FINAL REPORT

TO

THE RURAL INDUSTRIES RESEARCH AND DEVELOPMENT

CORPORATION

(For The Egg Industry Research and Development Committee)

Prevalence, genetic relationships and pathogenicity of intestinal spirochaetes infecting Australian poultry.

UMU 2E

By

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EXECUTIVE SUMMARY

This project was stimulated by reports from Europe and the USA that fastidious anaerobic intestinal spirochaetal bacteria had been found associated with diarrhoea and loss of production in laying birds. The bacteria were poorly defined, but were known to be distinct from the important pig pathogenic intestinal spirochaete, *Serpulina hyodysenteriae*, the agent of swine dysentery.

The project had three main aims:

i) To determine whether similar bacteria occurred in Australia, how common they were, and whether there presence was associated with disease,

ii) To isolate, characterise and identify Australian and overseas isolates, and to compare these,

iii) To test the pathogenic potential of selected isolates by experimentally inoculating chicks and laying hens, and recording the outcome.

These main aims and their subcomponents were successfully achieved.

To address the first aim a prevalence survey was conducted in Western Australia (WA), with 410 faecal samples cultured from birds in 37 layer flocks, and, for comparison, 157 samples cultured from 30 broiler breeder flocks. Spirochaetes were cultured on selective agar under anaerobic conditions. Colonisation with intestinal spirochaetes was shown to be common, with 35% of layer flocks and 53.3% of broiler breeder flocks being culture positive. Furthermore, a statistically significant association was found between colonisation and poor performance: the bacteria were isolated from 64% of 25 flocks recording signs of diarrhoea or reduced production, but only from 28% of 25 flocks considered clinically normal. The first aims of the project were therefore fulfilled.

To address the second aim, a total of 56 isolates were examined in detail. These comprised 39 isolates from WA, three from Queensland, seven from the Netherlands, six from the USA, and one from the UK. Each isolate was characterised by a series of appropriate phenotypic tests, and was analysed genetically using multilocus enzyme electrophoresis. The 16S ribosomal RNA gene sequences of 10 selected isolates also were determined, and aligned. The isolates were divided into 40 different electrophoretic types, distributed through six major genetic groups, each of which may represent a distinct species. Overseas isolates that have been considered to be pathogenic were heterogenous, belonging to three of these groups. 16S rDNA sequence results

confirmed these all belonged in the genus *Serpulina*. Clearly, poultry may be colonised by a diverse range of intestinal spirochaetes.

To address the third aim, seven Australian isolates were tested for their pathogenic potential in groups of 20 day-old broiler chicks, and one was also tested in 10 adult birds approaching lay. Three isolates of "*S. intemedia*", three from unnamed group d, one *S. pilosicoli* isolate and one control porcine *S. innocens* isolate were tested in chicks, and "*S. intermedia*" isolate HB60 was tested in layers. The chicks were killed after three weeks, and subjected to full postmortem examination and bacterial culture of their caecal contents. The adult birds were killed after 16 weeks, with daily egg production and weights recorded from 20 weeks of age.

Infection of chicks with "*S. intermedia*" strains and *S. pilosicoli* resulted in diarrhoea within 7-9 days of inoculation, and significant depressions in growth rate. Birds inoculated with the unnamed group developed diarrhoea only after 12-13 days, and did not show growth depression. *S. innocens* failed to colonise any bird. The *S. pilosicoli* strain attached by one cell end to the caecal epithelium, whilst the other strains were unattached in the caecal lumen and crypts. In adult birds the "*S. intermedia*" strain caused a significant increase in faecal moisture content, and a small reduction in body weight. Infected birds produced significantly fewer eggs, and these were lighter than those from uninfected control birds. "*S. intermedia*" was still present in the caecal contents of infected birds at slaughter.

These results demonstrate that intestinal spirochaetes from three different groups have pathogenic potential in chicks, causing diarrhoea, and that infection with "*S. intermedia*" can cause wet faeces and significant loss of egg production in laying birds. It was calculated that the value of national annual losses of egg production attributable to "*S. intermedia*" alone was estimated at $1,706,740.

