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Control of intestinal spirochaete infections in chickens

A report for the Rural Industries Research and Development Corporation

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Foreword

Avian intestinal spirochaetosis (AIS) is a condition of layers and broiler breeders resulting from infection of the large intestine with anaerobic intestinal spirochaetes. Colonisation can lead to wet litter and reduced egg production. The main causative species in Australia, *Brachyspira intermedia* and *Brachyspira pilosicoli*, are widespread, and present a constraint to efficient production. Currently little is known about how these organisms enter flocks and spread, nor about how to control them.

The purpose of this project was to develop improved means to control AIS. Studies were therefore undertaken to elucidate the on-farm epidemiology of these infections, and to assess the likely impact of farm management practices and biosecurity measures on the cycle of infection.

Experiments were also conducted in experimentally infected laying hens to investigate potential interactions between diet and colonisation with the two main spirochaete species in order to identify specific dietary risk factors for infection. In particular, possible interactions between the total and soluble non-starch polysaccharide (NSP) content of the diet, specific cereal grain effects, feed enzyme supplementation of the diet, digesta viscosity and colonisation with the two key spirochaete species were investigated.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to RIRDC's diverse range of over 1000 research publications, and forms part of our Chicken Meat Program. The Chicken Meat Program aims to support increased sustainability and profitability in the chicken meat industry by focussing on research and development on those areas which will enable the industry to become more efficient and globally competitive and which will assist in the development of good industry and product images. Funding was also provided by the RIRDC Egg Program, now the Australian Egg Corporation Limited.

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Simon Hearn
Managing Director
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Abbreviations

AIS	avian intestinal spirochaetosis
AME	apparent metabolisable energy
bp	base pair
MLEE	multilocus enzyme electrophoresis
mPa•s	miliPascals
NSP	non-starch polysaccharide
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
sNSP	soluble non-starch polysaccharide
iNSP	insoluble non-starch polysaccharide
tNSP	total non-starch polysaccharide

Contents

List of Tables

List of Figures

Executive Summary

Background

In recent years, intestinal spirochaetal bacteria of the genus *Brachyspira* (formerly *Serpulina*) have become recognised as being common inhabitants of the large intestines of commercial layer and broiler breeder chickens. Several distinct species of these bacteria infect poultry, some of which are pathogens. The organisms have fastidious growth requirements and need anaerobic conditions for their laboratory isolation, hence diagnosis of the infections is difficult. Despite an increased awareness of these bacteria, relatively few studies have been carried out on them. Work done in Europe and the United States has shown that infection with intestinal spirochaetes (avian intestinal spirochaetosis: AIS) is associated with wet droppings, delayed onset of egg laying, faecal staining of eggshells, reduced egg weights and reduced carotenoid content of eggs. Broiler chicks hatched from infected hens show reduced weight gain.

In Australia, studies of intestinal spirochaetal infections in commercial poultry flocks have showed that colonisation with the pathogenic species *Brachyspira intermedia* and *Brachyspira pilosicoli* is common. Moreover, these spirochaetes are recovered significantly more frequently from layer and broiler breeder flocks with diarrhoea and reduced egg production than from clinically normal flocks. Experimental infection studies in broilers and layer hens has shown that strains of both *B. intermedia* and *B. pilosicoli* isolated from Australian chickens can cause disease and loss of production.

The purpose of the current project was to seek means to improve control of infections with the two main species associated with disease in Australia. The study was a collaborative effort involving Professor David Hampson, Nyree Phillips, Tom La and John Pluske at Murdoch University in Western Australia, and Carol Stephens at the Toowoomba Veterinary Laboratory in Queensland.

The project had two main components. First, on-farm studies were conducted to investigate determinants of the infections, including identifying patterns of infection with different strains and species of the bacteria, looking for potential reservoirs of the bacteria and examining their survival in the environment of the production house. This new information should help when trying to devise means to break the cycle of infection on individual infected farms. Second, a serious of experimental studies were conducted in layer hens fed different diets and infected with the spirochaetes. This was done in an attempt to identify potential dietary influences on the infection. The ability to reduce or modify the infections by using specific diets, without having to recourse to antibiotics, would improve the capacity of individual producers to control the infections.

On-farm epidemiological studies

Detailed studies were undertaken on a broiler breeder farm and on a layer farm to investigate possible sources of spirochaete infection. The broiler breeder farm had a past history of a wet litter problem associated with infection with *B. pilosicoli.* Unfortunately, in both cross-sectional and cohort studies conducted on this flock in the current investigation, no spirochaetes were isolated. Furthermore, no spirochaetes were isolated from environmental samples examined in the sheds. The reason for the absence of spirochaetes in a previously-infected farm was unclear, especially as no specific measures had been instigated to control or eradicate the infection. It could be deduced that the infection is likely to cycle between groups of birds on a farm, and does not persist in the environment, nor often become introduced from outside sources such as from wild birds or rodents. These observations are very encouraging in relation to developing means to control AIS on infected farms.

Cross-sectional and cohort studies were conducted on a layer farm with a past history of wet litter associated with AIS. Birds were found to be commonly infected with *B. intermedia*, but there was also evidence of infection with *B. pilosicoli* and another species – which was subsequently identified

as the proposed species "*Brachyspira pulli*". Multiple strains of *B. intermedia* were recognised by using strain-typing techniques, and individual strains were not confined to single sheds of birds. Hence cross-infection between sheds appeared to be occurring. The youngest birds had the least infection, and this supported the idea that infection was initially acquired from older birds housed in other sheds on the farm. It appeared that infection with *B. intermedia* became amplified and more common the longer birds were kept in a shed. Again, no environmental sources of infection were identified, and it appeared that infected birds on the farm were the prime source of infection for new birds introduced to the farm.

Spirochaete survival

This part of the study aimed to evaluate the efficacy of some common disinfectants in inactivating *B. intermedia* and *B. pilosicoli*, and to examine spirochaete survival in chicken caecal faeces held at different temperatures. Six disinfectants were evaluated at their recommended working concentrations: alkaline salts, quaternary ammonium, iodine as an iodophor, chlorine from a chlorine-release agent, glutaraldehyde and hydrogen peroxide. All but alkaline salts inactivated two different concentrations of both spirochaete species in less than one minute in the presence of organic matter. Both spirochaete species at three different cell concentrations survived in caecal faeces at 37^{o}C for between 2 and 17 hours. *B. intermedia* tended to survive for longer than *B. pilosicoli*, but the maximum survival time for both species at 4^{o}C was only 72-84 hours. This part of the study demonstrated that avian intestinal spirochaetes are rapidly inactivated by several common disinfectants, and their survive time in chicken caecal faeces is much less than has been reported for porcine intestinal spirochaetes in porcine faeces. It should be relatively easy to break the cycle of infection between batches of laying birds by resting sheds for a few days, and by using disinfectants on any residual faecal matter. Prevention of subsequent cross-infection from older birds in other sheds by the use of appropriate biosecurity measures would then ensure that the new birds remained free of infection.

Dietary influences on spirochaete colonisation

Previous work in pigs and chickens has suggested that there may be dietary influences on spirochaetal colonisation, and a previous study showed that addition of exogenous dietary enzymes to diets may reduce the susceptibility of birds to infection. It was believed that dietary fibre (non-starch polysaccharide or NSP), particularly soluble NSP, increases the viscosity of the digesta in the intestinal tract, and that this increases colonisation with intestinal spirochaetes.

To explore these effects, four experiments were conducted using layer hens fed on different diets and then experimentally infected with spirochaetes. In the first experiment, birds received diets based on two different wheat varieties that varied in their NSP content. They were then infected with a strain of *B. intermedia*. A significant dietary effect on colonisation was observed. Birds fed wheat variety Westonia were more commonly colonised than those fed variety Stiletto (although this could be a wheat batch effect rather than a variety effect), and the ileal contents of the former birds were significantly more viscous than those of the latter. This suggested a link between increased digesta viscosity and increased colonisation with *B. intermedia*. Unexpectedly, the total sNSP values for the two diets were similar, and there was no obvious explanation for the differences in the viscosity of the ileal contents that were observed.

The second experiment was a repeat of the first, except that a strain of *B. pilosicoli* was used to infect the birds rather than *B. intermedia*. The diets of these birds also were supplemented with 50 ppm zinc bacitracin to enhance colonisation by *B. pilosicoli*. Again there was a significant dietary influence on colonisation, but in this case colonisation was more common in the birds fed wheat variety Stiletto. There were no significant differences in digesta viscosity, and no obvious explanation for the dietary effect on colonisation. The apparent different behaviour of *B. intermedia* and *B. pilosicoli* in the gastrointestinal tract in relation to the diet fed requires further investigation to confirm the effects seen.

The third experiment was designed to determine whether addition of dietary enzyme to the diet based on wheat variety Westonia would influence colonisation by *B. intermedia.* Unfortunately, in this experiment there was a very low colonisation rate in groups of birds either receiving or not receiving the dietary enzyme, and hence it was difficult to determine whether the enzyme preparation was useful in reducing colonisation. Interestingly, the enzyme had little effect on the viscosity of the ileal contents.

In the fourth experiment, three diets based on cereals with different NSP contents were used, each fed with or without the addition of commercial dietary enzymes (ie six groups of birds). The diets were based on wheat, barley or barley plus sorghum. The barley-based diets had around twice the sNSP content of the wheat-based diets, and resulted in the greatest viscosity of the ileal digesta. The barley plus sorghum diet had a little more sNSP than the wheat-based diet. All birds were experimentally infected with *B. intermedia*, and again a significant dietary effect on colonisation occurred. In this case birds fed both the wheat-based diets had more colonisation than birds on the other diets. Addition of enzymes to the mash diets had no significant effects on the viscosity of the ileal contents, or on colonisation rates, and there was no clear association between the total sNSP content of the diet and the ileal viscosity. This experiment suggested that diets based on wheat may predispose to infection with *B. intermedia*, and that the total sNSP content of these diets *per se* may not be responsible for this effect. Consistent with this, dietary enzymes may not have a significant effect on colonisation and cannot be recommended for use as a main means of controlling AIS caused by *B. intermedia*.

Recommendations

- In order to control AIS at the flock level, incoming birds should be from a source that is not infected with intestinal spirochaetes, sheds should be thoroughly cleaned, disinfected and rested between batches of birds, and strict biosecurity measures implemented to prevent transmission of infection from sheds of older birds to new flocks.
- In flocks with persistent problems with AIS consideration should be given to modifying the diets being used. However, the outcome would appear to be somewhat unpredictable. Where possible, cereals other than wheat should be used.
- Addition of dietary enzyme is not recommended as a sole means of controlling AIS.

1. Introduction

1.1 Background

Avian intestinal spirochaetosis (AIS) is a disease or disease complex affecting commercial layer and meat breeder chickens resulting from colonisation of the caecae and colon by one or more species of anaerobic spirochaetal bacteria (Davelaar *et al*., 1986; Swayne, 1997; Stephens and Hampson, 2001). These spirochaetes also can colonise the broiler chicks under experimental conditions, but colonisation and disease do not appear to occur in broiler flocks under field conditions. The causal spirochaetes were formerly placed in the genus *Serpulina*, but have been reassigned to the genus *Brachyspira* (Ochiai *et al.*, 1997). Seven species have been recorded as colonising poultry, but only three are currently considered to be pathogenic in poultry (*B. intermedia, B. pilosicoli* and *B. alvinipulli*) (McLaren *et al.*, 1997; Stanton *et al.*, 1998). AIS is associated with delayed and/or reduced egg production, and chronic diarrhoea in adult birds (Griffiths *et al*., 1987; Dwars *et al.*, 1989, 1990, 1992; Swayne *et al.*, 1992, 1995; Trampel *et al*., 1994). The diarrhoea leads to faecal staining of eggs, and the resultant wet litter presents problems in cage cleaning, odour emission and attraction of flies. Broiler chicks hatched from eggs of infected parents show a reduced performance compared to those of healthy parents (Dwars *et al*., 1993; Smit *et al*., 1998). Intestinal spirochaetes may go unnoticed in commercial poultry, and their significance may not be appreciated. Reasons include the facts that clinical signs may not be obvious, or may be mild and relatively non-specific; the spirochaetes do not always induce characteristic histological lesions; the spirochaetes stain poorly in histological sections; the spirochaetes can only be isolated using specialised media and techniques that are not generally available in diagnostic laboratories. Consequently, relatively few studies have been carried out on these organisms or on the pathogenesis, epidemiology and control of AIS.

Previously this research group has conducted a number of studies on avian intestinal spirochaetes in Australia (with the financial support of the RIRDC). This work has demonstrated that colonisation with intestinal spirochaetes is common (~50% flocks) in meat breeder and layer flocks both in Western Australia (McLaren *et al.*, 1996) and in the eastern States of Australia (Stephens and Hampson, 1999). We have identified several different spirochaete species in Australian poultry, amongst which *Brachyspira intermedia* and *Brachyspira pilosicoli* are the two main pathogenic species present (McLaren *et al.*, 1997; Stephens and Hampson, 1999). Both species are present in about 10% of layer and broiler breeder flocks, with some flocks infected with both pathogenic species (as well as with other species of uncertain pathogenic potential). Under experimental conditions, a strain of *B. intermedia* isolated in WA has been shown to increase faecal water content and reduce egg production in layers (Hampson and McLaren, 1999). Similarly, a chicken strain of *B. pilosicoli* delayed the onset of lay and overall egg production in experimentally infected meat breeders (Stephens and Hampson, 2002).

Despite the accumulated evidence that infections with *B. intermedia* and *B. pilosicoli* are common in Australia, and are likely to be causing significant losses of production in the egg and chicken meat industries, no specific recommendations concerning their control have been made. To assist in this endeavour, molecular diagnostic methods (polymerase chain reaction: PCR) have been developed by this group to aid in the identification of the infections, and a molecular strain typing technique (pulsed field gel electrophoresis – PFGE) has also been developed as a tool for studying the molecular epidemiology of the infections (Atyeo *et al.*, 1996, 1998, 1999; Suriyaarachichi *et al*., 2000). To date these techniques have not been systematically applied to study the epidemiology of AIS at the flock level, including issues such as persistence of the organisms in the environment of poultry houses. It was proposed that the current project examine some of these areas.

In relation to other means for control of AIS, the antimicrobial drug sensitivities of a collection of Australian intestinal spirochaetes from chickens have previously been examined by this group. These were relatively susceptible to a range of drugs used to control porcine intestinal spirochaete

infections (tetracycline; metronidazole; neomycin; ampicillin; lincomycin; tylosin; tiamulin). Treatment of two sheds of broiler breeders naturally infected with intestinal spirochaetes, using either lincomycin or tiamulin, demonstrated that both drugs were capable of temporarily removing the infection (Stephens and Hampson, 1999). Unfortunately, under these field conditions, re-infection occurred after several weeks. In experimental cage trials, treatment of layers infected with *B. intermedia* using tiamulin caused a temporary clearing of the infection. The use of 100 ppm zinc bacitracin in the diet also significantly impeded colonisation with the spirochaete (Hampson *et al.*, 2002). In contrast, using layers experimentally challenged with *B. pilosicoli*, the addition of 50 ppm zinc bacitracin to the diet had the opposite effect, and facilitated colonisation with this species (Jamshidi and Hampson, 2002). While the above drug sensitivities have been examined under experimental conditions, it should be noted that various antibiotic uses in commercial poultry flocks may be restricted under current APVMA regulations, hence the above remedial antibiotic treatments may not be practically available to the poultry producer in many (if not most) cases.

When the above results are taken together, it is evident that infection with intestinal spirochaetes can be manipulated and/or controlled by the use of antimicrobial agents. Unfortunately, this approach is limited by problems of antimicrobial residues in eggs, the lack of appropriate registered products for use in laying hens, and by a general desire to find alternative non-antimicrobial means of controlling enteric infections.

Previously, this group has shown that swine dysentery, an infection of pigs caused by the intestinal spirochaete *Brachyspira hyodysenteriae* can be prevented by dietary means (Siba *et al*., 1996; Pluske *et al*., 1996). Pigs fed diets low in fermentable substrate ('fibre': non-starch polysaccharides [NSPs], particularly soluble NSP [sNSP]), resist experimental colonisation by *B. hyodysenteriae*. It is believed that this is mediated either via changes in the resident microbiota (the activity of which is required to facilitate *B. hyodysenteriae* colonisation), or possibly by changes in the viscosity and/or hydration of the large intestinal contents. Levels of NSP components can be reduced by appropriate choice of dietary ingredients (eg. sorghum contains less NSP than wheat), by physical processing, or by enzymatic treatment. The original protective diet that was designed was based on cooked white rice and animal protein, with lesser levels of protection obtained with steam-flaked maize and sorghum. Since *B. intermedia* and *B. pilosicoli* strains are both genetically closely related to *B. hyodysenteriae*, and share a similar ecological niche in the large intestine, in the current study it is proposed to investigate whether it is possible to reduce colonisation of laying hens by these spirochaete species by feeding diets with low sNSP content. Initially the focus would be on wheat-based diets, and the use of exogenous enzymes to reduce NSPs. Addition of enzymes to some diets is known to reduce problems of wet litter in the field, and it may be that this is mediated through effects on growth of pathogenic intestinal spirochaetes in the caecae. Subsequently, diets with different sNSP contents would be studied, to see how these affect colonisation with intestinal spirochaetes.

