7.13 shows these data.

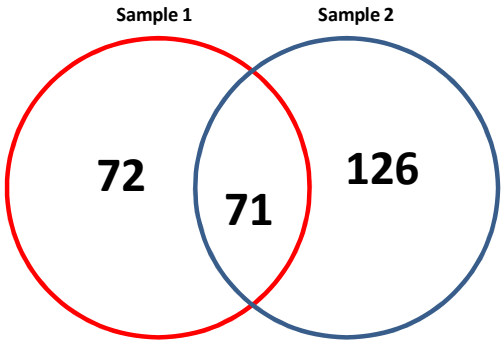


Figure 7.13: Venn diagram of the total proteins identified in the two gut scrape samples using LC-MS.

Each protein identified was further investigated to determine its potential functional location within the cell. This was achieved using the generated data and the linked website access site www.gpm.org. The peptide sequence was further analysed using either the UniProt or NCBI websites (www.uniprot.org and www.ncbi.nih.gov.org), where that specific protein was listed. The Gene Ontology website (www.geneontology.org) was used to confirm cellular location of the identified peptides and the standard nomenclature used by this site was subsequently used to define cellular location and function. Not all of the proteins had been identified in the *Gallus gallus* proteome databases and so the potential sites of protein function were taken from results in other species and linked to protein homology. Cellular locations of protein functions were allocated as follows; secreted, nucleus, cytoplasm, endoplasmic reticulum, Golgi apparatus, cytoskeleton, cell membrane, lysosome, ribosome, mitochondria and unknown.

7.6.4.2 Sample 1 (XH 7day old bird gut extract)

Seventy two different proteins were identified in sample 1 that were not present in sample 2 (Figure 7.13). Putative cellular locations/functions of these proteins are shown in Figure 7.14.

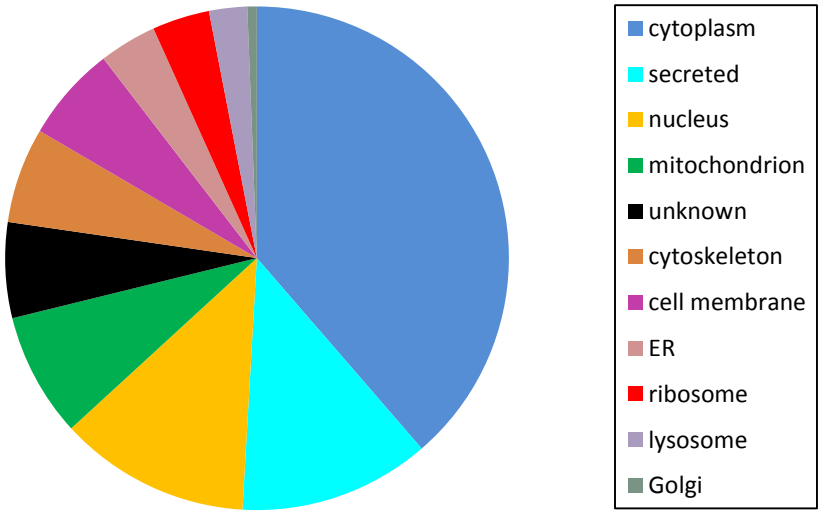


Figure 7.14: Putative cellular locations of the 72 proteins identified in gut mucosal scrape sample 1. ER= endoplasmic reticulum and Golgi= Golgi apparatus.

The majority of the proteins (39%) identified appear to perform their role within the cytoplasm, approximately 12% are secreted, 12% are nuclear located, 8% are located within the mitochondria, 6% have a role in the cytoskeleton, 6% are involved with the cell membrane, 4% are located in the endoplasmic reticulum, 4% form a component of ribosomes, 2% function inside lysosomes and 0.6% are associated with the Golgi apparatus. A total of 6% of the proteins, where mass spectrometry data was produced,

could not be identified by any of the bioinformatic tools described. Of the whole proteome identified with this technique, 35% of the proteins were associated with enzymatic activity.

7.6.4.3 Sample 2 (line X 0 day old bird gut extract)

According to the criteria outlined previously, a total of 126 proteins unique to sample 2 (line X 0 day old pooled gut scrapes) were identified. These proteins were classified as for sample 1 and the results are shown in Figure 7.15.

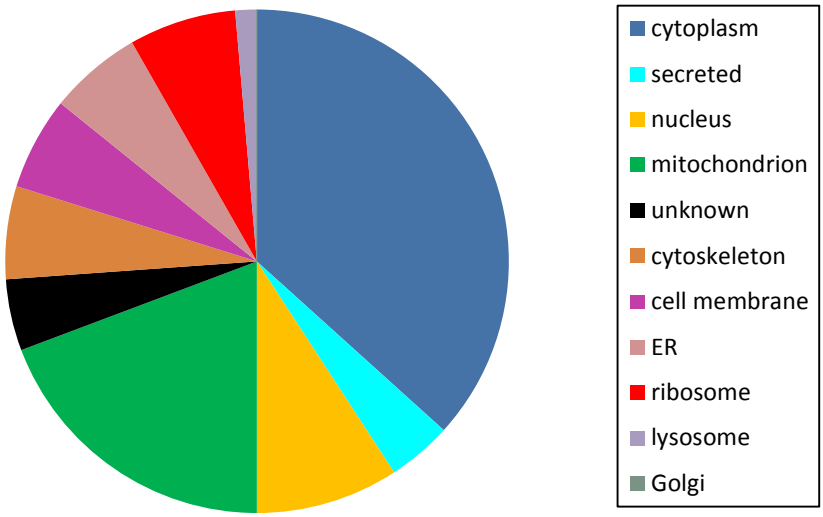


Figure 7.15: Putative cellular locations of the 126 samples identified in gut mucosal scrape sample 2. ER=endoplasmic reticulum and Golgi= Golgi apparatus.

Interestingly, more proteins were identified in sample 2 compared to sample 1 (Figure 7.13). Again, as for sample 1 the main site of activity of these proteins was within the

cytoplasm (37%). A total of 9% of proteins were predicted to be localised to the nucleus, 7% were localised to the ribosome, 6% of proteins were localised to the ER, cytoskeleton and cell membrane respectively, 4% were predicted to be secreted and 1% localised to the lysosome. Within this sample, 5% of the proteins could not be identified. Of all of the proteins identified in sample 2, 68% i.e. approximately double that of sample 1, were associated with enzymatic activity.

