Investigations of the Gut Innate Defences of Commercial Broilers

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Abstract

The E.U. ban on the use of anti-microbial growth promoters in poultry feed, introduced to counter global problems of bacterial antibiotic resistance, has increased the risk of enteric disease in commercially reared broiler chickens. Development of strategies to prevent such diseases requires further knowledge and understanding of avian gut defences and particularly the innate immune defences. In collaboration with Aviagen Ltd., the objective of this study was to investigate, through two farm trials, the effects of bacterial exposure on host avian β-defensin (AvBD) expression profiles and gut health of two commercial broiler lines (X and Y). Furthermore, two host defense peptides, avian beta defensin 1 (AvBD1) and 10 (AvBD10) were analysed *in vitro* for their anti-microbial efficacies.

In Trial 1, Lines X and Y, differing in their gut health, were exposed to one of three bacterial challenges on the day of hatch, namely a combination of *Bacteroides dorei* and *Barnesiella viscericola* (B/BV), *Lactobacillus johnsonii* (LJ) or a mixture of the two challenges (B/BV + LJ). At days 4, 7, 14, 21, 28 and 35, birds were scored for gut health using an industry approved system and digesta were sampled and analysed for microbiotae (pyrosequencing). The data revealed that, relative to control and LJ challenged birds, the B/BV challenge was associated with gut health deterioration. Furthermore, relative to Line X, there was a trend for the gut health of Line Y birds to be superior for all challenged groups. Although microbiome analyses did not reveal any clear differences between Lines X and Y, the data did suggest that birds with better gut health outcomes were associated with higher ileal *Lactobacillus* spp. levels at Day 4 and higher caecal levels of *Bacteroides* spp. at Day 21.

Despite less optimal gut health, Line X is important to the Aviagen Ltd. breeding programme. To understand the roles, if any, of the AvBDs in bird gut health, a second trial was performed in which gut AvBD1 and 10 gene expression were assessed in Line X birds following B/BV challenge. Relative to control birds, the B/BV challenge suppressed gene expression of AvBD1 in the duodenum/jejunum ($P < 0.05$) and AvBD10 in the duodenum/caecum ($P < 0.05$) and AvBD1 down-regulation was confirmed at the cellular level by data from an *in vitro* challenge model ($P < 0.001$). Interestingly, within the B/BV challenged group, birds with higher AvBD1 expression were associated with better gut health assessments.

The AvBD1 gene contains single nucleotide polymorphisms (SNP) within the region encoding region the mature peptide. Three AvBD1 variants were synthesised that were typical to Line X (NYH), Y (SSY) and another commercial Line, Z (NYY), and were assessed, together with AvBD10, for *in vitro* anti-microbial activities (AMAs) against a variety of gut bacterial isolates. Despite Line X displaying the least optimal gut health, the 'NYH' variant exhibited the greatest potency against all bacterial species. The data for AvBD10 revealed that, although bacterial growth was inhibited, this peptide had lower AMA than AvBD1, indicative of additional physiological functions. An in *vitro* examination of wound healing capacity using a scratch assay was inconclusive.

The *in vivo* data indicated that gut AvBD expression is susceptible to gene down-regulation by bacteria and that this, in turn, may have an adverse outcome on gut health. However, selectively breeding for birds able to maintain high AvBD expression presents a strategy to protect flocks against the threat of endemic gut health problems.

Dedication

I would like to dedicate this PhD to my partner Kate for all her love and support and my family for always being there for me. In addition, I would like to thank my supervisor Judith Hall for all her help, time and encouragement.

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- Dr Catherine Mowbray – For performing immunohistochemical staining for AvBD1, AvBD/IL primer design, endpoint RT-PCR tissue panels for AvBD1 and 10, GeNorm optimisation for the qPCR reference genes, tissue RNA extraction, and optimising and performing real-time qPCR assays on tissue extracted RNA.
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List of Abbreviations

ACTB - Beta-actin

AGP - Antimicrobial growth promoters

AMA – Anti-microbial activity

AMPs - anti-microbial peptides

AP-1- activating protein 1

AvBD - Avian beta-defensin

A.U. - arbitrary unit

B/BV - *Bacteroides*/*Barnesiella viscericola*

BCA - bicinchoninic acid

BD – *Bacteroides dorei*

bFGF - basic fibroblast growth factor

BHI - Brain heart infusion

BSA - Bovine serum albumin

cAMP - Cyclic adenosine monophosphate

C.F.E - cell free extract

CFU – colony forming unit

cLEAP - chicken liver-expressed antimicrobial peptide

chTLRs – Chicken Toll-like receptors

CFU – colony forming units

CP - crossing point

CPr – crude protein

CpG-ODN – CpG oligodeoxynucleotide

DAB - 3,3-diaminobenzedine tetrahydrochloride

DEFRA - Department for Environment, Food and Rural Affairs

DGGE - denaturing gradient gel electrophoresis

DMEM – Dulbecco's Modified Eagles Medium

DNA - deoxyribonucleic acid

dNTP - deoxyribonucleotide triphosphate

DTT – dithiothreitol

EDTA - Ethylenediaminetetraacetic acid

EEO - Electroendosmosis

ESTs - expressed sequence tags

E.U. – European Union

FCR – Feed conversion ratio

GALT - gut associated lymphoid tissue

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GF - Germ-Free

GGP - great-grandparent

GI - gastrointestinal

GP – grandparent

GST – Glutathione-S-transferase

hBD – human beta-defensin

HD-5 - human defensin-5

HDP - host defense peptide

HPLC – High Pressure Liquid Chromatography

IBDV - infectious bursal disease virus

IBD – inflammatory bowel disease

IBS - irritable bowel syndrome

IFN-ɣ - interferon-gamma

Ig – Immunoglobulin

IHC – immunohistochemistry

IL- interleukin

IPTG - Isopropyl thiogalactoside

IQR - inter-quartile range

ISD - In-Source Decay

KTN – Knowledge transfer network

LB - Luria-Bertani

LJ - *Lactobacillus johnsonii*

LTA – lipoteichoic acid

LPS – lipopolysaccharide

LX – Line Y

LY – Line Y

MCP-1 - monocyte chemotactic protein 1

ME – Metabolisable energy

MIC - Minimum inhibitory concentration

MMP7 - matrix metalloproteinase 7

M-MLV - Moloney murine leukemia virus

MRS - de Man, Rogosa and Sharpe

MTS - (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MW – molecular weight

NE - Necrotic enteritis

NFκB - nuclear factor kappa-light-chain-enhancer of activated B cells

NK - Natural killer

NSPs - Non starch polysaccharides

NMR - Nuclear magnetic resonance

OD – Optical density

PAMPs - pathogen-associated molecular patterns

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PMS - phenazine methosulfate

PRRs - pattern-recognition receptors

PS - parent stock

PSSs - positively selected sites

RNA – ribonucleic acid

rRNA- ribosomal ribonucleic acid

RT – reverse transcriptase

SCFA - short chain fatty acid

SDHA - Succinate dehydrogenase complex, subunit A

SEM – standard error of the mean

SF3A1 - Splicing factor 3 subunit 1

SFB - segmented filamentous bacteria

SNP - single nucleotide polymorphism

SPF - specific-pathogen free

S.T – *Salmonella typhimurium*

TBE - Tris/Borate/EDTA

Th – T helper

TLC – Thin layer chromatography

TLRs - Toll-like receptors

TGFs - transforming growth factors

Tm – annealing temperature

TNFSF - tumor necrosis factor superfamily

TSA - Tryptic soy agar

TSB - Tryptic soy broth

TYG - Tryptone yeast glucose

UBC – ubiquitin

UTR – untranslated region

WHO – World Health Organisation

YWHAZ - Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

Ethical statement on the use of animals in research

The trials reported in this thesis were carried out under ethical guidelines for the use of animals in research at Newcastle University. All birds were euthanized humanely using cervical dislocation by trained Aviagen Ltd. employees to allow for subsequent *post mortem* collection of tissue samples. Aviagen is a member of the European Forum of Farm Animal Breeders (EFFAB) and follows the Code of Good Practice Code-EFABAR and Principles of Sustainable Breeding. The trials were performed on an Aviagen commercial sib-testing farm in Scotland, UK that is routinely inspected by DEFRA and is part of Aviagen Ltd. compartment status indicating a good level of biosecurity and disease control. The farm trials and protocols were approved and managed through the Aviagen veterinary department.

1 Introduction

1.1 Overview of the Poultry Industry

The UK poultry meat industry has grown dramatically since the 1950s, from approximately 5 million birds produced per year to current figures of more than 900 million (DEFRA, 2014a). Poultry meat production continues to rise and figures released from the Department for Environment, Food and Rural Affairs (DEFRA) revealed that during 2013 broiler meat production in the UK rose by 5% to 1.4 million tonnes (DEFRA, 2014b). Increased production has been underpinned by decades of applied research, focussed on the genetic selection of individual birds that support a short but efficient production cycle. At present the typical time for a broiler chicken to go from hatchery to slaughter is 35- 40 days with the average weight at slaughter being over 2.25kg (DEFRA, 2014a). To remain competitive and profitable, the primary focus of the industry is to improve the conversion of feed to body mass, also known as the feed conversion ratio (FCR), without compromising bird welfare. In 2012 the Aviagen FCR for poultry was reported to be as low as 1.38 using Ross broilers (Aviagen, 2012) i.e. 1.38 kg of feed is required to increase bird mass by 1kg. Comparison of typical FCRs across the farming sector demonstrates that poultry is, agriculturally, an efficient source of meat protein production. For example, in 2011 the FCR for beef production was 8.8 (Wilkinson, 2011), 2.9 – 3.9 for lamb (Wilkinson, 2011) while 3 was the EU average for pigs (BPEX, 2012).

Although demand for free range chicken has increased over recent years (Martinez Michel et al., 2011), the vast majority of broiler chickens are reared intensively in purposely built barns, which house large numbers of birds, typically over 10,000. Although this type of intensive farming meets the high customer demand, the high stocking densities adopted can increase the susceptibility of birds to disease and facilitate the spread of infection, in addition to increasing welfare-related conditions such as footpad dermatitis and hock burn (Buijs et al., 2009; Estevez, 2007). However, despite the potential for disease, knowledge about the involvement & roles of the host immune defences in protecting birds in low hygiene situations, more reflective of conditions in commercial situations, is lacking. Hence for the UK to maintain a healthy & competitive poultry industry there is a requirement for the industry to start genetically selecting birds for disease resistance (Stear et al., 2001; Kaiser, 2010).

1.2 Genetic Selection in the Poultry Industry (Aviagen Ltd.)

According to their website, Aviagen Ltd. (www.aviagen.com) are the global leaders in poultry genetics and supply day-old chicks to customers in 130 countries worldwide under the brand names Ross, Arbor Acres and Indian River. Each brand has specific characteristics that are tailored to specific global markets. For example, Ross broilers are divided into Ross 308, said to be the world's most popular broiler with balanced and versatile characteristics, Ross 708 bred for high yields, and Ross PM3 suitable for farms in which feed and space are at a premium and uniform birds are required. More recently birds have been selected that are more suitable for free range and organic farming such as the Rowan Ranger®, a slower growing bird launched into the European market in 2013 (www.aviagen.com). The selective breeding programme adopted by Aviagen Ltd is structured as a hierarchal pyramid (Figure 1.1). Genetic improvement takes place in pure lines at the top of the pyramid (pedigree selection) where phenotypes are measured for a broad range of traits which can be summarised as broiler, breeder, health and welfare traits. For example, breast meat yield (bird size), FCR (feed efficiency) and leg strength (to support heavier birds) are three important broiler traits for intensive production. For these traits, breeding values are estimated using statistical tools such as Best Linear Unbiased Prediction (BLUP) which, in turn, informs multi-trait selection (personal correspondence, Dr Kellie Watson, Aviagen Ltd.).

As shown in Figure 1.1, it can take 4 – 5 years for the genetic improvements to be transmitted to the commercial broiler population. The pedigree birds are housed in tightly controlled high hygiene facilities to preserve the pathogen free status of the elite stock. However, it is vital that information is obtained on how they perform under conditions more reflective of commercial environments. It is likely that birds with different genotypes will respond to the environmental conditions in different ways and such genotype-by environment (G×E) interactions have been demonstrated in broilers. For example, G X E interactions have been demonstrated in low and high hygiene environments and linked to both growth and mortality (Ye et al., 2006; Long et al., 2008). Therefore, to investigate the robustness of birds with different genotypes under commercial farming conditions, the performance of the elite-stock siblings is assessed in lower hygiene environments (sib-testing).

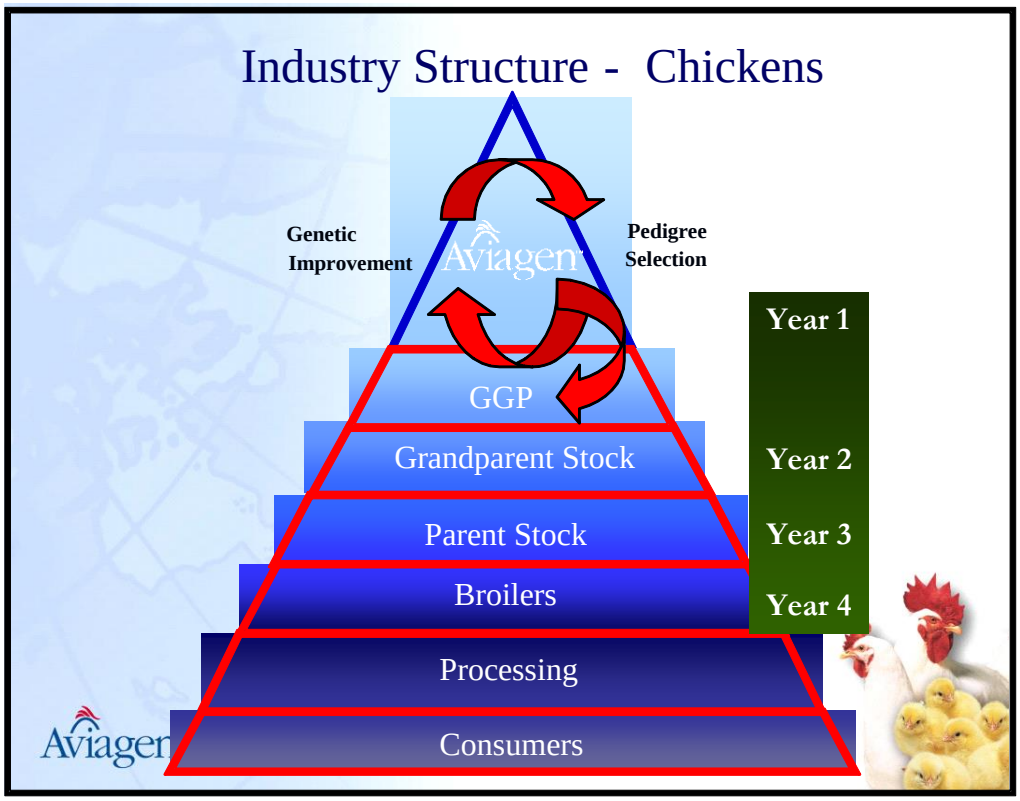


Figure 1.1: The selective breeding structure of the poultry industry

Figure provided courtesy of Aviagen Ltd (K. Laughlin) and adapted. GGP = great-grandparent stock.

1.3 Enteric Diseases in Poultry

Enteric diseases present serious health and welfare problems to the birds and are an economic challenge to the industry. Enteric problems in poultry can often be complex and are caused by pathogenic bacterial infections, parasites and viruses resulting in a physical, chemical or biological disturbance. Examples include necrotic enteritis (Timbermont et al., 2011), coccidiosis (Chapman, 2014) and more recently the syndrome dysbacteriosis (Teirlynck et al., 2011).

Necrotic enteritis (NE) was first described in 1961 (Parish, 1961) and has been reported in most poultry producing countries (McDevitt et al., 2006). The condition is characterised by severe intestinal lesions particularly located to the jejunum (Long et al., 1974). The disease is caused by *Clostridium perfringens* and the toxins produced cause the lesions typical with this disease. Infections by *Clostridium perfringens* may be acute (Shane et al., 1985), or subclinical resulting in decreased weight gain and reduced nutrient absorption leading to poor feed conversion ratios (Lovland et al., 2004). *Clostridium perfringens* is a normal part of the gut flora and, for the bacteria to cause pathogenesis, predisposing factors such as concurrent coccidiossis and diets high in Non Starch Polysaccharides (NSPs) and animal proteins such as fish meal are likely to be important (Kocher, 2003; Van Immerseel et al., 2004; McDevitt et al., 2006). NSPs increase the viscosity of the digesta leading to increased bacterial fermentation in the proximal gut (Choct et al., 1996), and diets that contain fish meal are high in amino acids glycine and methionine that stimulate *C. perfringens* proliferation (Kocher, 2003; Dahiya et al., 2007).

Coccidiossis is caused by *Eimeria*, a protozoan that infects birds via ingestion of oocysts found in the litter. In brief, the mechanism of action involves the attachment of sporocysts to the intestinal epithelium, which undergo asexual and then sexual reproduction to produce more oocysts that are released into the faeces. Due to the intensive nature of broiler production and the constant contact with litter the condition is spread rapidly. Such infections present as haemorrhagic diarrhoea, resulting in poor FCR and increased bird mortality. The first attempts at modelling the monetary costs to the broiler industry revealed losses due to the disease to be in excess of £38m per year in the UK (Williams, 1999) and $800m in the U.S (Williams, 1998). A recent review has estimated the global impact of coccidiosis to be in excess of $3 billion per year (Blake and Tomley, 2014).

The poultry industry controls coccidiosis using hygiene control and pen management, and broad-spectrum anticoccidial drugs such as monensin (Chapman et al., 2010).

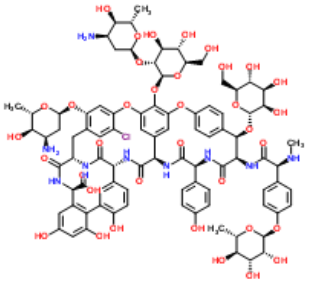
Dysbacteriosis is the term used to describe a digestive condition possibly caused by excess growth of intestinal microbiota resulting in non-specific enteritis (McMullin, 2004, Bailey, 2010). Interestingly, the incidence of dysbacteriosis has increased in recent years although the reasons are unclear. The result of this condition is a decrease in food absorption affecting the FCR and the production of wet litter, which is foamy and orange in appearance. Wet litter can lead to additional health problems including pododermatitis (Shepherd and Fairchild, 2010), and hock-burn (Hepworth et al., 2011). In turn these welfare problems impact upon the industry through increased production costs due to both the requirement for antibiotic treatment and decreased bird growth (Flemming, 2008). Although diagnosis is not straightforward, the assessment criteria includes a visual examination of the faeces, monitoring fluctuations in water content and checking for poor uniformity of growth across the hatch. An early detection method uses a scoring system based on water content in the faeces (Mortimer, 2002). Other monitoring techniques based on faecal water content include using a faecal fluid finder to obtain the ratio of solid matter to liquid (Bailey, 2010).

1.4 Antimicrobial Growth Promoters (AGPs)

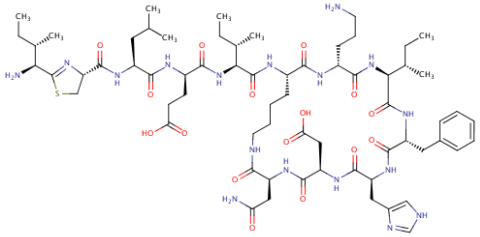
In the 1940's the by-product of tetracycline fermentation was fed to chicks as a potential source of vitamin B12 but produced an additional 'unintended' benefit of significantly improving bird growth (Moore et al., 1946; Stokstad et al., 1949). Although, the exact mechanism of action is controversial, one proposal is that these antimicrobial growth promoters (AGPs) suppress growth of the normal microflora leading to a reduction of microbial metabolites that would otherwise suppress growth (Visek, 1978; Knarreborg et al., 2004; Dibner and Richards, 2005). In the following 50 years AGPs were routinely added to livestock feed, a practice that helped drive and support the proliferation of intensive farming in the 1950s and 1960s. Indeed, although intensive farming increased the risk of disease and its spread, AGPs added to feed helped control outbreaks in gut health diseases. Examples of AGPs that were once commonly used in the E.U. are virginiamycin, avoparcin, tylosin, spiramycin, bacitracin, olaquindox, carbadox and tetracycline (Castanon, 2007) (Figure 1.2).

Despite the economic benefits in terms of disease control and improved FCR, opposition to the use of AGPs has arisen due to concerns over the proliferation of antibacterial resistant strains (Barton, 2000). For example, drug-resistant *Enterococcus faecium* strains have been isolated in chickens following administration of feed supplemented with sub-therapeutic levels of virginiamycin (Donabedian et al., 2003) and it has been shown that antibiotic resistant genes can be transmitted from animal to human microbiota (Greko, 2001). However, the relationship between AGP use in animal production and antibiotic-resistant infections in humans is still debated (McDermott et al., 2002; Phillips et al., 2004a; Phillips et al., 2004b; Walsh and Fanning, 2008). Nevertheless, following WHO recommendations the EU commission initiated a phased ban on many of these AGPs in 1999, which was fully operational by 2006 (EC Regulation No. 1831/2003). Denmark was the first country to voluntarily ban AGPs actually prior to the enforcement by the EU in 2006 and provided a good case study for the consequences of the ban. Interestingly, poultry productivity was not significantly affected by the ban, but from 1996 to 2002, it was linked to a doubling in the therapeutic use of the anticoccidial salinomycin, most likely reflecting an attempt to reduce the incidence of necrotic enteritis (WHO, 2003). Since the EU ban there has been, as seen in Denmark, an increase in poultry enteric problems, particularly necrotic enteritis and dysbacteriosis, associated with a corresponding increase in therapeutic antibiotic use throughout Europe to counteract these diseases (Hughes et al., 2008; Van Immerseel et al., 2009). Therefore, to protect against enteric disease, whilst circumventing the threat of antibiotic resistance, new strategies/agents are required.

Avoparcin (ChemSpider ID16736403)



Bacitracin (Drugbank DB00626)



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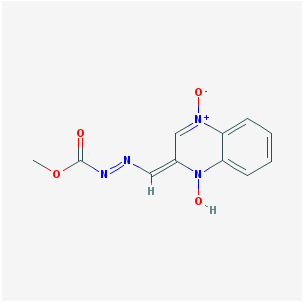
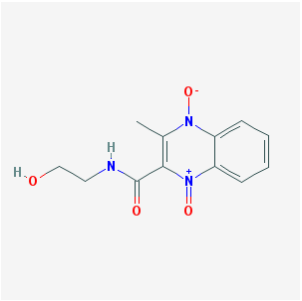
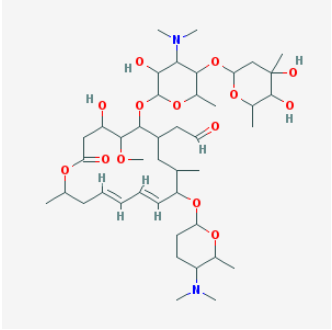


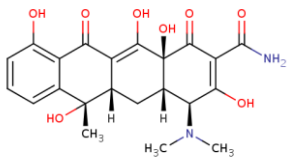
Olaquindox (PubChem CID71905)



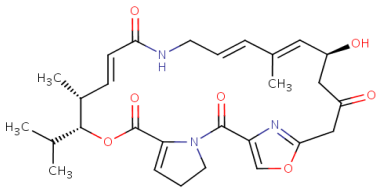
Spiramycin (PubChem CID 5356392)



Tetracycline (Drugbank DB00759)



Virginiamycin (Drugbank DB01669)



Vancomycin (Drugbank DB00512)

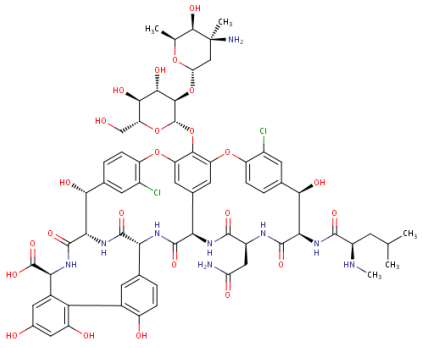


Figure 1.2: Chemical structures of a selection of antimicrobial growth promoters used in poultry feed prior to E.U. restrictions (EC Regulation No. 1831/2003).

Structural information taken from PubChem (https://pubchem.ncbi.nlm.nih.gov/), DrugBank (http://www.drugbank.ca/) and ChemSpider (http://www.chemspider.com/StructureSearch.aspx). Unique ID is shown for each compound.

1.5 Gut Microbiota

The formation of the gut microbiome, i.e. the establishment of a bacterial population within the host intestine, is an important step in protecting the host from gastrointestinal (GI) disease. Commensal bacteria can form protective biofilms, which prevent attachment of pathogenic bacteria to the underlying epithelia (Baranov and Hammarstrom, 2004; Granato et al., 2004) and facilitate mucosal immune development (Round and Mazmanian, 2009). Evidence from studies using germ-free (no gut flora) mice versus specific-pathogen free (SPF) or normal mice have illustrated the role of the normal gut flora in the development of a healthy and immunologically competent gut. Indeed, Germ-Free (GF) mice exhibited broad defects in the maturation of gut associated lymphoid tissue (GALT) such as isolated lymphoid follicles (Bouskra et al., 2008), poorly functioning Peyer's Patches, reduced numbers of IgA producing cells (Macpherson et al., 2001) and altered intestinal gene expression profiles (Hooper et al., 2001). Such defects were however, reversible by colonising GF mice with commensal bacteria from SPF mice (Macpherson and Harris, 2004). Although such studies were focussed in mammals it is possible that these data can be extrapolated to other vertebrates including birds.

Intensive poultry farming is unusual in relation to other livestock production in that chicks are hatched in the relatively sterile environment of a hatchery and have no direct parental contact. Therefore, the post-hatch development of the microbiota is likely to be facilitated by exposure to non-parental sources such as litter, transport conditions and human handling and furthermore, Stanley et al., (2013) have indicated these factors contribute to the high variability in microbiota composition between birds. Nevertheless, the broad pattern is for bacterial density to increase from the proximal (duodenum, jejunal) to the more distal regions of the gut reaching a maximum level in the caeca of 10^{12} per gram of digesta (Gong et al., 2002a). Furthermore, each section of the chicken gut has a distinctive population profile. In simple terms, the caecum hosts a wide variety of microflora (Mead, 1989) compared to the small intestine in which 98% of the bacteria is composed of *Lactobacilli* (Gong et al., 2007). In addition to the immediate post-hatch exposure, the composition of commensal bacteria is influenced by factors such as bird age, disease, antibiotic use and diet. The latter is thought to be a critical factor in determining the composition of the microbiota, with for example, diets high in barley shown to enhance the growth of *Lactobacillus*, while oat-based diets have been associated with an increase in *Escherichia* spp. (Apajalahti et al., 2004).

Gong et al., (2002b) compared the microbial populations of the bird ilea and caeca using molecular analysis of 16S rRNA genes and reported a more diverse bacterial population in the cecum. For both tissues, the major species identified were *Lactobacilli*, *Enterococcus cecorum* and various types of butyrate-producing bacteria, which the authors suggested may be a useful target for novel probiotics (Gong et al., 2002b). In a similar study, an examination of 16S rRNA genes through denaturing gradient gel electrophoresis (DGGE), revealed that as broilers age the number of bands on the DGGE gel increased indicating further complexity of the microbiota (van der Wielen et al., 2002).

Antibiotic treatments have been shown to alter the types of bacteria in the GI tract. An early study investigating the effect of AGPs on the broiler GI demonstrated that the AGP zinc bacitracin, in conjunction with salinomycin, decreased numbers of *C. perfringens* and inhibited the *Lactobacillus salivarius* throughout the length of the GI tract (Engberg et al., 2000). Crucially, a significant increase in bird growth was also observed with the combined treatment, which infers that high levels of *L. salivarius* may suppress broiler growth. Zhou and colleagues, using PCR-DGGE, also showed that the dietary antibiotics virginiamycin and bacitracin methylene disalicylate affected the chicken microbiota in a dose and age dependent manner, with three of the groups affected identified as *Klebsiella granulomatis*, *Enterococcus* sp. AK61 and *Lactobacillus salivarius* (Zhou et al., 2007). Quantitative real-time PCR revealed that *Lactobacillus salivarius* was particularly sensitive to dietary virginiamycin (22 ppm), being completely inhibited relative to the control group at 3, 7 and 14 days (Zhou et al., 2007).

Further advances in high-throughput sequencing, such as 454 pyro-sequencing, have now enabled the microbial populations to be phylogenetically profiled in diseased and challenged broilers (Stanley et al., 2012). A comparison of the microflora in birds challenged with *C. perfringens*, to induce necrotic enteritis, and non-treated control birds revealed distinct microbial communities populating the guts of the healthy and diseased birds (Stanley et al., 2012). In diseased birds, *C. perfringens* was increased, reducing the abundance of other Clostridia ($P < 0.05$). Significant changes were also observed within Lactobacillus spp., with challenged birds harbouring both higher (e.g. *L. crispatus, L. salivarius*), and lower (e.g. *L. johnsonii*) relative abundances compared to controls ($P < 0.05$). A key finding was that *Weissella confusa* was only present in healthy birds,

suggesting that this species disappears following *C. perfringens* infection (Stanley et al., 2012).

Although *C. perfringens* has been demonstrated to be the most important microorganism in the aetiology of necrotic enteritis, less is known about the microbial perturbations associated with other disease states. However, qualitative analyses using DGGE has revealed that birds with dysbacteriosis have an altered caecal and small intestinal microbiota characterised by the presence of various members of the Bacteroidetes (*Bacteroides dorei*, *Barnesiella viscericola*, *Bacteroides ovatus*, *Bacteroides vulgatus* and *Barnesiella viscericola*) in the caeca, and *Lactobacillus aviarius*, *Escherichia coli* and *Bacteroides vulgatus* in the small intestine (Bailey, 2010).

Such studies reveal that the composition of microbiota influences bird growth and thus, further exploration & knowledge of the microbiomes of different genetic lines raised in different environments may provide the poultry industry with new markers to select for robust birds with improved disease resistance.

1.6 Immunity in the Chicken GI tract

Due to the intensive rearing conditions increasing the risk of diseases, including those linked to the gastrointestinal tract, it is clear that a successful poultry industry relies upon an understanding of the chicken immune system. Of particular importance is the innate immune system as newly hatched chicks lack adaptive immunity (Korver, 2006). At this critical stage of development, chicks are also protected by maternally-derived antibodies which have been transferred via the egg yolk, until the endogenous immune system develops (Hasselquist and Nilsson, 2009). Although humoral or antibody-mediated immunity starts to develop at 5 days, birds utilise the maternal immunoglobulin Y up to day 13 (Rose and Orlans, 1981; Apanius, 1998). Maternal antibodies have been shown to protect against infectious bursal disease (Goddard et al., 1994), but their efficacy can be affected by the diet fed to young birds (Kidd, 2003; Leandro et al., 2011).

Protection against exogenous pathogens in the GI tract is conferred through the innate defences that are relatively non-specific and include the physical epithelial/mucosal barrier, mucin secretion, chemical factors such as pH and bile acids to lower pH, as well as innate effector molecules. As part of the innate defence system eukaryotic host cells express pattern-recognition receptors (PRRs) on their surface, which recognise pathogen-

associated molecular patterns (PAMPs) specific to microbes. Toll-like receptors (TLRs) are key sensory PRRs that are evolutionary conserved and appear to be widespread throughout the animal kingdom (Roach et al., 2005). These membrane proteins contain an extracellular ligand-binding domain that varies in its structure, and hence ligand specificity, between TLR forms (Kang and Lee, 2011). Bioinformatic comparisons of human and chicken TLRs (chTLRs) revealed a number of orthologs but also identified TLRs unique to chickens namely chTLR15 and chTLR21 (Temperley et al., 2008). No ortholog of the mammalian DNA binding TLR9 molecule is found in the chicken, but this function can be performed through chTLR21 (Brownlie et al., 2009; Keestra et al., 2010). PAMPS that have been demonstrated to activate TLR-signalling include lipopolysaccharide (LPS) (ligand for chTLR4), CpG-DNA (ligand for chTLR21), flagellin (ligand for chTLR5) and various forms of lipopeptide (ligands for chTLR2t1/chTLR16/ TLR2t1/TLR1LB) (Keestra et al., 2013). In mammalian cells PAMP ligands activate TLR signalling by inducing TLR dimerisation, which in turn activates a MyD88-dependent pathway leading to the up-regulation, often through NF-kappa B or activating protein 1 (AP-1) signalling, of pro- and anti-inflammatory molecules such as cytokines, chemokines and host defense peptides (HDPs) (Kawai and Akira, 2006; Kawai and Akira, 2007). It is probable although not proven that similar mechanisms exist in the chicken. A number of studies have utilised PAMP ligands to assess immune responses in chicken cells. Chicken heterophils have been shown to express many TLR sub-sets and the TLR 4 agonist lipopolysaccharide (LPS) was shown to induce the expression of pro-inflammatory cytokines such as the interleukins, IL-6, IL-8 and IL-1β (Kogut et al., 2005). This up-regulation of pro-inflammatory cytokine expression was also observed *in vivo* in the spleen (St Paul et al., 2011). These authors also revealed that the TLR21 agonist CpG-ODN can induce a Th1-like response evidenced by IL-13 suppression and interferon-gamma (IFN-ɣ) up-regulation (St Paul et al., 2011). In addition, TLR agonists have been investigated with the aim of priming the immune response against gut-related pathogens such as *Salmonella enteritidis* (Swaggerty et al., 2012) and *Eimeria acervulina* (Dalloul et al., 2004).

Analyses of the chicken genome has now identified the majority of immune signalling molecules including cytokines such as ILs, chemokines, IFNs, transforming growth factors (TGFs) and tumor necrosis factor superfamily (TNFSF) members (Kaiser et al., 2005). In broad terms, many of these molecules can be grouped into those that are pro-inflammatory including IL-1β, IL-6, IL-17, TNFs and IFN-ɣ and those that are anti-

inflammatory such as IL-10 and TGF-β. It is proposed that to maintain healthy gut host-commensal interactions a balancing act exists between pro- and anti-inflammatory signalling, (Brisbin et al., 2008), and yet is primed to respond effectively to exogenous pathogens (Lavric et al., 2008). For example, in response to Salmonella challenge the chemokines CXCLi1 and CXCLi2 were significantly up-regulated (Cheeseman et al., 2008) and furthermore, high constitutive expression of these genes was associated with Salmonella resistant lines (Swaggerty et al., 2014).

Many of the host-defense peptides synthesised by epithelia and antigen presenting cells were initially studied for their anti-microbial properties and designated as anti-microbial peptides (AMPs) (Boman, 2003). Following numerous studies which showed these peptides capable of stimulating other immune functions, the term host defense peptide (HDP) were adopted (Steinstraesser et al., 2011). A huge variety of HDPs have now been characterised across a variety of species and the majority exert a broad action against microbes through membrane disruption that is driven by their cationic (positively charged) and amphipathic nature i.e. spatially distinct regions of positively charged and hydrophobic residues (Ganz, 1999; Zasloff, 2002). Based on structural differences, three groups of AMPs or HDPs have been proposed: α-helical conformation e.g. cathelicidins; three disulphide bridges between cysteine residues e.g. defensins (Ganz, 2005); β-hairpin with one or two disulphide bonds e.g.hepcidin (Bulet et al., 2004).

1.7 Host defense peptides and microbiota

In mammalian cells it has been shown that human-defensin 5, secreted from Paneth cells in intestinal crypts, is able to influence the microbiota composition of the small intestine (Salzman et al., 2010). Using transgenic mice able to express human defensin-5 (HD-5) and MMP7 KO mice which lack matrix metalloproteinase 7 (MMP7) which is required to cleave the propeptide to produce active defensin, Salzman et al. (2010) were able to induce significant shifts in bacterial species. Analyses of the microbiota from mice which were able to express HD5 had lower percentage Firmicutes and higher percentage of Bacteroidetes. As part of the decrease in Firmicutes a complete loss of segmented filamentous bacteria (SFB) was found in the active HD5 expressing mice. SFB are unique in that they are in direct contact with epithelial cells (Snel et al., 1995) and have also been shown to stimulate Th17 cells (Ivanov et al., 2009), which have been implicated in tissue damage associated with autoimmune diseases (Steinman, 2007). To date this is the only

study on the relationship between microbiota composition and defensins and, in addition, this study was on human alpha-defensins which are not present in birds (van Dijk et al., 2008). Nevertheless, the work of Salzman indicated that host defence peptides exert influence on commensal bacteria and are not just important in protecting against exogenous pathogenic bacteria. Therefore, this suggests there may be a relationship between avian HDP expression and the GI microbiome but, to date, no studies have been performed to confirm this.

1.8 Avian Host defense peptides

Avian host defense peptides can be broadly grouped into two main families: cathelicidins (Xiao et al., 2006) and defensins (van Dijk et al., 2008), the latter of which are sub-divided into the beta-defensins (van Dijk et al., 2008) and the egg white expressed ovo-defensins (Gong et al., 2010). In addition, other individual peptides have also been identified and include the saposin-like NK-lysin (Hong et al., 2006), named due its presence in Natural Killer (NK) cells, and chicken liver-expressed antimicrobial peptide (cLEAP) (Townes et al., 2004).

1.9 Cathelicidins

Cathelicidins are alpha-helical cationic peptides that display both potent anti-microbial activity and a range of immunomodulatory properties (Zanetti, 2005). They exist as prepropeptides containing a signal peptide at the N-terminus which is cleaved prior to secretion, a cathelin-like domain and a C-terminal mature peptide (Figure 1.3). The mature peptide is activated once the pro-cathelin-like domain is cleaved. The initial discovery of Cathelicidin-1 in the chicken was through a bioinformatics approach that screened a library of chicken expressed sequence tags (ESTs) (Lynn et al., 2004). This was then followed by the identification of three other peptides; Cathelicidin-2 (van Dijk et al., 2005) Cathelicidin-3 (Xiao et al., 2006) and Cathelicidin-B1/Cathelicidin-4 (exclusive to the bursa of Fabricus) (Goitsuka et al., 2007). With the exception of CATH-B1/CATH-4, which is mainly expressed in the bursa of Fabricus, the cathelicidins are expressed across many tissues, including the GI tract (Achanta et al., 2012), although it was discovered that the CATH-2 peptide is located in heterophils that are recruited to the site of infection, rather than in intestinal epithelial cells (van Dijk et al., 2009b). CATH-1, 2 and 3 all display potent anti-bacterial activity against both gram-negative and positive strains (Xiao et al., 2006), and CATH-2 was shown to be active against gut isolates of *S.*

typhimurium, *S. enteritidis*, *E. coli* and *C. perfringens* (van Dijk et al., 2009b). In addition, CATH-1 and 2 exert immunomodulatory functions such as binding lippolysaccharide (LPS) preventing the LPS-mediated induction of pro-inflammatory cytokine expression (Xiao et al., 2006) and CATH-2 can also induce the expression of monocyte chemotactic protein 1 (MCP-1) (van Dijk et al., 2009a). The broad-spectrum anti-microbial activity coupled to their immune-boosting functions suggests that cathelicidin-based peptides are potential candidates as a long-term replacement for conventional antibiotics and AGPs (van Dijk et al., 2011).

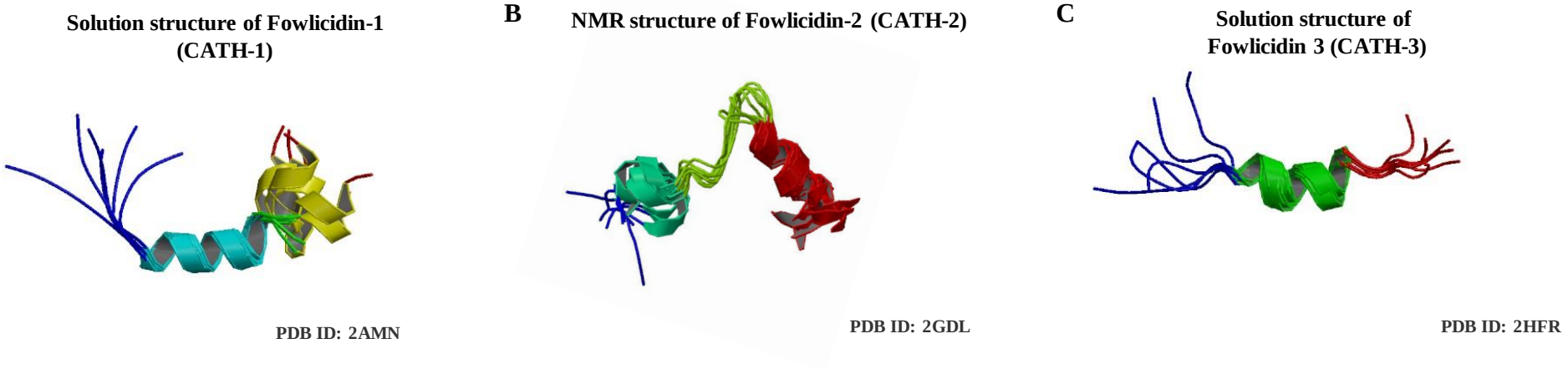


Figure 1.3: The 3D structures of the three chicken anti-cathelicidins

The three-dimensional structures of A) cathelicidin-1 (Xiao et al., 2006), B) cathelicidin-2 (Herrera et al., 2007) and C) cathelicidin-3 (Bommineni et al., 2007) as deposited in the RCSB protein data bank (http://www.rcsb.org)

1.10 Avian Defensins

Structurally, all defensins comprise of a β sheet-fold and six conserved cysteine residues that form three disulphide bridges (Ganz and Lehrer, 1994) (Figures 1.4 and 1.5). According to their cysteine to cysteine bonding patterns, defensins are grouped as alpha (α) (1–6, 2–4, 3–5), beta β (1–5, 2–4, 3–6), and theta (θ) (1-6, 2-5, 3-4) (Lehrer and Ganz, 2002). Both α and β defensins are found in mammals, θ-defensins are only active in primates (Tang et al., 1999) and interestingly avian species exclusively express β-defensins. Avian beta-defensin (AvBDs) gene sequences have been identified not only in the chicken (Lynn et al., 2004; Xiao et al., 2004), but also in the turkey (Zhao et al., 2001), mallard duck (Ma et al., 2012a), quail (Wang et al., 2010), king pigeon (GenBank: ABI20694.1), ostrich (Sugiarto and Yu, 2006) and king penguin (Thouzeau et al., 2003).

1.10.1 Chicken avian beta-defensins (AvBDs)

The first AvBDs were isolated from leukocytes and heterophils of the chicken, and were initially named chicken heterophil peptides CHP1 and CHP2 (Evans et al., 1994). In that same year, another group reported the isolation from chicken leukocytes of AMPs that had homology to the bovine β-defensins, and designated the peptides as 'gallinacins' (Harwig et al., 1994). The remaining AvBD genes were later identified using an *in silico* approach that first identified AvBD3 in 2001 (Zhao et al., 2001). Later, sequences for AvBDs 4 – 13, were reported in two key 2004 studies (Lynn et al., 2004; Xiao et al., 2004), closely followed by the discovery of the AvBD14 sequence (GenBank ref: AM402954.1)(Soulier, 2006). In 2007, a standard nomenclature was proposed based on the numbering system of Xiao et al. (2004), replacing the term 'gallinacin' with 'avian beta-defensin' (Lynn et al., 2007).

1.10.2 Ovo-defensins

Three forms of an *in ovo* defensin, Gallin, were identified that exhibited homology to egg white proteins from other species including the turkey (meleagrin) and swan (cygnin) and closely resembled the AvBD family (Gong et al., 2010). Indeed, Gallin consists of a conserved six-cysteine motif although the number of amino acids between cysteines differs from the classical AvBDs. Structural studies utilising Nuclear Magnetic Resonance (NMR) spectroscopy confirmed that Gallin shares all the structural features of the AvBDs, but contains an additional two stranded beta-sheet (Herve et al., 2014).

Moreover, the anti-microbial activity of Gallin was confirmed against *E. coli* although not against any of the other bacterial strains tested namely *S. enteritidis*, *S. typhimurium*, *S. aureus* and *L. monocytogenes* (Herve et al., 2014).

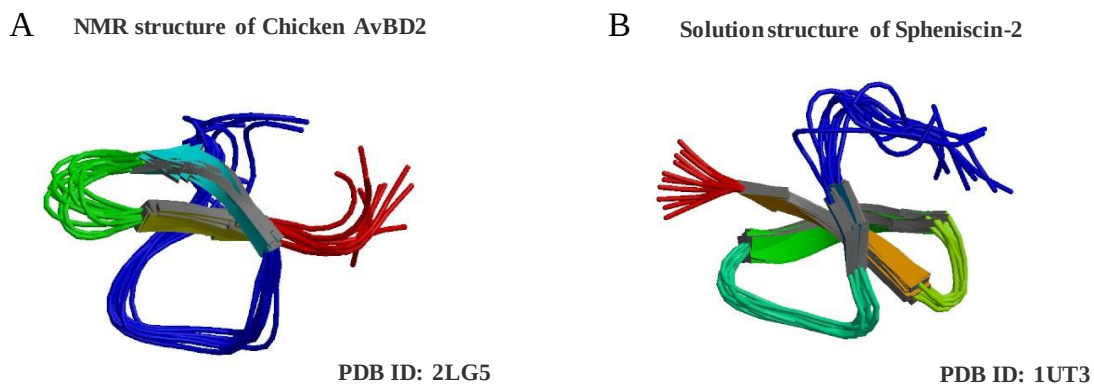


Figure 1.4: The 3D structures of two avian beta-defensins

The three-dimensional structures of A) chicken AvBD2 (Derache et al., 2012) and B) king penguin beta-defensin (spheniscin-2) (Landon et al., 2004) as deposited in the RCSB protein data bank (http://www.rcsb.org).

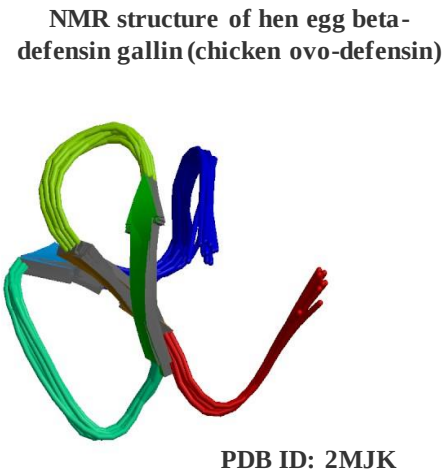


Figure 1.5: The three- dimensional structure of ovo-defensin

Three-dimensional structure was determined by NMR (Herve et al., 2014) and deposited in the RCSB protein data bank (http://www.rcsb.org)

1.10.3 Genomics and SNPs in the chicken AvBDs

All the AvBDs identified to date have been located to a single 86kb cluster on chromosome 3 (3q3.5–q3.7), and with the exception of AvBD12, contain four exons that are translated to produce AvBDs as prepropeptides containing a signal peptide, a propiece and a mature peptide. The signal peptide sequence is encoded by exon 2, the peptide propiece is encoded for by exons 2 and 3, and the mature peptide is encoded by exons 3 and 4 respectively (Figure 1.6).

Despite being evolutionary conserved, natural allelic variation has been identified within human defensins (Jurevic et al., 2003; Prado-Montes de Oca et al., 2006), and aided by the sequencing of the chicken genome (Wallis et al., 2004) single nucleotide polymorphisms (SNPs), have also been reported in the AvBDs. An analysis of five candidate defensins (AvBD2, 3, 4, 5 and 7) revealed the presence of SNPs at a mean rate of 13.2 per kb, and moreover, SNPs in AvBD3 and 7 were associated with antibody titres following *S. Enteritidis* vaccination suggesting that SNPs in the AvBDs may be markers of disease susceptibility (Hasenstein et al., 2006). In addition, a genome-wide study of three commercial broiler lines (X, Y and Z) revealed the presence of 15 SNPs within the AvBD genome including three SNPs in the mature peptide of AvBD1. To date, these three non-synonymous SNPs have yet to be fully investigated for their effects on anti-microbial activity, but notably were found at variable frequencies in birds that differ in susceptibility to enteric problems (Butler, 2010).

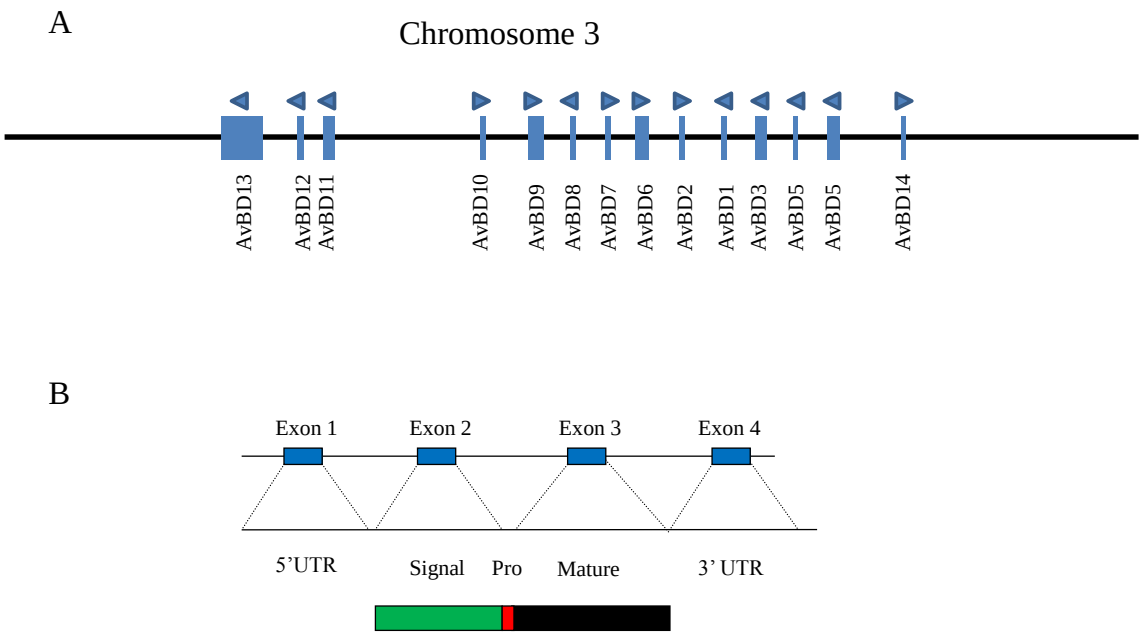


Figure 1.6: Genomic organisation of the chicken AvBDs

A) The relative positions of each AvBD (1 – 14) on chromosome 3 (3q3.5–q3.7) are shown. The width of each vertical block represents the size of each gene and the direction of transcription is arrowed.

B) AvBDs contain four exons that encode a signal peptide (green), pro-peptide (red) and mature peptide (black).

Figure adapted from Xiao et al. (2004) and Cuperus et al. (2013).

1.10.4 Structure of chicken AvBDs

A comparison of the primary structures of the 14 AvBD mature peptides reveals that they are 36 – 82 amino acids in length and all contain a six cysteine sequence motif: C-x_{2-4}-G-x_{1-2}-C-x_{3-5}-C-x_{9-10}-C-x_{5-6}-CC-x_n. Typically, these peptides are enriched for positively charged amino acids such as arginine and lysine resulting in cationic properties i.e. a positive net charge at physiological pH. The primary sequences and charge at pH7 for all the chicken AvBDs are shown in Table 1.1. AvBD11 is unusual in that it contains a large post-piece comprising a six cysteine defensin-like motif, postulated to have arisen from gene duplication (Herve-Grepinet et al., 2010). In addition, AvBD3 and 13 also contain large post-pieces relative to the other AvBDs, although their functions, if any, are unknown. Other functional differences between the AvBDs are likely to exist due to post-translational modifications, for example AvBD1 and 7, but not AvBD2 exhibit C-terminal amidation (Derache et al., 2009b). Amidation has been shown to stabilise structural features and enhance activity (Shalev et al., 2002) and provide resistance against proteolytic degradation (Stromstedt et al., 2009). The ability of defensins to form dimers has been revealed for hBD3 and is thought to be the reason for its increased anti-microbial potency against *S. aureus*, relative to hBD1 and 2 which are more likely to be monomeric (Schibli et al., 2002), although homology modelling has suggested hBD2 may also form dimers (Suresh and Verma, 2006). Therefore, it is likely that AvBDs, *in vivo*, may also form dimers, as shown for duck AvBD2 (Soman et al., 2009b) although, to date, dimerisation has not been reported for the chicken AvBDs. No X-ray crystallography structures have been reported for the AvBDs but Nuclear Magnetic Resonance (NMR) spectroscopy has allowed the tertiary structures, of AvBD103b (Landon et al., 2004), a king penguin defensin, and chicken AvBD2 (Derache et al., 2012) to be modelled. Both these peptides were revealed to form the characteristic three-stranded beta-sheet, and contained both hydrophobic and positive residues on the outer surface (Derache et al., 2012).

Table 1.1: Amino acid sequences for the mature peptide regions of AvBD1 - 14

AvBD	GenBank Protein ID	Amino acid sequence of the mature peptide	Net charge at pH 7
1	AAB30584	GRKSDCFRKSGFCAFLKCPSLTLISGKCSRFYLCCKRIWG	8
2	AAB30585	LFCKGGSCHFGGCPSHLIKVGSCFGFRSCCKWPWNA	4
3	Q9DG58	TQCRIRGGFCRVGSCRFPHIAIGKCATFISCCGRAYEVDALNSVRTSPWLLAPGNNPH	5
4	AAS99318	RYHMQCGYRGTFCTPGKCPYGNAYLGLCRPKYSCCRWL	6
5	AAS99320	GLPQDCERRGGFCSHKSCPPGIGRIGLCSKEDFCCRSRWYS	3
6	AAS99315	SPIHACRYQRGVCIPGPCRWPYYRVGSCGSGLKSCCVRNRWA	7
7	AAS99316	RPIDTCRLRNGICFPGICRRPYYWIGTCNNGIGSCCARGWRS	6
8	AAU07922	NNEAQCEQAGGICSKDHCFHLHTRAFGHCQRGVPCCRTVYD	0
9	AAS99317	ADTLACRQSHGSCSFVACRAPSVDIGTCRGGKLKCCKWAPSS	4
10	AAS99319	DPLFPDTVACRTQGNFCRAGACPPTFTISGQCHGGLLNCCAKIPAQ	1
11	AAT45551	LPRDTSRCVGYHGYCIRSKVCPKPFAAFGTCSWRQKTCCVDTTSDFHTCQDKGGHCVSPKIRCLEEQLGLCPLKRWTCCKEI	6
12	AAS99321	MRNLCFVFIFISLLAHGSTHGPDSCNHDRGLSRVGNCNPGEYLAKYCFEPVILCCKPLSPTPTKT	2
13	AAT48937	FSDSQLCRNNHGHCRRLCFHMESWAGSCMNGRLRCCRFSTKQPFSNPKHSVLHTAEQDPSPSLGGT	4
14	AM402954	MGIFLLFLVLLAVPQAAPESDTVTCRKMKGKCSFLLCPFFKRSSGTCYNGLAKCCRPFW	6

The conserved cysteine – cysteine disulfide bonding motif is highlighted for C1 – C5 (yellow), C2 – C4 (green) and C3 – C6 (blue). The GenBank protein I.D. is shown for each defensin. The net charge at pH7 was calculated using Innovagen Peptide Calculator (http://www.innovagen.com/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp).

1.10.5 Anti-microbial activities of chicken AvBDs

AvBDs have been shown to exhibit broad spectrum activity against numerous microbes as measured using a variety of techniques including radial diffusion assays based on zones of inhibition (Lehrer et al., 1991), colorimetric assays (Peck, 1985), microbroth dilution assays (van Dijk et al., 2007; Ma et al., 2008) and a time-kill assay based on colony counts at two different time-points (Townes et al., 2004). Minimum inhibitory concentrations (MICs) have been reported for AvBDs against various microbial strains, but generally, it is difficult to compare data between studies, as variations in assay methodologies exist; for example, information on the ratio of bacteria to peptide is often lacking and different AvBD preparations are often utilised including chemically synthesised, naturally extracted, recombinant, folded and unfolded forms. A summary of the *in vitro* investigations of chicken AvBDs against gram-positive and negative bacteria are summarised in Table 1.2 and these data, although sparse, show that the peptides are active against a wide range of organisms.

1.10.6 Mechanism of action

Due to their cationic nature (Table 1.1) it is thought that defensins, including AvBDs, exert their anti-microbial function by binding to negatively charged components found in microbial membranes, such as lipopolysaccharides (LPS) and lipoteichoic acid (LTA) (Ganz, 2003). It is thought that defensins are far less active against host eukaryotic membranes due to the presence of zwitterionic phospholipids and sterols and this has been modelled *in vitro* using liposomes to mimic peptide-membrane interactions (Mason et al., 2007; Dong et al., 2012). In addition, the hydrophobic residues, as part of the amphipathic surface of many AvBDs, are also thought to contribute to anti-microbial activity by facilitating interactions between the peptide and the microbial cell wall (Powers and Hancock, 2003). Following the initial electrostatic interactions, the membrane is disrupted via one of three potential models: the "carpet/wormhole" model (Figure 1.7) (Shai, 1995), the "barrel-stave" model (Figure 1.8) (Oren and Shai, 1998) or the "Toroidal pore" model (Figure 1.9) (Brogden, 2005). In all cases, a key target of the peptides is the bacterial cell membrane, although once inside the cell AMPs have been shown to bind to and interfere with DNA/RNA and protein function (Brogden, 2005; Nicolas, 2009).

Although the structural characteristics that govern killing capacity have not been fully determined, a number of structure-function studies have provided evidence for the importance of critical residues such as the relatively conserved C-terminal lysine (Derache et al., 2012) and positively selected sites (PSS), amino acid sites selected for by evolution (Higgs et al., 2007). A role for increased cationicity in exerting higher AMA has also been established using modified versions of AvBD8 that differ by their charge (Higgs et al., 2007), and through comparisons of the activities of AvBD1 (+8), AvBD7 (+6) and AvBD2 (+2) (Derache et al., 2009b). Although studies on human defensins have indicated that the three dimensional structure is more important for immunomodulatory functions rather than AMA (Wu et al., 2003), it is interesting that a recent study has reported that the folded variant of AvBD2 possesses higher AMA than the linearised form (Derache et al., 2012).

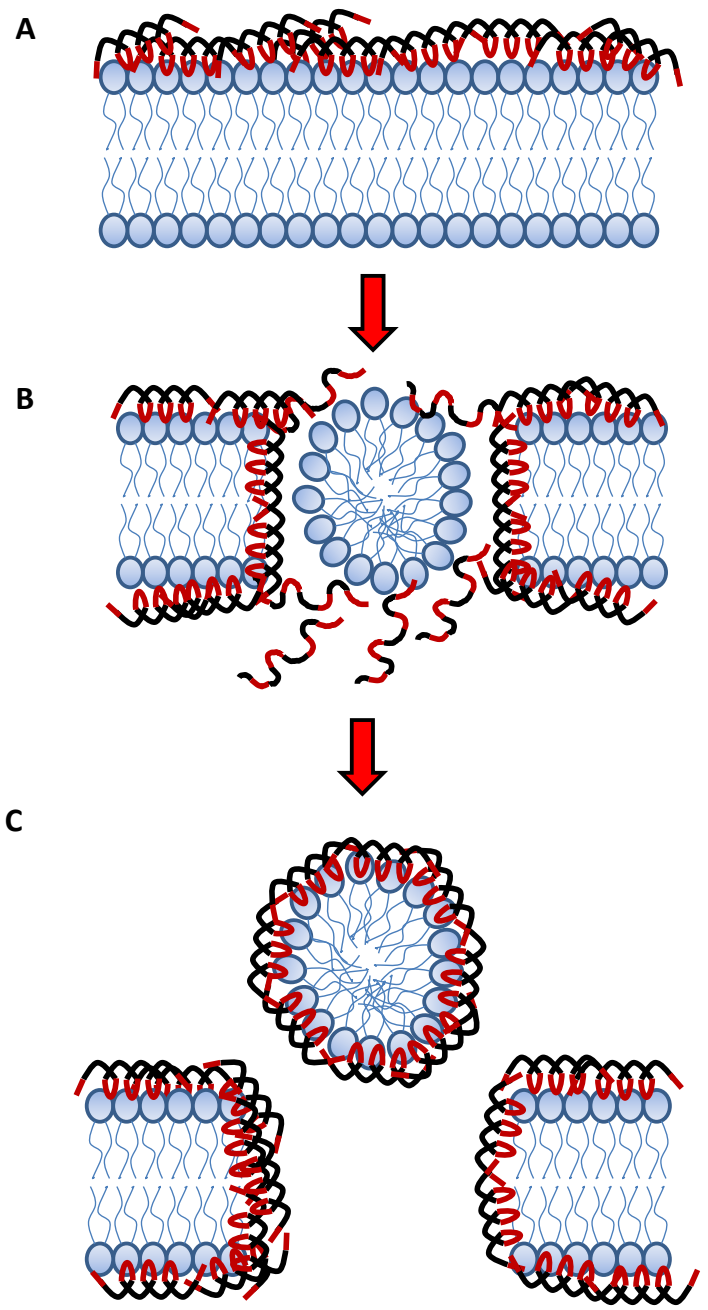


Figure 1.7: The carpet model of the action of AMPs against bacterial membranes.

A) The positively charged regions of the anti-microbial peptides form electrostatic interactions with negatively charged phospholipid head groups on the bacterial cell membrane leading to the formation of an extensive carpet-like layer.

B) At high concentrations the membrane is disrupted via a detergent-like mechanism.

C) The membrane is completely disrupted as micelles are formed.

The hydrophilic regions of the defensin peptide are shown in black and the hydrophobic regions are shown in red. Model described by Brogden (2005) and Oren and Shai (1998).

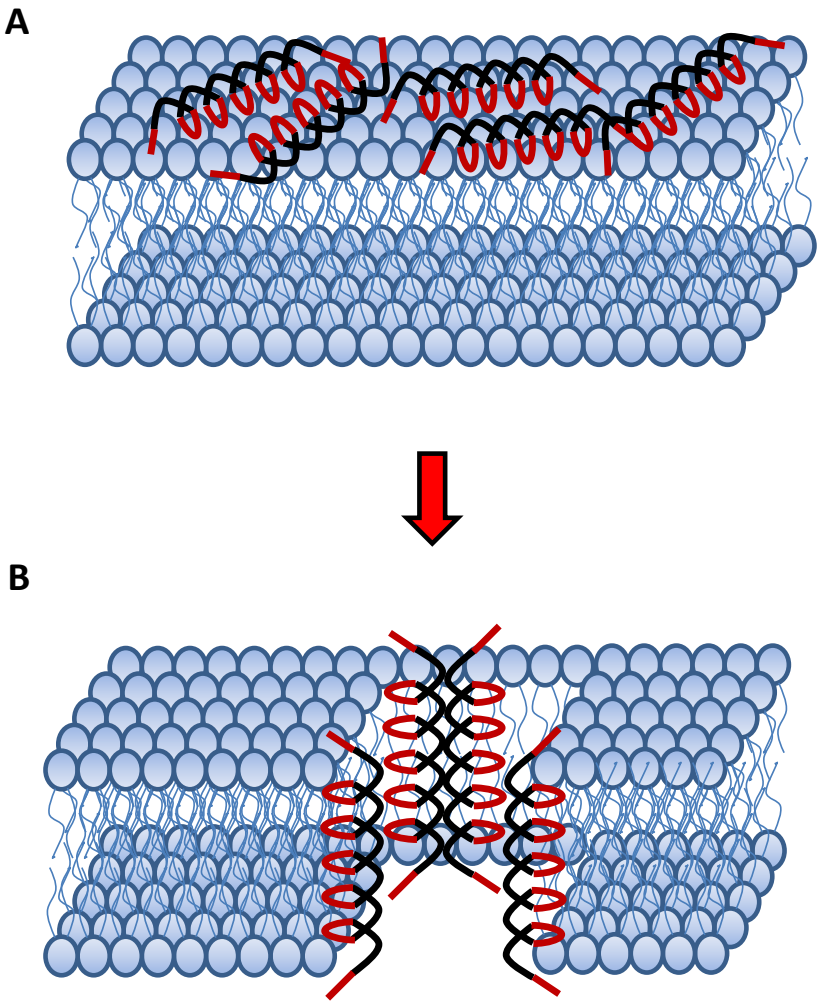


Figure 1.8: The barrel-stave model of the action of AMPs against bacterial membranes.

A) The positively charged regions of the anti-microbial peptides form electrostatic interactions with negatively charged phospholipid head groups on the bacterial cell membrane leading to peptide aggregation.

B) Peptides insert into the membrane forming a pore. The hydrophilic regions of the peptide form a hydrophilic interior of the pore whilst the exterior is formed by the interaction of the hydrophobic peptide regions with the phospholipids.

The hydrophilic regions of the defensin peptide are shown in black and the hydrophobic regions are shown in red. Model described by Brogden (2005) and Oren and Shai (1998).

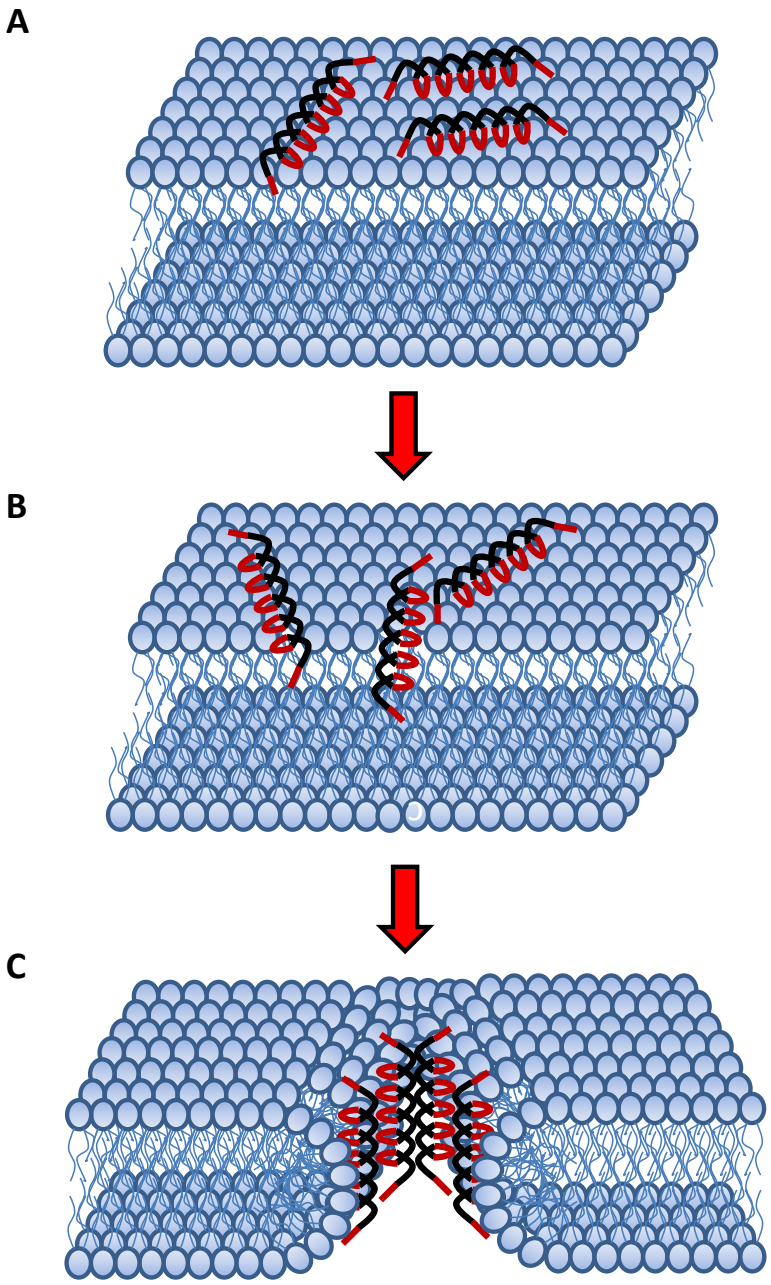


Figure 1.9: The toroidal pore model of the action of AMPs against bacterial membranes.

A) The positively charged regions of the anti-microbial peptides form electrostatic interactions with negatively charged phospholipid head groups on the bacterial cell membrane.

B) AMPs begin to insert into the phospholipid bilayer causing the top lipid monolayer to bend as the pore is formed.

C) A toroidal pore is formed as the two lipid monolayers connect resulting in a water core lined by phospholipid head groups in contact with the inserted AMPs.

The hydrophilic regions of the defensin peptide are shown in black and the hydrophobic regions are shown in red. Model described by Brogden (2005) and Huang et al. (2004).

Table 1.2: Summary of the anti-microbial activities of the chicken AvBDs

Bacteria		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Gram negative	*E. coli*	✔ ✔ ✔ ✔	✔ ✔ ✔ ✔	-	-	✔	-	✔	✔	✔	-	✔	-	X	-
	S. enteritidis	✔ ✔	✔ ✔ ✔	-	✔	✔	-	✔ ✔	-	-	-	✔	-	-	-
	S. typhimurium	✔ ✔	✔ ✔ ✔	-	✔	✔	-	✔ ✔	X	X	-	✔	-	✔	-
	S. pullorum	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C. jejuni	✔	✔	-	-	-	-	-	-	✔	-	-	-	-	-
	P. multocida	X	-	-	-	-	-	-	-	-	-	-	-	-	-
	P. aeroginosa	✔ ✔	X ✔	-	-	-	-	✔	-	X	-	-	-	-	-
	E. cloaca	✔ ✔	XX	-	-	-	-	✔	-	-	-	-	-	-	-
	K. pneumoniae	✔ ✔	✔ ✔	-	-	-	-	✔	-	-	-	-	-	-	-
	B. avium	✔	✔	-	-	-	-	-	-	-	-	-	-	-	-
Gram postive	*B. subtilis*	✔	✔	-	-	-	-	✔	-	-	-	-	-	-	-
	Lactobacillus	✔	-	-	-	-	-	-	-	-	-	-	-	-	-
	C. perfringens	✔	-	-	-	-	-	-	-	✔	-	-	-	-	-
	S. aureus	✔ ✔ ✔	✔ ✔	-	-	-	-	✔	X	X	-	✔	-	X	-
	B. cereus	✔ ✔	✔ ✔	-	-	-	-	✔	-	X	-	-	-	-	-
	L. monocytogenes	✔ ✔ ✔	✔ ✔ ✔	-	-	-	-	✔	✔	-	-	✔	-	✔	-
	S. haemolyticus	✔ ✔	✔	-	-	-	-	✔	-	-	-	-	-	-	-
	S. saprophytus	✔ ✔	✔	-	-	-	-	✔	-	-	-	-	-	-	-
	S. suis	-	-	-	-	✔	-	-	-	-	-	-	-	-	-
	S. pyogenes	-	-	-	-	-	-	-	✔	✔	-	-	-	X	-

✔ anti-bacterial, X – not anti-bacterial, - not tested.

References: **AvBD1** (Evans et al., 1994; Evans et al., 1995; Harwig et al., 1994; Derache et al., 2009b); **AvBD2** (Harwig et al., 1994; Evans et al., 1995; Derache et al., 2009b; Derache et al., 2012); **AvBD4** (*Milona et al., 2007*); **AvBD5** (*Milona et al., 2007*; *Ma et al., 2008)*; **AvBD7** (*Milona et al., 2007*; Derache et al., 2009b); **AvBD8** (Higgs et al., 2007); **AvBD9** (*van Dijk et al., 2007*); **AvBD10** ; AvBD11 (Herve-Grepinet et al., 2010); **AvBD13** (Higgs et al., 2005).

Normal font – AvBD peptide extracted and purified from bird tissues/cells; **underlined** – AvBD synthetic peptide; **italic** – AvBD recombinant peptide.

1.10.7 Other immune functions

A number of studies have shown that human defensins possess many immune-modulatory activities including functioning as chemoattractants (Yang et al., 1999), anti-toxins (Wang et al., 2006), facilitating wound repair (Yang et al., 2004), and suppressing the production of pro-inflammatory cytokines (Semple et al., 2011). Despite the likelihood of a role for immune regulation, few studies have investigated novel non-killing properties in the AvBDs. Duck T and B cell lymphocytes have been shown to exhibit chemotaxis towards AvBD2-containing media (Soman et al., 2009b) but no comparable experiments have been reported using chicken AvBDs. There are strong suggestions, however, from *in vivo* work, of immunomodulatory roles. A large-scale *in vivo* trial revealed that birds fed chicken AvBD13 immediately post-hatch produced significantly higher IgG, IgM and infectious bursal disease virus (IBDV) antibody titres, relative to control birds, following administration of the infectious bursal disease vaccine (Yang et al., 2007). In addition, AvBD1 has also been shown to be able to boost antibody titres when used as a vaccine adjuvant for IBDV (Zhang et al., 2010). The potential of AvBDs to link the innate and adaptive immune responses was shown in another study by Yang et al., who showed that the addition of AvBD13 to murine peripheral blood mononuclear cells activated NF-κB pathways and up-regulated IL-12 and IFNα through TLR-4 signalling (Yang et al., 2010).

1.10.8 Chicken AvBD gene expression

Discovery of the AvBD genes using a bioinformatics approach was supported by tissue expression studies that employed small numbers of birds and endpoint RT-PCR techniques that are, at best, semi-quantitative. Therefore, although the relative expression of each AvBD gene in chicken tissues has been reported, the data has to be viewed cautiously until large sample studies utilising quantitative real-time PCR have been performed. Nonetheless, the data so far does imply that the AvBD expression pattern in each tissue is unique and these data are shown in Table 1.3.

In relation to the GI tract expression has been revealed for all 14 AvBDs in the small intestine and also many AvBDs have been found expressed in the large intestine/caeca indicating that these peptides are important innate immune effectors which protect the gut from both exogenous pathogens and may also may have roles in modifying the host microbiota (Salzman, 2010). In the oesophagus, proventriculus and gizzard, fewer defensins appear to be expressed than in other regions although only a small number of studies have been performed (Lynn et al., 2004; Xiao et al., 2006). The crop has been shown to express AvBD1 – 7, 9 and 11 with increased AvBD9 expression reported in the crop in comparison with the small and large intestine (van Dijk et al., 2007).

1.10.9 Developmental chicken AvBD expression

Temporal and tissue specific differences in AvBD have been reported to occur prior to hatch during chicken embryogenesis (Meade et al., 2009a). Similarly, evidence exists for high levels of AvBD expression in the first few days post-hatch, which then decrease with increasing bird age (Bar-Shira and Friedman, 2006; Milona et al., 2007; Butler, 2010). This supports the hypothesis that AvBDs are of importance in the early innate immune response of young birds that are, as yet, unable to mount an effective antibody response (Bar-Shira et al., 2003).

Table 1.3: Chicken AvBD tissue expression

Tissues	Defensin													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Skin	X	X	✓	X	X	X	X	X	✓	✓	✓	X	X	-
Reproductive	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-
Spleen	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Kidney	X	X	✓	✓	X	✓	X	X	✓	✓	✓	✓	✓	-
Liver	X	✓	X	✓	X	✓	X	✓	✓	✓	X	X	✓	-
Lung	✓	✓	X	✓	✓	✓	✓	X	✓	✓	X	X	✓	-
Tongue	X	X	✓	✓	✓	X	X	X	✓	✓	X	X	✓	-
Esophagus	X	X	✓	✓	X	✓	X	X	✓	X	X	X	X	-
Crop	✓	✓	✓	✓	✓	✓	✓	X	✓	X	✓	X	X	X
Proventriculus	X	X	✓	X	✓	X	X	X	✓	X	X	X	✓	-
Gizzard	X	X	X	✓	X	X	X	X	✓	X	X	X	X	-
S. Intestine	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
L. Intestine	✓	✓	✓	✓	X	✓	✓	X	✓	X	X	✓	X	-
Caeca	✓	✓	✓	✓	X	✓	X	X	✓	X	X	X	X	-
Colon	-	-	-	X	X	X	X	X	✓	X	X	X	✓	-
Caecal tonsil	✓	✓	-	✓	-	✓	-	-	✓	-	-	-	-	-

✓ Present, X Not detected, - not tested.

References: AvBD1 (Lynn et al., 2004; Sadeyen et al., 2004; Sadeyen et al., 2006; Akbari et al., 2008; Mageed et al., 2008; Crhanova et al., 2011; Hong et al., 2012); **AvBD2** (Lynn et al., 2004; Sadeyen et al., 2004; Sadeyen et al., 2006; Akbari et al., 2008; Mageed et al., 2008; Crhanova et al., 2011; Hong et al., 2012) **AvBD3** (Zhao et al., 2001; Lynn et al., 2004; Mageed et al., 2008; Hong et al., 2012; Rodriguez-Lecompte et al., 2012) **AvBD4** (Lynn et al., 2004; Xiao et al., 2004; Milona et al., 2007; Akbari et al., 2008; Mageed et al., 2008; Crhanova et al., 2011; Hong et al., 2012); **AvBD5** (Lynn et al., 2004; Xiao et al., 2004; Milona et al., 2007; Ma et al., 2008; Mageed et al., 2008; Hong et al., 2012); **AvBD6** (Lynn et al., 2004; Xiao et al., 2004; Milona et al., 2007; Mageed et al., 2008; Crhanova et al., 2011; Hong et al., 2012; Rodriguez-Lecompte et al., 2012); **AvBD7** (Lynn et al., 2004; Xiao et al., 2004; Mageed et al., 2008; Hong et al., 2012); **AvBD8** (Xiao et al., 2004; Mageed et al., 2008; Hong et al., 2012) **AvBD9** (Lynn et al., 2004; Xiao et al., 2004; van Dijk et al., 2007; Mageed et al., 2008; Hong et al., 2012); **AvBD10** (Lynn et al., 2004; Xiao et al., 2004; Ma et al., 2008; Mageed et al., 2008; Hong et al., 2012); **AvBD11** (Xiao et al., 2004; Mageed et al., 2008; Hong et al., 2012) **AvBD12** (Xiao et al., 2004; Mageed et al., 2008; Hong et al., 2012); **AvBD13** (Xiao et al., 2004; Mageed et al., 2008; Hong et al., 2012); **AvBD14** (Hong et al., 2012).