1.2 Factors investigated

The work conducted to date has posed a number of questions that are of direct relevance to the control of AIS, which were to be investigated in the current project:

1.2.1 Source of infection and reinfection

In this group's previous survey work, it was found that infection with intestinal spirochaete species usually first appears around 15 weeks of age, and that the within-flock prevalence then increases with increasing bird age. The source(s) of this first infection, and of the re-infection in birds that have been medicated, is not known. Improved understanding of where the infection first comes from in individual flocks would facilitate development of improved control measures.

1.2.2 Dietary manipulation of intestinal spirochaete infections

Experiments to determine whether dietary sNSP has an influence on colonisation and/or disease associated with the pathogenic intestinal spirochaete species in adult chickens are needed. If a dietary influence on AIS exists, then manipulation of diet or the use of exogenous dietary enzymes potentially would be a useful means of helping to control the condition without having to resort to using antimicrobials.

2. Objectives and tasks

Two main tasks were identified for the current project:

- examination of critical aspects of on-farm epidemiology of relevance to the control of AIS; and
- examination of dietary influences on *B. intermedia* and *B. pilosicoli* infections.

2.1 Examination of critical aspects of on-farm epidemiology of relevance to the control of AIS

It was proposed to investigate details of the epidemiology of infection on two infected farms (one broiler breeder and one layer farm, since these are the two main categories of farms where AIS occurs).

2.1.1 Cross-sectional surveys

On each farm a cross sectional survey would be conducted in which a statistically representative number of birds from different areas of the sheds and of different ages would be sampled on a single day. Isolates would be identified to species level, and strain-typing methods used to try to identify individual strains that might be circulating.

2.1.2 Longitudinal surveys

At each site a cohort of birds would be sampled at monthly intervals throughout their productive life. All the samples would be cultured for spirochaetes, and these would be identified to the species level by PCR, and the individual strains present identified using PFGE. The data obtained from the two sorts of surveys would demonstrate the prevalence, incidence and dynamics of infection with the various species, including consideration of the number of strains circulating and their own dynamics.

2.1.3 Environmental sources of infection

To look for possible environmental reservoirs of infection, environmental samples (dried faeces, dander, dust, feedstuffs, water) from around the sheds would be cultured for spirochaetes, and wild rodents, birds and insects (potential vectors) would be sampled as available.

2.1.4 Duration of survival and resistance to disinfectants

It was proposed that the duration of environmental survival of the two spirochaete species would be determined in the laboratory by seeding different concentrations of a strain of each species into chicken faeces, storing these at different temperatures, and culturing from them until viable organisms could no longer be recovered.

The effectiveness of a range of commercial disinfectants in common use on the viability of the two main pathogenic spirochaete species would be examined in the laboratory. Chicken faeces seeded with different concentrations of spirochaetes would be exposed to different concentrations of the disinfectants for different lengths of time, and cultured for viable spirochaetes.

2.2 Examining dietary influences on *B. intermedia* and *B. pilosicoli* infections

It was initially proposed that two experimental infection studies would be conducted, one using a strain of *B. intermedia* and one using a strain of *B. pilosicoli*. Each experiment would last approximately four months and would utilise four groups of 12 birds of 18 weeks of age. Experimental layer diets based on wheat and animal protein would be used. The focus of this work would be to examine the influence of dietary non-starch polysaccharides (NSP) on colonisation by the two spirochaete species, and the potential influence of treating the diets with exogenous dietary enzymes on the birds' susceptibility to colonisation. Birds would be acclimatised to the diets, infected, and then monitored for colonisation and signs of disease. Depending on results achieved, the experiments might be repeated using more birds and/or different dietary treatments. Ultimately, after discussion with the representatives of the funding bodies, four experiments were conducted, the last of which involved feeding three different diets with different levels of NSP, and evaluating these in relation to their effects on spirochaete colonisation.

3. On-farm Epidemiological Surveys

3.1 Broiler breeder farm

3.1.1 Introduction

This study was conducted on a large commercial broiler breeder farm in South-Eastern Queensland. *Brachyspira pilosicoli* previously had been isolated and associated with a wet litter problem on the production unit of this farm (Stephens and Hampson, 1999). The rearer unit consisted of four newly constructed enclosed sheds with concrete floors. The young birds in these sheds were housed on the floor on wood shavings. The production unit was on a separate site from the rearer unit, and consisted of four wooden sheds with dirt floors and open wire-enclosed sides. Each shed contained 10,000 birds. Three sheds housed Cobb birds and the other contained Ross birds. Wooden nesting boxes were suspended from the ceilings in the production sheds, and were lined with disposable cardboard liners. The sheds were cleaned between batches of birds by mechanically scraping the floor, and then covering it with lime. Two days later, fresh wood shavings were added and the cardboard liners in the nests were replaced. Small birds, rodents and cane toads occasionally were able to enter the production sheds.

3.1.2 Methods

3.1.2.1 Cross-sectional survey

A full cross-sectional survey was planned at the production facilities just prior to introduction of the new flock that was being followed in the longitudinal survey. Unfortunately, due to heightened concerns about biosecurity by the management of the farm at the time the sampling was due to take place, this survey was limited to the collection of 30 caecal faeces samples (these being caecal squirts specifically identified and collected from the litter) from each of four sheds, repeated after two weeks (ie 240 samples in total). Samples were collected into individual sterile containers and immediately transported to the laboratory for processing.

In the week prior to the birds for the longitudinal survey being moved to the production facilities, 20 environmental samples (water, drinking cups, dust, residual litter) were taken from each of four empty sheds on the production farm, into which new birds would subsequently be moved. Sampling occurred after mechanical cleaning and liming, and just before new wood shavings were added to the floor.

3.1.2.2 Longitudinal survey

The birds that were to comprise the new flock were monitored from day-old (when they arrived at the rearing unit from a grandparent flock), at fortnightly intervals through to transfer to the production facilities at 17 weeks of age (ie nine sampling visits made to the rearing unit). Initially, faeces were collected from 120 day-old birds on arrival (30 per shed). Subsequently, fortnightly visits were made during which 30 faecal samples and ten environmental samples (water, drinking cups, litter) were taken from each of four sheds on each visit (ie 160 samples per visit). Once the birds arrived at the production unit, samples continued to be taken at fortnightly intervals until the birds reached approximately 36 weeks of age (ie ten visits). Sampling at each visit was done in a consistent manner. A floor plan of the sheds was drawn up, and the area divided into ten equal sectors. At each visit, three fresh faecal samples and one environmental sample were collected from each sector in each shed.

3.1.2.3 Detection of spirochaetes

All faecal and environmental samples obtained were cultured anaerobically on selective plates, following standard procedures for isolating intestinal spirochaetes from chickens (Hampson and Stephens, 1999). Samples were cultured on selective trypticase soy agar (Micro Diagnostics,

Australia), supplemented with 5% defibrinated bovine blood, 0.1% porcine mucin (Sigma, USA), 200 µg/mL spectinomycin and 6.25 µg/mL each of colistin and vancomycin (Sigma, USA). Plates were incubated in an anaerobic environment generated by Anaerogen sachets (Oxoid, UK), and growth examined by dark field microscopy after five and ten days.

3.1.3 Results and Discussion

No spirochaetes were isolated from any of the 3,400 samples collected in the cross-sectional or longitudinal studies, including from the environmental samples and there was no obvious wet litter problem overall in the flocks examined. Failure to detect spirochaetes was not likely to be associated with the culture methods used, as these had not been modified from the previous study in which spirochaetes were detected on this farm (Stephens and Hampson, 1999).

This study in the broiler breeder birds was frustrated by a lack of natural infection with intestinal spirochaetes. Replacement birds were not infected from their farm of origin, nor did they acquire infection in the rearing unit. This was perhaps not surprising, as the rearing unit was new and fully contained. More surprising was the lack of infection at the production facilities, despite serious infection having occurred in these sheds in previous years. No evidence was found for persistence of intestinal spirochaetes in environmental samples from sheds that had been cleaned. The sampling regimen in the production sheds, with 400 samples collected per shed of 10,000 birds over ten visits was such that it should have detected the spirochaetes if they were present. Collection of 40 samples per visit per shed would only detect a prevalence of colonisation of around 8% in a shed on that visit (with 95% confidence), but the sampling of four sheds and the repeated sampling at fortnightly intervals should have been capable of detecting a prevalence of colonisation of far below 1% of birds. Although, in view of the objectives of the study, it was disappointing not to find any spirochaetes colonising the birds, these results demonstrate that a farm that has been infected in the past may spontaneously become non-infected. No specific management changes directed at controlling intestinal spirochaetes had been made since the time that AIS had originally been identified, and it was apparent that wild birds and rodents still had occasional access to the production sheds.

No isolates were obtained from this part of the study for future identification and typing.

3.2 Layer farm

3.2.1 Introduction

This study was conducted concurrently with the study on the broiler breeder farm. It was undertaken on a layer farm in Western Australia, and was intended to determine whether there were likely to be differences in patterns of infection with intestinal spirochaetes between broiler breeder and layer farms. The farm had had an on-going problem with wet litter, and intestinal spirochaetes previously had been isolated from birds on the farm.

3.2.2 Methods

3.2.2.1 Cross-sectional survey

The farm that was studied had four sheds, three of which contained ISA-Brown layers at the time the cross-sectional study was undertaken. These birds had been reared on the floor and there were multiple groups present on the same site in different sheds. Each shed housed approximately 5,000 birds. These birds were held with three birds per cage, with five rows of approximately 350 adjacent cages running along the length of each shed. The sheds were enclosed, had dirt floors and wooden walls and rafters.

Fresh faecal samples were collected from under the cages, with two hundred samples collected from each shed from sampling sites that were evenly distributed through each shed (ie 600 samples collected in total). As there were three birds per cage, the samples from under each cage potentially could have been contributed to by up to three birds. At the time of sampling, birds in shed 1 were near the end of their production cycle, those in shed 2 were in the middle of their cycle and those in shed 4 were newly-introduced and at the beginning of lay. Shed 3 was empty.

At the time of the survey, pools of six different types of insect also were obtained from each of the three sheds. These were mechanically homogenised and individually cultured for intestinal spirochaetes. These insect groups were mites, beetles, spiders, adult flies, fly maggots and midges.

3.2.2.2 Longitudinal survey

Approximately two months after the cross-sectional survey a longitudinal survey was conducted in birds from sheds 1 and 3. Prior to this survey, shed 3 had been cleaned and then left vacant for three months in response to a persistent wet litter problem that had occurred in the shed. Before the new birds were introduced, a range of 70 environmental samples, including soil, dust, insects, cobwebs, wild bird droppings, rodent droppings, remaining feed, water supply and feathers were collected from throughout shed 3.

Shed 1 was emptied after the cross-sectional survey, and then immediately cleaned by physical removal of faecal matter and high-pressure hosing with hot water. One week later, 70 environmental samples were collected from this shed, as with shed 3, prior to introduction of new birds. Shed 1 was restocked approximately three weeks after shed 3.

Both sheds were restocked with Hyline Brown hens, from a different source than used previously. This was done by the owners in response to there being a wet litter problem on the farm, and was not part of the originally planned approach to investigating the association between the wet litter problem and colonisation with intestinal spirochaetes.

Sampling in sheds 1 and 3 was conducted at two to four week intervals, with 144 samples being taken from shed 3 and 156 samples taken from shed 1 at each visit. A total of 13 visits were made between 4th April and 26th September 2002. Each individual sample represented a pool of freshly-passed faeces from under a cage (ie from up to three birds).

3.2.2.3 Detection and identification of spirochaetes

Faecal samples were plated to selective agar for isolation of intestinal spirochaetes, consisting of trypticase soy agar containing 400 μg/ml spectinomycin and 25 μg/ml each of colistin and vancomycin (Jenkinson and Wingar, 1981). Plates were incubated at 37^{o}C in an anaerobic jar in an atmosphere of 94% H_2 and 6% CO_2 for seven days, and then were scored visually for the presence or absence of intestinal spirochaetes. Plates were subcultured, and isolates transferred to Kunkle's prereduced anaerobic broth (Kunkle *et al*., 1986), and grown on a rocking platform at 37°C for three to five days before further subculture. Growth and purity were monitored under a phase contrast microscope, and cells were harvested in mid-log phase. Cells were washed and re-suspended in phosphate buffered saline (pH 7.2). The cells were used in confirmatory PCR assays, and for pulsed field gel electrophoresis. Two ml quantities of broth containing actively growing spirochaetes were extracted with xylol, and then Kovács' reagent was added. The development of a red colour in the upper layer was indicative of indole production by the spirochaete isolate.

Growth on the primary isolation plates was subjected to specific polymerase chain reaction (PCR) amplification for the detection and identification of *B. intermedia* and *B. pilosicoli.* A cell-pick method was used to obtain spirochaetal DNA from the plates. The methodology has been described previously (Atyeo *et al.,* 1998). The tip of a sterile wooden toothpick was used to stab through the area of spirochete growth, and the material adhering to the tip was resuspended in 50 μl of ultra-pure water before being boiled for 30 seconds. A 2.5 μl volume was added to each of the separate *B. intermedia* and *B. pilosicoli* PCR reactions, which were designed to amplify portions of the NADH

oxidase and 16S rRNA genes respectively. For amplification of *B. intermedia*, primer pair Int1 (5'-AGAGTTTGATGATAATTATGAC-3') and Int2 (5'-ATAAACATCAGGATCTTTGC-3'), targeting a 567 base pair (bp) region on the NADH oxidase (*nox*) gene of *B. intermedia*, were used. For *B. pilosicoli*, primer pair P1 (5'-AGAGGAAAGTTTTTTCGCTTC-3') and P2 (5'-GCACCTATGTTAAACGTCCTTG-3'), targeting an 823 bp region of the 16S rRNA gene of *B. pilosicoli* were used. The primers were designed from gene sequences available from the GenBank database so as to give specific PCR products of easily distinguishable sizes. DNA was amplified by hot-start PCR in a 25 µl total volume using HotStar*Taq* DNA polymerase (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. Briefly, amplification mixtures consisted of 1 x PCR buffer (containing 1.5 mM of $MgCl_2$), 0.5 U of HotStar*Taq* DNA polymerase, 0.2 mM of each dNTP (Amersham Pharmacia Biotech AB; Uppsala, Sweden), 0.5 µM of the primer pair and 2.5 µl chromosomal template DNA. Cycling conditions involved an initial 15 minutes HotStar*Taq* DNA polymerase activation step at 95°C, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and a primer extension at 72°C for 1 minute. The PCR products were subjected to electrophoresis in 1.5% (w/v) agarose gels in 1 × TAE buffer (40 mM Tris-acetate, 1 mM ethylenediaminetetra acetic acid [EDTA]), stained with ethidium bromide and viewed over UV light.

3.2.2.4 Pulsed field gel electrophoresis (PFGE)

Twenty-four *B. intermedia* isolates and one unidentified spirochaete that were recovered during the cross-sectional survey were subjected to strain typing using PFGE. The protocol used was as previously described (Suriyaarachichi *et al*., 2000). Cells in mid-log phase were harvested from 300 ml volumes of broth by centrifugation (12000 x g, 20 minutes, 4°C, Beckman JA-14 rotor). The cells were resuspended in 100 mls of sterile PBS and re-centrifuged. This was repeated, then the cells were finally resuspended in 0.5 mls of 10% sucrose buffer pH 8.0 before being stored at -18°C. Frozen cell stocks were thawed and approximately 10^{10} cells were placed in a fresh sterile microfuge tube. The cells were centrifuged at 4500 x g for 5 minutes, and washed twice on ice with chilled sterile PBS. Cells were resuspended in 250 µl of sterile PIV buffer (10 mM Tris, 1 mM NaCl, pH 8.0), held at room temperature, mixed with an equal volume of 2% low-melt agarose (LMA) in PIV, and pipetted into plug moulds. Plugs were hardened at 4°C for 20 minutes, placed in 2 mls of ESP solution (0.5M EDTA, 10% sodium lauroyl sarcosine, 100 µg/ml proteinase K, pH 8.0), and the tubes incubated in a waterbath at 55°C for two hours with occasional mixing. After hardening the plugs at 4°C the two hour incubation was repeated with 2mls of fresh ESP and incubated at 55°C for 16 hours with occasional mixing. The plugs were hardened at 4°C and subjected to six washes with 4 mls of sterile TE buffer (10 mM Tris, imM EDTA, pH 8.0) at 37°C of one hour each. Plugs were then stored in sterile 0.5 M EDTA. Plugs were divided into portions (approximately 5mm x 5mm x 1mm) using a sterile blade. These small plugs were placed into two 1 ml changes of TE buffer containing 0.2 mM phenylmethylsulfonyl fluoride at 55°C for 30 minutes. Plugs were hardened at 4°C between washes. The plugs were then placed in three 30 minute changes of TE buffer on ice.