When the proteomes of the two samples were compared, similar sites of localisation were identified. For example sample 1 had 39% of proteins localised to the cytoplasm while the second sample had 37%; 4% of proteins in sample 1 were predicted to be localised in the endoplasmic reticulum compared to 6% in sample 2. Surprisingly none of the proteins identified in the second sample were found to be localised within the Golgi apparatus. Both samples had only 6% of the proteins predicted to be localised to the cell membrane and less than 2% of proteins were associated with the lysosome. The most dramatic difference in the sites of predicted protein activity was probably within the mitochondria, where 8% of sample 1 proteins were identified compared to 19% of sample 2 proteins.

7.6.4.4: Proteins identified in both samples 1 and 2

A summary of the data for the proteins identified in samples 1 and 2 and those proteins common to both is illustrated in Table 7.2 and Figure 7.16.

Location	Sample 1 (%)	Sample 2 (%)	Both samples (%)
Cytoplasm	39	37	38
Mitochondria	8	19	16
Nucleus	12	9	9
Secreted	12	4	6
Cell membrane	6	6	9
Cytoskeleton	6	6	8
Ribosome	4	7	5
Endoplasmic reticulum	4	6	5
Lysosome	2	1	1
Golgi apparatus	1	0	0
Unknown	6	5	3

Table 7.2: Putative cellular location of the proteins identified in the proteomes of sample 1 and 2 gut mucosal extracts.

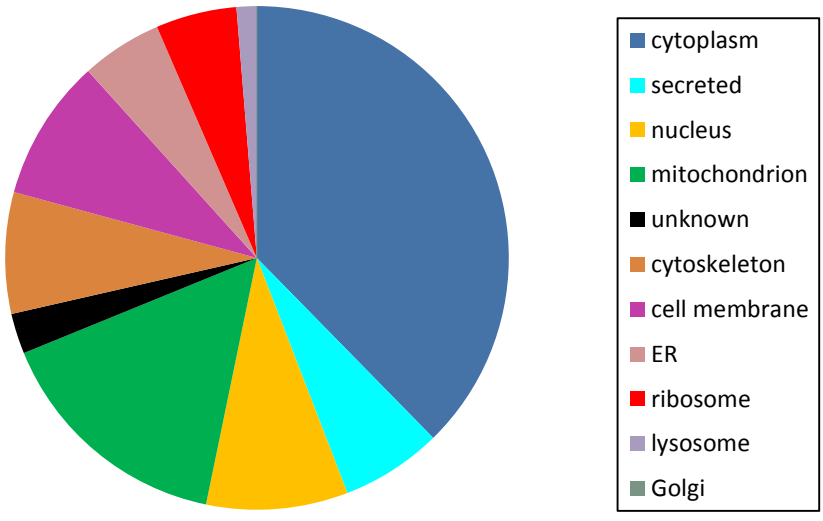


Figure 7.16: Putative sites of function for those proteins identified in both samples 1 and 2. ER= endoplasmic reticulum and Golgi= Golgi apparatus.

These data show that the cytoplasm is the most common site of protein activity. The mitochondria (16%), nucleus (9%) and cell membrane (9%) are also well represented.

7.6.4.5: Peptides with potential anti-microbial or immunological activities

Disappointingly no anti-microbial peptides were identified in either of the two samples despite gene expression of AvBD1, 4 and 10 being detected in the duodenal tissue samples (Chapter 5). The LC-MS data generated for sample 2, which showed evidence of anti-microbial activity, was further analysed to identify any peptides, linked to immunological functions, and six potential proteins were identified. These proteins were present in sample 2, but not 1 and include BCL2-like protein 15 (apoptosis), TNF receptor associated protein 1 (apoptosis), protective protein for beta-galactosidase (within lysosomes), peroxiredoxin (effects on oxidative stress), leukotriene A4 hydrolase (arachidonic acid metabolism) and Galectin 3 (inflammatory properties and apoptosis). The presence of each of these peptides in the samples was further validated by investigating the mass spectrum of each proposed protein.

In relation to Galectin 3, an example of the data generated for one of the three identified peptides is shown in Figure 7.17 A-C. B shows the mass error of the assigned b and y ions and the peaks all lie close to the zero line confirming peptide identification.

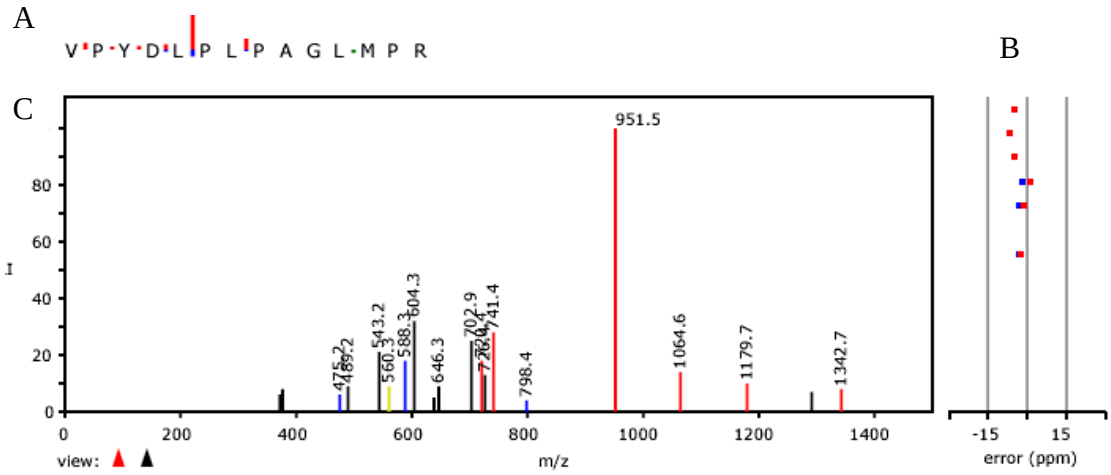


Figure 7.17A-C: Mass spectrum of peptide VPYDLPLPAGLMPR from Galectin 3. A: illustrates the fragmentation of the peptide. B: shows the mass error of the assigned b and y ions and C: shows all of the molecular weights of the fragments identified. In both B and C the y-ions are displayed as red peaks, b-ions as blue peaks and dehydrated b-ions in green. The black peaks are those which are unidentified.