1.10.10 AvBD regulation following bacterial challenge

A number of *in vivo* and *in vitro* studies have investigated the expression of AvBDs generally in GI and reproductive tract tissues following bacterial challenges, most commonly *Salmonella* spp, or bacterial PAMPs such as LPS. Table 1.4 summarises the findings from the *in vivo* challenges reported in the literature. The observed responses are variable with both up and down-regulation of AvBD expression reported, and generally related to the age of the bird, tissues analysed and microbial agents employed.

For example two day old birds challenged with *Salmonella enterica* serovar Typhimurium expressed AvBD1, 2, 4 and 6 at higher levels in the caecal tonsils (major lymphoid tissues in the avian caecum) relative to unchallenged birds and, interestingly, this up-regulation was suppressed via the delivery of a probiotic one day prior to infection (Akbari et al., 2008). In the small intestine, however, AvBD4 was not inducible by oral challenge with either *S. typhimurium* or *S. enteritidis* (Milona et al., 2007), and AvBD2 expression, similarly, was not affected in the caeca following *S. enteritidis* challenge (Cheeseman et al., 2008). These data suggest that defensin expression in the caecal tonsils is more sensitive to regulation. Comparison of the expression of all 14 AvBDs in the GI tract (duodenum, jejunum, ileum and caecum) of 3 day-old broilers following *S. pullorum* challenge revealed that groups of defensins were either up-regulated (AvBD3, 4, 5, 6 and 12), down-regulated (AvBD10, 11, 13 and 14) or unchanged (AvBD1, 2, 7, 8 and 9) (Ramasamy et al., 2012). Differential patterns of AvBD regulation have also been revealed between two commercial breeds (Cobb and Ross) in a necrotic enteritis model induced by co-infection with *C. perfringens* and *Eimeria maxima* (Hong et al., 2012).

The lack of a commercially available epithelial cell line to model the chicken gut has meant that most of the bacterial challenge studies in broilers have been performed using live birds. While more limited in number, *in vitro* experiments have also been performed on isolated primary cells and the data have generally supported the *in vivo* studies. Researchers using primary intestinal epithelial cells showed that AvBD1 is unchanged following *S. enteritidis* challenge while AvBD2 is up-regulated, although only in cells expanded from a *Salmonella* susceptible breed (Derache et al., 2009a). Using a chicken microarray, Chiang and colleagues discovered that following *Salmonella enteritidis* infection more immune genes were down-regulated in chicken heterophils expanded from a Salmonella susceptible line, including AvBD5, compared to birds from a resistant line (Chiang et al., 2008). Both the studies of Chiang et al. (2008) and Derache et al. (2009a)

indicate that regulation differs between the AvBD genes and is influenced, strongly, by host genetics.

1.10.11 Dietary supplementation to enhance AvBD expression

Comparison of AvBD expression in birds that are either susceptible or resistant to *Salmonella* spp. colonisation has indicated that enhanced constitutive AvBD expression is associated with a lower susceptibility to bacterial colonisation (Derache et al., 2009a). Therefore it is possible that enhanced AvBD expression could confer enhanced protection against potential gastro-intestinal pathogens and disease states. One possible strategy of inducing endogenous AMP expression has been to provide birds with dietary supplements. For example, vitamin D3 supplementation was shown to boost AvBD1 expression in the bursa of Fabricus, although the GI tract was not examined (Zhang et al., 2011). Butyrate is an important short chain fatty acid (SCFA) produced by the fermentation of undigested carbohydrate by numerous caecal bacteria including species belonging to the phylum Firmicutes such as *Butyricicoccus* spp. (Eeckhaut et al., 2008). Butyrate, when used as an in-feed additive, was shown to reduce Salmonella titres (Van Immerseel et al., 2005). It was later revealed that this beneficial effect was caused, at least in part, due to a significant up-regulation of the AvBDs, particularly AvBD9 (Sunkara et al., 2011). Furthermore, synergistic up-regulation of AvBD9 was observed *in vitro* using the cAMP agonist forskolin (Sunkara et al., 2014), which suggests that feeding both butyrate in conjunction with cAMP agonists may have potential benefits in protecting poultry against enteric disease, although further *in vivo* trials are necessary to confirm this.

Table 1.4: Regulation of AvBD expression following bacterial challenge

	S.T	References	*S.E*	References	*S.P*	References	*EM/CP*	
AvBD1	↑	(Akbari et al., 2008)	-		NC	(Ramasamy et al., 2012)	↑	(Hong et al., 2012)
AvBD2	↑	(Akbari et al., 2008)	NC	(Cheeseman et al., 2008)	NC	(Ramasamy et al., 2012)	NC	(Hong et al., 2012)
AvBD3	-		-		↑	(Ramasamy et al., 2012)	↓	(Hong et al., 2012)
AvBD4	NC, ↑	(Milona et al., 2007; Akbari et al., 2008)	NC	(Milona et al., 2007)	↑	(Ramasamy et al., 2012)	↓	(Hong et al., 2012)
AvBD5	NC, ↑	(Milona et al., 2007; Akbari et al., 2008)	NC	(Milona et al., 2007)	↑	(Ramasamy et al., 2012)	NC	(Hong et al., 2012)
AvBD6	NC, ↑	(Milona et al., 2007; Akbari et al., 2008)	NC	(Milona et al., 2007)	↑	(Ramasamy et al., 2012)	↑	(Hong et al., 2012)
AvBD7	-		-		NC	(Ramasamy et al., 2012)	NC	(Hong et al., 2012)
AvBD8	-		-		NC	(Ramasamy et al., 2012)	↑	(Hong et al., 2012)
AvBD9	-		-		NC	(Ramasamy et al., 2012)	NC	(Hong et al., 2012)
AvBD10	-		-		↓	(Ramasamy et al., 2012)	↑	(Hong et al., 2012)
AvBD11	-		-		↓	(Ramasamy et al., 2012)	↑	(Hong et al., 2012)
AvBD12	-		-		↑	(Ramasamy et al., 2012)	↓	(Hong et al., 2012)
AvBD13	-		-		↓	(Ramasamy et al., 2012)	↑	(Hong et al., 2012)
AvBD14	-		-		↓	(Ramasamy et al., 2012)	NC	(Hong et al., 2012)

S.T – *Salmonella typhimurium*, S.E – *Salmonella enteritidis*, S.P - *Salmonella pullorum*, EM/CP - *Eimeria maxima/Clostridium perfringens*

↑ up-regulation, ↓ down-regulation, NC no change, - not assessed. AvBD expression was compared between control and challenged birds determined by endpoint RT-PCR or real-time quantitative real-time PCR.

Experimental conditions:

Akbari et al. (2008): Real-time qPCR; broiler chickens challenged on day of hatch; gene expression 1, 3 and 5 days-post challenge.
Cheeseman et al. (2008): Real-time qPCR; challenged at 2 days of age; gene expression 7 days-post challenge.
Hong et al. (2012): Real-time qPCR; challenged at 14 days (*E. maxima*) and 18 days (*C. perfringens*); gene expression 2 days-post challenge.
Milona et al. (2007): semi-quantitative RT-PCR; challenged at 5 days; gene expression 4 days-post challenge.
Ramasamy et al. (2012): Real-time qPCR; challenged at 3 days; gene expression 1 day-post challenge.

1.11 Aims of the study

This study focussed on investigating potential links between bird gut health, microbial profile and AvBD expression. The project was performed in collaboration with Aviagen Ltd. and centred on two commercial broiler lines (Line X and Y) that differ in their gut health. The goals were first to assess and understand differences at the physiological, genetic and microbiome level, second to provide insight into why individual, and groups of birds, may be more prone to enteric disease and third to identify if innate immune genes, particularly the AvBDs, are useful biomarkers of gut health deterioration.

The first studies assessed and compared the gut health and microbiota (ileal/caecal) of bird Lines X and Y following environmental exposure to either *Bacteroides dorei*/*Barnesiella viscericola* (B/BV), or *Lactobacillus johnsonii* (LJ), or a mixture (B/BV + LJ). The objectives were to determine if changes in gut health were associated with shifts in the composition of the ileal and caecal microbiome.

The second studies examined AvBD expression in the GI tract of Line X birds, with the least robust gut health, following environmental exposure to a mix of *Bacteroides dorei* & *Barnesiella viscericola* (B/BV). The objectives were to determine the tissue expression of AvBD1 and 10 with particular focus on GI tract tissues, and to examine the regulation of expression following the B/BV challenge. In addition, AvBD expression was examined in relation to gut health at the individual bird and group level with the purpose of evaluating if AvBDs are useful biomarkers of gut health and/or if they have potential as candidate genes to include in the genetic selection of innate immune traits.

The final studies used an *in vitro* approach to further explore the AvBDs. The objectives were (i) to perform *in vitro* bacterial challenges to model AvBD gene regulation at the cellular level and evaluate the data in the context of the *in vivo* bird trials (ii) to assess the anti-microbial activity of peptides encoded by three SNP variants of AvBD1, that differ in their prevalence within the commercial breeding lines, and AvBD10 against chicken bacteria isolated from the gut of a bird with poor enteric health, and (iii) to explore novel properties for the AvBDs including wound healing and cell proliferation.

2. Materials and Methods

2.1 Consumables

Unless stated, acids and solvents were from Fisher Scientific (Loughborough, U.K.), all other reagents were supplied from Sigma-Aldrich (Poole, U.K.) and all plastics were purchased from Starlab (Milton Keynes, U.K.).

2.2 Farm Trials

2.2.1 Overview

In collaboration with Aviagen Ltd., two farm trials were performed at a site in Ayrshire, Scotland which reflected poultry rearing in commercial environments (Kapell et al., 2012). Trial 1 was performed during April – May 2011 and Trial 2 was performed during January – March 2012.

Two different genetic lines of broilers were used in this study (Lines X and Y), chosen due to their differing gut health. Compared to Line X, Line Y birds exhibit a more robust gut health and thus suffer a lower incidence of enteric health problems (Butler, 2010). Bird numbers, bacterial challenges and sampling protocols are reported in the appropriate Chapters. For each trial, three hatches of birds were reared in physically separate locations (either different barns or compartments within the barn). All birds were euthanized humanely by trained Aviagen Ltd. employees using cervical dislocation.

2.2.2 Bird rearing conditions

In Farm Trial 1, each hatch of birds was reared on fresh bedding. However, in Farm Trial 2, birds were reared on litter consisting of a 50% fresh bedding (top layer) and 50% litter from the previous hatch that had been mechanically conditioned. Where litter was re-used, appropriate management strategies were employed to ensure that litter was kept dry and friable on the surface at all times including de-caking between hatches, adding a fresh top layer of litter and monitoring ammonia levels (see Aviagen Brief: Reused Litter Treatments for Improved Bird Health, http://www.aviagen.com/assets/Tech_Center/Broiler_Breeder_Tech_Articles/English/AviagenBrief_LitterTreatment_Aug08.pdf). Each pen contained 100 birds reaching a maximum stocking density of 33kg per m^2.

All birds were provided *ad libitum* the same diet which consisted of 'crumble' containing a relatively low dietary content of maize, similar in quality to that used in commercial rearing environments. During the starter period (hatch - day 10) the diet contained 195g of crude protein (CPr)/kg providing 12 MJ of metabolisable energy (ME)/kg whilst during the grower period (day 11 - slaughter) contained 170 g of CP/kg providing 12.7 MJ of ME/kg. All birds underwent normal vaccination protocols such as that for infectious bursal disease (Appendix B). Environmental temperature was controlled at all times and reduced steadily from 35 to 24°C as birds aged. During the first week birds were reared in 23h light and 1h dark and from day 8 onwards the photoperiod was altered to 20h light and 4 h dark.

2.2.3 Bird weights

For Farm Trial 1, 100 birds were weighed per pen at 4, 7, 14, 21 and 28 days post-hatch and for Farm Trial 2, only the sampled birds were weighed prior to the gut health assessments and tissue sampling.

2.2.4 Tissue sampling

For Farm Trial 1, birds were sampled by Dr Richard Bailey (Aviagen Ltd.) at 4, 7, 14, 21 and 28 days and for Farm Trial 2, birds were sampled by myself and Dr Vanessa Armstrong at 0, 7, 14, 21 and 28 days. For each bird, a 5 – 10 cm section of jejunum was excised starting from the end of the folded duodenum, cut longitudinally and washed in PBS. The exposed mucosal surface was collected by scraping with a microscope slide and the contents placed in aluminium foil. The samples were snap-frozen in liquid nitrogen, transported to Newcastle and stored long-term at -80°C. Digesta was taken from the ileum and caecum and stored in 1.5ml microfuge tubes.

In Farm Trial 2, GI tissue sections (approximately 1 – 2cm in length) including duodenum, jejunum, ileum, caecum and caecal tonsil, plus the liver, kidney, thymus, spleen and bursa of Fabricus, were collected, placed in 1ml of RNA later solution, prior to long-term storage at -80°C.

2.2.5 Gut health assessments

The gut health assessment was performed by Dr Richard Bailey (Aviagen Ltd.) and is shown in Appendix A. The gut was analysed for redness of the gut surface, gut tone and

consistency of gut contents (water content) using scoring numbers 0 (normal), 1 (mildly abnormal) and 2 (severely abnormal).

2.2.6 DNA extraction from digesta and sequencing of the microbiota

DNA extraction from ileal and caecal digesta samples was performed by Dr Richard Bailey, (Aviagen Ltd.) using a protocol that was optimised for extracting DNA from chicken faeces using the DNAzol™ kit (Life Technologies Ltd, Paisley, UK).

Aliquots of 1g of faecal material were washed by placing in a centrifuge tube containing 150 µl of acid washed glass beads (Sigma-Aldrich, Poole, U.K.) and 5ml H_2O, vortexed for 5 min and then centrifuged (3000 x *g*). The supernatant was removed and the pellet was again washed as before. The faecal pellet was weighed and 200mg was added to a 1.5ml microfuge tube containing an equal volume of glass beads and 400 µl DNAzol™ mix. To facilitate lysis, the tubes were left overnight on a rotating box mixer. The next day the lysate was centrifuged (10 min 10,000 x *g*) and 400 µl of supernatant was mixed with 200 µl of 100% ethanol in a fresh microfuge tube. After leaving for 5 min the mixture was centrifuged as before and the supernatant removed to leave a pellet containing the DNA. The pellet was washed in 1ml of 80% ethanol, centrifuged as before and the supernatant discarded. The pellet was left to dry in air for 10 min and then re-suspended in 200 µl of TE elution buffer (Qiagen, UK). DNA concentration and purity was assessed by Nanodrop spectrophotometer (Fisher Scientific, Loughborough, U.K.)

The DNA samples were analysed commercially by the Animal Health and Veterinary Laboratories Agency (AHVLA) (Weybridge, U.K). Bacterial DNA was sequenced by 454-pyro sequencing (Roche, Indianapolis, U.S.A) at the V4-V5 region of the 16S ribosomal gene. The data was formatted commercially and sent to Aviagen Ltd. as percentage abundance of the overall microbiome for each identified species.

2.3 Analysis of jejunal gut scrapes

2.3.1 Total protein extraction and quantification

Gut samples were stored on ice during the extraction procedure. Each sample was placed in a 15ml Falcon tube containing 1ml of 10% acetic acid and homogenised using a TissueRupter® (Qiagen, Crawley, UK). The TissueRupter probes were carefully washed

between samples using 70% ethanol and 0.1M PBS. After homogenisation the samples were transferred to 1.5ml microfuge tubes and centrifuged at room temperature, 13000 x *g* for 10 min. Following centrifugation, the supernatant was removed from each sample, placed in a new microfuge tube and the sample was dried in a heat block at 56°C until all the acetic acid had evaporated leaving behind a dried pellet of protein. The pellet was reconstituted with 0.3ml – 1ml 0.1M PBS (depending on approximate size of gut scrape) and vortexed. Centrifugation was repeated at 13000 x *g* for 10 min to remove insoluble material and the supernatant was pipetted into a clean microfuge tube. Total protein concentration was measured using NanoDrop (Fisher Scientific, Loughborough, U.K.) and samples were diluted with 0.1M PBS to give a concentration of 4µg/µl. (Butler, 2010).

2.3.2 Thin layer chromatography

A pencil line was drawn 1cm from the end of a 10cm x 12cm siliconised aluminium plate (Millipore U.K. Limited, Hertfordshire, U.K.) and marked at 1cm intervals for sample spotting. On the left side of the plate 2µl of 10mM sugar standard (Megazyme, Co. Wicklow, Ireland) was spotted and dried with a hairdryer. Six microlitres of each sample was analysed (3µl spotted, dried and further 3µl was spotted). Plates were placed in a tank containing solvent (2:1:1, butanol:acetic acid:H_20) at 1cm depth for 1.5 h. Plates were dried, placed again in the solvent again for 1.5 h and re-dried. Plates were covered in developer (32.3ml sulphuric acid : 752.7ml ethanol: 215ml water : 1g orcinol) for 30 s, dried, placed in a drying oven at 70°C and monitored until bands were distinguishable (approx. 5min). Samples were assessed by comparing sample bands to the sugar standards.

2.3.3 High Pressure Liquid Chromatography (HPLC)

Prior to running samples and standards on HPLC, four sets of running buffer were prepared. Buffer A contained 3.5ml NaOH (46/51% soln. HPLC electrochemical grade) in 1L H_20; buffer B contained 3.5ml NaOH and 68g NaAc in 1l H_20; buffer C contained 1L H_20 and buffer D contained 28.4ml NaOH in 1L H_20. Buffers were filtered to remove impurities and de-gassed for 1hr. A series of sugar standards (mannose, arabinose, galactose) were diluted from 10mM stock to 0.3mM in a total volume of 200µl. The gut scrape samples were diluted 1:10 to a total volume of 200µl and 3 sets of standard + sample were prepared (6µl sugar standard + 20µl sample + 174µl H_20). In addition, three sets of 300µl water samples were prepared and all samples were placed in glass vials (Chromacol, Hertfordshire U.K) for HPLC analysis (UltiMate® 3000 HPLC, Dionex,

Thermo, Hemel Hempstead, U.K.). A water sample was injected onto the HPLC column, followed by the gut samples and two water samples to finish. The HPLC data was analysed using Chromeleon® software (Dionex, Thermo, Hemel Hempstead, U.K.).

2.4 Bacterial culture

2.4.1 Bacterial strains

Chicken isolates were sampled from a commercial Ross 308 broiler showing symptoms of enteric upset that resembled dysbacteriosis. The strains isolated and used for the microbial growth assays were from *Escherichia coli*, *Bacteroides dorei* (strain 1 and 2), *Barnesiella viscericola* and *Lactobacillus johnsonii*. In addition, a chicken isolate of *Enterococcus faecalis*, obtained in 2007 from the post-mortem of an Aviagen Ltd. bird, was used (Butler (2010).

The Salmonella strain utilized was *Salmonella* enterica serovar *Typhimurium* 1344 and was provided from Dr A. Khan, Newcastle University, U.K.

Competent cells (Promega, Southampton, U.K.) used for cloning, transformation, plasmid propagation and hyperexpression were: *E. coli* DH5α, *E. coli* JM109 and *E. coli* BL21(DE3)pLysS (protein hyperexpression only).

2.4.2 Growth media and agar

All media was purchased from Sigma-Aldrich and prepared using the manufacturer's instructions prior to sterilisation at 121ºC using an autoclave.

Luria-Bertani (LB) broth and agar was prepared from reagents from BD Biosciences (Oxford, UK). For each litre of broth, NaCl (10g), Bacto-tryptone (10g) and Bacto-yeast (5g) was dissolved in 1L of de-ionised water and the pH was adjusted to 7.4 with 1M NaOH. LB agar was prepared using the same method with the addition of 15g agar/1L LB.

Super optimal broth (SOC medium) was used for bacterial transformation.

Blood agar was used without the addition of heparinised horse blood and was used in *E. coli* and *E. faecalis* growth assays. Tryptic soy broth (TSB) and agar (TSA) were used to culture *S. typhimurium*.

Brain heart infusion (BHI) broth was used for the growth of *B. viscericola* and BHI agar used for plating out colonies of *B. viscericola* and *B. dorei*. De Man, Rogosa and Sharpe (MRS) broth and agar was used for culturing *L. johnsonii*.

B. dorei strains were grown in Tryptone Yeast Glucose (TYG) broth which was prepared in 100ml aliquots containing tryptone peptone (1g), bacto yeast extract (0.5g), glucose (0.2g), cysteine (free base) (0.05g), 1 M KPO_4 pH 7.2 (10ml), Vitamin K solution, 1mg/ml (1ml), TYG salts (4ml), 0.8% $CaCl_2$ (0.1ml), $FeSO_4$, 0.4 mg/ml (0.1ml), resazurin, 0.25 mg/ml (0.4ml) and H_2O (85ml). Prior to culturing, haematin (w/v %) was added to a final concentration of 0.1 % w/v.

For plating bacteria, *E. coli*, *E. faecalis* and *S. typhimurium* were grown overnight aerobically while *B. dorei*, *L. johnsonii* and *B. viscericola* were cultivated overnight on plates in an anaerobic jar (Anaerocult® system, VWR International, Leicestershire, U.K.).

2.4.3 Growth curves

For all bacterial species, growth curves were plotted to determine the optical density (OD_{600nm}) at which the bacteria enter the exponential growing phase. To ensure all bacteria tested were in the same growth phase, cultures were grown to mid-log phase prior to dilution and anti-microbial testing using either the colony counting, microbroth dilution or radial diffusion assays.

To prepare *E. coli* and *E faecalis* prior to their use in the anti-microbial assays a loop of bacteria was taken from a glycerol stock (50% glycerol stored at -80°C), streaked onto a blood agar plate and grown overnight. From this plate a single colony was selected and added to 5ml of LB broth. The bacteria were grown overnight (approx. 16 h) in an orbital shaker set at 200 rpm and 37°C. A sterile 30ml universal container containing 10ml LB and 100mM glucose was inoculated with 200µl of the overnight culture and the bacteria grown in the orbital shaker under the same conditions. The OD_{600nm} of the culture was

monitored using a spectrophotometer (Amersham Biosciences, Ultraspec 43000 pro, High Wycombe, UK) until the culture reached mid-log (OD_{600nm} 0.3 – 0.6).

For *B. dorei*, *B. viscericola* and *L. johnsonii* overnight cultures were prepared first by adding 50µl of glycerol stock to 5ml of the appropriate media. Once in liquid culture these bacteria were plated under the appropriate conditions.

2.4.4 Preparation of heat-killed bacteria for cell challenges

Bacteria were grown to exponential phase as previously described. At mid-log stage bacteria were centrifuged (1000 x *g*) for 10 min, the growth media removed, the cell pellet washed in PBS, re-centrifuged and pellets resuspended in 1ml of fresh PBS in a 1.5ml microfuge tube. A dilution series was performed and colonies were plated overnight prior to counting to provide the bacterial concentration in colony forming units per ml (CFU/ml). For heat inactivation the bacterial suspension was boiled for 5 min and to confirm cell death 50µl plated onto a selective medium and incubated overnight as appropriate.

2.4.5 Colony counting assay

Bacteria were grown to exponential phase as previously described (Section 2.4.3) and a working stock of bacteria was prepared by diluting the culture 1 in 1000 with 0.1M PBS (10µl of culture added to 4990µl of 0.1M PBS). The colony counting assays were modified from Milona et al. (2007). A series of tubes were prepared in triplicate for each sample containing 90µl of the diluted bacterial culture and either 10µl of gut scrape to a final concentration of 0.4µg/µl (gut scrape growth assays) or 10µl of AvBD peptide to a final concentration 0.1 - 50µg/ml (anti-microbial assay).

These test samples were analysed for bacterial growth by performing a dilution series at two time-points (0 h and 2 h) in 96-well microtitre plates and then plating out the colonies. At time-point 0h, 10µl was removed from the 1/1000 bacteria + AvBD/gut scrape sample and serially diluted four times to give final dilutions relative to the mid-log bacterial culture of 10^{-5} (dilution 1), 10^{-6} (dilution 2), 10^{-7} (dilution 3) and 10^{-8} (dilution 4). Aliquots of 10µl from each of these serial dilutions were plated onto individually labelled quarters on blood agar plates. After the AvBD/gut scrape and bacteria had been incubated for 2 h the serial dilutions and plating procedures were repeated. Following 16 - 18 h of

incubation, the C.F.U. were counted for each plate quarter, multiplied according to the dilution factor (10^5 – 10^8) and an average C.F.U/ml was calculated across the dilution series. The value at time 0 h was subtracted from the value at 2 h, to represent the extent of bacterial growth. As performed by Townes et al. (2004), the bacterial growth inhibited by each sample was presented relative to the PBS control, which was assigned a growth value of 100%. Therefore, a value of more than 100% indicated excess bacterial growth, i.e. a pro-microbial effect, values of 0 - 99% represented inhibition of bacterial growth and a value < 0% indicated that less colonies were found at 2 h than at 0 h i.e. bacterial killing.

2.4.6 Microbroth dilution assay

For each anti-microbial peptide or control (PBS), a single row of a 96-well microtitre plate was utilized. Each well contained 100µl of anti-microbial peptide serially diluted 1 in 2 with PBS across the row and starting at 125µg/ml. To each well, 100µl of diluted mid-log bacteria (1 in 20,000) was added and the plate was incubated for 3 h. At 3 h, 100µl of LB growth media was added and the plate was incubated for further 16 h in a plate reader (FluoSTAR Omega, BMG Labtech, Ortenberg, Germany) using a custom program that maintained the temperature at 37ºC, provided shaking at 200rpm every 20 min and measured the optical density at 600nm every 20 min. From these data, growth curves were plotted and the minimum inhibitory concentration (MIC) was determined. To confirm complete killing the contents of each well was plated out on an appropriate medium and checked for bacterial colonies.

2.4.7 Radial diffusion assay

The radial diffusion assay was performed as outlined by Schroeder and Wehkamp, (2011). A low nutrient 'underlay' gel was prepared to a volume of 50ml (5 petri dishes) by dissolving 0.5g low EEO-agarose in a solution containing 0.5ml TSB, 5ml 100mM phosphate buffer (mixture of 47.5 ml of monobasic sodium phosphate with 202.5 ml dibasic sodium phosphate) and 45ml dH_2O. Similarly, a higher nutrient 'overlay' gel was prepared by dissolving 0.5g EEO and 3g TSB powder in a solution containing 5ml 100mM phosphate buffer and 45ml dH_2O. Both 'underlay' and 'overlay' gel were autoclaved and left to cool. Bacteria was grown to mid-log from an overnight culture and pelleted by centrifugation (1000g) for 10 min. The media was removed, the pellet was washed with cold 10mM phosphate buffer, re-centrifuged, re-suspended to an OD_{600nm} of 0.1nm (against a buffer control) and kept on ice until required. Whilst bacteria were growing, both underlay and overlay gel were kept at 50ºC in a waterbath.

For each petri-dish, a 10ml aliquot of liquid 'underlay' gel was left to cool to 45 ºC and 150µl of diluted bacterial suspension (OD_{600nm} 0.1nm) was added. The aliquot was gently mixed and poured into a petri-dish and allowed to set. Holes measuring 3mm were punched into the gel and removed by suction to ensure the holes were clean. Peptide was applied at the required amounts eg 4, 2 and 1µg and appropriate controls were used (PBS – negative control, Cecropin/Lysozyme – positive controls). The plates were incubated for 3 h, 5ml of melted overlay was added to each plate, allowed to dry and then incubated overnight. The following day, zones of bacterial inhibition were photographed using a spImager (S&P Robotics Inc, Chennai, Tamil Nadu, India) and the relative size of each zone of inhibition was measured using ImageJ software (imagej.nih.gov/ij/, National Institutes of Health, Maryland, U.S.A.).

For the assessment of peptide activities against anaerobic bacteria (*B. dorei*, *B. viscericola* and *L. johnsonii*), the underlay and overlay aliquots also contained a reducing agent, namely dithiothreitol (DTT) (1mM), and a redox-indicator namely, resazurine (1µg/ml). Anaerobic bacteria were cultured under anaerobic conditions using the Anaerocult® system (VWR International, U.K.). In addition, the overlay gel contained 3g of the appropriate growth media i.e. BHI for *B. dorei* and *B. viscericola* and MRS for *L. johnsonii*.

2.5 Cell Culture

2.5.1 Overview

Chicken CHCC-OU2 (OU2) cells were donated by Professor Pete Kaiser and Dr Lisa Rothwell from the National Avian Research Centre, Roslin Institute, Edinburgh. Unless otherwise stated all plastics were supplied from Corning (Massachusetts, USA) and reagents were supplied from Sigma (Dorset, UK). Cells were grown and passaged using high glucose DMEM media containing 5% fetal calf serum, 1% chicken serum, 1% penicillin/streptomycin and 10% tryptose buffered phosphate. For challenge experiments, the media was prepared as stated but without the addition of the antibiotics. For maintenance and passaging, cells were seeded at 1 x 10^6 for 25 cm^2, 2 x 10^6 for 75cm^2, and 4 x 10^6 for 175cm^2 flasks. All experiments were performed under sterile conditions in a class II laminar flow cabinet (S@feflow 1.2, BIOAIR, Italy) and cells were cultured at 41ºC in 5% CO_2.

2.5.2 Bacterial challenge experiments

Cells were seeded at 2 x 10^5 in 12-well plates and grown overnight. At 24 h media was removed and replaced with challenge media (no antibiotics). At 48 h cells were challenged with heat-killed bacteria (10^2, 10^3, 10^4, 10^5, 2 x 10^6 C.F.U. per well) for up to 24 h. After the appropriate incubation time the media was removed, the cells washed twice in 1 x PBS and 125µl of RNA lysis solution (Promega, Southampton, U.K.) was added. The cells were then scraped off the wells with a cell scraper and stored at -20ºC prior to RNA extraction.

2.5.3 Wound healing assay

CHCC-OU2 cells were seeded into 6 well plates at a density of 1 x 10^6/well and cultured for up to 48 h or until confluent (cell to cell contact visable throughout). The media was removed and a straight vertical line was scratched in each well using a 200µl pipette tip. The cells were washed twice in PBS to remove debris and 1ml of media was added. To triplicate wells recombinant AvBD10 was added to a final concentration of 0.1, 0.5 or 1nM. In addition, three wells were set-up containing basic fibroblast growth factor (bFGF) (Life Technologies Ltd, Paisley, UK) at 1ng/ml as a positive control or PBS as a negative control. Images were recorded at 0, 24, 48 and 72 h of the same wound area, outlined originally using a china pencil. At 48 h, media was replaced containing the appropriate concentration of AvBD10 or control reagent. Images were analysed using ImageJ software (http://imagej.nih.gov/ij/) and percentage wound healing was calculated using pixel measurements as the total wound area (black pixels) minus the number of cells (represented by white pixels) that had migrated into the wound area.

2.5.4 Cell proliferation assays

The CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay was used to measure the number of metabolically active cells following challenge with heat-killed *Bacteroides dorei*, whilst the CellTiter-Blue® Assay was used to assess the number of viable cells relative to a PBS control following incubation with recombinant AvBD10. For both assays, reagents were supplied by Promega (Southampton, U.K.) and the assays were performed according to the manufacturer's protocol.

The CellTiter 96® AQeuos Non-Radioactive Cell Proliferation Assay utilises the tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) reagent. The MTS (20ml) and PMS (1ml) reagents were thawed and then mixed together prior to storing at -20ºC in 1ml aliquots. For the assessment of cell viability, CHCC-OU2 cells were seeded in wells in a 96-well plate at a density of 2 x 10^4 in 200µl of media. Cells were cultured to confluence (48 – 72 h) and then challenged with killed bacteria at a final concentration of 10^2 – 2 x 10^6 C.F.U/ml for 24 h. After challenge, the media was removed and cells were washed three times in warm PBS (41ºC) and allowed to equilibrate for 15 min in 100µl PBS. To each well, 20µl MTS/PMS solution was added, the plate was incubated for 4 h and the absorbance was read at 490nm using a micro-titre plate reader (FLUOstar Omega, BMG LabTech, Germany). The percentage viability of the challenged wells was compared to unchallenged CHCC-OU2 cells in PBS using standard curves.

For the CellTiter-Blue Reagent® Assay, cells were seeded in 24-well plates at a density of 1 x 10^5 and cultured for 48 h. At this time-point the media was replaced with antibiotic-free media and the cells were challenged with recombinant AvBD10, bFGF, mytomycin C, Bovine serum albumin (BSA) or PBS at a final concentration of 1, 5 and 10nM for 48 h. After 48 h of incubation, the media was removed and 100% methanol was added to two wells per plate and left for 2 – 3 min to kill the cells. Next the methanol was removed and to all wells 500µl of warm serum-free media was added together with 100µl of CellTiter-Blue® reagent. The plates were incubated for 2 – 4 h until a colour change from blue to pink was observable, indicating that the cells are metabolically active. Into a 96-well plate, a standard curve was set up in duplicate using the media from the methanol-killed cells (blue) and the media from the 100% viable PBS treated cells (pink). The standard curve covered the viability range 100%, 75%, 50%, 25% and 0%. For the remaining treated wells, 100µl of media was transferred to the 96-well plate. The 96 well plate containing media from both the standards and treated cells was read for absorbance at 570 and 600nm (FLUOstar Omega, BMG LabTech, Germany). The value at 600nm was subtracted from the value at 570nm and a standard curve plotted which was used to calculate the relative number of metabolically active cells relative to the PBS control.

2.6 Molecular Analyses

2.6.1 DNA extraction from CHCC-OU2 cells

Genomic DNA was extracted from bird tissues using the DNeasy® Blood & Tissue Kit (Qiagen, Crawley, UK) according to the manufacturers' instructions. Briefly, three triplicate wells from a 6 well plate containing 80 – 100% confluent cells (~ 5 x 10^6 cells in total) were removed by scraping, the cells were centrifuged for 5 min at 300 x g, the pellet re-suspended in 200µl of PBS, and 20µl proteinase K was added. To ensure RNA-free genomic DNA, 4 µl of RNase A (100mg/ml) was added. To this, 200 µl Buffer AL was added, the suspension vortexed and incubated at 56°C for 10 min. After incubation, 200µl ethanol was added, the sample mixed and transferred into a DNeasy Mini spin column. Following centrifugation for 1 min at 6000 x *g*, the flow-through was discarded, 500µl Buffer AW1 was added and the spin column re-centrifuged as described previously. To the spin column, 500µl Buffer AW2 was added and centrifuged for 3 min at 14,000 x *g*. Finally, 200µl of Buffer AE was pipetted onto the DNeasy membrane and, following 1 min of incubation, the column was centrifuged for 1 min at 6000 x *g* to elute the DNA.

2.6.2 Sequencing and analysis

Prior to sequencing, DNA concentration and purity (260/280 ratio ~1.8 – 2) was confirmed using a Nanodrop 2000 spectrophotometer (Fisher Scientific, Loughborough, U.K.). All gene sequencing was performed by Genevision, (Newcastle-Upon-Tyne, UK), using customised forward and reverse primers supplied with the sample. Sequence analysis was performed using FinchTV software (Geospiza, Seattle, USA), (http://www.geospiza.com/Products/finchtv.shtml). Query and subject sequences were manually aligned or the nucleotide sequences were searched for matches against the chicken genome (Gallus gallus) using BLASTn (http://www.ncbi.nlm.nih.gov/).

2.6.3 RNA extraction and quantification

RNA extraction from tissue samples was performed by Dr Catherine Mowbray using the SV Total RNA Isolation System (Promega, Southampton, U.K.) and according to the manufacturer's instructions. The tissue samples were removed from the -80°C freezer and a sample of 10 - 20mg homogenized in liquid nitrogen using a pestle and mortar. The homogenised sample was added to a microfuge tube containing 175µl lysis buffer, the

sample mixed by inversion and 350µl of RNA Dilution Buffer added. The tubes were inverted, heated for 3 min at 70°C using a hotblock and centrifuged for 10 min at 13,000 x *g*. The cleared lysate was transferred to a fresh tube, 200µl of ethanol (95%) added and, following mixing, the suspension transferred into a spin basket assembly. To the mixture, 600µl of RNA Wash Solution was added, the spin column centrifuged for 1 minute as previously described and the eluate discarded. To remove contaminating DNA, the spin column membrane was incubated with 50µl of DNAse mix (80% Yellow Core Buffer, 10% MnCl2, 10% 0.09M DNase I) for 15 min. To stop any further DNase activity, 200µl of DNase Stop Solution was added and the column re-centrifuged for 1 min. The membrane was washed twice in RNA Wash Solution and the RNA eluted in 50 - 100µl of nuclease-free water. For the extraction of RNA from CHCC-OU2 cells, the above method was followed except cells were homogenised by repeatedly passing them through a 0.8mm gauge needle (BD Microlance™ 3, VWR International, U.K). The quantity and purity of the RNA was determined using the Nanodrop 2000 spectrophotometer (Fisher Scientific, Loughborough, U.K.).

2.6.4 Reverse transcription

Reverse transcription was performed using a Px2 Thermal Cycler (Fisher Scientific, Loughborough, U.K.). Table 2.1 shows the reagents required for each RT reaction. All reagents were from Promega unless stated. For each sample, a PCR tube containing a total of 0.5µg of RNA was mixed with Milli-Q pure water (Millipore U.K. Limited, Hertfordshire, U.K.) to a total volume of 12.5µl. To each tube 1µl of random hexamers (0.5mg/ml) (Roche, Indianapolis, U.S.A) was added, the samples incubated at 65°C for 5 min and placed on ice for 2 min. To each tube, 12µl of mastermix was added containing 5µl MMLV buffer, 6.25µl dNTPs (Bioline, London, U.K.), and 0.25µl RNasin and 0.5µl MMLV RT enzyme. The samples were mixed by pipetting and incubated at 42°C for 2 h.

Table 2.1: Reagents required per tube for each reverse transcription reaction

Reagent	Volume (µl)	Supplier
RNA (0.5µg)	12.5	
Random hexamers (0.5mg/ml)	1	Roche, U.S.A
M-MLV Buffer	5	Promega, UK
dNTPs (2mM)	6.25	Bioline, UK
RNasin	0.25	Promega, UK
M-MLV reverse transcriptase	0.5	Promega, UK

2.6.5 Primer design for polymerase chain reaction (PCR)

Real-time qPCR primers for AvBD1 and 10 were designed and optimised by Dr Catherine Mowbray. All primers were designed to amplify across at least two exons. Primers were supplied by Integrated DNA Technologies (Leuven, Belgium). To confirm optimum primer annealing temperatures for both AvBD1 and 10 primers, a repeated set of PCR reactions were set up over a temperature gradient (57 - 62°C) and the annealing temperature was selected based on the presence of a strong single band with low levels of primer dimerisation. The same sets of primers were used for both the endpoint and quantitative real-time PCR reactions, as shown in Table 2.2.

Table 2.2: Primers, expected gene product sizes and annealing temperatures (Tm°C) for AvBD1, AvBD10, IL-6 and IL-1β

Gene	Forward 5' to 3'	Reverse 5' to 3'	cDNA Product Size (bp)	Tm °C
AvBD1 Genomic	GCGGATCGTGTACCTGCTC	TTGTGAAACCAGCAAGCCAG	911	60
AvBD1	TACCTCTGCTGCAAAAGAATATGG	GAGAAGCCAGGGTGATGTCC	70	60
AvBD10	CTGTTAAACTGCTGTGCCAAGATTC	TGTTGCTGGTACAAGGGCAAT	77	58
IL-6	CTTCGACGAGGAGAAATGCCT	ACTCGACGTTCTGCTTTTCG	110	58
IL-1β	CTCCAGCCAGAAAGTGAGGC	CTTGTAGCCCTTGATGCCCA	109	58

2.6.6 Endpoint RT-PCR

The reagents required for each endpoint reverse-transcriptase (RT) PCR reaction are shown in Table 2.3. The amplification protocol was performed using a thermal cycler (Techne, Bibby Scientific Limited, UK) and the following program was applied: 95°C for 2 min followed by 35 cycles consisting of 95°C for 30 s, 58/60°C for 30 s (annealing temperatures), 72°C for 30 s. To complete the reaction, a final extension step was performed at 72°C for 10 min followed by a hold step at 4°C.

Table 2.3: PCR reagents, suppliers and volumes per reaction (total volume 20µl)

Reagent	Volume (µl)	Supplier
cDNA	1.5	
5X Green GoTaq® Reaction Buffer	5	Promega, UK
GoTaq® G2 DNA Polymerase	0.2	Promega, UK
dNTPs (2mM)	2.5	Bioline, UK
F + R Primer Mix [10µM]	2.5	IDT, Belgium
Water	8.3	

2.6.7 Gel Electrophoresis

PCR products were electrophoresed using 1.5% w/v TBE-agarose gels at 70V for ~1 h. For each gel, 1.5g of agarose was dissolved in 1 X TBE buffer (54g Tris, 27.5g boric acid, 20ml 0.5M EDTA per 1L de-ionised water) by heating in a microwave. Gels were allowed to cool, ethidium bromide was added (5µg/ml) and gels were poured into a gel electrophoresis tank. The PCR reaction products were loaded to a volume of 15µl and the outside lanes flanking the samples were loaded with 10µl 100bp DNA Ladder.

2.6.8 Gel extraction and purification of cDNA bands

Gels were visualized on a U.V. illuminator and cDNA bands excised using a scalpel. The gel section was placed in a microfuge tube and the DNA purified using a QIAquick® Gel extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Following extraction, the gel slice was weighed and dissolved in Buffer QG (300µl of Buffer QG:100mg of gel) by incubating at 50°C. For each 100mg of gel, 100µl of isopropanol (100%) was added to the sample, and after mixing, transferred to a QIAquick spin column. The sample was centrifuged 1 min at 13,000 x *g* twice, discarding the eluate between each spin. To the spin column 500µl of Buffer QG was added and the column re-centrifuged. To wash the membrane, 750µl of buffer PE was added to the column, allowed to incubate for 2 min and the column re-centrifuged. Finally, 50µl of H_2O was added to the membrane and left to stand for 2 min prior to DNA elution by centrifugation for 2 min.

2.7 Cloning of cDNAs and screening

2.7.1 Competent cells

For transformation using plasmids for real-time PCR, the *E. coli* strain DH5α (*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR1*) was used (Promega, Southampton, U.K).

2.7.2 Preparation of ampicillin/IPTG/X-gal plates

LB agar was prepared and autoclaved as described in Section 2.4.2. Once the LB had cooled to ~50°C, ampicillin was added to a final concentration of 0.05mg/ml. Isopropyl thiogalactoside (IPTG) was prepared as a 0.1M solution and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) was prepared as a 20mg/ml solution in dimethyl sulfoxide (DMSO). After the plates had cooled, 40µl of both X-gal and IPTG were spread evenly over each plate. Plates were then allowed to dry prior to colony plating.

2.7.3 Vector ligation and transformation

For the production of plasmids for use in real-time qPCR standard curves, all cDNA sequences for the AvBDs (AvBD1, AvBD10), cytokines (IL-6, IL-1β), and housekeeping genes were cloned using the pGEM-T® easy vector according to the manufacturer's protocol (Promega, Southampton, U.K.). The ligation reagents were set up as outlined in Table 2.4, vortexed and incubated overnight at 4°C. The following day, competent cells were thawed for 5 minutes on ice, while 2µl of each ligation reaction was added to a microfuge tube. In another tube 0.1ng of uncut plasmid was added as a negative control. To each sample, 50µl of competent cell suspension was added and the microfuge tubes were gently flicked and then incubated on ice for 20min. The samples were then heat shocked for 45 s at 42°C and immediately returned to ice for a further 2 min. To each tube 950µl of SOC media was added and the samples were incubated for 1.5 h at 37°C with shaking. To screen for recombinant colonies, 100µl of culture was plated onto ampicillin/IPTG/X-gal plates (10µl for the uncut plasmid control) and the white colonies were selected after 24 h.

Table 2.4: Reagents for pGEM-T® easy ligation reactions for cDNA genes, positive control and background control.

Reagents	cDNA Reaction	Positive Control	Background Control
	Volume (µl)		
pGEM®-T Easy Vector (50ng)	1	1	1
2X Rapid Ligation Buffer, T4 DNA Ligase	5	5	5
T4 DNA Ligase (3 Weiss units/µl)	1	1	1
PCR product	3	-	-
Control Insert DNA	-	2	-
Milli-Q pure water to a final volume of	10	10	10

2.7.4 Colony PCR

As an additional screening method, white coloured colonies were selected and amplified using the primers of interest to confirm the presence of the gene insert. Up to four single colonies were picked using a sterile pipette tip for each colony and re-plated by lightly touching onto LB/ampicillin/IPTG/X-gal plates for colony preservation. Using the same pipette tip for each colony, an overnight culture was prepared in LB/ampicillin media and the following day a colony lysate was prepared by boiling the culture and centrifuging at 14,000 x *g* for 10 min. Using 2.5µl of supernatant, PCR reactions were set up as outlined in Table 2.5 and amplified using the usual endpoint PCR parameters (Section 2.6.6).

Table 2.5: Colony PCR reagents, suppliers and volumes per reaction (total volume 25µl)

Reagent	Volume (µl)	Supplier
Colony lysate (supernatant)	2.5	
5X Green GoTaq® Reaction Buffer	5	Promega, UK
GoTaq® G2 DNA Polymerase	0.2	Promega, UK
dNTPs (2mM)	2.5	Bioline, UK
F + R Primer Mix [10µM]	2	IDT, Belgium
Water	12.8	

2.7.5 Plasmid mini-prep

Following confirmation of the correct insert using colony PCR, each re-plated colony was grown overnight at 37°C with shaking in 5ml of LB broth supplemented with 5µl ampicillin (50µg/µl). After >16 h of growth each 5ml culture was centrifuged and the plasmid DNA extracted using QIAprep® Miniprep according to the manufacturers'

instructions. The DNA was eluted in 30µl of Milli-Q pure H_20. Plasmids were sent for sequencing (Genevision, Newcastle-Upon-Tyne, U.K.) prior to use in real-time PCR assays.

2.8 Quantitative real-time PCR (qRT-PCR)

2.8.1 Selection of reference genes using GeNorm Kit

The MIQE guidelines state that the selection of reference genes for qRT-PCR should be experimentally validated and that, in most cases, a minimum of two are required (Bustin et al., 2009; Bustin, 2010). The chicken GeNorm kit (Primerdesign Ltd, Southampton, U.K.) was used to select two appropriate reference genes to correct for variation in the amount of genetic material between samples. The kit allows the gene expression of 6 potential reference genes were selected in a representative set of samples to be determined. The reference genes were GAPDH (glyceraldehyde-3-phosphate dehydrogenase), YWHAZ (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta), ACTB (Beta-actin), UBC (ubiquitin C), SDHA (Succinate dehydrogenase complex, subunit A, flavoprotein variant) and SF3A1 (Splicing factor 3 subunit 1). For this study the reference genes were analysed alongside cDNA from both representative tissue samples (from challenge and unchallenged birds) and CHCC-OU2 cells. GeNorm software (PrimerDesign, Southampton, U.K.) was used to rank the stability of the reference genes and also select the minimum number of reference genes required. The most suitable genes for the *in vivo* tissue experiments were SDHA and SF3A1, and for the *in vitro* cell culture experiments were GAPDH and YWHAZ.

2.8.2 Real-time PCR reactions

The reaction mixtures were set up in 96-well plates (Roche, Basel, Switzerland) each in a total volume of 10µl (Table 2.6). Each sample was prepared in duplicate and each plate contained two plasmid dilutions to enable the relative quantification of the samples. The amplification program was performed using a Roche LightCycler 480 (Roche, Basel, Switzerland) and is shown in Table 2.7.

Table 2.6: Quantitative real-time PCR reagents, suppliers and volumes per reaction

Reagent	Volume (µl)	Suplier
Sybr green master mix	5	Roche, Basel, Switzerland
Diluted plasmid/RT product	2	
Molecular grade water	2.5	
F + R Primer Mix [10µM]	0.5	IDT, Belgium

Table 2.7: Quantitative real-time PCR protocol (Roche LightCycler 480)

Programme	**Number of cycles**	**Time (min:sec)**	**Temperature °C**
Pre-incubation	1	10:00	95
Amplification	45	00:10	95
		00:20	60 (AvBD1) 60 (SDHA) 60 (SF3A1) 60 (GAPDH) 60 (YWHAZ) 58 (AvBD 10) 58 (IL-6) 58 (IL-1β)
		00:01	72
Melting curve		00:05	95
		00:01	70
			97
Cooling	1	00:10	40

2.8.3 Relative quantification and analysis

Relative quantification was performed against a standard curve produced from a series of 1/10 dilutions of each plasmid-gene construct. Diluted plasmids were analysed together with sample cDNA to ensure the sample values fell within the range of the standard curve. A number of standard curves were completed for each gene of interest. In addition, the reference genes and standard curves were selected with an amplification efficiency of ~2 and an error rate of <0.05. Based on these standard curves, the crossing point (CP) value for each sample was assigned an arbitrary unit value (A.U). Relative quantification was performed by dividing the A.U. value for each sample by the A.U. geometric mean of the two appropriate reference genes.

2.9 Engineering of recombinant AvBD

2.9.1 Plasmids for hyperexpression

Engineering of the plasmids for hyperexpression was performed in collaboration with Dr Vanessa Armstrong, Newcastle University. Partial sequences of AvBD1 and 10 genes were cloned into the vector PGEX-6P-1 (GE Healthcare Life Sciences, Buckinghamshire, U.K.) using BamHI and EcoRI (Fisher Scientific UK Ltd, Loughborough, U.K.). The resulting plasmid constructs used for hyperexpression were PGEX-6P-1-AvBD10 and PGEX-6P-1-AvBD10.

2.9.2 Competent Cells and Transformation

For the initial cloning of the AvBD1 and 10 cDNAs, the PGEX-6P-1-AvBD constructs were transformed into JM109 (Promega, Southampton, U.K.), recombinants selected using blue-white colony screening (as described in Section 2.7.3) and sent for sequencing. However, the *E.coli* strain utilized for hyperexpression was BL21 Origami B (DE3):: plySs (Novagen, Darmstaft, Germany). BL21 strains, although efficient for hyperexpression, do not transform well and hence were not used for the initial cloning and propagation of the PGEX-6P-1-AvBD constructs. Moreover, the BL21 Origami B (DE3):: plySs cells were prepared fresh each time prior to transformation.

2.9.3 Preparation of fresh BL21 Origami B (DE3):: plySs competent cells

An overnight culture was set up by inoculating 10ml of LB broth containing chloramphenicol (0.03mg/ml dissolved in 100% ethanol) with 50µl of BL21 Origami B (DE3):: plySs from a glycerol stock and gently shaking overnight at 37°C in an orbital shaker. A two ml aliquot was taken and sub-cultured into 100ml of LB containing chloramphenicol until an OD_{600nm} of ~0.3 was reached. The 100ml culture was centrifuged at 3000 x *g* for 5min at 4°C, the supernatant removed and the pellet re-suspended in 4ml 0.1 M $MgCl_2$. The suspension was centrifuged as before, the supernatant removed and the pellet re-suspended in 4ml of 0.1M $CaCl_2$. The cells were left to incubate on ice for 2 h prior to transformation.

2.9.4 Transformation of BL21 Origami B (DE3):: plySs cells and screening

Transformation of BL21 Origami B (DE3):: plySs was carried out as described in Section 2.7.3. To screen for recombinants BL21 Origami B (DE3):: plySs cells were plated onto LB plates containing chloramphenicol (0.03mg/ml) and ampicillin (0.05mg/ml).

2.9.5 Sequencing of PGEX-6P-1-AvBD constructs

Prior to the hyperexpression experiments, the plasmid DNA was checked by sequencing using primers PGEX F and R at 3.4μM (GE Healthcare Life Sciences, Buckinghamshire, U.K.).

2.9.6 Hyperexpression

For hyperexpression, a single colony containing the plasmid-gene construct of interest was selected and grown overnight in 10 ml LB together with chloramphenicol (0.03mg/ml) and ampicillin (0.05mg/ml) in a shaking incubator at 37°C. For each colony, 1L of LB media (with appropriate antibiotics) was prepared and 10ml of overnight culture was added. Cells were cultured at 37°C to an OD_{600nm} of 0.8 and hyperexpression induced by the addition of 0.8M ITPG (isopropyl-beta-D-thiogalactopyranoside) to a final concentration of 0.08M. The cultures were grown for 3 h at 25°C then centrifuged at 5000 x *g* for 10 min (Avanti J centrifuge, Beckmann Coulter Inc, High Wycombe, U.K.). The supernatant was discarded and the pellets were stored at -80°C for up to 3 months until needed.

2.9.7 Sonication

Each bacterial pellet was thawed on ice, reconstituted in 20ml 1 x PBS, transferred to a 30ml centrifuge tube and the cell suspension was sonicated for 3 – 4 min on ice in 30 s bursts. Following sonication, the samples were centrifuged at 15,000 x *g* (Avanti J centrifuge, Beckmann Coulter Inc, High Wycombe, U.K.) and the supernatant containing the cytoplasmic proteins stored on ice prior to purification.

2.9.8 Protein Purification

Poly-Prep® Chromatography Columns (BioRad, Hertfordshire, U.K.) were rinsed with alcohol and 2ml of Amintra® glutathione sepharose (Expedeon, Cambridge, U.K.) loaded onto the column. The column was left to equilibrate for 1 – 2 min and 10ml of PBS was

added. The supernatant containing the cell free extract (C.F.E) was added slowly to maintain a flow rate of 0.2–1 ml/min until all the C.F.E had passed through the column. At every step in the purification process, a small aliquot was removed and analysed by NuPAGE to confirm the success of each purification step. The column was re-washed with up to 10ml of 1 x PBS. To remove the GST-tag from the AvBD peptide an on-column cleavage approach was adopted. The column was washed with 10ml of cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol DTT, pH 7.5). For each 1ml of glutathione sepharose, an enzymatic cleavage mix was prepared containing 80µl (160 units) of PreScission Protease (GE Healthcare Life Sciences, Buckinghamshire, U.K.) with 920µl of cleavage buffer. This enzymatic cleavage mix was loaded onto the column and left to incubate overnight at 4°C. The next day the column was eluted using 5-10ml of cleavage buffer and 1ml aliquots were collected. The majority of the GST and PreScission Protease attached to the column. To separate the cleaved AvBD peptides (~5kDA) from the remaining GST (26kDa) and PreScission Protease (46kDa), the eluate was passed through a Centrifugal concentrator with a molecular weight cut-off of 10kDa (Vivaspin 6, Fisher Scientific UK Ltd, Loughborough, U.K.). The flow-through containing the cleaved peptide was collected and buffer exchanged in PBS using a PD-10 Desalting Column (Sigma-Aldrich, Poole, U.K.). The AvBD peptide was passed through a centrifugal concentrator with a molecular weight cut-off of 5kDa (Vivaspin 6, Fisher Scientific UK Ltd, Loughborough, U.K.) and the flow-through discarded. The remaining liquid in the top of the column was recovered.

2.9.9 Determination of peptide concentration

AvBD peptide concentrations were determined using the Novagen® BCA Protein Assay Kit (Millipore U.K. Limited, Hertfordshire, U.K.) according to the manufacturers' instructions. In summary, a series of bovine serum albumin (BSA) standards were prepared at 0 - 1000µg/ml. Twenty-five µl of each BSA standard or AvBD sample were pipetted, in duplicate, into wells of a 96 well plate and 200µl of BCA working reagent (80µl 4% Cupric Sulfate + 4ml BCA solution) was added to each well. After a short mix, the plate was incubated for 30 min at 37°C and the absorbance measured at 562nm (FLUOstar Omega, BMG LabTech, Germany). Using the BSA concentrations, a standard curve was plotted and the peptide concentrations calculated.

2.9.10 Identification of peptides using polyacrylamide gel electrophoresis

Proteins were separated using the NuPAGE® system (Life Technologies Ltd, Paisley, U.K.). All running buffers and reagents required for electrophoresis were supplied by Life Technologies Ltd unless otherwise stated. To prepare the samples, a mixture containing 10.4µl of sample, 4µl of 4x buffer and 1.6µl of reducing reagent was heated to 70°C for 5 min. Running buffer (x1) was prepared by diluting 42.5ml of 20x buffer to 850ml using dH_20. From this running buffer, 200ml was dispensed into a separate measuring cylinder and 0.5ml anti-oxidant was added. The comb was removed from a 4-12 % Bis-Tris Pre-Cast gel which was placed into the XCell SureLock® Mini-cell gel running tank. The upper chamber, containing the inward facing wells, was filled with the 200ml 1 x running buffer containing the anti-oxidant. To each well, 15µl of sample was added and 10µl of Novex® Sharp standard was used as a size marker. The remainder of the chamber was filled with the 1 x running buffer and the gel was electrophoresed at 200V for 45 min. The gels were stained with InstantBlue (Expedeon, Cambridge, U.K.), a ready to use Coomassie® stain, for 30 min and photographed.

2.10 Synthesis of AvBD1 variants

Three variants of AvBD1 were synthesised by PeptideSynthetics (Hampshire, UK) termed 'NYH', 'SSY' and 'NYY' based on their corresponding primary sequence and shown in Table 2.8. The purity was >95% for all peptides (checked by RP-HPLC and electrospray mass spectrometry). For each AvBD1 variant 5 x 1mg lyophilised aliquots were prepared. The lyophilised aliquots were stored at -20°C and dissolved in 20µl of 10% acetic acid and the volume made up to 1ml using Milli-Q pure H_2O to give a working stock at a concentration of 1mg/ml.

Table 2.8: Primary sequences synthesised by PeptideSynthetics for three AvBD1 variants termed 'NYH', 'SSY' and 'NYY'.

AvBD Variant	Sequence
'NYH'	GRKSDCFRK**N**GFCAFLKCP**Y**LTLISGKCSRF**H**LCCKRIWG
'SSY'	GRKSDCFRK**S**GFCAFLKCP**S**LTLISGKCSRF**Y**LCCKRIWG
'NYY'	GRKSDCFRK**N**GFCAFLKCP**Y**LTLISGKCSRF**Y**LCCKRIWG

2.11 Circular dichroism (CD) spectrometry

Circular dichroism experiments and analyses were performed by Sherko Subhan, Newcastle University PhD student. The three synthetic AvBD1 peptides were reconstituted in either Sodium Phosphate buffer (50mM pH 7.4 or sodium dodecyl sulphate (1% SDS) to a final concentration of 0.25mg/ml, as determined by A280nm (Nanodrop). For CD analysis, 80 µl of peptide or control (Sodium Phosphate buffer 50 mM or 1% SDS) were added into a 0.2 mm cuvette. Far-UV was recorded over the range 250 – 185 nm (Jasco-810 CD spectropolarimeter) with settings of band width 0.2 nm, data pitch 0.5 nm, scanning speed 100 nm/min, response 10 sec and accumulation 10. Absorption units were calculated using Spectra Manager (JASCO UK, Ltd, Essex, U.K.) software and analysed in Microsoft Excel.

2.12 Generation of custom antibodies

Custom antibodies for AvBD1 and AvBD9 were produced by Cambridge Research Biochemicals (CRB) (Cleveland, U.K.). The primary amino acid sequences of the AvBDs were sent to CRB for antigenic prediction, to ensure no cross reactivity between defensins and to ensure good anti-genticity. A unique peptide antigen for AvBD1, GRKSDSFRKNGFC-amide, determined by the Company was used to raise rabbit polyclonal antibody to AvBD1.

2.13 Tissue immunohistochemistry (IHC)

All IHC developmental and staining work was performed by Dr Catherine Mowbray.

Avian tissue was harvested and fixed in 4% buffered formalin and subsequently stored in 70% ethanol before being processed into paraffin blocks. Tissue was sectioned at a thickness of 4 µm onto SuperFrost Plus slides (Fisher Scientific, Loughborough, U.K.) and allowed to dry for 24 to 48 hours before staining. For immunohistochemical staining, slides were de-waxed in xylene, rehydrated through graded alcohols to water and subjected to a hydrogen peroxide block (1.5% in water) for 10 minutes. All antibodies were assessed independently to determine the appropriate antigen retrieval method for use with each stain. Methods assessed were pressure cooking with citrate buffer (pH6.0), pressure cooking with EDTA (pH8.0), enzymatic digest with trypsin (pH7.8 at 38^{oC}) and no antigen retrieval. For the AvBD1 antibody, pressure cooking with EDTA worked most effectively. After antigen retrieval, staining was carried out using the Vectastain Elite ABC peroxidase kit (rabbit) (Vector Laboratories, Peterborough, U.K.) as per

manufacturers' instructions. Antibodies were used at dilutions of 1/250 in TBS (pH7.6) for 1 hour at room temperature for AvBD1. The reaction was developed using the peroxidase chromogen DAB (3,3-diaminobenzedine tetrahydrochloride) as per manufacturer's instructions, then the nuclei counterstained using Mayer's Haematoxylin and Scot's tapwater substitute. Sections were then dehydrated through graded alcohols and cleared in xylene before mounting using DPX.

2.14 Statistical Methods

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc, La Jolla, California, USA). Median values between two groups were compared using Mann-Whitney U test and median values between three or more groups were compared using Kruskal-Wallis test followed by Dunn's post test. Means were compared using one-way ANOVA followed by Dunn's multiple comparison post-test or two-way ANOVA followed by Bonferroni post-tests. The significance level was set at 5% ($P < 0.05$). To check for a normal distribution of data, the D'Agostino & Pearson (1973) omnibus normality test was performed.

Chapter 3: Farm Trial 1

3.1 Farm Trial 1 Overview

This trial was set up in collaboration Aviagen Ltd, and the aim was to explore and compare the effects of exposure to different residents of the normal chicken microbiota on the gastrointestinal health of two commercial breeding lines of Aviagen broilers. The two genetic lines, X and Y, differed in their gut function, with Line Y characterized by its increased gut efficiency. Other details about the genotype of these Lines cannot be provided for reasons of commercial confidentiality. The objectives of this trial was to identify potential factors that determine the differing gut function.

It is common practice within the poultry industry to administer probiotics (generally <5 species of defined bacteria) or competitive exclusion products (undefined bacterial composition >200 species) to growing broilers. This trial aimed to mimic the widespread commercial application of direct-fed microbials to aid the colonisation of the gut flora. Most probiotic products available contain members of the genus lactobacillus, such as *L. johnsonii,* due to their well-documented benefits to gut health (La Ragione et al., 2004; Van Coillie et al., 2007; Wegmann et al., 2009). Furthermore, since lactobacilli are the major component of the small intestine microbiome of the chicken, administration of a probiotic lactobacillus strain with proven benefits to intestinal health was expected to improve gut health. Competitive exclusion products are produced from mass culture of healthy chicken caecal contents clear of known pathogens. Administration of competitive exclusion products aims to not only prevent the colonisation of pathogens such as *E. coli* but also to aid the maturation of the caecal microbiota. Due to the undefined nature of competitive exclusion products containing hundreds of bacterial species, pure cultures of two bacterial species known to be residents of the adult chicken caeca were chosen as a means to aid colonisation of the caeca. *Bacteroides dorei* and *Barnesiella viscericola* were chosen as they had previously been isolated from healthy chicken caeca and represent normal residents of the adult chicken caecal microbiota (Sakamoto et al., 2007; Sergeant et al., 2014). A mixture of the lactobacillus with the BD and BV aimed to assist the colonisation and maturation of both the large and small intestine.

The trial was performed during April – May 2011 on an Aviagen commercial sib-testing farm in Scotland, UK, which is designed to represent the lower quartile of commercial

UK broiler farms in terms of standards of hygiene. Line X and Y birds were administered with either *Lactobacillus johnsonii* (LJ), *Bacteroides dorei/Barnesiella viscericola* (B/BV), or a mixture of both. Following arrival from the hatchery (Day 0), Line X and Y birds were assigned to one of four pens in which they were administered bacteria (treated) or just water (control) (Figure 3.1). In the three treated pens, birds were administered either LJ at a concentration of 10^{12} CFU/ml, B/BV spp. at 10^{9} CFU/ml or a mixture of the two (total 10^{12} CFU/ml). Following hatch the intestinal tract of a chicken undergoes rapid development which includes colonisation and succession of the microbiota along with maturation of the intestinal tissues (Yadav et al., 2010) and immune system (Crhanova et al., 2011). Therefore birds were sampled at multiple ages in order to map the temporal changes in the development of the gut environment. At 4, 7, 14, 21, 28 and 35 days post-hatch respectively, a random sample of five birds per pen were sacrificed and visually assessed for gut health using criteria designed and implemented by Aviagen Ltd. (see Appendix A). To determine the microbiome the gut contents were sampled and analysed using 454 pyrosequencing. To assess the ability of the gut to promote or inhibit microbial growth a mucosal jejunal scrape was taken from each of the five sampled birds. As a more general assessment of growth, each bird was weighed and an average weight for each pen (n = 100) was calculated. The entire process was repeated for a total of three hatches, each hatch in separate locations (either different barns or compartments of the same barn).

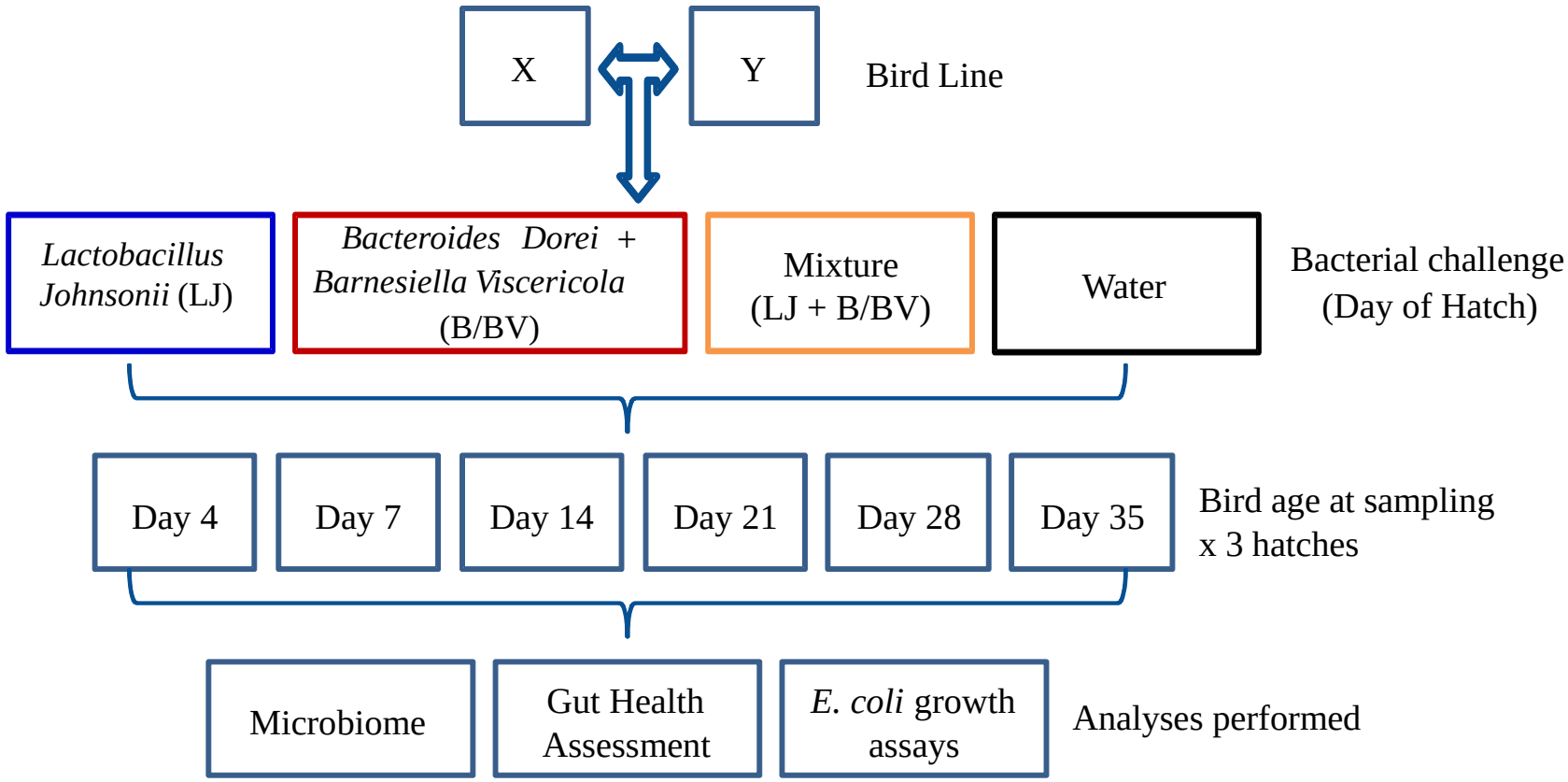


Figure 3.1: Flow-chart of Farm Trial and analyses performed.

Microbiome sampling and gut assessments were performed by Dr Richard Bailey (Aviagen Ltd). Jejunal gut scrapes were sent to Newcastle University for use in the *E. coli* growth assay.

3.2 Results

3.2.1 Overview of Gut Health Assessments

Figure 3.2 A-D illustrates the total gut health per challenged pen over six sampling timepoints for Hatches 1 and 2, respectively. This figure provides a simple overview of how the gut health varied with time, genetic line and bacterial challenge. Data for individual birds are shown in Figures 3.3 – 3.16 alongside mean bird weight and the caecal microbiota. Hatch 3 birds were affected by a low level coccidiosis outbreak thus the data relating to these birds was excluded from the final analyses.

For each pen at each time-point, five individual birds were assessed for gut health and assigned a score of 0 (normal), 1 (some abnormalities) or two (very abnormal) across three criteria (redness, water content and gut tone). These scores were summed for each group of five birds. For example, the worst possible gut health score per bird was 6, whereas a completely healthy bird was scored 0. These data revealed a trend for gut health to deteriorate over time with the worst gut health observed at 28 days post-hatch followed by recovery at 35 days. In general, Hatch 2 had worse gut health than Hatch 1, with an overall total gut score for all sampling time-points of 101 compared to 77, respectively.

Relative to the water control group, the B/BV challenged group displayed worse gut health, with the effects clearly observable at the later sampling time-points (Day 21 – 28), particularly in the Line X birds. In contrast, the LJ challenge was associated with beneficial effects in both lines and in both hatches. At 28 days post-hatch the total gut score per group was lower for the LJ challenge than for all other challenges, including the water control. In general, the trend was for the 'mix' challenge not to adversely affect gut health.

The total gut health data indicated that relative to Line X birds, Line Y birds were resistant to potentially adverse bacterial challenges such as B/BV. For example, in Hatch 1 at 28 days post-hatch the gut health of the Line X B/BV challenged birds had deteriorated markedly (water = 6, B/BV = 16) whereas this was not observed in Line Y (water = 4, B/BV = 5).

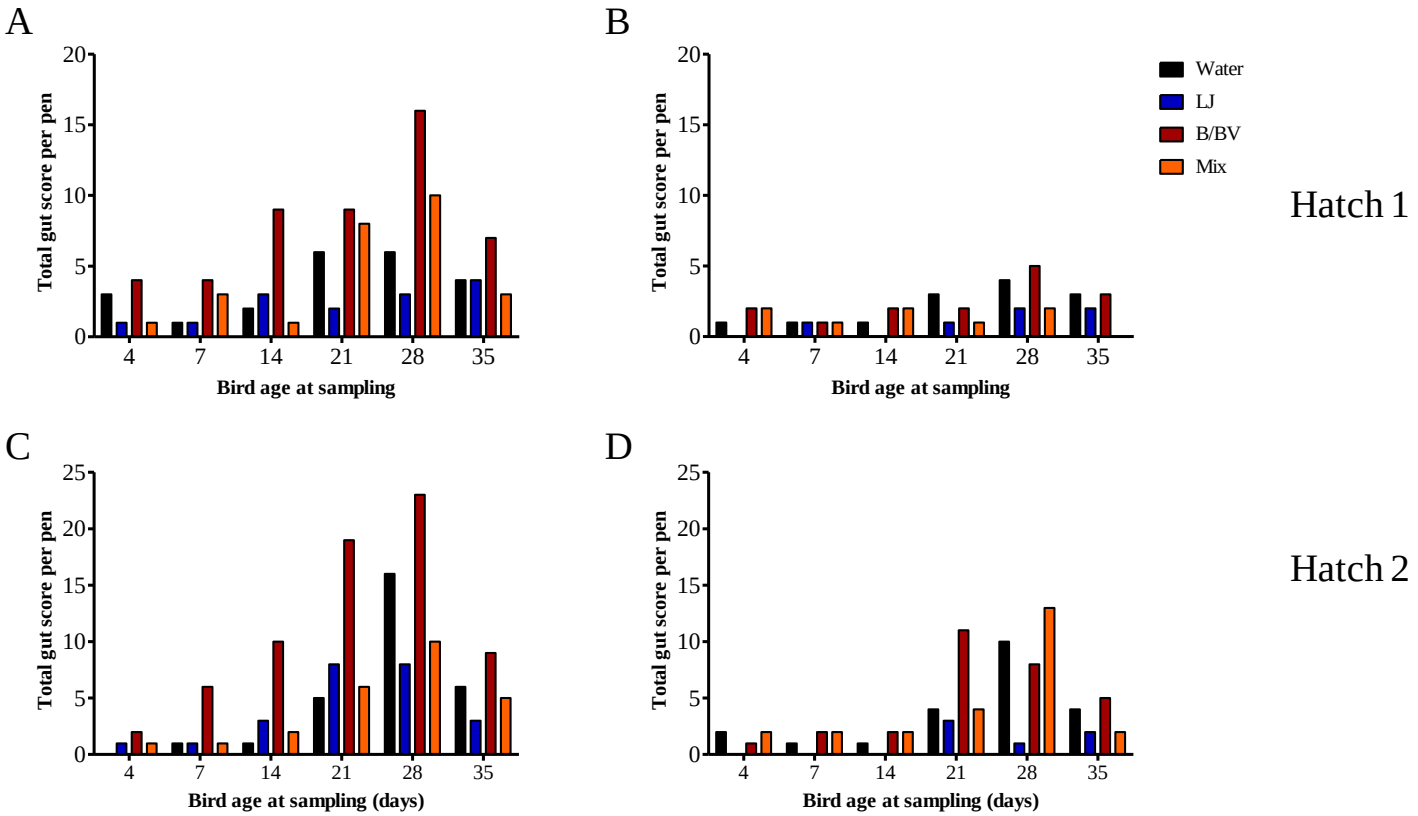


Figure 3.2: Total gut score per pen for Line X and Y birds.

Line X (A and C) and Y (B and D) birds were sampled at 4, 7, 14, 21, 28 and 35 days in Hatch 1 (A and B) and Hatch 2 (C and D) (n = 5 birds). Pens were challenged with water, *Lactobacillus johnsonii* (LJ), *Bacteroides/Barnesiella viscericola* (B/BV) or a mixture (B/BV + LJ). Each bird was assessed for redness, watery digesta and gut tone and assigned a score of 0 (normal), 1 (some abnormalities) or 2 (very abnormal) for each criteria. The total scores comprised of summed gut scores across the three gut health criteria for 5 birds.

3.2.2 Gut health, bird weight and caecal microbiota in Line X and Y birds

Figures 3.3 and 3.4 illustrate individual bird gut health at each sampling timepoint (A), the mean weight per bird (B) and the caecal microbiome in Line X (C) and Line Y control birds (D).

A comparison of the median gut health scores revealed no significant differences in gut health between Line X and Y birds at any of the sampling time-points for either hatch ($P > 0.05$, Dunn's multiple comparison test). However, the total gut health scores per group do show worse gut health in Line X birds (Hatch 1: 6 and Hatch 2: 16) compared to Y (Hatch 1: 4 and Hatch 2: 10). A significant age-dependent effect was observed in Hatch 2 only with bird gut health deteriorating over time for both Line X ($P < 0.01$) and Line Y ($P < 0.05$). Interestingly, Figures 3.4B and 3.5B highlight that Line X birds are significantly heavier than Line Y at all sampling time-points apart from 4 days post-hatch ($P <0.001$).

The caecal microbiota reveals that the most abundant bacterial genera at 4 days post-hatch are *Lactobacillus* spp. (Hatch 1: Line X, Hatches 1 and 2: Line Y) and *Faecalibacterium* spp. (Hatch 2: Line X). By 28 days post-hatch *Barnesiella* spp. were the dominant species (> 25%) in three of the four pens; the exception was Hatch 1: Line X in which *Lactobacillus* spp. remained dominant. Differences were observed between lines, although no obvious bacterial markers of adverse gut health were identified. In Hatch 1, Line Y had a higher abundance of *Lactobacillus* spp. (Day 4), *Bacteroides* spp. (Day 21), and *Barnesiella* spp. (Day 28) than Line X, but in Hatch 2 the differences were less obvious with similar levels of *Bacteroides* spp. and *Barnesiella* spp. identified.