Further work is now required to investigate means to control these common but previously unrecognised pathogens of Australian poultry.

GENERAL PROJECT INFORMATION

Project title: Prevalence, genetic relationships and pathogenic potential of intestinal spirochaetes infecting Australian poultry.

Project Number: UMU 2E

Background:

Intestinal spirochaetal bacteria colonise the large intestine of a variety of animal species. In the pig, the strongly haemolytic spirochaete *Serpulina hyodysenteriae* is the aetiological agent of swine dysentery (Taylor and Alexander 1971; Harris *et al* 1972; Stanton 1992), whilst the weakly haemolytic *Serpulina innocens* is considered to be a normal component of the intestinal microflora (Kinyon and Harris 1979; Stanton 1992). Other weakly haemolytic spirochaetes have been implicated in a condition called "intestinal spirochaetosis" in pigs (Taylor *et al* 1980; Lee *et al* 1993), human beings (Harland and Lee 1967; Sanna *et al* 1984; Lee and Hampson 1994), and dogs (Pindak *et al* 1965; Duhamel *et al* 1995). Other animal species, including rodents and primates, also may be colonised by intestinal spirochaetes (Takeuchi and Zeller 1972; Lee and Phillips 1978; Cowley and Hill 1986).

The colonisation of birds by intestinal spirochaetes was first reported in the USA in 1930 (Harris 1930). Three types of spirochaetes were described, differing mainly in size. A subsequent report described spirochaetes located in nodules in the caeca of turkeys, chickens, and pheasants (Mathey and Zander 1955).

Interest in intestinal spirochaetal infections of chickens was rekindled in the mid-1980s, following the publication of a series of reports implicating these bacteria as a cause of diarrhea, reduced egg production and/or fecal staining of eggshells in layer flocks in the Netherlands (Davelaar *et al*, 1986; 1989), England (Griffiths *et al*, 1987), and the USA (Swayne *et al*, 1992; Trampel *et al*, 1994). Remarkably, up until now these bacteria have remained poorly characterised, and it is not clear whether the various reports even refer to spirochaetes of the same species. Although some isolates have been used in experimental reproduction of disease in both Europe and the USA, again it was not known how these strains were related to each other, if at all.

Up until now no attempt has been made to look for intestinal spirochaetes infecting Australian poultry, and, were these organisms present, their exact identity and clinical significance would be uncertain. The current project was designed to resolve these questions which potentially are of considerable significance to the Australian egg industry.

Objectives:

The objectives of the project were to:

I) Determine whether intestinal spirochaetes naturally infect Australian chickens

ii) Determine the prevalence of such infections on a flock basis

iii) Assess disease associations where natural infection were found to occur

iv) Develop means to identify and characterise intestinal spirochaetes from chickens

v) Apply these techniques to Australian and overseas isolates to establish their identity and diversity

vi) Test the pathogenic potential of Australian isolates

Outcomes:

At the end of this project it was hoped to have a clearer understanding of whether and to what extent infection with intestinal spirochaetes was reducing production in the Australian egg industry, so that appropriate measures could be developed to prevent such losses.

CHAPTER ONE
THE PREVALENCE OF INTESTINAL SPIROCHAETES IN POULTRY FLOCKS IN WESTERN AUSTRALIA

1.1 Introduction.

Interest in intestinal spirochaetal infections of poultry was stimulated in 1986 when Davelaar and colleagues in the Netherlands used an unabsorbed fluorescent antiserum to *Serpulina hyodysenteriae* to demonstrate intestinal spirochaetes in the caeca of laying hens with diarrhoea (Davelaar *et al* 1986). These spirochaetes were weakly haemolytic on blood agar and did not produce indole. Subsequently, Griffiths *et al* (1987) reported spirochaetes in the caecal contents of laying hens showing reduced growth rates and delayed onset of egg production. Dwars *et al* (1989) used unabsorbed fluorescent antisera against two avian intestinal spirochaetes to demonstrate intestinal spirochaetes in samples from 37/134 (27.6%) flocks with diarrhoea or low production, but from only 2/45 (4.4%) flocks that had no signs of disease.