Figure 7.18A-C shows the data for the peptide, identified as TNF receptor associated protein 1, and again supports the identification of this protein in the gut extract.

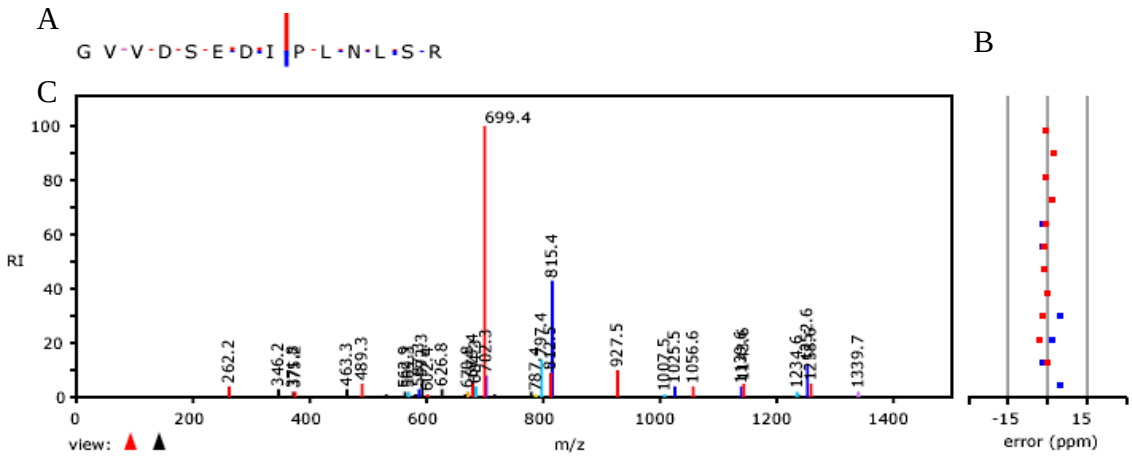


Figure 7.18A-C: Mass spectrum of peptide GVVDSEDIPLNLSR from TNF receptor associated protein 1. A illustrates the fragmentation of the peptide. B shows the mass error of the assigned b and y ions and C: shows all of the molecular weights of the fragments identified. In both B and C the y-ions are displayed as red peaks, b-ions as blue peaks and dehydrated b-ions in green. The black peaks are those which are unidentified.

All of the identities of the six peptides characterised in Sample 2 as having a possible immunological function were supported by mass spectrometry data.

7.6.4.6 Conclusions from mass spectrometry

The LC-MS analysis of the two gut extracts was very successful in identifying many proteins. Two unique proteomes were identified but disappointingly no anti-microbial peptides were identified in either proteome. Several unique proteins i.e. BCL2-like protein 15, protective protein for beta-galactosidase, Peroxiredoxin-1, leukotriene A4 hydrolase and galectin 3, were identified in sample 2, which may have roles in the immune response and, in vivo, play roles in the gut defense.

7.7: Discussion

To investigate and compare the gut anti-microbial properties of the three bird lines raised in the different environments, and in order to be able to correlate this data with the AvBD gene expression data in Chapter 5, duodenal mucosal scrapes taken from the birds in the trial were processed and analysed for their bacterial killing properties using an established time-kill assay. In all, 180 bird duodenal gut scrapes were extracted and analysed to correspond with those samples analysed in Chapter 5 for AvBD gene expression.

To compare the AMA data generated between the populations it was necessary to devise sampling and extraction techniques that were reproducible. This was especially important as bird population sizes within the different groups were limited i.e. maximum n=10 for any one group of birds. It is for this reason that the duodenal loop was selected for the site of mucosal scraping. The sampling of the 0 day old birds proved technically quite challenging, especially the removal of the mucosal layer due to the size and friable nature of the duodenal samples. Only a limited volume of gut extract was produced, thus limiting analysis. In addition, whilst care was taken not to apply too much force while scraping the tissues it is highly likely that the scrape was somewhat deeper into the tissue when compared to those of the older birds.

As the day 0 birds were taken straight from the hatchery to the laboratory for sampling, the duodenum was empty of ingesta. Meyer-Hoffert, Hornef et al. (2008) found that the

secreted anti-bacterial activity of the intestinal mucosa was largely confined to the mucus layer compared to minimal activity noted in the luminal contents, and so attempts were made in obtaining mucus containing gut scrapes from all ages of birds. It is feasible that 0 day old bird scrapes had a larger mucus content relative to luminal contents, and this may have had a potential effect on the proteins actually extracted. The older bird guts were rinsed to reduce the amount of luminal contents, but this may not have been 100% successful in removing ingesta. Sugars were detected in a selection of 7 day old bird gut samples, but not in the day 0 samples, as shown by the TLC (Figure 7.9). This may have been due to sugars present in the partially digested feed i.e. the presence of luminal contents contaminating the protein extracts. The presence of such sugars may also have aided bacterial survival in the assays i.e. lead to the pro-microbial effects reported.

The protein extraction procedure adopted in this study was crude and involved no further attempts at purification or isolation of particular proteins and/or peptides. The use of 10% acetic acid had previously been shown, using HPLC, to extract anti-microbial peptides from bird gut scrapes (Nile, Townes et al. 2006), and so extraction, was performed, using this technique in an attempt to maximise the inclusion of AMPs in the gut extracts. Fractionation of samples using HPLC was considered but was not performed because of the number of samples, (>100), included in the study. No commercial AvBD antibodies are available and so detection and/or quantitation of the gut peptides using techniques such as ELISA was also not possible.

The data from the individual bacterial time-kill assays was found to be reproducible. All samples were tested in triplicate within each assay, and any sample felt to be anomalous to other samples within the group was re-assayed. The time-kill assay had been developed and used in the laboratory over the past six years and was deemed appropriate for such an investigation. Similar protocols have been used in determining the potency of recAvBD8 (Higgs, Lynn et al. 2007) and recAvBD6 (van Dijk, Veldhuizen et al. 2007). Alternative methods of measuring the anti-microbial effects of samples might involve measuring a zone of inhibition (Ma, Liu et al. 2008), and are similar to assays measuring antibiotic susceptibility, but can be more subjective and less accurate.