Differences in gut health observed between hatches were however reflected by different microbiota compositions. The caecal digesta from the Hatch 2 water control pens, which had worse gut health than in Hatch 1, contained higher abundances of *Barnesiella* spp. at Day 28 compared to Hatch 1. This pattern was observed for both Line X (11% vs. 32%) and Line Y (21% vs. 27%). In addition, at Day 4 higher abundances of *Butyricicoccus* spp, *Coprococcus* spp. and *Faecalibacterium* spp. were found in Hatch 2 compared to Hatch 1. It is of interest that the guts of Line X: Hatch 2 birds were healthier at this early sampling time-point (all 5 birds normal), supporting these bacterial species to be associated with beneficial effects. Furthermore, it was revealed that *Bacteroides* spp. form

part of the Day 4 microbiota in Hatch 1 (figure 3.3) but are not present in Hatch 2 at this timepoint (Figure 3.4). In Line Y, the most notable difference in the Hatch 2 pens was a much lower abundance of *Lactobacillus* spp. at Day 4 (30% lower than Hatch 1).

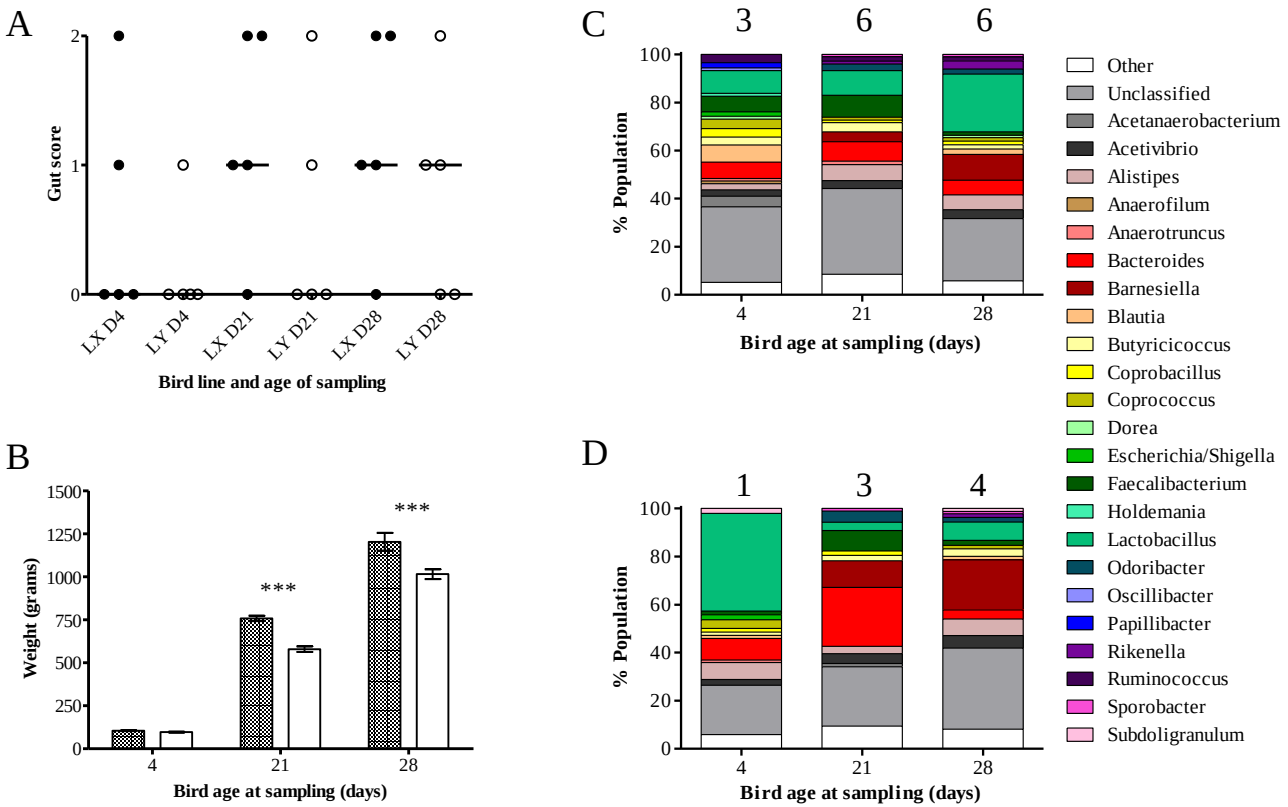


Figure 3.3: Gut health, caecal microbiota and bird weight analysis for Hatch 1 water control LX and LY birds.

Birds were sampled at 4, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LX and LY birds (n = 5 birds per pen), filled circles = Line X birds, open circles = Line Y birds. **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = Line X birds, open columns = Line Y birds. **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of Hatch 1 Line X birds (C) and Hatch 1 Line Y birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

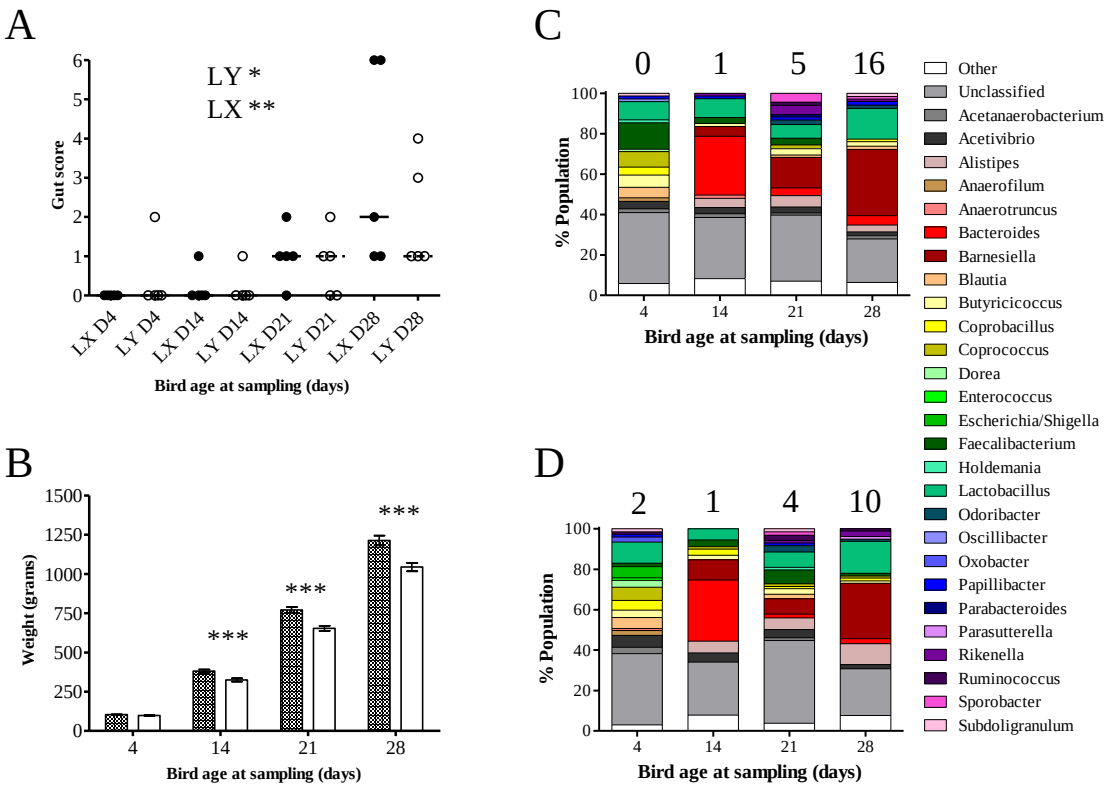


Figure 3.4: Gut health, caecal microbiota and bird weight analysis for Hatch 2 water control LX and LY birds.

Birds were sampled at 4, 14, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LX and LY birds (n = 5 birds per pen), filled circles = Line X birds, open circles = Line Y birds. **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = Line X birds, open columns = Line Y birds. **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of Hatch 1 Line X birds (C) and Hatch 1 Line Y birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

3.2.3 The effects of Lactobacillus johnsonii challenge on gut health, bird weight and caecal microbiota

3.2.3.1 Line X

Figures 3.5 and 3.6 illustrate individual Line X bird gut health (A), the mean weight per bird (B) and the caecal microbiome of control birds (C), and LJ challenged birds (D) at each sampling time-point.

When individual bird gut scores for Hatch 1 (Figure 3.5 A) and Hatch 2 (Figure 3.5 B), were plotted and median values compared no significant differences in gut health were observed between control and LJ challenge pens ($P > 0.05$, Dunn's multiple comparison test). However, if birds were grouped and the total gut scores per pen calculated then a beneficial effect of the LJ challenge was evident. For example, the water control pens had scores of 6 and 16 and the LJ challenge pens had scores of 3 and 8, for Hatch 1 and 2 respectively. The median gut score of the Line X: LJ challenged pen in Hatch 2 differed significantly between sampling time-points highlighting that gut health worsened with age ($P < 0.05$). In Hatch 2, the mean weight of the water control birds was significantly lighter than the LJ challenged birds at Days 14, 21 and 28 ($P < 0.01$, Bonferroni post-tests) though no differences in bird weight were observed in Hatch 1.

The caecal microbiota data showed that in both hatches the relative abundance of *Lactobacillus* spp. at Day 28 was lower in the LJ challenged pens i.e. 15% compared to 6% in Hatch 1 and 24% compared to 11% in Hatch 2.

3.2.3.2 Line Y

Figures 3.7 (Hatch 1) and 3.8 (Hatch 2) illustrate individual Line X bird gut health (A), the mean weight per bird (B) and the caecal microbiome of control birds (C) and LJ challenged birds (D) at each sampling time-point.

Comparison of median gut health in Hatch 1, shown in Figure 3.7A, revealed no significant effects of the LJ challenge on gut health ($P > 0.05$, Two-way ANOVA). However in Hatch 2, the LJ challenged birds had significantly lower gut scores at 28 days post-hatch than the water control group ($P < 0.05$, Dunn's multiple comparison test). Moreover, in Hatch 2 only 1/5 LJ challenged birds had an abnormal gut score in

comparison with 5/5 in the water control pen indicating that the Line Y birds respond to probiotic LJ intervention , at least in this sampled hatch.

In Hatch 1, the mean weight of the water control birds was significantly heavier than the LJ challenged birds by Day 28 ($P < 0.01$, Bonferroni post-tests) though no differences in bird weight were observed in Hatch 2.

Analysis of the caecal microbiota revealed that compared to the water control group the LJ challenged group had higher percentage abundances of *Butyriccoccus* spp. at Day 4 and *Bacteroides* spp. at Day 21. Another notable effect of LJ challenge was that the sampled microbiome from the LJ challenged birds in Hatch 2 contained a higher percentage of *Barnesiella* spp. at 21 days post-hatch compared to the water control birds (8% in the water control group compared to 34% in the LJ challenged group).

As observed in the Line X birds, the caecal microbiome of the LJ challenged birds contained a lower percentage of *Lactobacillus* spp. at Day 28 than the water control birds.

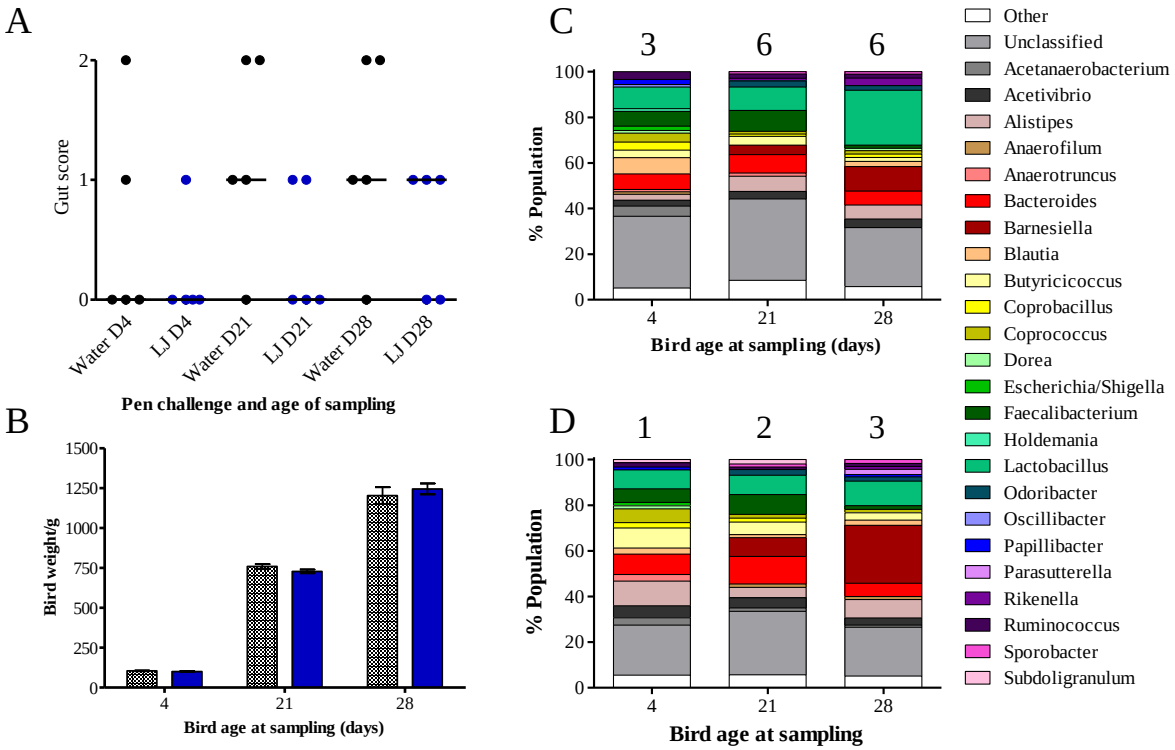


Figure 3.5: The effects of *Lactobacillus johnsonii* (LJ) challenge on Line X birds in Hatch 1

Line X birds were challenged with water or Lactobacillus johnsonii (LJ) at Day 0 and sampled at 4, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LX birds (n = 5 birds per pen), black circles = water challenge, blue circles = LJ challenged birds. **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, blue columns = LJ challenged birds (*** P < 0.001, Bonferroni post-test following Two-way ANOVA). **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and LJ challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each timepoint is shown above each column.

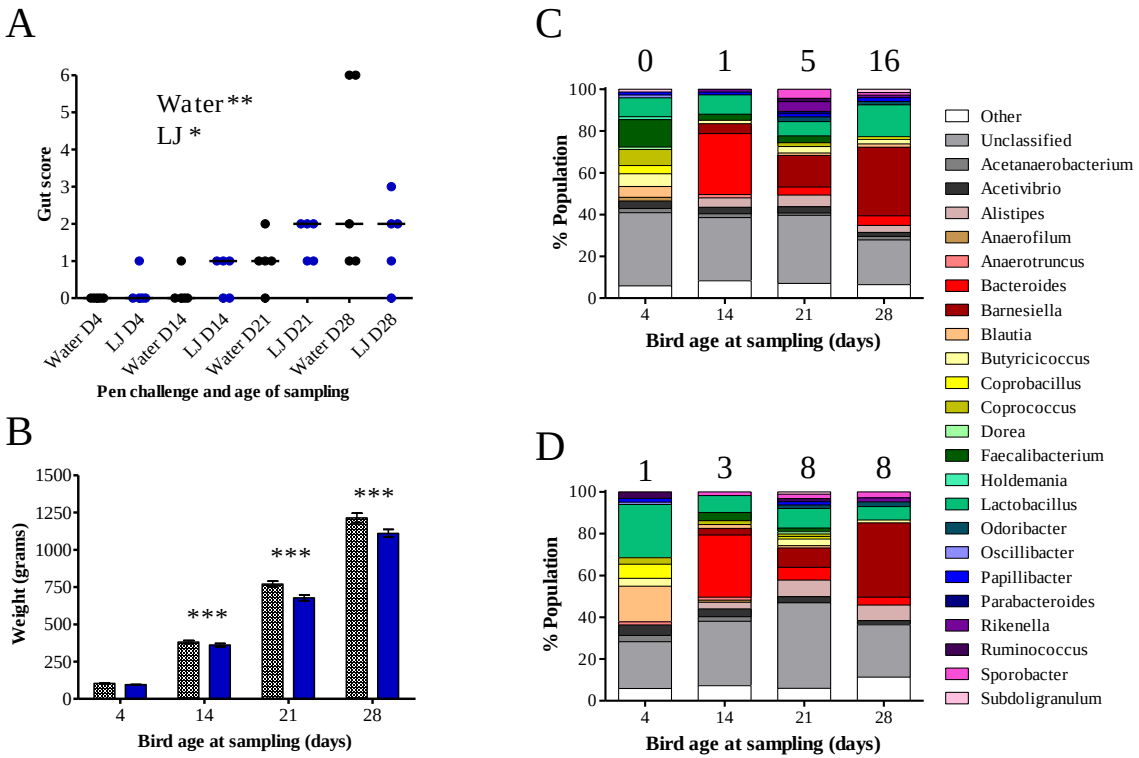


Figure 3.6: The effects of *Lactobacillus johnsonii* (LJ) challenge on Line X birds in Hatch 2

Line X birds were challenged with water or *Lactobacillus johnsonii* (LJ) at Day 0 and sampled at 4, 14, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LX birds (n = 5 birds per pen), black circles = water challenge, blue circles = LJ challenged birds. (* $P < 0.05$, ** $P < 0.01$ Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, blue columns = LJ challenged birds (*** $P < 0.001$, Bonferroni post-test following Two-way ANOVA). **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and LJ challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each timepoint is shown above each column.

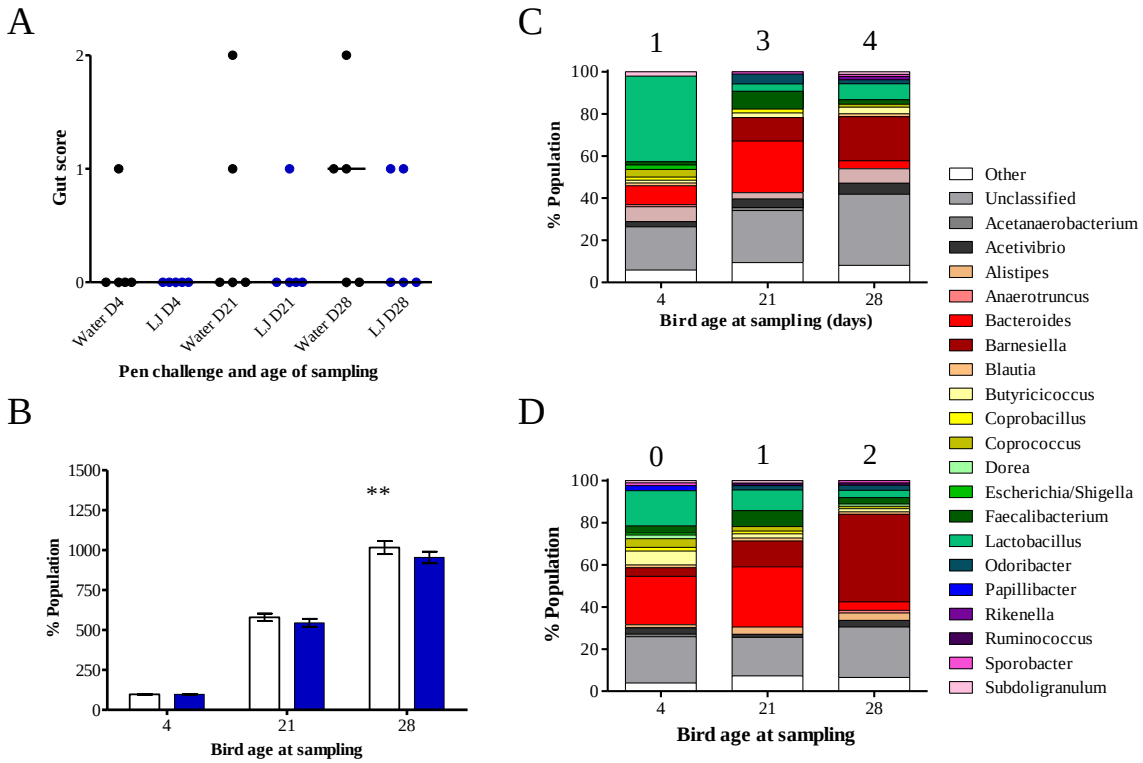


Figure 3.7: The effects of *Lactobacillus johnsonii* (LJ) challenge on Line Y birds in Hatch 1

Line Y birds were challenged with water or *Lactobacillus johnsonii* (LJ) at Day 0 and sampled at 4, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LY birds (n = 5 birds per pen), black circles = water challenge, blue circles = LJ challenged birds. **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, blue columns = LJ challenged birds (** P < 0.01, Bonferroni post-test following Two-way ANOVA). **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and LJ challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

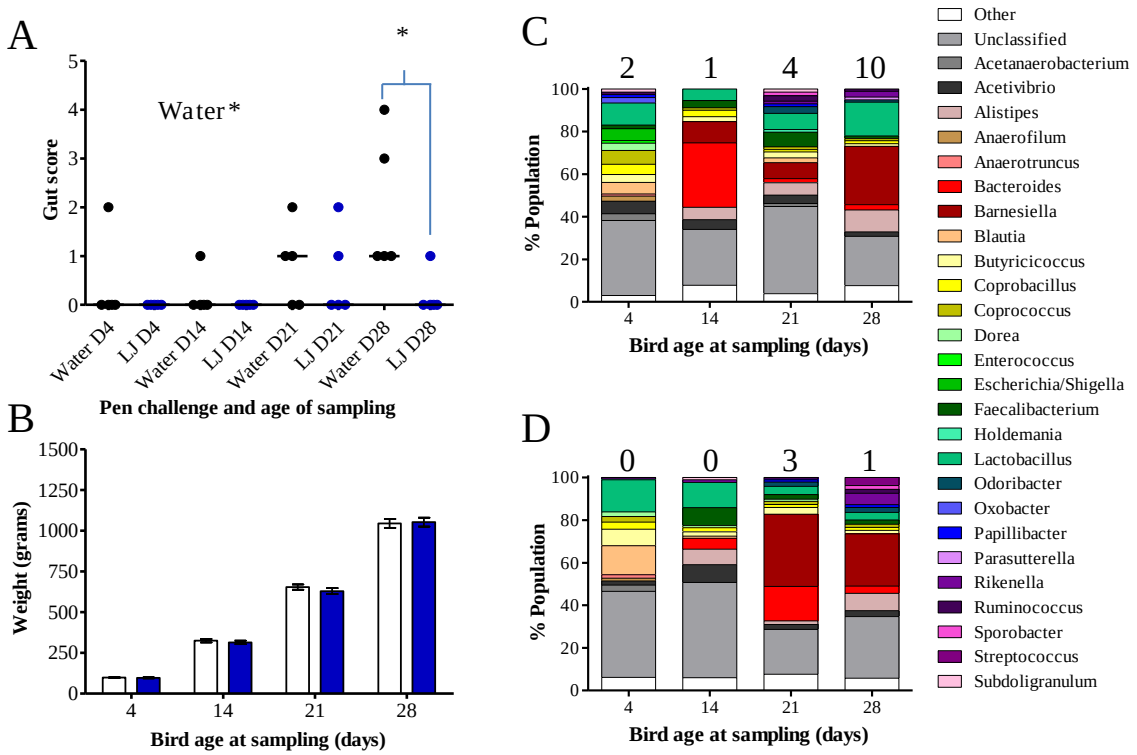


Figure 3.8: The effects of *Lactobacillus johnsonii* (LJ) challenge on Line Y birds in Hatch 2.

Line Y birds were challenged with water or *Lactobacillus johnsonii* (LJ) at Day 0 and sampled at 4, 14, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LYbirds (n = 5 birds per pen), black circles = water challenge, blue circles = LJ challenged birds. . (* $P < 0.05$, Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, blue columns = LJ challenged birds. **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and LJ challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

3.2.4 Effects of Bacteroides spp. /Barnesiella viscericola spp. challenge (B/BV) on gut health, bird weight and caecal microbiota

3.2.4.1 Line X

Figures 3.9 and 3.10 illustrate individual Line X bird gut health at each sampling timepoint (A), the mean weight per bird (B), the caecal microbiome of water control birds (C) and B/BV challenged birds (D) for hatches 1 and 2, respectively.

For both hatches, higher median gut score values were observed in the B/BV challenged groups at 21 and 28 days-post hatch, suggesting an adverse effect of B/BV challenge on gut health although no statistically significant differences were revealed ($P > 0.05$; Dunn's multiple comparison test) (Figures 3.9A and 3.10A). Significantly different gut scores were observed between sampling time-points for the B/BV challenged group in Hatch 1 ($P < 0.05$) and Hatch 2 ($P < 0.01$) indicating a deterioration in gut health up to Day 28.

In Hatch 1, the water control birds aged 21 days were significantly heavier than the B/BV challenge groups ($P <0.05$, Bonferroni post-tests), although there were no differences observed in this pen 1 week later at Day 28. No weight differences were observed in Hatch 2.

In Hatch 2 the Day 4 caecal microbiome in the B/BV challenged group contained *Bacteroides* spp. at a relative abundance of 40%, but this was not replicated in the water control pen. Relative to the water control birds, the microbiota from the Line X B/BV challenged groups contained higher abundances of *Butyricicoccus* spp. at Day 4 (Hatch 1: 3% vs. 8%; Hatch 2: 6% vs. 11%), *Lactobacillus* spp. at Day 21 (Hatch 1: 7% vs. 16%; Hatch 2: 10% vs. 16%) and *Alistipe*s spp. at Day 28 (Hatch 1: 6% vs. 11%; Hatch 2: 3% vs. 9%). Relative to the water control birds the B/BV challenged group had lower abundances at Day 4 of *Blautia* spp. (Hatch 1: 7% vs 4%; Hatch 2 5% vs. 3%) and *Faecalibacterium* spp. (Hatch 1: 6% vs. 5%; Hatch 2: 13% vs. 7%) and at Day 28 lower relative abundances of *Lactobacillus* spp. were found (Hatch 1: 24% vs. 12%; Hatch 2: 15% vs. 8%).

3.2.4.2 Line Y

Figures 3.11 and 3.12 illustrate the individual gut health scores of Line Y birds at each sampling time-point (A), the mean weight per bird (B), the caecal microbiome of water control birds (C) and the B/BV challenge birds (D) for hatches 1 and 2, respectively.
There were no significant differences in gut scores between the B/BV challenged pens and the water pens at any of the sampling time-points in either hatch 1 or 2 ($P > 0.05$; Dunn's multiple comparison test). This was reflected in the total gut scores per sampling group i.e. the gut scores for the water control birds were 4 and 10 compared to 5 and 8 for the B/BV challenged birds for hatch 1 and 2, respectively. In addition, no significant differences were found in mean bird weight for the water and the B/BV challenged groups for either hatch.

A significant age effect was found in Hatch 2 for both the B/BV and the water challenge pen ($P < 0.05$; Kruskal-Wallis test), indicating that the gut health was worse at the later sampling time-points, irrespective of challenge.

In general, the Line Y birds were resistant to the B/BV challenge in that their gut health was robust and not adversely affected. At Day 4 the microbiota of the B/BV challenged pens differed to their corresponding control pen by showing reduced levels of *Lactobacillus* spp. (Hatch 1: 41% vs. 3%; Hatch 2: 10% vs. 3%). The abundance of *Bacteroides* spp. was higher in the B/BV challenged group in Hatch 1 at Day 21 (24% vs. 33%) and at Day 14 in Hatch 2 (30% vs. 37%). At Day 21 in Hatch 2 a much larger proportion of the microbiota in the B/BV challenged group consisted of *Barnesiella* spp. in comparison with the control pen (8% vs. 48%) but no obvious differences in *Barnesiella* spp. were seen in Hatch 1.

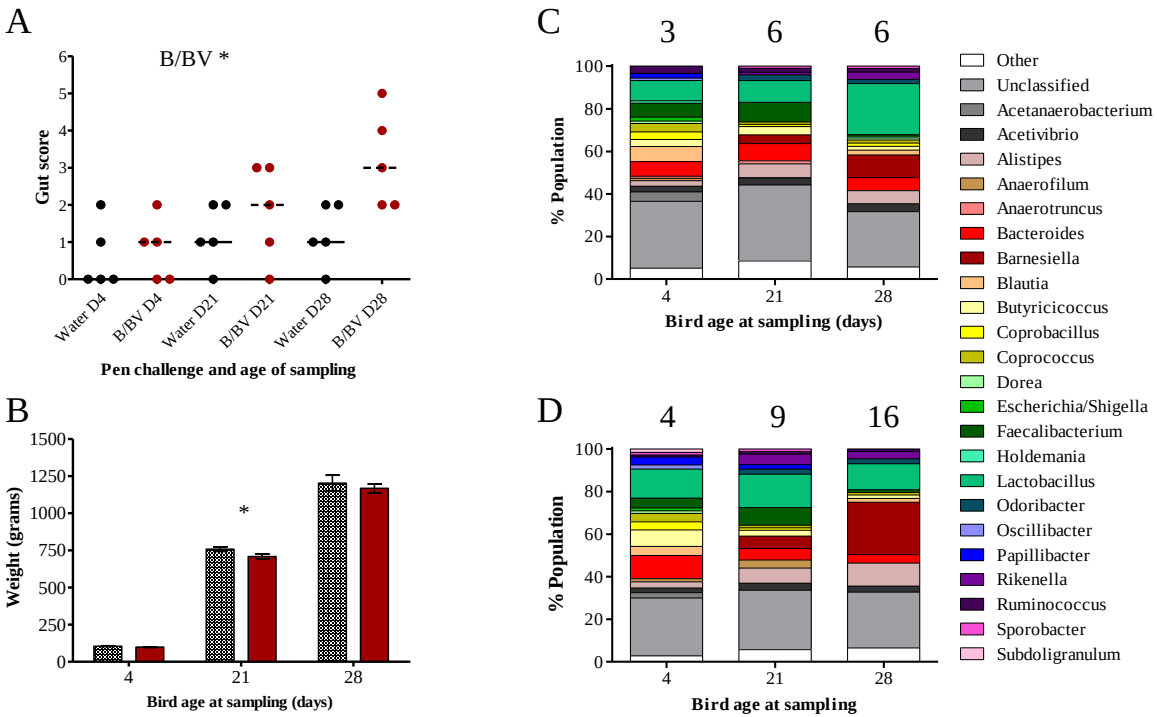


Figure 3.9: The effects of *Bacteroides dorei* + *Barnesiella viscericola* challenge on Line X birds in Hatch 1.

Line X birds were challenged with water or *Bacteroides dorei* + *Barnesiella viscericola* (B/BV) at Day 0 and sampled at 4, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LX birds (n = 5 birds per pen), black circles = water challenge, red circles = B/BV challenged birds, (* $P < 0.05$, Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, red columns = B/BV challenged birds (*$P < 0.001$, Bonferroni post-test following Two-way ANOVA). **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and B/BV challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling time-point is shown above each column.

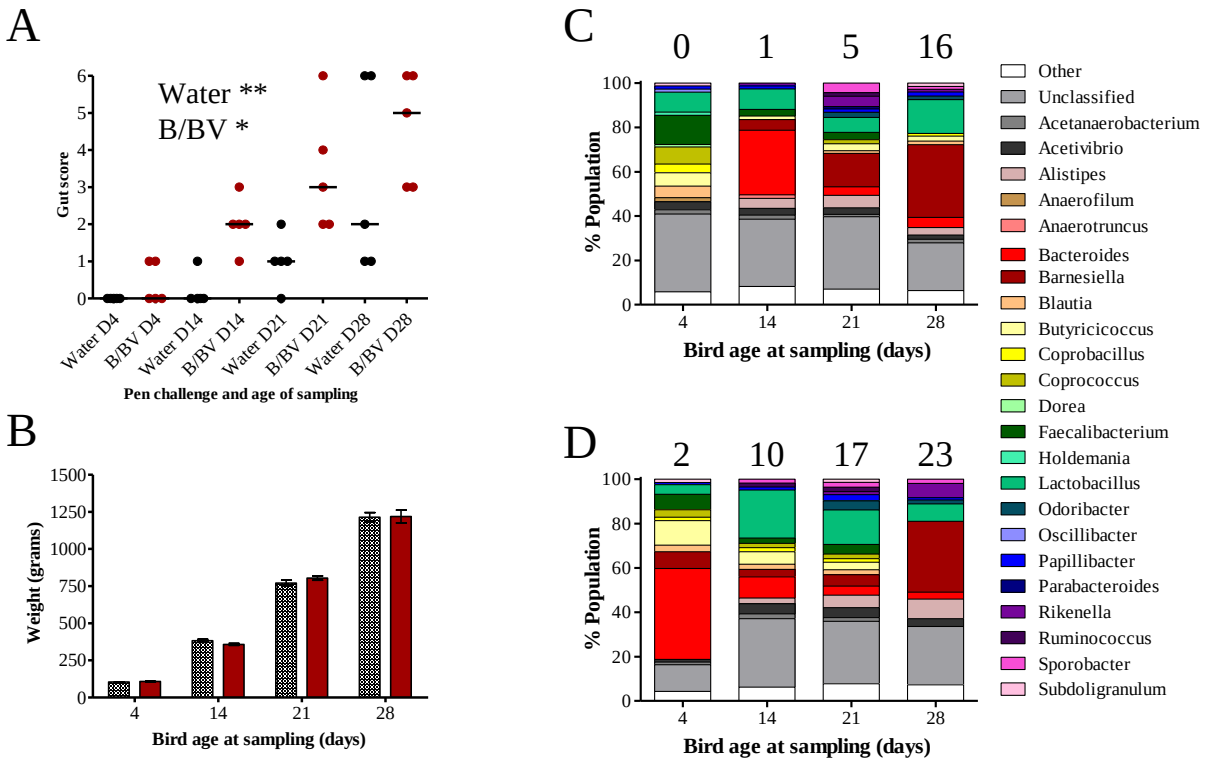


Figure 3.10: The effects of *Bacteroides dorei* + *Barnesiella viscericola* challenge on Line X birds in Hatch 2.

Line X birds were challenged with water or *Bacteroides dorei* + *Barnesiella viscericola* (B/BV) at Day 0 and sampled at 4, 14, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LX birds (n = 5 birds per pen), black circles = water challenge, red circles = B/BV challenged birds, (* $P < 0.05$, ** $P < 0.01$; Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, red columns = B/BV challenged birds.**C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and B/BV challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling time-point is shown above each column.

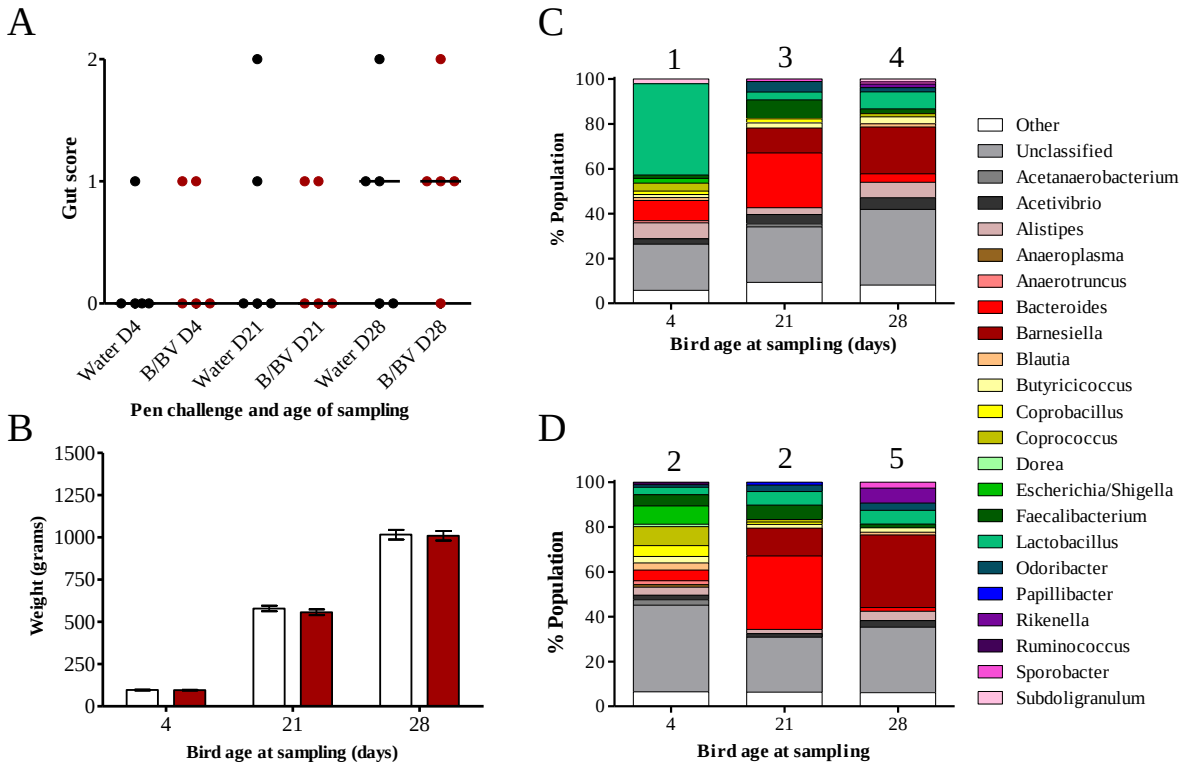


Figure 3.11: The effects of *Bacteroides dorei* + *Barnesiella viscericola* challenge on Line Y birds in Hatch 1.

Line Y birds were challenged with water or *Bacteroides dorei* + *Barnesiella viscericola* (B/BV) at Day 0 and sampled at 4, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LY birds (n = 5 birds per pen), black circles = water challenge, red circles = B/BV challenged birds, (* $P < 0.05$, Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of variation % (n = 100); filled columns = water challenged, red columns = B/BV challenged birds. **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and B/BV challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

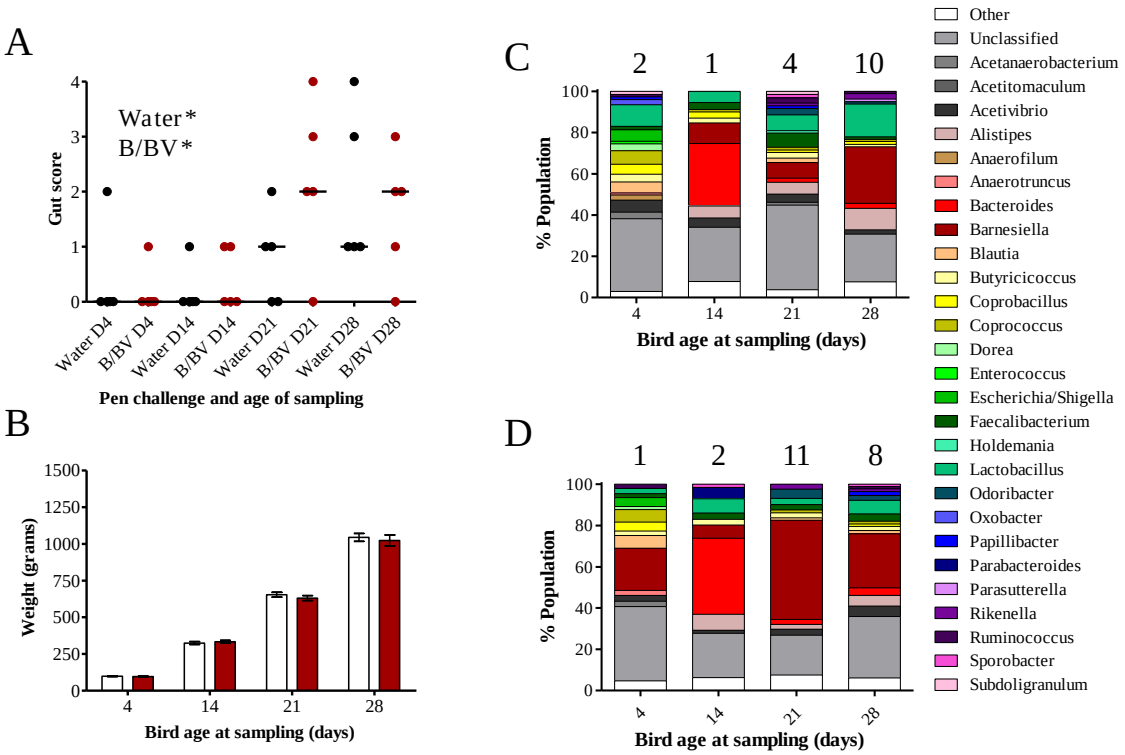


Figure 3.12: The effects of *Bacteroides dorei* + *Barnesiella viscericola* challenge on Line Y birds in Hatch 2.

Line Y birds were challenged with water or *Bacteroides dorei* + *Barnesiella viscericola* (B/BV) at Day 0 and sampled at 4, 14, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LYbirds (n = 5 birds per pen), black circles = water challenge, red circles = B/BV challenged birds, (* $P < 0.05$, Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, red columns = B/BV challenged birds. **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and B/BV challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

3.2.5 Effects of mix challenge (B/BV + LJ) on gut health, bird weight and caecal microbiota

3.2.5.1 Line X

Figures 3.13 and 3.14 illustrate individual Line X bird gut health scores at each sampling timepoint (A), the mean weight per bird (B), the caecal microbiome of water control birds (C) and mix challenged birds (D) for hatches 1 and 2, respectively.

For Hatches 1 and 2, no significant differences in gut health were observed between the water and mix challenge pens ($P > 0.05$, Dunn's multiple comparison test), although at Day 28 for Hatch 1, the median gut health for the mix challenge was two compared to one in the water control group. For Hatch 1, a significant effect of sampling age on gut health was observed in the mix challenged group indicating that gut health worsens at the later sampling time-points; this did not reach statistical significance in the water challenged pens ($P < 0.05$, Kruskal-Wallis test). Despite the gut health worsening over time, seeding the pens with mixed bacteria had no significant effect on mean Line X bird weight for either hatch.

In comparison to the water control birds , the caecal microbiome of the Line X mix challenge birds had a higher abundance of *Barnesiella* spp. at Day 4 (Hatch 2: 0% vs. 18%), Day 21 (Hatch 2: 15% vs. 23%) and Day 28 (Hatch 1: 11% vs. 23%). The relative abundance of *Lactobacillus* spp. at Day 4 was higher in the 'mix' challenged birds compared to the water control birds (Hatch 1: 10% vs. 18%; Hatch 2: 9% vs. 16%). In contrast, *Lactobacillus* spp. was less abundant in the 'mix' challenge pens at Day 28 (Hatch 1: 24% and 10%; Hatch 2: 15% vs. 9%).

3.2.5.2 Line Y

Figures 3.15 and 3.16 illustrate individual Line Y bird gut health at each sampling timepoint (A), the mean weight per bird (B), the caecal microbiome of water control birds (C) and B/BV challenged birds (D) for hatches 1 and 2, respectively.

No significant differences in gut health scores were observed between the water controls and 'mix' challenge birds ($P > 0.05$, Dunn's multiple comparison test). Consistent to both hatches was that the 'mix' challenge appeared to result in lighter birds than the water

challenge pen (Hatch 1: mean weight 1016g vs. 982g; Hatch 2: 1044g vs. 1029g)($P < 0.01$ and $P < 0.001$, Bonferroni post-tests following Two-way ANOVA).

Compared to the water control pens the caecal microbiome of the 'mix' challenged birds contained higher relative abundances of *Bacteroides* spp. at Day 21 (Hatch 1: 24% vs. 39%; Hatch 2: 2% vs. 14%) and Day 28 (Hatch 1: 4% vs. 6%; Hatch 2: 2% vs. 12%). Lower abundances of *Barnesiella* spp. were found in the 'mix' challenged birds at Day 28 (Hatch 1: 21% vs. 14%; Hatch 2: 27% vs. 22%). Noteworthy differences found in single hatches were that relatively high levels of *Escherichia/shigella* spp. were found in Hatch 1 mix challenged birds (17%), and, as described earlier, high *Lactobacillus* spp. levels were also found in the water control pen in this hatch (40%).

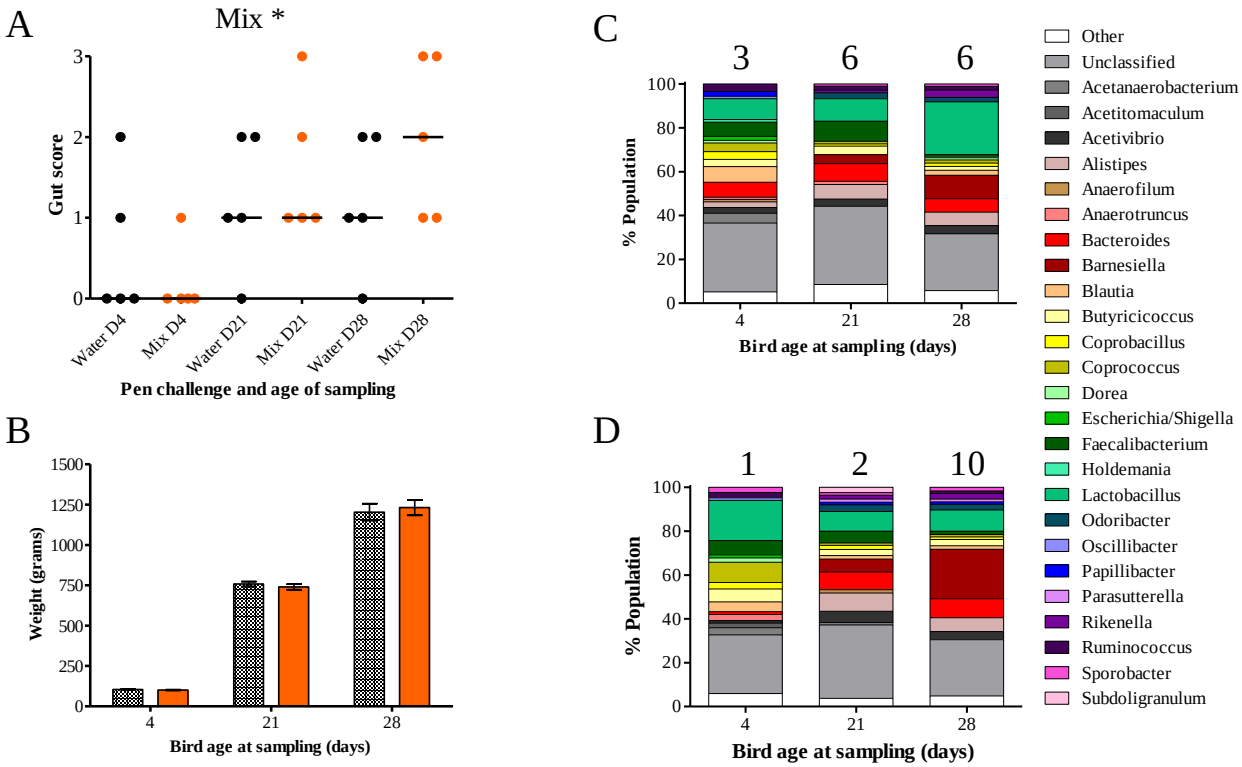


Figure 3.13: The effects of mix challenge (B/BV + LJ) on Line X birds in Hatch 1.

Line X birds were challenged with water or mixture (B/BV + LJ) at Day 0 and sampled at 4, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LX birds (n = 5 birds per pen), black circles = water challenge, orange circles = Mix challenged birds, (* $P < 0.05$, Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, orange columns = B/BV challenged birds. **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and Mix challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

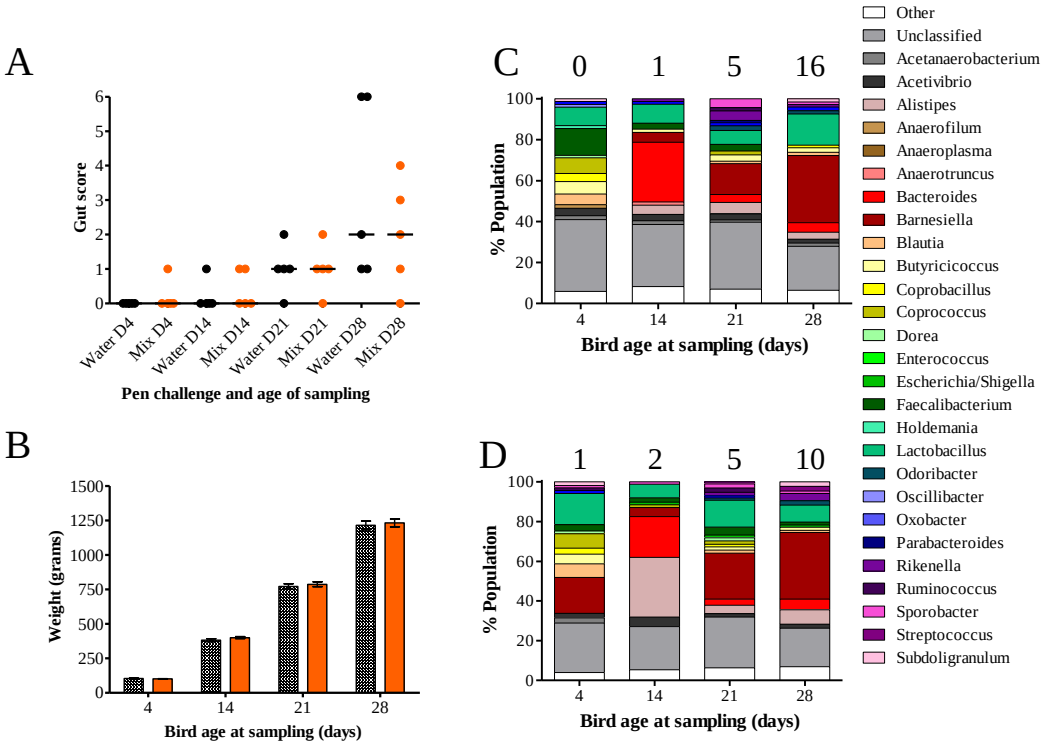


Figure 3.14: The effects of mix challenge (B/BV + LJ) on Line X birds in Hatch 2.

Line X birds were challenged with water or mixture (B/BV + LJ) at Day 0 and sampled at 4, 14, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LX birds (n = 5 birds per pen), black circles = water challenge, orange circles = Mix challenged birds, (* $P < 0.05$, Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, orange columns = Mix challenged birds (*** $P < 0.001$, Bonferroni post-test following Two-way ANOVA). **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and Mix challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

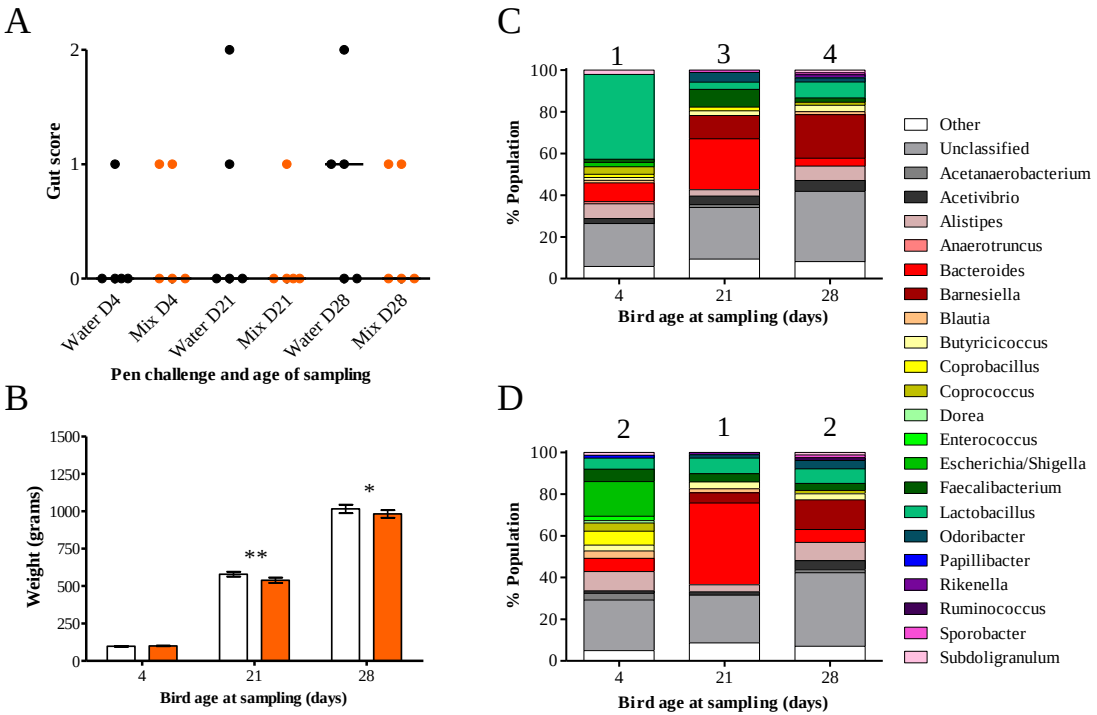


Figure 3.15: The effects of mix challenge (B/BV + LJ) on Line Y birds in Hatch 1.

Line Y birds were challenged with water or mixture (B/BV + LJ) at Day 0 and sampled at 4, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LY birds (n = 5 birds per pen), black circles = water challenge, orange circles = Mix challenged birds, (* $P < 0.05$, Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, orange columns = B/BV challenged birds (* $P < 0.05$, ** $P < 0.01$, Bonferroni post-test following Two-way ANOVA). **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and Mix challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

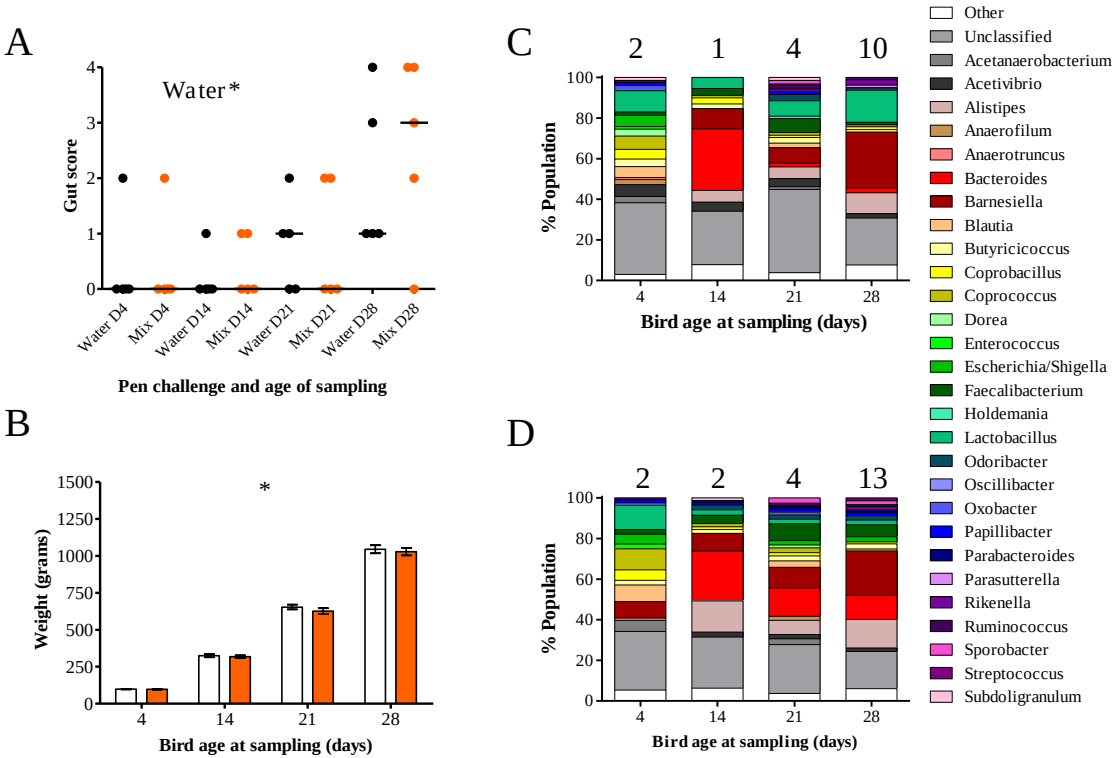


Figure 3.16: The effects of mix challenge (B/BV + LJ) on Line Y birds in Hatch 2.

Line Y birds were challenged with water or mixture (B/BV + LJ) at Day 0 and sampled at 4, 14, 21 and 28 days post-hatch. **A**: Gut health assessments for individual LY birds (n = 5 birds per pen), black circles = water challenge, orange circles = Mix challenged birds, (* $P < 0.05$, Kruskal-Wallis test). **B**: Average bird weight per pen ± co-efficient of Variation % (n = 100); filled columns = water challenged, orange columns = Mix challenged birds (* $P < 0.05$, Bonferroni post-test following Two-way ANOVA). **C & D**: Relative abundance of bacterial genus (% population) in caecal digesta of water challenged (C) and Mix challenged birds (D) (n = 5 birds per pen). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

3.2.6 Summary of caecal microbiotae at the phylum level

Although a relatively large percentage of bacterial species could not be identified, the composition of the caecal microbiota for all groups of birds could be broadly categorised at the phylum level into Firmicutes (predominantly *Lactobacillus* spp., *Faecalibacterium*, *Blautia*, *Butyricicoccus* and *Coprobacillus*) and Bacteroidetes (predominantly *Alistipes* spp, *Bacteroides* spp. and *Barnesiella* spp.). Figure 3.17 summarises the composition of the caecal microbiotae at the phylum level and shows the effect of bird age and the type of bacterial challenge. In brief, at 4 days following challenge there were few trends that were consistent across Hatches 1 and 2, although the Hatch 2 B/BV challenge did result in a large increase (>40%) in species belonging to the Bacteroidetes phyla for both Lines X and Y. The microbiotae data at Day 28 showed no obvious effect of bacterial challenge although the microbiotae had shifted away from the Firmicutes found at Day 4 to Bacteroidetes particularly *Barnesiella* (as shown in Figures 3.3 – 3.16).

3.2.7 The relationship between gut health score and caecal microbiome composition

Taking both Lines X and Y into account, no single microbial shift could be identified that was linked to deterioration of gut health. Analysis of all healthy/normal pens at the Day 4 sampling timepoint (Line Y LJ challenged pens in both hatches and Line X Hatch 2 control) revealed no consistent composition of microbiota that was different to pens containing abnormal birds. At Day 28, although large differences in gut health between pens was observed (total pen score 1 – 23), the microbiotas at this time-point were similar with *Barnesiella* spp. as the dominant species. However, a significant correlation was found between the relative abundance of *Bacteroides* spp. at Day 21 and the gut health of these birds at Day 21 and Day 28 (Figure 3.18A and B).

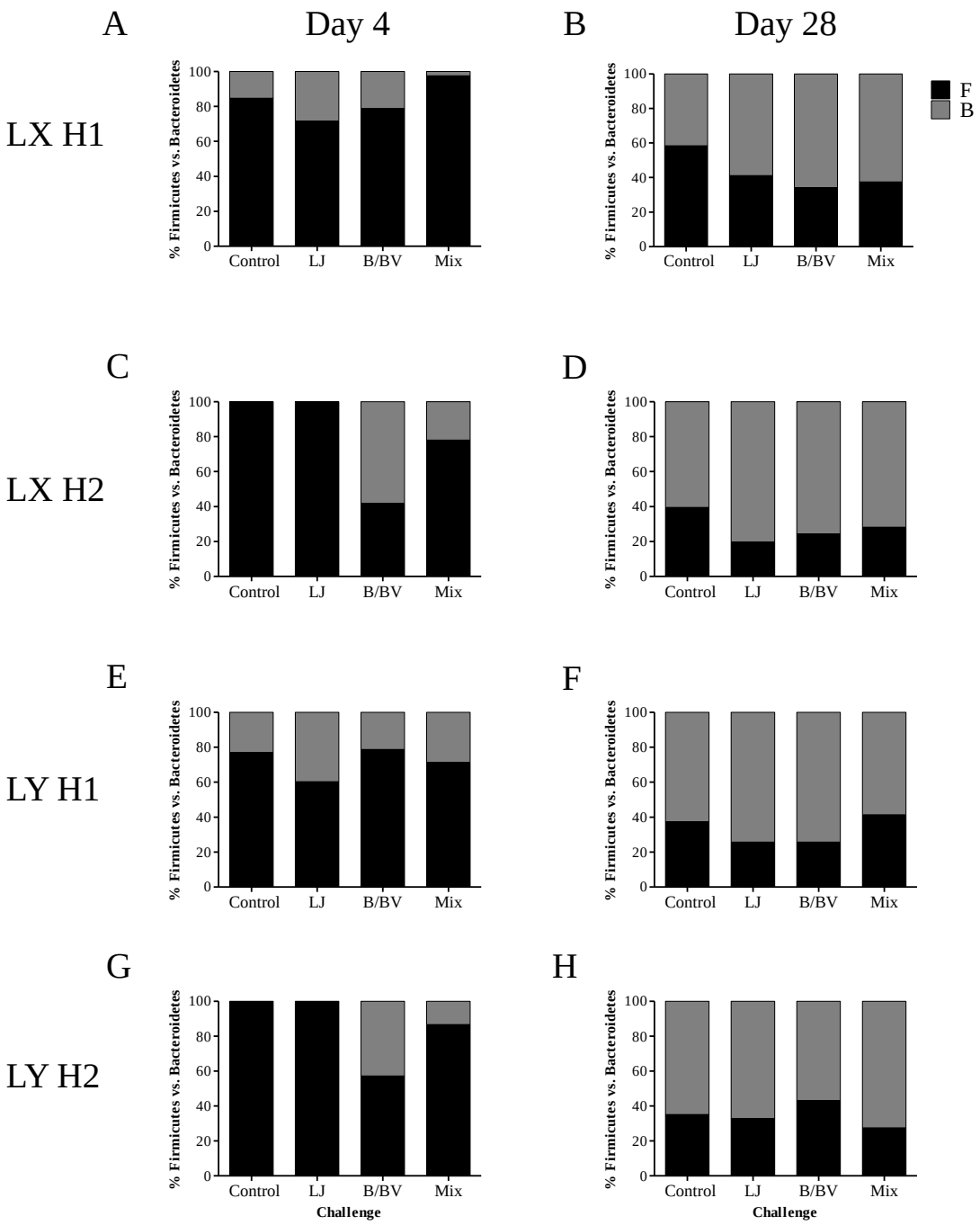


Figure 3.17: The effect of bird age and species of bacterial challenge on caecal microbiotae at the phylum level

The relative bacterial abundances (%), taking into account only Firmicutes (black) and Bacteroidetes (grey), are shown for groups of birds (n = 5) challenged with water (control), *Lactobacillus johnsonii* (LJ), *Bacteroides dorei/Barnesiella viscericola* (B/BV) and mixture (LJ + B/BV) at hatch and sampled at Day 4 (A, C, E and G) and Day 28. LX – Line X, LY – Line Y, H1 – hatch 1, H2 – hatch 2.

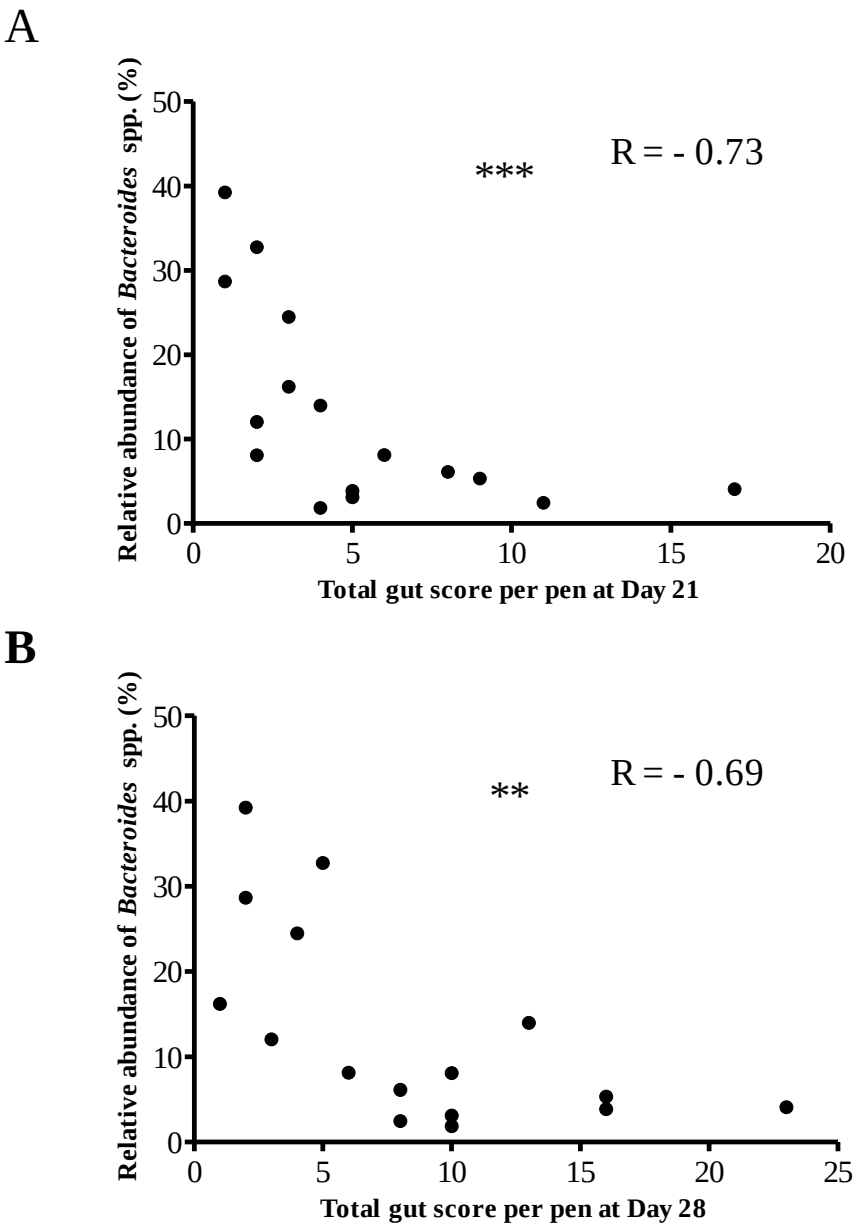


Figure 3.18: The correlation between relative abundance of *Bacteroides* spp. in the caecal digesta and total gut score.

The data shows the abundance of *Bacteroides* spp. (%) in the digesta from birds sampled at 21 days post-hatch and the total gut score per pen at 21 days (A) and 28 days post-hatch (B) (*** $P < 0.001$; ** $P < 0.01$; Spearman Rank correlation).

3.2.7 Ileal microbiome of challenged broilers

The relative abundance of bacterial genera from ileal digesta for Line X and Y birds are shown in Figures 3.19 – 3.22, respectively.

Lactobacillus was the most abundant species found in all sampled groups apart from the Line X mix challenged pen sampled at Day 14 (Hatch 2) in which *Escherichia/shigella* spp. were found to dominate at 69% (Figure 3.21D). This high level of *Escherichia/shigella* spp. appeared to have no detrimental effect on gut health (group gut score 2) compared to other Line X Hatch 2 pens. This unusual microbiome appeared to be short-lived as no *Escherichia/shigella* spp. were found in the ileal digesta of birds sampled seven days later at 21 days post-hatch.

For both hatches, Line Y birds had increased bacterial diversity compared to Line X birds. The highest number of bacterial genera was found in digesta from 21 day-old birds and diversity was highest in Line Y *Lactobacillus johnsonii* challenged birds. For example, digesta from Line Y *Lactobacillus johnsonii* challenged birds (Hatch 1) contained bacteria from 8 genera at low abundances of 1 – 4% (*Weissella* spp., *Streptococcus* spp., *Staphylococcus* spp., *Corynebacterium* spp., *Brevibacterium* spp., *Brachybacterium* spp. and *Atopostipes* spp.) in contrast to the equivalent Line X group in which *Lactobacillus* spp. were the only bacterial species identified. However, by 28 days-post hatch little variation in the microbiome was observed and *Lactobacillus* spp. levels had increased to over 89% for all pens.

The Line X: B/BV challenged group in Hatch 2 displayed the worst gut health and contained lower levels of *Lactobacillus* spp. and higher levels of *Enterococcus* spp. than the LJ and mix challenged pens, which had better gut health. Plotting the relative abundance of *Lactobacillus* spp. found in digesta at 4 days post-hatch in Line X birds against total gut health per pen at Day 28 identified a significant inverse correlation ($P < 0.05$) (Figure 3.23). However, no relationship between early *Lactobacillus* spp. colonisation and gut health was found in Line Y birds ($P > 0.05$). Irrespective of line, a comparison of the pen with the worst gut health (Line X H2: B/BV challenge, Figure 3.21C) and the best gut health (Line Y H2: LJ challenge, Figure 3.22B) revealed no differences in *Lactobacillus* spp. levels at Day 4 with relative abundance levels of 75% for both pens.

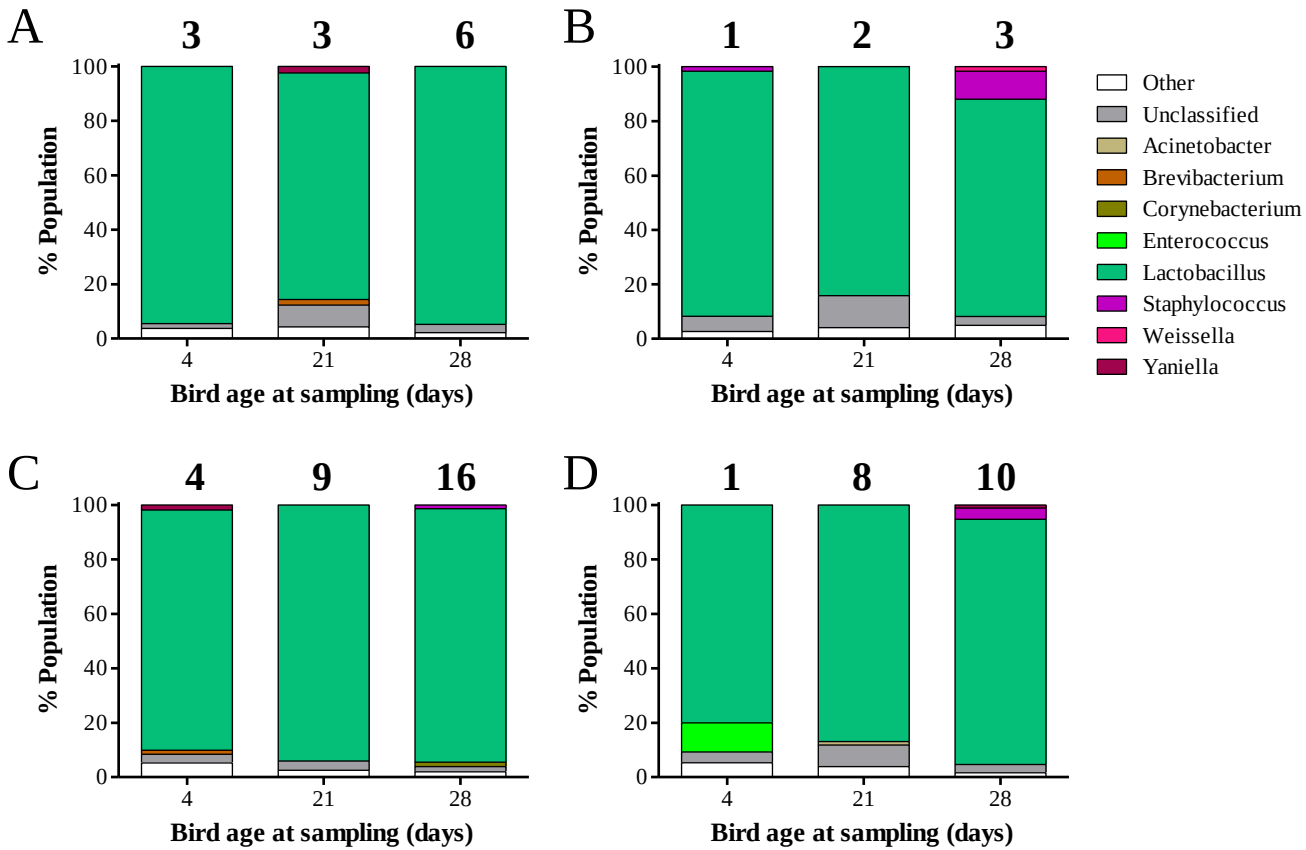


Figure 3.19: The ileal microbiome of Line X birds in Hatch 1.

Relative abundance of bacterial genus (% population) in ileal digesta of Hatch 1 Line X birds (n = 5) sampled at 4, 21 and 28 days post-hatch from pens challenged with A) water, B) *Lactobacillus johnsonii* (LJ), C) *Bacteroides dorei/Barnesiella viscericola* (B/BV), D) Mixture (B/BV + LJ). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

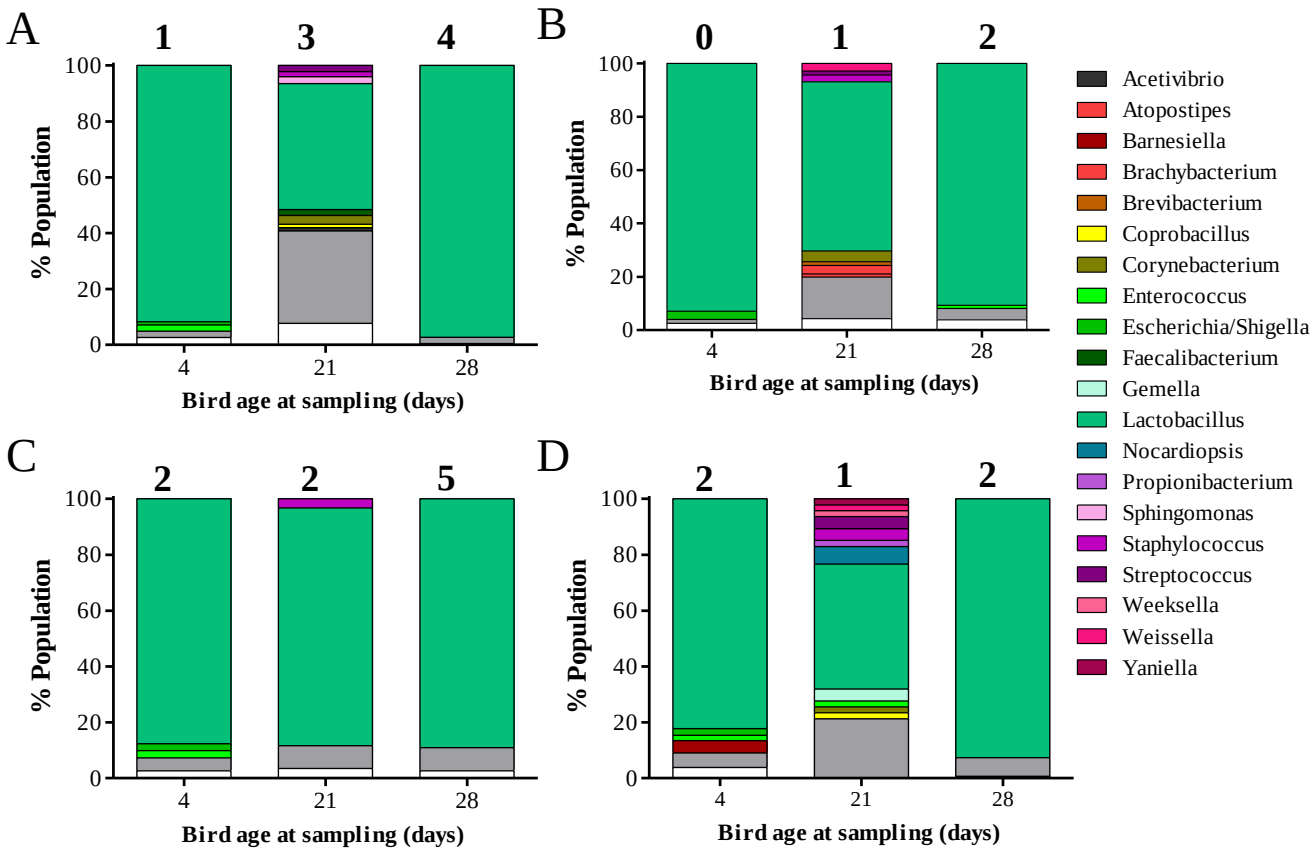


Figure 3.20: The ileal microbiome of Line Y birds in Hatch 1.