Two recent reports have described the isolation of intestinal spirochaetes from laying flocks. In the first, the spirochaetes were located in a tangled mass in the caecal crypts and lumen (Swayne *et al* 1992). Infected birds had wet faeces surrounding the vent, and produced eggs that were stained with faeces, although egg production was not affected. In the second report, spirochaetes were attached end-on to the caecal enterocytes of 25 % of chickens belonging to a large flock showing a 5% decrease in egg production (Trampel *et al* 1994). A spirochaete also has been described in the caeca of rheas (*Rhea americana*) with typhlocolitis (Sagartz *et al* 1992). To date, no investigations have been conducted to determine whether intestinal spirochaetes colonise birds in poultry flocks in Australia. The current study was designed to determine the prevalence of infection by these bacteria in flocks in Western Australia (WA).

1.2. Materials and methods.

Samples of caecal faeces were collected from chickens on farms in WA, in collaboration with field staff from Agriculture Western Australia. In total, 410 faecal samples were obtained from 37 layer flocks, and 157 faecal samples from 30 broiler breeder flocks. Forty whole caeca from a broiler

breeder flock in WA, and 13 whole caeca from a broiler breeder flock with reduced production in New South Wales (NSW), also were submitted for testing**.** The number of samples received from each flock ranged from 10 to 40 for broiler breeder flocks and 10 to 20 for layer flocks. The occurrence of diarrhoea or decreased production in the flocks at the time the samples were collected was recorded, where this information was available.

Faecal material or caecal mucosal scrapings were plated onto Trypticase Soy agar (BBL) containing 5% defibrinated bovine blood, 400 µg/mL spectinomycin and 25 µg/mL each of colistin and vancomycin, and were incubated at 37^{o}C in an atmosphere of 94% N_2/6% CO_2. Plates were examined for spirochaetal growth after 5 and 7 days. The presence of spirochaetes was confirmed by phase contrast microscopy. The amount of beta-haemolysis on blood agar was determined by comparison with reference strains of *S. hyodysenteriae* and *S. innocens*.

Selected isolates were subcultured into pre-reduced Trypticase Soy broth (BBL) supplemented with 2% foetal calf serum and a 1% cholesterol solution (Kunkle *et al* 1986). To test for indole production, 1 to 2 mL of xylene and 4 drops of Kovac's reagent were added to 5 mL of broth culture and shaken. A red upper phase indicated that the isolate produced indole. Isolates in broth also were stored at -80^{o}C for later analysis.

2.3 Results.

No spirochaetes were isolated from the samples from the NSW flock. The results of culture of the WA samples are presented in Table 1. Intestinal spirochaetes were found to often colonise poultry in WA, with 16/30 broiler breeder flocks and 13/37 layer flocks being positive for intestinal spirochaetes when samples were cultured on the media described above (Table 1). One layer flock was tested three times, and one broiler breeder flock was tested twice, with samples taken up to four months apart. Intestinal spirochaetes were isolated from both of these flocks on each occasion. For individual flocks that tested positive for spirochaetes, the proportion of positive samples ranged from 2/10 to 38/40 for broiler breeder flocks and from 1/10 to 9/10 for layer flocks.

Intestinal spirochaetes were isolated from 6/12 (50%) layer flocks showing signs of either diarrhoea or reduced production, compared to only 5/23 (21.7%) flocks that were apparently disease free (Table 2). This

difference just failed to reach significance in the chi-square test, with chi^2=2.92 (P<0.10). Unfortunately, information on the occurrence of disease was not available for 15/30 broiler breeder flocks in this study, so there were too few flocks for statistical analysis. When the results for both layer and broiler breeder flocks for which an adequate history was available were combined, however, intestinal spirochaetes were isolated from 16/25 (64%) flocks with disease signs, compared to only 7/25 (28%) flocks which appeared clinically normal. This difference was statistically significant in the chi-square test (P<0.02). A similar relationship between colonisation with intestinal spirochaetes and signs of disease was found in the Dutch study mentioned earlier (Dwars *et al* 1989), although the prevalence of colonisation in that study was lower than found in WA. This difference may have been influenced by the different methods used to detect the spirochaetes (that is, the use of immunofluorescence compared to culture in the current study).