Salmonella species were chosen for use within the time-kill assays because they are human pathogens associated with food-borne disease, and poultry products are often implicated in their carriage. *S. enterica* serovar Typhimurium phoP was chosen due to the increased susceptibility this strain shows to the actions of AMPs, as a result of an engineered mutation (Behlau and Miller 1993). The argument was that this mutation allowed the effects of AMPs within the gut scrapes to be more easily detected than if a wild-type *Salmonella* strain was used. On reflection the use of the wild-type avian clinical isolates may have represented a more useful approach as such strains represent the microbes that actually challenge the birds *in situ*.

The most notable findings were the anti-microbial effects of the gut extracts from 0 day old birds and the very pro-microbial properties of the 7 day old XH bird gut extracts.

Further strains of *Salmonella* (wild-types) were analysed in the time-kill assays to confirm whether similar responses to those observed for *S. enterica* serovar Typhimurium phoP, again pro-microbial effects, although reduced, were observed.

The data produced from the time-kill assays for the five groups of 7 day old birds were very interesting in that there were statistically significant differences between the groups of birds. In particular the gut samples relating to the XH group of birds showed very pro-microbial effects when *S. enterica* serovar Typhimurium phoP was used as the target strain compared to the other four groups of 7 day old birds. Here both bird line i.e. genetics and rearing environment appear to have influenced the data. The YH group (Figure 7.3) also appeared to support microbial growth compared at least to the YL group, which like the line X birds emphasises the effects of the rearing farm environment on the bird gut anti-microbial properties. Diet and other environmental parameters such as the bacterial bedding count, temperature and humidity all contributed to the differences observed, presumably via altering the physiological and immunological responses of the bird GI tracts. In addition, the gut commensal populations would be expected to be different across the groups of birds.

The diets fed to the birds on the two farms were very different. The high hygiene birds were fed, in the form of a crumble, a wheat-based diet containing high levels of nutrients and supplemented with enzymes, with the effects of lowering feed viscosity levels, and increasing the feed conversion rates presumably through beneficial changes in intestinal morphology (Shakouri, Iji et al. 2009). This same diet was also in the form of a crumble

and contained a high level of nutrients. Faster growth rates are therefore observed in birds fed this type of diet. In contrast the LH diet was maize-based as is the diet of commercially reared broilers in the UK. The LH diet was in fact 100% maize (10-20% only in commercial diets) and this has the effect of leading to the ingesta having poor viscosity in comparison to the HH diet with subsequent effects on growth rates and gut morphology. The LH diet was made more challenging and viscous by the fineness of the crumbs. The diet could have had an effect on the proteins that are present in the mucosal layer and therefore the gut extracts by affecting the local gut environment or dietary nutrients may be responsible. It is also feasible that the source of cereal and nature of the diet may potentially have had an effect on the commensal population of these birds in the duodenum with subsequent effects on the proteins secreted into the mucosal layer. Repeating the trial using groups of birds in the two environments but fed the same diet would allow the effects of diet alone on the gut extract proteomes to be determined.

Establishment of a commensal population within the gastrointestinal tract will not have taken place in the day 0 birds since they were taken directly from the hatchery. Thus mucosal contents of the newly hatched birds provided a more hostile environment to microbes, observed as anti-microbial activity against *S. enterica* serovar Typhimurium phoP in the time-kill assays. Following challenge and colonisation of the guts with micro-flora, a more pro-microbial gut mucosa establishes, as was the case with the 7 day old bird scrapes. To support this theory it would be interesting to identify and quantify gut

bacteria in birds aged 0-7 days using techniques including 16S Denaturing Gradient Gel Electrophoresis, DGGE, (Collins, Verdu et al. 2009) and sequencing could be utilised.

The role of the commensal population in gut immunity has been investigated by many groups but the importance of such microbes is emphasised by the finding that germ-free animals have a higher susceptibility to intestinal infection (O'Hara and Shanahan 2006). In addition, these commensal bacteria have been shown to aid development of the gut-associated lymphoid tissue (GALT) (Rhee, Sethupathi et al. 2004). The main genera of bacteria within the chicken small intestine are *Lactobacillus*, *Enterococcus* and *Clostridium*, with some strains of *Enterobacteriaceae* (Salanitro, Blake et al. 1978; Amit-Romach, Sklan et al. 2004). Additional functions of this population include their ability to directly affect the innate and adaptive immune systems, to maintain immune homeostasis by suppression of unnecessary inflammatory responses (Moal and Servin, 2006), and in food metabolism. Future studies could investigate the gut proteomes of lines raised in different environments in relation to their bacteria populations and establish any potential relationships. Apajalahti, Kettunen et al. (2001) found that both the feed source and the local feed amendment changed the bacteriological profile of the caecum but that farm hygiene had little effect on this population. This groups finding again emphasizes the potential effects that diet has compared to the environmental conditions that birds are faced with.

The wild-type Salmonella strains were used only to investigate the duodenal AMAs of the 7 day old bird samples because of the marked pro-microbial activities of the XH bird gut extracts in the assays with *S. enterica* serovar Typhimurium phoP. Interestingly the 'high' pro-microbial effects were not observed when *S. enterica* serovar Typhimurium SL 1344 strain was used in the assay with the line X HH bird gut extracts. It is feasible that there is a peptide or another component of the XH 7 day bird extracts that selects for the growth of the *S. enterica* serovar Typhimurium phoP bacteria and this may somehow be directly related to the phoP/phoQ mutation. Alternatively it may be that the XH gut extracts contain lower levels of AMPs as indicated by the expression data for duodenal tissues and AvBD1 as indicated in Chapter 5, Figure 5.14 (50 fold difference between XL group and XH birds, the former being the highest mean). However, this finding was not supported by the LC-MS analysis but may be a result of technique and small number of samples analysed by this technique. For the groups reared in the LH environment, it was in the assays using *S. enteriditis* where the most pro-microbial effects were seen. Although speculative, this strain of bacteria may *in vivo* function as a 'commensal' organism with its survival supported by the repertoire of peptides produced and secreted into the duodenal mucosal layer.