Relative abundance of bacterial genus (% population) in ileal digesta of Hatch 1 Line Y birds (n = 5) sampled at 4, 21 and 28 days post-hatch from pens challenged with A) water, B) *Lactobacillus johnsonii* (LJ), C) *Bacteroides dorei/Barnesiella* viscericola (B/BV), D) Mixture (B/BV + LJ). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

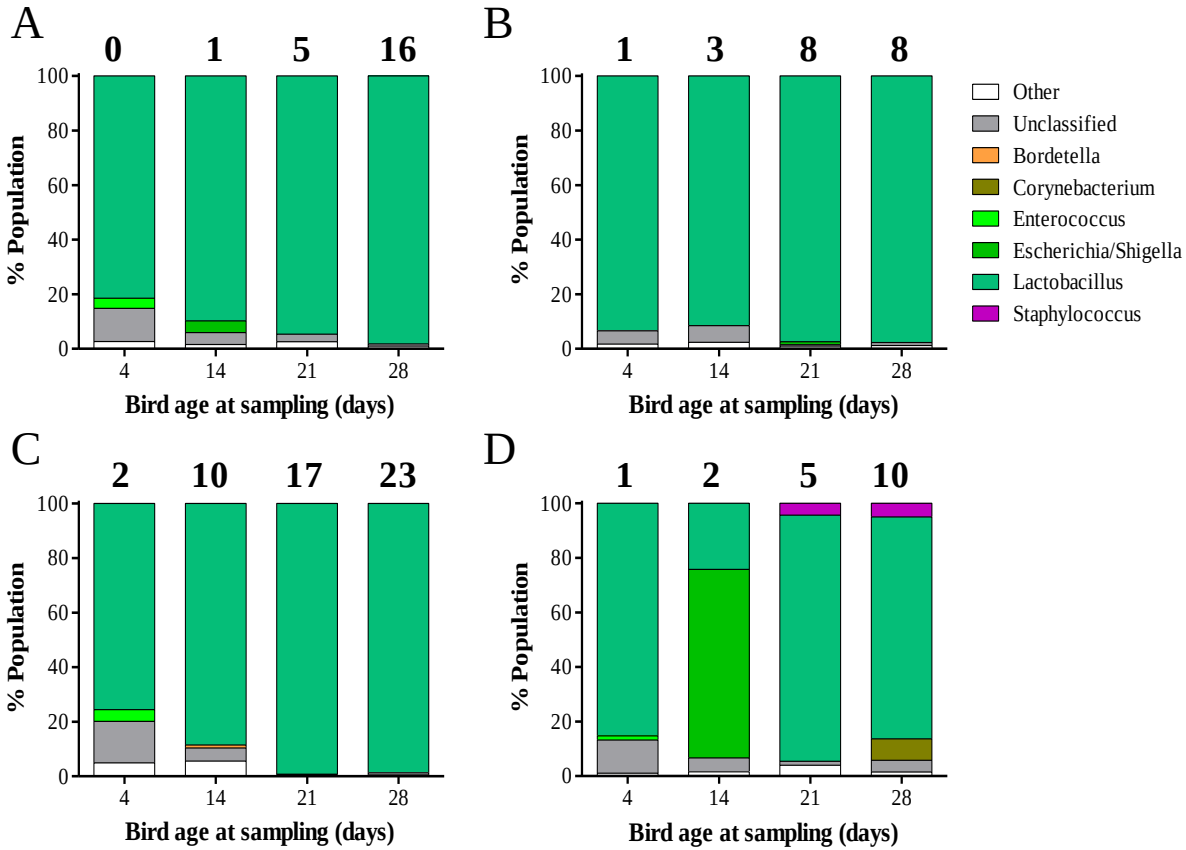


Figure 3.21: The ileal microbiome of Line X birds in Hatch 2.

Relative abundance of bacterial genus (% population) in ileal digesta of Hatch 2 Line X birds (n = 5) sampled at 4, 21 and 28 days post-hatch from pens challenged with A) water, B) *Lactobacillus johnsonii* (LJ),C) *Bacteroides dorei/Barnesiella viscericola* (B/BV), D) Mixture (B/BV + LJ). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

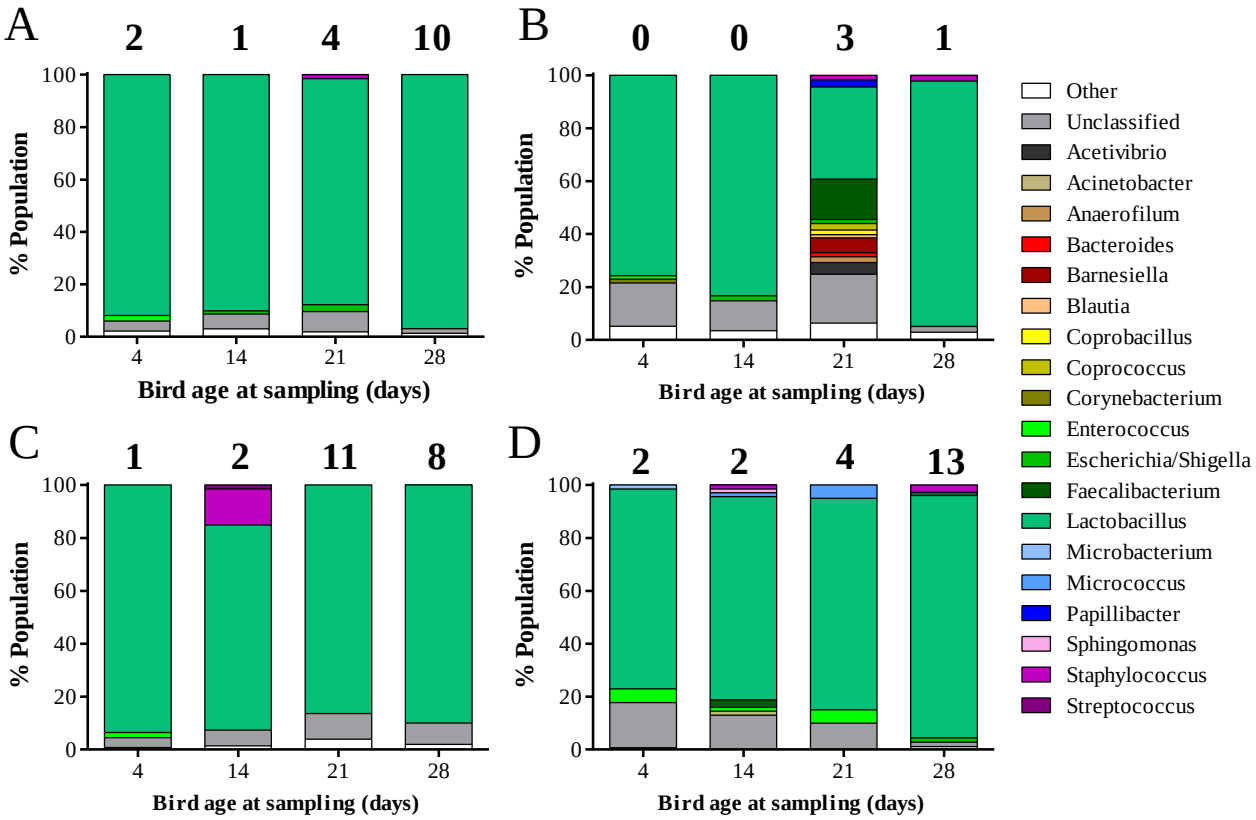


Figure 3.22: The ileal microbiome of Line Y birds in Hatch 2.

Relative abundance of bacterial genus (% population) in ileal digesta of Hatch 2 Line Y birds (n = 5) sampled at 4, 21 and 28 days post-hatch from pens challenged with A) water, B) *Lactobacillus johnsonii* (LJ),C) *Bacteroides dorei/Barnesiella viscericola* (B/BV), D) Mixture (B/BV + LJ). Corresponding total gut health score per sampled group (n = 5) at each sampling timepoint is shown above each column.

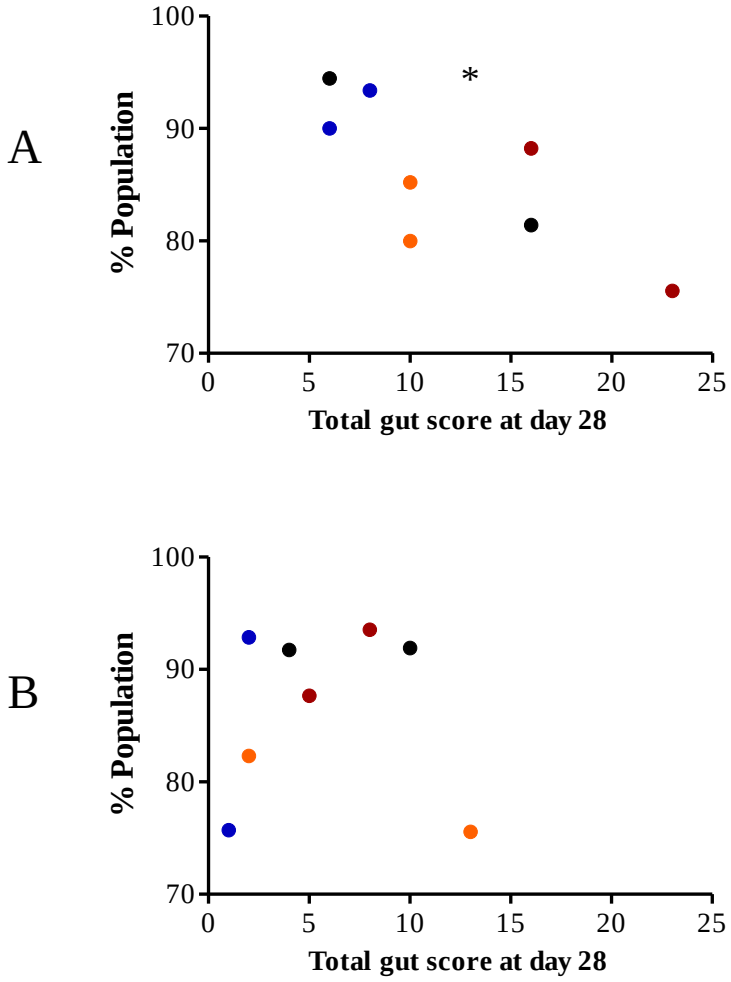


Figure 3.23: The relationship between the abundance of ileal *Lactobacillus* spp. at Day 4 and the total gut score at Day 28.

The abundance of *Lactobacillus* spp. identified in the ileal digesta of 4 day-old birds and the total gut score for 28 day-old birds is shown for A) Line X and B) Line Y birds. Black circles: water control, Blue circles: *Lactobacillus johnsonii* challenge, Red circles: *Bacteroides/Barnesiella viscericola* challenge and Orange circles: Mix challenge. Each data point represents 5 sampled birds (n = 5). * $P < 0.05$; One-tailed Spearman rank correlation.

3.2.8 Microbial activities of jejunal mucosal scrapes

Although the microbiome of the small intestine is mainly populated by *Lactobacillus* spp. (Gong et al., 2007), it has been revealed that intestinal overgrowth of bacterial species that are normally resident in the gut has been implicated in enteric diseases in poultry such as necrotic enteritis (Long et al., 1974) and dysbacteriosis (Bailey, 2010). To investigate the ability of the host intestinal mucosa to facilitate the growth of potentially opportunistic pathogens, in this case *E. coli*, total protein extracts from jejunal gut scrapes were prepared from the same birds that were assessed for microbiota and gut health.

Microbial growth assays for sampled jejunal scrapes from Line X and Line Y birds are shown in Figures 3.24 and 3.27 - 3.29, respectively. Data from Line X and Y water control groups in Hatches 1 and 2 are shown in Figure 3.24. For Hatch 1 no significant effect of bird line on *E. coli* growth was identified. When the Line X birds were analysed in isolation the gut scrapes from birds at day 21 post-hatch facilitated significantly less % *E. coli* growth than the Day 4 birds ($P < 0.01$, Students T-test). For Hatch 2 a significant effect of line was observed with mean % *E. coli* growth for the Line Y gut scrapes supporting higher mean % *E. coli* growth for all three sampling time-points, and this was found to be highly significant at Day 7 ($P < 0.001$, Bonferonni post-tests).

Figure 3.25A shows that Day 4 jejunal scrapes from control birds with abnormal gut health supported significantly higher % *E. coli* growth than scrapes from birds with healthy guts. When all bird data, irrespective of challenge, were pooled the same result was observed with higher % mean *E. coli* growth found in the abnormal group, however there was complete overlap between the normal and abnormal groups (Figure 3.25B). The *E. coli* growth (%) induced by gut scrapes from birds aged 21 days is presented in Figures 3.26A and B. No significant relationship was found between gut score and *E. coli* growth (%) for the either the water control birds (3.26A) or all challenged birds (3.26B).

A comparison of jejunal scrapes from Line X and Y birds from control and *Lactobacillus johnsonii* pens is shown in Figure 3.27. For Hatch 1: Line X (Figure 3.27A), there was a significant interaction between challenge and sampling time-point indicating that the challenge did not have the same effect at each time-point (Two-way ANOVA, $P < 0.01$). At Day 4 the gut scrapes from the LJ challenged birds induced significantly lower mean % *E. coli* growth than the water group ($P < 0.05$, Bonferonni post-tests). Similarly, the scrapes from the LJ challenged Hatch 1 Line Y birds also suppressed *E. coli* growth

relative to the water control (Figure 3.27B)(Two-way ANOVA, $P < 0.05$). However, in Hatch 2 the gut scrapes from Line X LJ challenged birds supported higher mean % *E. coli* growth than the water control groups at all sampling time-points (Figure 3.27C) (Two-way ANOVA, $P < 0.05$). Higher mean % *E. coli* growth was also observed in Line Y birds, but only for the gut scrapes taken from birds at 7 days post-hatch ($P < 0.05$, Bonferonni post-tests), which suggested that the response was age-dependent (interaction between sampling timepoint and effect of challenge; $P < 0.01$, Two-way ANOVA). At Day 4 and 21 there was a suggestion of reduced *E. coli* growth in Line Y: LJ challenged birds, but this was not statistically significant.

Figures 3.28A-D illustrate the differences in % *E. coli* growth induced by jejunal gut scrapes between B/BV and water challenged pens. The data from Hatch 1 shows that gut scrapes sampled from Line X: B/BV challenged birds supported significantly higher % *E. coli* growth than samples from the water challenged pen (Figure 3.28A)($P < 0.05$, Two-way ANOVA). Furthermore, the gut scrapes from Line X birds sampled at 21 days post-hatch were associated with lower *E. coli* growth than the Day 4 gut scrapes ($P < 0.05$, Two-way ANOVA) (Figure 3.28A). In Hatch 2, significantly higher E. *coli* growth was supported by gut scrapes from the Line X ($P < 0.05$) and Line Y B/BV challenged birds ($P < 0.01$) sampled at 7 days post-hatch (Figures 3.28C and D).

Figures 3.29A-D show the differences in % *E. coli* growth associated with jejunal gut scrapes from water and mix challenged Line X and Line Y birds. For Hatch 1 no significant differences in *E. coli* growth between birds from mix challenged and control pens were observed for either Line X (Figure 3.29A) or Line Y (Figure 3.29B). However, for Hatch 2, gut scrapes from Line X birds facilitated higher mean % *E. coli* growth than the water control birds across all three sampling time-points ($P < 0.01$, two-way ANOVA). Moreover, a significant effect of bird age was found. Gut scrapes from birds sampled at Day 4 supported higher mean % *E. coli* growth than jejunal scrapes taken at 7 and 21 days post-hatch ($P < 0.05$). There was no effect of challenge in Line Y birds for this hatch although % *E. coli* growth did vary significantly with age ($P < 0.01$, Two-way ANOVA).

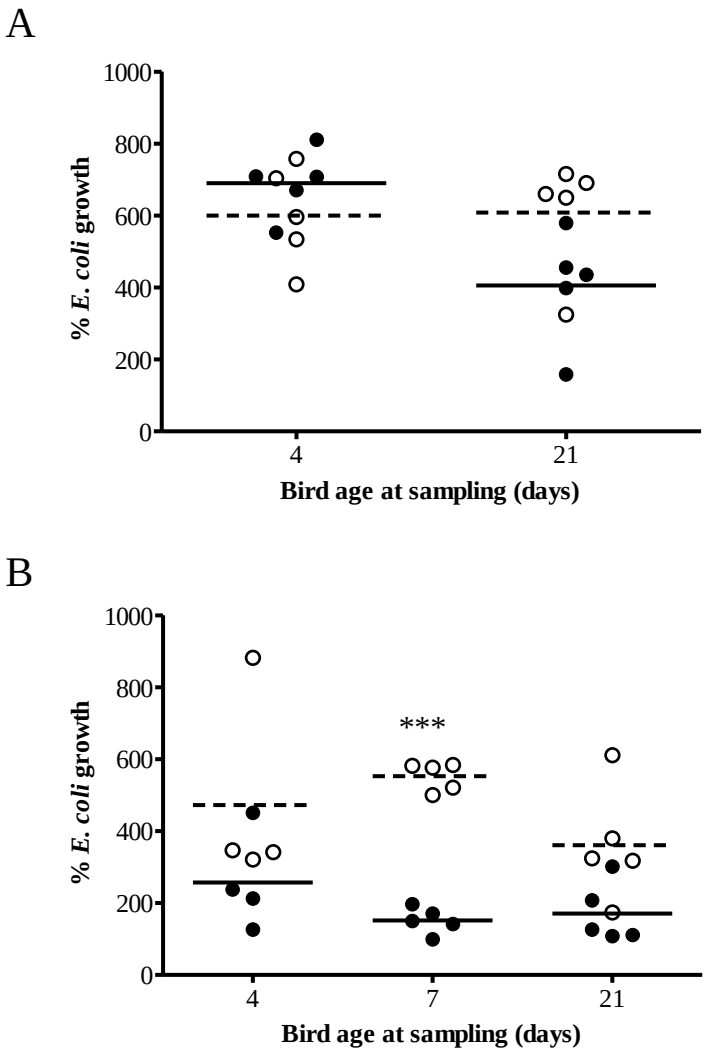


Figure 3.24: The effect of jejunal protein extracts from Line X and Y birds on % *E. coli* survival.

Microbial growth assay (colony counting) data shows the mean and individual % *E. coli* survival facilitated by jejunal gut extracts from Line X and Y birds aged 4, 7 and 21 days for Hatch 1 (A) and Hatch 2 (B) (n=4/5 for all groups). Line X – solid circles, Line Y – open circles, Solid line – Line X mean. Dotted line – Line Y mean.

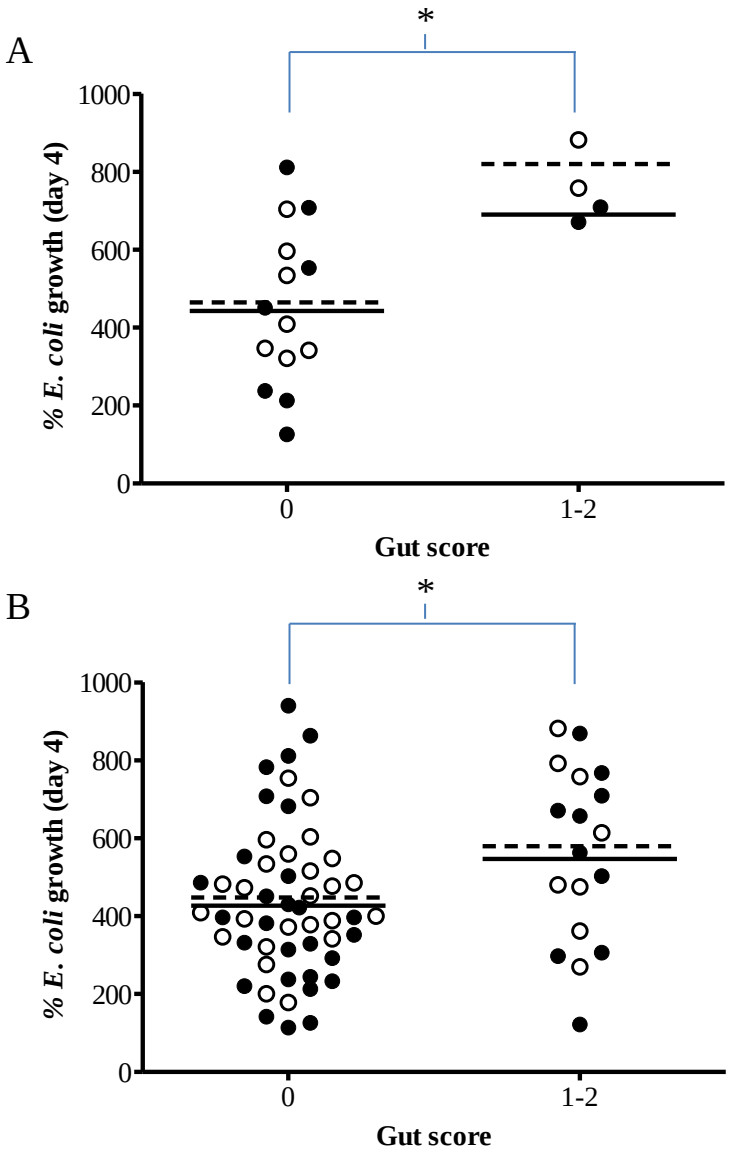


Figure 3.25: Jejunal protein extracts from abnormal birds aged 4 days-old support higher % *E. coli* growth.

E. coli growth (%) was calculated in a colony counting assay following incubation with jejunal mucosal proteins extracted from Line X and Y birds aged 4 days-old with normal and abnormal gut scores. Birds were sampled from (A) water control pens (B) all pens irrespective of challenge. Gut score 0 = normal gut health; Gut score 1-2 = abnormal gut health. Line X – solid circles, Line Y – open circles, Solid Line – Line X mean, Dotted Line – Line Y mean.

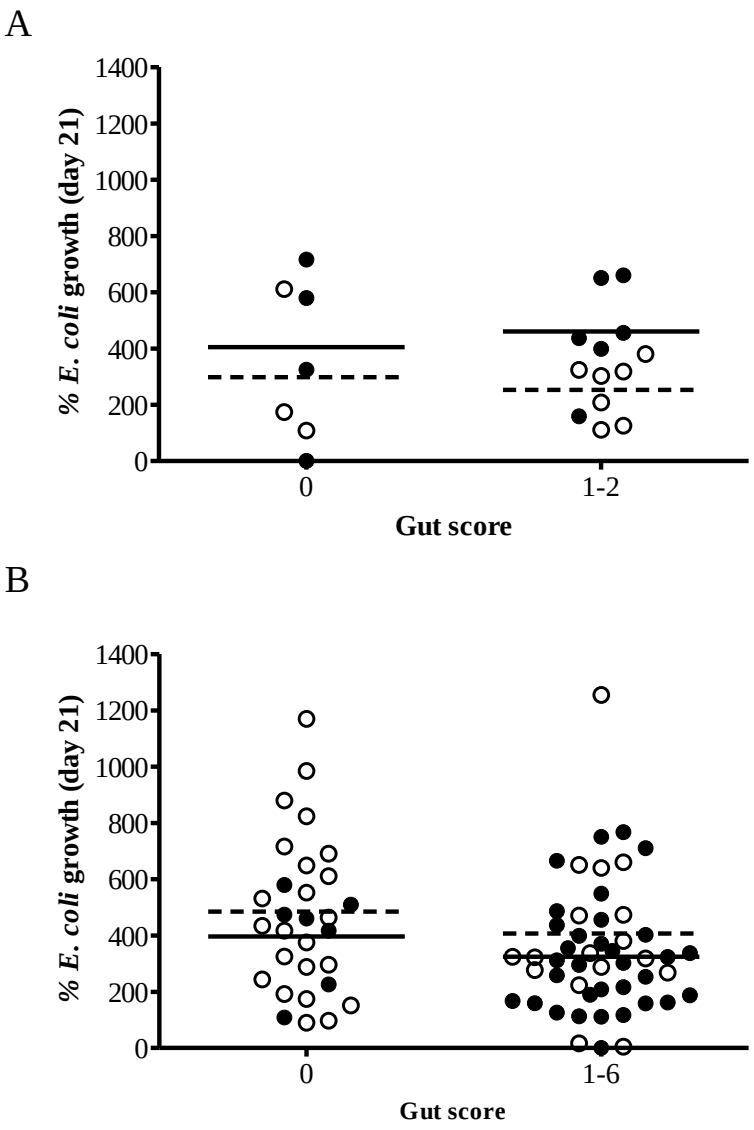


Figure 3.26: Jejunal protein extracts from normal and abnormal birds aged 21 days-old support similar levels of % *E. coli* growth.

E. coli growth (%) was calculated in a colony counting assay following incubation with jejunal mucosal proteins extracted from Line X and Y birds aged 21 days-old with normal and abnormal gut scores. Birds were sampled from (A) water control pens (B) all pens irrespective of challenge. Gut score 0 = normal gut health; Gut score 1-6 = abnormal gut health. Line X – solid circles, Line Y – open circles, Solid Line – Line X mean, Dotted line – Line Y mean.

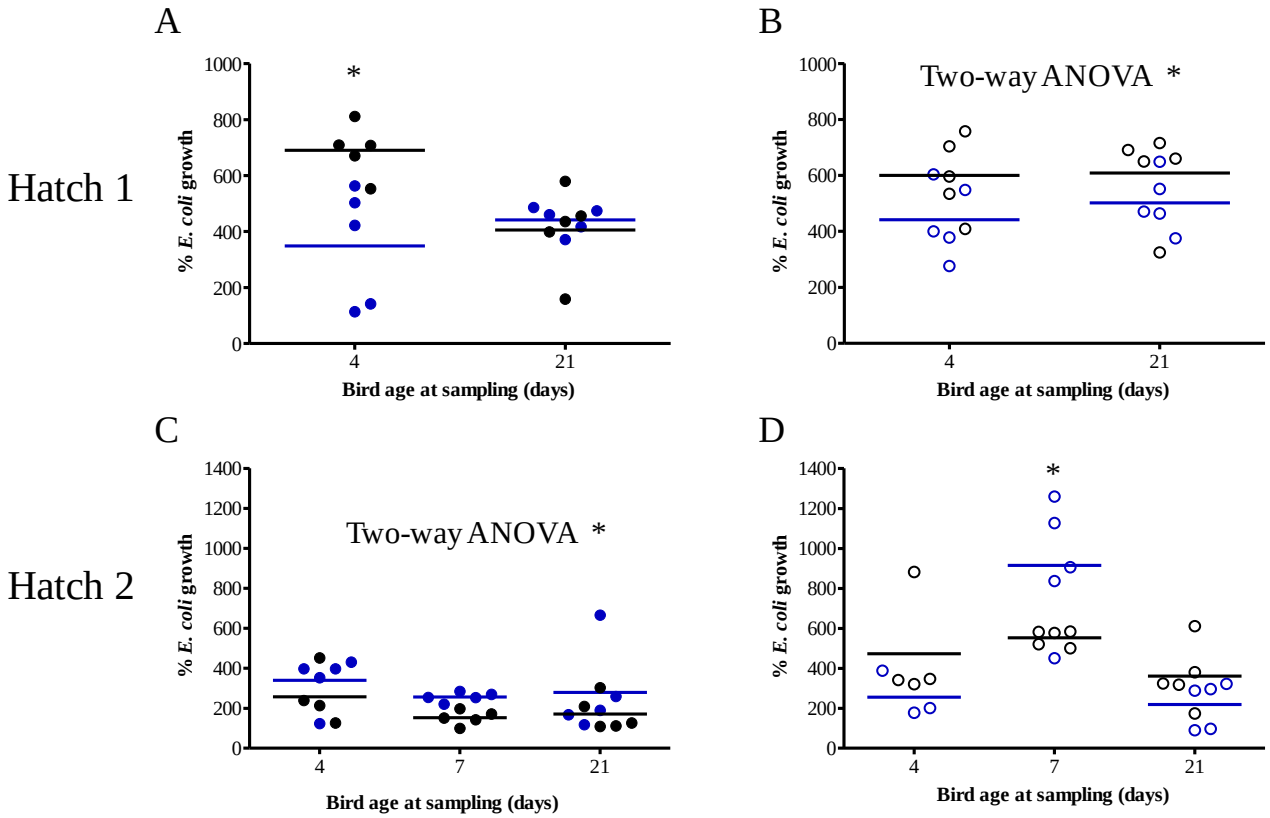


Figure 3.27: The effect of *Lactobacillus johnsonii* challenge on the ability of jejunal protein extracts to support *E. coli* growth.

Mean and individual % *E. coli* survival facilitated by jejunal protein extracts was assessed using the microbial growth assay (colony counting). Data is shown for Line X birds from Hatch 1 (A) and 2 (C) (solid circles) and Line Y birds from Hatch 1(B) and 2 (D) (open circles). Samples were analysed from birds aged 4, 7 and 21 days-old (n=3 - 5 for all groups). Water challenged – black circles, *Lactobacillus johnsonii* challenged – blue circles. Black line – water challenged mean, Blue line - *Lactobacillus johnsonii* challenged. (* = $P < 0.05$, Two-way ANOVA followed by Bonferonii post-tests specific to sampling timepoint).

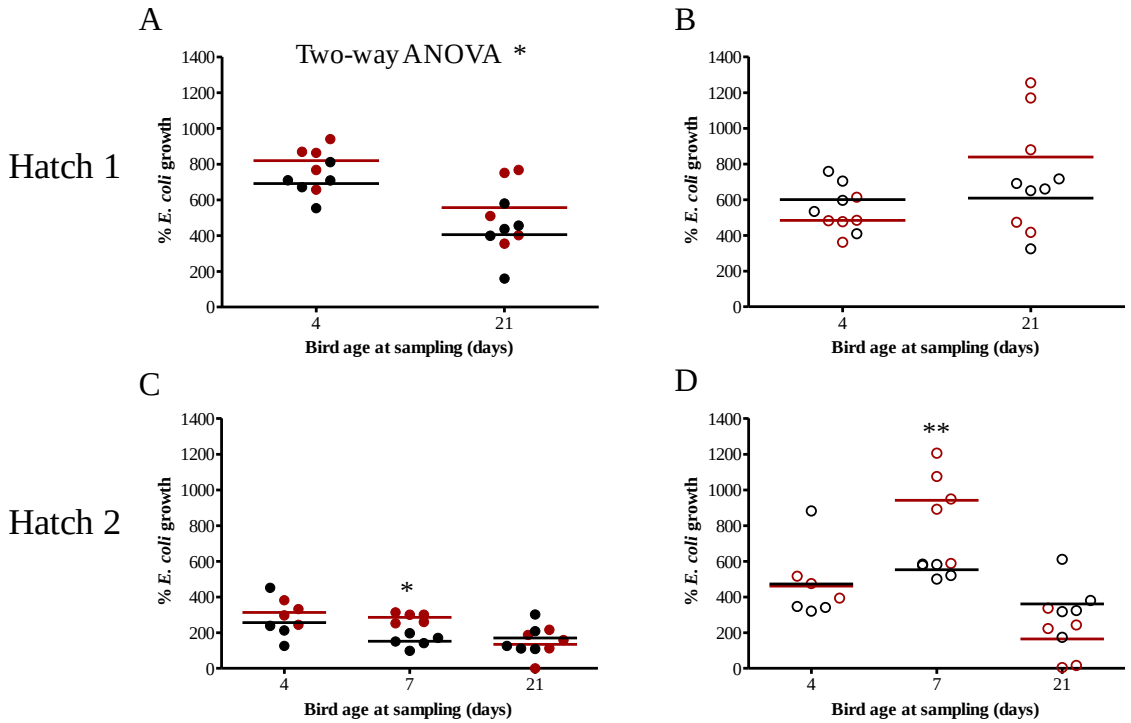


Figure 3.28: The effect of *Bacteroides dorei/Barnesiella viscerocola* challenge on the ability of jejunal protein extracts to support *E. coli* growth.

Mean and individual % *E. coli* survival facilitated by jejunal protein extracts was assessed using the microbial growth assay (colony counting). Data is shown for Line X birds from Hatch 1 (A) and 2 (C) (solid circles) and Line Y birds from Hatch 1(B) and 2 (D) (open circles). Samples were analysed from birds aged 4, 7 and 21 days-old (n=3 - 5 for all groups). Water challenged – black circles, *Bacteroides dorei/Barnesiella viscerocola* challenged – red circles. Black line – water challenged mean, Red line - *Bacteroides dorei/Barnesiella viscerocola* challenged. (* = P <0.05, ** = P < 0.01 Two-way ANOVA followed by Bonferonni post-tests) (P values indicated are for the effect of B/BV challenge on % *E. coli* growth only; age-related effects and age-challenge interactions are discussed in the text).

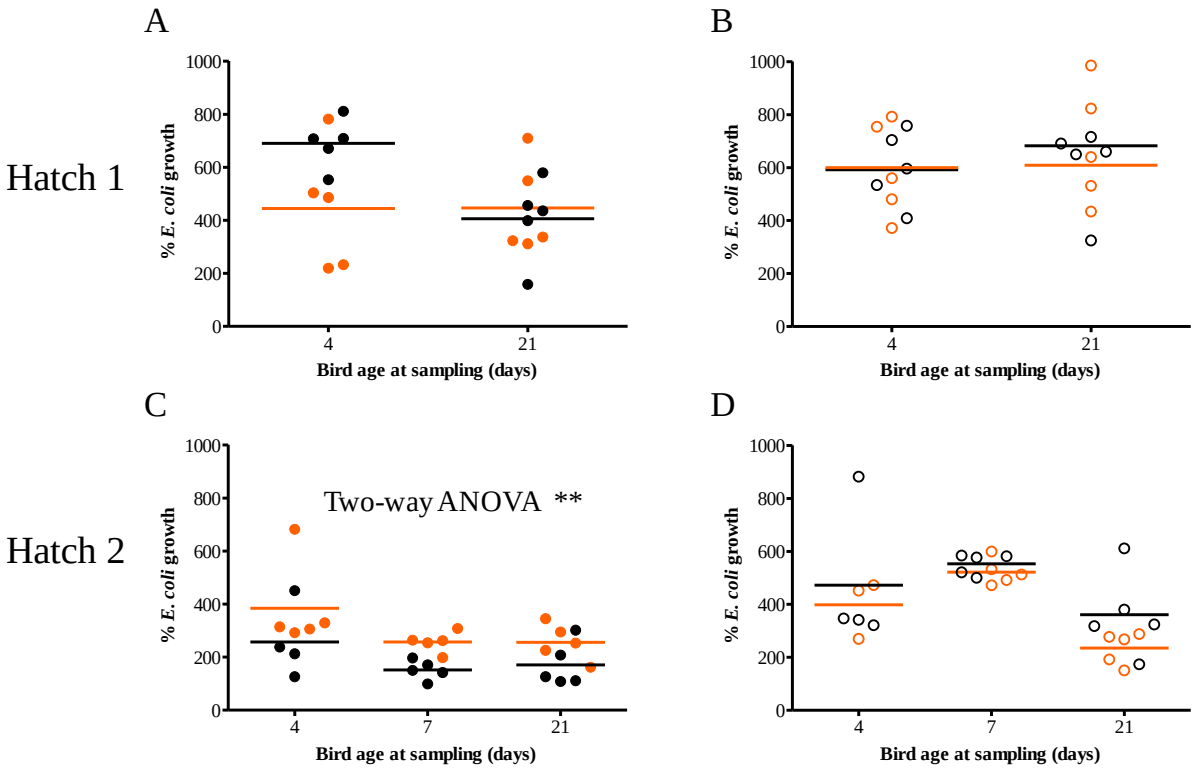


Figure 3.29: The effect of mix challenge (B/BV + LJ) on the ability of jejunal protein extracts to support *E. coli* growth.

Mean and individual % *E. coli* survival facilitated by jejunal protein extracts was assessed using the microbial growth assay (colony counting). Data is shown for Line X birds from Hatch 1 (A) and 2 (C) (solid circles) and Line Y birds from Hatch 1(B) and 2 (D) (open circles). Samples were analysed from birds aged 4, 7 and 21 days-old (n= 3 - 5 for all groups). Water challenged – black circles, Mix challenged – orange circles. Black line – water challenged mean, Orange line – Mix challenged. (* = P <0.05, two-way ANOVA followed by Bonferonni post-tests) (P values indicated are for the effect of mix challenge on % *E. coli* growth only; age-related effects and age-challenge interactions are discussed in the text).

3.2.9 Identification of sugars in gut samples

To investigate whether differences in *E. coli* growth were influenced by sugar content in the gut scrapes, thin layer chromatography was performed. Initially, a series of sugar standards were spotted in conjunction with a single gut scrape (Figure 3.30) and these data suggested that the most prominent sugar band corresponded to galactose. To confirm, a series of sugar standards were analysed in conjunction with the gut scrape sample by HPLC. The result, shown in Figure 3.31, demonstrated that the sugar associated with this TLC band was galactose.

To explore potential differences between the bird lines, ages and treatments, gut scrapes sampled at Days 4 and 7 from Line X and Y birds challenged with either LJ or B/BV were analysed by TLC (Figure 3.32). The pattern of sugar bands revealed a switch from a strong galactose signal in Line X day 4 birds to a strong galactose signal in Line Y, Day 7 birds. That this occurred in the control birds, as well as in the LJ and B/BV challenged groups indicated that it was intrinsic to these two bird lines rather than an effect of the bacterial challenge. Moreover, these results taken with the microbial growth data reveal that the presence of sugar is linked to bird line and age, but not to *E. coli* survival. For example, in Line X the mean *E. coli* growth (%) at Day 4 and Day 7 were similar (314% vs. 285%) even though clear sugar differences were observed.

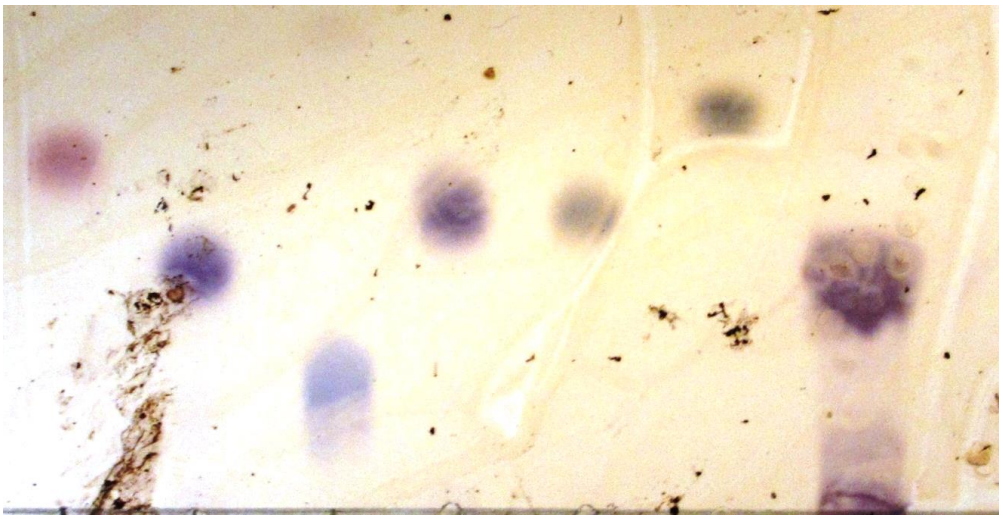


Figure 3.30: Thin Layer Chromatography showing 6 sugar standards.

(F – Fucose, G – galactose, GA - glucaronic acid, M – mannose, A – arabinose, X – Xylose) and one gut scrape extract from hatch 2 Line Y bird at 7 days-post hatch. The sugar standard for galactose migrates the same distance as the sample.

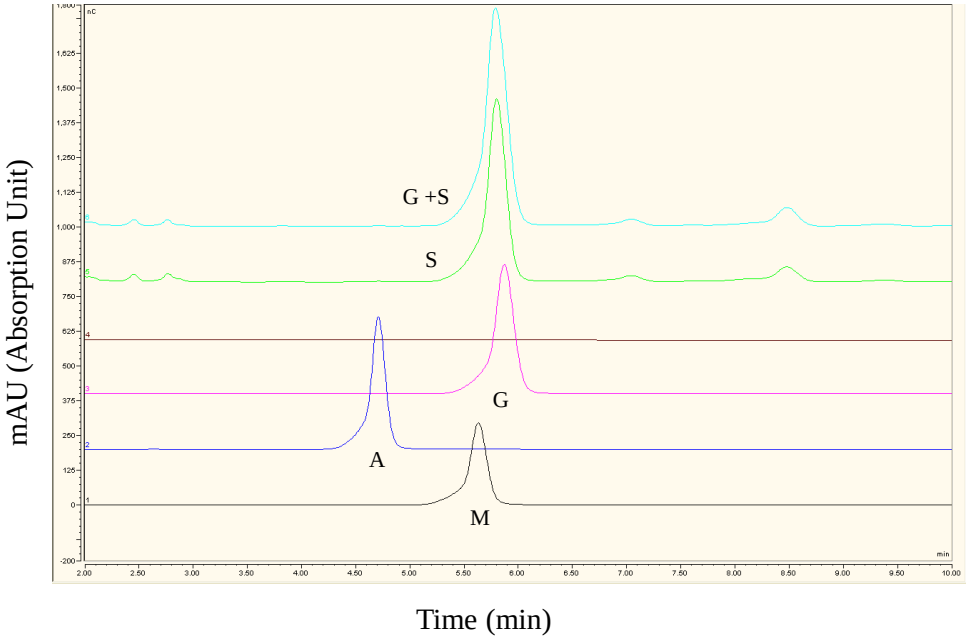


Figure 3.31: High Pressure Liquid Chromatography chromatogram reveals that galactose is the predominant sugar in the sample.

3 sugar standards (A - Arabinose , M - Mannose and G - Galactose), sample H2 D7 (S) , and Galactose standard + sample H2 D7 (Gal + S).

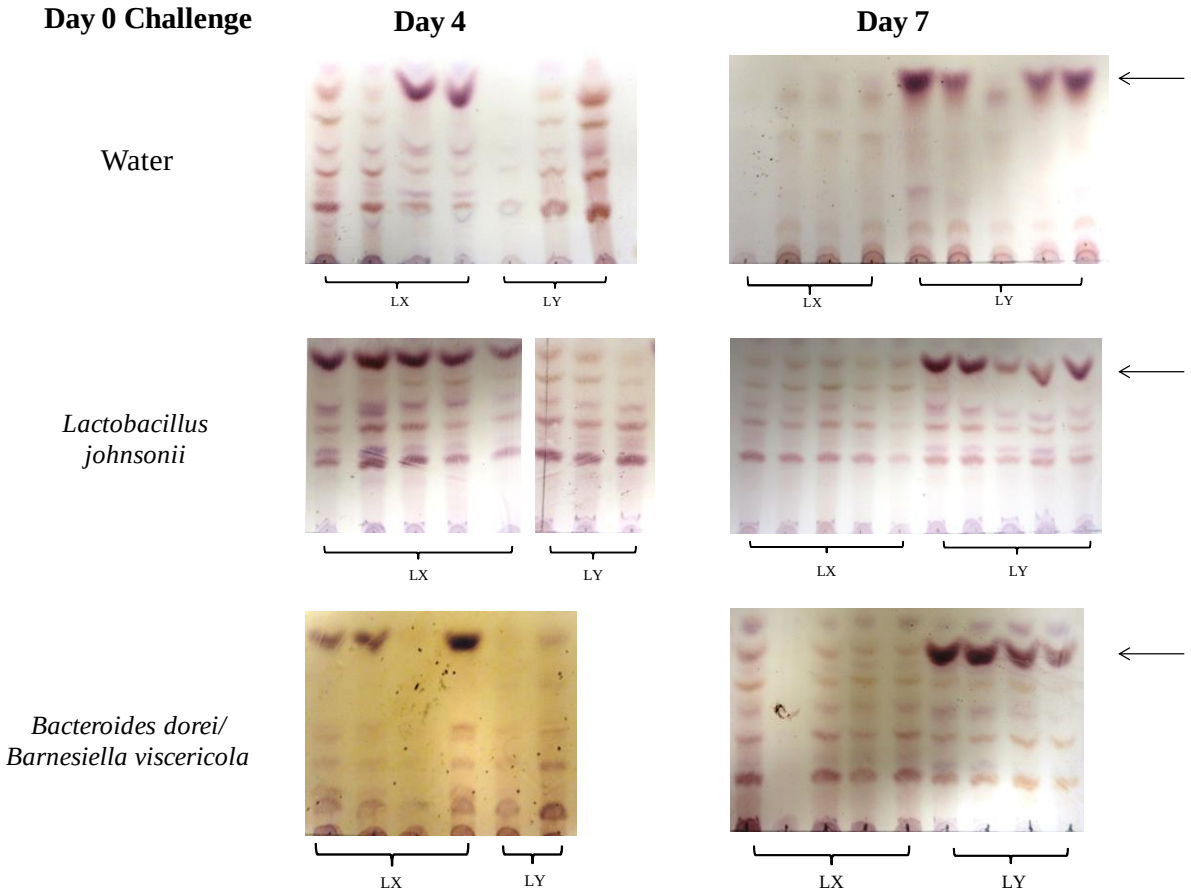


Figure 3.32: Age and line-dependent shifts in jejunal galactose content.

TLC of extracted gut scrapes showing sugar differences between Line X and Line Y birds challenged with water, *Lactobacillus johnsonii* and *Bacteroides dorei/Barnesiella viscericola.* LX – Line X, LY – Line Y. The band corresponding to galactose is indicated.

3.3 Discussion

Analysing the causes of enteric disease and implementing strategies to improve bird gut health has grown in importance over recent years, particularly since the E.U ban on anti-microbial growth promoters (Van Immerseel et al., 2004). The aim of this trial was to investigate the gut health differences between two genetically different commercial breeding lines, namely X and Y, and to assess their response to further bacterial challenge with the long-term objectives of genetically selecting for improvements in disease susceptibility. Indeed, genetic loci have already been identified as a mechanism to help reduce the incidence of gut associated conditions including ascites (fluid accumulation in abdominal cavity) (Krishnamoorthy et al., 2014), coccidiosis (Pinard-van der Laan et al., 2009) and Salmonella susceptibility (Calenge et al., 2009; Thanh-Son et al., 2012).

3.3.1 Gut health

This trial was unique in that comparisons were made not only between two genetic lines of birds but also between the birds in two different hatches. Assessments from both hatches indicated gut health deterioration from the Day 4 sampling time-point up to Day 28 followed by marginal improvement at Day 35**.** However, comparison of Day 28 control bird gut data produced unexpected findings in that the overall gut health was worse in Hatch 2 than Hatch 1 birds. The reasons for this hatch difference remain unknown as the rearing conditions were comparable. It is recognised that stress-related hormones such as adrenaline and cortisol can lower immune function, which may in turn increase the risk of intestinal problems (Mayer, 2000). Thus unique environmental stresses experienced by Hatch 2 birds including those of temperature, humidity and bedding may have induced hormonal responses that adversely affected their gut immune defences. It has however been established that enteric disease is more closely linked to lower hygiene conditions, particularly caused by poor litter management (Dawkins et al., 2004). In fact, the litter on which the birds are reared not only helps form the intestinal microbiota, but is a major source of potentially pathogenic microbes including parasites (Fries et al., 2005; Hermans et al., 2006). A comparison of bacterial loads and diversity in the bedding and the drinking water from the two hatches was not undertaken in this study, but these data may have helped unravel the exact cause of the inferior enteric health observed in Hatch 2 birds.

In this study, birds were challenged with either LJ or B/BV, and a combination of the two (B/BV + LJ). The rationale for using a LJ challenge was that *Lactobacillus* spp. function as probiotics (Torres-Rodriguez et al., 2007a; Brisbin et al., 2011; Mappley et al., 2011), and so the aim was to assess if any gut health improvements linked to LJ challenge were consistent between the bird lines. Probiotics function through a combination of factors including competitive exclusion, production of beneficial metabolites particularly butyric acid, production of bacteriocins and a lowering of environmental pH all of which prevent gut colonization by exogenous pathogenic bacteria or overgrowth of the normal commensal microbiota (Ng et al., 2009; Sherman et al., 2009). At 28 days post-hatch, when poor gut health was readily observed, the total gut health score per sampled group was lower in all LJ challenged groups than the water control groups (i.e. indicative of better gut health). This observation indicated that an immediate probiotic intervention following hatch does improve the gut health of birds in a commercial rearing environment, even those birds that are more susceptible to enteric problems such as Line X. However, it is also worth noting the number of birds per group that were healthy and those that had abnormalities. In Hatch 2, the guts of all five sampled Line Y birds from the water control pen were abnormal (>1 gut score), but only one abnormal bird was observed in the LJ challenged group (Figure 3.8). In contrast, four Line X birds from Hatch 2 suffered gut abnormalities in the LJ challenged group compared to five in the water challenged group (Figure 3.6). These data indicated that the Line Y birds responded more favourably to LJ as a probiotic than Line X birds. Interestingly, examination of two probiotic preparations, previously successful in piglets, revealed that Ross 308 broilers fed a diet supplemented with *Lactobacillus casei* had significantly improved feed conversion efficiency relative to control groups whilst Sasso X40 broilers did not (Fajardo et al., 2012). This variation in response between breeds therefore indicates that bird performance following probiotic intervention is, at least, partially-dependent on host genetics.

A study which analysed the broiler gut microbiome using denaturing gradient gel electrophoresis (DGGE) identified the presence of bands in birds with dysbacteriosis, which were not found in healthy birds (Bailey, 2010). Sequencing analysis revealed that two of these bands corresponded to *Bacteroides dorei* and *Barnesiella visericola*. Although resident

in the digesta of healthy birds, the organisms are present at low levels in comparison to the diseased birds (Bailey, 2010). This observation led to the hypothesis that overgrowth of these two species is linked to the dysbacteriosis phenotype. In the present study, *Bacteroides dorei* and *Barnesiella visericola*, referred to as B/BV, were used to challenge the Line X and Y birds. A key finding was that the gut health of the Line Y birds did not deteriorate upon B/BV challenge whereas the gut health of the Line X birds was visibly worse, again supporting that bird genetics has a strong influence on host-bacterial interactions that shape the microbiome. However, the addition of LJ to B/BV as part of the mix challenge (LJ + B/BV) did result in lower overall gut scores relative to the B/BV groups alone (Figure 3.2), suggesting that LJ can alleviate some of the effects of the B/BV challenge, at least when administered concurrently. It was noted that in many of the challenged pens, including those challenged with "probiotic-like" LJ, had birds with lower mean weight than birds from the control pens. The reason for this is unknown but it did not appear to relate to actual gut health. However if reproducible then there is a potential commercial impact that warrants further investigation.

3.3.2 Ileal microbiota

In this trial the ileal and caecal microbiota of control and challenged birds were sampled at various timepoints up to 28 days post-hatch and the composition, and development, of the microbiota assessed by 454 sequencing. Analyses of the caecal and ileal microbiomes were performed on groups of five birds that represented the microbiome of the entire pen. Due to differences in gut health within sampled groups, particularly in the first week of life, there is, with hindsight, a strong argument for performing such analyses on individual birds and comparing data from normal (gut score 0) and abnormal birds (gut score >1). Indeed, the individual sequencing approach may be more likely to indicate whether, and if so, which bacterial species are useful markers of gut health. It would be interesting, for example, to know whether the high *Escherichia/Shigella* spp. found in Hatch 2 Line X, 'mix' challenge birds aged 14 days (Figure 3.21) were specific to the digesta of the two birds with abnormal guts or whether they also inhabited the ileal microbiota of the three healthy birds. Individual birds within a sampled group have a unique microbiota (van der Wielen et al., 2002), thus correlating individual microbiomes with observable phenotypes such gut health status and nutrient absorption may actually be necessary to link microbial composition to desirable or non-desirable breeding characteristics. However, pooling of samples to reduce variation and

sequencing costs is common practice and allowed initial comparisons between treatment groups to be performed.

Factors in addition to rearing environment that influence the chicken microbiota are diet (Apajalahti et al., 2001; Knarreborg et al., 2002) and age (Lu et al., 2003). In this trial the diet was the same for both hatches allowing the effects of age on the microbiome to be assessed. The microbiome data from this trial mirrored the reported patterns of ileal microbiota formation in broilers. It has been shown previously that over 70% of 16s rRNA sequences are related to *Lactobacillus* spp. with 6.5% of sequences corresponding to *Streptococcus* spp. and *Enterococcus* spp. (Lu et al., 2003). These values correlated with the bacterial profiles found in this farm trial as the ileal microbiomes, with one exception that had a high *E. coli* profile, contained over 75% of sequences corresponding to *Lactobacillus* spp. Interestingly, fluctuations in the composition of the ileal microbiota could not be linked to gut health outcomes. For example, *Bacteroides* spp. associated with dysbacteriosis (Bailey, 2010), were only found in the ileal contents of a single group (<2% abundance) which had the healthiest gut health score. Moreover, *E. coli* levels were extremely high in the Day 14 Line X birds (69%) and yet no adverse effect on gut health was observed. There was, however, a strong suggestion in Line X birds (Figure 3.23), that a high abundance of *Lactobacillus* spp. at day 4 was protective allowing the gut to mature without suffering inflammation, increased watery contents or weakening of the gut tone. Intestinal overgrowth of endogenous bacterial commensals has been reported in human irritable bowel syndrome (IBS) (Madden and Hunter, 2002). If the poor gut health displayed by birds in this trial is analogous to human IBS, then it is possible that an overall higher count of bacteria were present in the ilea of such birds. However, only relative abundances were determined and culture-based methods would be required to determine actual bacterial densities.

However the gut scrape assay data (Figure 3.28), suggested that the B/BV challenge affected the jejunal mucosa of birds so that it was able to support excess bacterial growth relative to unchallenged birds. This was in contrast to the mucosa of the LJ challenged birds that, on the whole, inhibited bacterial proliferation relative to the control birds (Figure 3.27). Furthermore, jejunal scrapes from four day-old birds with abnormal gut health also supported higher *E. coli* growth (Figure 3.25), suggesting that small intestinal bacterial overgrowth may

be linked to adverse gut health outcomes. However, it was also noted that higher *E. coli* growth was supported by gut scrapes from seven day-old Line Y birds compared to Line X birds and, as previously shown, the Line Y birds have fewer gut abnormalities. Furthermore, correlations between absolute % *E. coli* growth values and gut health scores did not reveal any clear links to gut health (data not shown). In practice, such assays would have little commercial use for determining the susceptibility of a pen to gut health problems, but do hint at physiological changes to the jejunal gut mucosa resulting from the LJ and B/BV challenges.

3.3.3 Caecal microbiota

The caecal microbiome is of huge interest because it not only provides nutrients via bacterial fermentation of polysaccharides but also functions as a reservoir for zoonotic food borne pathogens such as *Salmonella* spp. and *Campylobacter* spp., and pathogens adversely affecting bird gut health such as *E. coli* and *C. perfringens* (Clench and Mathias, 1995, Stanley et al., 2014). Previous pair-wise comparisons of the broiler ileal and caecal microbiomes have shown that the caecal microbiota at 3 days of age is not significantly different to the ileum being dominated by genera such as *Clostridia* spp. and *Lactobacilli* spp. However, from 21 days onwards the caecal microbiota differs significantly showing increased complexity, and comprising of *Fusobacterium* spp., *Bacteroides* spp., and various species from the family *Clostridiaceae* (Lu et al., 2003).

Caecal microbiota profiling using DGGE revealed an increase in species diversity in birds with dysbacteriosis compared to healthy birds, although it was the presence of two bands that corresponded to *Bacteroides dorei* and *Barnesiella viscericola*, respectively, that appeared to mark the enteric health status of the birds (Bailey, 2010). In the present study the microbiota were analysed at the genus level only so it was possible that excess growth of these two species occurred. Exposing newly hatched chicks with *Bacteroides dorei* and *Barnesiella viscericola* was predicted to induce intestinal overgrowth of bacterial species and enteritis. However, while it was clear that these species induced gut health deterioration, the microbiota data indicated no consistent ileal or caecal microbial shifts. It should be noted that unclassified bacteria were often the most abundant group found and thus it cannot be

excluded that species, of as yet unclassified, bacteria are influential in causing gut health changes.

The microbiota of the birds sampled for this trial showed that *Bacteroides* spp. dominated prior to formation of a stable caecal microbiome, then decreased in relative abundance. Moreover, closer analyses hinted that a high level of *Bacteroides* spp. in very young birds was detrimental to gut health. For example, in the pen with the worst gut health (Hatch 2: Line X, B/BV challenged) *Bacteroides* spp. were the dominant caecal species at Day 4. This was not observed in Line Y (Figure 3.12). Analyses of the caecal microbiota from the healthiest group of birds (LJ challenged Line Y: Hatch 2) revealed no *Bacteroides* spp. at Day 4 and a relatively low abundance at 14 days post-hatch (5%) (Figure 3.8), and this may have been crucial in the continued good gut health that followed (only 1 abnormal bird gut recorded at Day 28). In contrast, in the Hatch 2 Line X birds the relative abundance of *Bacteroides* spp. at Day 14 was 29% in both the LJ and the water control groups, and the number of birds suffering gut abnormalities by Day 28 was 4 and 5, respectively (Figure 3.6). In swine *Bacteroides* spp. are linked to the production of ammonia and amines (Gaskins, 2001). It is therefore feasible that the presence of high levels of *Bacteroides* spp., and such metabolites, in the first two weeks post-hatch when the bird gut is still developing, may contribute to adverse gut health outcomes. It is also possible that the presence of *Bacteroides* spp. is indicative of protein malabsorption in the small intestine leading to an excess of protein in the caeca, conditions that actually favour *Bacteroides* spp. growth. Paradoxically however, data from the current trial (Figure 3.18) also showed that a high abundance of *Bacteroides* spp. in birds, aged 21 and 28 days, was associated with better gut health. *Bacteroides* spp. have been shown to confer many benefits to the host and have even been described as mutualistic (Backhed et al., 2005). For example, they have been shown to be involved in the activation of host T-cell mediated responses (Mazmanian, 2008), and the exclusion of potentially pathogenic bacteria (Mazmanian et al., 2008). Crucially, they are known to contribute to dietary energy via uptake of short-chain fatty acids, such as butyrate, derived from bacterial fermentation of complex polysaccharides (Flint et al., 2008; Martens et al., 2011). Therefore, the relationship between bird gut health and the presence of *Bacteroides* spp. is not simplistic but it is possible that the age at which *Bacteroides* spp. colonises the bird gut is critical in determining the effects of the colonisation on gut health.

3.3.4 Carbohydrate utilisation

In this trial, TLC analysis revealed an interesting age and line-dependent pattern of galactose in jejunal gut scrapes. Poultry feed contains cereal grains of which the hemicellulose component contains galactan and galactomannan (Hsiao et al., 2006). Birds, like mammals, do not possess the enzymes able to break down complex polysaccharides and instead these dietary fibres are degraded in the caeca by species such as Bacteroides to produce short chain fatty acids (Mead 1989; Bolam and Sonnenburg 2011; Martens et al., 2011). However, many species of Lactobacilli, which are found at high levels in the bird crop, proventriculus and gizzard, have the ability to break down more complex sugars using a variety of glycosyl hydrolases (O'Donnell et al., 2013), which may in turn explain the intestinal galactose.

Comparisons revealed that gut scrapes from Line X birds contained galactose at four days post-hatch, but not at seven days, whereas, in Line Y galactose was only found in birds aged seven days. Therefore, it is reasonable to speculate that the absence of galactose in Line X birds is linked to their less robust gut health. Birds lack lactase and are thus unable to break down lactose; the sugar has, however, been utilized as a prebiotic because it can be metabolized by caecal anaerobes resulting in a reduction in the hindgut pH and decreased proliferation of potentially pathogenic bacteria (Hume et al., 1992). Low levels of galactose (2 -4 %) have also been shown to increase broiler body weight (Douglas et al., 2003), while lactose supplementation is associated with an increase in the weight of normal healthy turkey hen poults (Torres-Rodriguez et al., 2007b), and those given a Salmonella challenge (Vicente et al., 2007). Specific benefits relating to enteric disease have also been noted with a 2.5% lactose supplementation reducing necrotic enteritis lesions following *C. perfringens* challenge (McReynolds et al., 2007). Therefore, the presence of jejunal galactose in older Line Y birds, and from whatever source, may inadvertently function to maintain good gut health.

3.3.5 Immune response to host microbiota

Part of the rationale for using *Lactobacillus* spp. as a probiotic is that these species may help prevent excessive immune responses in young birds, such as those associated with gut inflammation, whereas *Bacteroides* spp. do not. Indeed, in murine models, various Lactobacillus species have been shown to produce cytokines that help modulate immune

responses (Christensen et al., 2002), and such modulation has also been reported for *in vitro* and *in vivo* chicken models (Brisbin et al., 2010; Brisbin et al., 2011). A study by Tsuda et al., (2007) comparing the cytokine stimulating properties of *Lactobacillus johnsonii* and *Bacteroides acidofaciens* on antigen presenting cells showed that the former induced lower levels of pro-inflammatory cytokines IFN-γ and IL-6 and higher levels of anti-imflammatory cytokines IL-10 and IL12p40 than *Bacteroides acidofaciens* (Tsuda et al., 2007). Although *Bacteroides* spp. are regarded as normal bacteria in the gut flora, some species such as *Bacteroides vulgatus* have been associated with inflammatory gut conditions in transgenic rats (Rath et al., 1996), and guinea pigs (Onderdonk et al., 1981). Within the GI tract the precise role(s) of *Bacteroides* spp. in the induction and/or maintenance of an inappropriate immune response have not been fully described but various strains have been implicated in enteric diseases. For example high antibody titres against *B. vulgatus* surface antigens in ulcerative colitis have been described (Bamba et al., 1995) and it has been suggested that enterotoxigenic *B. fragilis* may cause inflammation and damage to epithelial cells in inflammatory bowel disease (Wu et al., 1998; Wu et al., 2004).

Lines X and Y are commercial broiler breeds genetically selected for optimal broiler performance i.e. efficiency in converting feed to muscle mass. However, the selective breeding processes employed to produce Line X birds has resulted in a bird with a compromised gut health. This facet is not uncommon because the selective breeding process *per se* has not been tailored towards robust gut immunity (van der Most et al., 2011). For this trial no markers of immune response were measured, but it is possible that the increased inflammation in Line X birds was driven by an unregulated gut epithelial response to the host microbiota. Indeed, further analysis investigating the expression of immune effectors including host defense peptides and cytokines may help explain the susceptibility of such birds to enteric disease.

3.3.6 Summary

In summary, Line X birds had sub-optimal gut health relative to Line Y birds, were less robust to bacterial challenge and responded less favourably to the probiotic intervention although there were suggestions from the microbiome data that a high abundance of *Lactobacillus* spp. in the ileum of young birds did help protect the gut. Of interest was the

observation that colonization of the young bird gut with *Bacteroides* spp was linked with reduced gut health whereas high abundances in older birds was associated with improved gut health. Clear differences in jejunal galactose content between Lines X and Y warrants deeper investigation as sugar utilization may be linked to microbiome stability. Finally, bacterial overgrowth in the small intestinal is thought to cause dysbiosis, which can lead to enteric problems. B/BV treated birds were characterized by reduced gut health and interestingly microbial growth assays revealed that the gut scrapes from B/BV challenged birds supported increased *E. coli* growth, which reinforced the bacterial overgrowth and dysbiosis theory.

Chapter 4: Farm Trial 2

4.1 Overview

Line X is an important part of the breeding programme at Aviagen Ltd. and the data resulting from Trial 1, Chapter 3, indicated that Line X bird gut health is less robust than that of Line Y. The data, however, indicated that bird gut health differences were particularly marked following a B/BV bacterial challenge at Day 0, i.e. the day of hatch. To further examine this observation, a second farm trial was performed to characterise, specifically, the gut innate defences of Line X birds following a B/BV challenge. The gut pathology of Line X birds makes it a good model to investigate bird gut immunity with the aim of improving gut health and therefore, maximising the potential of the Line X birds for commercial breeding purposes.

An overview of this trial is shown in Figure 4.1. The aims were first to identify individual and/or groups of birds whose gut health was robust to bacterial exposures representative of commercial rearing environments, secondly to investigate potential relationships between gut health and AvBD gene expression, and thirdly to identify potential biomarkers of gut health. On arrival from the hatchery (Day 0), five male and female Line X birds were sacrificed, and tissue samples taken for AvBD gene expression (AvBD1 and 10) and immunohistochemical analyses. In addition, a jejunal gut scrape was taken from each bird for microbial growth assay analysis. The remaining birds were assigned to one of six pens so each pen contained 100 birds. Birds were provided with either acute (high B/BV seeding 10^9 CFU/bird), prolonged (low B/BV seeding 10^9 CFU/water container) or water control challenges. At 7, 14, 21, and 28 days post-hatch a random sample of three birds per pen were individually weighed, sacrificed and visually assessed for gut health as in Trial 1. As described for the newly hatched chicks, tissue samples, jejunal scrapes and digesta were collected.

All birds were fed the same diet as in Trial 1 and underwent the normal vaccination protocols (section 2.2.2). In contrast to Trial 1, birds were put into pens containing re-conditioned ‘old litter’ with fresh bedding layered on top to represent conditions found on commercial farms outside of the U.K.

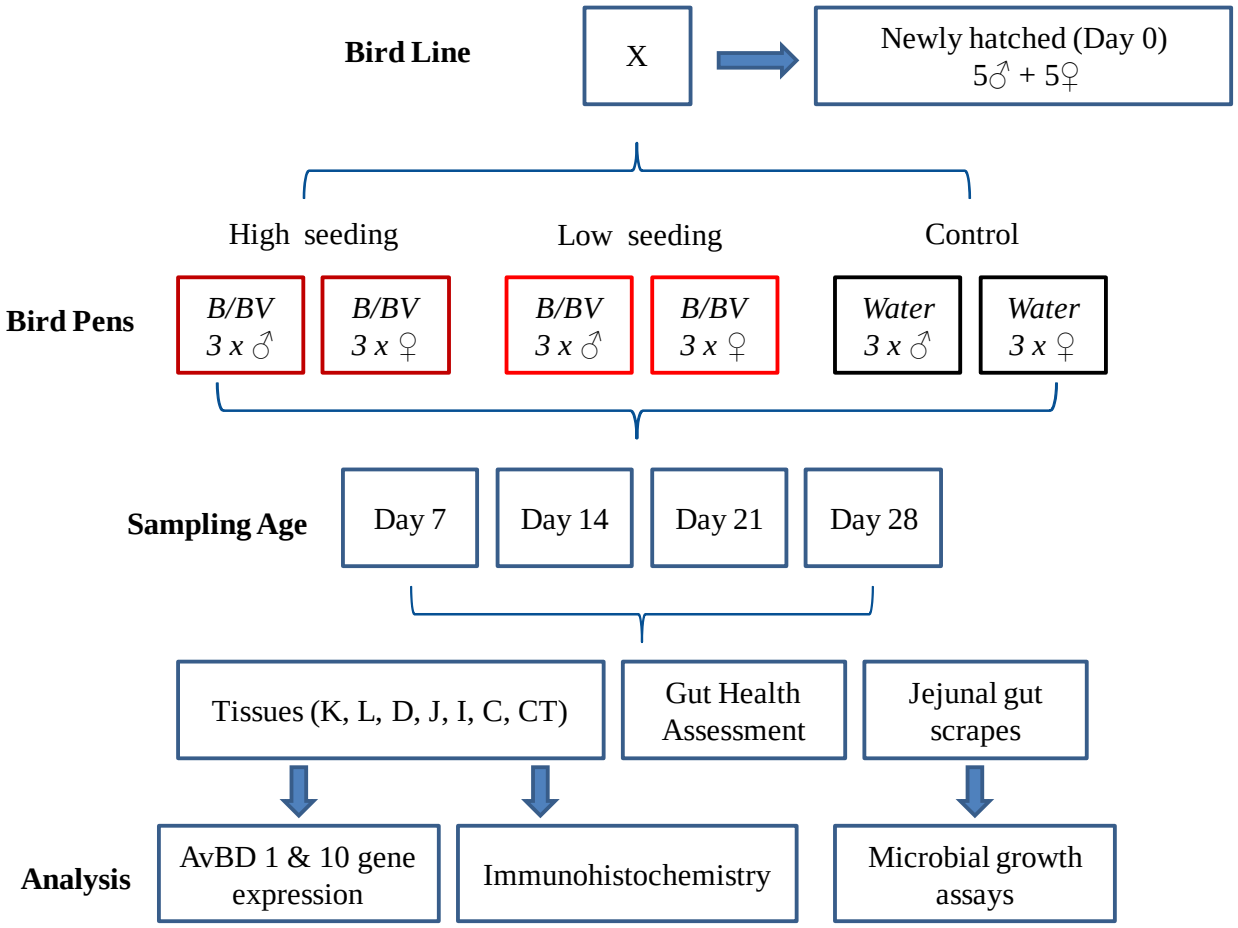


Figure 4.1: Farm trial 2 overview.

Line X birds were separated into males and females and reared in six pens that were given one of three treatments: water (control), high seeding of B/BV (~10^9 C.F.U per individual) or low seeding B/BV (10^9 C.F.U per drinking water container). The trial was repeated for three hatches. Bacterial challenges and gut health assessments were performed by Aviagen Ltd.

4.2 Gut Health Assessments

Gut health assessments were performed by Dr Richard Bailey and Mr Johnny Begley (Aviagen Ltd.).

Figure 4.2A-C shows the gut health scores per sampled group (n = 3) at 7, 14, 21 and 28 days, respectively, for three separate hatches following the B/BV challenges. The mean data for all three hatches, Figure 4.2D, shows that the bird gut health scores increased up to 21 days post-hatch, supporting deterioration in gut health, but decreased at 28 days post-hatch, suggesting some improvement. For male birds the challenged groups had significantly higher gut scores at all sampling time-points whereas in females the adverse effect of challenge was delayed until 21 days post-hatch ($P < 0.001$, Two-way ANOVA, Bonferroni post-tests). A comparison of low with high seeding challenge groups revealed no significant differences in gut health score between the groups ($P > 0.05$).

4.3 Effects of B/BV challenge on bird weight

Figure 4.3A-C illustrates the effect of low and high B/BV seeding on mean bird weight per sampled group (n = 3) for hatches 1, 2, 3 and all three hatches combined at 7, 14, 21 and 28 days (Figure 4.3D). No significant differences in bird weight were observed between control and challenged groups at days 7, 14 and 21. However, by day 28, both male and female birds that underwent prolonged challenge (low seeding) were significantly lighter (Males: $P< 0.01$, Females $P < 0.05$), than the birds from the control pens. When all the hatch data was combined no significant effects of the acute challenge (high seeding) on bird weight were identified although if Hatch 1 and Hatch 3 birds were considered separately the high seeding female birds were significantly lighter than the three sampled birds from the water control pen ($P < 0.05$).

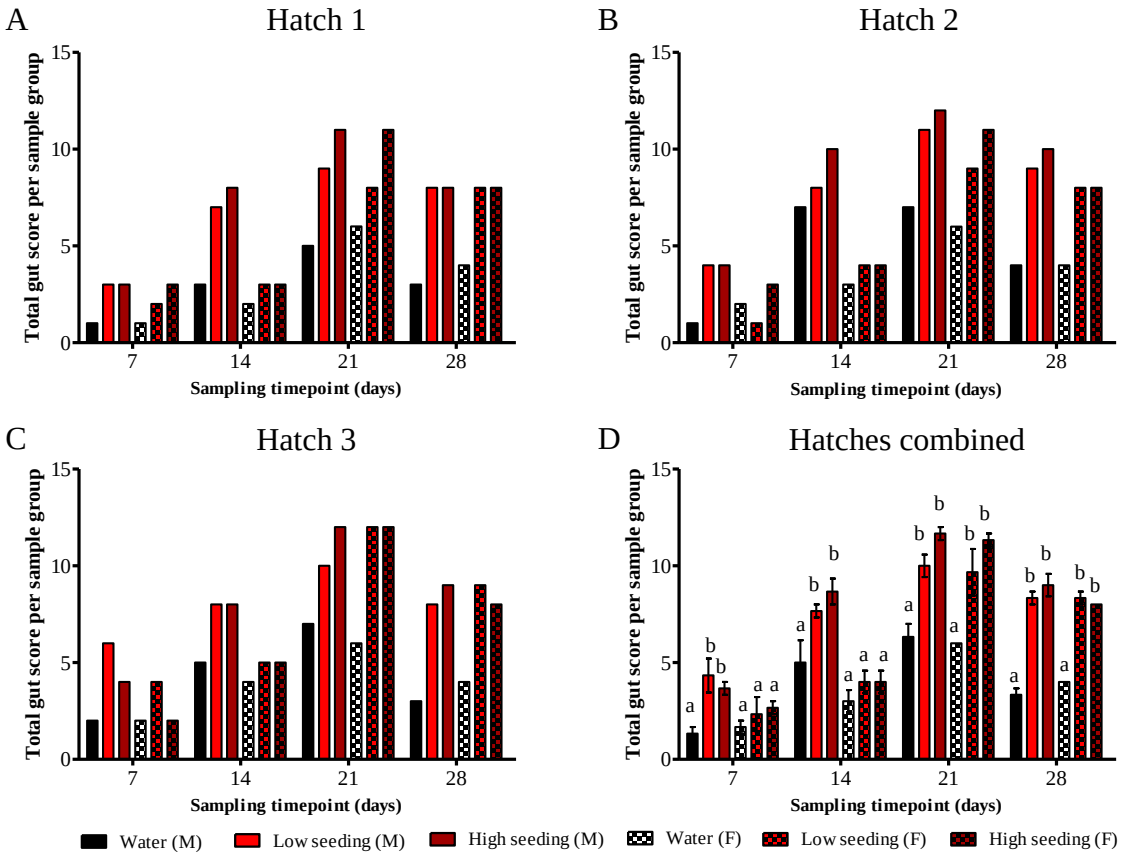


Figure 4.2: The effect of low and high *Bacteroides* spp/*Barnesiella* spp. (B/BV) seeding on the gut health of male and female Line X birds.

Birds were sampled at 7, 14, 21 and 28 days post-hatch and the data are displayed for Hatch 1 (A), Hatch 2 (B), Hatch 3 (C), and Hatches 1-3 combined (D). Bars not sharing letters are significantly different according to Bonferronii post-tests ($P < 0.05$). Only the significant effects of challenge are shown. Significant differences relating to sampling age and gender are not shown and instead are discussed in the text. Gut health analyses were performed by Dr Richard Bailey (Aviagen Ltd.).

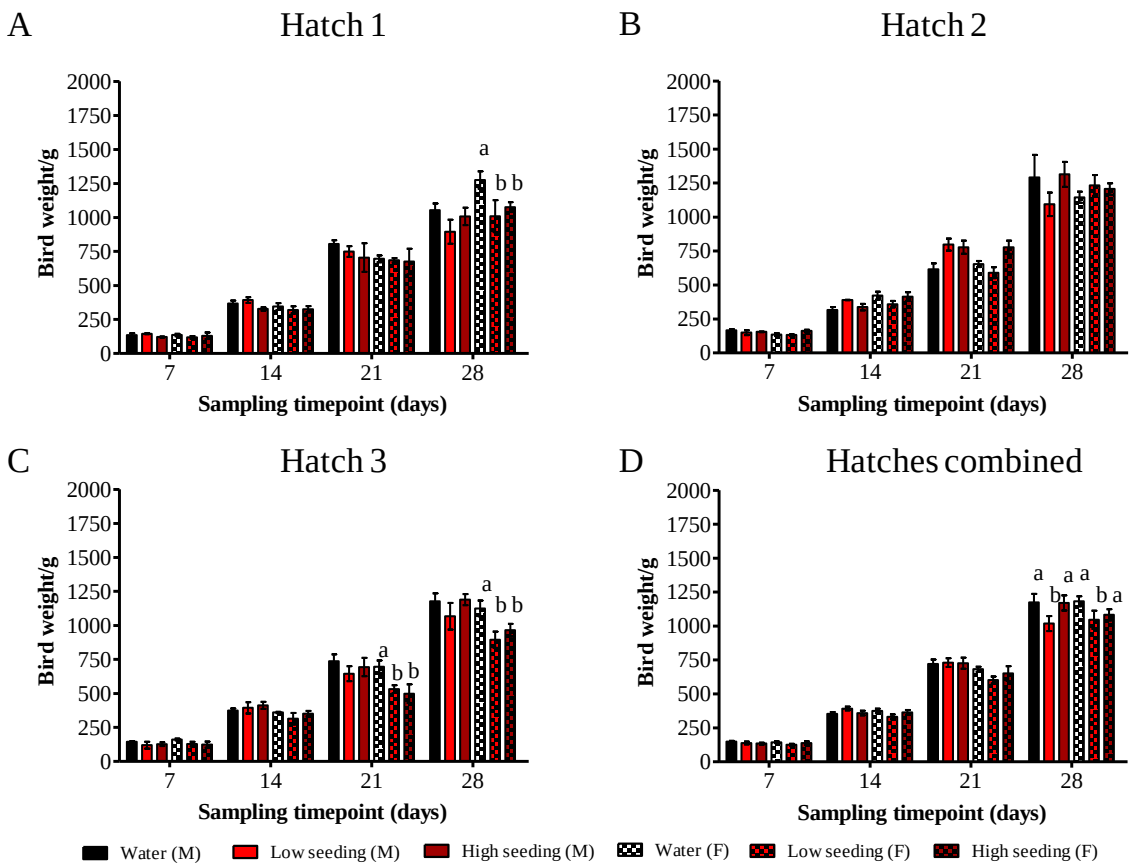


Figure 4.3: The effect of low and high *Bacteroides* spp/*Barnesiella* spp. (B/BV) seeding on the mean bird weight of male and female Line X birds. Birds were sampled at 7, 14, 21 and 28 days post-hatch and the mean weights **(±** SEM**)** are displayed for Hatch 1 (A), Hatch 2 (B), Hatch 3 (C), and Hatches 1-3 combined (D). A – C: n = 3 birds per pen; D: n = 9. Bars not sharing letters are significantly different according to Bonferronii post-tests ($P < 0.05$). Only the significant effects of challenge are shown. Significant differences relating to sampling age and gender are not shown and instead are discussed in the text.

4.4 AvBD1 gene expression

Avian beta-defensins (AvBD) are hypothesised to be important effectors of the innate immune system, particularly during the first week of life when birds are vulnerable to bacterial infections (Bar-Shira et al., 2003; Bar-Shira and Friedman, 2006). In this study the effects of the B/BV challenges on AvBD 1 and 10 gene expression and the links, if any, to gut health were examined. AvBD1 was studied as the gene carried by Line X birds contains SNPs within the mature peptide coding sequence that may impact on its microbial killing capacity (Butler, 2010). AvBD10 contains a SNP in the 5'UTR region (Rs14411785), which may affect expression and, in addition, previous work had hinted at an unusual expression profile with expression detected not only in tissues of the GI tract but also at high levels in the kidney and liver (Butler, 2010).

4.4.1 RT-endpoint PCR tissue panels (AvBD1)

Initially, endpoint RT-PCR was performed on RNA extracted from the ten sampled tissues to determine tissue expression profiles prior to performing extensive real-time quantitative PCR analysis. AvBD1 was expressed in numerous tissues of newborn chicks (Day 0) and older birds (Figure 4.4) but real-time quantitative PCR analyses was limited to the gut tissues including duodenum, jejunum, ileum, caecum and caecal tonsil as well as kidney and liver. As this study was primarily an investigation of gut immunity and gut health analysis of the spleen, bursa of Fabricus and thymus were not performed.