Restriction digestion of the DNA in the plugs was carried out by adding 2 µl restriction endonuclease *Mlu*1 (New England Biolabs Inc) and 78 µl of 10% restriction buffer and incubating for 16 hours at 37°C. The digested plugs were stored at 4°C prior to loading into 0.5 x TBE (0.09M Tris, 0.09 M borate, 2 mM EDTA, pH 8.0) 1% agarose gels. The plugs were sealed into the agarose gel using 1% LMA in 0.5 x TBE, and the gels were chilled at 4°C for 6 hours before being subjected to electrophoresis. A lambda ladder molecular weight marker was included in one or more lanes of each gel. PFGE was carried out using a clamped homogeneous electric field device (CHEF DR11: Bio-Rad). Electrophoresis was at 200V for 20 hours at 14°C, with the pulse ramped from 1-40 seconds. Mid-range lambda ladder PFG and wide-range PFG molecular mass markers (New England Biolabs) were included in lanes at both ends of each gel, and were used to normalise isolates. Each strain was tested at least twice. The gels were stained in ethidium bromide solution, photographed over UV light, and analysed by scanning using a Biorad Gel Doc 2000+ and analysing using Biorad Molecular Analyst Software. The program created a dendrogram from a matrix of Pearson correlation

coefficients by the unweighted pair group method of arithmetic averages (UPGMA) clustering fusion strategy.

3.2.2.5 16S rDNA sequencing

The five isolates which were determined not to be *B. intermedia* or *B. pilosicoli* following the use of the diagnostic PCRs were investigated by PCR amplification and partial sequencing of their 16S rDNA genes. Two general *Brachyspira* primers that annealed to complementary sequences at the 5'-terminus and 3'-terminus of the 16S rRNA gene were designed and optimised for PCR amplification and sequence analysis. The primers used were Brachy-16S-F (5'-TGAGTAACACGTAGGTAATC-3') and Brachy-16S-R (5'-GCTAACGACTTCAGGTAAAAC-3'). A 1,311 bp portion of the gene was amplified by PCR in a 50 µl total volume using *Taq* DNA polymerase (Biotech International) and *Pfu* DNA polymerase (Promega). The amplification mixture consisted of 1 x PCR buffer (containing 1.5 mM of $MgCl_2$), 0.5 U of *Taq* DNA polymerase, 0.05 U *Pfu* DNA polymerase, 0.2 mM of each dNTP (Amersham Pharmacia Biotech), 0.5 µM of the primer pair (Brachy-16S-F, Brachy-16S-R), and 2.5 µl chromosomal template DNA. Cycling conditions involved an initial template denaturation step of 5 minutes at 94°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 15 seconds, and primer extension at 68°C for 2 minutes.

Following agarose gel electrophoresis to confirm that the PCR product was the correct molecular weight, the products were purified using the UltraClean PCR Clean-up Kit (QIAGEN), according to the manufacturer's instructions. Sequencing of the PCR product was performed in duplicate using the above primers. Each sequencing reaction was performed in a 10 µl volume consisting of 50 ng of PCR product, 2 pmol of primer, and 4 µl of the ABI PRISM® Dye Terminator Cycle Sequencing Ready Reaction Mix (PE Applied Biosystems). Cycling conditions involved a 2 minute denaturing step at 96°C, followed by 25 cycles of denaturation at 96°C for 10 seconds, primer annealing at 50°C for 5 seconds, and primer extension at 60°C for 4 minutes. Residual dye terminators were removed from the sequencing products by precipitation with 95% (v/v) ethanol containing 120 mM sodium acetate (pH 4.6), and vacuum dried. The sequencing products were analysed using an ABI 373A DNA Sequencer. Sequence results were edited, compiled and compared using SeqEd v1.0.3 and Vector NTI version 6.

The 16S rDNA sequences obtained for the five avian isolates then were compared and a 1,250 bp nucleotide sequence aligned with the 16S rDNA sequences available in the GenBank sequence database and the 16S rDNA sequences available from the Murdoch University database. A total of 48 sequences were used including those from nine strains of *Brachyspira hyodysenteriae*, six strains of *B. pilosicoli*, two strains of *B. intermedia*, two strains of *Brachyspira murdochii*, eight strains of *Brachyspira innocens*, 12 strains of "*Brachyspira pulli*", four strains of "*Brachyspira canis*", one strain of *Brachyspira alvinipulli* and four strains of *Brachyspira aalborgi*. The sequences were aligned and the phenogram drawn using ClustalX.

3.2.3 Results

3.2.3.1 Cross-sectional survey

Results of the cross-sectional survey are summarised in Table 3.1. Overall there was a high prevalence of colonisation of birds by intestinal spirochaetes, as observed by the presence of spirochaetal growth on the isolation plates (~20%). Only 8.5% of the samples were positive with one or the other of the two diagnostic PCRs that were used, and *B. intermedia* was much more common than *B. pilosicoli* (overall prevalence 7.2% compared to 1.3%). The overall prevalence of intestinal spirochaetes and the prevalence for *B. pilosicoli* were similar in the three sheds, even though the sheds housed birds of different ages. Colonisation rates for *B. intermedia* were highest (11%) in shed 2 where the birds of intermediate age were housed, and lowest in shed 4 (3.5%) containing the youngest birds.

Table 3.1 Visual assessment on the primary isolation plates and subsequent PCR results obtained from a collection of 200 fresh faecal samples from each of three individual sheds on a layer farm in Western Australia (600 samples in total)

		PCR results		
	Spirochaetes seen on plate (growth from plates then tested by PCR)	*B. intermedia*	*B. pilosicoli*	PCR negative
1	40 (20%)	14 (7%)	2 (1%)	24 (12%)
2	45 (22.5%)	22* (11%)	3* (1.5%)	20 (10%)
4	36 (18%)	7 (3.5%)	3 (1.5%)	26 (13%)
Total	121 (20.2%)	43 (7.2%)	8 (1.3%)	70 (11.7%)

*2 samples positive for both spirochaete species – ie evidence of mixed infection

None of the environmental samples that were taken at the farm yielded any spirochaetal growth on the primary isolation plates.

After repeated subculture of the plates inoculated with chicken faeces, 29 intestinal spirochaete were subsequently isolated in pure culture. These were tested for indole production (a feature of *B. intermedia* and the swine pathogen *B. hyodysenteriae*), and re-tested with the PCRs. The results are shown in Table 3.2. Twenty four isolates were *B. intermedia* and the remaining five isolates did not react in the PCRs, and were indole negative. No isolates of *B. pilosicoli* were recovered.

Table 3.2 Isolates obtained following culture of a collection of 200 fresh faecal samples from each of three individual sheds on a layer farm in Western Australia (600 samples in total)

	Spirochaetes isolated	
Shed	*B. intermedia** (indole positive)	Unknown spirochaetes (indole negative)
1	6	2
2	16	2
4	2	1
Total	24	5

*Identity confirmed by PCR

3.2.3.2 Longitudinal survey

Both new flocks developed a severe wet litter problem within two to three weeks of being introduced. Faeces failed to cone under the cages, the sheds had a strong smell of ammonia, and individual birds were passing liquid faeces and some had prolapsed intestines. At this time, spirochaetes started to be isolated from some of the faecal samples (Table 3.3). Between the visits of 17th May and 31st May, the wet litter problem disappeared. During this period between visits the sheds had been cleaned, the diet changed from a crumble to a mumble, and it appeared likely that antibiotics had been administered to the birds (although this could not be confirmed). Subsequently, spirochaetes were not isolated from any sample until 23rd August, when two samples from shed 1 were found to be positive (Table 3.3).

Although spirochaetes were seen growing on the isolation plates from some samples (Table 3.3), only one sample from shed 1 (17/5/02) was obtained in pure culture. This was identified as *B. intermedia* by PCR. The other spirochaetes present could not be isolated or identified by PCR from the primary growth.

Table 3.3 Visit schedule and results of faecal culture for intestinal spirochaete culture for sheds 1 and 3

Collection Date	Positive samples from Shed 1 (156 samples/visit)	Positive samples from Shed 3 (144 samples/visit)
5/4/02	NA	0
19/4/02	NA	0
26/4/02	1	0
3/5/02	3	3
17/5/02	5	4
31/5/02	0	0
14/6/02	0	0
28/6/02	0	0
12/7/02	0	0
27/7/02	0	0
9/8/02	0	0
23/8/02	2	0
26/9/02	0	0

3.2.3.3 PFGE results

The 24 *B. intermedia* isolates from the cross-sectional survey could be divided into four major PFGE patterns. Three isolates consistently gave smeared profiles, and could not be assigned to a subtype. Major pattern 1 could be subdivided into four subtypes based on minor banding differences (patterns 1a to 1d). A single unidentified spirochaete isolate ("*B. pulli*") that was examined gave a distinct pattern (pattern 5). Some representative gel patterns are shown in Figure 3.1, and the distribution of the various PFGE patterns through the three sheds at the time of the cross-sectional survey are shown in Table 3.4.

Table 3.4 Analysis of PFGE banding patterns and distribution of isolates through the sheds

PFGE pattern	Shed	Number of isolates	Species determined by PCR or 16SrRNA sequencing
1a	1	1	*B. intermedia*
1a	2	6	*B. intermedia*
1a	4	1	*B. intermedia*
1b	2	1	*B. intermedia*
1c	1	1	*B. intermedia*
1d	2	1	*B. intermedia*
2	1	2	*B. intermedia*
3	2	2	*B. intermedia*
4	2	2	*B. intermedia*
5	1	1	"*B. pulli*"

3.2.3.4 Identification of PCR-negative avian spirochaetes by 16S rDNA sequencing

The dendrogram expressing the genetic differences among these spirochaetes is shown in Figure 3.2. The unidentified avian isolates (designated MMM and marked with an arrow) shared the greatest genetic identity with the proposed chicken spirochaete "*Brachyspira pulli*" (McLaren *et al.*, 1997; Stephens and Hampson, 1999). Each of the five isolates had a slightly different 16S rDNA sequence.

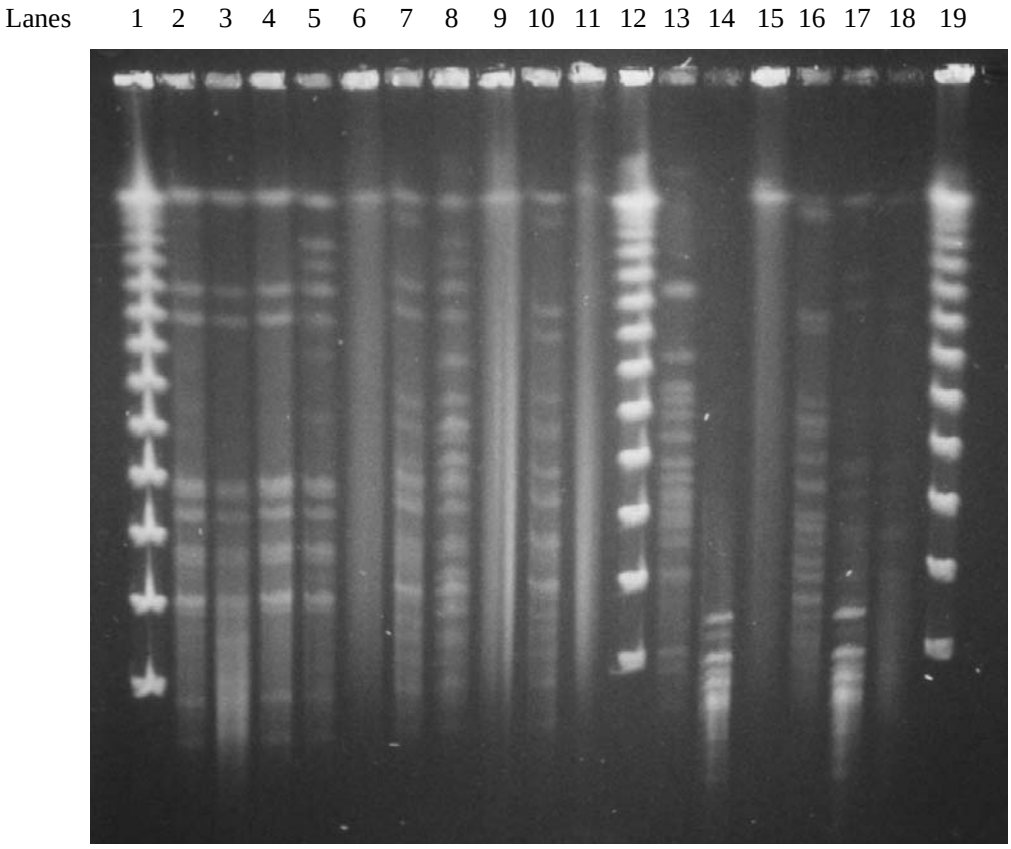

Figure 3.1 PFGE patterns of B. intermedia isolates obtained in the cross-sectional survey
Notes: Lanes 1, 12 and 19: lambda ladders. Lanes 2, 3 and 4: pattern 1a. Lane 5: pattern 1b. Lanes 6 and 11: smears. Lane 7: pattern 1c. Lane 8: pattern 1d. Lane 9: smear. Lanes 10 and 18: pattern 2. Lanes 13 and 15: pattern 3 (lane 15 smeared). Lanes 14 and 17: pattern 4. Lane 16: pattern 5.

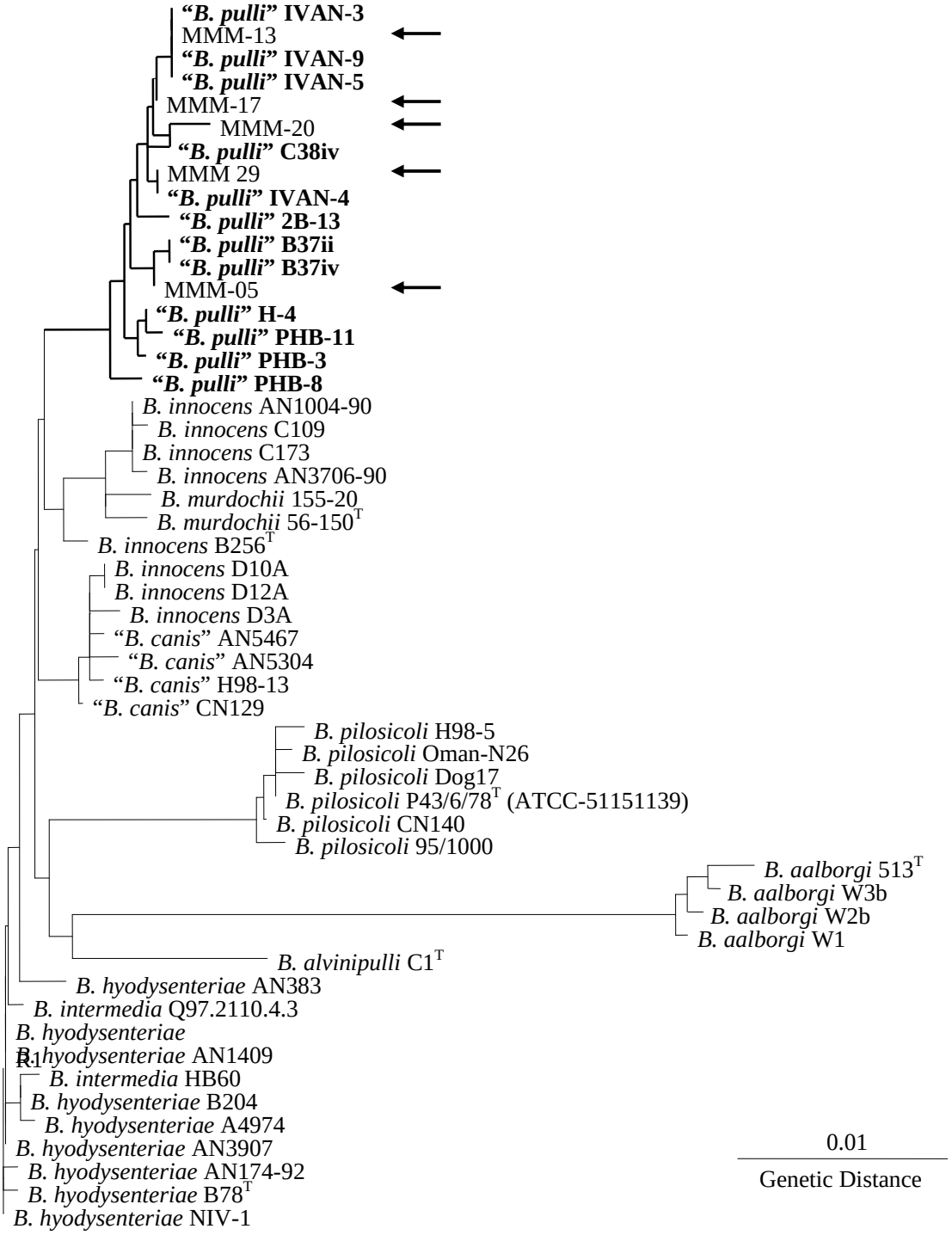

Figure 3.2 Phylogenetic differences among 48 intestinal spirochaetes from swine, birds, dogs and human beings

Notes: Different strains of the proposed chicken spirochaete species “*Brachyspira pulli*” are outlined in bold. The un-identified isolates obtained from the cross-sectional study are indicated with the arrows, and appear to be *“B. pulli”* isolates.