The data obtained for the day 14 birds showed that it was the line XL gut extracts that showed the most pro-microbial effects. This result hi-lighted a seven day lag in maximal pro-microbial activity of the gut scrapes between line X birds reared in the LH and HH environments. The bird weight data for the birds reared in the two environments

(Chapter 3), indicated that there was a significant weight difference between birds reared on the two farms. The average bird weights (mean ±SEM) at 7 days of age were 129g (±6.0) for XL and 199g (±4.2) for XH birds. At day 14 bird weights (mean ±SEM) had increased to 328g (±16.75) for XL birds and 518g (±14.91) for the XH group. The bird weights may be an indirect marker of gastro-intestinal development in the birds affecting GALT, tissue robustness and the repertoire of proteins present in the gut mucosal layer. Analysis of further gut scrapes from line XH birds during the first seven days would allow the age at which the gut extract 'switches' from anti-microbial to pro-microbial to be determined. Equally, analysis of the XL bird gut scrapes between 7 and 14 days of age would allow determination of a possible age when the pro-microbial effects peak. Correlating such findings from these two studies with bird weight may show that the pro-microbial effects occur when the birds reach a specific weight. Bird weight and development may have effects on the immune system and/or other parameters. In particular, obtaining data from line XL birds when they reach the same weight as the XH 7 day old birds would be of particular interest. Again the nutritional status of the two groups could be a major contributory factor rather than the environmental differences. This could be related to gut development or something contents and bioavailability of the two diets.

By day 35 the results (Figure 7.5) show that there were no marked differences in bacterial survival across the five groups of birds, but again pro-microbial activity was observed.

Attempts were made to identify and characterize the proteomes of the gut samples. SDS-PAGE analysis proved insensitive and inconsistent, MALDI-TOF analysis failed, but LC-MS proved relatively successful. This technique revealed very different protein populations within the scrapes collected from the different groups of birds, although admittedly only two samples were analysed i.e. an anti-microbial sample (0 day old bird extract) and ii) a pro-microbial sample (7 day old XH bird extract). A total of 143 proteins were identified in the pro-microbial sample compared to 196 in the anti-microbial sample. No AMPs were identified in either the day 0 or day 7 samples despite AvBD1, 4 and 10 expression being identified in the duodenal tissue (Chapter 5). However six potential proteins hypothesized to have a role in the defence of the GI tract were identified in the anti-microbial, day 0, sample (sample 2).

Anti-microbial peptides by their nature are very difficult to identify, as they are small in size, hydrophobic and positively charged. Studies performed by other groups but using mammals and focusing on the more distal small intestine have identified gut AMPs (Ouellette 2004; Meyer-Hoffert, Hornef et al. 2008). In such regions of the gastro-intestinal tract it is the alpha defensins, which have been characterised. However, only the beta defensins have been shown to exist within the chicken genome. It could be argued that taking mucosal scrapes from more distal regions of the gut would have led to the detection of AMPs via LC-MS, but for reproducibility of sample taking, and to compare and correlate results with tissue gene expression, the duodenum was selected.

The smallest peptide identified was 4kDa in size and this was identified as a fragment of a larger protein. Only a total of three proteins were identified in both samples which fitted the size of standard AMPS (approximately 4-6kDa in size). In addition to LC-MS, two- dimensional SDS-PAGE gel electrophoresis could have been considered as a form of proteome analysis (O'Farrell, 1975). Potentially more gut extracts could have been analysed and proteomes compared however, it is also questionable as to whether small peptides, such as AMPs would have been detected using 2D gels.

Of the six proteins with a potential defence–related function, Galectin 3 provided an interesting result. Galectins are beta-galactoside-binding lectins that are involved in several biological processes including modulation of immune and inflammatory responses. For example, Galectin 3 expression levels have been shown to be greatly reduced in rabbit enterocytes following their exposure of Shiga toxins from enterohaemorrhagic *Escherichia coli* (EHEC), and such reduced levels may be detrimental by contributing to the diarrhea via the impairment of structural proteins and transporters including sodium-proton exchanger 2 (Laiko, Murtazina et al. 2009). Galectin 3 has also been shown to have a modulatory role on neutrophils in mice infected with *Toxoplasmosis* by affecting Nphi activity (intra-peritoneal neutrophils) (Alves, Silva et al. 2009). The commercially available 44K Agilent chicken microarray includes Galectin 3 and work carried out by Sarson, Wang et al. (2009), showed that Galectin 3 expression levels increased in the spleen of birds infected with the necrotic enteritis inducing bacteria *Clostridium perfringens* when the birds were fed antibiotic-supplemented diets compared to those that received antibiotic-free feed. A further study

on this protein found that Galectin 3 knockout mice showed reduced phagocytosis of macrophages and neutrophils compared to normal mice, again stressing the importance of this protein as an immune-modulator (Sano, Hsu et al. 2003).

As Galectin 3 was only identified in the gut samples of the 0 day old birds (anti-microbial), it could be postulated that its presence is more important in the newly hatched birds prior to gut colonisation and before an adequate adaptive immune system has developed. Utilisation of the aforementioned microarray could prove an exciting tool for further studies to determine Galectin 3 gene expression patterns and determine whether there were any significant differences in levels between the groups of birds.

Two of the other five identified proteins with possible immune function, BCL-2, Tumour necrosis factor receptor associated protein 1, have been linked to apoptotic activity (Kilpatrick, Sun et al. 2004; Zhai, Jin et al. 2008). These may well aid the host in evading epithelial colonization and invasion by facilitating apoptosis following microbe recognition.

In conclusion, the results show very distinctive findings for the different lines, ages and rearing conditions of the birds included in this study. The 0 day old bird gut extracts were found to be the most potent with regard to anti-microbial activity followed by the LH 7 day old bird samples. All other samples were shown to be pro-microbial and line X birds produced the most interesting findings. The AMA results using the *S. enterica* serovar

Typhimurium phoP strain were different compared to those using the two wild-type strains for the 7 day old bird groups. LC-MS was shown to be the most appropriate technique for characterisation of the gut proteomes and two different protein populations were determined following analysis of two gut extracts. However, these analyses were limited and further analyses of gut mucosal scrapes is required to confirm whether patterns exist between different lines and ages, and whether specific proteins can be linked to the results of the time-kill assays. The findings for the line X birds may help to explain why they are susceptible to gastro-intestinal problems, which could potentially be of commercial interest.