Endpoint RT-PCR tissue panels for AvBD1 and 10 were performed by Dr Catherine Mowbray, Newcastle University.

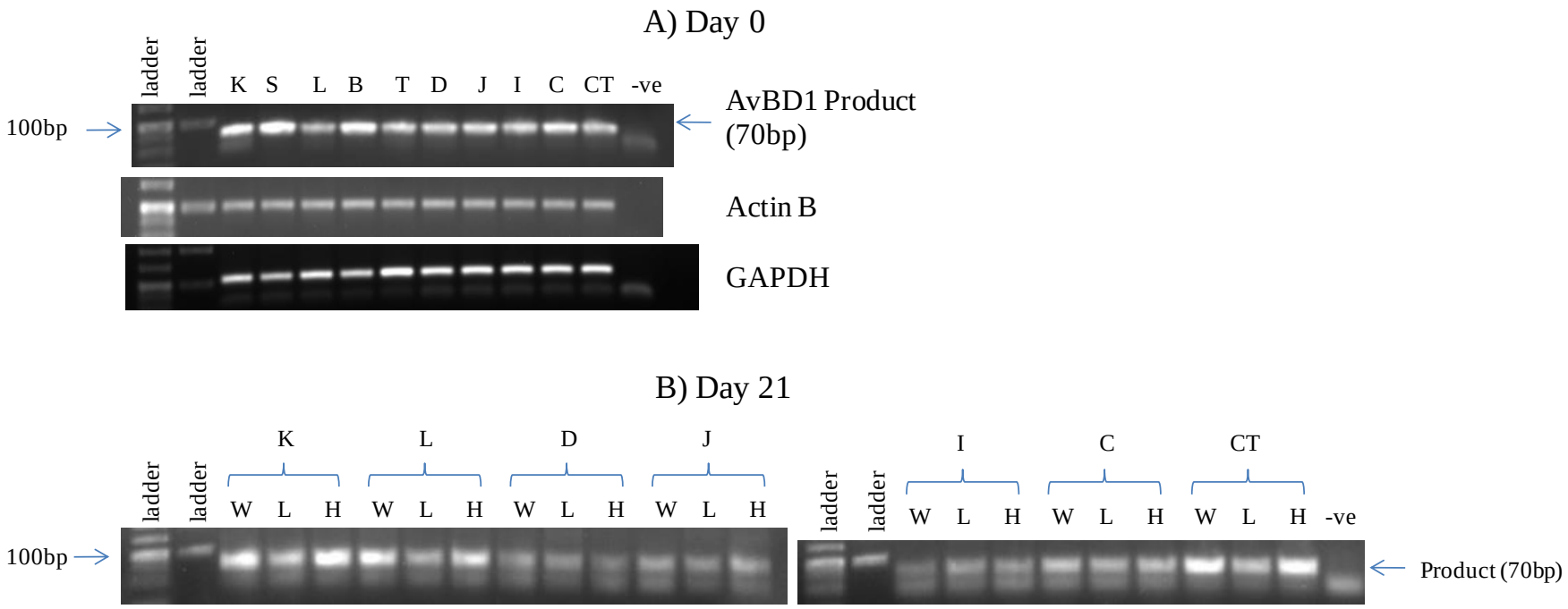


Figure 4.4: Endpoint PCR tissue panels showing AvBD1 gene expression at Day 0 and Day 21 for control and challenged birds.

A) AvBD1 tissue panel utilising a single newly hatched bird for each tissue and for B) three birds aged 21 days, each one sampled from either the water control pen (W), the low seeding B/BV challenge pen (L) or the high seeding B/BV challenge pen (H). RNA was extracted from Kidney (K), Spleen (S), Liver (L), Bursa (B), Thymus (T), Duodenum (D), Jejunum (J), Ileum (I), Caecum (C) and Caecal Tonsil (CT). –ve = negative control. Data supplied by Dr Catherine Mowbray, Newcastle University.

4.4.2 Housekeeping genes

Quantitative PCR requires the use of housekeeping genes for data normalisation i.e. to enable variations in yields of RNA extraction and subsequent reverse transcription to be controlled between samples. Guidelines released as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) suggest that the use of a single housekeeping gene is not normally acceptable and all chosen genes must be experimentally validated (Bustin et al., 2009).

The chicken GEnorm kit (PrimerDesign, UK) was used to select suitable housekeeping genes for sample normalisation. Genes assessed for suitability were GAPDH, YWHAZ, ACTB, UBC, SDHA and SF3A1. Figure 4.5 shows the output from the GEnorm software, which indicates the average expression stability value (M) of the six potential reference genes. The lower the M value the more stable the gene expression. The software indicated that SDHA and SF3A1 were the most suitable housekeeping genes for avian tissue.

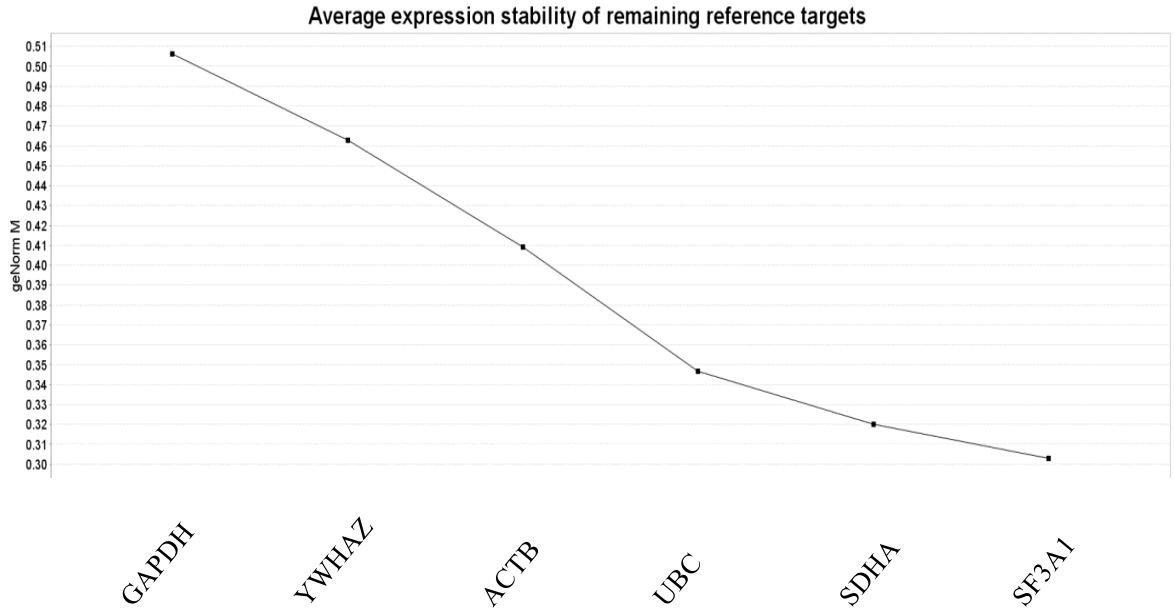


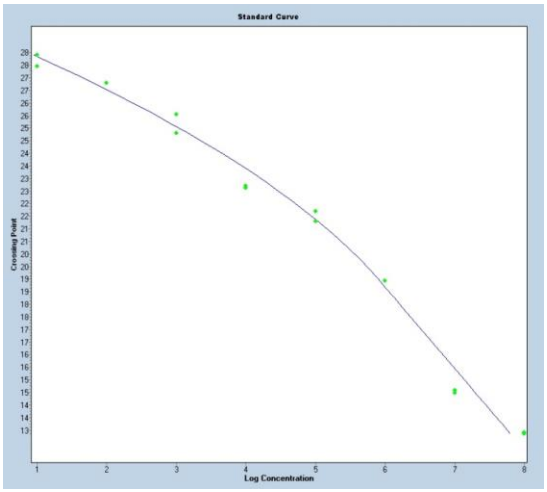
Figure 4.5: GeNorm analysis to determine the most appropriate genes for real-time qPCR in avian tissues.

The average expression stability value (M) of six chicken reference genes in the GeNorm kit (PrimerDesign); GAPDH, YWHAZ, ACTB, UBC, SDHA and SF3A1. The expression stability increases from left to right.

4.4.3 AvBD1 standard curve and melt curve

Using a 1:10 dilution series of AvBD1 plasmid, real-time qPCR reactions were performed to provide a large range of crossing point (CP) values that could be used for relative quantification of individual samples. This procedure was repeated at least three times to ensure reproducibility, an amplification efficiency of ~2 and a low error value for sample replicates ($P < 0.05$). The standard curve used for relative quantification of AvBD1 is shown in Figure 4.6A. For each sample, melt curves were checked for a single peak at 83°C which ensured AvBD1 primer specificity. A series of melt curves for AvBD1 PCR products from gut tissue cDNAs is shown in Figure 4.6B.

A) Standard curve



B) Melt curves

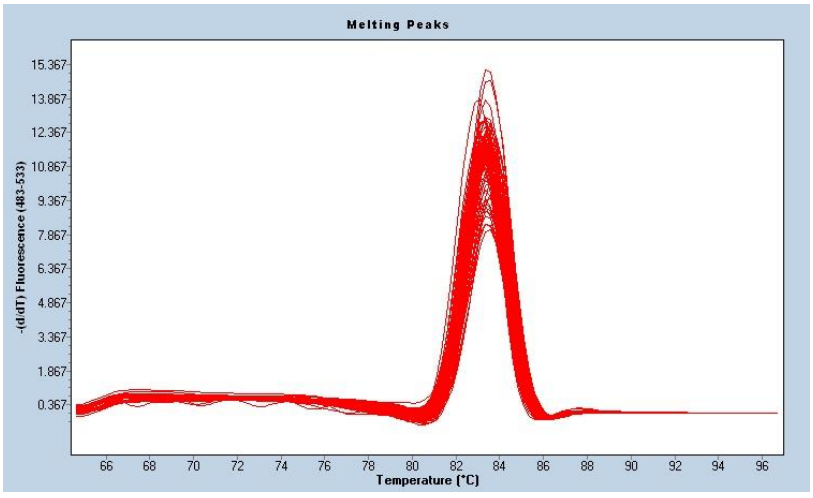


Figure 4.6: Real-time qPCR standard and melt curve for AvBD1.

A) Standard curve for serial dilutions of AvBD1 plasmid against calculated CP values. B) Melt curve for AvBD1 PCR products of gut tissue samples (n = ~ 40 samples). A single peak for each product was observed at the melting temperature of ~83.3°C.

4.4.4 Summary of AvBD1 gene expression in tissues of birds aged 0, 7 and 21 days

A total of 236 birds from three hatches were sampled for this study, but unfortunately no samples were available for Hatch 1, Day 0 birds. As this was considered to be an important time-point, subsequent analyses focussed on data resulting from Hatch 2 and 3 birds only. Real-time qPCR analyses for Farm Trial 2 were performed in collaboration with Dr Catherine Mowbray, Newcastle University.

Figure 4.7 shows the AvBD1 gene expression profiles of tissues from Hatch 2 (A, C and E) and Hatch 3 (B, D and F) birds aged 0, 7 and 21 days. It is clear from these data that newly hatched chicks exhibit intrinsically large variation in AvBD1 expression, and for all tissue groups a small number of birds have extremely high expression levels (hence necessitating the use of split Y-axis graphs). To ascertain if the expression values were sampled from a Normal (Gaussian) distribution, the D'Agostino & Pearson omnibus normality test was performed. Many of the tissue groups tested suggested that the data was not normally distributed. However, with this taken into consideration and the use of non-parametric statistical tests, comparison of median AvBD1 expression levels revealed significant differences between tissue types at all sampling time-points in both Hatch 2 and 3, indicating that expression is tissue dependent. In summary, on the day of hatch there was a pattern of increased AvBD1 expression in the distal gut (ileum/caecum/caecal tonsil) compared to the proximal gut tissues (duodenum/jejunum). In both hatches, AvBD1 expression had decreased markedly by the Day 7 sampling time-point (Panels C and D) with only caecal tonsil expression being maintained. Interestingly, by Day 21 although AvBD1 expression was low throughout, a single bird in each hatch expressed a relatively high level of the AvBD1 gene in the caecal tonsils (Panels E and F).

At Day 0 a comparison of the median AvBD1 values between Hatch 2 and Hatch 3 revealed statistically significant differences in AvBD1 expression ($P < 0.001$, Two-way ANOVA). For this reason, all subsequent data were presented according to the individual hatches.

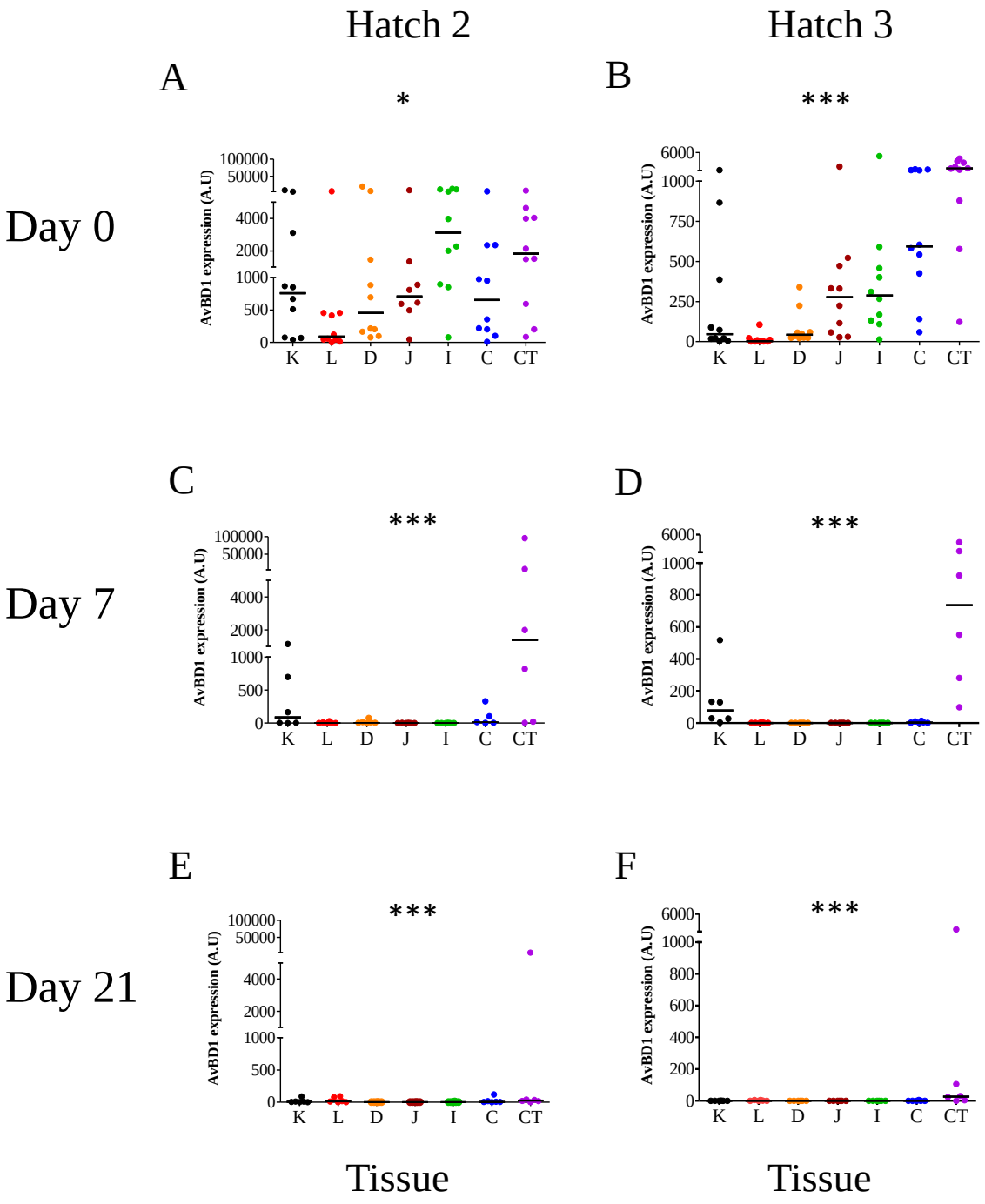


Figure 4.7: AvBD1 gene expression in tissues of newly hatched chicks (Day 0) and birds aged 7 and 21 days

Gene expression is shown in arbitary units for newly hatched chicks in Hatch 2 (A) and Hatch 3 (B), birds aged 7 days in Hatch 2 (C) and Hatch 3 (D), and birds aged 21 days in Hatch 2 (E) and Hatch 3 (F). K – kidney, L – liver, D – duodenum, J – jejunum, I – ileum, C – caecum, CT – caecal tonsil. Solid line indicates the median expression level. (n = 6 - 10 birds). (* $P < 0.05$, *** $P < 0.001$; Kruskal-Wallis test compares the median values of all tissue types).

4.4.5 AvBD1 expression in the kidney and liver

Figures 4.8 and 4.9 show the kidney and liver AvBD1 expression data for Hatch 2 (A) and 3 (B) in control birds aged 0, 7 and 21 days and, specifically, 7-day old control and B/BV challenged birds from Hatches 2 (C) and 3 (D), respectively.

Examination of bird age on kidney and liver AvBD1 expression indicated that newly hatched birds exhibited higher median expression values than older birds aged 7 or 21 days, with the exception of Hatch 3 birds that exhibited relatively high kidney expression at 7 days of age. For Hatch 2 this difference in expression was statistically significant ($P < 0.01$) in the kidney between birds aged 0 days and 21 days (Figure 4.8), but it did not reach statistical significance in the liver. However in Hatch 3 the Day 0 birds i.e. newly-hatched had significantly higher liver AVBD1 expression than birds aged 7 days (Figure 4.9).

A comparison of water control groups with the B/BV challenged groups revealed no consistent patterns, but it was noted that in Hatch 3 birds the kidney AvBD1 expression in the control group was significantly higher ($P< 0.05$) than in the high seeding group.

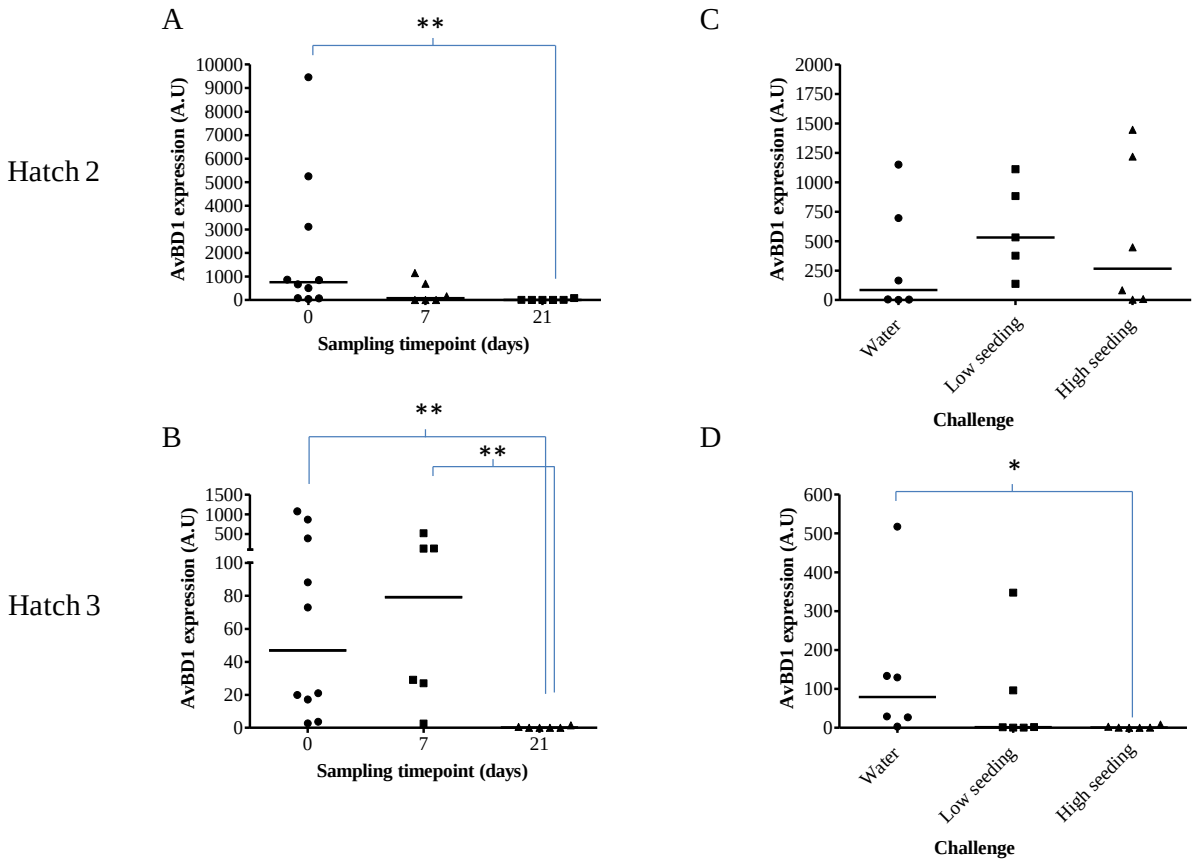


Figure 4.8: The effects of age of sampling and bacterial challenge on kidney AvBD1 gene expression in Line X birds.

AvBD1 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD1 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, ** $P < 0.01$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

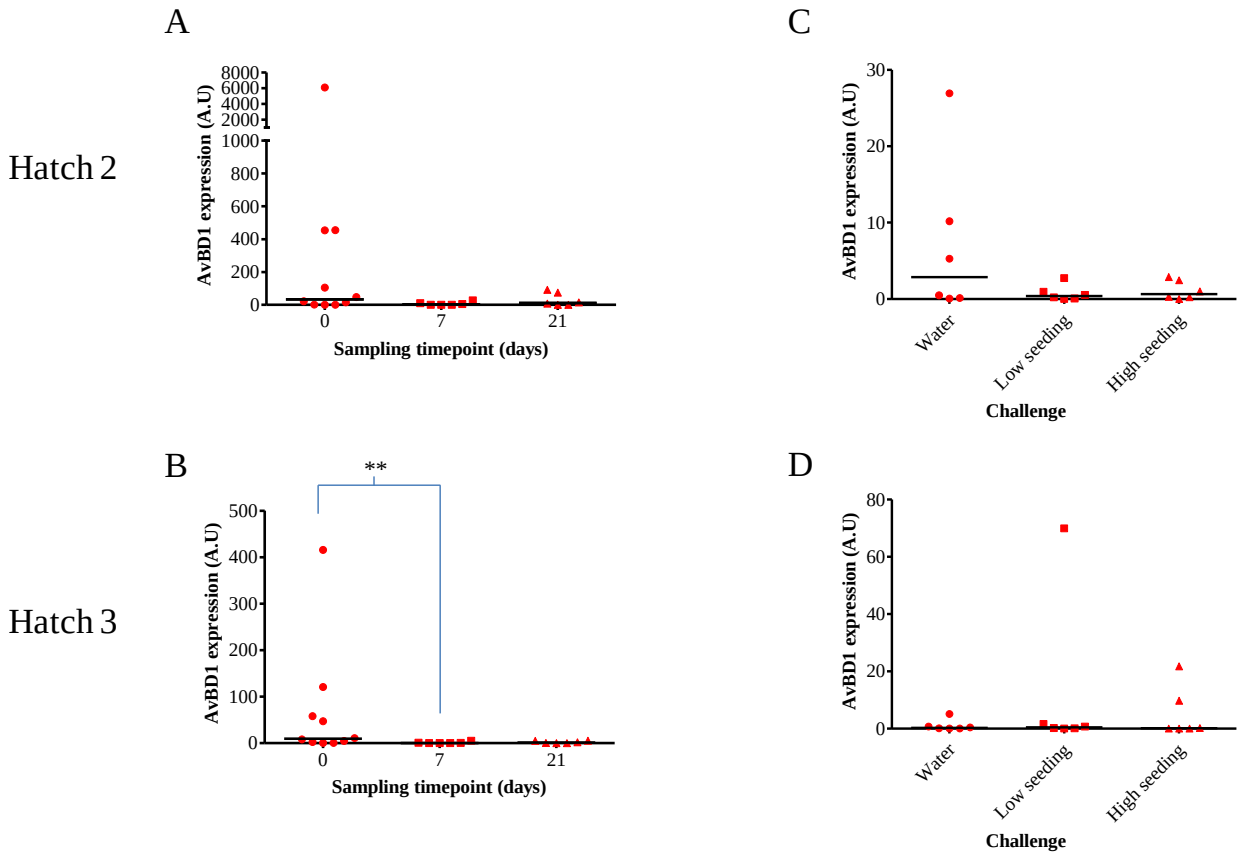


Figure 4.9: The effects of age of sampling and bacterial challenge on liver AvBD1 gene expression in Line X birds.

AvBD1 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD1 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. ** $P < 0.01$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

4.4.6 AvBD1 expression in the GI tract

AvBD1 gene expression is illustrated in the duodenum (Figure 4.10), jejunum (Figure 4.11), ileum (Figure 4.12), caecum (Figure 4.13) and caecal tonsil (Figure 4.14) of control birds aged 0, 7 and 21 days in Hatches 2 (A) and 3 (B), respectively. Also shown in each Figure is AvBD1 gene expression of the 7 day old birds sampled from the control and B/BV seeded pens of Hatch 2 (C) and 3 (D), respectively.

Duodenal expression for both Hatch 1 and 2 showed a significant effect of bird age on AvBD1 expression. Compared to Day 0, the trend was for the median AvBD1 expression to be lower at the 7 day time-point and to remain low in the 21 day-old birds. This trend was consistent between hatches and was statistically significant between birds aged 0 and 21 days ($P < 0.05$). This pattern of age-dependent AvBD1 expression was repeated for the jejunum (Figure 4.11), ileum (Figure 4.12), caecum (Figure 4.13) and caecal tonsil (Figure 4.14).

The expression data relating to the B/BV challenged birds was less clear. The duodenal data showed that seeding newly-hatched chicks with B/BV (low and high) resulted in significantly lower AvBD1 expression in the 7 day-old challenged birds compared to the water control birds (Figure 4.10 C and D). This bacterial challenge effect was not, however, observed in Hatch 3 although the expression values recorded for the challenged Hatch 3 birds were reduced by over 150-fold compared to Hatch 2. Similarly, in the jejunum, B/BV seeded groups had lower AvBD1 expression in Hatch 2 than control birds and this reached statistical significance between control and high-seeding birds ($P < 0.05$). As described for duodenal tissues, no effect of challenge was seen in Hatch 3 although the expression values were extremely low (<0.02 A.U) for all birds. No significant differences between control and B/BV challenged groups were observed in the ileum (Figure 4.12), caecum (Figure 4.13) and caecal tonsil (Figure 4.14). Generally these data suggest that the B/BV challenge was associated with a further down-regulation of AvBD1 expression, particularly in the proximal gut tissues.

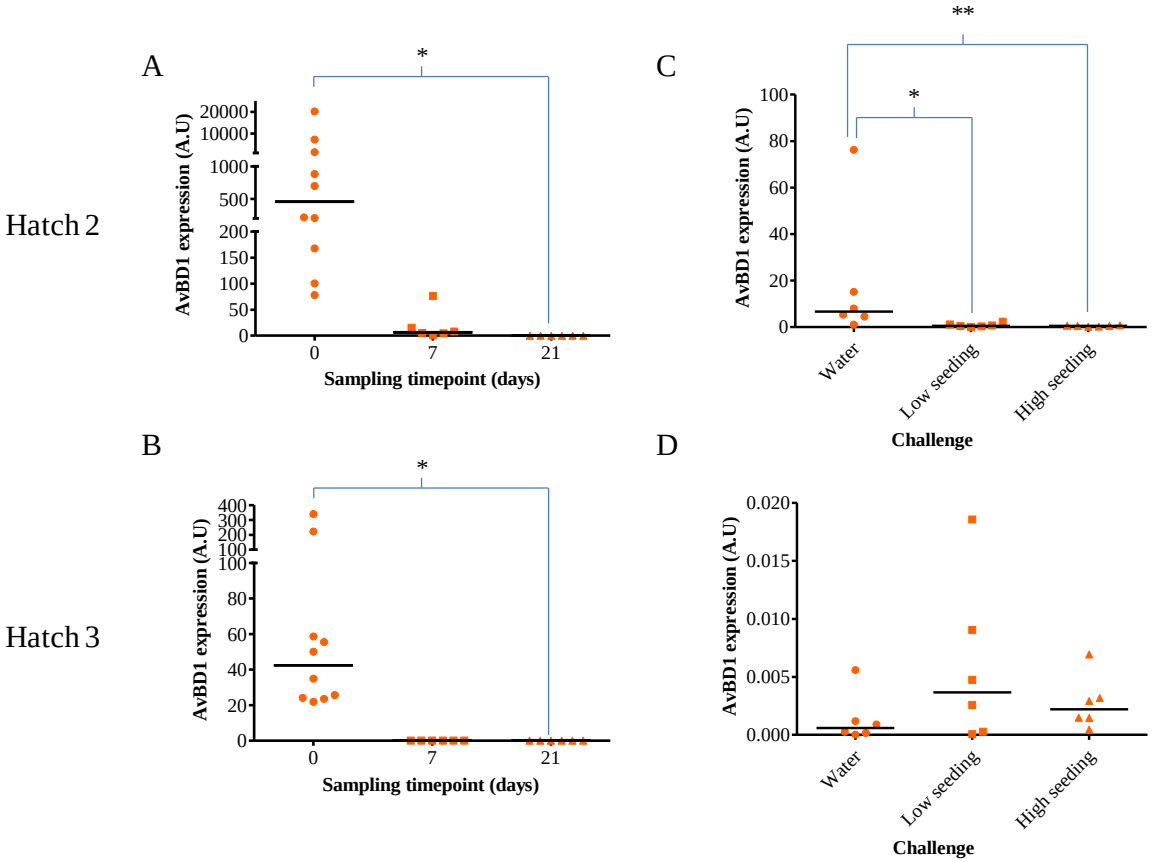


Figure 4.10: The effects of age of sampling and bacterial challenge on duodenal AvBD1 gene expression in Line X birds.

AvBD1 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD1 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, ** $P < 0.01$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

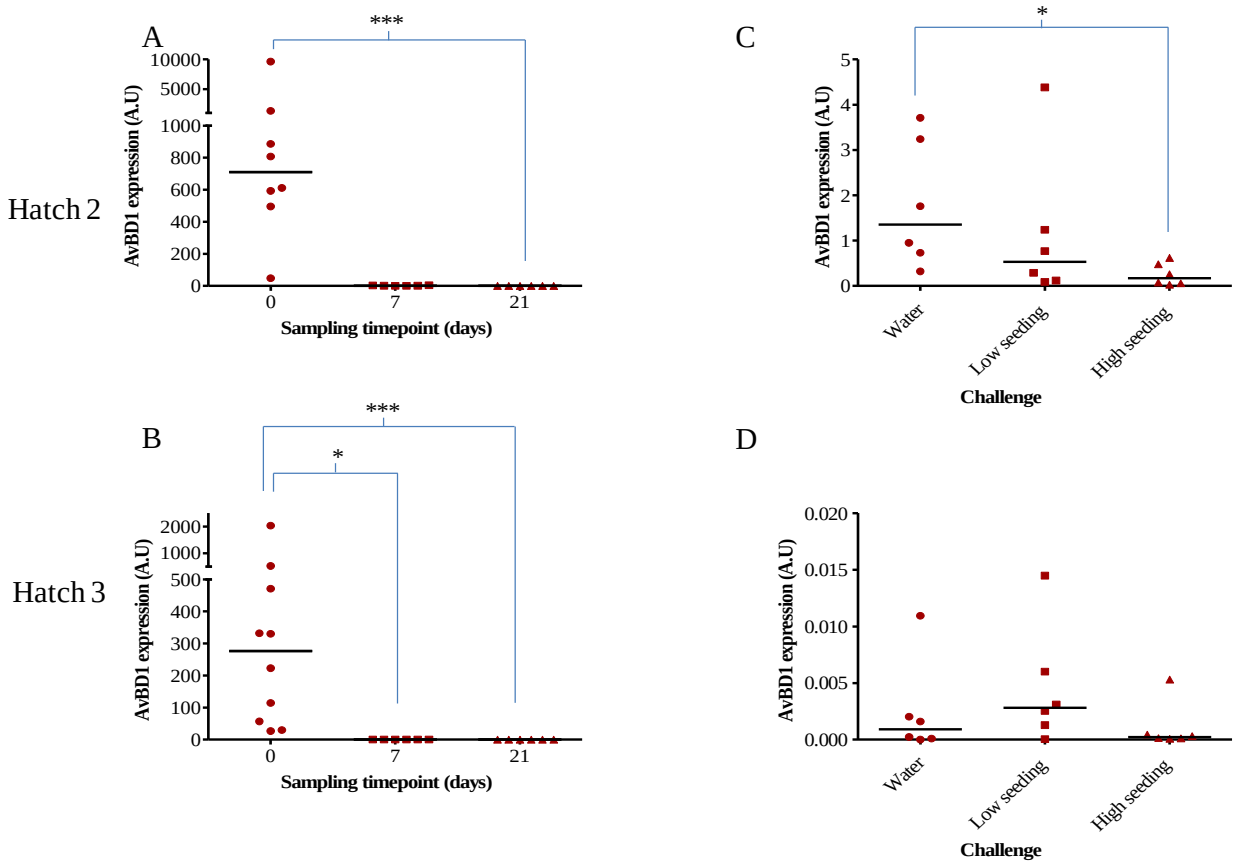


Figure 4.11: The effects of age of sampling and bacterial challenge on jejunal AvBD1 gene expression in Line X birds.

AvBD1 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD1 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, *** $P < 0.001$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

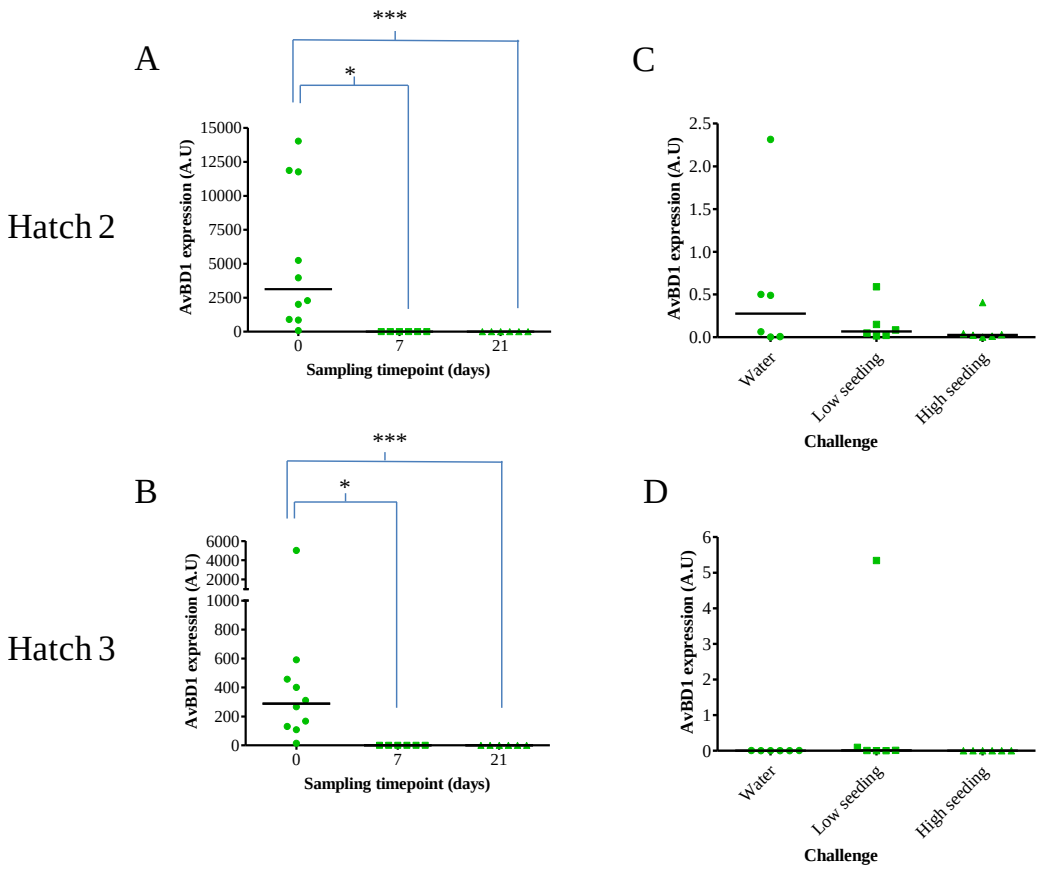


Figure 4.12: The effects of age of sampling and bacterial challenge on ileal AvBD1 gene expression in Line X birds.

AvBD1 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD1 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, *** $P < 0.001$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

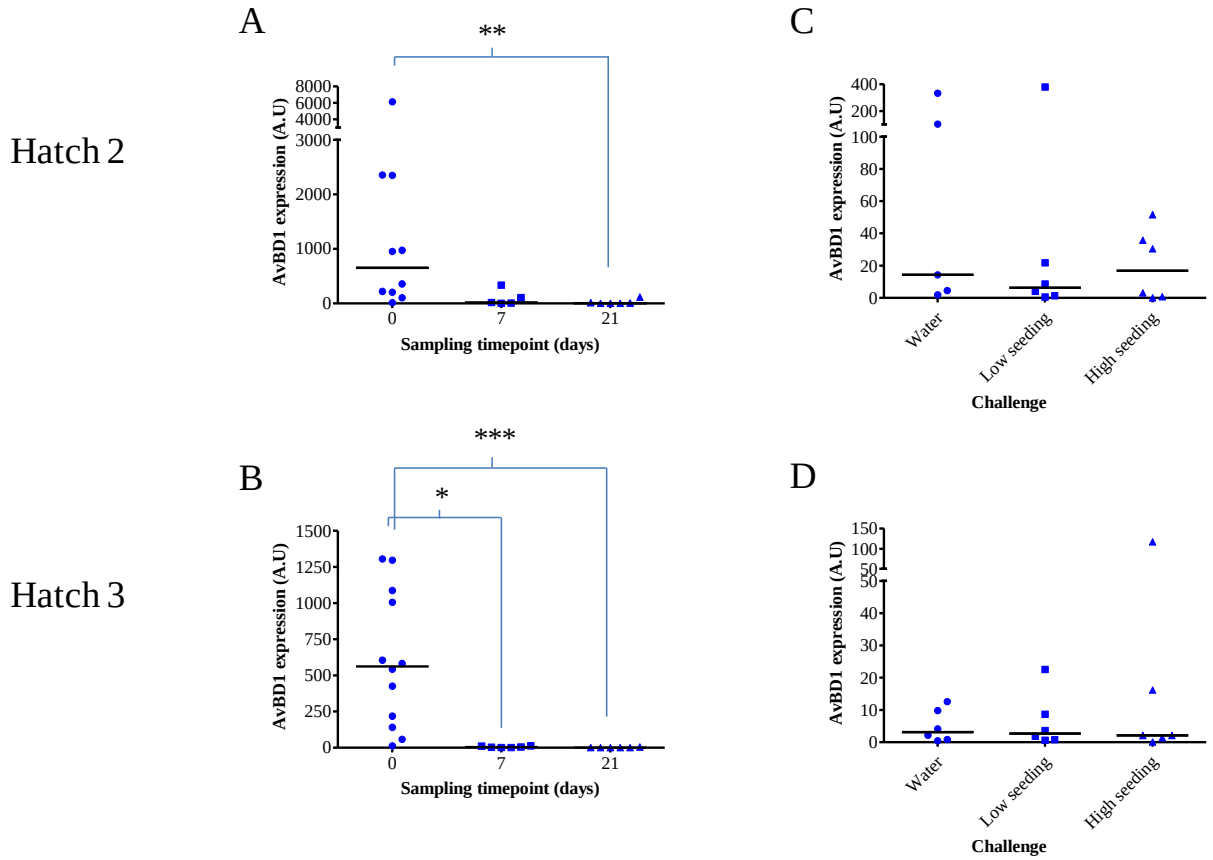


Figure 4.13: The effects of age of sampling and bacterial challenge on caecal AvBD1 gene expression in Line X birds.

AvBD1 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD1 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

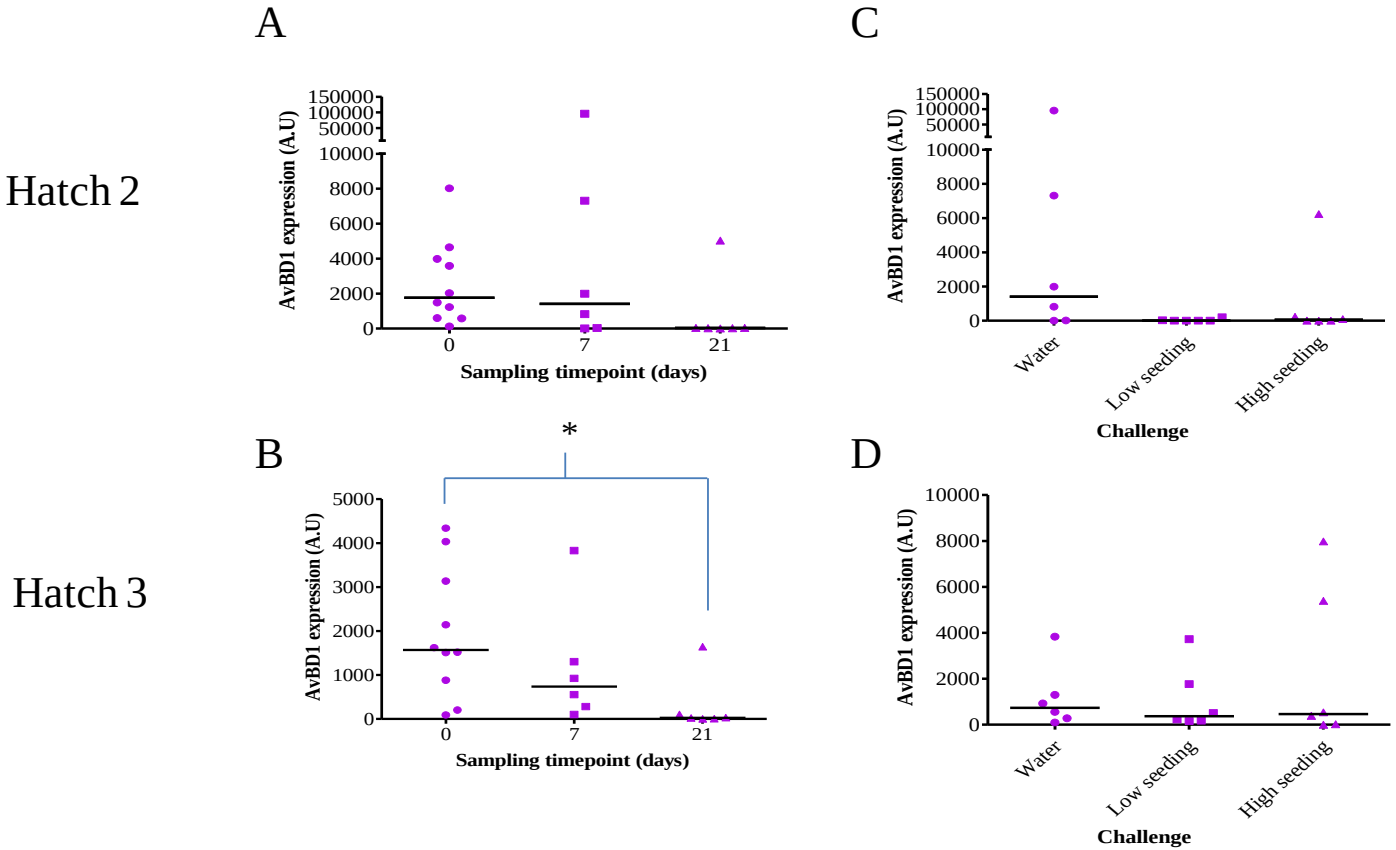


Figure 4.14: The effects of age of sampling and bacterial challenge on caecal tonsil AvBD1 gene expression in Line X birds.

AvBD1 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD1 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparison test.

4.4.7 Summary of AvBD1 expression data

Tables 4.1 and 4.2 summarise the statistically significant differences in AvBD1 expression that were found when considering the effect of age and bacterial seeding, respectively. Table 4.1 shows that, compared to birds at Day 0, AvBD1 gene expression was significantly lower at Day 7 in tissues sampled from birds in Hatch 2 (liver, ileum) and Hatch 3 (kidney, jejunum, ileum, caecum). This age-dependent decrease in expression relative to Day 0 was also observed at the Day 21 sampling time-point in which significantly lower AvBD1 expression was found in all tissues accept the liver (Hatch 2 and 3) and the caecal tonsil (Hatch 3).

Table 4.1: The effect of age on AvBD1 expression (compared to Day 0)

	Day 7		**Day 21**	
	Hatch 2	**Hatch 3**	**Hatch 2**	**Hatch 3**
Kidney	NS	↓ $P < 0.01$	↓ $P < 0.01$	↓ $P < 0.01$
Liver	↓ $P < 0.01$	NS	NS	NS
Duodenum	NS	NS	↓$P < 0.05$	↓$P < 0.05$
Jejunum	NS	↓ $P < 0.01$	↓ $P < 0.001$	↓ $P < 0.001$
Ileum	↓ $P < 0.05$	↓ $P < 0.05$	↓ $P < 0.001$	↓ $P < 0.001$
Caecum	NS	↓ $P < 0.05$	↓ $P < 0.01$	↓ $P < 0.001$
Caecal tonsil	NS	NS	NS	↓ $P < 0.05$

P value represents significant differences between birds at hatch and birds aged 7 and 21 days.
NS – no significance, ↓ - lower gene expression than Day 0

Table 4.2 shows that, compared to control birds, those that were seeded with B/BV at low levels had significantly lower AvBD1 expression in the duodenum (Hatch 2). When comparing to birds exposed to high B/BV seeding, significantly lower AvBD1 expression was found in the kidney, duodenum and jejunum (Hatch 3). No consistent effect was observed across both hatches for either low or high seeding.

Table 4.2: The effect of seeding type on AvBD1 expression in birds aged 7 days

	Low seeding		**High seeding**	
	Hatch 2	**Hatch 3**	**Hatch 2**	**Hatch 3**
Kidney	NS	NS	NS	↓ $P < 0.05$
Liver	NS	NS	NS	NS
Duodenum	↓ $P < 0.05$	NS	↓ $P < 0.01$	NS
Jejunum	NS	NS	↓ $P < 0.05$	NS
Ileum	NS	NS	NS	NS
Caecum	NS	NS	NS	NS
Caecal tonsil	NS	NS	NS	NS

P value represents significant differences between control and seeded birds
NS – no significance. ↓ - lower gene expression than control birds.

4.5 Immunolocalisation of AvBD1

IHC staining was performed by Dr Catherine Mowbray, Newcastle University.

To explore the gut localisation of AvBD1 a custom polyclonal AvBD1 antibody was commercially produced and gut tissue sections (duodenum and caecum) from Hatch 2 birds aged 0 and 7 Days were stained for AvBD1 (Figure 4.16). These data revealed that the AvBD1 peptide was present in birds at both sampling ages and, at the protein level, no clear differences were discernible. Moreover, Figure 4.15 C and D showing the AvBD1 stain at a higher magnification (x400), indicates that AvBD1 is present throughout the tissue and not localised to a specific region.

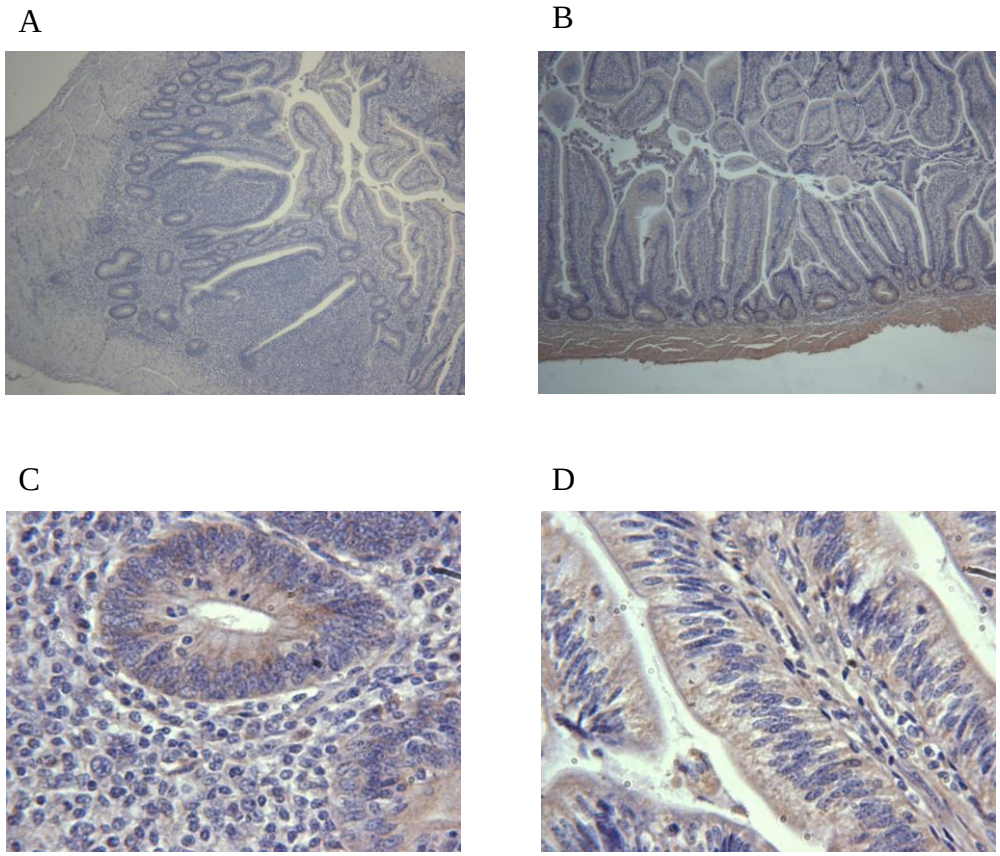


Figure 4.15: Antibody staining for AvBD1 peptide in caecum.

Tissue was provided from a single bird (Bird 79). A) Negative control – No primary antibody, x 40. B) 1/250 dilution of AvBD1 antibody in EDTA, x 40 C) and D) 1/250 dilution of AvBD1 antibody in EDTA, x400. Data supplied by Dr Catherine Mowbray, Newcastle University.

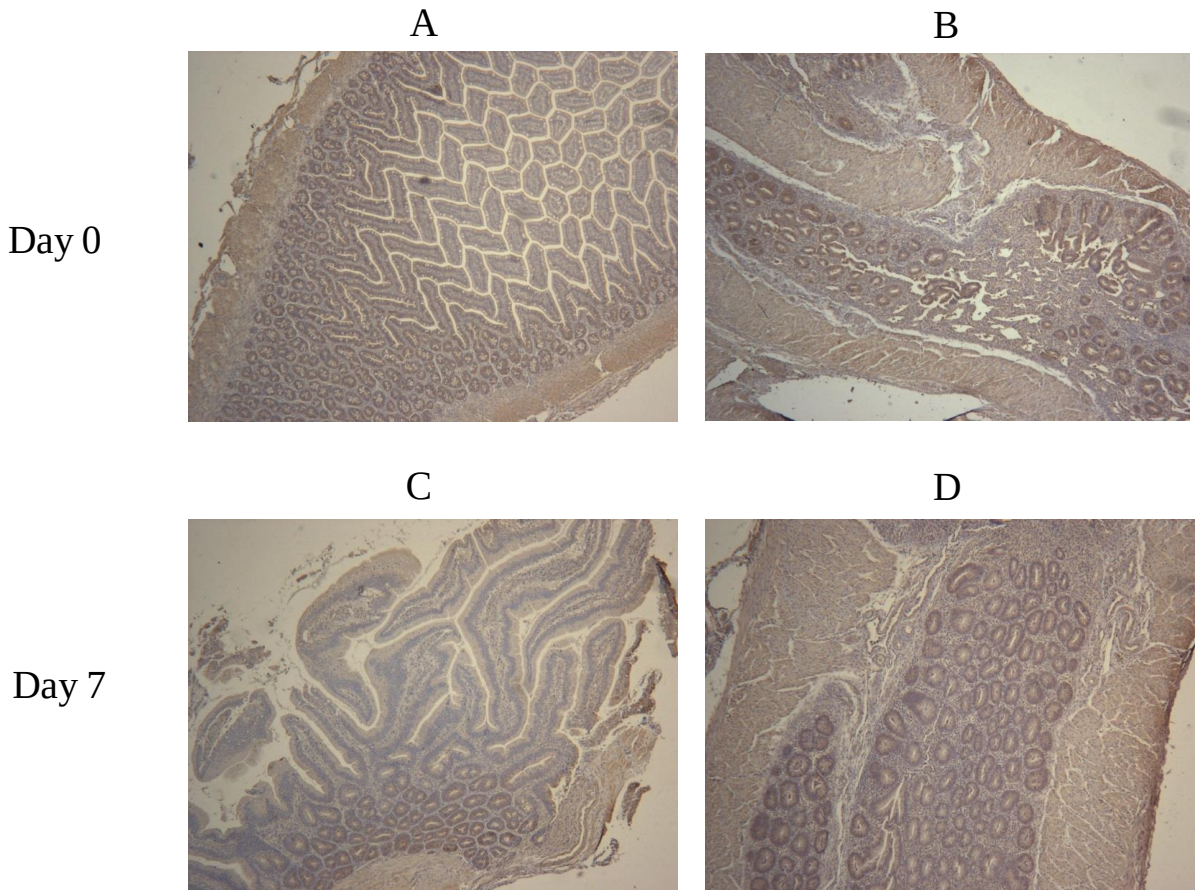


Figure 4.16: AvBD1 peptide is present in the GI tract of birds aged 0 and 7 days.

Sections of gastrointestinal tract from Line X birds at Day 0 (A and B) and Day 7 (C and D) immunostained for AvBD1; A) and C) duodenum, B) and D) caecum. Data supplied by Dr Catherine Mowbray, Newcastle University. Brown staining indicates AvBD1 presence.

4.6 AvBD10 gene expression

4.6.1 RT-endpoint PCR tissue panels (AvBD10)

Figure 4.17 shows endpoint RT-PCR AvBD10 expression for the kidney, liver and the GI tract (duodenum, jejunum, ileum, caecum and caecal tonsil) for A) day 0 i.e. newly-hatched chicks and B) 21 day-old control and B/BV challenged birds. The data, although not quantitative, shows AvBD10 expression throughout all tissues in newly hatched chicks, but also suggests marked liver and kidney expression in control and challenged 21 day-old birds.

4.6.2 AvBD10 standard curve and melt curve for real-time qPCR

A real time qPCR assay was developed to quantitate expression. Figure 4.18A shows the standard curve produced from qPCR reactions using AvBD10 primers and a 1:10 dilution series of cloned plasmid containing the AvBD10 partial gene sequence. As performed for AvBD1, this procedure was repeated at least three times and a curve with an amplification efficiency of 2.02, and an error value of 0.045 for sample replicates was chosen to enable relative quantification of cDNA samples. In addition, for each sample, melt curves were checked for a single peak at 83°C which ensured specificity of the AvBD10 primers. An example of a series of melt curves for AvBD10 PCR products from a range of GI tissue cDNA is shown in Figure 4.18B.

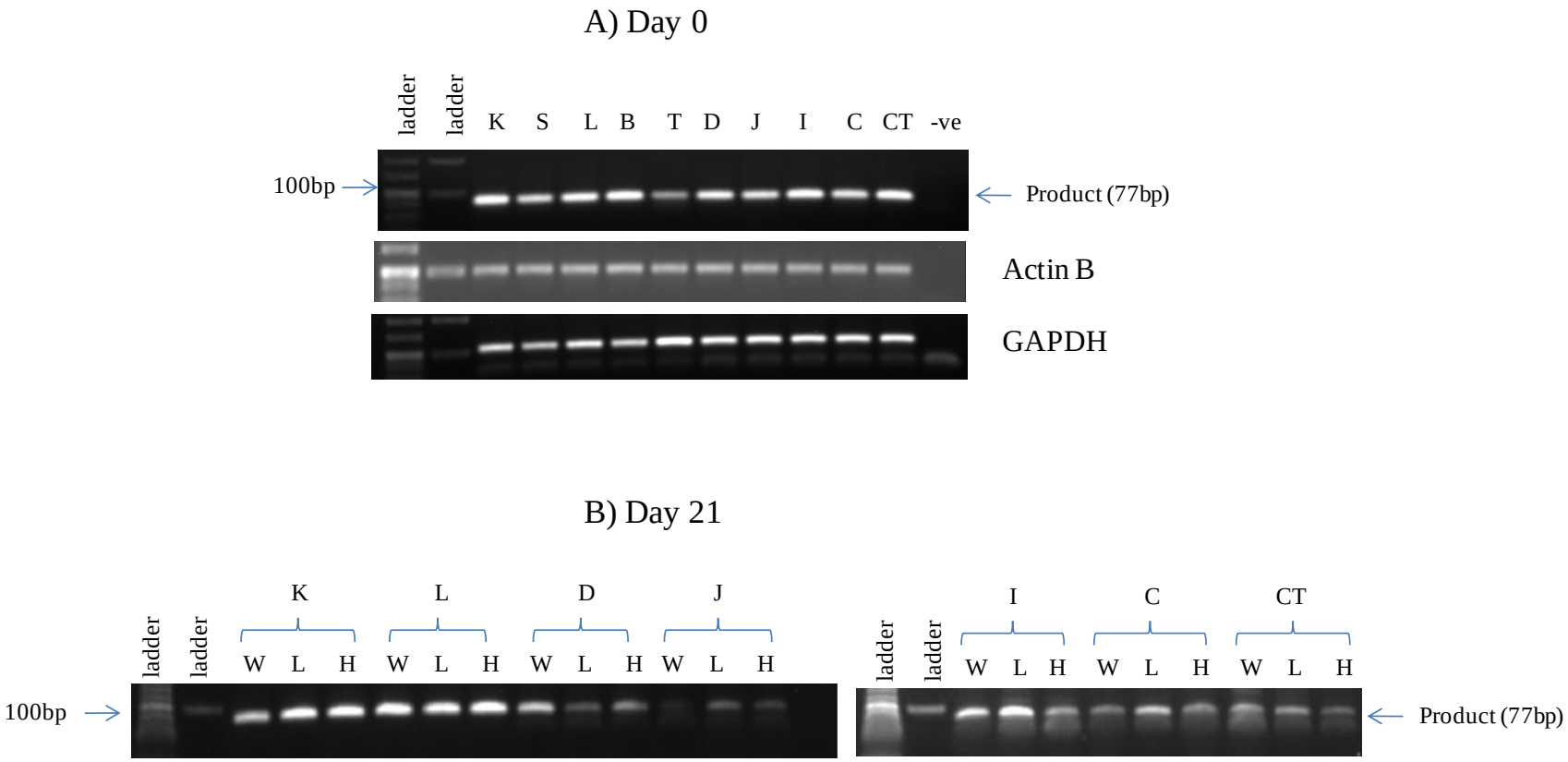
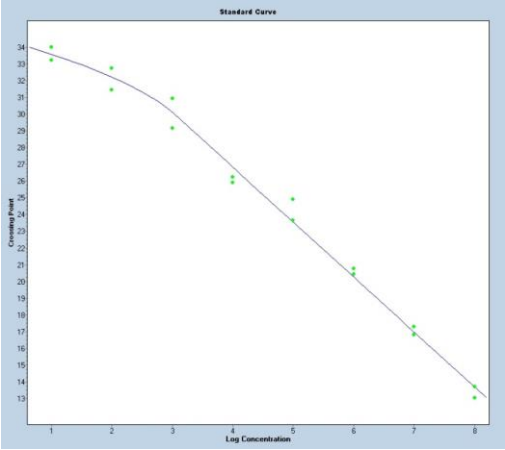


Figure 4.17: Endpoint PCR tissue panels showing AvBD10 gene expression at Day 0 and Day 21 for control and challenged birds.

A) AvBD10 tissue panel for a newly hatched bird and for B) three birds aged 21 days, each one sampled from either the water control pen (W), the low seeding B/BV challenge pen (L) or the high seeding B/BV challenge pen (H). RNA was extracted from Kidney (K), Spleen (S), Liver (L), Bursa (B), Thymus (T), Duodenum (D), Jejunum (J), Ileum (I), Caecum (C) and Caecal Tonsil (CT). –ve = negative control. Data supplied by Dr Catherine Mowbray, Newcastle University.

A) Standard curve



B) Melt curves

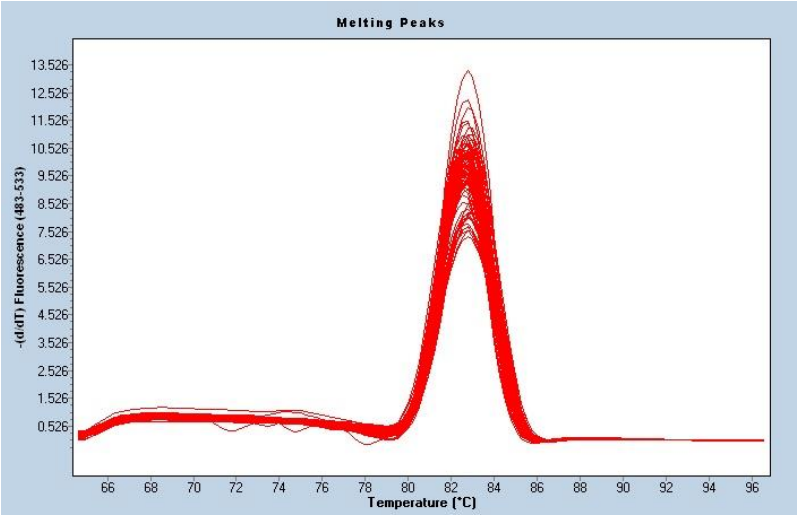


Figure 4.18: Real-time qPCR standard and melt curve for AvBD10.

A) Standard curve for serial dilutions of AvBD10 plasmid against calculated CP values. B) Melt curve for AvBD10 PCR products of gut tissue samples (n = ~ 40 samples). A single peak for each product was observed at the melting temperature of 83°C.

4.6.3 Summary of AvBD10 gene expression in tissues of birds aged 0, 7 and 21 days

Figure 4.19 shows the AvBD10 gene expression profiles of tissues from Hatch 2 (A, C and E) and Hatch 3 (B, D and F) birds aged 0, 7 and 21 days.

As for AvBD1, significant within-group variability was observed and the data at the Day 0 sampling time-point was skewed indicating a non-Gaussian distribution for the kidney, duodenum, ileum and caecum in Hatch 2 and all GI tissues in Hatch 3 (Panel A and B) ($P < 0.001$, D'Agostino & Pearson omnibus normality test). A comparison of the median AvBD10 expression between sampled tissue groups revealed that expression patterns were tissue-dependent in both Hatch 2 and 3 ($P < 0.001$). In particular, AvBD10 expression was elevated in the liver and kidney tissues compared to those of the GI tract. Interestingly, these relatively high levels of kidney/liver expression remained at the Day 7 and Day 21 sampling time-points, in contrast to the gut tissues which displayed a pattern of decreasing AvBD10 expression with time.

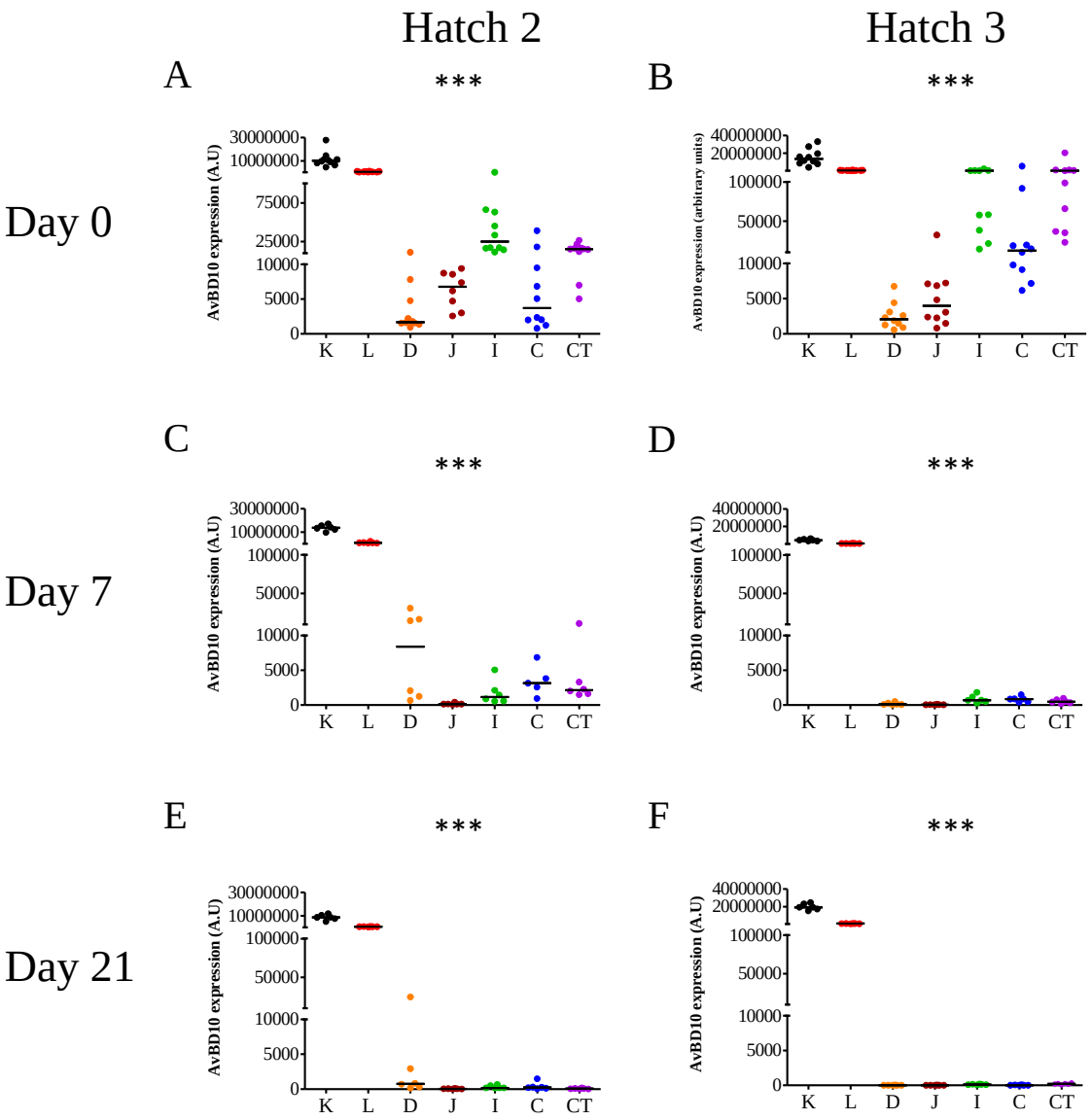


Figure 4.19: AvBD10 gene expression in tissues of newly hatched chicks (Day 0) and birds aged 7 and 21 days

Gene expression is shown in arbitary units for newly hatched chicks in Hatch 2 (A) and Hatch 3 (B), birds aged 7 days in Hatch 2 (C) and Hatch 3 (D), and birds aged 21 days in Hatch 2 (E) and Hatch 3 (F). K – kidney, L – liver, D – duodenum, J – jejunum, I – ileum, C – caecum, CT – caecal tonsil. Solid line indicates the median expression level. (n = 6 - 10 birds). (* $P < 0.05$, *** $P < 0.001$; Kruskal-Wallis test compares the median values of all tissue types).

4.6.4 AvBD10 expression in the kidney and liver

Figures 4.20 and 4.21 show the kidney and liver AvBD10 expression data for control birds aged 0, 7 and 21 days, respectively for Hatch 2 (A) and 3 (B), and for 7 day-old birds sampled from the control and B/BV seeded pens in Hatch 2 (C) and 3 (D).

Analyses of the data shows that, although individual variation in AvBD10 expression was observed, the general pattern was for both liver and kidney expression levels to be maintained at relatively high values throughout the sampling period.

For Hatch 2, no effect of B/BV seeding on AvBD10 expression was found in either the kidney or liver. In Hatch 3, no differences between control and challenged groups were found in the kidney, but in the liver, the expression in the 'high' seeding group was significantly lower than in the control group ($P < 0.01$, Dunn's multiple comparison test).

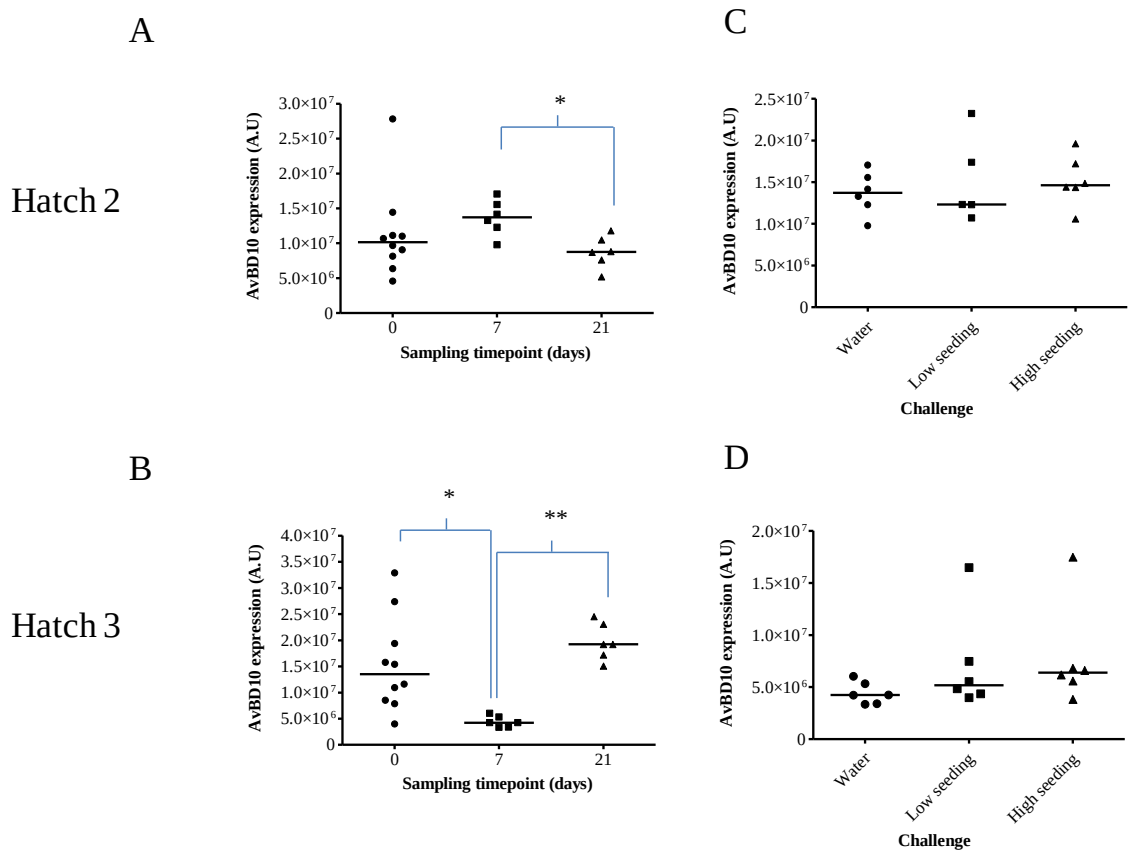


Figure 4.20: The effects of age of sampling and bacterial challenge on kidney AvBD10 gene expression in Line X birds.

AvBD10 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD10 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, ** $P < 0.01$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

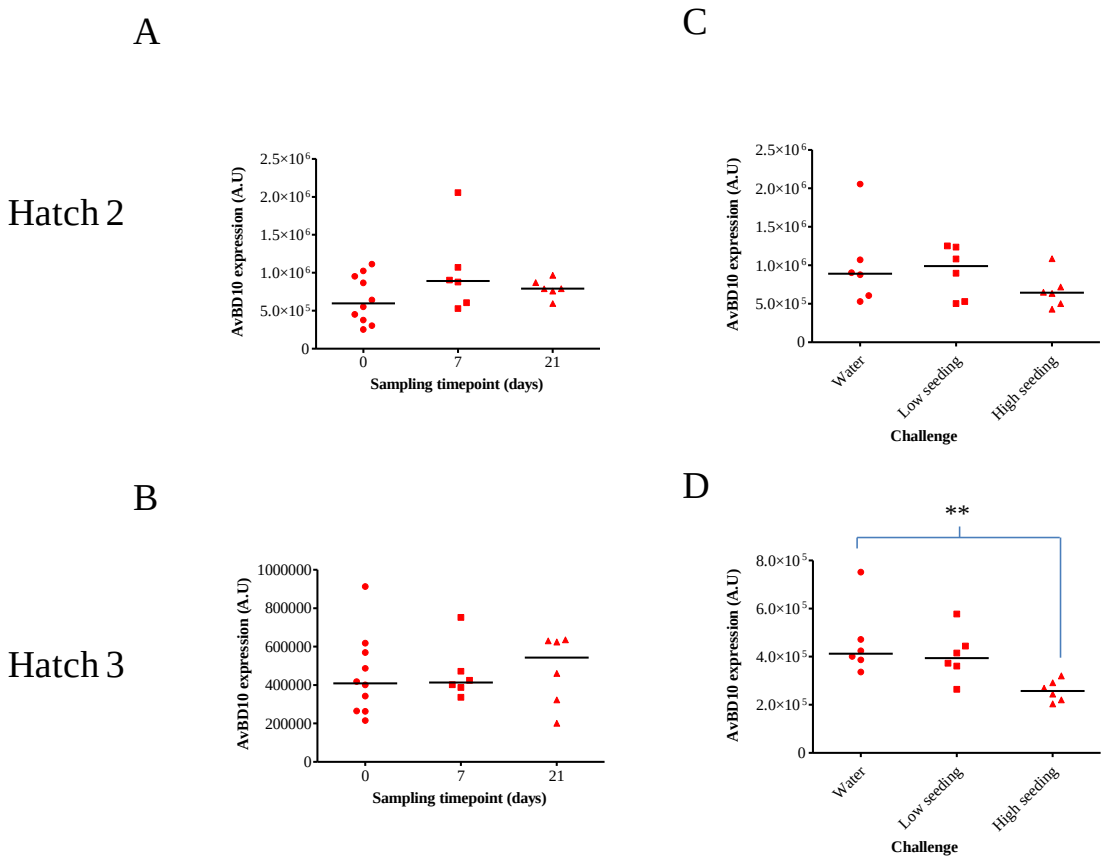


Figure 4.21: The effects of age of sampling and bacterial challenge on liver AvBD10 gene expression in Line X birds.

AvBD10 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD10 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. ** $P < 0.01$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

4.6.5 AvBD10 expression in the GI tract

Figures 4.22 to 4.26 illustrate AvBD10 gene expression in the duodenum, jejunum, ileum, caecum and caecal tonsil of Hatch 2 (A) and 3 (B) birds aged 0, 7 and 21 days, respectively, and specifically birds aged 7 days sampled from the water control pens or pens with low and high B/BV seeding in Hatch 2 (C) and 3 (D).

Age was found to impact significantly on AvBD10 expression. Higher median AvBD10 expression was found in the newly-hatched chicks compared to 7 and 21 day-old birds and this reached statistical significance for many of the groups sampled (Duodenum H3, $P < 0.001$; jejunum H2/H3, $P < 0.001$; ileum H2/H3, $P < 0.001$; caecum H2, $P < 0.01$; caecum H3, $P < 0.001$; caecal tonsil H2/H3, $P < 0.001$).

Interestingly the data indicated that birds from Hatch 2 and Hatch 3 responded differently to B/BV seeding. Hatch 3 data, with the exception of ileal expression, indicated higher duodenal, jejunal, caecal and caecal tonsil expression in challenged than control birds and this reached statistical significance in the caecal tonsil (low seeding, $P < 0.05$; high seeding, $P < 0.001$) (Figure 4.26). In contrast, data from Hatch 2 illustrated that birds from B/BV seeded pens had lower median AvBD10 expression than the control birds, and this was statistically significant for duodenum (high seed, $P < 0.05$) and caecum (low seed, $P < 0.05$; high seed, $P < 0.01$), although there were no differences in AvBD10 expression between control and challenged groups for jejunal, ileal and caecal tonsil tissues.