3.2.4 Discussion

The cross-sectional survey demonstrated that birds on this layer farm, where wet litter problems had been a major on-going constraint, were commonly colonised by intestinal spirochaetes. Around 20% of samples from each of the sheds yielded some spirochaetal growth on the primary isolation plates. About 12% of samples had spirochaetes of unknown identity, whilst about 7% were *B. intermedia* and 1% were *B. pilosicoli*. *B intermedia* was less common amongst the younger birds in shed 4 than in the other sheds, suggesting that after infection was acquired in a shed it slowly increased in prevalence over the course of several months. The source of infection was uncertain, but the spirochaetes were not detected in environmental samples. The most likely source of initial infection therefore would be from older infected birds in other sheds.

Even though many samples were positive by PCR, it was difficult to isolate spirochaetes in pure culture. This may reflect a relatively low level of colonisation, but also could have been because of the presence of contaminating flora that was resistant to the antibiotics used in the isolation plates.

The PFGE analysis of the *B. intermedia* isolates that were obtained indicated that there were several strains circulating on the farm. These strains were not necessarily shed-specific, as strain pattern 1a was found in all three sheds. Had more strains been isolated, it is likely that even more cross-contamination of sheds would have been identified. This dissemination of strains again suggests that they were being moved between sheds, perhaps in faecal contamination of the boots or overalls of the staff. Clearly, in order to reduce such cross-infection, better on-farm biosecurity measures would be needed.

As about half the spirochaetes detected in samples from the farm were not *B. intermedia* or *B. pilosicoli*, it was thought useful to try to identify them further. The sequencing and alignment of the 16S rDNA genes from five isolates clearly placed them together with other chicken isolates in a new group provisionally called "*Brachyspira pulli*". This species was initially identified in a study using multilocus enzyme electrophoresis (MLEE) to genetically characterise intestinal spirochaetes from Western Australian chickens (McLaren *et al.*, 1997), where the spirochaetes were called the "group d" spirochaetes. Subsequently, Stephens and Hampson (1999) used the provisional name "*B. pulli*" for spirochaetes of this type. The pathogenic potential of these spirochaetes has not been determined, and further work is required to clarify this issue. Minor differences in the 16S rDNA sequence of the five isolates suggest that they represent a number of different strains.

The longitudinal survey failed to achieve the desired outcomes of monitoring a build-up of infection with intestinal spirochaetes and helping to identify the source of infection. Not only did the producer use different birds from a new source at the time the longitudinal survey was conducted, but their diets were changed and they may well have received antimicrobial treatment in response to the clinical problem. It was even more unfortunate that these actions were taken without forewarning the investigators. Treatment was necessary on production and welfare grounds but unfortunately it disrupted the experiment. There did appear to be a build up in spirochaete numbers after the new birds were introduced, and this correlated with the development of severe wet litter problems. The management changes and possible treatment did successfully stop the wet litter problem, and resulted in removal of the spirochaetes. This provided some further circumstantial evidence that the spirochaetes were the cause of the clinical problems on the farm.

4. Survival of Intestinal Spirochaete Strains in the Presence of Disinfectants and in Faeces held at Different Temperatures

4.1 Introduction

To date there has been a lack of basic data available about the likely survival time of pathogenic intestinal spirochaetes in chicken faeces, as well as the effectiveness of some common disinfectants that might be used to remove them from the environment of poultry sheds. The current study was undertaken to provide some of this basic information.

4.2 Methods

4.2.1 Spirochaete strains and culture techniques

The two pathogenic avian spirochaete strains used were *B. intermedia* strain HB60 (Hampson and McLaren, 1999) and *B. pilosicoli* strain CPSp1 (Stephens and Hampson, 2002). Both were obtained from the culture collection held at the Reference Centre for Intestinal Spirochaetes at Murdoch University. They were grown to mid-log phase in Kunkle's pre-reduced anaerobic broth (Kunkle *et al*., 1986), and enumerated using a counting chamber.

4.2.2 Survival in the presence of disinfectants

Survival of the two spirochaete strains was tested in the presence of six disinfectants commonly used in poultry houses. These were alkaline salts (Active Dot, Peerless Emulsion Products Pty Ltd, Australia), quaternary ammonium (Quickmaster Lemon, Kwikmaster Products, Australia), iodine as an iodophor (Vetadine, PharmTech Pty Ltd, Australia), chlorine from Chloramine T (Halamid, Akzo Nobel Chemicals, the Netherlands), glutaraldehyde (Sigma, USA), and hydrogen peroxide (BDH, Australia). The disinfectants were tested at the concentration recommended by the manufacturers (Table 4.1). For each disinfectant, two cell concentrations of each spirochaete strain were tested (10^8 and 10^6 cells/ml) at four different exposure time intervals (one minute, five minutes, ten minutes and 30 minutes). Control strains grown in the absence of disinfectant were also included, and these were handled in the same way as for the treated samples. The spirochaetes were thoroughly mixed with the respective disinfectants in 10ml of anaerobic trypticase soy broth containing 10% foetal calf serum. One ml volumes of the broths containing the spirochaetes were then removed after the set time intervals, and added to 10 ml of fresh broth. After mixing, these tubes were centrifuged at 2,500 *g* for 15 minutes, and the supernatant containing any residual disinfectant discarded. A swab was then placed in the pellet and this was plated to trypticase soy agar containing 5% defibrinated ovine blood, and incubated anaerobically (94% H_2 and 6% CO_2) at 37°C for seven days. Subsequently, growth on the plates was examined, scraped off, and observed under a phase contrast microscope.

4.2.3 Survival in faeces at different temperatures

Chicken caecal faeces (which, for the purposes of this work, describes caecal squirts specifically identified and collected from the litter) that were tested negative for intestinal spirochaetes by culture, and by *B. pilosicoli* 16S rDNA PCR (La *et al*., 2003) and *B. intermedia* NADH oxidase PCR (Atyeo *et al*., 1999) applied to growth on the primary plate, were pooled, thoroughly mixed and then divided into 20 g aliquots. Samples were seeded with different concentrates of either *B. intermedia* or *B. pilosicoli* cells (10^5, 10^7 and 10^9 cells/g faeces), or with an equivalent volume of sterile broth, and

were thoroughly mixed in a blender. These samples were held in sealed containers at either 4°C, 25°C or 37°C, and cotton-tipped bacteriology swabs were inserted into these at times 0 and 2 hours, and then once every 9-15 hours until 14 days had elapsed. The swabs were plated to a selective agar for isolation of intestinal spirochaetes, consisting of trypticase soy agar containing 400 μg/ml spectinomycin and 25 μg/ml each of colistin and vancomycin (Jenkinson and Wingar, 1981). Plates were incubated at 37°C in an anaerobic jar in an atmosphere of 94% H_2 and 6% CO_2 for seven days, and spirochaete growth on the plate was recorded as being either present or absent.

4.3 Results

The results for survival times of *B. intermedia* and *B. pilosicoli* in the presence of the disinfectants are presented in Table 4.1. Both species were still viable in the control broths without disinfectants after 30 minutes. The two species showed similar sensitivities to the disinfectants, except that the *B. pilosicoli* strain was slightly more susceptible to the alkaline salts. Except in the case of the alkaline salts, increasing the cell concentration did not increase the survival time. Apart from alkaline salts, the other five disinfectants all inactivated the spirochaetes in less than one minute.

Table 4.1 Survival time of *B. intermedia* and *B. pilosicoli* strains at two cell concentrations in the presence of six disinfectants

		Survival time (minutes)[a]			
		B. intermedia		*B. pilosicoli*	
Disinfectant active constituent (stock conc.)	Working dilution in broth	10^8 cells per mL	10^6 cells per mL	10^8 cells per mL	10^6 cells per mL
Alkaline Salts 61g/L	1:25	>10 < 30	>1 <5	>1 < 5	<1
Quaternary Ammonium 15g/L	1:20	<1	<1	<1	<1
Iodine (active) 16g/L	1:167	<1	<1	<1	<1
Chlorine 250g/Kg	1:200	<1	<1	<1	<1
Glutaraldehyde 25% v/v	1:25	<1	<1	<1	<1
Hydrogen peroxide 30 % v/v	1:30	<1	<1	<1	<1

[a]Both species at both concentrations still viable in the control broths after 30 minutes.

The survival times for *B. intermedia* and *B. pilosicoli* cells held in chicken caecal faeces at different temperatures are shown in Table 4.2. The two species had broadly similar survival times, although both concentrations of *B. pilosicoli* cells survived for a slightly shorter period than the *B. intermedia* cells at 25°C, and survived for a shorter time at 10^5 cells per gram when held at 4°C.

Table 4.2 Survival time of three different concentrations of *B. intermedia* or *B. pilosicoli* cells suspended in 20g of chicken caecal faeces held at three different temperatures

	Survival time (hours)					
	B. intermedia			*B. pilosicoli*		
Temp	10^5 per gram	10^7 per gram	10^9 per gram	10^5 per gram	10^7 per gram	10^9 per gram
4°C	> 60 < 72	> 72 < 84	> 72 < 84	> 41 < 50	> 72 < 84	> 72 < 84
25°C	> 50 < 59	> 59 < 74	> 59 < 74	> 17 < 26	> 41 < 50	> 41 < 50
37°C	> 2 < 17	> 2 < 17	> 2 < 17	> 2 < 17	> 2 < 17	> 2 < 17

Survival time for both species was considerably reduced at 37°C compared to the two other temperatures, being only between 2 and 17 hours, and was longer at 4°C than at 25°C. Survival times for both species at 4°C and 25°C, but not at 37°C, tended to increase slightly at the higher cell concentrations. The maximum survival time for both species was between 72 and 84 hours at higher cell concentrations in faeces held at 4°C.

4.4 Discussion

This study indicates that pathogenic intestinal spirochaetes infecting chickens are relatively susceptible to disinfectants in common use, even in the presence of organic matter. The related intestinal spirochaete *B. hyodysenteriae*, the agent of swine dysentery, has similarly been shown to be rapidly inactivated in a faecal slurry by a number of common disinfectant solutions (Chia and Taylor, 1978). In the current study, the disinfectants were tested against the spirochaetes in trypticase soy broth containing 10% serum, with this medium acting as a standard controlled source of organic matter. Disinfectants are normally inactivated by direct contact with organic matter and hence their efficacy is variably impacted by organic loads. Therefore, they should be tested in the presence of sources of this material to evaluate their likely efficacy under field conditions. The use of a broth medium, which is rich in organic material, previously has been recommended for use when testing disinfectants against other avian pathogenic bacteria (Ruano *et al*., 2001). The alternative of testing disinfectant efficacy on faeces seeded with bacteria can be problematic, since it is difficult to remove residual disinfectants when transferring the bacteria to suitable growth medium for subsequent evaluation of their viability. Despite using a standardised broth method of evaluation in this study, the possibility remains that spirochaetes could have survived for longer if they were in the centre of a faecal bolus, where active disinfectants could not penetrate.

Both species of spirochaete were relatively short-lived in chicken caecal faeces. Previous studies on intestinal spirochaete survival have concentrated on species isolated from pigs, particularly *B. hyodysenteriae*. Survival in these studies has generally been much longer than the survival times found here. For example, Chia and Taylor (1978) showed that *B. hyodysenteriae* survived for 48 days in dysenteric faeces held between 0 and 10°C, although it only survived for seven days at 25°C, and less than 24 hours at 37°C. In another study, *B. hyodysenteriae* survived for ten days in soil held at 10°C, but this increased to 78 days in soil in the presence of 10% pig faeces, and was 112 days in pure pig faeces (Boye *et al*., 2001). In the same study, *B. pilosicoli* was found to be more resistant, surviving for 119 days in pure soil, and 210 days both in soil with 10% pig faeces and in pure pig faeces. Oxberry *et al.* (1998) also found *B. pilosicoli* to be relatively resistant, surviving in lake water at 4°C for 66 days, although its survival was reduced to four days at 25°C. The results from the current study confirm that both *B. pilosicoli* and *B. intermedia* survive for shorter periods at warmer than colder temperatures. Overall, their survival times in chicken caecal faeces was greatly reduced compared to the survival time of spirochaetes held in pig faeces, or in water, being less than 84 hours even at 4°C. Chicken faeces tend to be acidic and relatively dry, and this adverse environment may be responsible for the short survival times. It is possible that the methodology used for seeding faeces with spirochaetes may have affected the viability of the organisms, and the distribution of spirochaetes through the seeded faeces was unlikely to be completely representative of the distribution in the faeces of naturally-infected birds. In the latter case, it is likely that there are

accumulations of spirochaetal cells in parts of the faeces, and these may survive for longer than cells homogeneously distributed through seeded faeces.

The practical implications of the findings of the study are that avian intestinal spirochaetes are likely to be relatively fragile in the environment of a chicken shed. It should be easy to break cycles of infection between batches of layer birds by resting sheds and equipment for only a few days, and applying common disinfectants to contaminated equipment after cleaning to remove organic matter. The results also suggest that where infection occurs in new batches of birds, it is unlikely to arise from spirochaetes from a previous batch of birds surviving in the shed. Infection is more likely to arise by cross-contamination from sheds of older birds on the same site, or from exogenous sources.

5. Experimental Infection Studies

A series of four experimental infection studies were carried out in the animal holding facilities at Murdoch University. The purpose of these studies was to look for possible dietary effects on colonisation of layer hens with avian intestinal spirochaetes, with a particular emphasis on the role of soluble NSP (sNSP) and digesta viscosity.

5.1 Experiment 1: Infection of laying hens with *B. intermedia*

5.1.1 Introduction

In a previous study the authors demonstrated that inclusion of a commercial dietary enzyme supplement (Avizyme® 1303) to a wheat-based diet fed to layer hens reduced colonisation with a chicken strain of *B. intermedia* following experimental infection (Hampson *et al*., 2002). It was considered that the protection might be associated with the influence of the enzyme in degrading dietary sNSP, and reducing the viscosity of the digesta – which in turn inhibited colonisation. The purpose of the current experiment was to determine whether layer hens fed diets based on wheats differing in their NSP content would show different extents and/or patterns of colonisation following experimental inoculation with cultures of *B. intermedia.* Attempts would be made to determine whether differences in colonisation were related to the digesta viscosity. The hypothesis being tested was that increased digesta viscosity arising from the presence of sNSP in the diet would enhance colonisation with *B. intermedia*.

5.1.2 Methods

This experiment was conducted with the approval of the Murdoch University Animal Ethics Committee.

5.1.2.1 Experimental birds

Fifty ISA-Brown laying pullets were purchased from a commercial breeder at 18 weeks of age, and were housed in individual cages with mesh floors located in an environmentally-controlled (25^{o}C) facility. The birds were subjected to 12 hours artificial light each day. Thirty birds that were to be experimentally infected were housed in one room, and twenty that were to be uninfected control birds were housed in another separate room. Experimental infection commenced at 22 weeks of age, once the birds were all in lay. The experiment was conducted in November/December 2001.

5.1.2.2 Experimental diets

The birds were fed *ad lib* on one or other of two mash diets differing only in their wheat component, this being of two different varieties both of which were from the year 2000 harvest. Details of the diets are shown in Table 5.1.

In early 2001, the non-starch polysaccharide (NSP) contents of the wheats were measured in the laboratory of Professor Mingan Choct in the School of Rural Science and Agriculture at the University of New England, using the standard methodology described in the text by Spiller (1993). Details of the NSP contents at that time can be found in the publication by Kim *et al*. (2003).

5.1.2.3 Experimental infection

Brachyspira intermedia strain HB60 was obtained as a frozen stock culture from the collection held by the Reference Centre for Intestinal Spirochaetes at Murdoch University. The strain was originally isolated from a Western Australian layer flock with a wet litter problem (McLaren *et al.*, 1996), and had previously been used to experimentally infect layer hens (Hampson *et al.*, 2002). The strain was thawed and grown in Kunkle's anaerobic broth medium (Kunkle *et al.*, 1986) at 37^{o}C on a rocking platform until early log-phase growth was achieved, when the spirochaetes were actively motile.

Growth and absence of contamination was monitored by examining aliquots taken at daily intervals under a phase contrast microscope. At 22 weeks of age, fifteen birds in each dietary group were orally inoculated via a crop tube with 4 mL of the actively growing culture, on three consecutive days. The broths contained approximately 10^8-10^9 bacterial cells per mL. The twenty uninfected control birds (ten on each diet) in the other room were sham-inoculated with sterile broth.