Chapter 8: Final discussion

The poultry industry in recent years has been challenged by high profile disease outbreaks particularly avian influenza (2006) and Newcastle Disease (2006), and the risk of pandemic status is increased significantly by large-scale production systems and world-wide shipment of birds. Although recent outbreaks have been contained successfully there is a constant threat of disease, which ultimately may transmit to humans, and thus justifies the need for strict biosecurity protocols in order to limit potential risk. More common disease challenges also face the birds such as dysbacteriosis, necrotic enteritis and skin lesion. Large-scale commercial broiler production involves high stocking densities of birds, relatively closed air spaces and large groups of fast growing birds being reared in close proximity and these combined factors can add to the challenge. Moreover not only is it the health of the individual birds that needs consideration, but that of the flock that is of vital importance. Thus limiting disease occurrence and potential spread is very important both at a welfare level and also commercially.

In addition to the health of the birds, the ability of poultry to carry human pathogens such as *Salmonella* and *Campylobacter* in their GI tract often leads to the contamination of poultry products and consumer infections. A survey performed by the Food Standards Agency revealed 65% of chicken meat products tested were contaminated with *Campylobacter* while 6% carried *Salmonella* species (www.food.gov.uk- 03.10). This information high-lights the threat of poultry meats per se as a source of disease in the

human population. A decline in the incidence of clinical cases of Salmonella has been reported over the last 18 years although this is countered by the rise in reported cases of *Campylobacter* (www.hpa.org.uk- 03.10). However, the mechanisms that allow poultry to carry potential human pathogenic bacteria are still obscure but may be directly related to the birds immune status.

To date broiler breeding programmes have focused on shortening the time taken from hatching birds to their purchase by the consumer, with birds showing good feed conversion rates, fast growth rates, general survival, cardiovascular fitness and skeletal strength elected preferentially. Thus far the immune system of the birds has been neglected as a direct selection measure except Mareks disease. However, the need for producing birds with superior immune systems is increasing based on potential disease risks to flocks, the banning of the use of prophylactic antibiotics and the emerging risk of birds functioning as a reservoir of potentially pathogenic human microbial agents (Persoons, Van Hoorebeke et al. 2009).

Aviagen Ltd is the worlds' largest chicken breeding company and collaboration with the company has allowed a good insight into the industry and the requirements of their clients across the world. Moreover one of their interests, driven in part by the arguments above, is in investigating and enhancing the endogenous immune defences of their commercial livestock.

Three pure lines of chickens were used throughout the study and they were selected as they form a focus for broiler breeding programs within the company. Studies have shown

that the lines X, Y and Z despite originally originating from the same breed of bird, are now phylogenetically distinct (Andreescu, Avendano et al. 2007). Bird line selection was also based on perceived differences in gut health, line X birds having the poorest health and line Z birds fewer problems. Wet litter had been reported by the company to be one of the major problems with the line X birds and subsequent problems such as foot lesions are potentially more of an issue with this line.

The role and importance of AvBD peptides in the chickens immunity has been suggested by many authors (Evans, Beach et al. 1995; Xiao, Hughes et al. 2004; van Dijk, Veldhuizen et al. 2008), particularly given the absence of myeloperoxidase in chicken heterophils, and a reliance by these immune cells on non-oxidative mechanisms to fight infection. It has been suggested that the repertoire of AMPs produced in the gastro-intestinal system may also have an effect in controlling both the species and number of bacteria that colonise the guts with a subsequent effect on pathogenicity (Salzman, Chou et al. 2003). To date a total of 14 AvBD peptides have been identified, the majority being identified via a bioinformatics approach and presence of signature motifs i.e. six encoded cysteine residues. Three of these peptides; AvBD1, 4 and 10 were selected for investigation within this study. The farm trial conceived with Aviagen Ltd and involving a total of 180 birds was designed to enable the effects of bird age, genetics, and the bird rearing conditions on both AvBD tissue expression and gut anti-microbial activities. Disappointingly due to the rearing patterns of the different lines of birds, and increased bio-security due to the threat of avian influenza when the trial was performed, it was not possible to sample line Z birds in the HH rearing conditions. This therefore meant that

no direct comparison of this line could be made across the two farms (LH and HH). Logistically it was not possible to obtain data from every bird included in the study thus a focus was placed on samples from 7 day old birds. This was defended in that innate immunity has been shown to be particularly important during the first week of a birds life (Bar-Shira, Sklan et al. 2003), a time when it is being exposed to the outside environment and potential challenges that might impact on its survival.

The study of single nucleotide polymorphisms and linking their presence with specific traits forms part of a large ongoing genomics initiative by Aviagen Ltd. The use of SNPs in assessing both innate and adaptive immunity is gaining popularity (Biscarini, Bovenhuis et al. 2010), particularly in relation to its use as a potential tool for breeding programmes. The presented studies form a part of this initiative with the presence of polymorphisms associated with the AvBD genes being investigated. Previous studies have linked the presence of polymorphisms within the AvBD locus to disease resistance (Lamont, Kaiser et al. 2002).

A panel of 49 possible SNPs within the AvBD locus was formed to coincide with a large study organised by Aviagen Ltd to determine the occurrence of polymorphisms within the lines of birds of interest to the company. These SNPs had been identified by bioinformatic searches and the updated second version of the chicken genome (2006) was used throughout. None of the readily available data from such sources included the occurrence of polymorphisms within the three lines of birds investigated in this study. The published data on SNPs in chickens is very limited with the focus being on non-

broiler birds. One study showed a reported SNP rate of 13.2 SNPs per kilobase of DNA in the 3.25kb region of AvBDs 2,3,4,5 and 7 (Hasenstein, Zhang et al. 2006). It could therefore be argued that a larger panel should have been entered for analysis however, fifteen SNPs were found to be present within the three lines of bird selected for study. Published work on the presence of polymorphisms within the defensins has focused on those present within the introns (Lamont, Kaiser et al. 2002), but in this study SNPs were identified within exonic regions, thus potentially affecting the actual structure and functional activities of the encoded peptides.

Of the 14 reported AvBDs identified in the chicken, three genes were selected for further investigation i.e. AvBD1, 4 and 10. These three genes were chosen based on the identification of SNPs and in addition AvBD10 was selected because of its unusual tissue expression (kidney, testicle and liver) and lack of characterisation. The investigation of a larger repertoire of AvBDs was considered but was not possible within the time frame of this study. However, future studies could be performed using the RNA and DNA samples extracted from the population of birds used in the trial.