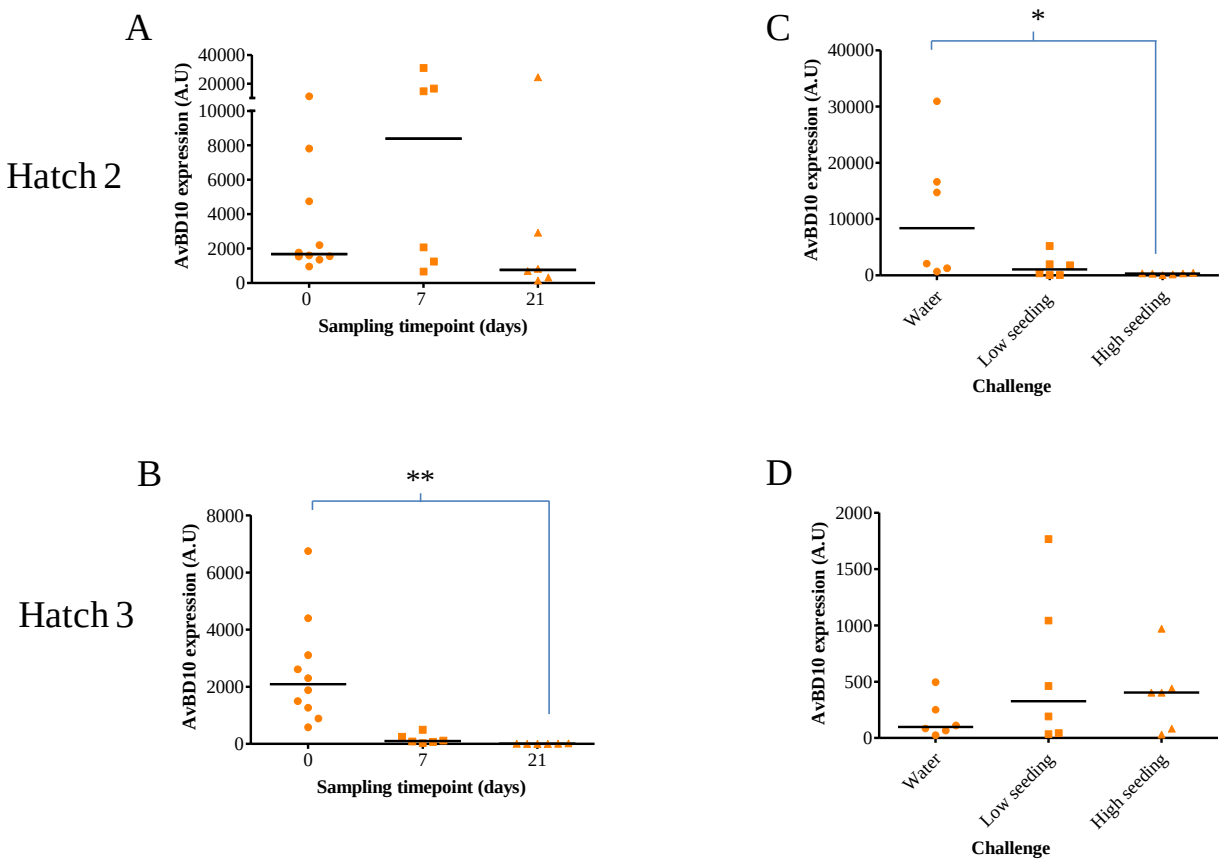


Figure 4.22: The effects of age of sampling and bacterial challenge on duodenal AvBD10 gene expression in Line X birds.

AvBD10 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD10 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, ** $P < 0.01$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

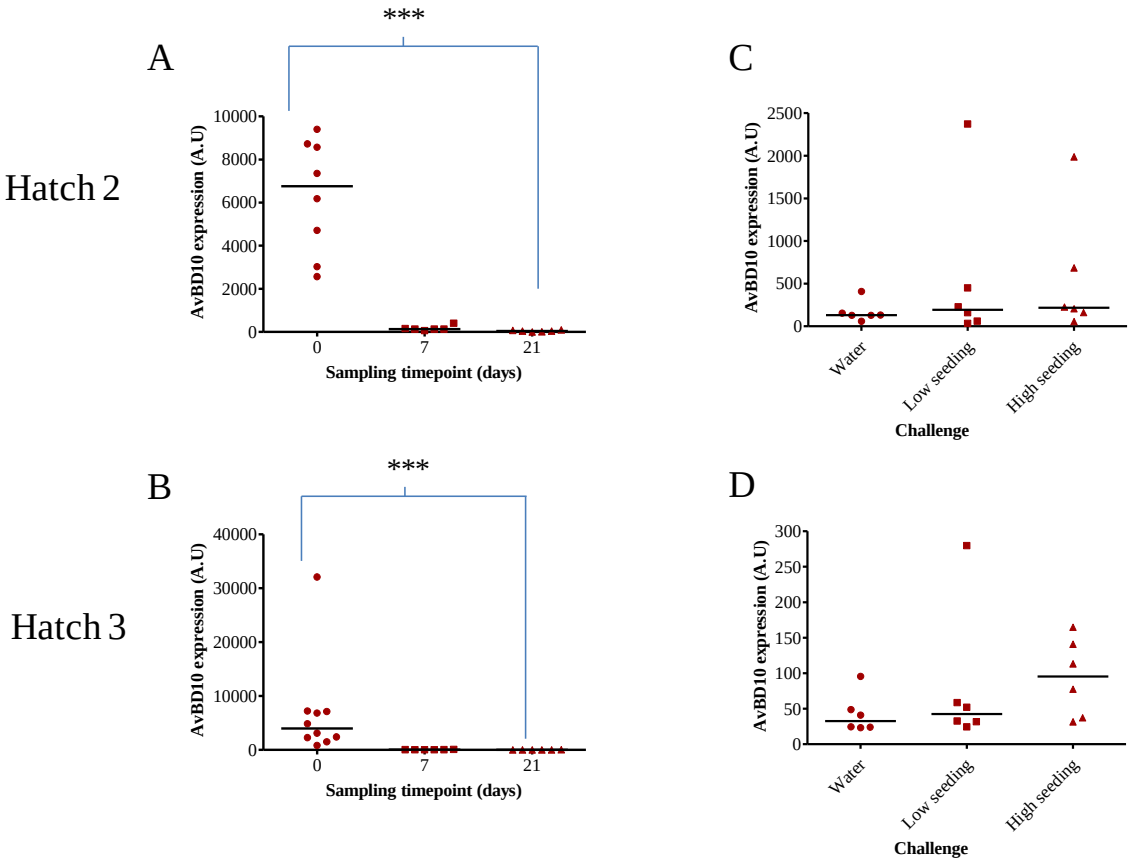


Figure 4.23: The effects of age of sampling and bacterial challenge on jejunal AvBD10 gene expression in Line X birds.

AvBD10 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD10 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, *** $P < 0.001$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

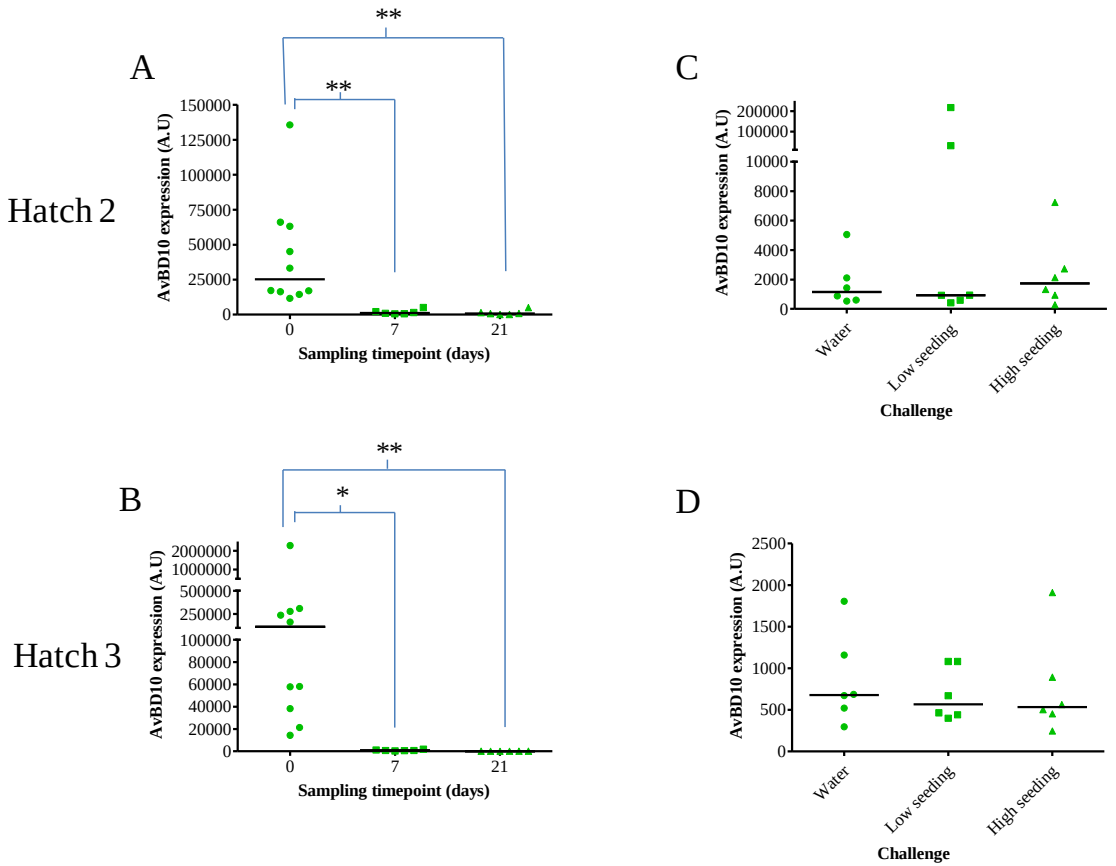


Figure 4.24: The effects of age of sampling and bacterial challenge on ileal AvBD10 gene expression in Line X birds.

AvBD10 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD10 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, ** $P < 0.01$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

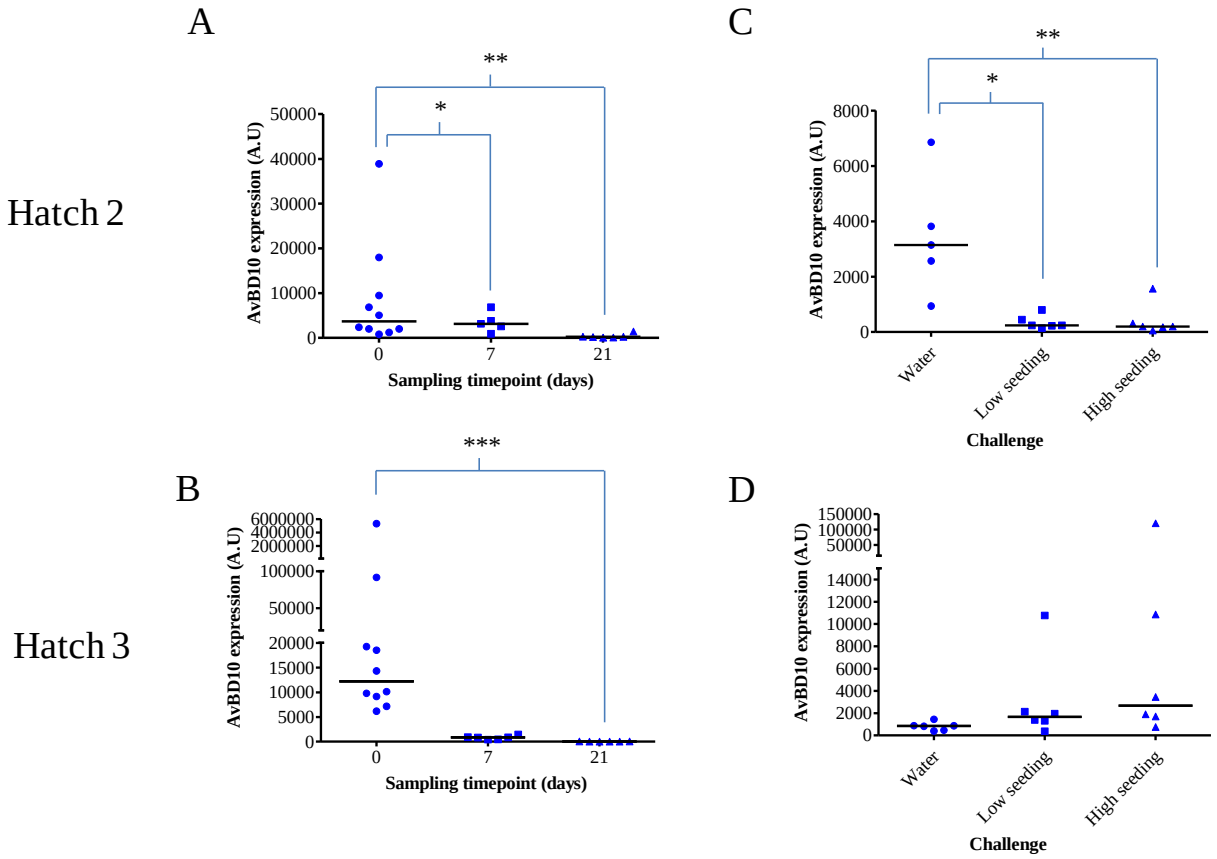


Figure 4.25: The effects of age of sampling and bacterial challenge on caecal AvBD10 gene expression in Line X birds.

AvBD10 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD10 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. * $P < 0.05$, **$P < 0.01$, *** $P < 0.001$; Kruskal-Wallis test followed by Dunn's multiple comparison test.

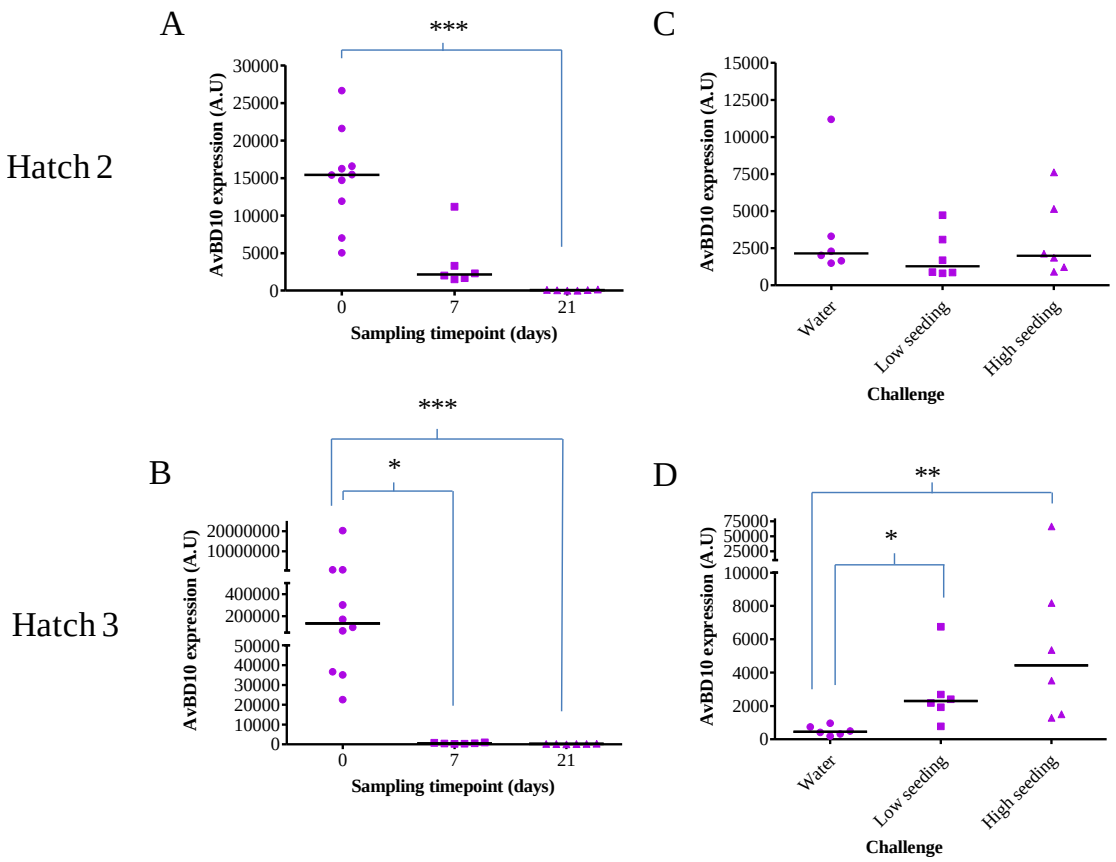


Figure 4.26: The effects of age of sampling and bacterial challenge on caecal tonsil AvBD10 gene expression in Line X birds.

AvBD10 gene expression in arbitrary units (A.U) is shown for birds aged 0, 7 and 21 days sampled from water control pens for Hatch 2 (A) and Hatch 3 (B). AvBD10 gene expression is shown for birds aged 7 days sampled from water control pens and pens with low and high *Bacteroides/Barnesiella visericola* (B/BV) seeding in Hatch 2 (C) and Hatch 3 (D). Sampled groups comprised of 5 males/5 females for 0-day old birds and 3 males/3 females at other sampling timepoints. *P < 0.05, ** P < 0.01, ***P < 0.001; Kruskal-Wallis test followed by Dunn's multiple comparison test.

4.6.6 Summary of AvBD10 expression data

Tables 4.3 and 4.4 summarise the statistically significant differences in AvBD10 expression that were found when considering the effect of age and bacterial seeding, respectively. Table 4.3 shows AvBD10 gene expression in the kidney and liver was not age-dependent. In contrast, distal gut tissues sampled from birds in Hatch 2 (ileum, caecum, caecal tonsil) and Hatch 3 (ileum) were significantly lower at Day 7 than at Day 0. In addition, by Day 21 significantly lower expression was maintained in the ileum, caecum and caecal tonsil for both hatches, a finding which was also observed in the proximal gut tissues in Hatch 2 (jejunum) and Hatch 3 (duodenum and jejunum).

Table 4.3: The effect of age on AvBD10 expression (compared to Day 0)

	Day 7		**Day 21**	
	Hatch 2	**Hatch 3**	**Hatch 2**	**Hatch 3**
Kidney	NS	NS	NS	NS
Liver	NS	NS	NS	NS
Duodenum	NS	NS	NS	↓ $P < 0.01$
Jejunum	NS	NS	↓ $P < 0.001$	↓ $P < 0.001$
Ileum	↓ $P < 0.01$	↓ $P < 0.05$	↓ $P < 0.01$	↓ $P < 0.01$
Caecum	↓ $P < 0.05$	NS	↓ $P < 0.01$	↓ $P < 0.001$
Caecal tonsil	↓ $P < 0.05$	NS	↓ $P < 0.001$	↓ $P < 0.001$

P value represents significance differences between birds at hatch and birds aged 7 and 21 days.
NS – no significance. ↓ - lower gene expression than Day 0

Table 4.4 shows that, compared to control birds, those that were seeded with B/BV at low levels had significantly lower AvBD10 expression in the caecum (Hatch 2) but significantly higher AvBD10 expression in the caecal tonsil (Hatch 3). The high B/BV seeding resulted in significantly lower AvBD10 expression in the liver (Hatch 3), duodenum (Hatch 2) and caecum (Hatch 2) and, as observed for low seeding, higher AvBD10 expression was found in the caecal tonsil tissues from birds sampled from Hatch 3. No consistent effect was observed across both hatches in any tissue for either low or high seeding.

Table 4.4: The effect of seeding type on AvBD10 expression in birds aged 7 days

	Low seeding		**High seeding**	
	Hatch 2	**Hatch 3**	**Hatch 2**	**Hatch 3**
Kidney	NS	NS	NS	NS
Liver	NS	NS	NS	↓ $P < 0.05$
Duodenum	NS	NS	↓ $P < 0.01$	NS
Jejunum	NS	NS	NS	NS
Ileum	NS	NS	NS	NS
Caecum	↓ $P < 0.05$	NS	↓ $P < 0.01$	NS
Caecal tonsil	NS	↑$P < 0.05$	NS	↑$P < 0.01$

P value represents significance differences between control and seeded birds
NS – no significance. ↓ - lower gene expression than control birds; ↑ - higher gene expression than control birds.

4.7 The relationship between GI tract AvBD expression and gut health.

Bird tissue data for both AvBD1 and 10 consistently demonstrated that expression reduced during the first week and by 21 days host-hatch the expression levels were often negligible. For this reason no investigation of the relationship between AvBD expression and gut health was undertaken in 21 day-old birds. However, data from 7 day-old birds will be reported as gut health abnormalities were observed at this time-point. Bird guts were scored for redness (measure of inflammation), water content and tone. Figures 4.10 – 4.14 (AvBD1) and 4.22 – 4.26 (AvBD10) suggested that a B/BV challenge was associated with the down-regulation of AvBD expression. To further explore whether down-regulation was linked to poor gut health. The AvBD1 and 10 expression data for birds with healthy (gut score 0) and inflamed guts (gut score 1) were analysed (Figures 4.27 and 4.28).

Overlaps in expression values existed between birds with healthy or inflamed guts, but comparison of AVBD expression values did uncover some consistent patterns. For AvBD1, median expression values for all tissues in the birds with inflamed guts were lower than for healthy birds and this reached statistical significance (P,<0.05) in the caecum. Similarly, significantly lower AvBD10 levels were observed in the ileum ($P < 0.05$) for inflamed birds and lower median values were also observed in the duodenal and jejunal tissues of the inflamed birds without reaching statistical significance.

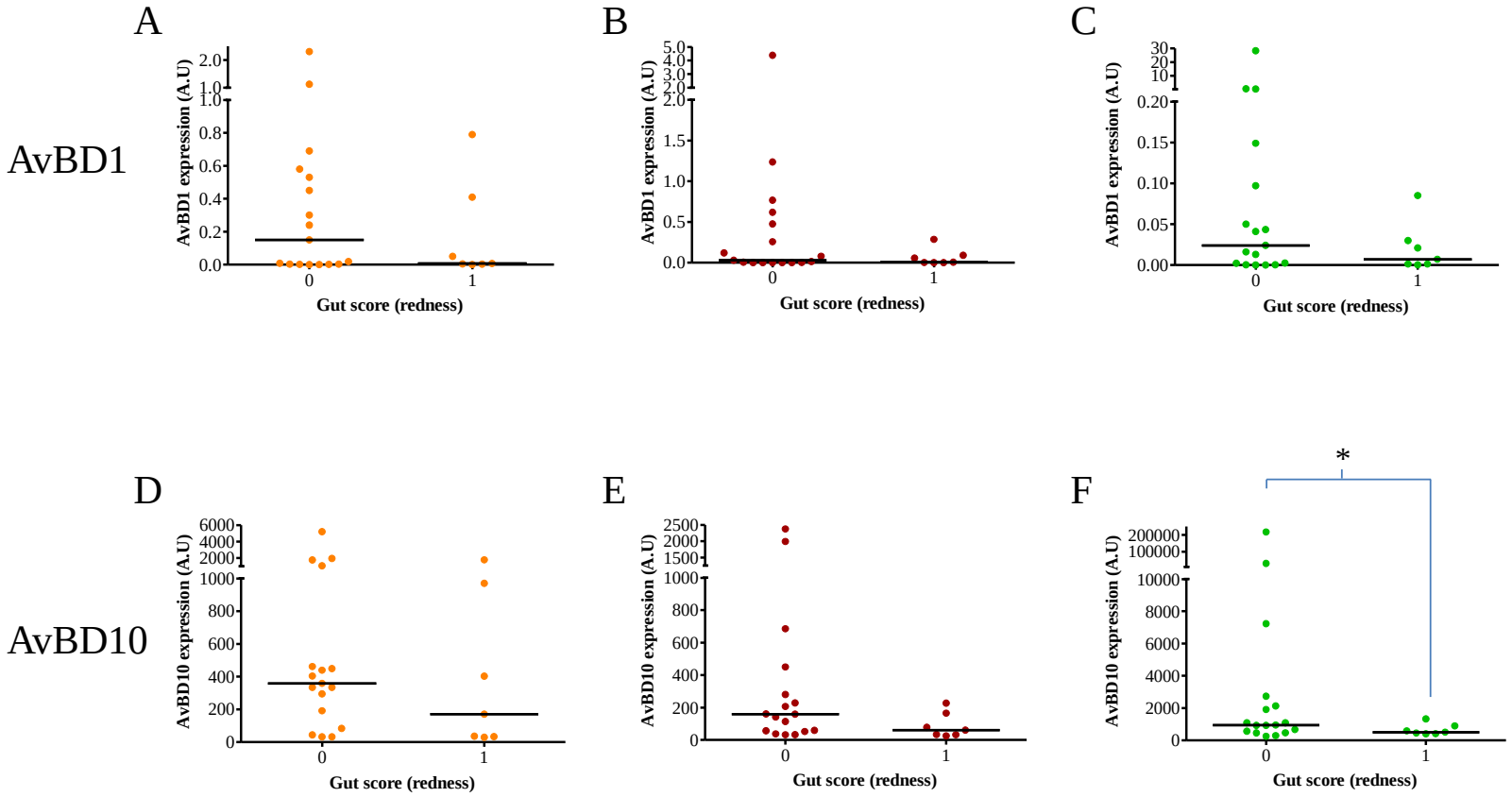


Figure 4.27: AvBD1 and 10 gene expression in birds with healthy and inflamed guts (duodenum, jejunum and ileum).

AvBD1 (A, B & C) and AvBD10 (D, E & F) expression in 7 day-old birds challenged with B/BV (low and high seeding) is shown in birds that have normal gut health (gut score 0) and birds that have inflamed guts (gut score 1). Solid line is the median value for all sampled birds and includes both control and challenged birds from Hatches 2 and 3. Expression was analysed in duodenal (A & D), jejunal (B and E) and ileal (C & F) tissues. * $P < 0.05$, Mann-Whitney U test.

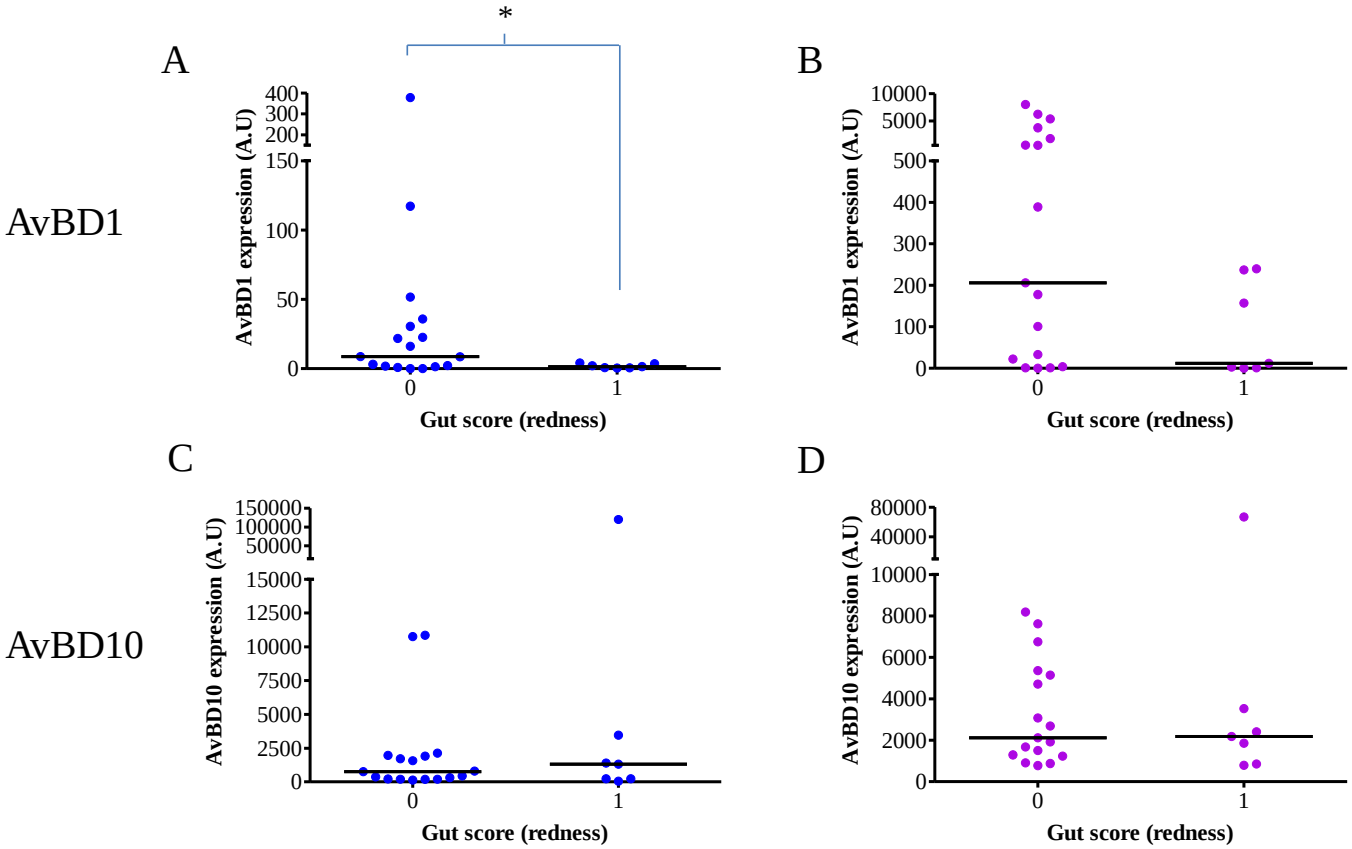


Figure 4.28: AvBD1 and 10 gene expression in birds with healthy and inflamed guts (caecum and caecal tonsil).

AvBD1 (A and B) and AvBD10 (C and D) expression in 7 day-old birds challenged with B/BV (low and high seeding) is shown in birds that have normal gut health (gut score 0) and birds that have inflamed guts (gut score 1). Expression was analysed in caecal (A & C) and caecal tonsil tissues (B and D). Solid line is the median value for all sampled birds and includes both control and challenged birds from Hatches 2 and 3. * $P < 0.05$, Mann-Whitney U test.

4.8 Identification of individual birds with low and high AvBD10 expression in the GI tract

Gene expression data has revealed marked variability within sampled groups and it was noted that some birds had expression values many fold higher than the median. A facet of this trial was to identify individual birds within the challenged pens that had relatively high AvBD gene expression values and to ascertain if these birds had improved gut health outcomes. To address this, individual birds were ranked against each other according to their tissue (duodenum, jejunum, ileum, caecum and caecal tonsil) AvBD expression. Figure 4.29 shows the ranked AvBD1 expression for each individual bird within the challenged pens in Hatch 2 (A) and 3 (B), and ranked AvBD10 expression in the challenged pens for Hatch 2 (C) and 3 (D).

Examination of the gut health scores in conjunction with the ranked AvBD1 expression indicated that birds with relatively low AvBD1 expression were characterised by high gut scores, indicating gut abnormalities, whereas birds with relatively high AvBD1 expression such as 158 and 152 (Hatch 3) had completely normal gut health despite the B/BV challenge. Indeed, dividing the birds into two groups of six based on the ranked AvBD expression values showed the higher expressing birds returned combined gut scores of 4 and 5 (Hatch 2 and 3, respectively) compared to 8 and 11 for the group of lower expressing birds.

For AvBD10, there was no clear evidence that high expression protected against gut problems and birds that were revealed to have relatively high expression throughout the GI tract such as 66 and 75 (Hatch 2) and 147 and 156 (Hatch 3) had abnormal gut health.

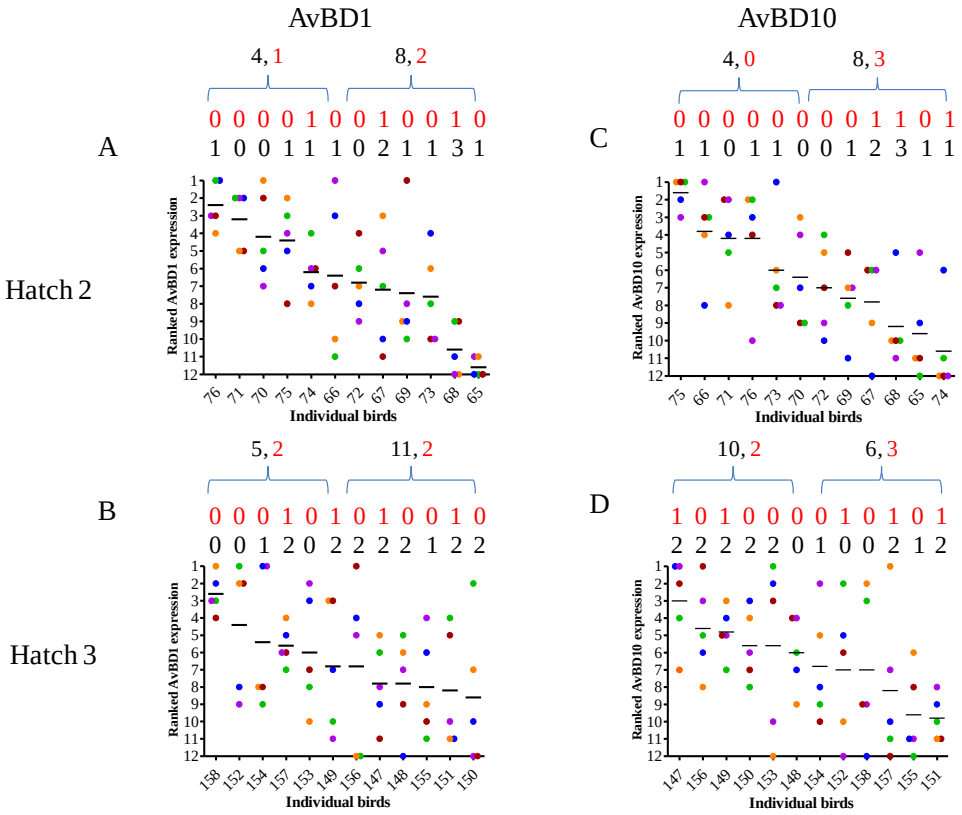


Figure 4.29: Individual birds ranked for GI AvBD1 and 10 expression and their corresponding gut health scores.

The data is displayed as the relative AvBD1 (A & B) and AvBD10 (C & D) expression in each tissue for B/BV challenged birds ranked within the group for Hatch 2 (A & C) and Hatch 3 (B & D). The highest expression value for a given tissue is ranked 1 and the lowest expression is ranked 12. Solid lines indicate the mean of the ranked expression and provide an indication of the overall AvBD expression throughout the GI tract relative to each bird. Orange – duodenum, Red – jejunum, Green – ileum, Blue – caecum and Purple – caecal tonsil. Overall gut scores for each bird are shown above each column (black), alongside scores for redness (red). Total gut scores and redness scores for two groups of six birds are shown above.

4.9 Microbial growth assays using jejunal gut scrapes.

Assay data reported in Chapter 3 suggested that the B/BV challenge resulted in a gut mucosa that supported increased *E. coli* growth. Moreover, gut scrapes from 4 day-old birds that had abnormal gut health also supported higher *E. coli* growth than healthy birds. To explore this further and determine if any relationship exists between gut health and bacterial growth, jejunal gut scrapes from birds in this trial were analysed to assess their ability to support the growth of *Salmonella typhimurium,* a human pathogen found in the bird caecum, and *Lactobacillus johnsonii,* a potential probiotic.

Figure 4.30 shows *S. typhimurium and L. johnsonii* growth supported by jejunal scrapes from birds aged 7 and 21 days, respectively. The data for *S. typhimurium* was very variable with no distinct patterns observed due to some gut scrapes proving to be anti-microbial, and thus inhibiting *S. typhimurium* growth, while others were extremely pro-microbial supporting *S. typhimurium* growth. In addition, scrapes sampled from 7 day-old birds supported higher microbial growth than those from birds aged 21 days. Comparison of gut scrapes from healthy birds aged 7 days with those from birds with inflamed guts revealed that there were no significant differences in *S. typhimurium* growth. However, by Day 21, the mean growth supported by the gut mucosa of birds with inflamed guts appeared elevated, although this did not reach statistical significance ($P > 0.05$). Unlike *S. typhimurium*, the data for *L. johnsonii* showed no age-dependent effects although a similar pattern of higher mean growth was observed when reviewing the scrape data from Day 21 birds with inflamed guts.

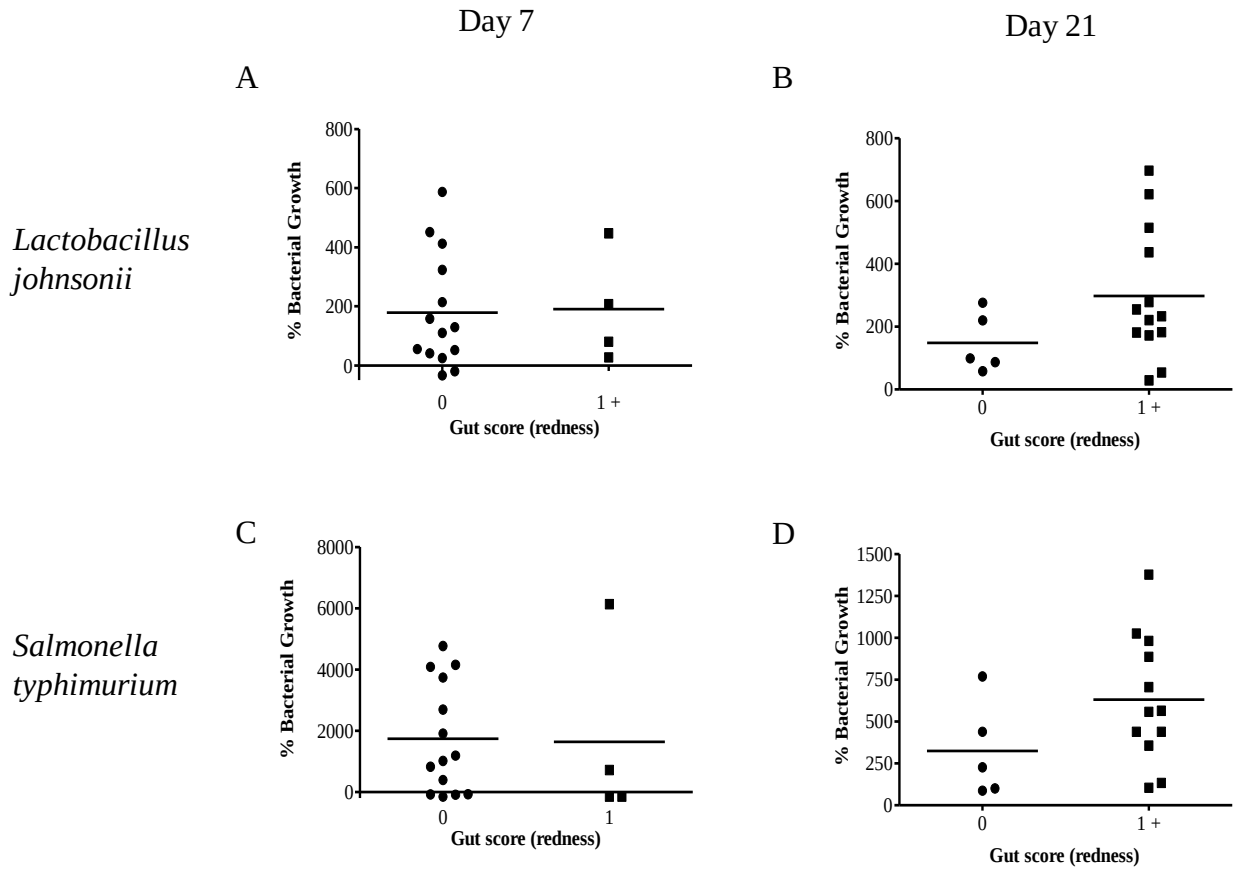


Figure 4.30: The effect of jejunal gut scrape protein extracts on *Lactobacillus johnsonii* and *Salmonella typhimurium growth*.

Data is presented as the percentage growth of *Lactobacillus johnsonii* (A & B) and *Salmonella typhimurium* facilitated by total protein extracts from jejunal gut scrapes in Line X birds (all treatment groups combined). Data are shown for gut scrapes from birds aged seven days (A and C) and 21 days (B & D).

4.10 Discussion

To further characterise the gut health deterioration observed in birds following early B/BV exposure, a second trial was performed utilising an acute (high seeding) and prolonged (low seeding) challenge of Line X birds, characterised by their increased susceptibility to gut issues. Gut health assessment data revealed no differences in bird gut health between the two types of bacterial challenge, but each challenge did result in birds that displayed significantly higher gut scores at the later sampling time-points than control birds indicating poorer gut health (Figure 4.1). The second trial was performed at the same location and under the same environmental parameters as Trial 1 (Chapter 3), but crucially in Trial 2 the birds were reared on re-used and not fresh litter. The use of recycled litter, despite a top layer of fresh litter, may have exposed the newly hatched chicks to numerous microbial species at high densities (10^{10}/g) and such bacterial exposures through bedding have been reported to contribute up to 90% of the host microbiota (Bolan et al., 2010). For Trial 2, neither the bacterial loads in the bedding or the host microbiome were determined, but it is probable that the 'old litter' used in Trial 2 introduced an earlier and more varied bacterial challenge than in Trial 1. Interestingly, a comparison of the gut health assessment data between the two trials (Figures 3.1 and 4.1) indicated that the birds in Trial 2 exhibited a more rapid deterioration in gut health (gut health worse at Day 21) than birds from Trial 1 (gut health worse at Day 28), suggesting that re-used litter may be detrimental, supporting excess bacterial growth in the small intestine and leading to a 'dysbacteriosis'-type phenotype (Bailey, 2010). To determine if the jejunal mucosa of 'unhealthy' birds with gut inflammation supported excess microbial growth, colony counting assays were performed to assess the growth of *Lactobacillus johnsonii*, a potential probiotic and *Salmonella typhimurium*, a potential human pathogen. No assays were performed using *E. coli* as this had been explored in Chapter 3. Analyses of the gut scrape data, despite some overlap, revealed a trend for birds aged 21 days with inflamed guts to support increased microbial growth (relative to 'healthy' mucosal scrapes) of both *L. johnsonii* and *S. typhimurium* (Figure 4.30). Despite the large variability within groups, data from Chapter 3 also revealed that gut scrapes from unhealthy birds facilitated increased microbial growth (*E. coli*) and overall this assay, irrespective of bacterial species utilised, appears to offer some support to the theory that excess growth of proximal gut bacteria is linked to a poor gut health phenotype.

In Trial 2, individual birds were weighed prior to tissue sampling and their gut health assessed to enable the relationship between bacterial challenge, gut health and weight to be determined. Birds aged 28 days given a prolonged bacterial challenge with B/BV were significantly lighter (Figure 4.3). Interestingly, Chapter 3 showed that a bacterial challenge, irrespective of the gut health outcome, has the potential to decrease the mean bird weight per pen. Thus, it is possible that microbe challenged birds divert energy away from muscle mass gain and towards immune functions (Korver, 2006).

An aim of the tissue sampling in Trial 2 was to quantitatively assess AvBD gene expression in three hatches of Line X birds over a number of time-points and to investigate AvBD gene expression following B/BV challenge. AvBD expression was examined in relation to gut health to evaluate if AvBDs are potential biomarkers of gut health at the group or individual bird level. Quantitative expression was determined for the AvBD1 and 10 genes, chosen specifically due to the presence of SNPs with potential to affect gene expression and/or the anti-microbial activity of the encoded products (Chapter 6). In this study, ten tissues were sampled for each individual bird and the endpoint PCR data (Figures 4.4 and 4.17) revealed that in newly hatched chicks, AvBD1 and 10 transcripts were present in all tissues analysed (kidney, spleen, liver, bursa, thymus, duodenum, jejunum, ileum, caecum and caecal tonsil) supporting the importance of these two genes in the innate defences of the young birds. Although not quantitative, and only performed on a single bird, the PCR data suggested a different expression pattern in the older (21 day-old) birds. In fact AvBD1 and 10 exhibited differences in tissue expression, with AvBD1 linked to relatively strong caecal tonsil expression and AvBD10 showing high kidney and liver expression (Figure 4.7 and 4.17). The first reported studies of AvBD expression, using an endpoint PCR approach, also revealed that AvBD10 was prominently expressed in the kidney and liver, but interestingly did not report any GI tissue expression (Lynn et al., 2004; Xiao et al., 2004). However, the tissues analysed in these early studies were from two month-old chickens (Xiao et al., 2004) and a single three week-old chicken (Lynn et al., 2004), which is consistent with the loss of gut AvBD expression in older birds. A later study did indicate that AvBD10 is prominently expressed in the small intestine, in addition to the liver (Ma et al., 2008); this is consistent with the PCR panels reported in this chapter and supports a role for AvBD10 in protecting the chicken gut against pathogenic invasion.

Due to time constraints, and because the focus of this study was gut health, only seven tissues were analysed using quantitative real-time PCR (qPCR). These were the five gut tissues and the kidney and liver, chosen due to the apparently high constitutive AvBD10 expression as evident in the endpoint PCR panels. For newly-hatched birds (Day 0), the qPCR analysis revealed firstly, that AvBD expression was highly variable within groups of sampled birds and, secondly, expression was significantly different between tissue types. As suggested by the endpoint PCR data, AvBD10 expression was particularly interesting in that, relative to the GI tissues, high kidney and liver expression were identified. In Hatch 2, for example, in newly-hatched chicks AvBD10 expression was over 20 and 400-fold higher in the kidneys and livers, respectively, than in the highest expressing gut tissue (ileum). Within the gut tissues there was a general trend for higher expression in the distal tissues (ileum, caecum and caecal tonsil) relative to the proximal tissues (duodenum and jejunum), which is perhaps reflective of the higher bacterial load and increased species diversity found in the hind gut (Gong et al., 2002b), necessitating the increased protection of the epithelium from bacterial attachment and invasion.

For both genes analysed, differences in expression were observed between hatches, most notably, the lower AvBD1 tissue expression of Hatch 3 birds. This was surprising and the reasons unknown. It was not a technical issue as the Hatch 3 samples were processed blind alongside those from Hatch 2. It may have resulted from the bird genetics, but this was unlikely as the birds used throughout all the trials originated from the same parent stocks. The most likely factor was that it was linked to an as yet unidentified environmental and/or microbial parameter influential on the day of arrival from the hatchery and responsible for gene up/down-regulation.

Comparison of the three sampling time-points 0, 7 and 21 days, revealed that across all GI tissues AvBD1 and 10 gene expression were higher in the newly-hatched chicks than at either of the later sampling time-points. This pattern of decreasing expression with bird age has also been shown in a study on AvBD4, but using semi-quantitative RT-PCR. The authors noted high expression in birds aged 4-days but no PCR product was observed at 17 and 38 days-post hatch indicative of gene down-regulation (Milona et al., 2007). These data support the

idea that AvBDs are defence molecules, functioning as vital effectors of innate immunity in the first week post-hatch, but are of less importance in older birds with a functioning adaptive immune system. In support, researchers have shown that broilers aged less than seven days cannot mount an effective adaptive antibody response due to functionally immature B and T lymphocytes (Bar-Shira et al., 2003) and instead utilise maternal anti-bodies, pro-inflammatory cytokines (IL-1β and IL-8) and defensins throughout the first week in response to bacterial and environmental antigens (Bar-Shira and Friedman, 2006). This 2006 study also revealed that expression of AvBD1 and 2 in duodenal, ileal and caecal tissues decreased significantly during the first week of life although the fold differences were in the region of 3 – 10, compared to the ≥100 fold differences reported in the present sudy. Differences could be due to sensitivity of assays used in the 2006 study, employing less sensitive semi-quantitative methods. The study by Bar-Shira and Friedman (2006) also showed that in the second week of life, AvBD1 and 2 expression increased reaching a peak at 14 days post-hatch, arguing that the response was linked to the increase in pro-inflammatory cytokine expression at the end of the first week of life (Bar-Shira and Friedman, 2006). In Trial 2 no samples were analysed for AvBD gene expression at the Day 14 time-point, but it is worth stating that the expression values were already falling at Day 7. In retrospect, the addition of additional early sampling time-points would have provided more information on when AvBD expression starts to fall.

Many *in vivo* studies have investigated the effects of bacterial challenge, usually human pathogens, especially *Salmonella spp*., on AvBD gene expression although no consistent patterns of regulation have been identified and the data are likely to be influenced by bird age, genetic line, and the tissues examined (Sadeyen et al., 2004; Sadeyen et al., 2006; Milona et al., 2007; Cheeseman et al., 2008; Derache et al., 2009a; Ramasamy et al., 2012). Interestingly, challenge of 5-day old chicks with *Salmonella* spp. did not induce a significant up-regulation of AvBD4, 5 or 6 in the small intestine (Milona et al., 2007), and similarly, another study showed no changes in AvBD2 expression at 1 week post-inoculation (Cheeseman et al., 2008). In contrast, increased caecal tonsil expression of AvBD1, 2, 4 and 6 has been shown in *S. typhimurium* challenged birds at 3 and 5 days post-infection (Akbari et al., 2008), and *S. pullorum* was shown to both up-regulate (AvBD3, 4, 5, 6 and 12) and down-regulate (AvBD10, 11 and 13) expression (Ramasamy et al., 2012). In comparison

with other challenge studies, the trial reported here was novel, in that the bacteria utilised (B/BV) are classed as gut commensals, and the challenge was performed on newly-hatched chicks.

Comparison of B/BV challenged and control birds at 7 days revealed that the challenged birds had significantly lower AvBD expression in the duodenum (AvBD1/10) (Figures 4.10 and 4.22), jejunum (AvBD1) (Figure 4.11) and caecum (AvBD10) (Figure 4.25). This suggested interactions between the exogenous B/BV bacteria and the small, and large, intestinal epithelia. For example down-regulation of anti-microbial peptide expression could be a bacterial strategy adopted to evade the immune response, which provides time for exogenous bacterial species to colonise and establish a microbial niche within the host. Indeed, it has been reported that 4-day-old broilers challenged with *Camplylobacter jejuni* had 2-fold lower cathelicidin-(CATH-2) expression in the small intestine than control birds (van Dijk et al., 2012). These data suggest the functioning of microbial specific factors that interact with the bird innate defences to facilitate gut colonisation. *C. jejuni* is a potential zoonotic pathogen, but its presence is less detrimental to bird than human health (van Gerwe, 2012) and it is often viewed, albeit controversially (Humphrey et al., 2014), as a bird commensal. Commensal bacteria have evolved mechanisms to reduce or prevent host recognition involving concealing themselves from the impact of innate immune effectors. For example, *Bifidobacterium* spp, a normal part of the human gut microbiome, suppresses the pro-inflammatory cytokine IL-8 in an intestinal epithelial cell line (Jijon et al., 2004). Studies have also revealed the down-regulation of anti-microbial peptides by pathogenic species. In patients infected with *Shigella* spp. infections, human cathelicidin LL-37 and hBD1 transcription were down-regulated and loss of epithelial LL-37 peptide was confirmed using IHC (Islam et al., 2001). In mice challenged with live *Salmonella typhimurium* the expression of cryptdin, an alpha defensin, and lysozyme were down-regulated three-fold in comparison to controls, as measured by Northern blot analysis (Salzman et al., 2003).

As previously mentioned, Hatch 3 birds were characterised by lower AvBD1 tissue expression than Hatch 2 birds. For AvBD10, a hatch dependent pattern of regulation was also observed. Higher median expression was found in Hatch 3 challenged birds compared to controls, in contrast to Hatch 2, in which AvBD10 down-regulation was found in the

duodenal and caecal tissues. The reason why AvBD10 remained high following challenge in one hatch but decreased in another hatch is open to conjecture, as no differences between the hatches were noted. As mentioned earlier it was possible that the environmental challenges in Hatch 2 and 3 were different resulting in hatch specific microbiotas that impacted uniquely on AvBD tissue expression patterns.

To explore a potential link between gut health and AvBD gene expression, 7 day-old challenged birds were pooled into groups of 12 and ranked for AvBD1 and 10 expression for all five GI tissues (Figure 4.29). This type of analysis enabled birds with high/low AvBD gene expression to be identified. For AvBD1, the general trend in both hatches was for the birds with low GI tract expression to present with worse gut health supporting a potential protective effect of AvBD1. This pattern was repeated for AvBD10 in Hatch 2 but not Hatch 3, perhaps suggesting that AvBD1 is more important than AvBD10 in maintaining healthy gut tissue. Further support for the hypothesis that birds with high levels of AvBD expression are more protected against bacterial challenge was shown by data in Figures 4.27 and 4.28, in which birds challenged with B/BV yet were inflammation free at Day 7 had higher AvBD1 and 10 expression than those with inflamed guts. A number of studies have shown that birds with higher AvBD expression are less prone to *Salmonella* spp. colonisation, which supports the theory that birds, which are able to maintain relatively high levels of AvBDs are, on the whole, less likely to suffer from enteric upset. For example, a 2009 study showed that primary intestinal cells isolated from a Salmonella-resistant bird line constitutively expressed higher AvBD1 and 2 than cells expanded from a Salmonella-susceptible chicken line, suggesting high AvBD expression contributes towards the prevention of *Salmonella* spp. colonisation (Derache et al., 2009a). Furthermore, Sunkara et al. (2011) showed that butyrate supplementation, resulted in a significant up-regulation of AvBD9 leading to significantly decreased caecal *S. enteriditis* titres (Sunkara et al., 2011). Higher constitutive AvBD expression has also been revealed in a certain strain of commercial Ross broilers compared to Cobb strain and, interestingly, these lines differed in their susceptibility to necrotic enteritis (NE) (Hong et al., 2012) with the former more resistant. Thus genetically divergent bird lines exhibit different patterns of constitutive and induced AvBD expression and such differences appear linked to disease susceptibility.

In humans, defensin regulation has been shown to be an important factor in inflammatory conditions such as Crohn's disease and Ulcerative Colitis (Ramasundara et al., 2009). For example, the ileal mucosa of patients with Crohn's disease showed significantly lower gene expression of the alpha defensins, HD5 and HD6, compared with healthy mucosa although this down-regulation was found irrespective of the level of inflammation (Wehkamp et al., 2005). Moreover, the authors state that it is difficult to determine if defensin down-regulation either predisposes the gut to inflammation or is a consequence of the diseased state. Similarly, in birds further studies are required to determine if decreased AvBD expression contributes towards a 'poor gut health' phenotype or is a marker of enteric disease. Regarding the B/BV challenge model, future work could compare AvBD expression between Line X ('B/BV susceptible-phenotype') and Line Y ('B/BV resistant-phenotype') under control and challenged conditions.

To date, the majority of studies have used a PCR-based molecular approach to examine AvBD gene expression. The lack of commercially available antibodies, has meant that studies evaluating AvBD expression at the protein level have been limited to AvBD3, 11 and 12 in the reproductive tract and reported by a single research group (Mageed et al., 2009; Mageed et al., 2011; Abdelsalam et al., 2012). Examination of AvBD localisation in the oviduct of 400-day old laying hens using custom antibodies to AvBD3, 11 and 12, chosen due to their up-regulation following LPS challenge (Mageed et al., 2008), showed that these defensins were found throughout the oviduct in epithelial cells and in the eggshell and eggshell membrane (Mageed et al., 2009). As part of the tissue analysis in the current experiment, a customised polyclonal antibody to AvBD1 was produced. The real-time qPCR data for AvBD1 revealed that at the later sampling time-points, particularly 21 days-post hatch, gene expression had decreased markedly in the gastro-intestinal tissues compared to tissues sampled from newly-hatched chicks. However, IHC analyses of the tissues indicated that the AvBD1 peptide was present at all sampling time-points and an age-dependent decrease in protein levels could not be confirmed. A possible hypothesis could be that high levels of gene expression observed in the young birds reflect high transcript turnover due to continuous peptide secretion, while in the older birds the low expression reflects peptide storage in epithelial cells.

To summarise, Chapter 4 has demonstrated that AvBD expression is tissue, age and hatch-dependent but can be down and up-regulated by bacterial challenge. Importantly, there was a trend for birds with relatively high GI tract AvBD1 expression to exhibit lower inflammation and better gut health, indicating the potential importance of the AvBD family in the innate gut defenses. The gut health assessment data from both farm trials have established that the B/BV challenge does adversely affect the gut health of Line X birds, and, as shown by the additional gut scrape mucosal analysis, this could be partially through supporting intestinal bacterial over-growth.

Chapter 5: *In vitro* bacterial challenge model

5.1 Overview

The *in vivo* data from Trial 2 (Chapter 4) indicated that the challenge of Day 0 i.e. newly hatched chicks with B/BV was associated with the down-regulation of AvBD expression in the bird GI tract. Moreover, these data also suggested that the down-regulation of expression was linked to an increased susceptibility to gut inflammation. Although persuasive, the bird data was variable and the intent of this chapter was to use an *in vitro* cell model to further explore and compare the effects of bacterial challenges, namely *Bacteroides dorei (BD), Lactobacillus johnsonii* (LJ) and *Salmonella typhimurium* (ST) on AVBD expression profiles. *B. dorei (BD)* and *L. johnsonii* (LJ) were chosen to reflect the *in vivo* challenges of Farm Trials 1 and 2 while *S. typhimurium* was used to expand the study and investigate the effects of a human pathogenic strain.

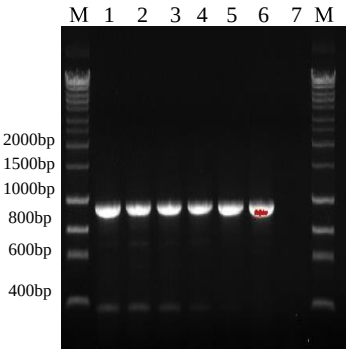
The *in vivo* data from Chapter 3 showed that birds challenged with LJ exhibited lower gut scores, indicative of healthy guts, and therefore lower redness (inflammation), than birds challenged with B/BV. Therefore to investigate this *in vitro,* the expression patterns of pro-inflammatory cytokines, namely IL6 and IL1β were also analysed following the bacterial challenges.

To date, no commercially available immortalised avian gut epithelial cell lines are available so the *in vitro* experiments were performed using chicken embryo cells designated CHCC-OU2 (Ogura and Fujiwara, 1987). These cells have been used in a number of chicken immunological studies, defending their use as a suitable model to study the regulation of innate immune gene expression. For example, CHCC-OU2 cells pre-treated with IFN-ɣ have been shown to inhibit the development of one of the intra-cellular parasites, *Eimella tenella,* associated with coccidiossis (Lillehoj and Choi, 1998, Heriveau et al., 2000). Other studies have investigated the latency of Marek's disease virus (Abujoub and Coussens, 1997) and cytokine expression (IL6/8) following challenge with chicken interleukin-17D (Hong et al., 2008).

5.2 AvBD1 gene and CHCC-OU2 cells

As indicated earlier, the AvBD1 gene carried by Aviagen bird lines is characterised by SNPs, which impact on the primary structure of the encoded peptide. To identify which allelic version was expressed in the OU2 cell line, the genomic DNA was isolated, subjected to PCR using AvBD1 genomic primers and the PCR product sequenced (Figure 5.1 A & B). The sequencing data showed that the AvBD1 gene codes for a SNP variant that is common to the Line X birds and designated 'NYH'.

A



B

CCCTTTCTTCTGGACAGGGTGCTGCAGGTGAGGTGTGAGTTCTGTGGGGTTCTCCATATCCCAGGAGGTGGCTTGTCAG
GGATGGGTAACGACTAGGAGGGCTCTGATCAGTTGGTTCAGGAGGGAGGGAAGATTTAGGTTGGATATCAGGGGGAAGT
TCTTTACAGAGAGAGAGGTGAGGTGCTGGAACAGCTGCCCAGAGAGGCTGTGGATGCCCCGTCCATCCCTGGAGGTGTTC
AAGGCCAGGTTGGATGGGGCCCTGGGCAGCCTGGGCTGGTGTTAGATGTGGAGGTTGGTGGCCCTGCCTGTGGTGGGTGG
GTTGGAGCTTCATGATCCTTGAGGTCCCTTCCAACCCAACCATTCTGTGATTCTGTGGTTTGGATGAGTGGCTGGGCTTTTG
GGTTTGGTGCTTTGTGCACGTGTTAGACTGAGATCCATGGGACAGCCACTCTAGAACCACACACAGCTTGTACAGGTATCC
CACACTCATTTTCTTTTGGTCTGTGCAG**GATCCTCCCAGGCTCTAGGAAGGAAGTCAGATTGTTTTCGAAAGAATGGC**
TTCTGTGCATTTCTGAAGTGCCCTTACCTCACTCTCATCAGTGGGAAATGCTCAAGATTTCACCTCTGCTGCAAAAG
GTAAGCTTTGGAATTAGGGATGAAATTGGATCTGCTACCACGATGGCAGAAATAGCTGTTGTTGTGTTTGATCCCCAAACC
TAGCTACTGGCTTTGGGCTATATATGATCCAGGGCAGGGGCTTGGGGAGGAAAGGAGAAGGTGCTAGGACCGGTCCTTTA
AAGGAACTGGAGGAACCCCAGATCAGACACTGGCCTCCCCATTGCCCTCAGTTACACGGGGCTGCCTGGCTTTGGGGTTTT

Figure 5.1: Genomic endpoint PCR and sequencing of the AvBD1 gene in CHCC-OU2 cells

A) Endpoint PCR (55 – 64°C temperature gradient) for AvBD1 primers against genomic DNA extracted from chicken OU2 cells. AvBD1 product size is 911 b.p. M – hyperladder 1, Lane 1 – 6: 55.5°C - 64°C, 7 – negative control (no DNA).

B) Sequence of extracted cDNA from PCR (Panel A) showing 98% sequence identity with the 'NYH' variant of the mature peptide that is most prevalent in Line X birds. Codons that confer the 'NYH' SNP are shown in yellow and the SNP is underlined; AAT – N, TAC – Y and CAC – H. Partial sequence for Exon 1 and full sequence for Exon 2 are indicated in bold.

5.3 Housekeeping genes for real-time qPCR in CHCC-OU2 cells

The reliability of housekeeping genes has been shown to vary according to the samples used and thus, the recommended practice is that housekeeping genes are experimentally validated prior to selection (Bustin et al., 2009; Bustin, 2010).

As performed for the *in vivo* tissue expression studies, the chicken geNorm kit (PrimerDesign, U.K.) was used to select suitable housekeeping genes (GAPDH, YWHAZ, ACTB, UBC, SDHA and SF3A1) for sample normalization in the CHCC-OU2 *in vitro* model. Figure 5.2 shows the output from the geNorm software (Vandesompele et al., 2002), which indicates the average expression stability value (M) of the six potential reference genes. The lower the M value the more stable the gene expression. The software indicated that, in contrast to the tissue samples, YWHAZ (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) were the most suitable housekeeping genes for CHCC-OU2 gene expression analysis.

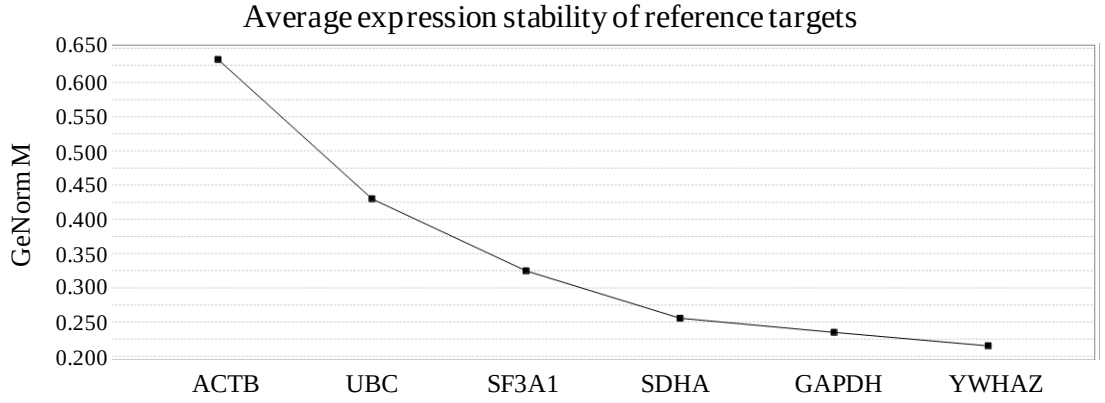


Figure 5.2: GeNorm analysis to determine the most appropriate genes for real-time qPCR in chicken CHCC-OU2 cells.

The average expression stability value (M) of six chicken reference genes in the GeNorm kit (PrimerDesign) is shown; GAPDH, YWHAZ, ACTB, UBC, SDHA and SF3A1. The expression stability increases from left to right indicating that YWHAZ is the most stable reference gene.

5.4 AvBD1 and 10 expression in CHCC-OU2 cells

In vitro experiments were performed to explore whether AvBD1 and 10 were expressed in CHCC-OU2 cells and if expression was linked to cell growth and, hence, cell density.

Figure 5.3 shows the AvBD1 and 10 mean gene expression data at 24, 72, 96 and 120 h respectively, following CHCC-OU2 seeding at 2 x 10^5 cells per 12-well plate. These data indicated that for both AvBDs, gene expression was significantly altered as a function of time (One-way ANOVA, $P < 0.05$). For AvBD1, expression peaked at 72 h post-seeding ($P < 0.01$) and decreased at the later time-points, whereas, mean AvBD10 expression increased over time and was significantly higher at 96 h ($P < 0.01$) and 120 h ($P < 0.001$) post-seeding. Therefore, to address any potential changes in expression due to cell growth, all challenge data was presented as fold or percentage change relative to an appropriate time-point control.

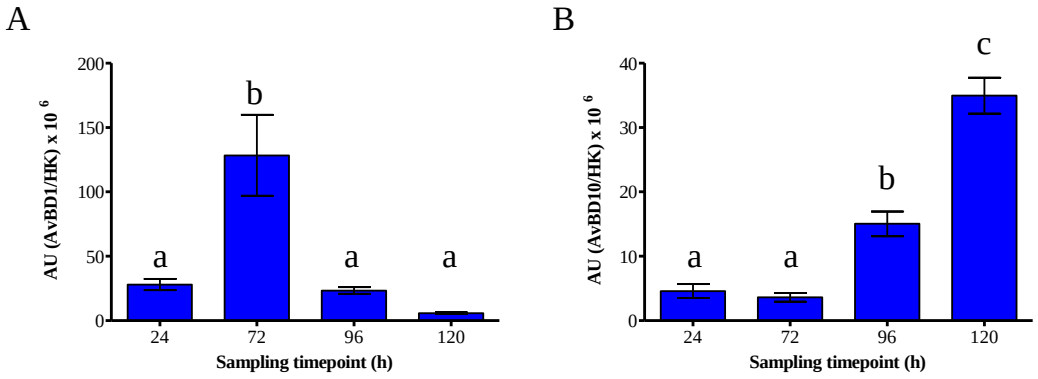


Figure 5.3: Mean AvBD expression (A.U) ± S.E.M in unchallenged CHCC-OU2 cells.

AvBD1 (A) and 10 (B) were assessed at 24, 72, 96 and 120 h post-seeding at 2 x 10^5 cells in 12 – well plates. Bars with different letters have means that are significantly different from each other. N = 2 experiments, n = 6 replicate wells.

5.5 AvBD1 expression in CHCC-OU2 cells challenged with bacteria

Challenge experiments were performed using heat killed bacteria at 2 x 10^6 colony forming units (C.F.U) per well of a 12 well plate, and OU2 cells seeded at 2 x 10^5 at 80%-100% confluence. Figure 5.4 shows AvBD1 expression following 4, 8 and 24 h challenge of the CHCC-OU2 cells with two clinical strains of BD, ST and LJ. These data show that at 4 h, aside from ST, which caused significant gene up-regulation ($P < 0.05$), the mean AvBD1 expression was lower in the challenged wells reaching statistical significance for BD 1 ($P < 0.05$). At the 8h time-point all bacterial species caused a significant down-regulation of AvBD1 expression relative to control cells ($P < 0.001$). Similarly, at 24 h sampling the two BD and ST challenges, but not the LJ challenge, caused significantly lower AvBD1 expression ($P < 0.001$). To confirm that down-regulation was not the result of decreased cell viability, a MTS assay was performed (see section 2.5.4) that compared the viability of control and challenged cells (Figure 5.5). No significant relationship between the number of C.F.U utilized and cell viability was found, with > 95% viability observed even when a high 2 x 10^6 bacterial inoculum was employed.

To explore if the down-regulation, observed using 2 x 10^6 C.F.U, was responsive to bacterial numbers the challenges were repeated using a range of bacterial C.F.U. (10^2 - 10^5) at the 8 h time-point (Figure 5.6). For BD strain 1, AvBD1 was significantly down-regulated relative to the control wells at all C.F.Us tested ($P < 0.001$). A similar pattern was found for ST treated cells, although statistical significance was only reached at 10^2 C.F.U ($P < 0.05$). At 10^2 C.F.U. no effects of LJ on AvBD1 expression were noted, but at higher inocula gene down-regulation was hinted although this was not statistically significant ($P > 0.05$, One-way ANOVA followed by Dunnett's test).

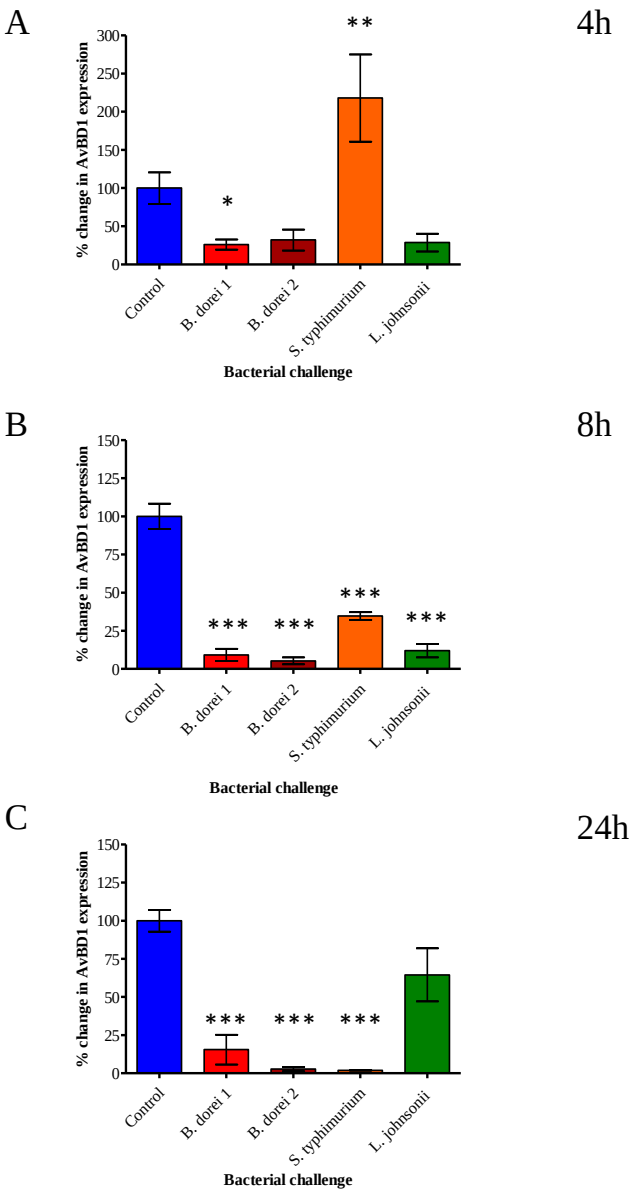


Figure 5.4: The effect of high C.F.U bacterial challenge for 4, 8 and 24 h on AvBD1 expression

The mean expression values (± SEM) are shown as percentage change relative to mean control expression at each sampling time-point. CHCC-OU2 cells were challenged with 2 x 10^6 heat-killed colony-forming units (C.F.Us) per well and sampled at 3 timepoints: A) 4h, B) 8h and C) 24h (N = 1 - 3 experiments, n = 3 - 9 wells). Unchallenged control – blue bars, *Bacteroides dorei* strain 1 – red bars, *Bacteroides dorei strain* 2 – dark red bars, *Salmonella typhimurium* – orange bars, *Lactobacillus johnsonii* – green bars. Significance values are for comparisons of means between control and challenged groups (One-way ANOVA followed by Dunnett's Multiple Comparison Test * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). For control cells, N = 3 experiments, n = 8 replicates. For BD and LJ challenged cells, N = 2 experiments, n = 6 replicates. For ST, N = 1 experiment, n = 3 replicates.

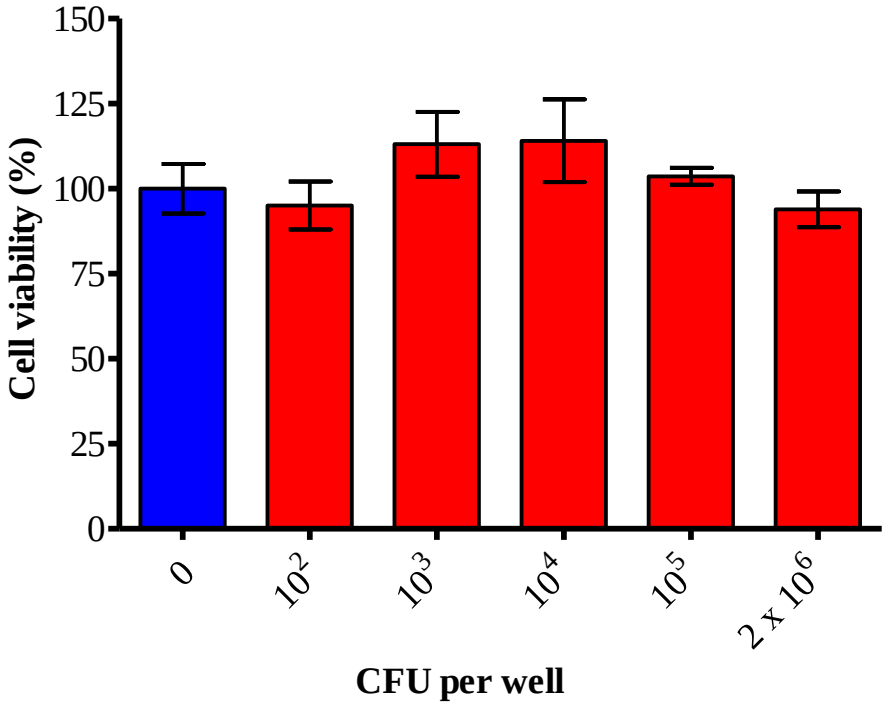


Figure 5.5: Cell viability (%) of CHCC-OU2 cells following 24 h incubation with *Bacteroides dorei.*

The MTS assay data shows the percentage viability of CHCC-OU2 cells relative to PBS control (Mean ± SEM) following 24 h incubation with *Bacteroides dorei* at 10^2, 10^3, 10^4, 10^5 and 2 x 10^6 C.F.U per well. N = 1 experiment, n = 5 replicate wells.

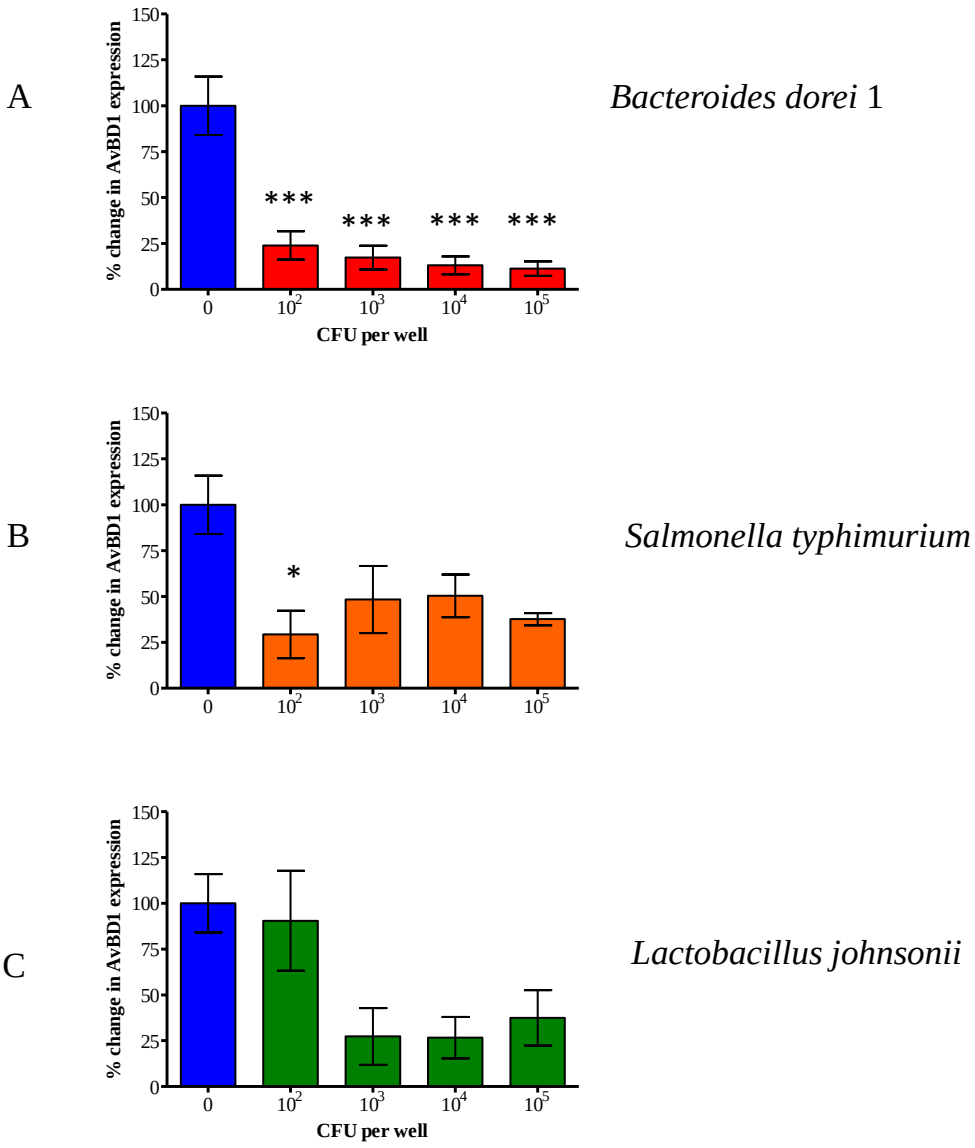


Figure 5.6: The effect of C.F.U. number on mean AvBD1 expression in CHCC- OU2 cells challenged for 8 h.

The mean expression values (± SEM) are shown as percentage change relative to mean control expression. Wells were challenged with heat-killed colony-forming units (CFUs) at 10^2- 2 x 10^6 per well of A) *Bacteroides dorei* strain 1 (red columns) B) *Salmonella typhimurium* (orange columns), and C) *Lactobacillus johnsonii (*green columns). Significance values are for comparisons of means between control and challenged groups (One way ANOVA followed by Dunnett's Multiple Comparison Test * $P < 0.05$, *** $P < 0.001$). N = 2 experiments, n = 3 - 6 replicates.