Table 5.1 Diet fed to layer hens in experiments 1 to 3

ingredient	**%**
wheat (either variety Stiletto or Westonia)	65.96
lupin kernel	10.00
soya-bean meal	4.00
meat and bone meal	9.20
vegetable oil	1.00
limestone	9.00
salt	0.12
sodium bicarbonate	0.10
dl-methionine	0.14
l-lysine HCl	0.08
household layer premix*	0.30
choline 10% premix	0.10
calculated analysis	**% (unless otherwise stated)**
apparent metabolisable energy (kcals/kg)	2,779
protein	18.22
calcium	4.38
total phosphate	0.69
available phosphate	0.45
methionine	0.39
total sulphur amino acids	0.71
lysine	0.85
threonine	0.57
tryptophan	0.19
linoleic acid	1.37
crude fibre	2.81
sodium	0.14

*Containing minerals and vitamins to meet the nutritional requirements of birds of this age and weight (NRC, 1994)

5.1.2.4 Experimental monitoring

The birds were weighed on entry to the experiment at 18 weeks of age and at weekly intervals thereafter. Eggs were collected daily and weighed. At weekly intervals, starting at 21 weeks, aluminum foil was placed under the cage of each bird, and after one hour individual faecal samples were collected. Approximately 1 gram portions of the samples were weighed, then dried to constant weight in a hot air oven to determine the faecal moisture content. At weekly intervals, starting immediately before the experimental infection, cloacal swabs were taken from each bird at the time of weighing. A proportion of the faeces was re-suspended in phosphate buffered saline, and examined under a phase contrast microscope. Detection and identification of spirochaetes by culture and PCR from the primary isolation plate were as described in section 3.2.2.3.

5.1.2.5 Post-mortem examination

Three weeks after the experimental infection (week 25) the birds were killed by cervical dislocation, and subjected to post-mortem examination. The caeca and colon were opened to look for evidence of gross changes, a section of one caecum was placed in 10% neutral buffered formalin as a fixative for subsequent histological examination, and a swab was taken from the wall of the other caecum for

spirochaete culture. These were processed as for faecal samples. The fixed tissue was processed through to paraffin blocks, sectioned at 4 µm and stained with haematoxylin and eosin.

5.1.2.6 Viscosity of ileal contents

Ileal contents were collected from each of the birds at post-mortem, and immediately frozen at –80°C. Subsequently, these were thawed and measurements of the viscosity performed using a Brookfield LVDV-II+ cone plate (CP40) rotational viscometer (Brookfield Engineering Laboratories, Inc., MA, USA). The samples were diluted 1:1 (v/v) with distilled water, mixed and centrifuged at 12,000 g for 8 minutes. The viscosity of 0.5 ml supernatant fraction was measured at 25°C, applying a shear rate of 60 s^{-1} (McDonald *et al*., 2001).

5.1.2.7 Statistical analysis

Comparisons were made between the four groups of birds. For the two infected groups, the number of days that faecal samples were positive for *B. intermedia* compared to the total number of days that samples were taken were compared using Chi-squared tests. Weekly group bird weights, faecal moisture content, egg numbers and egg weights were compared using one-way analysis of variance. Means were compared using the Tukey-Kramer multiple comparisons test, and significance was accepted at the 5% level.

5.1.3 Results

5.1.3.1 NSP content of the wheats

On an as-fed basis, wheat variety Stiletto contained 9.9 g per kg sNSP, 69.2 g per kg iNSP and 79.1 g per kg total NSP. The ratio of sNSP to iNSP was 0.14:1. Variety Westonia contained 8.0 g per kg sNSP, 73.4 g per kg iNSP and 81.4 g per kg total NSP. The ratio of sNSP to iNSP was 0.11:1. Thus, wheat variety Westonia had a slightly higher total NSP content than variety Stiletto, although the ratio of sNSP to iNSP in Westonia was lower than in Stiletto.

5.1.3.2 Colonisation with B. intermedia

For the two experimentally-infected groups of birds, the proportion of samplings that were made over the period when the birds were 23-25 weeks old that were positive for *B. intermedia* are presented in Table 5.2. The birds receiving the Westonia diet shed the spirochaete in their faeces significantly more often and for longer than the birds fed the Stiletto variety ($P = 0.0001$; 95% confidence interval 0.4033 to 0.7772). All 15 birds on the Westonia diet were colonised at some point, whilst only 11 of 15 birds on the Stiletto diet were colonised during the experimental period.

Table 5.2 Colonisation of birds experimentally infected with *B. intermedia*, as assessed between the age of 23 and 25 weeks

Age of birds (weeks)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
23-25	28/135	58/135	**0.0001**

*infected with *B. intermedia* strain HB60 following sampling at 22 weeks

5.1.3.3 Faecal water contents

The faecal water contents of the four groups of birds are presented in Table 5.3. The control birds fed the Westonia diet had significantly drier faeces than the birds in the two infected groups in week 21, prior to infection. Significant differences were also seen in weeks 23 and 24, with the control birds fed Westonia having drier faeces than the infected birds fed Westonia (week 23), and the control birds fed Stiletto having drier faeces than the infected birds fed Stiletto (week 24).

Table 5.3 Weekly group mean and standard deviation faecal water content following allocation of birds into four groups

Age (weeks)	Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
21	$75.86^{ab} \pm 4.67$	$72.65^{a} \pm 4.62$	$78.75^{b} \pm 4.71$	$78.19^{b} \pm 4.91$	**0.0142**
22	74.89 ± 6.72	72.18 ± 13.13	77.82 ± 3.1	76.90 ± 5.14	0.2743
23	$73.58^{ab} \pm 3.15$	$69.51^{a} \pm 6.99$	$75.62^{ab} \pm 4.09$	$79.43^{b} \pm 13.32$	**0.0432**
24	$68.03^{a} \pm 14.80$	$75.74^{ab} \pm 4.52$	$76.48^{b} \pm 2.54$	$75.12^{ab} \pm 3.97$	**0.0368**
25	63.54 ± 3.29	65.5 ± 5.78	67.65 ± 4.82	62.12 ± 11.54	0.2328

* infected with *B. intermedia* strain HB60 following sampling at 22 weeks
[ab] Group means with different superscripts differ at the 5% level of significance

5.1.3.4 Bird weights, egg numbers and egg weights

Group mean and standard deviation of bird weights, weekly egg numbers and weekly egg weights are presented in tables 5.4, 5.5 and 5.6, respectively. No significant differences between groups were found in any of these production measurements.

Table 5.4 Weekly group mean and standard deviation of body weight (kgs) following allocation of birds into four groups

Age of birds (weeks)	Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
21	1.70 ± 0.22	1.78 ± 0.19	1.82 ± 0.14	1.73 ± 0.16	0.3354
22	1.71 ± 0.22	1.83 ± 0.21	1.77 ± 0.14	1.75 ± 0.19	0.3938
23	1.71 ± 0.21	1.80 ± 0.20	1.84 ± 0.13	1.78 ± 0.14	0.3078
24	1.73 ± 0.21	1.85 ± 0.10	1.86 ± 0.13	1.79 ± 0.15	0.1667
25	1.76 ± 0.20	1.89 ± 0.09	1.91 ± 0.14	1.81 ± 0.15	0.0974

*infected with *B. intermedia* strain HB60 following sampling at 22 weeks

Table 5.5 Weekly group mean and standard deviation of egg numbers following allocation of birds into four groups

Age (weeks)	Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
21	6.00 ± 2.21	6.20 ± 1.87	6.47 ± 1.06	6.14 ± 2.07	0.9283
22	6.44 ± 0.73	5.90 ± 1.45	6.47 ± 1.12	6.43 ± 1.65	0.7130
23	6.30 ± 2.21	6.10 ± 1.59	6.73 ± 0.46	6.43 ± 1.09	0.7091
24	6.10 ± 1.85	6.89 ± 0.33	6.87 ± 0.35	7.00 ± 0.00	0.0809
25	6.60 ± 0.70	6.30 ± 1.89	6.73 ± 0.59	6.93 ± 0.27	0.4715

*infected with *B. intermedia* strain HB60 following sampling at 22 weeks

5.1.3.5 Post-mortem findings

No specific pathological changes were found in the birds at post-mortem, and there was no gross or microscopic evidence of typhlitis/colitis in any of the birds. Spirochaetes were not observed associated with the fixed caecal tissue.

5.1.3.6 Viscosity of ileal contents

The mean and standard deviation of viscosity of the ileal samples in the four groups are presented in Table 5.7. Group differences were not significant. When the values for the uninfected and infected birds on each diet were pooled, the mean ± standard deviation for the birds receiving Stiletto was 3.18 ± 1.29, and for Westonia it was 4.16 ± 1.99. This difference was significant in a *t*-test ($P = 0.0483$). Hence the viscosity was higher in the birds fed wheat variety Westonia.

Table 5.6 Weekly group mean egg weight and standard deviation following allocation of birds into four groups

Bird age (weeks)	Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
21	53.31 ± 4.23	54.51 ± 4.14	54.45 ± 3.14	54.79 ± 3.38	0.8104
22	54.75 ± 3.40	56.11 ± 5.80	56.24 ± 3.22	56.87 ± 3.66	0.6751
23	56.26 ± 3.50	57.60 ± 4.58	58.06 ± 4.34	57.12 ± 3.49	0.7509
24	55.57 ± 5.30	57.96 ± 4.18	59.70 ± 4.51	59.17 ± 5.08	0.1879
25	57.72 ± 5.01	59.74 ± 4.49	60.11 ± 4.47	59.43 ± 3.90	0.6018

*infected with *B. intermedia* strain HB60 following sampling at 22 weeks

Table 5.7 Mean and standard deviation of viscosity of ileal contents in mPa•s at post-mortem

Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
3.43 ± 1.80	4.22 ± 1.37	3.04 ± 0.92	4.12 ± 2.36	0.2475

*infected with *B. intermedia* strain HB60 following sampling at 22 weeks

5.1.4 Discussion

In this experiment a significant dietary influence on colonisation with *B. intermedia* was observed, with birds fed wheat variety Westonia having significantly more colonisation following experimental infection than those receiving variety Stiletto. Furthermore, the viscosity of the digesta in the ileum of birds fed variety Westonia was greater than that of the birds fed variety Stiletto, and this difference became significant if data for the infected and uninfected birds was pooled. This observation supported the hypothesis that increased viscosity of the digesta would result in increased spirochaete colonisation. Although viscosity was measured in the ileum, and spirochaetal colonisation occurs in the caeca and colon, these diet-related changes in the microenvironment of the ileum are likely also to reflect changes more distally in the tract.

The link between NSP content of the diets and digesta viscosity was les obvious than the positive association between ileal viscosity and colonisation rates. Although variety Westonia had a slightly higher total NSP content than variety Stiletto, its sNSP content was less. The sNSP component of the diet is considered to contribute most to elevated viscosities of the digesta (Bedford and Schulze, 1998). One explanation for the unexpected viscosity results could be that the diets were stored for nearly nine months between the NSP analysis and their use. NSP values are known to alter with storage time, although not in a predictable way (Kim *et al*., 2003). Hence the NSP values recorded may not accurately reflect the true NSP content at the time the diets were fed.

It was not possible to exclude other possible reasons for the different colonisation rates on the two diets. Had additional measurements of fermentation in the large intestine, or other aspects of the large intestinal lumenal environment been made, these too may have been associated with differences in colonisation rates.

Birds that were experimentally infected with *B. intermedia* did not show any significant differences in production parameters (body weight and egg production) compared to the control birds, nor did they show specific pathological changes in their caeca at post-mortem. Under field conditions *B. intermedia* may show more evidence of pathogenic potential (Stephens and Hampson, 1999), and previously this same strain (HB60) has caused mild production losses in experimentally infected layers (Hampson and McLaren, 1999). Although colonised birds did have significantly wetter faeces than one or other of the control groups at one and two weeks after experimental infection, they also had significantly wetter faeces than the control Stiletto group before the birds were experimentally infected. In part, these differences may arise as a result of the small numbers of birds in each group, with changes in faecal water content of two or three birds greatly influencing the group result. Again,

to obtain a better understanding of the influence of *B. intermedia* on faecal water content it would be necessary to test much larger groups of birds.

5.2 Experiment 2: Infection of laying hens with *B. pilosicoli*

5.2.1 Introduction

The purpose of this study was to determine whether the dietary effects seen with *B. intermedia* in experiment 1 also occurred with an avian strain of *B. pilosicoli*. Hence experiment 1 was repeated using an experimental infection with *B. pilosicoli* strain CPSp1, a strain that has been shown to reduce egg production in broiler breeders (Stephens and Hampson, 2002). The same diets were used, except that 50 ppm zinc bacitracin was added to these diets as this is known to facilitate colonisation of birds with *B. pilosicoli* (Jamshidi and Hampson, 2002).

5.2.2 Methods

5.2.2.1 Experimental birds
Fifty birds of the same age and source as used in experiment 1 were used in the current experiment. Housing was as in experiment 1. Experimental infection commenced when the birds were 20 weeks of age, and they were killed at 26 weeks of age (ie the birds were infected two weeks earlier than birds in experiment 1, and overall were kept for two weeks longer).

5.2.2.2 Experimental diets
The diets were formulated to be the same as in experiment 1, except that 50 ppm zinc bacitracin was added to both diets to facilitate colonisation with *B. pilosicoli*. This experiment was conducted in April/May 2002, hence the two wheats that were used had been in storage for an additional six months since the previous experiment.

5.2.2.3 Experimental infection
Brachyspira pilosicoli chicken strain CPSp1 was used to infect the birds (Stephens and Hampson 2002). For experimental infection, the spirochaetes were grown to mid-log phase in Kunkle's broth (Kunkle *et al*., 1986), as with *B. intermedia*. The 4 mL inocula used to infect the birds contained approximately 10^8-10^9 actively motile bacterial cells per mL, as determined by direct counting of spirochaetes in a counting chamber placed under a dark field microscope. The birds were dosed once daily for five days. Monitoring of faecal shedding of spirochaetes, faecal water content, bird weight and egg production were as in experiment 1. The identity of *B. pilosicoli* on the isolation plates was confirmed by PCR, as described in section 3.2.2.3. Post-mortems were carried out when the birds were 26 weeks of age, and the same samples were collected for analysis as in experiment 1.

5.2.3 Results

5.2.3.1 Colonisation with B. pilosicoli
For the two experimentally-infected groups of birds, the proportion of samplings that were made over the period when the birds were 21-26 weeks old that were positive for *B. pilosicoli* are presented in Table 5.8. The birds receiving the Stiletto diet shed the spirochaete in their faeces significantly more often and for longer than the birds fed the Westonia variety (P = 0.0153; 95% confidence interval 1.063 to 1.534). Fourteen of the 15 birds on the Stiletto diet were colonised at some point, whilst only 12 of 15 birds on the Westonia diet were colonised during the experimental period.

Table 5.8 *B. pilosicoli* colonisation of infected birds between the age of 21 to 26 weeks

Age of birds (weeks)	Positive/no. of samples taken (Stiletto)	Positive/no. of samples taken (Westonia)	*P*-value
21-26	85/225	60/225	0.0153

5.2.3.2 Faecal water content

The water contents of the faeces in the four groups of birds are presented in Table 5.9. The only significant differences occurred on week 22, when the infected birds fed Watsonia had significantly drier faeces than the experimentally infected birds fed variety Stiletto.

Table 5.9 Weekly group mean faecal water content and standard deviation following allocation of birds into four groups

Age of birds (weeks)	Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
20	71.75 ± 5.08	76.27 ± 4.15	72.37 ± 4.15	71.53 ± 5.43	0.0864
21	72.41 ± 5.12	74.68 ± 4.43	74.08 ± 4.82	71.55 ± 4.45	0.3160
22	75.06^{ab} ± 3.87	75.66^{ab} ± 4.80	75.76^{a} ± 3.79	71.55^{b} ± 4.69	**0.0374**
23	72.88 ± 4.69	71.69 ± 3.88	73.00 ±3.55	72.35 ± 4.54	0.8732
24	73.34 ± 5.14	73.16 ± 4.86	73.20 ± 3.36	74.56 ± 4.24	0.7996
25	71.28 ± 7.95	68.97 ± 6.71	71.23 ± 4.09	72.48 ± 4.90	0.5357
26	71.55 ± 9.94	69.96 ± 7.70	72.43 ± 4.36	72.18 ± 5.14	0.8169

*Infected with *B. pilosicoli* strain CPSp1 following sampling at 20 weeks of age

[ab] Group means with different superscripts differ at the 5% level of significance

5.2.3.3 Bird weights and egg production parameters

The bird group weights are presented in Table 5.10, egg numbers in table 5.11 and egg weights in Table 5.12. There were no significant effects on body weight. For egg numbers there was an overall significant difference at 24 weeks of age. The control birds tended to produce fewer eggs, although there were no significant differences detected between individual groups at this time. A significant effect also occurred at 26 weeks of age, with the infected birds on the Stiletto diet producing significantly more eggs than the uninfected birds on the same diet. No significant group differences were detected for egg weights.