The technique of sequencing pooled genomic DNA to determine SNP frequency proved successful and generated comparable results to the sequencing obtained from Illumina, and individual bird cDNA sequences. The most striking finding was the presence of three non-synonymous polymorphisms within the cDNA encoding the mature AvBD1 peptides, and each of the three lines having distinct polymorphic patterns. The AvBD4 SNP was located in the 5'UTR region and a possible transcription factor binding site

(Thing1-E47), was identified in line X and Y birds at this location. However, this is not a TFBS that has been identified to function in *Gallus gallus* so its significance remains unresolved. The AvBD10 polymorphisms were all located within non-coding regions but differences in their occurrence were identified across the three lines of birds. Again the significance of this is not known but as stated previously (Hasentein and Lamont 2007) the presence of such SNPs may have an effect on the activities of other genes. The use of microarrays following SNP identification may prove a useful follow-up study to confirm whether any other genes (linked to innate immunity or otherwise) show altered expression levels based on the presence or absence of these SNPs in the AvBD locus.

The line X birds encoded the amino acid combination NYH, line Y birds predominately NYY and line Z birds SSY. To investigate whether the presence of the different amino acids affected peptide functionally, the encoded peptides were synthesised *in vitro* using a bacteria hyper-expression system. In addition, a single form of AvBD10 was prepared and the anti-microbial properties of the peptides analysed using a bacterial time-kill assay. Potential differences in the AMAs of these three peptides were hinted by the amounts of each peptide synthesised, with the greastest amount of the A form (NYH) and least amount of the C form (SSY) being synthesised. However, the inability to produce large amounts and lack of purification meant direct comparisons were not possible. In fact, only low concentrations of peptides were generated throughout (nM), which was disappointing as other studies reported µM amounts (van Dijk, Veldhuizen et al. 2007; Ma, Liu et al. 2008). Limited success was achieved with peptide purification however, all recombinant peptides, including AvBD10 a novel result, were shown to

have anti-microbial activity. The data suggested that the AvBD1-A form (NYH) was the least anti-microbial of the three recombinant forms, and this was the encoded peptide identified in the line X population of birds, characterised interestingly by their poor gut health. However, to allow the activities of these peptides to be determined particularly against other strains of bacteria, including human pathogens and clinical isolates, peptide purification is necessary, it would also allow further investigation into the structure and function of the peptides.

Real time PCR was utilised to compare gene expression levels across the groups of birds as an indirect measure of the protein production. As shown in Chapter 5, marked inter and intra-group variability in the gene expression levels was a common finding. Statistically significant differences in gene expression were apparent but in the main only trends could be identified. The most notable results were the higher level of AvBD1 gene expression in the day 7 old XL bird duodenal samples. This could be related to the NYH A form of the peptide and the previous suggestion that this form may not be as 'potent' as the other two AvBD1 peptides. The environment could well be the challenge for this higher level of expression as a similar finding is not seen in the XH group of similarly aged birds. This perceived 'up-regulation' of AvBD1 gene expression in the XL group may help confer superior gut immunity for these birds living in the more challenging environment. The finding that the YH 7 day old group had significantly higher levels of AvBD10 gene expression is more difficult to explain at the levels of SNPs due to the intronic location of the polymorphisms in the gene locus. Again the

complex relationship of response to environment and genetics is suggested with such findings, and the performance of future investigations is warranted.

The mucosal scrapes analysed in AMAs were taken from the same region of the duodenum as analysed for AvBD gene expression so that the two sets of data could be directly compared. It was found that the samples from the 0 day old birds were anti-microbial, but as the birds aged, less potent effects were observed and a switch from bacterial killing to bacterial survival was seen. This finding did not correspond with AvBD gene expression in the duodenum where peak levels were generally seen in the day 7 old gut samples and the inability to identify AMPs in the mucosal samples of the samples identified via LC-MS suggests that AvBDs may not be having the effect on anti-microbial activity of the samples analysed in this study.

The most surprising finding was the very pro-microbial effects seen in the assays where the XH 7 day extracts were used against *Salmonella enterica* serovar Typhimurium phoP although when other strains of *Salmonella* were used in the assays the data generated was found to be very different. A possible explanation for this could be that due to low levels of environmental bacteria, the proteome repertoire of the gut mucosa of this line of birds is different to those of similar genetics but reared in the LH conditions. This may be particularly important as this is the line of birds that is recognised as having the poorer gut health. Whether the phoP mutation is somehow having an effect on how the proteome interacts with the bacteria in the time-kill assay compared to the results observed for the wild-type strains can only be speculated. Further analysis of mucosal

samples via LC/MS would help answer this question as well as the use of further strains of bacteria. Another possibility is the presence of something in the pro-microbial samples other than protein and such things could include metal ions.

To identify AvBDs and other potential anti-microbial proteins in the gut scrapes LC/MS was used although the analyses were limited to those of a day 0 line X bird and a 7 day old XH bird. Disappointingly no AMPs were identified in either sample, which may have been due to the limits of the technique based on the size of peptides or that levels were so low in this region that they were undetectable. A large amount of data was generated using this technique (72 in sample 1, 126 peptides in sample 2 and 71 peptides common to both extracts, Appendix III- CD Rom). In both samples the putative cellular location of the identified peptides was focused on the cytoplasm but approximately twice the number of peptides identified in sample 2 (0 day sample) had enzymatic functions compared to those identified in sample 1. Six proteins with potential defense functions were identified in the day 0 sample, Galectin 3 being probably the most exciting. However, the sample number was small and analysis of furtherer gut extracts from the birds of different lines, ages and environments are needed to generate the data needed to investigate this further. In addition the effects of diet alone on the proteomes also needs further investigation as this was one of the major differences in the two rearing environments.

From the results chapters it can be seen that it is the line X birds which repeatedly showed different findings and this is the bird group reported to have the poorest gut health. For example the SNPs in the AvBD1 gene encoding potentially a peptide with

reduced anti-microbial activity, higher levels of AvBD1 gene expression in the duodenum of the XL 7 day group and the marked pro-microbial effects of gut extracts in both the XH 7 day group and XL 14 day old bird groups. The use of an innate immune gene specific microarray would be a good starting point to determine if the line X birds do generally exhibit very different gene expression patterns compared to the other two lines of birds. Potentially additional AvBD genes could be included to provide a more detailed picture. The interest in anti-microbial peptides is increasing as links are made with specific disease are elucidated and the full role of these peptides becomes clearer. Commercial interest is also increasing with the development of products impregnated with AMPs being developed and tested. Further investigation into the full role and benefits of AMPs needs to be performed so that successful treatments and breeding strategies can be applied to the livestock setting but this study has given further insight into the role and function of such peptides in chicken broilers.