5.6 AvBD10 expression in CHCC-OU2 cells challenged with bacteria

AvBD10 expression in CHCC-OU2 cells following 4, 8 and 24 h of challenge with the two BD strains and LJ (2 x 10^6 C.F.U.) is shown in Figure 5.7. Although these data were characterized by large SEMs, all the 8h challenges were typified by a significant decrease in gene expression ($P < 0.001$). At 24h post-challenge, no statistically significant differences between challenged and control groups were detected although the LJ challenge did suggest the up-regulation of AvBD10 gene expression.

The effect of the bacterial inoculum count on AvBD10 expression is shown in Figure 5.8. These data suggested that bacterial challenges over a range lower than 2 x 10^6 C.F.U (10^2 – 10^5 C.F.U.) were associated with AvBD10 up-regulation as the mean AvBD10 expression was higher than the controls for all bacterial species at all C.F.U used. However, due to the large variability in expression a statistically significant effect was only observed for ST ($P < 0.05$, one-way ANOVA) and at 10^4 C.F.U the mean AvBD10 expression was significantly higher than control ($P < 0.05$).

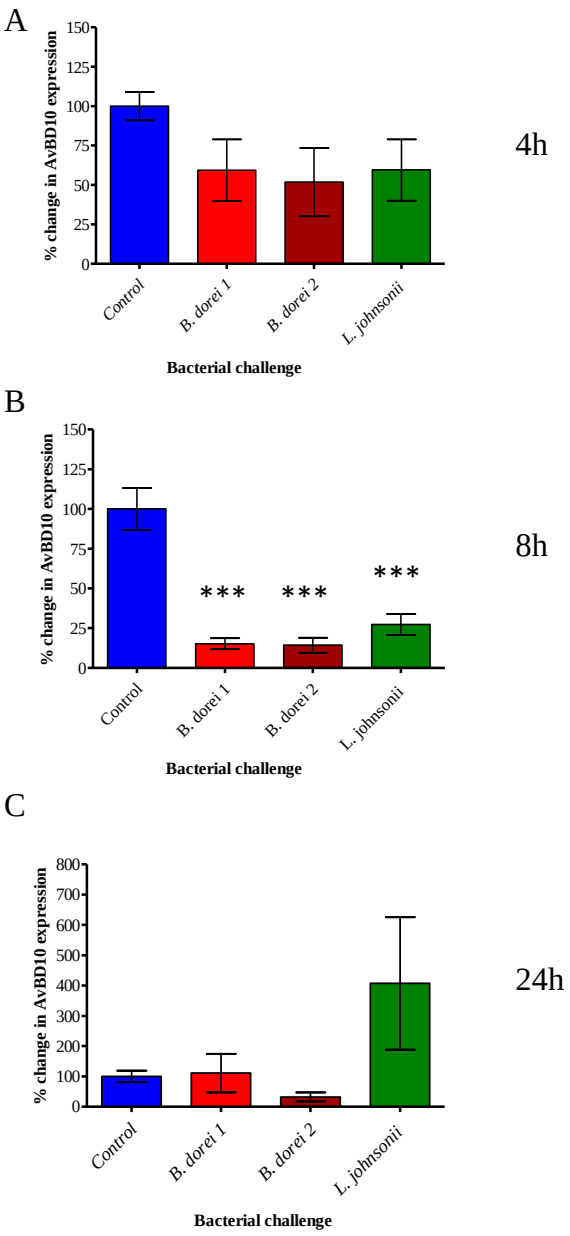


Figure 5.7: The effect of high C.F.U bacterial challenge for 4, 8 and 24 h on mean AvBD10 expression.

The mean expression values (± SEM) are shown as percentage change relative to mean control expression at each sampling time-point. Cells were challenged with 2 x 10^6 heat-killed colony-forming units (C.F.Us) per well and sampled at 3 timepoints: A) 4h, B) 8h and C) 24h (N = 1 - 3 experiments, n = 3 - 9 wells). Unchallenged control – blue bars, *Bacteroides dorei* strain 1 – red bars, *Bacteroides dorei* strain 2 – dark red bars, *Lactobacillus johnsonii* – green bars. Significance values are for comparisons of means between control and challenged groups (One-way ANOVA followed by Dunnett's Multiple Comparison Test *** $P < 0.001$). For control cells N = 3 experiments, n = 8 replicates. For BD and LJ challenged cells, N = 2 experiments, n = 6 replicates.

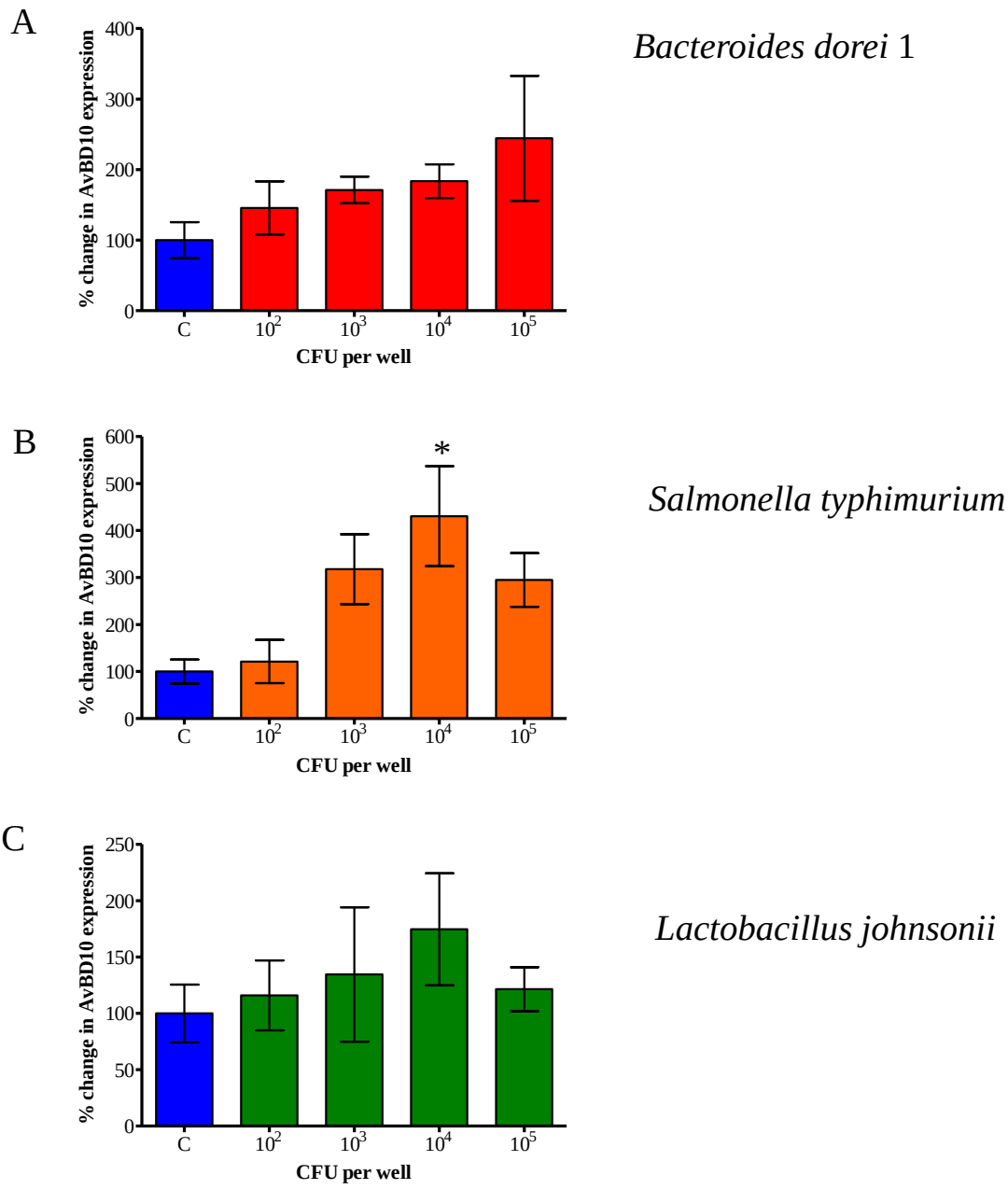


Figure 5.8: The effect of C.F.U. number on mean AvBD10 expression in CHCC- OU2 cells challenged for 8 h.

The mean expression values (± SEM) are shown as percentage change relative to mean control expression. Cells were challenge with heat-killed colony-forming units (CFUs) at 10^2 – 2 x 10^6 per well of A) *Bacteroides dorei* strain 1 (red columns) B) *Salmonella typhimurium* (orange columns), and C) *Lactobacillus johnsonii (*green columns). Significance values are for comparisons of means between control and challenged groups (One-way ANOVA followed by Dunnett's Multiple Comparison Test * $P < 0.05$). N = 2 experiments, n = 3 - 6 replicates.

5.7 Cytokine standard curves and melt curves for real-time qPCR

Figures 5.9 and 5.10 show the standard curves used for relative quantification of cDNA samples (Panel A) and melt curves (Panel B) produced from qPCR reactions using IL-1β and IL-6 primers, respectively. As described for the AvBDs, the standard curve was checked for amplification efficiency of ~2 and for each sample melt curves were performed to ensure primer specificity.

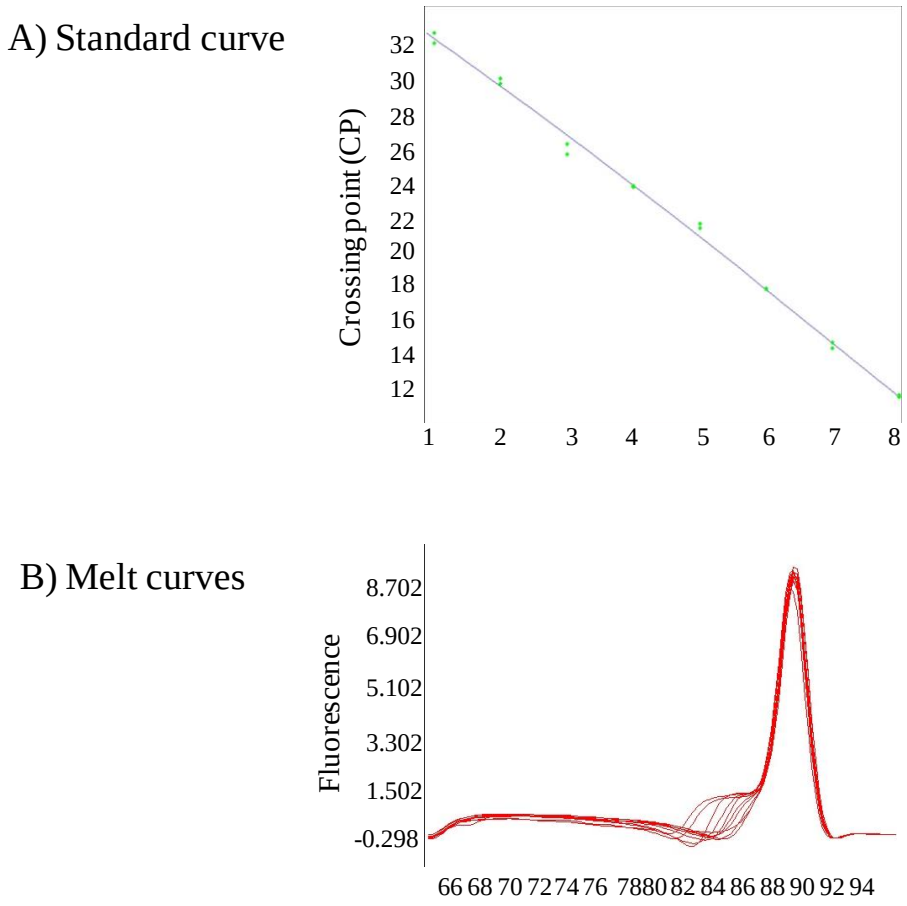
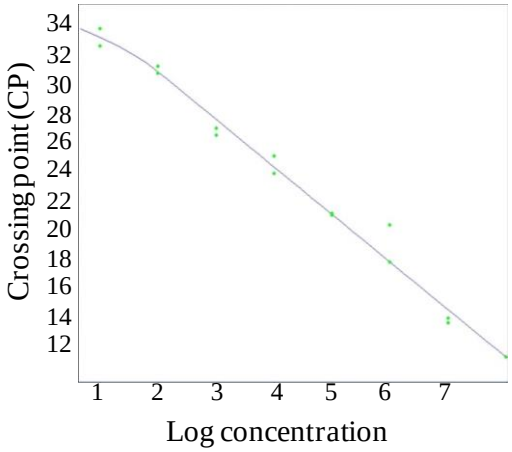


Figure 5.9: Real-time qPCR standard and melt curve for IL-1β.

A) Standard curve for serial dilutions of IL-1β plasmid against calculated CP values. B) Melt curve for IL-1β PCR products of gut tissue samples (n = ~ 20 samples). A single peak for each product was observed at the melting temperature of 89°C.

A) Standard curve



B) Melt curves

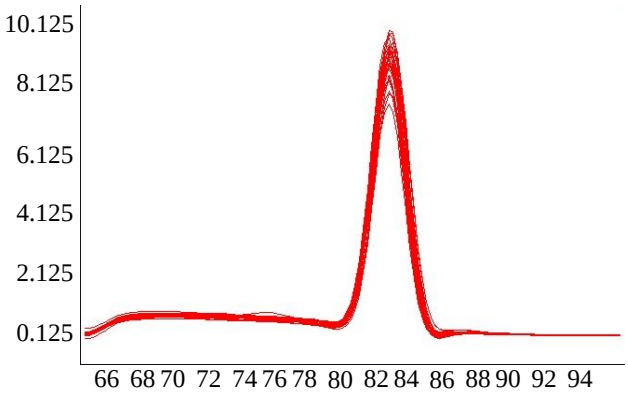


Figure 5.10: Real-time qPCR standard and melt curve for IL-6.

A) Standard curve for serial dilutions of IL-6 plasmid against calculated CP values. B) Melt curve for IL-6 PCR products of gut tissue samples (n = ~ 20 samples). A single peak for each product was observed at the melting temperature of 83°C.

5.8 Cytokine expression in CHCC-OU2 cells challenged with bacteria

The *in vivo* data (Chapter 3 and 4) revealed that the B/BV challenge resulted in a progressive deterioration in gut health, including gut inflammation, whereas the LJ challenge appeared to be linked to anti-inflammatory effects. Therefore, pro-inflammatory cytokine expression was compared *in vitro* in response to BD, LJ and ST challenges (2×10^6 C.F.U).

IL-6 and IL-1β expression were significantly up-regulated (up to >300 fold) following the challenge (Figure 5.11), with expression maximal at the 4 h sampling time-point. At 24 h sampling, gene expression was reduced although still above control values with fold-changes for IL-6 and IL-1β, of greater than 5-fold ($P < 0.05$) and 15-fold ($P < 0.001$), respectively.

Figure 5.12 illustrates the effects of increasing the inoculum count on cytokine expression. Although compromised by large SEMs, these data clearly show that as the number of BD and ST C.F.Us. increased so did the expression of IL-6 and IL-1β. In contrast, this dose-response type relationship was not as pronounced in the LJ challenged cells. Moreover, IL-6 and IL-1β expression were reduced in the LJ compared to the BD and ST challenged cells, and this was statistically significant at the higher C.F.U. inocula (10^4, $P < 0.05$; 10^5, $P < 0.001$).

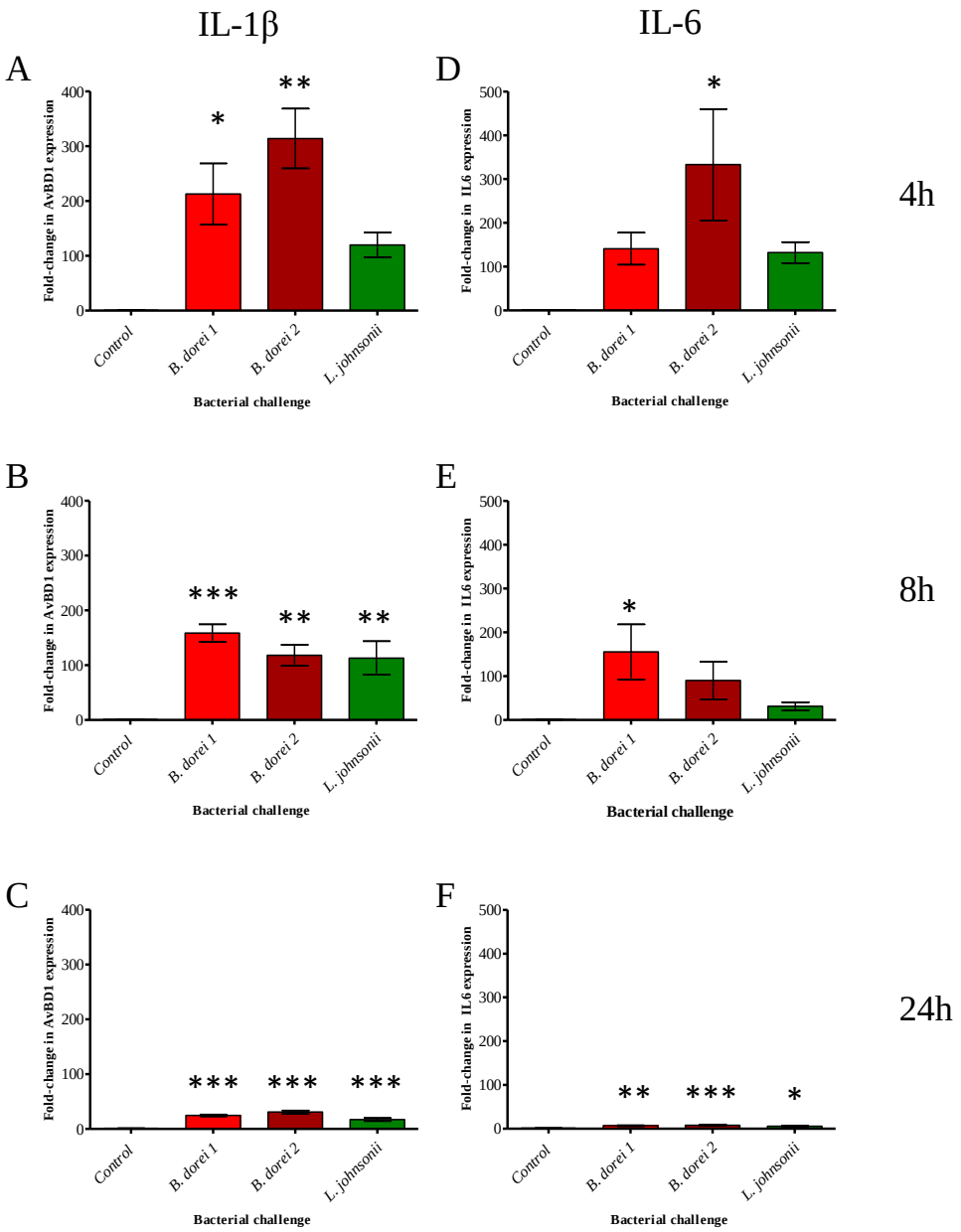


Figure 5.11: The effect of bacterial challenge for 4, 8 and 24 h on IL-1β and IL-6 gene expression.

Gene expression is shown as mean fold-change ± SEM relative to control cells, for IL-1β (A, B and C) and IL-6 (D, E and F). Cells were challenged with 2 x 10^6 heat-killed colony-forming units (CFUs) per well and sampled at 3 timepoints: 4h (A and D), 8h (B and E) and 24h (C and F) (N = 1 - 2 experiments, n = 2 - 6 replicate wells). Unchallenged control – blue bars, *Bacteroides dorei* strain 1 – red bars, *Bacteroides dorei* strain 2 – dark red bars, *Lactobacillus johnsonii* – green bars. Significance values are for comparisons of means between control and challenged groups (One-way ANOVA followed by Dunnett's Multiple Comparison Test * P < 0.05, ** P < 0.01, *** P < 0.001). N = 1 – 2 experiments, n = 2 – 6 total replicates.

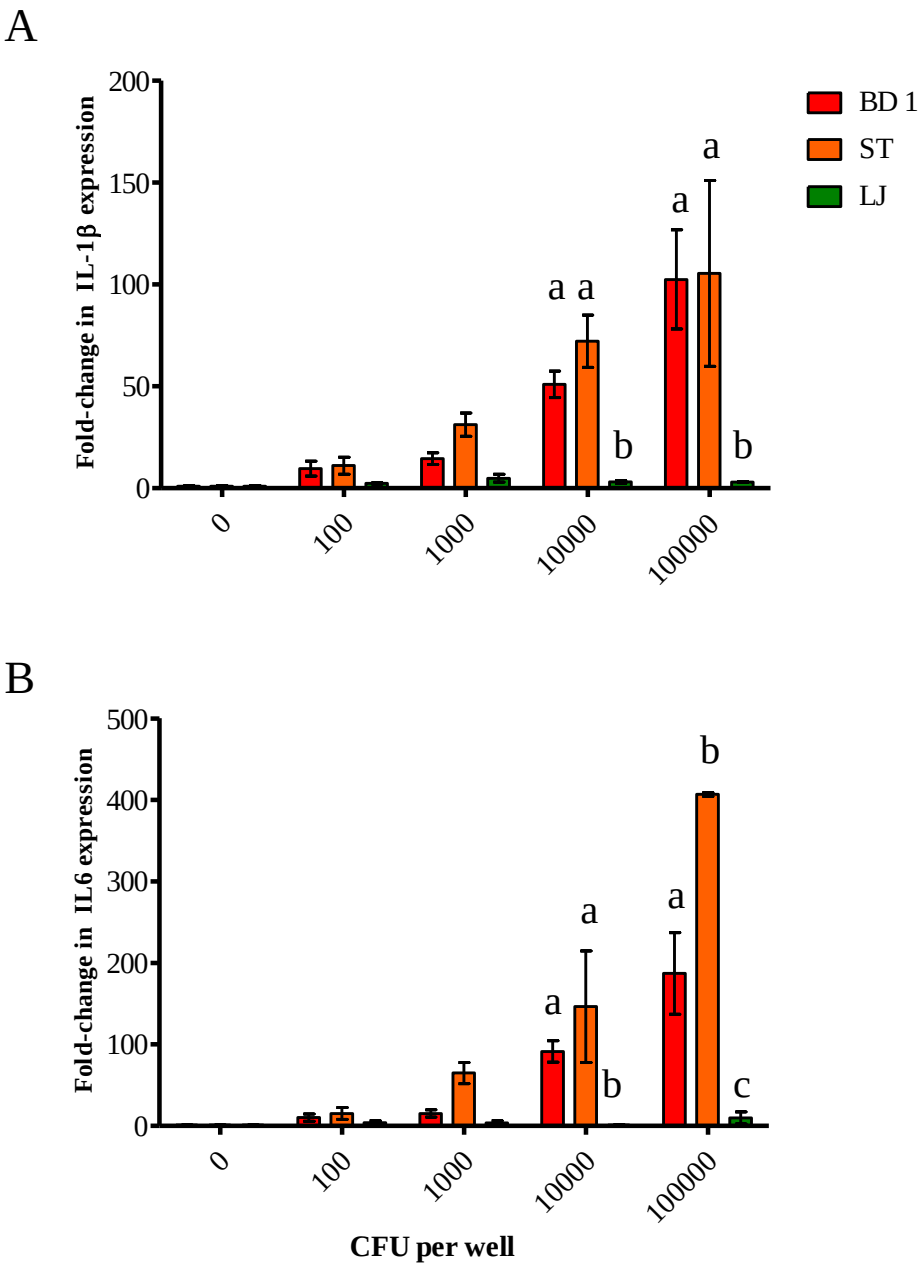


Figure 5.12: IL-1β and IL-6 gene expression induced by 8 h challenge with three species of bacteria over a range of C.F.U.

Gene expression is shown as mean fold-change ± SEM relative to control cells, for IL-1β (A) and IL-6 (B). Chicken OU2 cells were challenged for 8h with heat-killed colony-forming units (CFUs) at 10^2- 10^5 per well of *Bacteroides dorei* strain 1 (BD1) (red columns), *Salmonella typhimurium* (ST) (orange columns), and *Lactobacillus johnsonii* (LJ) (green columns). Bars with different letters have means that are significantly different from each other ($P < 0.05$, Two-way ANOVA). N = 2 experiments, n = 3 - 6 replicate wells.

5.9 Discussion

The *in vivo* data from Chapter 4 revealed that the B/BV challenge was linked to a significant reduction in the expression of AvBD1 and 10 in a number of bird gut tissues. However, as no tissues were taken for gene expression analysis in Farm Trial 1, data on the effect of the LJ challenge were lacking. The aim of this chapter was to further explore the effects of gut associated bacteria on AvBD and inflammatory cytokine gene expression, but using an *in vitro* model. Bacterial challenges were performed using the commensal species utilised in Farm Trials 1 and 2: *Bacteroides dorei* (BD) and *Lactobacillus johnsonii* (LJ), in addition to, *Salmonella enterica* serovar Typhimurium 1344 (ST). The latter was chosen due to its potentially pathogenic nature (Christenson, 2013).

The preferred *in vitro* model to investigate such challenges was a chicken gut epithelial line but, to date, none are commercially available. A number of groups have utilised primary cells from tissues such as the caecal tonsil (Brisbin et al., 2008), and intestine (Derache et al., 2009a). These primary cell systems are, however, technically difficult to prepare, requiring a constant supply of birds, as well as strict quality control criteria to address reproducibility issues. As an alternative to primary cell culture, this study utilised the CHCC-OU2 cell line, which has been used by a number of groups to investigate avian immune responses (Heriveau et al., 2000; Hong et al., 2008). Moreover, genomic sequencing revealed that the DNA of the CHCC-OU2 cells used in this study encoded the 'NYH' version of the AvBD1 peptide that is predominantly expressed by Line X birds. This strongly supported the use of the CHCC-OU2 cells as an appropriate in *vitro* model and moreover enabled any results obtained using the *in vitro* work to be compared to the *in vivo* study data.

Real-time PCR analysis, utilising a new set of house-keeping genes (GAPDH/YWHAZ), showed that AvBD1 and 10 genes were constitutively expressed by the cells under the growth conditions employed. Challenging the cells with bacteria did affect AvBD1 and 10 gene expression, although the data were often characterized by large SEMs that masked statistically significant changes. Nevertheless, the overall trend in relation to AvBD1 expression was that all the bacterial challenges down-regulated AvBD1 expression relative to the control even when low (10^2) bacterial doses were employed (Figure 5.6). These data indicated that the mechanism of AvBD1 down-regulation in the CHCC-OU2 cells was highly

sensitive and did not require high bacterial loads with MOI of less than one producing an effect. Interestingly, the CHCC-OU2 cells appeared more tolerant to LJ as down-regulation was only observed following a challenge of ≥1000 C.F.U. This contrasted to the AvBD10 expression data where significant down-regulation was only observed at the 8 h time-point and when a bacterial challenge of 2 x 10^6 C.F.U., which did not affect CHCC-OU2 cell viability, was used. There was however a strong suggestion of AvBD10 up-regulation in the ST challenged cells, which reached statistical significance at 8h.

The *in vivo* data shown in Chapter 4 indicated that AvBD1 was down-regulated in the gut tissues of 7 day-old birds and was not up-regulated following bacterial challenge. Therefore, both the *in vitro* and *in vivo* data suggest that AvBD1 expression is prone to down regulation, which perhaps reflects an immune evasion strategy by the bacterial strains associated with the gut epithelia. A similar observation was reported by investigators using primary intestinal cells expanded from a Salmonella susceptible bird line who reported that challenge with *S. enteritidis* (SE), did not significantly affect AvBD1 expression (Derache et al., 2009a). This pattern was also observed in an *in vivo* challenge model utilising commercial broilers in which *C. jejuni* and *S. typhimurium* challenges had no effect on AvBD1 regulation; yet *C. jejuni* down-regulated AvBD3, 4, 8, 13 and 14 and *S. typhimurium* infection significantly up-regulated AvBD3, 10 and 12 (Meade et al., 2009b). *In vitro* experiments have also revealed that the fatty acid butyrate, a major hind gut metabolite arising from bacterial fermentation, has no effect on AvBD1 expression but does up-regulate the expression of AvBD3, AvBD4, AvBD8, AvBD9, AvBD10, and AvBD14 in a time and dose-dependent manner (Sunkara et al., 2011). It is clear therefore, that differences exist in the regulation of AvBD expression with AvBD1 being more recalcitrant to up-regulation and prone to down-regulation although the reasons for this are not known. Although the mechanisms of HDP down-regulation have not been explored in avian species, the down-regulation of human HDPs has been demonstrated in an *in vitro* cell line model. RT-PCR and western-blot analyses revealed that the expression of LL-37 at the mRNA transcript and protein level is completely abrogated in epithelial cells (HT-29) by infection with the dysentery causing bacterium, *Shigella dysenteriae* (Islam et al., 2001). To ascertain the PAMP from S. *dysenteriae* that was responsible for the down-regulation, the authors challenged a monocyte cell line with two forms of LPS (*E. coli* and *Shigella* spp.), and two sonicated *S. dysenteriae* lysates, one

containing bacterial DNA and the other treated with DNase. The data confirmed that LL-37 suppression was facilitated by bacterial DNA only (Islam et al., 2001). Therefore, it may be possible that in the BD, ST and LJ challenges, outlined in Chapter 5, the bacteria also utilised bacterial CpG DNA to suppress AvBD1 expression, putatively through chicken TLR21 signalling but further work is required to explore this hypothesis. Other studies have suggested that the ability of *Helicobacter pylori* to persist in the human stomach is associated with its ability to down-regulate the human defensins, hβD1, which is constitutively expressed in non-infected individuals (Patel et al., 2013), and hBD3 (Bauer et al., 2014). For both genes the bacterial protein CagA is delivered into the host cell via a type IV secretion system which activates cell signalling cascades. For hBD1, it was shown that blocking NFκB expression using small interference RNA resulted in significant increases in hBD1 peptide relative to the *H. pylori* WT strain (Patel et al., 2013), whilst in another *in vitro* study hBD3 expression was down-regulated via blocking epidermal growth factor (EGFR) activation (Bauer et al., 2014). The ability of bacterial toxins to down-regulate hBD1 expression in intestinal epithelial cells was shown by *Vibrio cholerae* and enterotoxigenic *E. coli* (ETEC) and the signalling mechanisms were through ERK MAPKinase, protein kinase A (PKA), and Cox-2 pathways (Chakraborty et al., 2008). In summary, the data from these *in vitro* human cell line models highlight that no single pathway exists for the suppression of human HDPs. This is also likely to be the case in regards to AvBD signalling and could explain why following a specific microbial challenge some AvBDs are up-regulated whilst some are down-regulated, presumably through multiple signalling pathways.

It is recognised that cytokines and chemokines are important effectors of chicken innate, as well as, adaptive immunity (Kaiser et al., 2005). Chapter 4 identified potential links between inflammation and AvBD expression thus the expression of the pro-inflammatory cytokines IL-6 and IL-1β were also examined following the *in vitro* bacterial challenges. The data presented in this chapter indicated that the three bacterial challenges all induced IL-6 and IL-1β expression. In addition, a dose-dependent response was observed in relation to the BD and ST challenges, but interestingly not for the LJ challenge. Further, and, most strikingly, the LJ challenge was associated with significantly lower IL-6 and IL-1β expression than the BD and ST challenges at the intermediate inoculums used (10^4 and 10^5 C.F.U.). The reasons for this are not known but as discussed in Chapter 3, LJ is regarded as a probiotic organism

and studies have shown that probiotic strains have a decreased ability to up-regulate pro-inflammatory cytokines. For instance, in contrast to the *E.coli* strain Nissle 1917, *Lactobacillus* and *Bifidobacterium* have been shown not to induce the pro-inflammatory cytokine IL-8, as measured by ELISA, in gut associated HT29 cells that were incubated with bacterial cell debris for 32 h (Lammers et al., 2002). Microarray data has also shown that chicken caecal tonsil mononuclear cells, challenged with the isolated cell envelope from *Lactobacillus acidophilus* did not result in up-regulation of any innate genes but was characterized by the repression of the pro-inflammatory cytokine IL-17 gene, which the authors suggest may represent the adaptive response of the gut to normal host microbiota (Brisbin et al., 2008). These studies, alongside the *in vitro* data presented in this chapter, indicate that *Lactobacillus* spp., such as LJ can modulate the immune response in part by not activating the release of pro-inflammatory cytokines such as IL-6/IL-1β and hence reducing inflammation.

Bacteroides are commonly classed as gut commensals (Wexler, 2007). However, heat in-activated strains of *Bacteroides* spp. such as *Bacteroides fragilis* have been shown to stimulate the release of pro-inflammatory cytokines IL-6 and TNF from human mononuclear cells and whole blood (Nagy et al., 1998). This could indicate that outside of its normal microbial niche in the caeca, B*acteroides* spp. functions as an opportunistic pathogen resulting in an epithelial response and the up-regulation of pro-inflammatory cytokine genes. Accordingly, it is possible to hypothesise that the B/BV species ingested by the birds as part of the challenges outlined in Chapters 3 and 4, induced an excessive inflammatory response upon contact with the proximal gut epithelia whereas the LJ challenge did not. If BD also induces high levels of inflammation *in vivo* this could be potentially damaging to the immature gut structures of a bird that is newly hatched.

The *in vivo* data in Chapter 4 suggested that individual birds maintaining relatively high AvBD1 expression despite the B/BV challenge suffered less GI inflammation and were less prone to poor gut health. This suggested the importance of the defensins in protecting the bird gut. The *in vitro* data presented in this chapter provides further evidence that even at low numbers, BD can down-regulate AvBD1 expression at the cellular level, which is

indicative of an immune evasion strategy employed by opportunistic pathogens such as *Bacteroides* spp.

The *in vitro* data also supported AvBD10 down regulation in response to 10^6 CFU of supposedly commensal bacteria (Figure 5.7), which again suggests the potentially pathogenic characteristics of such bacteria. The *in vitro* data did however suggest that Lactobacillus species such as LJ, induce lower levels of pro-inflammatory cytokines and may be less able to down-regulate important innate associated genes such as the AvBDs. These findings provide a possible cellular mechanism that explains why the *in vivo* LJ challenge, as shown in Chapter 3, helped to maintain good enteric health, in contrast to the deterioration in gut health induced by B/BV. Overall, the CHCC-OU2 bacterial challenge model, as outlined by the data presented in this chapter, is a useful tool for evaluating innate immune gene regulation, at least until commercially available epithelial gut lines become available.

Chapter 6: Properties of three AvBD1 variants and AvBD10

6.1 Overview

Chapter 4 demonstrated the expression of AvBD1 and 10 in chicken GI tissues, particularly newly-hatched chicks, which supports their importance in protecting young birds from exogenous pathogens, and shaping, potentially, the composition of the early gut microbiota. To date fourteen AvBDs have been identified and studies have reported the anti-microbial properties of a number of the peptides against numerous bacterial species (van Dijk et al., 2008; Cuperus et al., 2013). While genes encoding the defensins tend to be conserved, natural allelic variation has been reported and linked to an altered potency. For example, single nucleotide polymorphisms (SNPs) in the great tit AvBD7 (Hellgren et al., 2010) as well as the chicken NK-lysin gene (Lee et al., 2012) have been shown to confer differences in anti-microbial activity (AMA). In this chapter the production of AvBD1 and 10 peptides is described, in addition to, their anti-microbial potency against microbes associated with the chicken GI tract.

Although originally investigated for anti-microbial activities, it is has been established that host-defense peptides expressed in avian and mammalian species modulate immune functions by inducing cytokine production as well as stimulating wound healing (Otte et al., 2008; Steinstraesser et al., 2011; Choi et al., 2012) Therefore, in addition, to investigating anti-microbial activity, AvBD1 and 10 were assessed for cell proliferative properties and AvBD10 for wound healing capacity.

6.2 SNPs within AvBD1 locus

The SNP study commissioned by Aviagen Ltd. to identify allelic variation within the commercial breeding lines has been previously reported (Butler, 2010). In summary, three non-synonymous SNPs were identified in the AvBD1 mature peptide coding region and shown in Figure 6.1. The three AvBD1 variants designated 'NYH', 'SSY' and 'NYY', according to the differences in their primary amino acid sequences were targeted for synthesis. ‘NYH’ represented the major AvBD1 form synthesized in Line X birds, ‘SSY’ typified AvBD1 in Line Y birds, while ‘NYY’ represented Line Z, which has the most robust gut health, but was not investigated in this PhD.

A ‘NYH’ Line X

GGA AGG AAG TCA GAT TGT TTT CGA AAG AAT GGC TTC
G R K S D C F R K N G F

TGT GCA TTT GTG AAG TGC CCT TAC CTC ACT CTC ATC
C A F L K C P Y L T L I

AGT GGG AAA TGC TCA AGA TTT CAC CTC TGC TGC AAA
S G K C S R F H L C C K

AGA ATA TGG GGC TGA
R I W G STOP

B ‘SSY’ Line Y

GGA AGG AAG TCA GAT TGT TTT CGA AAG AGT GGC TTC
G R K S D C F R K S G F

TGT GCA TTT GTG AAG TGC CCT TCC CTC ACT CTC ATC
C A F L K C P S L T L I

AGT GGG AAA TGC TCA AGA TTT TAC CTC TGC TGC AAA
S G K C S R F Y L C C K

AGA ATA TGG GGC TGA
R I W G STOP

C ‘NYY’ Line Z

GGA AGG AAG TCA GAT TGT TTT CGA AAG AAT GGC TTC
G R K S D C F R K N G F

TGT GCA TTT GTG AAG TGC CCT TAC CTC ACT CTC ATC
C A F L K C P Y L T L I

AGT GGG AAA TGC TCA AGA TTT TAC CTC TGC TGC AAA
S G K C S R F Y L C C K

AGA ATA TGG GGC TGA
R I W G STOP

D

Genetic Line	SNP 1 (N or S)	SNP 2 (Y or S)	SNP 3 (Y or H)
X	N (100%)	Y (76%)	H (86%)
Y	S (89%)	S (96%)	Y (90%)
Z	N (49%)	Y (30%)	Y (61%)

Figure 6.1: Three single nucleotide polymorphisms (SNPs) are present within the coding region of AvBD1.

Nucleotide sequences are shown in black and the corresponding translated amino acids are shown underneath. The three non-synonymous coding SNPs are identified in green, red and blue. The AvBD1 peptides were designated A) ‘NYH’, B) SSY’ and C) ‘NYY’ according to the polymorphisms which are present. The prevalence of each SNP form in each genetic line is shown in D). Percentage values are for homozygous alleles (TT or GG) and were calculated using data from the Aviagen Ltd. SNP study performed by Illumina (San Diego, U.S.A) in combination with sequencing of pooled bird DNA (n = 120) (Butler 2010).

6.3 Production of recombinant AvBDs

6.3.1 Overview

The system chosen for AvBD peptide production was the Glutathione S-transferase (GST) fusion system (GE Healthcare) and is described fully in Chapter 2. Briefly, cDNA sequences corresponding to the mature peptide of AvBD1 'NYH', 'SSY' and 'NYY' and AvBD10 were cloned into the pGEX-6P1 vector followed by transformation into competent JM101 cells. After sequence confirmation, pGEX-6P1-AvBD plasmids were transformed into *E. coli* BL21(DE3)pLysS cells for hyperexpression and the GST-AvBD fusion proteins purified.

6.3.2 Engineering of AvBD1 and 10 hyperexpression vectors

Cloning was performed in collaboration with Dr Vanessa Armstrong, now School of Biomedical Sciences, Newcastle University. Briefly, primers incorporating the restriction enzyme sites BamHI and EcoR1 were designed to the mature peptide DNA sequences for AvBD1 and 10, and PCR was used to amplify cDNA from Line X and Y birds (Gel electrophoreses not shown). The cDNA sequences were cloned into the expression vector PGEX6p-1, transformed into JM101, plasmids prepared and sequenced (GeneVision, UK). To illustrate, the sequencing results for AvBD10 is shown in Figure 6.2.

GTGAGCTTGATGTGGCGACATCCTCCAAATCGGATCTGGAAGTTCTGTTCCAGG
GGCCCCTGGGATCC**GACCCACTTTTCCCTGACACCGTGGCATGCAGGACTC**
AGGGGAATTTCTGCCGTGCTGGGGCATGCCCCCCCACCTTCACCATCTCT
GGGCAGTGCCATGGGGGGCTGTTAAACTGCTGTGCCAAGATTCCGGCGCA
GTAAGAATTCCCGGGTCGACTCGAGCGGCCGCATCGTGACTGACTGACGATCT
GCCTCGCGCGTTT

Figure 6.2: The nucleotide sequence for the AvBD10-GST construct plasmid.

The DNA sequence encoding the mature peptide is shown in bold underlined, the location of the restriction enzyme sites BamH1 are in yellow and EcoR1 in blue, the DNA encoding the GPLGS amino acid sequence following GST-tag removal is in pink and stop codon is in red.

6.3.3 Hyperexpression and Purification of AvBD1 and 10

Aliquots at each stage of the hyper-expression and purification procedures were sampled and analysed by NuPAGE gel electrophoresis. Figure 6.3 shows gel electrophoresis of aliquots from each stage of the AvBD1 protein purification procedure. Lane 1 showed that that the majority of protein appeared as inclusion bodies in the bacterial pellet indicating that the GST-AvBD1 fusion protein had poor solubility in the cell free extract. The lack of GST-AvBD1 in the soluble fraction (Lane 2) resulted in less GST-fusion available for enzyme cleavage and thus only a small amount of peptide was produced (red box in Lane 7). Crucially, no separation using a 10kDa MW cut-off column was observed (Lane 8), and the cleaved AvBD1 peptide appeared in the >10kDa fraction alongside the GST tag, cleavage protease and other contaminants (Lane 9). An additional attempt to separate cleaved AvBD1 from higher M.W proteins using size exclusion chromatography failed to yield AvBD1 peptide. However at this time a PhD top-up grant from the Knowledge Transfer Network (KTN) for £5,000 was awarded, and this funded the commercial synthesis of the three versions of AvBD1 as linear peptides.

In contrast, comparison of Lanes 1 and 2 in Figure 6.4 illustrated that the AvBD10 fusion protein (~30kDa), was at a higher concentration in the soluble cell free extract (CFE) compared to the insoluble pellet. In addition, following GST-AvBD10 cleavage, relatively high levels of peptide were found corresponding to the correct M.W ~ 5kDa (lanes 5 – 9). The single band at ~5kDa observed in Lane 11 in the <10kDA fraction following size separation demonstrated that this strategy enabled high levels of purification.

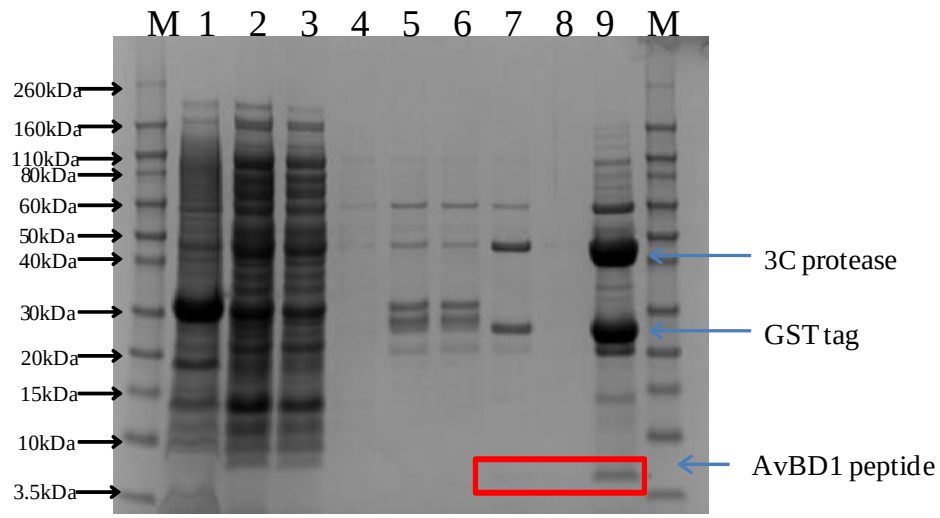


Figure 6.3: AvBD1: InstantBlue™ NuPAGE® 4-12% Bis-Tris Gel

Aliquots of each step of hyperexpression and purification for AvBD1 were as follows: M – Novex® Sharp molecular marker. 1 – re-suspended pellet; 2 - cell free extract; 3 – PBS wash No. 1; 4 – PBS wash No. 10; 5 – elution of GST-AvBD1; 6 – buffer exchange into PBS; 7 - enzyme cleaved GST-AvBD1 peptide; 8 - Collection of <10kDa proteins; 9 - Collection of >10kDa proteins. The cleaved AvBD1 peptide is highlighted by the red box.

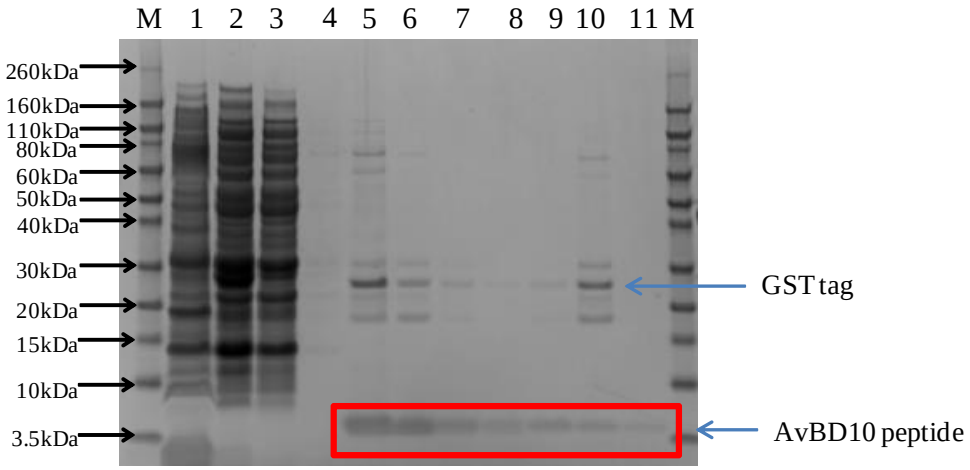


Figure 6.4: AvBD10: InstantBlue™ NuPAGE® 4-12% Bis-Tris Gel

Aliquots of each step of hyperexpression and purification for AvBD10 were as follows: M – Novex® Sharp molecular marker. **1** – re-suspended pellet; **2** - cell free extract; **3** – PBS wash No. 1; **4** – PBS wash No. 10; **5 – 9**: 1ml elutions of cleaved peptide + GST + enzyme; **10** – Collection of >10kDa proteins; **11** - Presumed recAvBD10 peptide. The cleaved AvBD10 peptide is highlighted by the red box.

6.3.4 Predicted properties of AvBD1 and AvBD10

The predicted properties for the three AvBD1 mature peptides and recombinant AvBD10 with additional cleavage tag (GPLGS) at the N-terminus were determined using online software (Innovagen, http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp), and are shown in Table 6.1. The predicted properties revealed that unlike many cationic anti-microbial peptides, AvBD10 has a low net charge at pH7 and an iso-electric point close to neutral pH (7.64).

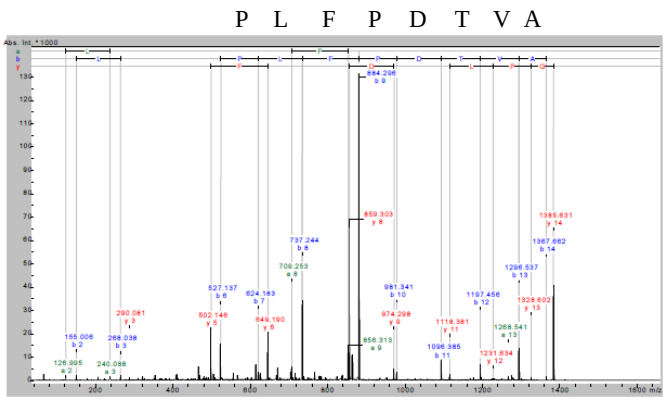
Table 6.1: Predicted properties of three AvBD1 variants ('NYH', 'SSY' and 'NYY') and recombinant AvBD10 + GPLGS tag.

	AvBD10	**AvBD1 (NYH)**	**AvBD1 (SSY)**	**AvBD1 (NYY)**
Molecular weight (g/mol)	5193	4644.6	4567.6	4670.7
Extinction coefficient ($M^{-1}cm^{-1}$)	0	6970	6970	8250
Iso-electric point (pH)	7.64	9.92	9.92	9.81
Net charge at pH 7	0.8	7.8	7.7	7.7
Est. water solubility	Poor	Good	Good	Good

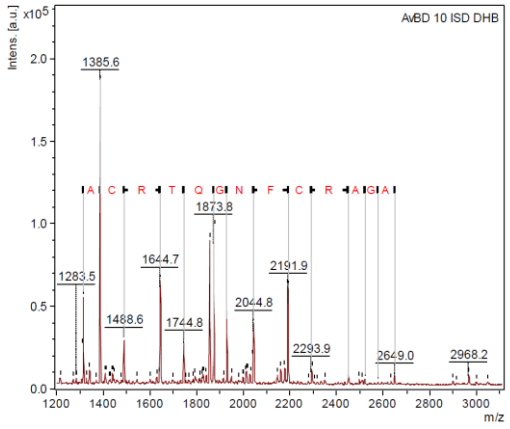
6.3.5 Peptide sequencing of AvBD10 using Mass Spectrometry

To authenticate the purified AvBD10 peptide an aliquot was sent to the Proteomics Laboratory, York University; peptide sequencing was performed using MALDI-MS/MS and In-Source Decay (ISD). A combination of MS/MS fragmentation (Figure 6.5A) and ISD fragmentation (Figure 6.5B) revealed the first 26 amino acids of AvBD10 (in bold) attached to GPLGS from the GST-tag used for purification (Figure 6.5C). Although the remaining 25 amino acids in the sequence could not be identified, a signal of mass (5189.5 *m/z*.), similar to predicted expected full length mass of 5193g/mol (Table 6.1), was observed in the full MALDI-ISD spectra suggesting the full length peptide was present.

A



B



C

GPLGSDPLFPDTVACRTQGNFCRAGACPPTFTISGQCHGGLLNCCAKIPAQ

Figure 6.5: Identification of AvBD10 sequence using MALDI-MS/MS and In-Source Decay (ISD).

A) MALDI-MS/MS spectrum of precursor at 1385.6 m/z with DHB matrix, annotated with the *de novo* derived sequence PLFPDTVA. B) MALDI-ISD spectrum of AvBD10 with overlaid sequence ACRTQGNFCRAGA**.** ISD was used to fragment the entire component of the sample and read back to the N-terminus. C) Total sequence of AvBD10 identified using both using MALDI-MS/MS and In-Source Decay (ISD) is shown in bold.

6.4 Anti-microbial activities of AvBD1 and 10

The AvBD1 and 10 peptides were used in anti-microbial assays and their activities tested against bacteria linked to the chicken GI tract. The assays employed both gram negative (E*scherichia. coli*, *Salmonella typhimurium, Barnesiella viscericoli, Bacteroides dorei*) and gram positive (*Enterococcus faecalis, Lactobacillus johnsonii*) isolates. The assays included a colony counting time-kill assay (Townes et al., 2004; Milona et al., 2007), a radial diffusion assay (Lehrer et al., 1991; Schroeder et al., 2011) and microbroth dilution assay (van Dijk et al., 2007). The colony counting assay, developed in the Hall Laboratory (Townes et al., 2004), provides a quantitative method to assess the activity of peptide against a diluted bacterial broth over a 2 – 3 h incubation period. Similarly, the microbroth dilution assay performed in a microtitre plate utilises a 1 in 2 dilution series of peptide (125 – 0.1μg/ml) against a set dilution of bacteria with the aim of determining the minimum inhibitory concentration (MIC), which is the lowest concentration at which there is no visible growth. Typically, the assays are performed overnight and checked for growth the following day; for the assays reported herein the plates were incubated overnight in a plate reader and OD^{600nm} was plotted every 20 min to produce a growth curve at all concentrations. Finally, the radial diffusion assay was set up, which enabled a straightforward visual comparison of the AMA of different peptides, visualized as a zone of inhibition, against a bacterial lawn. This assay can be used semi-quantitatively by measuring the diameter of inhibition or, as reported in the results, a percentage area was calculated using imaging software. The colony counting and microbroth dilution assays were unsuccessful for assessing peptide AMA against *Bacteroides* spp., and so a modified version of the radial diffusion assay for anaerobic bacteria was adopted as previously outlined (Schroeder and Wehkamp, 2011).

6.4.1 Anti-microbial activities of sAvBD1 'NYH', 'SSY' and 'NYY'

Figure 6.6 shows the anti-microbial activity of the AvBD1 'NYH', 'SSY' and 'NYY' peptides against *E. coli* (clinical isolate) using the colony counting (A), microbroth dilution (B) and the radial diffusion (C) assays. The data from the colony counting assays revealed that at 10μg/ml the 'NYH' form of the peptide exhibited *E. coli* killing activity whereas at the same concentration, the SSY and NYY peptides were associated with *E. coli* survival, 18% and 22%, respectively. To determine the MIC for each peptide the microbroth dilution assay was employed. These data, shown in Panel B, supported the colony counting data in that 'NYH' peptide was revealed to be the most active peptide form with complete *E. coli* killing demonstrated up to 15.6μg/ml (MIC 15.6μg/ml); 'SSY' killed at 125μg/ml (MIC 125μg/ml) but 'NYY' did not kill *E. coli* at any of the concentrations tested (MIC > 125μg/ml). These data confirmed that 'NYH' is the most active of the three AvBD1 variants against *E. coli* and suggested that the 'SSY' form is more active than 'NYY' (Panel B). To examine the effects of these peptides at a high concentration against a bacterial lawn, the radial diffusion assay was employed. At 1μg concentration the relative size of the inhibition zone was 51% for the 'NYH' variant compared to 35% and 31% for 'SSY' and 'NYY', respectively; again demonstrating that 'NYH' is the most potent peptide of the three different forms (panel C).

Figure 6.7 shows the anti-microbial activity of AvBD1 'NYH', 'SSY' and 'NYY' against *Enterococcus faecalis* using the colony counting (A), microbroth dilution (B) and radial diffusion (C) assays. Colony counting assays showed a lower mean *E. faecalis* growth (%) at 5, 10 and 25μg/ml for the 'NYH' variant, although this did not reach statistical significance (panel A). The microbroth dilution assay produced MIC values of 1.98, 3.9 and 3.9μg/ml for 'NYH', 'SSY' and 'NYY' respectively, establishing that the 'NYH' variant was more active against *E. faecalis* than the 'SSY' and 'NYY' forms. Further evidence for the enhanced potency of 'NYH' was shown by the radial diffusion assay that illustrated larger zones of inhibition at 1 and 2μg for the 'NYH' form compared to either the 'SSY' or 'NYY' variants. In summary for *E. faecalis,* all three assays supported that 'NYH' was the most potent peptide although no difference in activities between 'SSY' and 'NYY' could be established.

Figure 6.8 shows that all forms of AvBD1 completely inhibited *Salmonella typhiumurium* growth, at concentrations of >7.5µg/ml, but at 2.5 and 5µg/ml the 'NYH' peptide resulted in significantly lower *S. typhiumurium* growth than the SSY and NYY peptide forms ($P < 0.001$). These data were corroborated by the radial assay results that showed larger inhibition zones for 'NYH' than 'SSY' and 'NYY' at all concentrations tested (Panel B). Furthermore, a comparison between 'SSY' and 'NYY' at 2µg illustrated a larger inhibition zone area for 'SSY' (74%) than 'NYY' (48%), suggesting increased 'SSY' potency. However, no MIC values were determined for any of the variants to support the radial diffusion assay data. An initial attempt at the microbroth assay failed, due to contamination between wells, and was not re-attempted due to time constraints.

The anaerobic bacteria, both facultative (LJ, BV) and obligate (BD) were difficult to culture and experiments utilising the colony counting and microbroth methodologies were only partially successful in that colonies were counted for BV and LJ. Radial assays performed under anaerobic conditions were however, successful for all three anaerobes. The colony counting assay data showed that 'NYH' was the most anti-microbial of the three variants against LJ (Panel A). At the highest concentration of 50µg/ml the 'NYH' form completely inhibited growth while the 'SSY' and 'NYY' variants did not. This result was confirmed by the radial diffusion assay, as well as illustrating larger areas of inhibition for the 'SSY' (2µg 64%, 1µg 51%), than the 'NYY' (2µg 47%, 1µg 27%), peptide (Figure 6.9C).

As shown for the other bacterial strains tested, the 'NYH' variant was more potent than the 'NYY' form against BV (Figure 6.10) and BD (Figure 6.11). In addition, for BV, at 2µg/ml the 'SSY' variant appeared to be less potent than 'NYH', with clearing areas of 48% and 72% determined, respectively. However, no clear differences between the 'NYH' and 'SSY' forms could be demonstrated for BD (Figure 6.11).

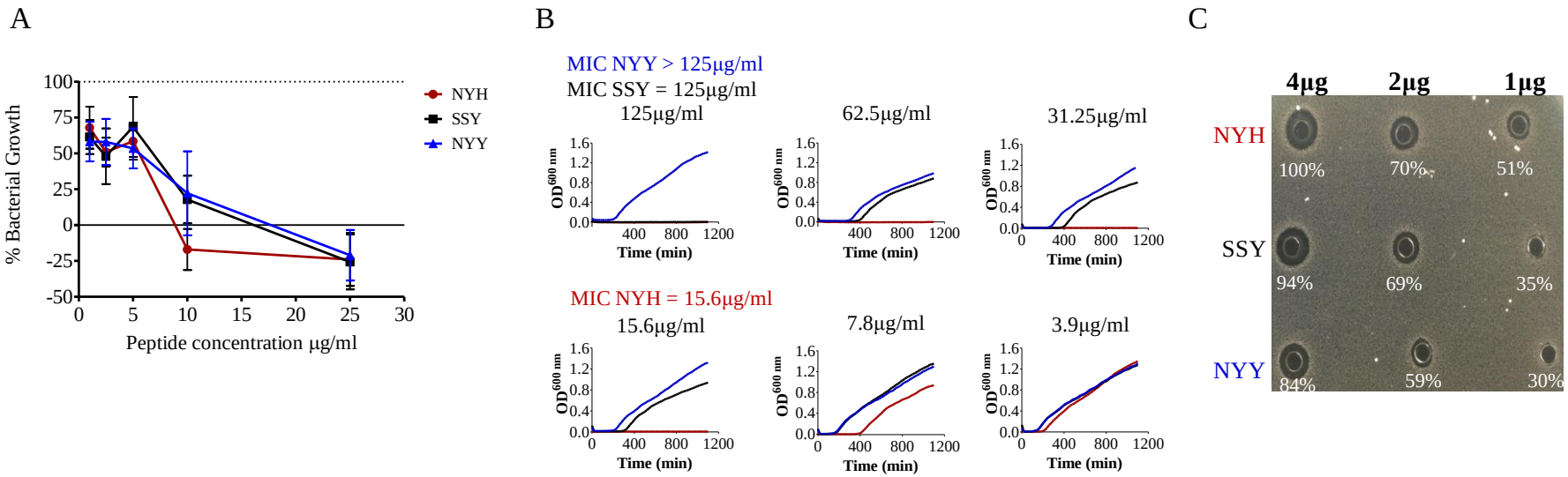


Figure 6.6: The anti-microbial activities of three AvBD1 variants (NYH, SSY and NYY) against avian *Escherichia coli.*

A) Colony-counting assay showing percentage bacterial growth (*E. coli*) following 2 h incubation of mid-log bacteria (1/1000 dilution) with three AvBD1 peptides (NYH, SSY and NYY) at a final concentration of 1 - 25µg/ml. All percentage growth is shown relative to PBS + bacteria control (dotted line). Percentage values < 0% indicate fewer colonies after 2 h than at 0 h and hence, indicate bacterial killing. Each point shows the mean ± SEM from three experiments (N = 3).

B) Growth curves for the microbroth dilution assay showing growth *of E. coli* (mid-log diluted 1/20000) in LB media as a measure of OD^{600nm} over time (min) following the addition of AvBD1 NYH, SSY and NYY at a final concentration of 125 – 3.9µg/ml. The Minimum Inhibitory Concentration (MIC) is indicated for each peptide next to the relevant growth curve (N = 1 experiment).

C) Radial diffusion assay showing the inhibitory effect of AvBD1 variants 'NYH', 'SSY' and 'NYY' at 4, 2 and 1µg. The largest zone of inhibition is assigned a value of 100% and relative size of each inhibition zone (%) was determined using ImageJ software (N = 1 experiment).

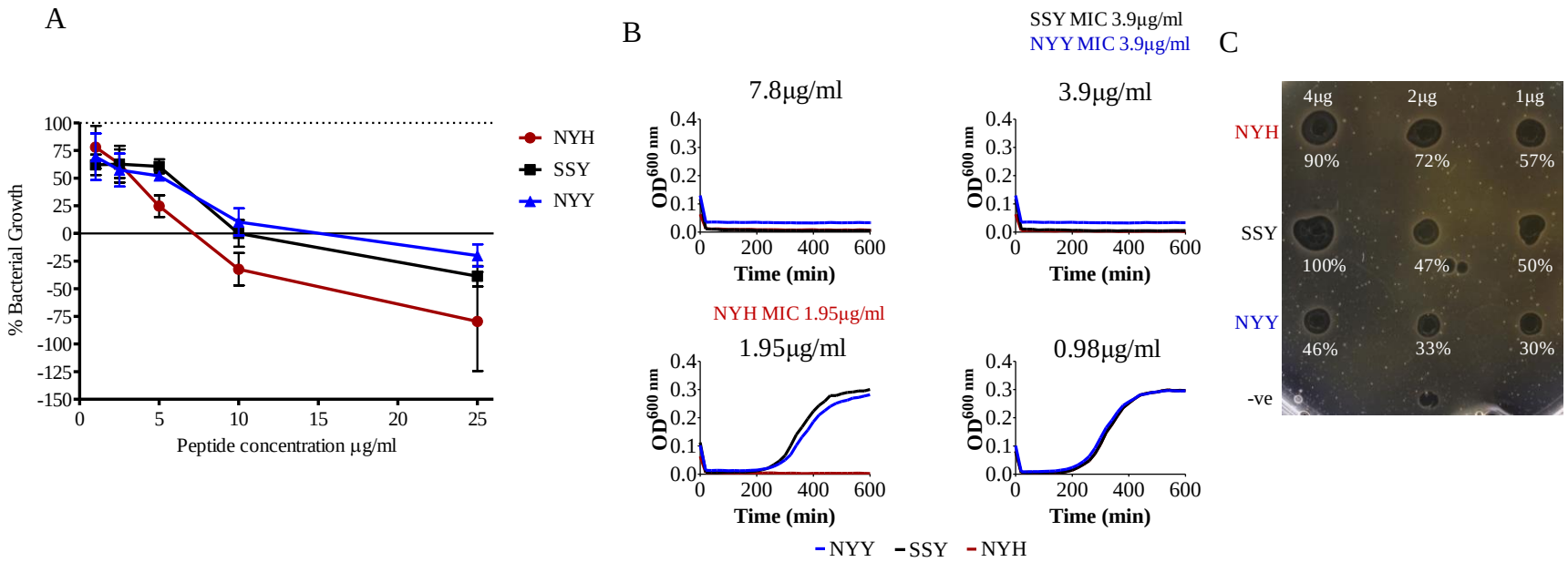


Figure 6.7: The anti-microbial activities of three AvBD1 variants (NYH, SSY and NYY) against avian *Enterococcus faecalis.*

A) Colony-counting assay showing percentage bacterial growth (*E. faecalis*) following 2 h incubation of mid-log bacteria (1/1000 dilution) with three AvBD1 peptides (NYH, SSY and NYY) at a final concentration of 1 - 25µg/ml. All percentage growth is shown relative to PBS + bacteria control (dotted line). Percentage values < 0% indicate fewer colonies after 2 h than at 0 h and hence, indicate bacterial killing. Each point shows the mean ± SEM from three experiments (n = 3).

B) Growth curves for the microbroth dilution assay showing growth *of E. faecalis* (mid-log diluted 1/20000) in LB media as a measure of OD^{600nm} over time (min) following the addition of AvBD1 NYH, SSY and NYY at a final concentration of 7.8 – 0.98µg/ml. The Minimum Inhibitory Concentration (MIC) is indicated for each peptide next to the relevant growth curve (N = 1 experiment).

C) Radial diffusion assay showing the inhibitory effect of AvBD1 variants 'NYH', 'SSY' and 'NYY' at 4, 2 and 1µg. The largest zone of inhibition is assigned a value of 100% and the relative size of each inhibition zone (%) was determined using ImageJ software.

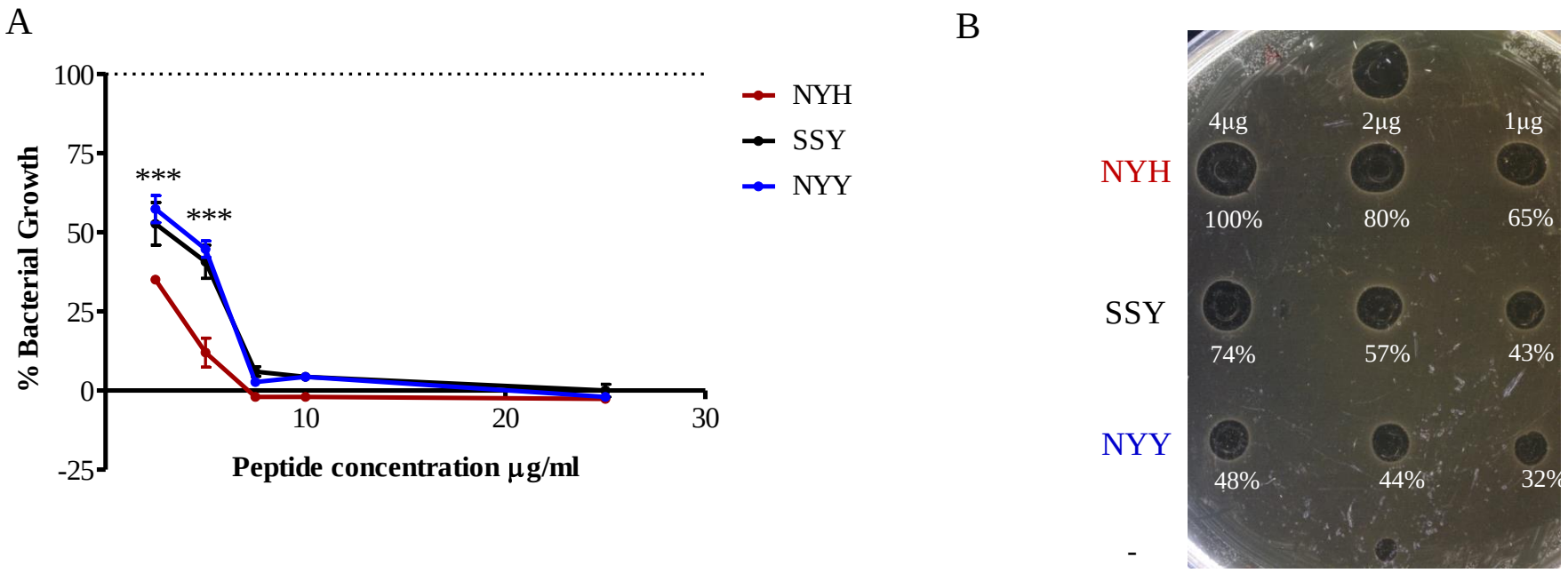


Figure 6.8: The anti-microbial activities of three AvBD1 variants (NYH, SSY and NYY) against avian *Salmonella typhimurium*.

A) Colony-counting assay showing percentage bacterial growth (*S. typhimurium*) following 2 h incubation of mid-log bacteria (1/1000 dilution) with three AvBD1 peptides (NYH, SSY and NYY) at a final concentration of 1 - 25µg/ml. All percentage growth is shown relative to PBS + bacteria control (dotted line). Each point shows the mean ± SEM from three replicates (n = 3). *** $P < 0.001$; Bonferroni post-tests.

B) Radial diffusion assay showing the inhibitory effect of AvBD1 variants 'NYH', 'SSY' and 'NYY' at 4, 2 and 1µg. The largest zone of inhibition is assigned a value of 100% and the relative size of each inhibition zone (%) was determined using ImageJ software.

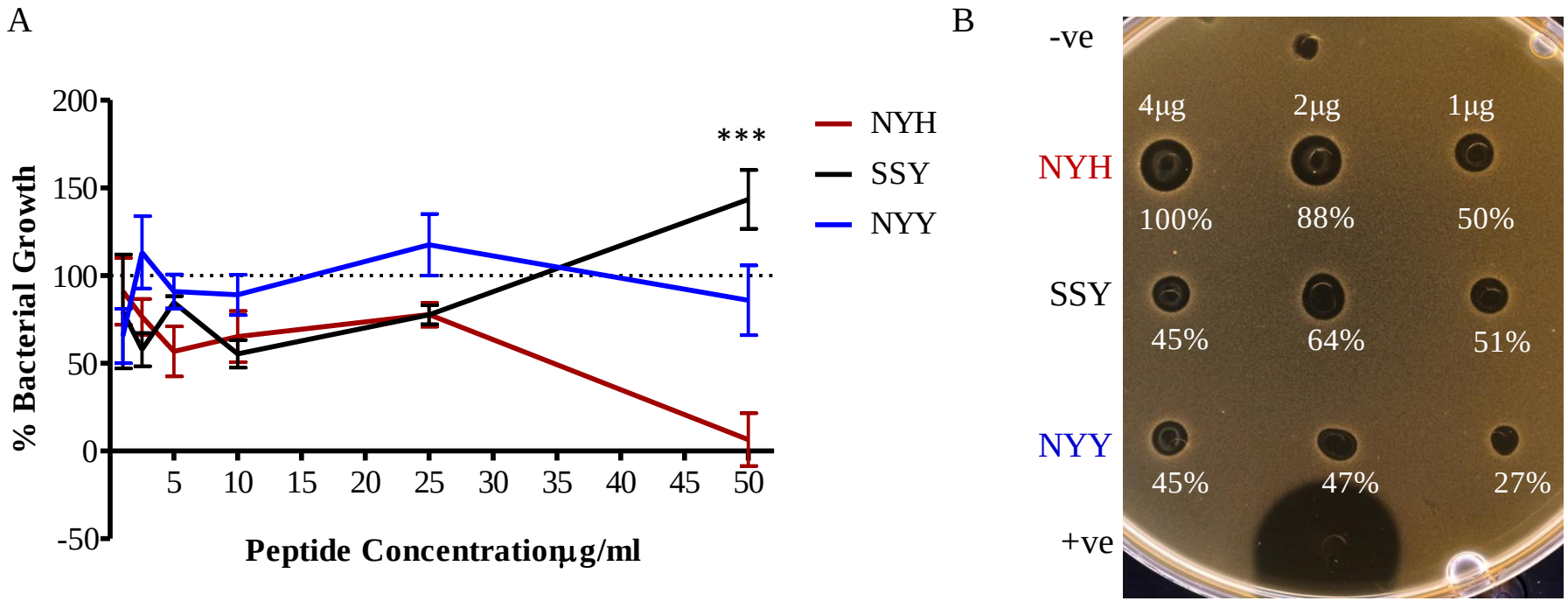


Figure 6.9: The anti-microbial activities of three AvBD1 variants (NYH, SSY and NYY) against avian *Lactobacillus johnsonii*.

A) Colony-counting assay showing percentage bacterial growth (*L. johnsonii*) following 3.5 h incubation of mid-log bacteria (1/1000 dilution) with three AvBD1 peptides (NYH, SSY and NYY) at a final concentration of 1 - 50µg/ml. All percentage growth is shown relative to MRS media (5%) + bacteria control (dotted line). Each point shows the mean ± SEM from three experiments (n = 3). *** $P < 0.001$; Bonferroni post-tests comparing 'NYH' to 'SSY' and 'NYY'.

B) Radial diffusion assay showing the inhibitory effect of AvBD1 variants 'NYH', 'SSY' and 'NYY' at 4, 2 and 1µg. The largest zone of inhibition is assigned a value of 100% and the relative size of each inhibition zone (%) was determined using ImageJ software.

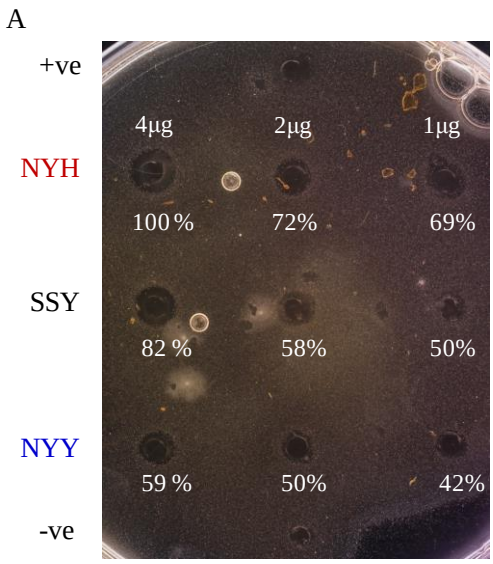


Figure 6.10: The anti-microbial activities of three AvBD1 variants (NYH, SSY and NYY) against avian *Barnesiella viscericola.*

A) Radial diffusion assay showing the inhibitory effect of AvBD1 variants 'NYH', 'SSY' and 'NYY' at 4, 2 and 1µg. The largest zone of inhibition is assigned a value of 100% and the relative size of each inhibition zone (%) was determined using ImageJ software.

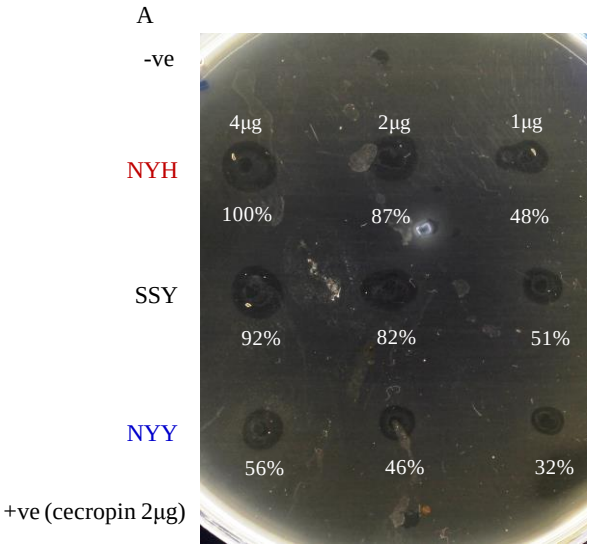


Figure 6.11: The anti-microbial activities of three AvBD1 variants (NYH, SSY and NYY) against avian *Bacteroides dorei.*

A) Radial diffusion assay showing the inhibitory effect of AvBD1 variants 'NYH', 'SSY' and 'NYY' at 4, 2 and 1µg. The largest zone of inhibition is assigned a value of 100% and the relative size of each inhibition zone (%) was determined using ImageJ software.

6.4.2 Effects of lipid-AvBD1 interactions on secondary structure

The three SNP peptide variants were synthesised commercially as linear peptides. It has been shown by using circular dichroism (CD) that AMPs can change confirmation, for example, from a random to a helical structure in the presence of anionic phospholipids (Lee et al., 2012; Yeaman and Yount, 2003). To determine if such a change in peptide secondary structure was observed in the three AvBD SNP variants, CD experiments were performed in the presence and absence of SDS, the latter mimicking a bacterial phospholipid membrane. The data shown in Figure 6.12A indicated that all three peptides formed disorganised structures in aqueous solution, although the NYY variant also formed a partial beta-sheet-like structure (negative band at 217nm and a positive band below 200nm). However, the data in Figure 6.12B demonstrated that following the addition of SDS, all three peptides change to an alpha-helical conformation, which was probably linked to their anti-microbial activities.

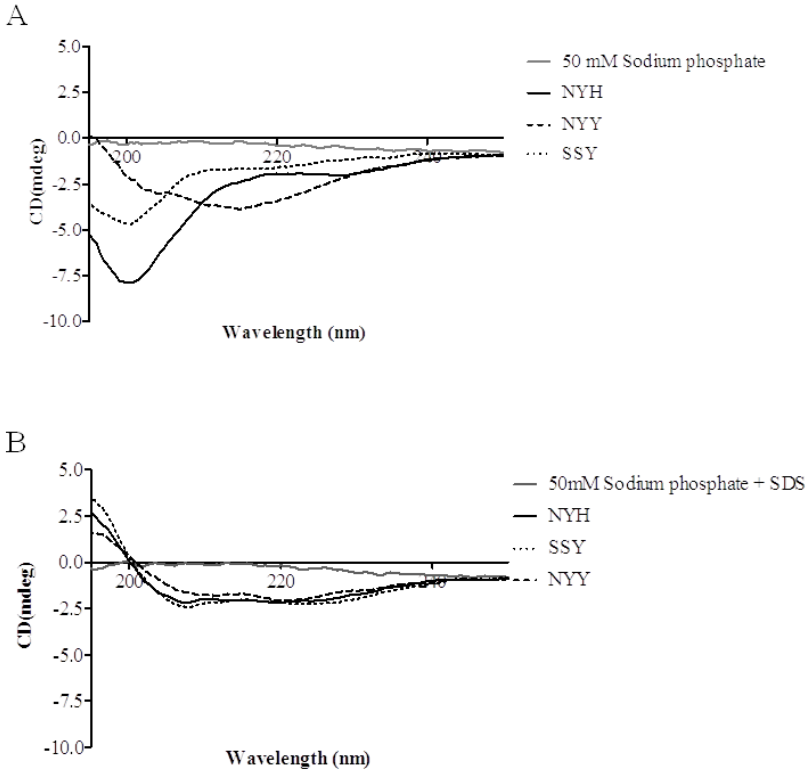


Figure 6.12: CD spectra of three AvBD1 variants (NYH, SSY, NYY)

A) In 50mM sodium phosphate buffer the AvBD1 variant NYY shows beta-sheet-like structures in contrast to random coil structures for NYH and SSY.