Table 5.10 Weekly group mean and standard deviation body weight (kgs) following allocation of birds into four groups

Bird age (weeks)	Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
20	1.65 ± 0.19	1.67 ± 0.11	1.69 ± 0.15	1.66 ± 0.14	0.9318
21	1.73 ± 0.16	1.73 ± 0.10	1.77 ± 0.13	1.72 ± 0.11	0.7418
22	1.70 ± 0.13	1.77 ± 0.10	1.75 ± 0.13	1.74 ± 0.11	0.5525
23	1.79 ± 0.16	1.76 ± 0.12	1.82 ± 0.15	1.82 ± 0.12	0.6414
24	1.82 ± 0.18	1.82 ± 0.12	1.86 ± 0.17	1.89 ± 0.14	0.5687
25	1.86 ± 0.20	1.83 ± 0.12	1.88 ± 0.15	1.95 ± 0.11	0.2292
26	1.85 ± 1.20	1.89 ± 0.13	1.94 ± 0.14	2.00 ± 0.14	0.0900

*Infected with *B. pilosicoli* strain CPSp1 following sampling at 20 weeks of age

5.2.3.4 Post-mortem findings

No pathological changes were found in any of the birds at post-mortem, and there was no histological evidence of typhlitis or end-on attachment of spirochaetes to the caecal epithelium.

5.2.3.5 Viscosity of ileal contents

The viscosity values of the ileal contents are shown in Table 5.13. There were no significant differences between the groups. When the results for the uninfected and infected birds on each diet were pooled and compared, again there was no significant dietary effect ($P = 0.8399$).

Table 5.11 Weekly group mean and standard deviation egg numbers following allocation of birds into four groups

Bird age (weeks)	Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
20	4.70 ± 2.63	5.80 ± 1.03	5.53 ± 2.00	3.67 ± 2.69	0.0715
21	6.10 ± 0.99	6.00 ± 1.41	6.20 ± 1.90	5.27 ± 2.52	0.5462
22	6.00 ± 1.05	5.50 ± 1.65	6.20 ± 1.32	6.33 ± 1.80	0.5720
23	6.80 ± 0.42	6.40 ± 1.58	6.33 ± 1.05	6.60 ± 1.30	0.7701
24	6.40 ± 0.84	6.10 ± 1.37	6.87 ± 0.35	6.87 ± 0.35	**0.0464**[#]
25	5.70 ± 1.83	5.30 ± 2.58	6.33 ± 1.11	6.80 ± 0.41	0.0958
26	5.40 ± 2.46[a]	5.70 ± 1.42[ab]	6.93 ± 0.26[b]	6.73 ± 0.80[ab]	**0.0159**

*Infected with *B. pilosicoli* strain CPSp1 following sampling at 20 weeks of age
[#]No individual differences
[ab] Group means with different superscripts differ at the 5% level of significance

Table 5.12 Weekly group mean and standard deviation egg weights following allocation of birds into four groups

Bird age (weeks)	Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
20	45.64 ± 16.83	48.85 ± 3.54	44.98 ± 13.46	43.76 ± 12.69	0.8017
21	53.58 ± 3.17	52.49 ± 4.43	52.32 ± 3.71	51.26 ± 4.07	0.5663
22	56.03 ± 4.66	53.42 ± 6.44	54.33 ± 3.51	50.28 ± 14.28	0.4152
23	56.12 ± 4.42	55.58 ± 3.88	55.49 ± 2.81	54.99 ± 3.47	0.8947
24	58.09 ± 3.54	56.15 ± 4.51	56.22 ± 4.51	56.57 ± 4.30	0.7003
25	59.29 ± 6.05	50.73 ± 18.32	56.04 ± 2.79	57.50 ± 4.39	0.1751
26	59.0 ± 6.03	55.96 ± 5.15	56.62 ± 2.33	58.21 ± 4.31	0.3650

*Infected with *B. pilosicoli* strain CPSp1 following sampling at 20 weeks of age

Table 5.13 Mean and standard viscosity measurements (in mPa•s) of the ileal contents at post-mortem (26 weeks of age)

Control (Stiletto)	Control (Westonia)	Infected* (Stiletto)	Infected* (Westonia)	*P*-value
4.38 ± 3.11	4.27 ± 2.12	4.19 ± 1.22	3.98 ± 2.23	9.765

*Infected with *B. pilosicoli* strain CPSp1 following sampling at 20 weeks of age

5.2.4 Discussion

In this experiment a dietary influence on *B. pilosicoli* colonisation was demonstrated, but in this case colonisation was more common in birds fed the diet containing wheat variety Stiletto than on those fed the diet containing variety Westonia. Hence, this dietary effect was opposite to that found in the previous *B. intermedia* infection trial, where again a clear dietary influence on colonisation was observed. The relative differences in colonisation rates between diets seen for *B. intermedia* were greater than those seen with *B. pilosicoli.* Apparently these two distinct but related spirochaetal pathogens are influenced in different and perhaps opposite ways by the diet. If this is correct, then it will make development of dietary means to control AIS extremely difficult, and highly dependant on

having a good diagnostic capacity to identify the species of intestinal spirochaete present in a given flock. Moreover, individual flocks may be colonised by both species (see results of the cross-sectional study on the layer farm, Table 3.2), making dietary control even more complicated.

In the current experiment there was no difference in the viscosity of the ileal digesta in birds on the two diets, so there was no obvious link between increased viscosity and increased colonisation, as was seen with *B. intermedia*. The lack of a dietary group viscosity effect in the current experiment compared to the previous experiment may have resulted from inclusion of zinc bacitracin in the diet (necessary to facilitate colonisation by *B. pilosicoli*), or could have been associated with the prolonged storage of the wheat and associated changes in NSP content before it was used in the current experiment. The fact that the birds were two weeks older at post-mortem compared to the previous experiment seems unlikely to have had an important influence on the digesta viscosity.

Colonisation with *B. pilosicoli* did not have any significant negative influence on egg production, body weight or faecal moisture content, nor were any pathological changes found at post-mortem. The significant improvement in egg numbers in colonised groups at some ages was unexpected and is difficult to explain. In the field *B. pilosicoli* infection has been associated with an increase in faecal water content and a reduction in egg production (Trampel *et al.*, 1994; Stephens and Hampson, 1999), but this has only occasionally been reproduced under experimental conditions (Stephens and Hampson, 2002). The difference may be due to a more extensive colonisation of individual birds held under commercial conditions, associated with increased stress and the crowding of birds which may facilitate bird-to-bird transmission.

5.3 Experiment 3: Infection with *B. intermedia* in the presence of exogenous dietary enzyme

5.3.1 Introduction

In experiment 1 a dietary effect on colonisation with *B. intermedia* was observed. Increased colonisation on birds fed the Westonia wheat variety was correlated with an elevated viscosity of the ileal contents compared to birds fed the Stiletto variety. The purpose of the present experiment was to repeat experiment 1, but in this case feeding all the birds on the Westonia diet, with half receiving a commercial exogenous enzyme designed to degrade the NSP in the wheat. It was hypothesised that the enzyme treatment would both reduce digesta viscosity and limit the extent of colonisation by *B. intermedia.* In a previous experiment, exogenous enzyme added to a wheat diet did result in reduced colonisation with *B. intermedia* following experimental infection (Hampson *et al*., 2002).

5.3.2 Methods

5.3.2.1 Diets

This experiment used the same Westonia diet that had been used in experiments 1 and 2. Half the birds received this mash diet (previously shown to enhance colonisation with *B. intermedia*), and half received the same diet to which a commercial enzyme preparation (Avizyme® 1302, Danisco: guaranteed minimum activity of 5000 U/g endo-1,4-beta-xylanase EC 3.2.1.8 and 1600 U/g subtilisin [protease]) was added at the manufacturer's recommended rate (256g/tonne).

5.3.2.2 Birds

The experiment was a replicate of the previous experiments, using 50 birds of the same age from the same source. The experiment was conducted in September/October 2002. The infected birds were kept to 27 weeks of age, but the two control groups were removed at 24 weeks of age because the experimental diets were beginning to run out, and it was necessary to keep the infected birds as long as possible to maximise the chance of detecting infection.

5.3.2.3 Experimental infection

The *B. intermedia* strain HB60 was again used to infect the birds. The same protocol as used in experiment 1 was followed, except that dosing with the challenge strain was repeated daily for five consecutive days.

5.3.2.4 Experimental monitoring

Spirochaetal shedding, bird health and productivity was monitored as in the previous experiments.

5.3.3 Results

5.3.3.1 Colonisation with B. intermedia

Colonisation occurred in both dietary groups following experimental infection, but in both groups it was relatively transient, and there were no differences between the groups (Table 5.14). In the group without enzyme, ten of 15 birds were colonised at some point, whilst 12 of 15 birds receiving enzyme were colonised at some point.

Table 5.14 Colonisation of infected birds between 21 to 27 weeks of age

Age of birds (weeks)	Positive/number of samples taken (No enzyme)	Positive/number of samples taken (Enzyme)	*P*-value
21-27	19/315	21/315	0.8705

5.3.3.2 Faecal water content

The mean faecal water contents of the birds in the four groups are presented in Table 5.15. At week 21 the uninfected birds receiving enzyme had significantly drier faeces than the infected birds that were not receiving enzyme. At week 22 the uninfected birds not receiving enzyme had wetter faeces than the other three groups. No other differences were significant.

Table 5.15 Weekly group mean and standard deviation faecal water content following allocation of birds into four groups

Bird age (weeks)	Control (No Enzyme)	Control (Enzyme)	Infected* (No Enzyme)	Infected* (Enzyme)	*P*-value
20	71.93 ± 5.27	75.03 ± 6.31	74.58 ± 5.41	75.65 ± 4.84	0.5156
21	76.28^{ab} ± 7.98	68.86^{a} ± 3.87	76.68^{b} ± 5.73	74.68^{ab} ± 4.40	**0.0076**
22	76.29^{a} ± 8.02	68.86^{b} ± 4.24	70.14^{b} ±5.06	66.61^{b} ± 4.45	**0.0022**
23	75.45 ± 7.24	73.24 ± 9.01	72.68 ± 5.92	71.93 ± 3.40	0.6091
24	69.94 ± 8.41	69.95 ± 5.88	73.46 ± 3.57	69.04 ± 4.39	0.2445
25	NA	NA	71.05 ±5.14	68.79 ± 5.84	0.3725
26	NA	NA	67.54 ± 3.79	68.20 ± 5.88	0.9580
27	NA	NA	71.49 ± 3.16	69.44 ± 5.34	0.2248

* Infected with *B. intermedia* strain HB60 following sampling at 20 weeks

[ab] Group means with different superscripts differ at the 5% level of significance

NA = not available

5.3.3.3 Bird weights and egg production parameters

Details of bird weights, egg production and egg weights are presented in Tables 5.16, 5.17 and 5.18 respectively. No significant group differences were found.

Table 5.16 Weekly group mean and standard deviation body weight (kgs) following allocation of birds into four groups

Age of birds (weeks)	Control (No Enzyme)	Control (Enzyme)	Infected* (No Enzyme)	Infected* (Enzyme)	*P*-value
20	1.82 ± 0.13	1.85 ± 0.17	1.84 ± 0.17	1.87 ± 0.18	0.9078
21	1.81 ± 0.11	1.83 ± 0.26	1.87 ± 0.17	1.90 ± 0.18	0.5090
22	1.82 ± 0.10	1.80 ± 0.21	1.88 ± 0.18	1.90 ± 0.21	0.4552
23	1.86 ± 0.15	1.72 ± 0.18	1.89 ± 0.18	1.91 ± 0.26	0.1606
24	1.92 ± 0.15	1.90 ± 0.18	1.96 ± 0.18	1.96 ± 0.21	0.7315
25 [a]	NA	NA	NA	NA	NA
26	NA	NA	1.98 ± 0.18	2.01 ± 0.19	0.6579
27	NA	NA	NA	NA	NA

* Infected with *B. intermedia* strain HB60 following sampling at 20 weeks
[a] Control chickens were killed at the start of week 25 due to a lack of feed
NA = not available

Table 5.17 Weekly group mean and standard deviation egg numbers following allocation of birds into four groups

Age of birds (weeks)	Control (No Enzyme)	Control (Enzyme)	Infected* (No Enzyme)	Infected* (Enzyme)	*P*-value
20	5.60 ± 2.01	6.00 ± 1.63	5.53 ± 1.77	6.00 ± 1.73	0.8562
21	6.63 ± 1.06	5.40 ± 2.17	6.07 ± 1.69	6.21 ± 1.76	0.5036
22	5.67 ± 1.66	6.13 ± 0.83	5.60 ± 2.23	5.86 ± 1.75	0.9172
23	4.60 ± 1.71	6.25 ± 1.04	5.29 ± 1.54	6.00 ± 1.36	0.0620
24	6.25 ± 0.71	5.33 ± 2.06	6.15 ± 0.99	5.86 ± 1.61	0.5228
25	NA	NA	5.43 ± 1.99	5.29 ± 1.86	0.8458
26	NA	NA	6.00 ± 1.54	5.64 ± 1.39	0.5401
27	NA	NA	4.87 ± 2.80	4.73 ± 2.34	0.8885

* Infected with *B. intermedia* strain HB60 following sampling at 20 weeks
NA = not available

Table 5.18 Weekly group mean and standard deviation egg weight following allocation of birds into four groups

Age of birds (weeks)	Control (No Enzyme)	Control (Enzyme)	Infected* (No Enzyme)	Infected* (Enzyme)	*P*-value
20	60.01 ± 2.97	59.43 ± 3.45	58.76 ± 3.46	60.27 ± 3.72	0.6588
21	60.10 ± 4.17	59.97 ± 3.37	59.71 ± 2.34	60.76 ± 4.40	0.8893
22	60.68 ± 2.79	61.02 ± 2.18	60.28 ± 3.29	62.38 ± 4.07	0.3815
23	59.96 ± 3.65	61.18 ± 2.02	60.48 ± 2.95	62.21 ± 2.83	0.1139
24	60.29 ± 3.45	60.28 ± 4.39	61.50 ± 2.64	62.80 ± 3.13	0.1937
25	NA	NA	62.64 ± 3.48	62.55 ± 4.39	0.9067
26	NA	NA	63.15 ± 3.63	63.43 ± 4.52	0.6873
27	NA	NA	62.97 ± 4.25	61.50 ± 3.89	0.5202

* Infected with *B. intermedia* strain HB60 following sampling at 20 weeks
NA = not available

5.3.3.4 Post-mortem findings

No pathological changes were found in the birds at post-mortem, and there was no histological evidence of typhlitis.

5.3.3.5 Viscosity of ileal contents

The viscosity of the ileal contents in the four groups of birds is presented in Table 5.19. There were no significant differences between the groups overall. Also there were no significant differences between the two control groups, or between the two infected groups, when these were separately compared using *t*-tests.

Table 5.19 Mean and standard viscosity measurements (in mPa•s) of the ileal contents at post mortem in birds aged 24 weeks (controls) or 27 weeks (infected birds)

Control (No Enzyme)	Control (Enzyme)	Infected* (No Enzyme)	Infected* (Enzyme)	*P*-value
2.28 ± 1.20	2.35 ± 1.36	2.82 ± 0.92	2.06 ± 0.63	0.4199

* Infected with *B. intermedia* strain HB60 following sampling at 20 weeks

5.3.4 Discussion

The lack of a consistent and prolonged colonisation in both groups of infected birds made it impossible to determine, in the context of this diet, whether the enzyme preparation could have had any protective effect on colonisation. There was no obvious explanation for why the colonisation rate was lower than in experiment 1. Indeed, in the current experiment, the birds were dosed daily for five days compared to only three days in experiment 1, which should have considerably increased their exposure to the *B. intermedia* strain. The cultures used for infection appeared equally as active as previously, the birds were from the same source, and the base diet was the same.

Given the relatively low incidence of colonisation, it is not surprising that the infection did not influence any of the measured production parameters. Equally, addition of enzyme to the diet did not cause any significant or obvious changes in these parameters. Furthermore, addition of enzyme did not influence the viscosity of the ileal contents. This finding is consistent with the results of a recent Australian study where a number of different commercial enzyme products failed to significantly alter digesta viscosity in the jejunum or ileum of layer hens (Roberts, 2003).

There was an interaction in faecal water content with infected/not infected and +/- enzyme, but the reason for this is not easy to explain.

Overall, this experiment did not answer the specific research question posed. This was largely because of a lack of successful experimental infection, but also may have been contributed to by the relatively low NSP value of the diet used.

5.4 Experiment 4: Infection with *B. intermedia* using other diets

5.4.1 Introduction

Following concerns about the relatively low levels of NSP in the wheat-based diets used in experiments 1-3, it was decided to examine the effects of other diets that would be predicted to have greater NSP contents on colonisation with *B. intermedia*.

5.4.2 Methods

5.4.2.1 Birds

In this experiment, 72 birds of 18 weeks of age were obtained from the same source as in experiments 1-3 were used. They were divided into six groups of 12, each housed in adjacent cages in the same facility as used in the previous experiments.