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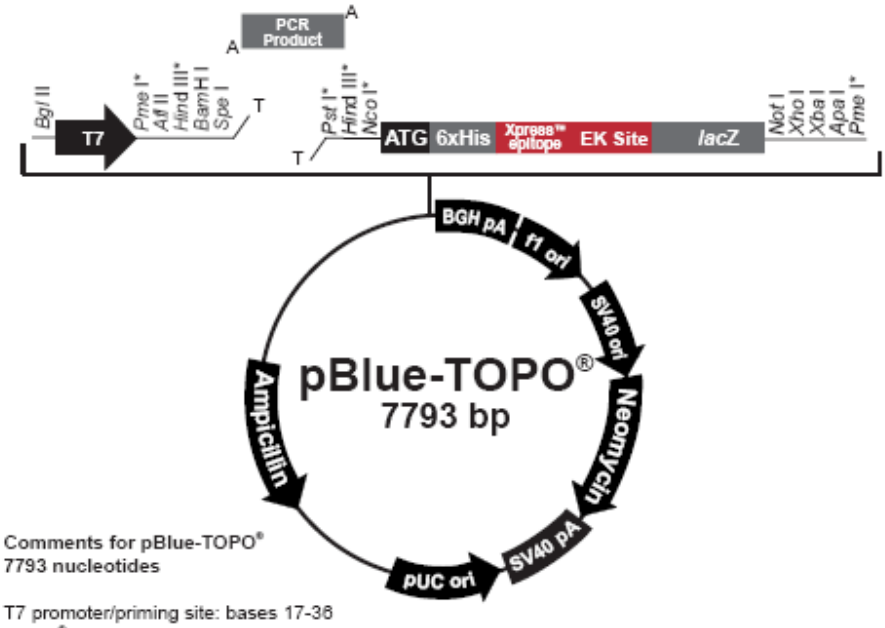
Appendices:

Appendix I: Plasmid maps

A) pBlue TOPO used for cloning 18S gene for qRT-PCR (Invitrogen)

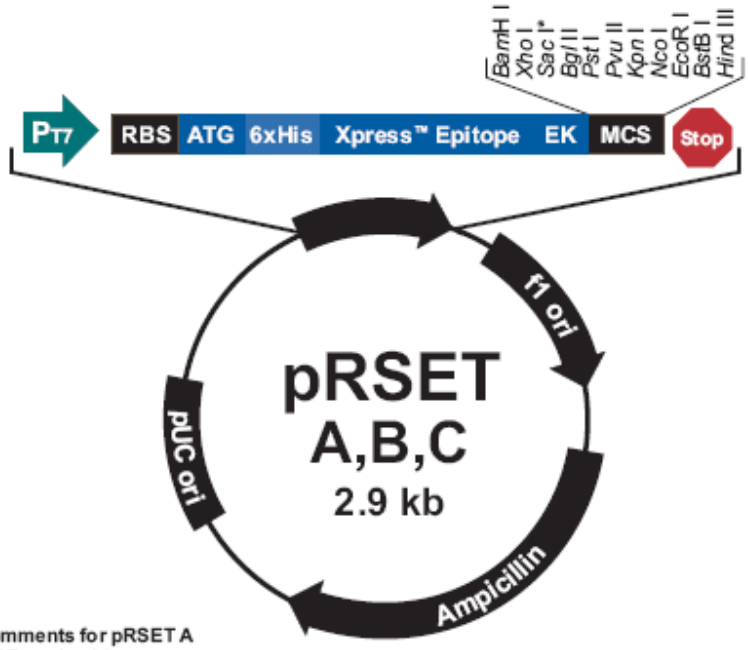


Comments for pBlue-TOPO®
7793 nucleotides

T7 promoter/priming site: bases 17-36
TOPO® Cloning site: bases 116-117
ATG initiation codon: bases 143-145
Polyhistidine region: bases 155-172
LacZ Reverse priming site: bases 173-191
Xpress™ epitope: bases 212-235
Enterokinase recognition site: bases 221-235
LacZ ORF: bases 264-3313
BGH polyadenylation sequence: bases 3386-3613
f1 origin: bases 3659-4087
SV40 promoter and origin: bases 4141-4423
Neomycin resistance gene: bases 4498-5292
SV40 polyadenylation sequence: bases 5466-5596
pUC origin: bases 5979-6652 (complementary strand)
Ampicillin resistance gene: bases 6797-7657 (complementary strand)

* These sites are not unique but may be used to excise the PCR product. The *Pme* I sites may be used to excise the reporter cassette, providing there are no *Pme* I sites in the PCR product.

B) pRSETA used for cloning AvBD1 and 10 for hyper-expression (Invitrogen)



Comments for pRSET A
2897 nucleotides

*Version C does not contain *Sac* I

T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Xpress™ epitope: bases 169-192
Multiple cloning site: bases 202-248
T7 reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 2047-2720 (C)

Appendix II: Vaccination protocols

A) Low hygiene environment

Name of vaccine	Age administered (days)	Route of administration	Disease	Manufacturer
Paracox 5	0	In feed	coccidiosis	Intervet,Schering Plough Animal Health Corporation
Bursine 2	15	In water	Infectious Bursal Disease	Fort Dodge
MA5	22	Aerosol	Infectious bronchitis	Intervet, Schering Plough Animal Health Corporation
HB1	22	Aerosol	Newcastle Disease	Lohmann Animal Health

B) High hygiene environment

Name of vaccine	Age administered (days)	Route of administration	Disease	Manufacturer
Rismavac	0	subcutaneously	Mareks disease	Intervet, Schering Plough Animal Health Corporation
Paracox	5	In water	coccidiosis	Intervet, Schering Plough Animal Health Corporation
Nobilis Rhino CV	11	Aerosol	Avian pneumo viruses	Intervet, Schering Plough Animal Health Corporation
IB primer	21	Aerosol	Infectious bronchitis	Fort Dodge
HB1	21	Aerosol	Newcastle Disease	Lohmann Animal Health
TAD Gumboro	25	In water	Infectious Bursal Disease	Lohmann Animal Health

Appendix III: LC/MS data from two samples analysed

This data is with the live links to mass spectrometry findings is on the enclosed CD Rom.