A higher rate of colonisation was found in birds from layer flocks with wet litter (5/8), than in those with both wet litter and reduced production (1/4) (Table 2). The significance of this result is uncertain as only a small number of flocks with disease problems were available for testing.

There was no significant difference in the ages of the flocks from which spirochaetes were recovered, compared to those from which spirochaetes were not recovered. The significance of treatment with antibiotics prior to sample collection could not be adequately assessed as this information was available for only a few flocks.

With one exception, the spirochaetes isolated were weakly beta-haemolytic on blood agar; the other was strongly beta-haemolytic, and was isolated from a broiler breeder flock. The production of indole by these isolates varied, with 19 of 52 (36.5%) isolates tested being indole positive, including the strongly beta-haemolytic isolate. Strong beta-haemolysis on blood agar and the production of indole are characteristics of *Serpulina hyodysenteriae* (Stanton 1992). Previously, *S hyodysenteriae* has been shown to be pathogenic for day-old chicks in experimental infections (Sueyoshi and Adachi 1990), but has never been recovered from commercial poultry.

Given the high prevalence of infection with intestinal spirochaetes in WA poultry flocks, it is possible that some of the spirochaetes isolated may be

commensals, and not responsible for any disease condition. This is the case with many porcine spirochaetal isolates (Kinyon and Harris 1979; Lee *et al* 1993), and in part may obscure the significance of the relationship between pathogenic spirochaetes and disease. The fact that spirochaetes were isolated from two flocks on several occasions up to four months apart suggests that spirochaetal infections may persist for some time.

1.4. Discussion.

This is the first published study that has used culture to determine the prevalence of infection with intestinal spirochaetes in poultry. The results of this study show a high prevalence of infection, and a significant relationship between infection and signs of diarrhoea and decreased production in poultry flocks in WA. Samples from the NSW flock did not contain spirochaetes, although intestinal spirochaetes have been isolated from poultry in Queensland (CP Stephens 1994, personal communication). It is not certain that the spirochaetes isolated in this study are the cause of the intestinal problems observed in the flocks from which they were recovered, nor is it known whether the spirochaetes that have been recovered represent a single species. However, the recovery of an isolate resembling the porcine pathogen *Serpulina hyodysenteriae* from a flock with production problems is of particular interest. In subsequent chapters, studies involving genetic characterisation of the isolates, and experimental infection of day-old and adult birds, are described.

Table 1 Results of the culture of faecal samples from both layer and broiler breeder flocks for the presence of intestinal spirochaetes.

	Number of flocks tested	Number culture positive
Broiler breeder	30	16 (53.3%)
Layer	37	13 (35.1%)
Total	67	29 (43.3%)

Table 2 Association between the isolation of intestinal spirochaetes and signs of disease*.

		Broiler breeder flocks		Layer flocks	
		Number tested	Number culture positive	Number tested	Number culture positive
Disease signs	- wet litter	3	2 (66.6%)	8	5 (62.5%)
	- reduced production	6	5 (83.3%)	0	0
	- both	4	3 (62.5%)	4	1 (25%)
Total		13	10 (76.9%)	12	6 (50%)
No disease		2	2 (100%)	23	5 (21.7%)

* Only those flocks for which information on disease signs was available were included in this analysis.

CHAPTER TWO

GENETIC ANALYSIS OF INTESTINAL SPIROCHAETES ISOLATED FROM CHICKENS, AND THE ASSOCIATION OF THREE GENETIC GROUPS WITH DISEASE.

2.1 Introduction

A variety of poorly characterised spirochaetes inhabit the gastrointestinal tract of poultry, and infection appears to be common. For example, Dwars *et al* (1989) demonstrated intestinal spirochetes in 37 of 134 (27.6 %) samples from European layer flocks with diarrhoea or reduced production, although only in two of 45 (4.4%) samples from flocks with no disease signs. In the study in Western Australia (chapter 1), faecal samples from 16 of 30 (53.3%) broiler breeder and 13 of 37 layer flocks (35.1%) contained spirochaetes, and again those flocks with a history of either diarrhoea or reduced production were significantly more likely to contain birds infected with spirochaetes than were apparently healthy flocks (McLaren *et al*, 1996).