5.4.2.2 Diets
Three diets were fed, with each dietary group being divided into the diet with or without an appropriate commercial dietary exogenous enzyme being added (ie six groups in total). The diets were formulated by a commercial poultry nutritionist, and the specifications (eg AME, amino acids) were typical for layer birds of this age. The cereal sources in the three diets were wheat, barley and barley+sorghum respectively (Table 5.20). The NSP contribution from all the non-cereal sources in the diet were balanced across diets, so that the only difference in NSP content between diets was due to the cereal component. The calculated sNSP levels in the wheat and in the barley+sorghum diets was about 1.05% as fed (10.5 g/kg), while the sNSP in the barley diet was about 2.3% as fed (23 g/kg). The sNSP values were subsequently established by testing at the University of New England (Table 5.21). The diets were fed as mash diets and, as such, were not steam pelleted.

Dietary enzymes from Roche Vitamins, NSW, were added to duplicates of the diet. Ronozyme WX at 200g/tonne was added to the wheat-based diet. This contained endo-1-4 β xylanase (IUB No. 3.2.1.8) with a minimal activity of 1000 FXU/g. Ronozyme A at 200g/tonne was added to the barley-based diet. This contained α-amylase (IUB 3.2.1.1) with a minimum activity of 200 KNU/g, and endo-1-3;1,4 β gluconase (IUB 3.2.1.6) with a minimum activity of 350 FBG/g. For the barley+sorghum diet, both Ronozyme WX and Ronozyme A were added, both at 100 g/tonne.

5.4.2.3 Experimental infection
At 20 weeks of age all birds were experimentally infected with *B. intermedia* strain HB60 by crop tube, as in the previous experiments. The birds were dosed daily over five days.

5.4.2.4 Experimental monitoring and post-mortem
The birds were monitored for eight weeks, as previously described, and subjected to post-mortem analysis as in the previous experiments. Viscosity measurements were made on both the ileal and colonic contents.

5.4.3 Results

5.4.3.1 Colonisation with B. intermedia
The results for faecal excretion of *B. intermedia* are presented in Table 5.22. Although colonisation rates were generally low, there was a significant overall effect of diet on colonisation ($\chi 2 = 18.60$; $P = 0.0023$). Subsequent pair-wise comparison of colonisation rates between dietary groups indicated the birds on both the wheat-based diets had significantly more colonisation than birds on barley + enzyme ($P = 0.0181$ and $P = 0.0001$ for the wheat diets with and without enzyme respectively). The birds receiving wheat without enzyme also had significantly more colonisation than the birds fed barley+sorghum without enzyme. For the wheat and the barley diets, addition of enzyme was associated with less colonisation, but the reverse occurred with the barley+sorghum diet.

5.4.3.2 Faecal water content
Group faecal water contents are presented in Table 5.23. No significant group effects were identified.

5.4.3.3 Body weights and egg production parameters
The body weights, total eggs produced and egg weights are presented in Tables 5.24, 5.25 and 5.26 respectively. The only significant group differences in body weight occurred towards the end of the experiment. There was an overall effect when the birds were 26 weeks of age, with birds fed barley + sorghum with enzyme being heavier than the other groups, although not significantly so. At 27 weeks of age this group was significantly heavier than those fed barley without enzyme. At 23 weeks of age the group receiving barley without enzyme produced significantly more eggs than the group receiving wheat without enzyme. No other group differences in egg numbers or weights were significant at any other age.

Table 5.20 Diets used in experiment 4

Raw material	wheat diet %	barley diet %	barley+sorghum diet %
wheat	49.24	.	.
barley	.	50.58	24.65
sorghum	.	.	25.76
choline chloride 75%	0.01	0.01	0.01
groats	15.000	15.000	15.000
lupin kernel	7.00	7.00	7.00
soya-bean meal	11.20	10.00	10.00
meat and bone meal	2.00	2.35	3.50
fishmeal (peruvian)	1.00	1.00	1.00
canola oil	2.00	2.15	1.00
limestone fine	10.40	10.00	10.45
dicalcium phosphate	1.20	1.05	0.75
salt	0.31	0.23	0.24
dl-methionine	0.13	0.13	0.14
l-lysine HCl	0.01	.	.
enzymes premix	0.25	0.25	0.25
household layer premix*	0.25	0.25	0.25
total	**100.00**	**100.00**	**100.00**

Nutrient	analysis	analysis	analysis
soluble NSP from wheat, barley and sorghum (g/kg)	10.59	20.74	10.57
apparent metabolisable energy (kcals/kg)	2,738	2,739	2,735
protein (%)	17.28	17.14	17.00
calcium (%)	4.45	4.29	4.50
total phosphate (%)	0.63	0.61	0.63
available phosphate (%)	0.42	0.40	0.40
Methionine (%)	0.37	0.37	0.37
total sulphur amino acids (%)	0.68	0.68	0.66
lysine (%)	0.79	0.80	0.78
threonine (%)	0.60	0.61	0.60
tryptophan (%)	0.19	0.18	0.18
leucine (%)	1.22	1.20	1.33
fat (%)	4.76	5.10	4.33
linoleic acid (%)	2.11	2.29	1.73
crude fibre (%)	3.03	3.87	3.13
sodium (%)	0.16	0.14	0.16

*Containing minerals and vitamins to meet the nutritional requirements of birds of this age and weight (NRC, 1994)

Table 5.21 NSP analysis of diets used in experiment 4

		g/kg								
Sample ID		Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	
	Sugars	0.17	0.00	0.08	0.30	0.16	1.75	7.34	15.06	**24.87**
wheat+E	sNSP	0.12	0.07	0.13	4.05	5.09	0.31	3.24	7.21	**17.79**
	iNSP	0.64	0.48	0.30	16.04	17.23	2.29	16.60	22.42	**67.72**
	tNSP	0.76	0.55	0.43	20.09	22.32	2.60	19.84	29.63	**85.51**
	Sugars	0.17	0.00	0.08	0.28	0.14	1.58	6.96	13.61	**22.82**
wheat-E	sNSP	0.12	0.04	0.07	3.24	3.78	0.27	3.62	6.66	**15.66**
	iNSP	0.70	0.50	0.33	18.50	20.80	2.50	18.09	23.84	**75.93**
	tNSP	0.82	0.54	0.40	21.74	24.58	2.77	21.71	30.50	**91.59**
	Sugars	0.16	0.00	0.07	0.25	0.10	1.83	7.15	16.93	**26.48**
barley+E	sNSP	0.12	0.05	0.11	2.62	2.73	0.70	2.22	25.61	**30.41**
	iNSP	0.63	0.58	0.38	17.66	26.88	3.00	15.41	31.78	**85.78**
	NSP	0.75	0.63	0.49	20.28	29.61	3.70	17.63	57.39	**116.19**
	Sugars	0.17	0.11	0.08	0.30	0.12	1.83	7.14	16.52	**26.23**
barley-E	sNSP	0.14	0.06	0.11	2.69	2.65	0.59	2.70	25.49	**30.65**
	iNSP	0.70	0.47	0.40	19.25	29.30	3.15	17.78	34.38	**93.90**
	NSP	0.84	0.53	0.51	21.94	31.95	3.74	20.48	59.87	**124.55**
	Sugars	0.18	0.00	0.08	0.36	0.13	1.79	6.92	15.22	**24.68**
barley/sorg+E	sNSP	0.09	0.07	0.08	1.88	1.77	0.49	2.50	16.76	**20.98**
	iNSP	0.65	0.47	0.33	14.58	17.14	2.64	16.57	26.24	**70.12**
	NSP	0.74	0.54	0.41	16.46	18.91	3.13	19.07	43.00	**91.10**
	Sugars	0.17	0.20	0.06	0.25	0.08	1.40	7.04	14.35	**23.45**
barley/sorg-E	sNSP	0.12	0.06	0.08	1.67	1.37	0.38	2.66	16.69	**20.44**
	iNSP	0.61	0.40	0.26	13.79	15.47	2.44	16.90	23.69	**65.61**
	tNSP	0.73	0.46	0.34	15.46	16.84	2.82	19.56	40.38	**86.05**

Notes:
Sugars = Free sugars
E = enzyme
Last column (bold) shows the total NSP values for each row

Table 5.22 Colonisation of infected birds with *B. intermedia* in samples taken between 21 to 27 weeks of age

Wheat + enzyme	Wheat - enzyme	Barley + enzyme	Barley - enzyme	Barley/sorghum + enzyme	Barley/sorghum - enzyme	*P*-value
30/315	42/315	14/315	26/315	27/315	20/315	**0.0023**

5.4.3.4 Post-mortem findings

No pathological changes were found in the birds at post-mortem, and there was no histological evidence of typhlitis.

5.4.3.5 Viscosity of the digesta

Group results for digesta viscosity in the ileum are presented in Table 5.27, and results for the colon in Table 5.28.

There was a highly significant dietary effect at the ileum, with birds fed barley with enzyme having significantly more viscous digesta than birds in all other groups, except for those receiving barley without enzyme. There were no other significant differences between groups. Addition of enzyme was associated with a lower group mean ileal digesta viscosity in birds fed wheat and barley + sorghum, but a higher viscosity in birds fed barley. A regression analysis of sNSP content of the six diets against mean ileal viscosity gave an r squared value of 0.4555, and the slope was not significantly different from zero ($P = 0.1423$).

Viscosity values in the colon were lower than in the ileum, and no significant group differences were detected. Addition of enzyme was associated with a lower mean colonic viscosity in birds fed wheat, and a slightly lower value in those fed barley, but there were no numerical differences for the birds fed barley + sorghum with or without enzyme.

Table 5.23 Weekly group mean and standard deviation faecal water content following allocation of birds into six groups

Age (weeks)	Wheat + enzyme	Wheat - enzyme	Barley + enzyme	Barley - enzyme	Barley+sorghum + enzyme	Barley+sorghum - enzyme	*P*-value
20*	72.54 ± 3.39	75.66 ± 6.57	75.45 ± 6.08	77.72 ± 3.25	75.23 ± 3.26	71.26 ± 7.44	0.0610
21	73.46 ± 9.05	76.28 ± 6.03	77.15 ± 6.02	77.54 ± 5.53	73.78 ± 4.54	75.09 ± 5.01	0.4722
22	76.38 ± 5.52	77.79 ± 5.06	77.02 ± 5.02	79.19 ± 3.55	78.39 ± 3.55	79.88 ± 7.03	0.5534
23	73.95 ± 4.41	76.69 ± 5.16	77.02 ± 3.36	76.98 ± 3.36	75.45 ± 4.60	76.57 ± 5.44	0.5327
24	75.06 ± 6.34	76.70 ± 5.55	73.94 ± 3.85	74.86 ± 4.80	73.80 ± 8.65	74.74 ± 4.36	0.8568
25	73.91 ± 4.69	77.80 ± 4.79	78.08 ± 3.73	73.96 ± 6.70	74.17 ± 5.81	78.36 ± 5.30	0.0679
26	77.97 ± 8.22	75.70 ± 5.22	77.93 ± 2.12	73.60 ± 3.40	76.37 ± 3.91	76.47 ± 4.39	0.2774
27	73.89 ± 6.38	74.87 ± 5.72	76.99 ± 3.83	77.27 ± 6.99	74.85 ± 6.78	75.23 ± 4.86	0.6880

* All birds infected with *B. intermedia* strain HB60 following sampling at 20 weeks

Table 5.24 Weekly group mean and standard deviation body weight (kgs) following allocation of birds into six groups

Bird age (weeks)	Wheat + enzyme	Wheat - enzyme	Barley + enzyme	Barley - enzyme	Barley/sorghum + enzyme	Barley/sorghum - enzyme	*P*-value
20*	1.80 ± 0.15	1.78 ± 0.14	1.76 ± 0.13	1.71 ± 0.07	1.76 ± 0.16	1.73 ± 0.20	0.8105
21	1.83 ± 0.17	1.80 ± 0.14	1.80 ± 0.13	1.76 ± 0.09	1.78 ± 0.16	1.80 ± 0.15	0.8576
22	1.88 ± 0.15	1.88 ± 0.13	1.88 ± 0.12	1.78 ± 0.09	1.87 ± 0.15	1.85 ± 0.18	0.4608
23	1.90 ± 0.10	1.85 ± 0.10	1.88 ± 0.12	1.80 ± 0.09	1.84 ± 0.29	1.85 ± 0.17	0.7763
24	1.90 ± 0.10	1.85 ± 0.11	1.88 ± 0.14	1.82 ± 0.10	1.92 ± 0.14	1.88 ± 0.17	0.5439
25	1.94 ± 0.10	1.88 ± 0.12	1.93 ± 0.13	1.87 ± 0.11	2.00 ± 0.13	1.93 ± 0.16	0.1885
26	1.98 ± 0.09	1.90 ± 0.11	1.98 ± 0.12	1.90 ± 0.11	2.03 ± 0.11	1.96 ± 0.13	**0.0415**[#]
27	2.01 ± 0.08	1.95 ± 0.12	2.03 ± 0.13	1.92[a] ± 0.12	2.07[b] ± 0.12	2.00 ± 0.13	**0.0257**

* All birds infected with *B. intermedia* strain HB60 following sampling at 20 weeks
[ab] Group means with different superscripts differ at the 5% level of significance
[#] Overall significance but no significance between individual columns

Table 5.25 Weekly group mean and standard deviation egg numbers following allocation of birds into six groups

Bird age (weeks)	Wheat + enzyme	Wheat - enzyme	Barley + enzyme	Barley - enzyme	Barley+sorghum + enzyme	Barley+sorghum - enzyme	*P*-value
20*	1.67 ± 2.33	2.08 ± 2.94	1.83 ± 3.13	3.08 ± 3.0	1.67 ± 2.10	2.33 ± 2.90	0.6488
21	1.92 ± 3.00	3.00 ± 3.30	3.25 ± 3.17	4.33 ± 3.31	2.33 ± 2.99	3.67 ± 3.42	0.4845
22	4.33 ± 3.26	3.83 ± 3.43	4.25 ± 3.17	6.08 ± 1.83	3.58 ± 3.03	5.08 ± 2.50	0.3256
23	5.08^{ab} ± 2.64	4.33^{a} ± 2.42	5.42^{ab} ± 2.11	6.83^{b} ± 0.39	4.92^{ab} ± 2.61	6.58^{ab} ± 1.16	**0.0274**
24	5.42 ± 1.93	5.75 ± 1.29	5.92 ± 1.78	6.83 ± .0.39	5.83 ± 2.72	6.58 ± 0.67	0.2905
25	6.33 ± 1.44	6.44 ± 1.42	6.33 ± 1.72	7.00 ± 0.00	5.67 ± 2.67	6.42 ± 1.24	0.5409
26	6.42 ± 1.24	6.75 ± 0.45	6.17 ± 1.99	6.92 ± 0.29	5.75 ± 2.70	6.42 ± 2.02	0.6057
27	5.67 ± 2.15	6.58 ± 0.67	6.75 ± 0.45	6.75 ± 0.45	6.08 ± 2.11	6.42 ± 2.02	0.4613

* All birds infected with *B. intermedia* strain HB60 following sampling at 20 weeks
[ab] Group means with different superscripts differ at the 5% level of significance

Table 5.26 Weekly group mean and standard deviation egg weight following allocation of birds into six groups

Bird age (weeks)	Wheat + enzyme	Wheat - enzyme	Barley + enzyme	Barley - enzyme	Barley+sorghum + enzyme	Barley+sorghum - enzyme	*P*-value
20*	53.86 ± 7.95	47.40 ± 4.10	47.94 ± 7.25	49.04 ± 5.11	43.22 ± 3.68	48.21 ± 2.41	0.1024
21	55.80 ± 5.66	52.26 ± 3.61	47.73 ± 4.49	51.25 ± 6.41	49.75 ± 4.47	50.93 ± 5.74	0.2378
22	54.69 ± 3.73	53.31 ± 2.09	52.30 ± 4.26	51.99 ± 6.21	51.41 ± 3.73	51.99 ± 4.34	0.6968
23	56.22 ± 4.65	56.41 ± 2.90	54.51 ± 3.96	55.01 ± 4.31	54.23 ± 4.25	54.51 ± 3.56	0.6584
24	56.69 ± 3.41	56.73 ± 3.62	56.89 ± 3.27	56.98 ± .3.82	58.07 ± 3.98	55.84 ± 3.46	0.8239
25	60.07 ± 3.77	58.56 ± 3.67	57.06 ± 3.62	58.92 ± 4.45	60.22 ± 4.93	58.24 ± 4.23	0.4551
26	62.79 ± 7.47	60.03 ± 3.28	57.76 ± 3.74	59.66 ± 3.24	60.77 ± 3.33	59.27 ± 3.40	0.1586
27	60.72 ± 3.45	61.56 ± 3.43	58.99 ± 4.26	60.29 ± 4.16	60.64 ± 3.38	59.71 ± 3.38	0.6399

* All birds infected with *B. intermedia* strain HB60 following sampling at 20 weeks

Table 5.27 Viscosity of ileal contents at post-mortem

Wheat + enzyme	Wheat - enzyme	Barley + enzyme	Barley - enzyme	Barley+sorghum + enzyme	Barley+sorghum - enzyme	*P*-value
$1.69^{a} \pm 0.28$	$1.95^{a} \pm 0.22$	$2.41^{b} \pm 0.70$	$2.01^{ab} \pm 0.13$	$1.61^{a} \pm 0.20$	$1.84^{a} \pm 0.40$	**<0.0001**

[ab] Group means with different superscripts differ at the 5% level of significance

Table 5.28 Viscosity of colon contents at post-mortem

Wheat + enzyme	Wheat - enzyme	Barley + enzyme	Barley - enzyme	Barley+sorghum + enzyme	Barley+sorghum - enzyme	*P*-value
1.48 ± 0.21	1.77 ± 0.45	1.62 ± 0.41	1.72 ± 0.24	1.48 ± 0.36	1.47 ± 0.25	0.1499

5.4.4 Discussion

In this experiment, six diets were used that were predicted to differ in their NSP content. In particular, a diet containing barley was selected, since it was calculated that this should contain about twice as much sNSP as the wheat-based diet and the barley + sorghum diet that were also used. In the event, the sNSP content of the barley-based diet as assessed by chemical analysis was about twice that of the wheat based diet, whilst the sNSP content of the barley + sorghum diet was a little higher than that in the wheat diets (Table 5.21). In comparison to the sNSP contents, the insoluble NSP and the total NSP contents of the barley-based diet were only about 20% higher than those of the other two diets. Hence, in relation to NSP, the difference in the diets mainly resided in their sNSP content. Interestingly, addition of the enzymes to the diets had little or no effect on their NSP contents, possibly because the enzymes are not fully activated in the absence of moisture.