B) Addition of SDS induces a conformational change in all three AvBD1 variants from a random to an alpha-helical structure.

Experiment performed by Sherko Subhan, PhD student, Newcastle University.

6.4.3 Anti-microbial activity of recombinant AvBD10

Figures 6.13 – 6.17 show the anti-microbial activities of recombinant AvBD10 against *E. coli*, *E. faecalis*, *B. viscericola*, *L. johnsonii* and *B. dorei*, respectively.

The colony counting assay data for *E. coli* showed that as peptide concentration increased from 0.5 to 10μg/ml, *E. coli* growth was inhibited to approximately 70% of the PBS control (Figure 6.13). No further growth inhibition was observed when the AvBD10 concentration was increased from 10 to 25μg/ml. The radial diffusion however, showed a clear zone of inhibition at 1μg that increased in size at 2μg, indicating *E. coli* killing (Figure 6.13B). Colony counting assay data for *E. faecalis* (Figure 6.13A), showed that recAvBD10 (0.5 – 5μg/ml) inhibited the bacterial growth to 30 - 40% of the PBS control and to less than 20% at 10μg/ml. No further decrease in bacterial growth was observed at the highest concentration of 25μg/ml, suggesting that AvBD10 is not able to completely kill the bacteria (Figure 6.14A). The lack of *E. faecalis* killing capacity by AvBD10 was confirmed by the microbroth dilution assays (Figure 6.14B). These data did, however, support a concentration-dependent inhibition of growth. For example, at 7.8μg/ml measurable growth was delayed for approximately 400min, while at 125μg/ml the bacterial growth was inhibited until >900min.

Similar to that observed with *E. coli* and *E. faecalis*, the colony counting assay data for *B. viscericola* (Figure 6.15), showed a decrease in bacterial growth as the peptide concentration was increased up to 25μg/ml. A further increase in AvBD10 concentration up to 50μg/ml did not result in further inhibition, a pattern also observed with *E. faecalis* and *E. coli*.

Figures 6.16 and 6.17 show the results of the radial diffusion assays for AvBD against *L. johnsonii* and *B. dorei*, respectively. A thinning of the bacterial lawn was observed for *L. johnsonii* suggesting inhibition, although a complete zone of clearing, as seen for lysozyme, was not observed (Figure 6.16). For *B. dorei* no zones of inhibition were observed although lysozyme/cecropin (positive controls) also did not inhibit at 2μg. Zones of inhibition were only observed for lysozyme at 20μg (Figure 6.17).

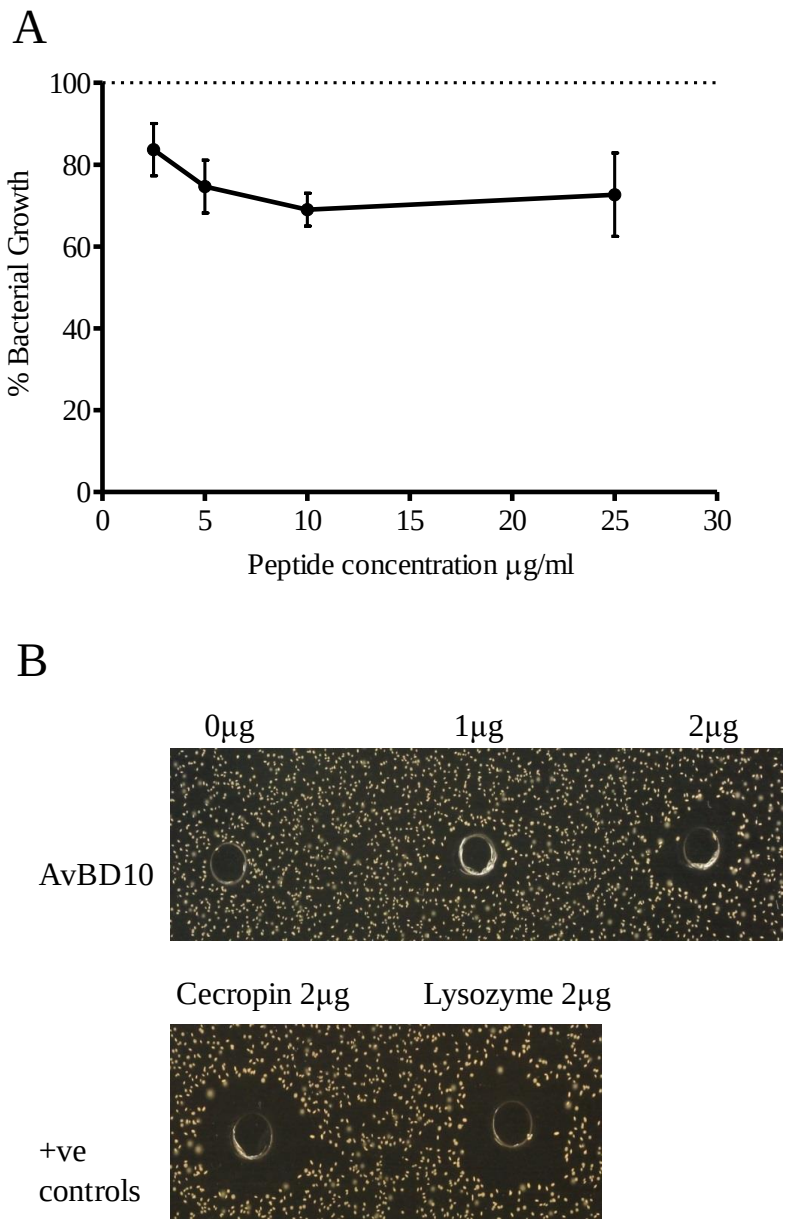


Figure 6.13: The anti-microbial activity of recombinant AvBD10 (recAvBD10) against avian *Escherichia coli.*

A) Colony-counting assay showing percentage bacterial growth (*E. coli*) following 2.5 h incubation of mid-log bacteria (1/1000 dilution) with recAvBD10 (1 - 25µg/ml). All percentage growth is shown relative to PBS + bacteria control (dotted line). Each point shows the mean ± SEM from three experiments using different protein purification batches (n = 3).
B) Radial diffusion assay showing the inhibitory effect of recAvBD10 at 1µg and 2µg, PBS (0µg) and positive controls (cecropin/hen-egg lysozyme at 2µg) on *E. coli*.

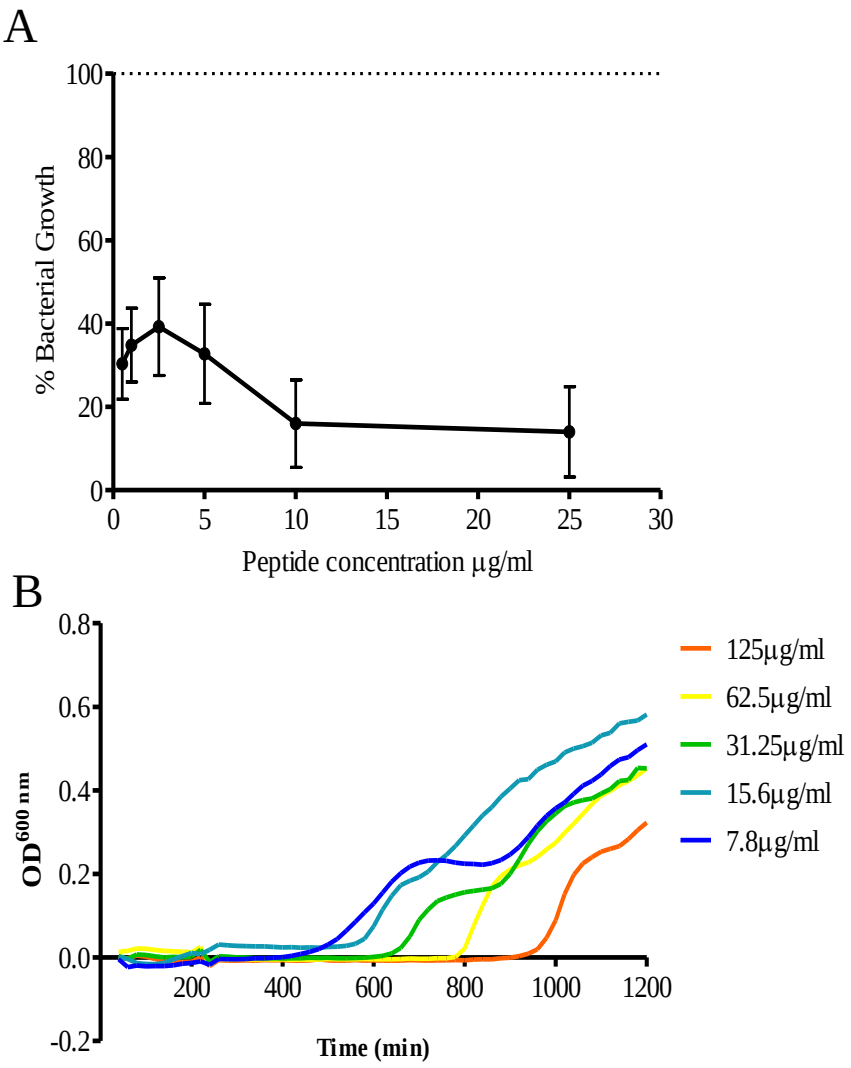


Figure 6.14: The anti-microbial activity of recombinant AvBD10 (recAvBD10) against avian *Enterococcus faecalis.*

A) Colony-counting assay showing percentage bacterial growth (*Enterococcus faecalis*) following 2.5 h incubation of mid-log bacteria (1/1000 dilution) with recAvBD10 (1 - 25µg/ml). All percentage growth is shown relative to PBS + bacteria control (dotted line). Each point shows the mean ± SEM from four experiments using different protein purification batches (n = 4).
B) Microbroth dilution assay showing *Enterococcus faecalis* growth as a measure of OD^{600nm} with the addition of recAvBD10 at 8.5, 17, 34, 67.5 and 125µg/ml.

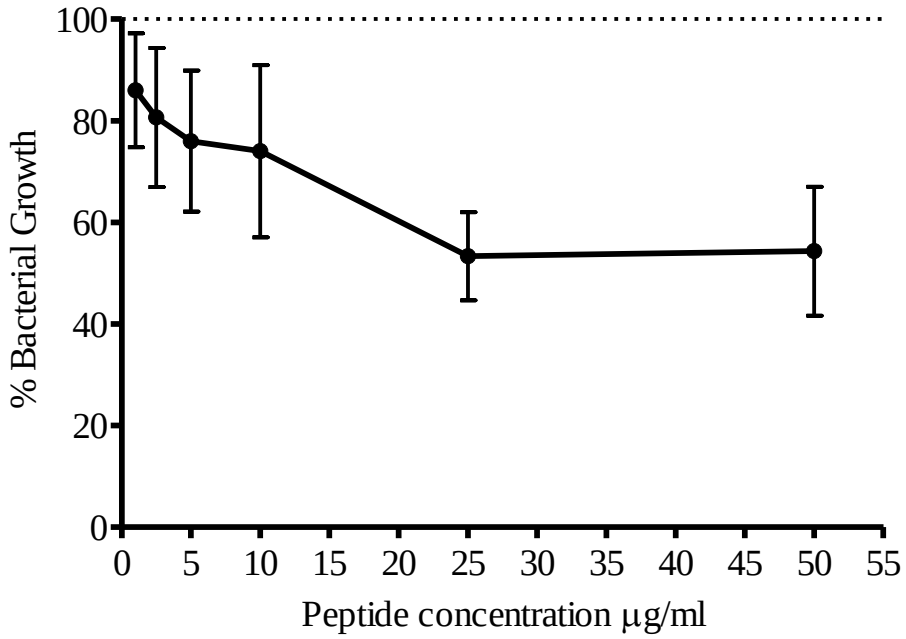


Figure 6.15: The anti-microbial activity of recombinant AvBD10 (recAvBD10) against avian *Barnesiella viscericola.*

A) Colony-counting assay showing percentage bacterial growth (*Barnesiella viscericola*) following 2.5 h incubation of mid-log bacteria (1/1000 dilution) with recAvBD10 (1 - 50µg/ml). All percentage growth is shown relative to PBS + bacteria control (dotted line). Each point shows the mean ± SEM from three replicates within a single assay (n = 1).

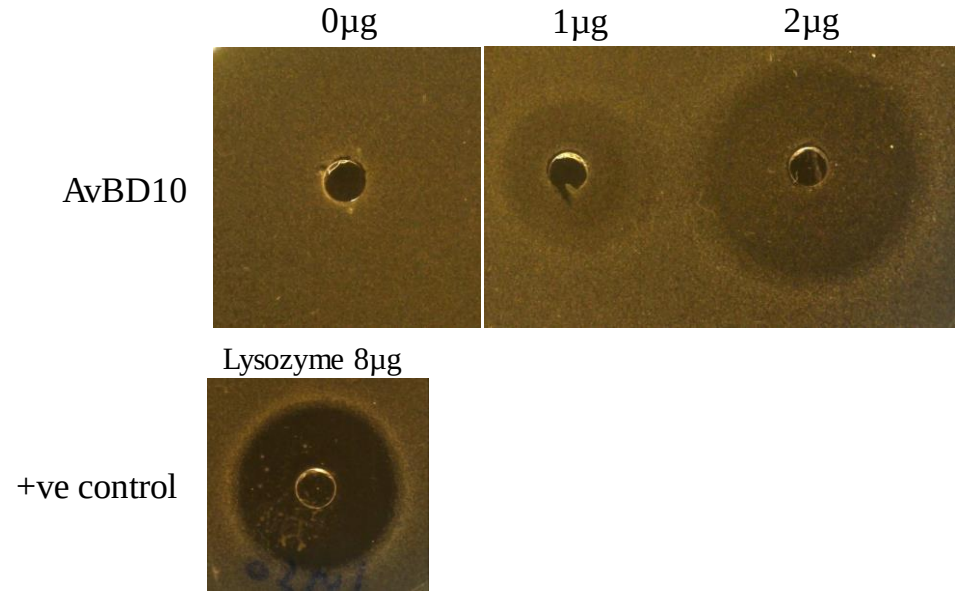


Figure 6.16: The anti-microbial activity of recombinant AvBD10 (recAvBD10) against avian *Lactobacillus johnsonii*.

A) Radial diffusion assay showing the effect of PBS (0µg), recAvBD10 (1 and 2µg/µl) and hen-egg lysozyme (+ve control) on *Lactobacillus johnsonii*.

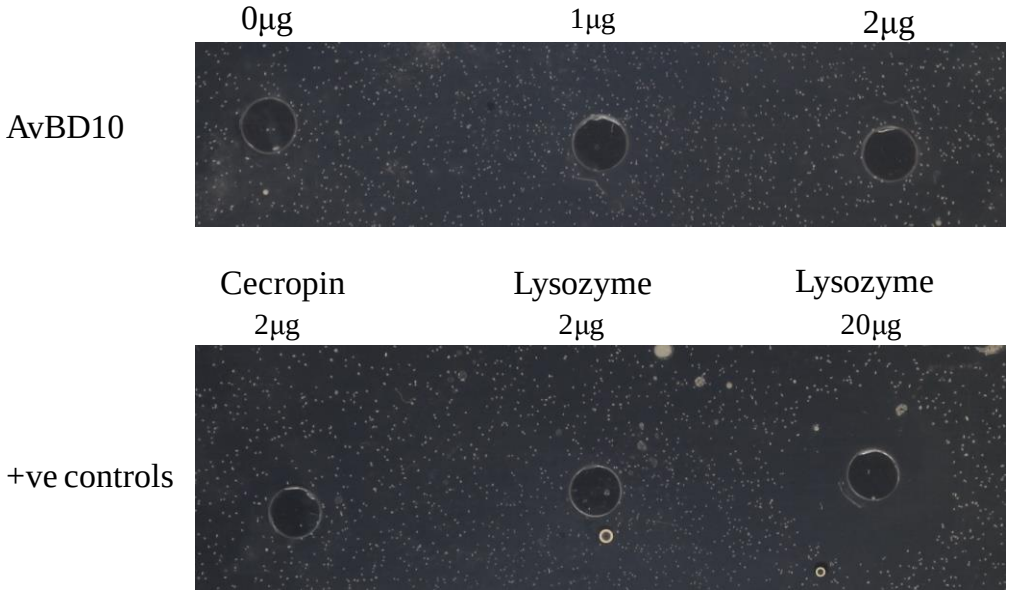


Figure 6.17: The anti-microbial activity of recombinant AvBD10 (recAvBD10) against avian *Bacteroides dorei*.

A) Radial diffusion assay showing the effect of recAvBD10 (1 and 2µg/µl), cecropin (+ve control) and hen-egg lysozyme (+ve control) on *Bacteroides dorei*

6.5 Comparison of the anti-microbial activity of AvBD1 and AvBD10

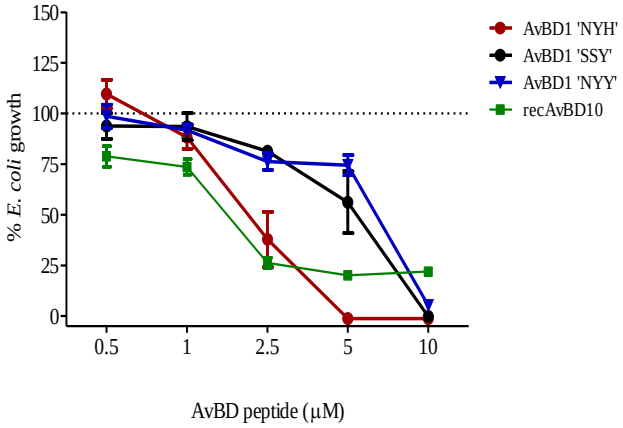
The data presented in Figures 6.17 and 6.18 show data from the colony counting assays that compare the anti-microbial activity of synthetic variants of AvBD1 and recombinant AvBD10 (0.5 - 10μM) against *E. coli* in high nutrient conditions of 10% LB media (Figure 6.18) and low nutrient conditions of PBS (Figure 6.19).

In the high nutrient assay (Figure 6.18), at the lowest concentration tested (0.5μM) the recombinant AvBD10 reduced *E. coli* growth to 75%, which was significantly different to the 'NYH' ($P < 0.05$), but not the other two AvBD1 forms. As the peptide concentration increased, *E. coli* growth decreased and differences in the antimicrobial activities of the peptides became apparent. At 2.5μM and 5μM respectively, incubation with both AvBD1 'NYH' and AvBD10 resulted in significantly lower *E. coli* (%) growth than either AvBD1 'SSY' or 'NYY' ($P < 0.01$). No significant differences between the 'SSY' and 'NYY' forms were found using this assay. At 10μM all AvBD1 variants completely inhibited *E. coli* growth, whereas, interestingly, AvBD10 only reduced growth to ~20% of the control (10% LB media).

Figure 6.19 shows a comparison of the activity of AvBD1 'NYH' and AvBD10 against *E. coli* grown in the low nutrient conditions of PBS. At 0.5 and 1μM no significant differences were observed, but the data indicated that at concentrations greater than 2.5μM, AvBD1 'NYH' treatment, in contrast to AvBD10, resulted in complete inhibition of *E. coli* growth, (2.5μM: $P < 0.05$, 5μM: $P < 0.001$, 10μM: $P < 0.01$).

‘High’ nutrient conditions (10% LB)

A



B

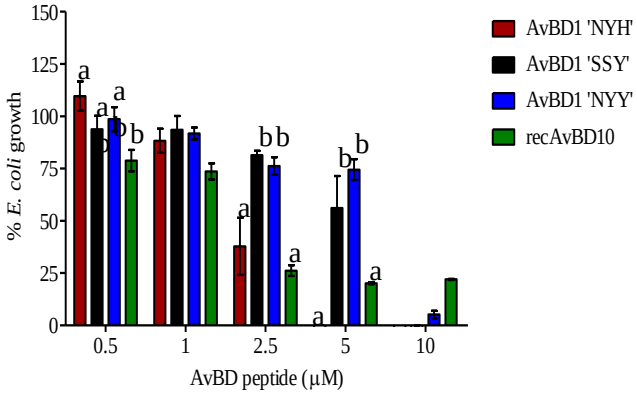


Figure 6.18: A comparison of the anti-microbial activity of three AvBD1 variants with recombinant AvBD10 under high nutrient conditions (10% LB media).

The data is illustrated as a line graph (A) and histogram (B).

A) Colony-counting assay showing percentage bacterial growth (*E. coli*) following 2 h incubation of diluted mid-log bacteria (1/1000) with three AvBD1 peptides (NYH, SSY and NYY) and recombinant AvBD10 at a final concentration of 0.5 - 10μM. All percentage growth is shown relative to the control of 10% LB media + bacteria (dotted line). Each point shows the mean ± SEM from three replicates in a single experiment (n = 1).

B) Significant differences between peptides at each concentration are presented as a histogram. Bars not sharing the same letters are significantly different according to Two-way ANOVA followed by Bonferroni's Multiple Comparison Test ($P < 0.05$).

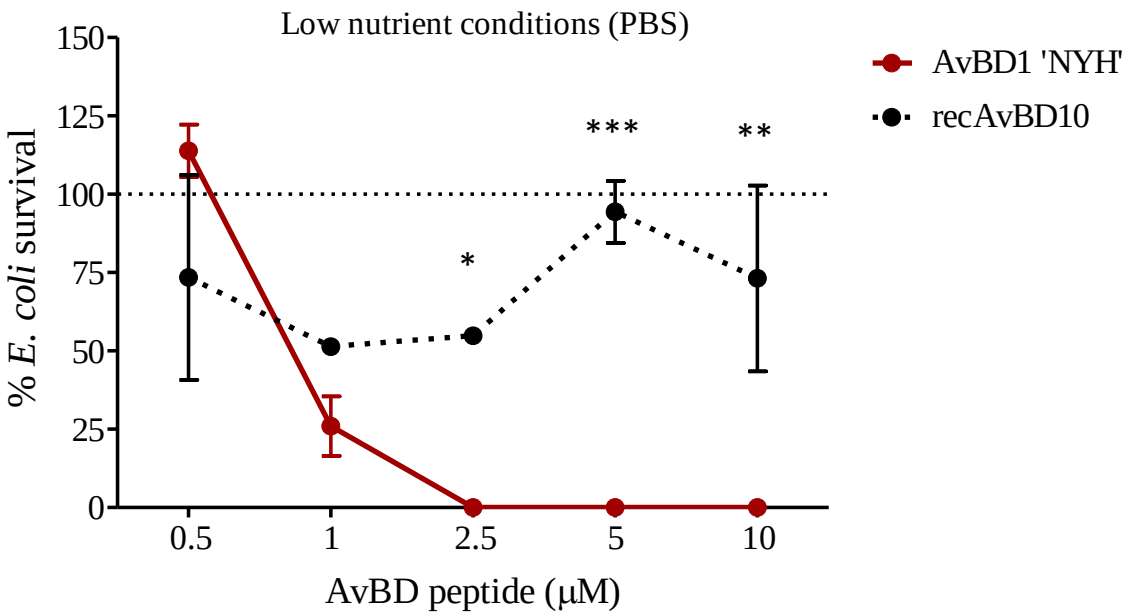


Figure 6.19: Colony-counting assay showing percentage bacterial growth (*E. coli*) in low nutrient conditions (PBS buffer).

AvBD1 peptide 'NYH' and recombinant AvBD10 were incubated for 2 h with diluted mid-log *E. coli* (1/1000) at a final concentration of 0.5 - 10μM. All percentage growth is shown relative to PBS + bacteria control (dotted line). Each point shows the mean ± SEM from three replicates in a single experiment (n = 1). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Bonferroni post-tests.

6.6 Novel properties of AvBD1 and AvBD10

Data from Chapter 4 indicated that relative to AvBD1, AvBD10 showed different patterns of tissue expression (high kidney/liver expression relative to gut tissues). The data from this chapter indicated that AvBD10 had an inhibitory rather than bactericidal function. Therefore, due its high constitutive tissue expression but low AMA, AvBD10 appeared to be a good candidate to investigate for further functions including those of cell proliferation wound healing. CHCC-OU2 cells were used for such experiments.

Cell proliferation, relative to a PBS control was examined for CHCC-OU2 cells incubated with one of the four AvBDs (AvBD1 NYH/SSY/NYY and AvBD10), or Fibroblast growth factor (FGF) – a positive control, or Bovine Serum Albumin (BSA)- a generic protein source or Mitomycin C - a anti-proliferative control (Figure 6.20). Due to lack of chicken peptide, human basic FGF was used as the positive control; however, at the concentrations used it did not significantly affect cell proliferation, although at 1nM FGF it did produce a higher mean cell proliferation, 117%, compared to control. The results using the AvBD peptides were also negative in that 48 h incubation of the CHCC-OU2 cells with either AvBD10 or the two AvBD1 variants ('NYH' and 'SSY') at 1, 5 and 10nM did not significantly affect cell proliferation, as measured by Cell Titre-Blue assay, relative to the PBS control. Mitomycin C was used as an anti-proliferative control and was shown to significantly inhibit cell proliferation at 5 ($P < 0.05$) and 10nM ($P < 0.01$)

The effects of AvBD10 on CHCC-OU2 cell wound healing were examined by performing scratch assays. Following 'wounding' the confluent cell layer with a pipette tip and incubating the wells at peptide concentrations of 0.1, 0.5 and 1nM, the closure of the wound was imaged over a 72 h time period (Figure 6.21). At 0.1nM and 0.5nM AvBD10 induced higher mean wound healing at each sampling time-point relative to control but this did not reach statistical significance. For example, at 0.1nM of AvBD10, percentage wound closure was 19% (24h), 45% (48h) and 43% (72h) compared to the PBS control that had wound closure measured at 9% (24h), 27% (48h) and 31% (72h).

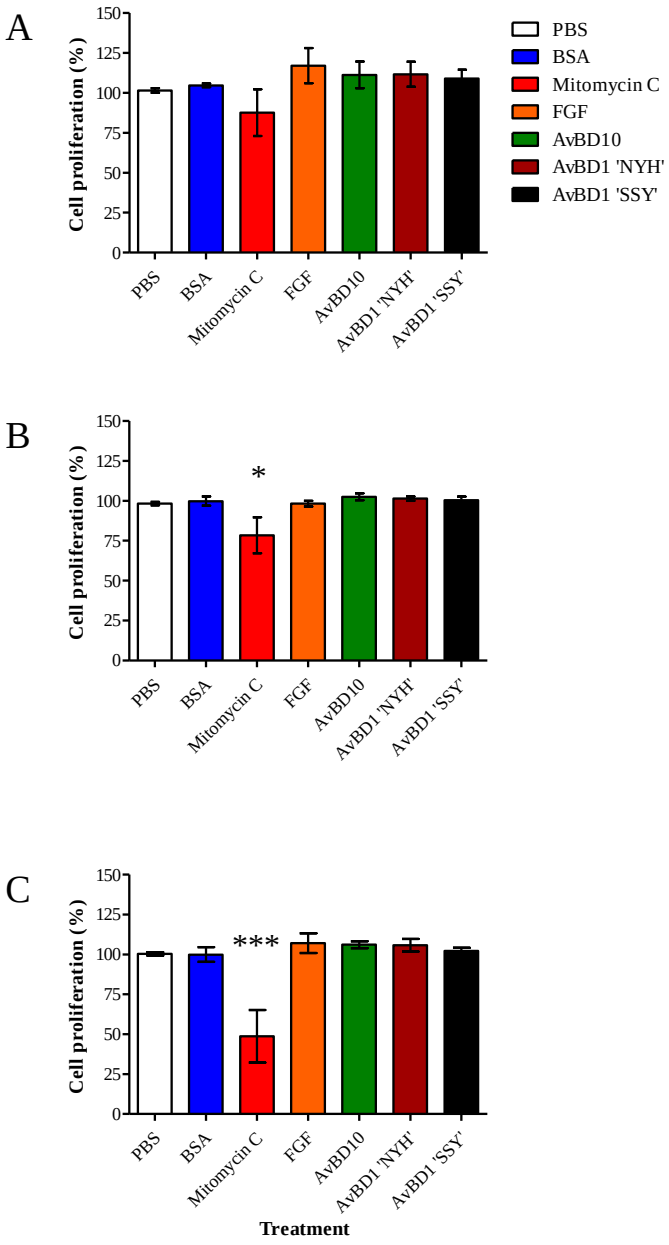


Figure 6.20: No significant effect of AvBD incubation on cell viability.

Viability is shown relative to PBS control (Mean ± SEM) following 48 h incubation with Bovine Serum Albumin (BSA), Mitomycin C, fibroblast growth factor (FGF), AvBD10, AvBD1 'NYH', and AvBD1 'SSY' at A) 1nM, B) 5nM and C) 10nM. After 48 h incubation, wells were incubated with CellTiter-Blue® Reagent for 2 -3 h. P values show comparisons of PBS control with treated wells. * $P < 0.05$, *** $P < 0.001$; Dunnett's Multiple Comparison Test. N = 3 experiments.

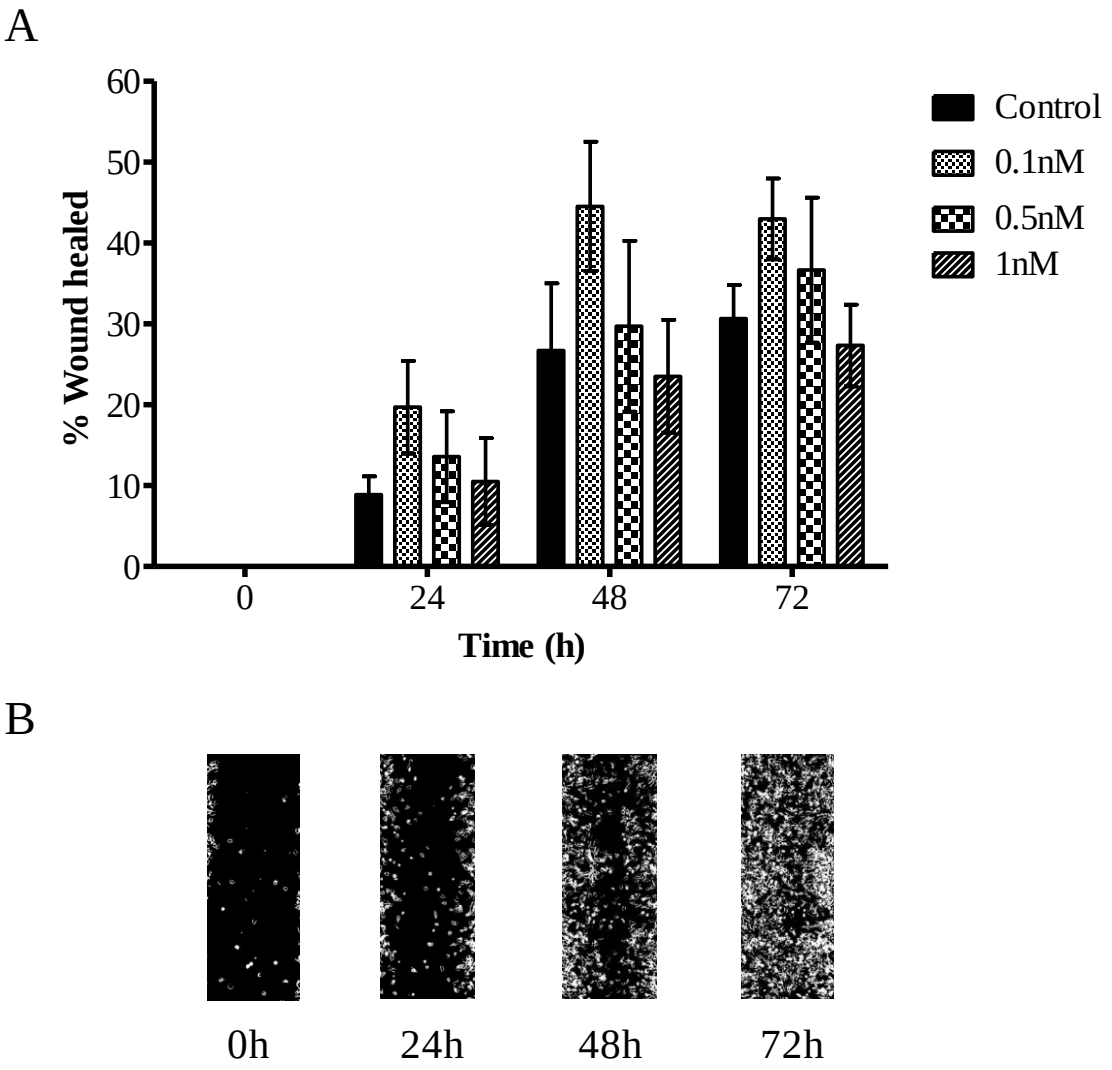


Figure 6.21: Effect of AvBD10 on wound healing.

For each concentration (0, 0.1, 0.5 and 1nM) three wells containing confluent cells were scratched and incubated with AvBD10 for 0, 24, 48 and 72 h. Photographs were taken at each time-point and percentage wound healing was calculated as the total wound area (black pixels) minus the number of cells (represented by white pixels) that had migrated into the wound area (example shown in panel B). The mean ± SEM for three wells were taken for each experiment. N = 3 experiments.

6.7 Discussion

Avian, unlike many human defensins, are not commercially available, and had to be produced 'in house'. To obtain biologically active peptide for anti-microbial testing, many methods have been employed e.g. AvBDs have been either directly extracted from leukocytes (Harwig et al., 1994) or produced using bacterial (Ma et al., 2012a), human cell (van Dijk et al., 2007), and yeast (Cao et al., 2012) expression systems or chemically synthesised (Higgs et al., 2007; Hellgren et al., 2010). Many of the assays using recombinant peptides have been performed using tagged peptides that have molecular weights much larger than those found *in vivo* and although the peptides have been shown to have anti-microbial activity, such properties may not be biologically representative.

To enable the production of peptides that are comparable to those found *in vivo*, this study utilized the GST fusion system (GE Healthcare Lifesciences), and following purification, removed the GST fusion tag by protease cleavage. The results shown in Figures 6.3 and 6.4 indicate that hyperexpression was successful for both AvBD1 and 10. However, in contrast to AvBD10 in which the majority of the GST fusion was in the cell free extract, the majority of GST- AvBD1 was found in insoluble fusion bodies. For AvBD10, this enabled a simple purification strategy of GST tag removal followed by size separation using spin columns with a 10kDa molecular weight cut-off, but solubility and/or charge issues meant it did not work for AvBD1. The likelihood of hyperexpressed proteins, including AvBD1, accumulating in inclusion bodies is high and protocols, including commercially available kits, are available to solubilize inclusion bodies in denaturing buffers such as guanidine hydrochloride or urea, followed by a re-folding step (Burgess, 2009; Yang et al., 2011). However, this is a technically demanding and time-consuming process which does not guarantee biologically active protein that is correctly folded (Panda, 2003). The successful production of a number of recombinant duck AvBD peptide GST-fusions in *E. coli* followed by purification using a refolding kit from Novagen has been described (Ma et al., 2009; Ma et al., 2012a), and this strategy was considered. Following a KTN PhD top-up award, the decision was taken to synthesise, chemically, the three AvBD1 variants. All three peptides were produced to >95% purity, which enabled a controlled assessment of how single amino acid changes altered their anti-microbial activities. Circular dichroism (CD) experiments have revealed that two synthetic SNP variants of NK-lysin are in an unorganised form in aqueous solution but, when in contact with dipalmitoylphosphatidyglycerol (DPPG) liposomes, used to mimic bacterial membranes, change to a helical structure (Lee et al., 2012). Figure 6.12

confirmed that a similar conformational change in secondary structure also occurred in the AvBD1 SNP variants in the presence of SDS, an amphiphilic detergent, mimicking bacterial membranes.

Data from the three anti-microbial assays employed (radial diffusion, colony counting and microbroth dilution), revealed that all three AvBD1 variants displayed anti-microbial activity against all the chicken gut bacterial isolates tested: *E. coli*, *E. faecalis*, *L. johnsonii*, *B. viscericola* and *B. dorei*. Moreover, the majority of data indicated that 'NYH' was the most potent AvBD1 variant. In addition, a number of the experiments revealed that the 'SSY' form was more potent than the 'NYY' form. In summary the overall trend for AMA was 'NYH' > 'SSY' > 'NYY'. It can be assumed that the potency is linked to interaction of peptide with bacterial membrane, but future CD analyses are required to verify this.

The linear AvBD1 SNP variants were synthesized to compare the properties of the AvBD1 peptides produced by the three Aviagen commercial breeding lines, that differ in their susceptibility to enteric problems: X ('NYH'), Y ('SSY') and Z ('NYY'). The gut health assessments detailed in Chapter 3 and 4 revealed that Line Y has more optimal gut health compared to Line X, with Line Z showing the most robust gut health of the three lines (Aviagen Ltd., personal communication). The AMA data presented in this chapter therefore indicated a trend for the less potent AvBD1 peptides to be associated with better gut health. The genetic and physiological determinants of bird gut performance are likely to be complex and are, therefore, unlikely to be driven by the activity of a single gut peptide. Nevertheless, Chapter 4 indicated that AvBD1 gene expression is at its highest immediately post hatch and it is possible that the increased potency of the NYH peptide may actually be disruptive in the early formation of the gut microbiota. Indeed, the NYH peptide was the most potent against LJ (Figure 6.9), a known gut probiotic organism. Thereby, it could be speculated that Line X birds may actually inhibit early *Lactobacillus* spp. colonisation, and, in doing so, expose the gut to other species that cause gut damage. Some support for this theory was shown by the Farm Trial 1 data which showed that the relative abundance of *Lactobacillus* spp. in the microbiome of Line X birds was only 10% compared to 40% in Line Y (Chapter 3; Figure 3.3).

The properties which govern differences in anti-microbial potency between the AvBDs have yet to be fully elucidated. Interestingly, however, data from the AvBD1 studies

indicated that the single substitution of H ('NYH': Line X) to Y ('NYY': Line Z) dramatically reduced the anti-microbial effectiveness of the peptide against all bacterial species examined. As part of the initial bioinformatic investigations of the AvBDs, a number of positively selected sites (PSS), amino acid positions which mutate at a higher rate than would be expected under neutral evolution, were identified (Lynn et al., 2004). For AvBD1, positions corresponding to SNP 2 (Y/S) and SNP 3 (Y/H) were identified as PSS (Lynn et al., 2004), and the anti-microbial data from this study demonstrates that these sites are important in determining activity. The functional importance of these putative PSS was further demonstrated by another study, which engineered mutant forms of AvBD8 and assessed their antimicrobial potencies (Higgs et al., 2007). A comparison of two AvBD8 variants showed that substituting valine for arginine at a PSS in the C-terminal of AvBD8 conferred a specific and potent activity against *E. coli* but not other bacteria, whereas an isoleucine to arginine substitution at a non-PSS position in the N-terminal did not enhance activity against *E. coli* (Higgs et al., 2007). Both these AvBD8 variants had the same charge (+2.7), as did the AvBD1 variants tested for this study (+7.7 to +7.8), highlighting that the specific amino acid, which is present at a PSS and not necessarily the cationicity of the peptide, is important. Further support for the evolution of anti-microbial specificity comes from a study which identified a SNP (arginine/isoleucine), in the great-tit gene encoding AvBD7 (Hellgren et al., 2010). In Hellgren's study, the two SNP variants were both potent against *E. coli*, but only the isoleucine allelic form strongly inhibited *S. aureus* despite this peptide having lower charge (+3.7 versus +4.7) (Hellgren et al., 2010). In contrast the anti-microbial data detailed in this chapter indicated that changes in the AvBD1 SNPs do not direct killing activities against specific species, but instead alter the activity of the encoded peptides against a broad spectrum of bacteria.

Two studies utilising GST-tagged AvBD peptides, have demonstrated the anti-microbial activity of chicken AvBD10, goose AvBD10 (82% amino acid homology) and quail AvBD10 (84% amino acid homology) (Wang et al., 2010,;Ma et al., 2012b). The anti-microbial assay data for chicken AvBD10 from this study revealed bacterial inhibition against *E. coli*, *E. faecalis*, *B. viscericola* and *L. johnsonii,* although not complete bacterial killing; and no activity was detected against *B. dorei* using a radial diffusion assay. Interestingly, cecropin the positive control peptide also failed to produce a clearing zone, hinting that that *Bacteroides* spp. isolates may be more resistant to anti-microbial peptides than the other bacteria tested, and possibly linked to *Bacteroides* spp. functioning

as a gut commensals. When AvBD10 was compared to AvBD1 'NYH' using an *E. coli* colony counting assay under low nutrient conditions (PBS), it was apparent that AvBD1 had far higher anti-microbial activity than AvBD10 (Figure 6.18). Similarly under high nutrient conditions (10% LB media), AvBD10 was able to inhibit *E. coli* growth but even at the highest concentration utilised (10μM), AvBD10 could not induce the complete inhibition that was observed for all three AvBD1 variants (Figure 6.19). This suggests that the anti-bacterial mechanisms of AvBD1 and 10 are different with AvBD1 more appropriately classed as bactericidal and AvBD10 as bacteriostatic. Examination of the AvBD primary sequences reveals that AvBD10 alongside AvBD8, 12, and 14, do not contain a C- terminal tryptophan. It has been reported the tryptophan residues are important in membrane disruption due to their hydrophobic nature and human β-defensin-3 studies have shown that replacing tryptophan residues can decrease the anti-microbial activity of the peptide (Kluver et al., 2005). Similar observations have been reported in relation to the antibiotic agent L-K6 (Bi et al., 2013). Thus, it is possible that AvBD1, in part due to its C terminal tryptophan, may be more able to disrupt the bacterial membrane than AvBD10.

It is proposed that positive residues on AMPs are attracted to, and bind, negatively charged phospholipids present in bacterial cell walls and this initial binding is a crucial step prior to membrane disruption (Zasloff, 2002). Therefore, there is some support for the hypothesis that an increase in cationic charge increases antimicrobial potency (Bessalle et al., 1992; Matsuzaki et al., 1997; Higgs et al., 2007). AvBD8 shares similarities with AvBD10 in that it is expressed at high levels in liver and is a weakly charged peptide (Higgs et al., 2005). However, modified versions of the AvBD8 peptide in which two native amino acids (isoleucine and valine) are substituted for arginines not only confers an increased charge (+2.7 vs. +0.7), but is associated with improved bacterial killing relative to the original mature peptide (Higgs et al., 2007). These data support a role for cationicity as an important determinant of anti-microbial activity and, considering the charge discrepancies as outlined in Table 6.1, may be one of the key reasons for the AMA differences observed between AvBD1 'NYH' and 10 (Figure 6.19). However, not all studies support this theory. For example, in geese, recombinant AvBD10 was shown to have higher bactericidal activity than both AvBD2 and AvBD5, which is perhaps surprising as, like in chicken, AvBD10 is a relatively uncharged peptide (Ma et al., 2012b).

The large range of cationic charges found in the 14 AvBDs (Table 1.1) coupled to the differences in the location of expression (Table 1.3, pg 31) may help group the AvBDs into those which are more likely to have a primary role against bacterial invasion and those which primarily exert novel non-killing immune functions. In regards to sites of action outside of the gut, previous studies have indicated that AvBD1 does not appear to play a role in kidney and liver function (Table 1.3). Although this thesis did find expression levels in the kidney and liver that were similar to the gut tissues at Day 0, expression decreased significantly by Day 7 (Figure 4.7). Instead, AvBD1 expression was maintained in the caecal tonsil after 7 days (Figure 4.7), suggesting a role for modulating the innate immune response in the gut. This was supported by data from the current chapter which revealed that AvBD1 has potent activity against bacterial genera that are normally resident in the small intestine, namely *Lactobacillus* spp., and the large intestine, namely *Bacteroides* spp., whereas AvBD10 was only weakly bacteriocidal against these commensals. It is reasonable, therefore, to suggest that AvBD1 may be more important than AvBD10 in shaping the composition of the gut microbiota from hatch and protecting against harmful exogenous pathogens. In contrast to AvBD1, at hatch AvBD10 was expressed at far higher levels in the kidney and liver than in the gut and, unlike AvBD1, remained high throughout the sampling period (Figure 4.19). Due to this unusual expression pattern in tissues not usually in contact with bacteria, AvBD10 was assessed for cell proliferation and wound healing function. Although these results were inconclusive, further exploration of wound healing ability is warranted, particularly as studies have also shown that AvBD10 but not AvBD1 is expressed in chicken skin (Table 1.3). Although this thesis has been primarily concerned with gut health, skin-associated problems such as hock burn and pododermatitis are damaging to bird welfare (Buijs et al., 2009; Estevez, 2007) and as such, skin-expressed AvBDs such as 3, 9, 10 and 11 (Table 1.3) may be potential targets to select for birds that are robust to skin problems.

The majority of investigations into the novel properties of host defense peptides have focussed on the human beta-defensins and the cathelicidin hCAP-18/LL-37. Human beta-defensins 1 – 4 have been shown, to varying degrees, to act as chemoattractants (Yang et al., 1999; Wu et al., 2003), up-regulators (Jin et al., 2010) and suppressors of pro-inflammatory cytokines (Semple et al., 2010), and inducers of cell migration, and wound healing (Otte et al., 2008; Vongsa et al., 2009). Few studies have been undertaken in relation to the AvBDs though one study has shown that chemotaxis of B and T cell splenocytes is stimulated by duck AvBD2 (85% sequence homology to chicken) (Soman

et al., 2009a; Soman et al., 2009b). In this study cell proliferation was examined using Cell-Titre Blue assays but these failed to show any effects of either AvBD1 or 10 (Figure 6.20). The positive control for this assay was human fibroblast growth factor (FGF) which shares 84% homology with chicken FGF. However, no significant effects of the positive control were observed perhaps suggesting human FGF is not specific to the CHCC-OU2 cells. Cell proliferation, measured by BrDU incorporation, could not be demonstrated in human intestinal epithelial cells incubated with hBD2 (Otte et al., 2008), but the authors did show that hBD2 treatment enhanced HT-29 cell migration in an *in vitro* wound healing model. Figure 6.21 demonstrated that incubation of CHCC-OU2 cells with AvBD10 at 0.1nM resulted in higher mean wound healing than control wells at all imaging time-points, but this did not reach statistical significance. Nonetheless, the results shown in Figure 6.20 were tantalizing and these data probably justify further experimentation. The hBD2 study utilized concentrations of 1 and 5µg/ml, which correspond to 200nM and 1µM, so it is possible that the AvBD10 concentrations used, at 0.1 – 1nM, were too dilute to support an effect. Yet as the data actually indicated that the lower concentrations of AvBD10 were inducing wound healing, it would probably be worthwhile to also examine wound healing at concentrations from 0.05nM to 1µM. Gut epithelial wound healing is likely to be an important facet in prevalent GI diseases such as necrotic enteritis and coccidiossis due their associated lesions. Therefore, it is possible that at damaged epithelial surfaces, AvBDs may serve dual functions, combining anti-microbial activity and supporting wound healing. It has been demonstrated for hBD3 that an intact defensin secondary structure is important for its chemotaxis, compared to its antimicrobial, properties (Wu et al., 2003) and therefore, it is possible that a folded structure is important for wound healing. The recombinant AvBD10 produced in this study has not been confirmed as containing the cysteine-cysteine bonding typical of β-defensins, which may also be required to observe an optimal effect.

The data outlined in this chapter has shown that natural allelic variation within AvBD1 can have a profound effect on the antimicrobial function of the encoded peptides, at least *in vitro*. Although the potential *in vivo* effects of such peptides are difficult to quantify the variations are associated with genetic lines of birds that differ in their gut microbial colonization patterns and susceptibility to enteric disease, suggesting that the peptides may play a role in the establishment of the gut microbiota. In addition to differential tissue expression (Chapters 4 and 5), AvBD1 and 10, also exhibit different AMAs against gut bacterial isolates, with AvBD1 associated with bacterial killing compared to the

bacteriostatic properties of AvBD10. The charge and primary amino acid sequence of AvBD10, particularly the lack of a C-terminal tryptophan, suggested other physiological functions for AvBD10, including wound healing, however these could not be confirmed in this study.

7 Final Discussion

Bird health and hence welfare is a major focus for those involved in intensive poultry production and a particular concern is the increased incidence and impact of pathogenic diseases that affect the bird gastrointestinal tract. These diseases include coccidiosisis (Chapman, 2014), and necrotic enteritis (Timbermont et al., 2011), as well as the less well-defined syndromes characterised by non-specific enteritis such as dysbacteriosis (Teirlynck et al., 2011) and malabsorption syndrome (Zekarias et al., 2005). Since prophylactic antibiotics in feed are now outlawed in the E.U., effective strategies to halt and/or reverse the prevalence of such syndromes are still lacking (Dibner and Richards, 2005). Moreover the development of such strategies actually requires further knowledge and understanding of the bird gut defences. To help address this, the studies described in this thesis aimed to explore potential relationships between a specific bacterial challenge, the gut innate AvBD response and gut health in two commercial poultry Lines, X and Y.

The deterioration in gut health described in the trials reported in Chapters 3 and 4 was facilitated by exposing birds to two bacterial species, namely *Bacteroides dorei* (BD) and *Barnesiella viscericola* (BV), isolated from birds suffering 'dysbacteriosis'. For both Trials 1 and 2 the patterns of gut health deterioration were consistent with the highest gut health scores i.e. the worst gut health, characterized by increased watery gut contents and gut thinning (poor tone), observed in older birds. These clinical symptoms were typical of the syndrome 'dysbacteriosis' (Teirlynck et al., 2011), which is associated with shifts in the microbiome and small intestinal microbial overgrowth (Bailey, 2010). In support, jejunal gut scrape data from both farm trials illustrated a trend for gut scrapes from birds with abnormal gut health to support higher *in vitro* bacterial growth. These data provided further evidence that the poor gut health was a 'dysbacteriosis-like' condition characterised by intestinal microbial over-growth.

Trial 1 attempted to explore if actual shifts in the gut microbiota were associated with the 'dysbacteriosis' phenotype. To achieve this Line X and Y birds were challenged with either B/BV, *Lactobacillus johnsonii* (LJ) or a mix (B/BV + LJ), and gut health assessments performed. As discussed, the B/BV challenged birds had worse gut health, while the LJ challenged birds exhibited better gut health, than the water control birds and this was consistent for both lines, X and Y. *Lactobacillus* spp., such as LJ, function as probiotics and have been investigated *in vivo* for their potential immunomodulatory

properties in preventing enteric disease (Nava et al., 2005). Samples from the bird trials were not assessed for inflammatory markers but the *in vitro* challenge model, using CHCC-OU2 cells, demonstrated that *Bacteroides dorei* induced significantly higher pro-inflammatory cytokine expression than LJ. If these findings were replicated *in vivo* in the newly-hatched chicks, then it could be argued that the B/BV challenged birds suffered more inflammation induced damaged or an up-surge in inflammation that was not properly resolved, resulting in a persistent inflammatory state. Yet despite the challenges clearly affecting gut health directly, this was not reflected by the 'global' caecal and ileal microbiome data as no consistent shifts in measurable microbial species were revealed. However, the studies did reveal that the age of the bird at which *Bacteroides* spp. predominate in the caeca may be an important factor in gut health; indeed, high abundances of *Bacteroides* spp. were associated with better gut health in older birds (3 - 4 week old) and worse gut health in young birds (4-days old). These data suggest that *Bacteroides* spp. can exert both beneficial and damaging effects that appear dependent on the maturity of the gut. Traditionally, B and BV spp. are considered as gut commensals, but observations made during the studies reported in this thesis, indicate that in young birds they can function as opportunistic pathogens. Hence it can be argued that the hierarchy of gut colonisation is important and exposure of very young birds to high numbers of a particular bacterial species including B & BV, through environmental exposure including litter and/or drinking water, may impact negatively on the epithelial defences and hence the physiology of the bird gut. Aside from the microbiome data, it was interesting to note that there were clear differences in galactose content in the gut scrapes of the X and Y bird lines, with galactose associated particularly with the Line X mucosa of young birds (4 day-old). The origin of the galactose was presumably the grain based diet, fed to both lines, and although no further investigative analyses were performed to help explain these observations, future studies could explore the gut sugar content in the digesta in relation to gut epithelial transport mechanisms, the gut microbiota and gut health.

Line X birds are an important part of the Aviagen Ltd. breeding programme but compared to some of the other commercial breeding lines there was a perceived issue of sub-optimal gut health (Aviagen Ltd.). In Trial 1, the use of a simple gut health scoring system, developed by Aviagen Ltd. enabled the perceived differences in gut health between Lines X and Y, to be quantitated, and the data confirmed that Line X birds had an increased susceptibility to gut health deterioration, particularly following challenge with B/BV.

Therefore, Trial 1 indicated the importance of bird genetics in the immune response to environmental, and particularly, a B/BV weighted challenge. Line X birds can be considered a 'dybacteriosis'-susceptible line whereas Line Y is not. In a mouse model of inflammatory bowel disease it was shown that gavage with *Bacteroides* spp. induced significant pathology in genetically-susceptible mice, but had no effect in non-susceptible mice (Bloom et al., 2011). Interestingly, challenge with *Enterobacteriaceae,* which are usually enriched in IBD, produced no pathology in either of the mice genotypes despite colonising the hind gut. Therefore, Bloom et al., (2011) have shown that the host-response to specific species such as Bacteroides is governed by genotype, which is critical in inducing the diseased state, rather than an altered susceptibility to colonisation of IBD enriched-species. Similarly, the microbiome data presented in Chapter 3 did not indicate large shifts in the microbiome between Line X and Y and *Bacteroides* spp. colonisation was found at some stage following challenge in both lines. Perhaps then, *Bacteroides* spp. is neither intrinsically 'bad' or 'good' and induces a different response dependent on whether the gut colonisation occurs in Line X and Y birds. Interestingly, the IBD-susceptible mice utilised in the study by Bloom et al. (2011), had defects in TGF-β and IL-10 signalling and further studies are required to ascertain if such disparities in expression and signalling pathways are also found in Line X and Y birds.

To further explore the innate immune response in the Line X birds, Trial 2 was performed (Chapter 4) to evaluate gut AvBD gene expression, a reflection of the innate defences, in these birds following BD/BV challenge. While admittedly the gut AvBD expression values within sampled groups were variable, bird age was shown to be important in AvBD expression with high constitutive levels detected immediately following hatch, supporting the importance of the encoded peptides in the innate protection of the gut. In the 7 day-old birds however, AvBD1 expression was consistently lower in the B/BV challenged group than the control, indicating that B/BV species caused AvBD down-regulation, and hence exposed the gut to microbial associated damage. These *in vivo* findings were supported by the *in vitro* data that supported down-regulation of AvBD1 expression in response to a B/BV challenge even at a low multiplicity of infection (MOI < 1). To ascertain if this AvBD suppression was associated with gut health status, further analyses were performed. Birds with inflamed guts had, on average, lower AvBD1 and 10 expression than healthy birds and, crucially, ranking the challenged birds for expression in each GI tissue revealed that birds that were able to maintain relatively high AvBD1 expression, despite B/BV challenge, exhibited better gut health than birds with low

expression. In contrast to AvBD1, no conclusive link between AvBD10 and gut health could be confirmed. None-the-less *in vivo* data from this study showed that AvBD10 is present throughout the GI tract of Line X birds and is also subject to regulation via bacterial challenge, and therefore, presumably plays a role in the gut defences.

It is acknowledged that this project focussed on AvBD1 and 10 expression, and analyses of other defensins needs to be performed to provide information on whether all or just a selection of the responses can be linked to bird gut health. A potential aim is to use AvBD expression as a biomarker to predict bird gut health. However, an obvious limitation of using AvBD tissue expression as a 'predictive gut health biomarker' is that the analyses requires tissue and hence bird sacrifice, which means individual birds, particularly those with extremely high constitutive expression at hatch, cannot be followed longitudinally i.e. throughout their life-span. However, AvBD expression in peripheral blood leukocytes (PBLs) has been shown to be responsive to oral challenges with *S. typhimurium* and *C. jejuni* (Meade et al., 2009b). If, therefore, AvBD expression in PBLs from individual birds following B/BV challenge links to gut health, this would allow individual birds to be followed from hatch to slaughter. Such studies are yet to be performed.

Overall these data strongly suggested that in birds with relatively high AvBD expression, the innate defences were functioning and were able to respond and control the microbial challenge, which helped to protect the GI tract from microbial assault. The link between innate immune gene expression and resistance to gut pathogens has already been demonstrated by recognition of Salmonella-resistant bird lines that exhibit increased AvBD (Derache et al., 2009a) and cytokine/chemokine levels (Swaggerty et al., 2009). Moreover, this knowledge has been utilised in a small-scale breeding trial that produced progeny with enhanced expression of pro-inflammatory mediators (IL-6, CXCLi2, and CCLi2), that have improved resistance to *Salmonella* spp. colonisation (Swaggerty et al., 2014). It should be possible, therefore, to identify individual birds with higher than average constitutive AvBD expression, and through knowledge of parental breeding stocks begin to genetically selected for birds with a 'dysbacteriosis'-resistant phenotype. However, as an alternative to genetic selection, improving the GI health status of broiler flocks may be possible by boosting endogenous AvBD expression through, for example, using butyrate-supplemented feed (Sunkara et al., 2011) or by adding AMPs directly to feed (van Dijk et al., 2011). Realistically, the large-scale routine use of chicken AMPs

such as the AvBDs and cathelicidins is likely to be limited by production costs, although pilot scale bacterial fermentation methods are being developed (Bommarius et al., 2010).

Chapter 6 outlined the small scale production of recombinant AvBD10 which revealed that AvBD10 had antimicrobial activity against an array of chicken gut isolates, except interestingly *Bacteroides* spp., which again may help explain the success of Bacteroides as an opportunistic pathogen in young birds. Previous work had identified the presence of three coding SNPs in the AvBD1 gene and based on the prevalence of these alleles in Line X, Y and a further line with robust gut health, Z, three variants of AvBD1 peptide were synthesised. The 'NYH' (Line X) peptide was, consistently, the most potent form against all the gut bacterial isolates tested *in vitro*. Although *in vivo* the effects of the expressed 'NYH' peptide are unknown, one could postulate that its increased AMA could in fact impact on and alter the gut microbiota. Interestingly it has been shown that the introduction of human alpha-defensin HD5 into the mouse gut alters the microbiota resulting in losses of Firmicutes such as Clostridia, Bacilli and segmented filamentous bacteria, and increases in Bacteroidetes (Salzman et al., 2010). In addition to anti-microbial properties, AvBD10, due to its elevated gene expression in kidney and liver tissues, was investigated for novel physiological functions, not associated with bacterial-host interactions. Preliminary experimental data hinted at AvBD10 involvement in wound healing, as has been shown for human β-defensin 2 (hBD2) (Otte et al., 2008) and hBD3 (Kiatsurayanon et al., 2014), but confirmation necessitates further experiments utilising higher concentrations of defensin peptide.

As set out in the aims of this thesis, the data has demonstrated that bacterial challenges can be both beneficial (LJ) and detrimental to gut health (B/BV). Furthermore, gut health outcome following bacterial exposure has been shown to be strongly influenced by host genetics particularly in the response to the initial B/BV challenge and possibly the subsequent immune-tolerance of such species during gut colonisation. Within the 'dysbacteriosis'-susceptible line (Line X), high AvBD expression was shown to be associated with better gut health supporting the inclusion of the innate immune genes as part of the selective breeding process. Future work will evaluate the expression of the remaining AvBDs throughout the GI tract and confirm the association with gut health. The opportunity of identifying birds with uniquely high GI AvBD expression is of commercial interest to poultry breeders such as Aviagen Ltd. as such birds may have huge potential as candidates to select for a healthy gut phenotype.

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Appendix A

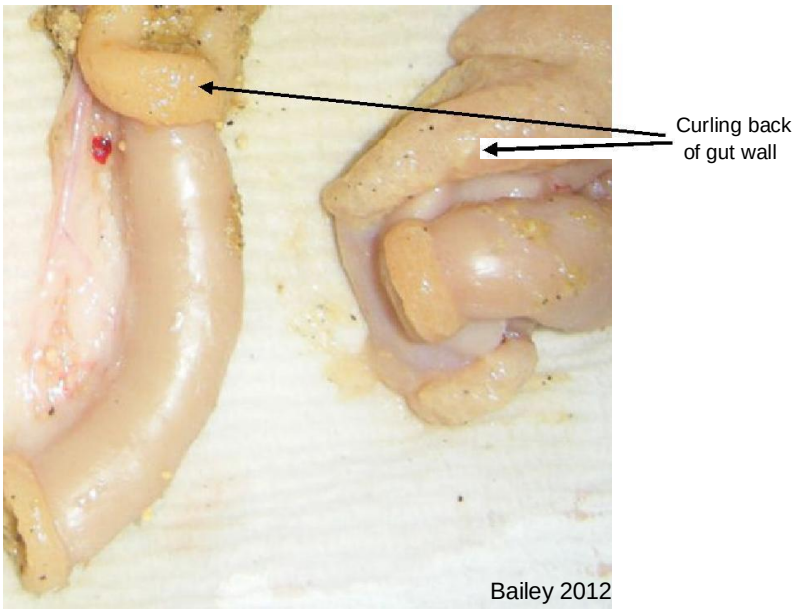
Gut Health Assessment Guide

The aim of this guide is to provide a quick and simple method of assessing gut health in the field without the need for any in depth analyses. The basic principle is to assess the tone of the gut wall, the colour of the gut surface and the consistency of the contents. By characterising these aspects of the gut at different ages of bird one can judge how well the gut is developing and whether it is under any challenge. The scoring system is based on a scale of 0, 1 and 2 where '0' is normal, '1' is mildly abnormal and '2' is severely abnormal.

Gut Tone

In normal circumstances when the gut wall is cut into the tissues will fold back immediately onto themselves to form rolls.

Score 0
On cutting into the gut the walls immediately curl back

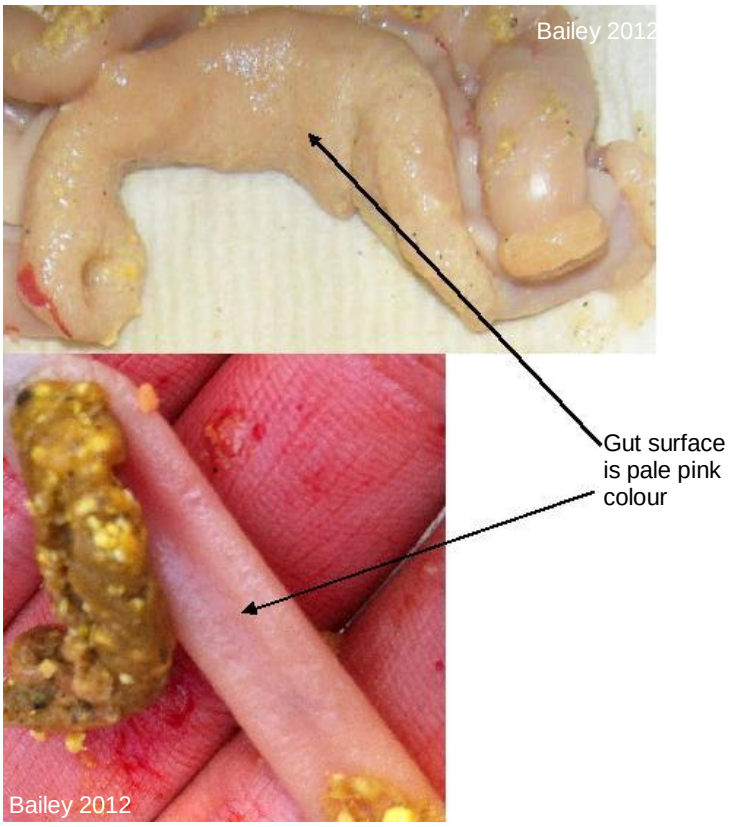


Score 1
The gut curls back on itself as above but it does not occur immediately and there is a delay (more than 2 seconds) in the wall moving.

Score 2
The gut wall fails to curl back on itself

<u>Colour of the gut surface (Redness)</u>

Score 0
In normal circumstances the gut wall should be a pale pink colour.



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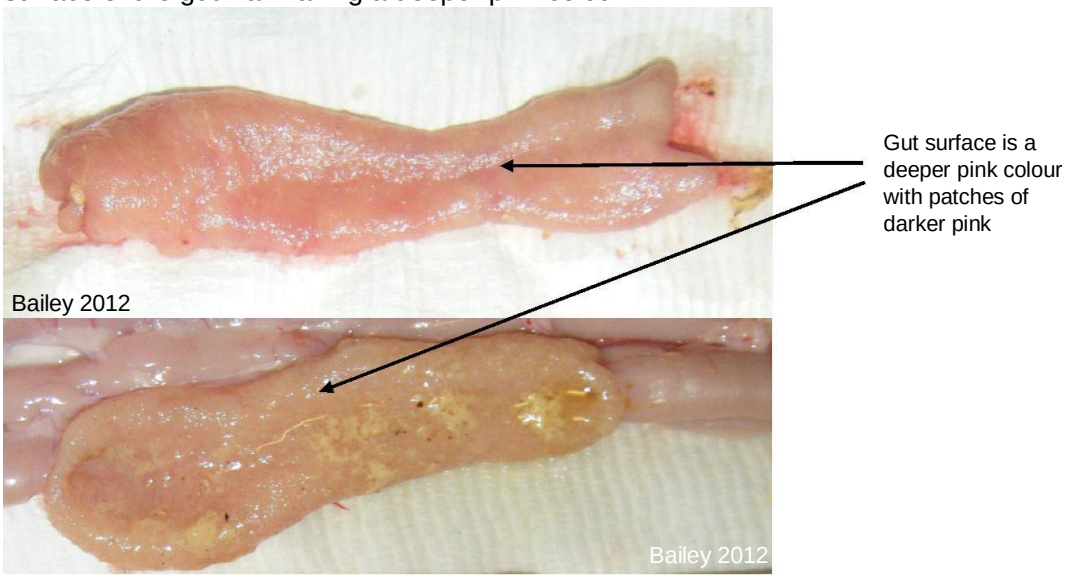
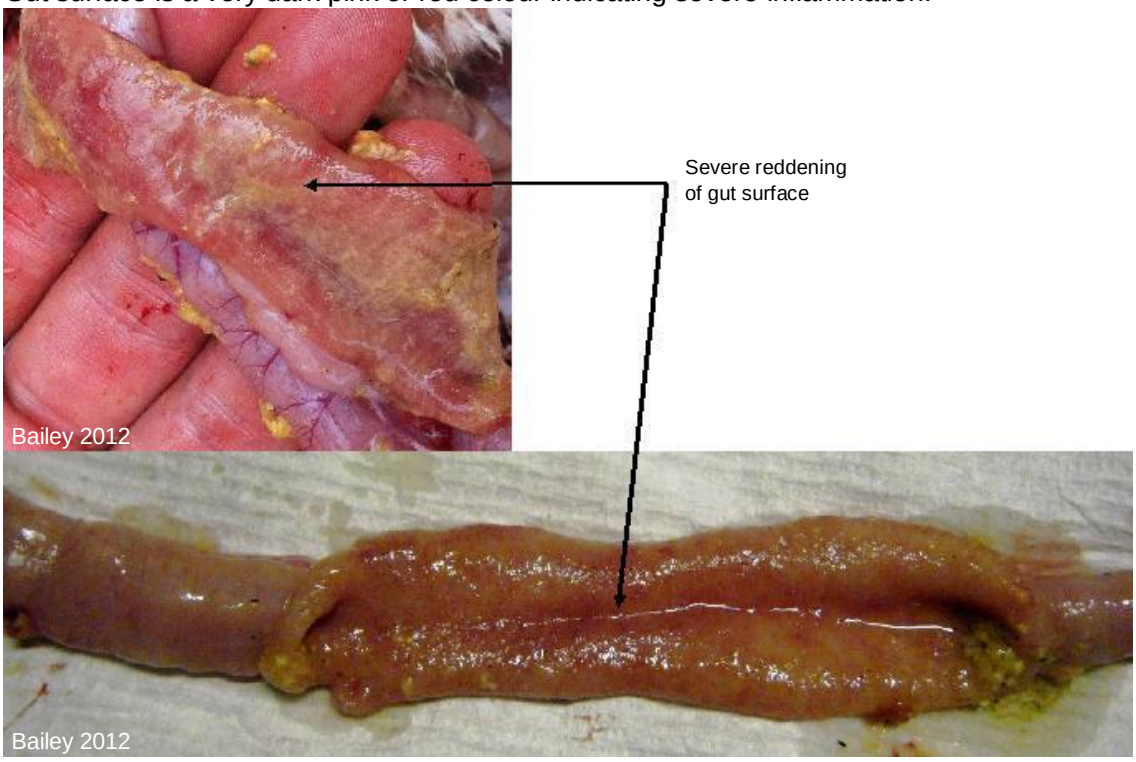
Score 1

This indicates a mild irritation/inflammation in the gut. This is characterised by the surface of the gut wall having a deeper pink colour.



Score 2

Gut surface is a very dark pink or red colour indicating severe inflammation.



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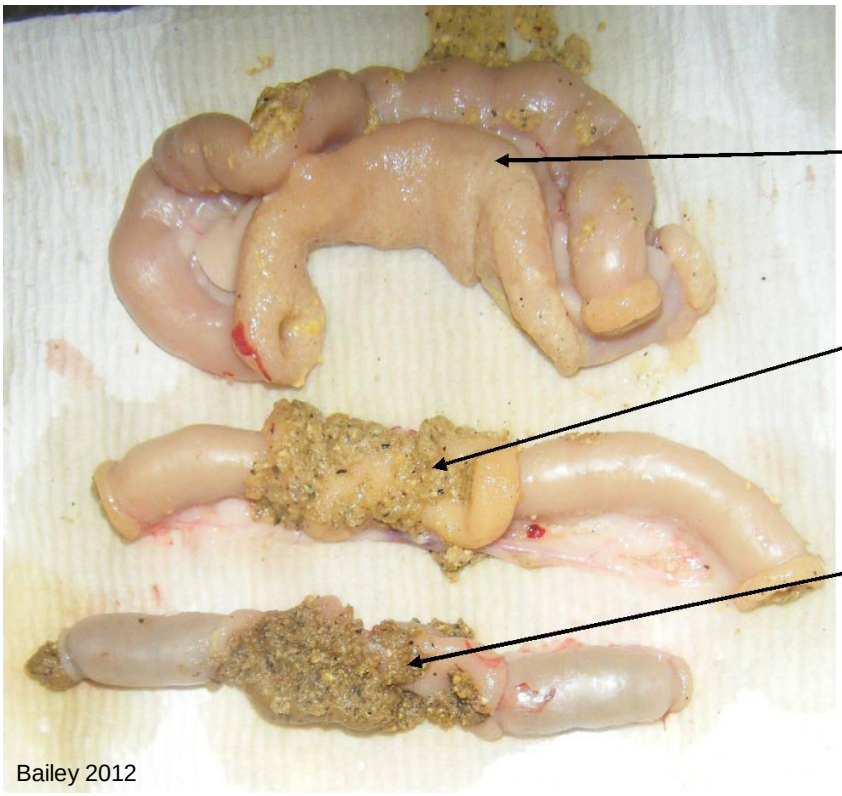
Consistency of contents (Watery contents)

Score 0

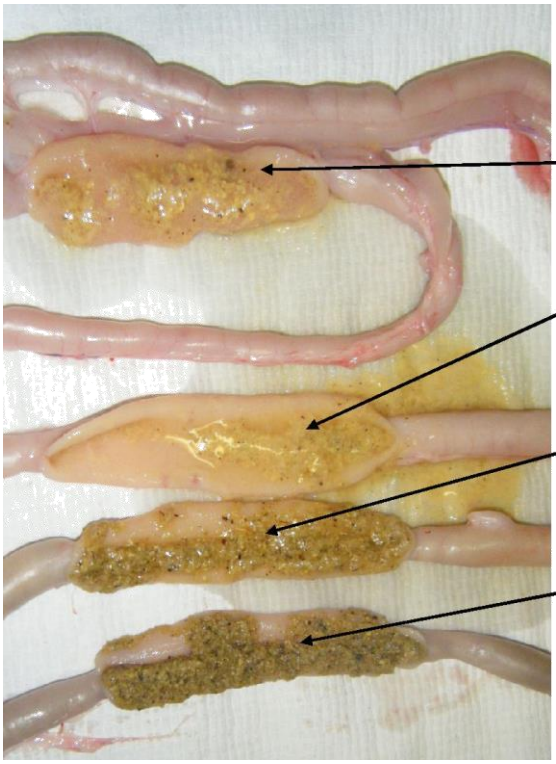
In normal circumstances as the digesta passes through the gut water is absorbed resulting in a faecal pellet. Thus in each region of gut the consistency should be different with the duodenum being the wettest and the ileum being the driest – the key point here is that the contents at each region should be homogenous in consistency.



On cutting into the duodenum it is normal for the contents to spill out however in the jejunum and ileum the contents should generally hold together when you cut into the gut.

Score 1

When cutting into the gut if the contents that are present are not uniform in consistency, i.e. a separation of water and solids, then it implies that the contents are abnormal.



Score 2

This is when on cutting into the small intestine there is predominately fluid and very little solid present which leaks out straight away. In the lower ileum the contents are generally very soft with little bolus formation.

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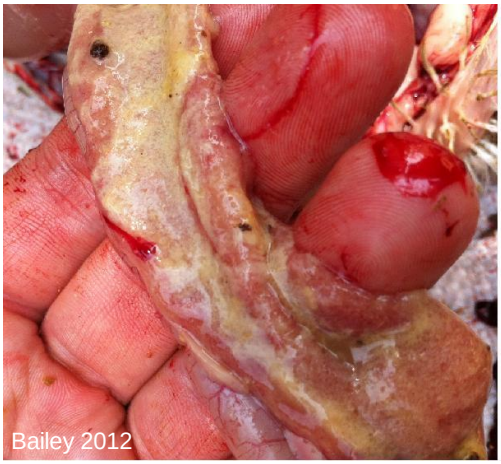
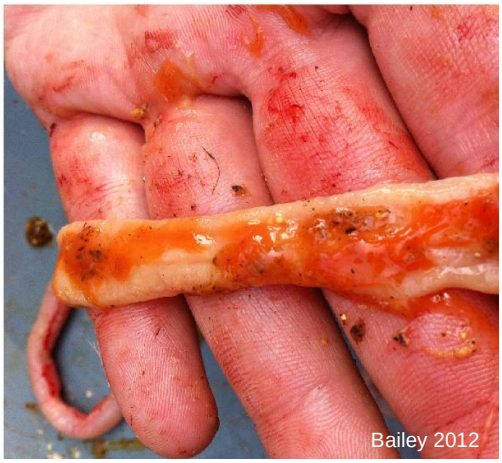
Mucus production

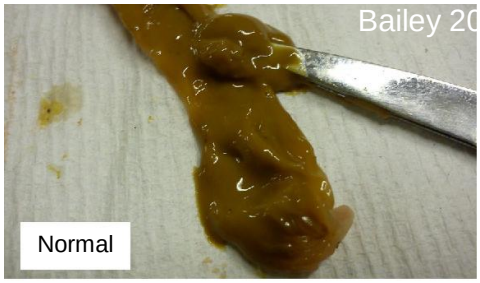
Mucus production in the gut is a normal phenomenon as it aids the passage of food through the intestine and forms a protective barrier on the mucosal surface. However in cases of infection and irritation excess mucus can be produced. As a rule of thumb if you can easily see mucus on the gut surface then it is excessive production.

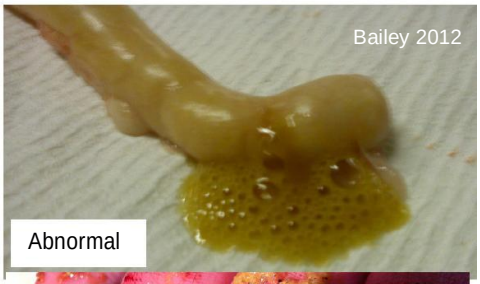




Caecal Health

The caeca should contain a dark viscous material with no gas bubbles. Normal caecal contents will generally slowly ooze out of a cut in the caecal wall. During an enteric upset it is common for the caecal contents to become watery, light in colour and contain gas bubbles. During a caecal upset when the caecal wall is cut the contents will empty very quickly





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Other gut lesions/issues

Gizzard erosion

The inner surface of the gizzard should be continuous with no breaks. In case of gizzard erosion clear breaks can be seen:

Mark on the form a Yes or No if they are present

Coccidiosis

Lesions can be seen in the small intestine or the caeca. Make a note if any lesions seen and where.

Feed passage

The ingesta in the ileum and colon should not contain any whole feed. If present record on form.

Appendix B

Vaccination protocols for chicks at the Aviagen, sib-testing farm, Scotland, U.K.

Name of vaccine	**Age administered (days)**	**Disease**	**Manufacturer**
Paracox 5	0	coccidiosis	Intervet,Schering Plough Animal Health Corporation
Bursine 2	18/19	Infectious Bursal Disease	Fort Dodge
MA5	22	Infectious bronchitis	Intervet, Schering Plough Animal Health Corporation
HB1	22	Newcastle Disease	Lohmann Animal Health