The barley-based diets, with the greatest sNSP content, also resulted in the greatest viscosity of the ileal digesta. The values for the barley + enzyme diet were significantly higher than those of the other groups, except for barley without enzyme. This positive association between high sNSP content and high ileal digesta viscosity was expected, although over all six diets the relationship between sNSP content and ileal viscosity was not linear. For example, the wheat-based diet without enzyme had about half the sNSP content of the barley diet without enzyme, but the viscosity of the ileal contents in birds on the two diets was very similar. It may be that specific sub-fractions of the sNSP, or other factors or components of the diet besides sNSP, apparently can influence digesta viscosity values in the ileum of layer birds.

Another important observation was that the addition of the recommended dietary enzymes at the recommended doses to the three diets had no significant effect on the viscosity of the digesta in the ileum or colon. This is consistent with the findings for the wheat diet in experiment 3, and with the results of Roberts (2003). There was a tendency for birds fed the wheat diet to have lower viscosities in the ileum and colon if their diets were supplemented with xylanase, but in the case of the barley diet viscosities were actually higher in the ileal contents in birds receiving α-amylase and β-gluconase than in those not receiving enzymes. It should be noted that the diets used in this work were not steam pelleted, and hence the absence of a clear effect of exogenous enzyme supplementation on viscosity (and other parameters) may have been due to the dominant effect of endogenous enzyme activity already present in the grains. Further work is required in the area of the effect of enzyme supplementation on colonisation, preferably concentrating on examining the optimisation of individual enzymes for individual diets. This sort of study should be done using much larger groups of birds.

A highly significant dietary effect on colonisation with *B. intermedia* was found in this experiment, with birds fed wheat, with or without enzyme added, being more commonly colonised than birds fed other diets, with this difference being significant compared to birds fed barley and enzyme. Hence, the birds with the lowest dietary sNSP (wheat) had the greatest colonisation, and those with the highest sNSP (barley) had the least colonisation. Regression analysis of sNSP content against colonisation (number of sample days culture positive) gave an r squared value of 0.4731, with the slope of the curve being not significantly different from zero ($P = 0.1310$). The sNSP content of the diet therefore did not predict colonisation rates. The wheat diet did result in a relatively high ileal viscosity, but an overall regression of ileal viscosity values against colonisation also did not reveal a significant relationship between the two. The r squared value for the regression was 0.2832 ($P = 0.3649$). Hence this experiment failed to show a clear relationship between viscosity of the ileal contents and colonisation with *B. intermedia*, but it did suggest a link between wheat-based diets and spirochaete colonisation. The specific properties of the wheat-based diets that have this effect remain unknown, and require more detailed analysis. It is possible that wheat has some other rather specific effects on physicochemical properties of the intestinal tract, or on components of the intestinal microflora, which in turn predispose to colonisation by intestinal spirochaetes. A better understanding of these interactions could greatly assist the development of control strategies for these bacteria, and possibly even for other enteric pathogens.

It should be noted that the dietary effects that were observed in this experiment may have been a response to variable feed intake, which was not recorded.

The dietary enzyme preparation used with the wheat diet in the current experiment did not significantly influence colonisation. This finding was in agreement with the results of experiment 3, but contrary to previous experimental findings (Hampson *et al.,* 2002). In both the previous study and in experiment 3, a different enzyme preparation was used (Avizyme® 1302: which included a protease with the xylanase) to that used in the current experiment. Although enzyme differences in these three experiments might have influenced the outcomes, the mixed results even when using Avizyme® 1302 suggest that addition of dietary enzymes is unlikely to give effective and reproducible control of *B. intermedia* infections in layers fed mash diets. A better recommendation might be to try to avoid diets using wheat, or to try to reduce the wheat content of layer diets on farms where AIS is a problem.

Neither infection with *B. intermedia* nor feeding the various dietary combinations had much influence on production parameters in the current experiment. This may reflect the small group sizes, the relatively stress-free and uncrowded environment, the effects of endogenous enzymes in the grains included in the mash diets fed and the relatively short period over which the experiment was run. Had the experiments been conducted under commercial conditions, greater differences may have been seen.

6. Implications

This study has shown that flocks can vary greatly in their AIS status, and that infection with intestinal spirochaetes can rapidly disappear from previously infected flocks. The spirochaetes are very labile in the environment and are sensitive to the commonly used disinfectants, hence it should be easy to remove potential environmental sources of infection. The main source of infection appears to be from older flocks of birds on the same site, and it should be possible to prevent spread of infection to new flocks, and indeed to entirely eradicate the infection from a site, by paying close attention to biosecurity measures designed to prevent cross-transmission of infection from older to younger flocks.

This project demonstrated that there are dietary influences on infection of layer hens with both *B. intermedia* and *B. pilosicoli*. Hence, where AIS is a problem it is worth considering modifying the diet to try to reduce the level of infection in the flock. Diets based on wheat appear to predispose to infection with *B. intermedia*, and so substitution of wheat with other cereals in the diets of such flocks should be considered. It appears that different wheats may have different influences on *B. intermedia* and *B. pilosicoli*, although this observation needs to be confirmed. The sNSP content of the diet is not the main influence on colonisation, and the viscosity of the ileal contents is also not a good predictor of whether or not birds will be susceptible to infection. Similarly, the sNSP content of the diet is not a good predictor of the ileal viscosity. Addition of dietary enzymes did not significantly affect the viscosity of the ileal contents in layer hens under the experimental conditions used, and addition of dietary enzyme was not obviously helpful in reducing the susceptibility of the birds to infection with *B. intermedia.*

7. Recommendations

- In order to control AIS at the flock level, incoming birds should be from a source that is not infected with intestinal spirochaetes, sheds should be thoroughly cleaned, disinfected and rested between batches of birds, and strict biosecurity measures implemented to prevent transmission of infection from sheds of older birds to new flocks.
- In flocks with persistent problems with AIS consideration should be given to modifying the diets being used. However, the outcome would appear to be somewhat unpredictable. Where possible, cereals other than wheat should be used.
- Addition of dietary enzyme is not recommended as a sole means of controlling AIS.

8. References

Atyeo, R.F., Oxberry, S.L. and Hampson, D.J. (1996). Pulsed-field gel electrophoresis for sub-specific differentiation of *Serpulina pilosicoli* (formerly "*Anguillina coli*"). *FEMS Microbiology Letters* **141**: 77-81.

Atyeo, R.F., Oxberry, S.L., Combs, B.G. and Hampson, D.J. (1998). Development and evaluation of polymerase chain reaction tests as an aid to the diagnosis of swine dysentery and intestinal spirochaetosis. *Letters in Applied Microbiology* **26**: 126-130

Atyeo, R.F., Stanton, T.B., Jensen, N.S., Suriyaarachichi, D.S. and Hampson, D.J. (1999). Differentiation of *Serpulina* species by NADH oxidase gene (*nox*) sequence comparisons and *nox*-based polymerase chain reaction tests. *Veterinary Microbiology* **67**: 47-60.

Bedford, M.R. and Schulze, H. (1998). Exogenous enzymes for pigs and poultry. *Nutritional Research Reviews* **11**: 91-114.

Boye, M., Baloda, S.B., Leser, T.D. and Moller, K. (2001). Survival of *Brachyspira hyodysenteriae* and *B. pilosicoli* in terrestrial microcosms. *Veterinary Microbiology* **81**: 33-40.

Chia, S.P. and Taylor, D.J. (1978). Factors effecting the survival time of *Treponema hyodysenteriae* in dysenteric faeces. *Veterinary Record* **103**: 68-70.

Davelaar, F.G., Smit, H.F., Hovind-Hougen, K., Dwars, R.M. and van der Valk, P.C. (1986). Infectious typhlitis in chickens caused by spirochaetes. *Avian Pathology* **15**: 247-258.

Dwars, R.M., Smit, H.F., Davelaar, F.G. and Veer, W van T. (1989). Incidence of spirochaetal infections in cases of intestinal disorder in chickens. *Avian Pathology 18,* 591-595.

Dwars, R.M., Smit, H.F. and Davelaar, F.G. (1990). Observations on avian intestinal spirochaetosis. *Veterinary Quarterly* **12**: 51-55.

Dwar, R.M., Davelaar, F.G. and Smit, H.F. (1992). Influence of infection with avian intestinal spirochaetes on the faeces of laying hens. *Avian Pathology* **21**: 427-429.

Dwars, R.M., Davelaar, F.G. and Smit, H.F. (1993). Infection of broiler parent hens (*Gallus domesticus*) with avian intestinal spirochaetes: effects on egg production and chick quality. *Avian Pathology* **22**: 693-701.

Griffiths, I.B., Hunt, B.W., Lister, S.A. and Lamont, M.H. (1987). Retarded growth rate and delayed onset of egg production associated with egg production in pullets. *Veterinary Record* **121**: 35-37.

Hampson, D.J. and McLaren, A.J. (1999). Experimental infection of laying hens with *Serpulina intermedia* causes reduced egg production and increased faecal water content. *Avian Pathology* **28**: 113-117.

Hampson, D.J., Phillips, N.D. and Pluske, J.R. (2002). Dietary enzyme and zinc bacitracin inhibit colonisation of layer hens by the intestinal spirochaete *Brachyspira intermedia. Veterinary Microbiology* **86**: 351-360.

Jamshidi, A. and Hampson, D.J. (2002). Zinc bacitracin enhances colonisation by the intestinal spirochaete *Brachyspira pilosicoli* in experimentally infected layer hens. *Avian Pathology* **31**: 293-298.

Jenkinson, S.R. and Wingar, C.R. (1981). Selective medium for the isolation of *Treponema hyodysenteriae*. *Veterinary Record* **109**: 384-385.

Kim, J.C., Mullan, B.P., Simmins, P.H. and Pluske J.R. (2003). Variation in the chemical composition of wheats grown in Western Australia as influenced by variety, growing region, season, and post-harvest storage. *Australian Journal of Agricultural Research* **54**: 541-550.

Kunkle, R.A., Harris, D.L. and Kinyon, J.M. (1986). Autoclaved liquid medium for propagation of *Treponema hyodysenteriae*. *Journal of Clinical Microbiology* **24**: 669-671.

La, T., Phillips, N.D. and Hampson, D.J. (2003). Development of a duplex PCR assay for detection of *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* in pig feces. *Journal of Clinical Microbiology* **41**: 3372-3375.

McDonald, D.E., Pethick, D.W., Mullan, B.P. and Hampson, D.J. (2001). Increasing the viscosity of the intestinal contents alters small intestinal structure and intestinal growth, and stimulates proliferation of enterotoxigenic *Escherichia coli* in newly weaned pigs. *British Journal of Nutrition* **86**: 487-498.

McLaren, A.J., Hampson, D.J. and Wylie, S.L. (1996). The prevalence of intestinal spirochaetes in poultry flocks in Western Australia. *Australian Veterinary Journal* **74**: 319-320.

McLaren, A.J., Trott, D.J., Swayne, D.E., Oxberry, S.L. and Hampson, D.J. (1997). Genetic and phenotypic characterisation of intestinal spirochetes colonizing chickens, and allocation of known pathogenic isolates to three distinct genetic groups. *Journal of Clinical Microbiology* **35**: 412-417.

NRC (1994). Nutrient Requirements of Poultry: Ninth Revised Edition. National Research Council. National Academy Press, Washington D.C. USA.

Ochiai, S., Adachi, Y., and Mori, K. (1997). Unification of the genera *Serpulina* and *Brachyspira*, and proposals of *Brachyspira hyodysenteriae* comb. nov., *Brachyspira innocens* comb. nov. and *Brachyspira pilosicoli* comb. nov. *Microbiology and Immunology* **41**: 445-452.

Oxberry, S.L., Trott D.J. and Hampson, D.J. (1998). *Serpulina pilosicoli*, water birds and water: potential sources of infection for humans and other animals. *Epidemiology and Infection* **121**: 219-225.

Pluske, J.R., Siba, P.M., Pethick, D.W., Durmic, Z., Mullan, B.P. and Hampson, D.J. (1996). The incidence of swine dysentery in pigs can be reduced by feeding diets that limit fermentation in the large intestine. *Journal of Nutrition* **126***:* 2920-2933.

Roberts, J.R. (2003). Effects of commercial feed enzymes in wheat-based diets on egg and egg shell quality in imported strains of laying hen. Publication No. 03/02, Australian Egg Corporation Limited, Hurstville, NSW.

Ruano, M., El-Attrache, J. and Villegas, P. (2001). Efficacy comparisons of disinfectants used by the commercial poultry industry. *Avian Disease* **45**: 972-977.

Siba, P.M., Pethick, D.W. and Hampson, D.J. (1996). Pigs experimentally infected with *Serpulina hyodysenteriae* can be protected from developing swine dysentery by feeding them a highly digestible diet. *Epidemiology and Infection* **116**: 207-216.

Smit, H.F., Dwars, R.M., Davelaar, F.G. and Wijtten, A.W. (1998). Observations on the influence of intestinal spirochaetosis in broiler breeders on the performance of their progeny and on egg production. *Avian Pathology* **27**: 133-141.

Spiller, G.A. (1993). CRC handbook of dietary fibre and human nutrition. Boca Raton, Florida: CRC Press, Inc.

Stanton, T.B., Postic, D. and Jensen, N.S. (1998). *Serpulina alvinipulli* sp. nov., a new *Serpulina* species enteropathogenic to chickens. *International Journal of Systematic Bacteriology* **47**: 1007-1012.

Stephens, C.P. and Hampson, D. J. (1999). Prevalence and disease association of intestinal spirochaetes in chickens in eastern Australia. *Avian Pathology* **28**: 447-454.

Stephens, C.P. and Hampson, D.J. (2001). Intestinal spirochaete infections in chickens: a review of disease associations, epidemiology and control. *Animal Health Research Reviews* **2**: 83-91.

Stephens, C.P. and Hampson, D.J. (2002). Experimental infection of broiler breeder hens with the intestinal spirochaete *Brachyspira* (*Serpulina*) *pilosicoli* causes reduced egg production. *Avian Pathology* **31**: 169-175.

Suriyaarachichi, D.S., Mikosza, A.S.J., Atyeo, R.F. and Hampson, D.J. (2000). Evaluation of 23S rDNA polymerase chain reaction assay for identification of *Serpulina intermedia*, and strain typing using pulsed-field gel electrophoresis. *Veterinary Microbiology* **71**: 139-148.

Swayne, D.E. (1997). Avian intestinal spirochaetosis. In: B.W. Calneck (Ed.), Diseases of Poultry. Tenth Edition. Ames: Iowa State University Press pp 325-332.

Swayne, D.E., Bermudez, A.J., Sagartz, J.E., Eaton, K.A., Monfort, J.D., Stoutenberg, J.W. and Hayes, J.R. (1992). Association of cecal spirochaetes with pasty vents and dirty eggshells in layers. *Avian Diseases* **36**: 776-781.

Swayne, D.E., Eaton, K.A., Stoutenburg, J., Trott, D.J., Hampson, D.J. and Jensen, N.S. (1995). Identification of a new intestinal spirochete with pathogenicity to chickens. *Infection and Immunity* **63**: 430-436.

Trampel, D.W., Jensen, N.S. and Hoffman, L.J. (1994). Cecal spirochaetosis in commercial laying hens. *Avian Diseases* **38**: 895-898.