Despite the potential clinical importance of these isolates, only two appear to have been studied in detail. Swayne *et al* (1995) compared the morphology, biochemical characteristics and rRNA gene restriction patterns of chicken isolates C1 and C2, isolated from the same flock, with those of the type strains of *Serpulina hyodysenteriae* (B78^T), the aetiologic agent of swine dysentery (Stanton, 1992), and *S. innocens* (B256^T), a commensal of swine (Kinyon and Harris, 1979). In the same study, multilocus enzyme electrophoresis (MEE) was used to compare these two strains with 188 porcine intestinal spirochaetes. The morphology of the chicken strains was very similar to that of the porcine *Serpulina* strains, and they were identical to B256^T in the biochemical tests conducted, being weakly ß-haemolytic, indole negative and lacking a-galactosidase and a-glucosidase activities. However, they differed in rRNA gene restriction pattern, and the results of the MEE analysis showed that they belonged to their own distinct genetic group (Swayne *et al*, 1995). A separate study using MEE and 16S rRNA gene sequence analysis also placed C1 in its own genetic group in the genus *Serpulina*, with sequence similarities of 98.9% and 98.6% respectively with B78^T and B256^T (Stanton *et al*, 1996).

The purpose of the current study was to characterise a large collection of intestinal spirochaetes isolated from commercial chicken flocks in Australia, the United States and Europe, so as to determine their diversity and, where

information was available, the association of specific genetic groups with disease. The isolates examined included a number previously shown to be pathogenic in experimental infections. The Western Australian (WA) isolates were selected as representative isolates of those collected in the prevalence survey of intestinal spirochetes in WA poultry flocks (McLaren *et al*, 1996; chapter one), whilst the other isolates were selected on the basis of their availability in culture collections. The results of MEE analysis, 16S ribosomal RNA gene sequence analysis, biochemical tests, and transmission electron microscopy on these isolates are described.

2.2 Materials and methods

Spirochaetes. Intestinal spirochaetes examined in this study were isolated either from faecal samples, caecal mucosal scrapings or cloacal swabs from chickens in commercial flocks. Fifty six intestinal spirochaetes were analysed: 39 from WA, three from Queensland, seven from the Netherlands, six from the United States and one from the United Kingdom (UK) (Table 3). Thirty of the 39 (77%) WA isolates were recovered from birds in flocks with either diarrhoea (which caused problems of wet litter, and/or faecal staining of egg shells) or reduced production (McLaren *et al*, 1996), as were all of the isolates from the United States (Trampel *et al*, 1994; Swayne *et al*, 1995). Strain 1380 was isolated from a bird in a broiler flock with diarrhoea in the Netherlands (Dwars *et al*, 1990). No information was available concerning the disease status of the flocks from which the other strains from the Netherlands, the strains from Queensland, or the strain from the UK were isolated. Isolates from the Netherlands were provided by H. Smit, Trepo Ltd., Wilnis, the Netherlands, isolates 308.93 and 42167 from the USA by N. S. Jensen, National Animal Disease Center, Ames, Iowa, the isolates from Queensland by C. P. Stephens, Regional Veterinary Laboratories, Toowoomba, Queensland, and the isolate from the UK by R. Sellwood, Institute for Animal Health, Compton, England.

Primary isolation of spirochaetes was either on Trypticase Soy agar (BBL) plates, designed initially for the isolation of porcine intestinal spirochetes (Davelaar *et al*, 1986; McLaren *et al*, 1996), or on Brucella agar (Swayne *et al*, 1992). These media contained 5% (or 10% for the isolates from the USA) defibrinated ovine blood, 400 µg/ml spectinomycin (Sigma), and, for the WA and UK isolates, 25 µg/ml each of colistin and vancomycin (Sigma). Plates were incubated for up to five days at either 37°C in an atmosphere of 94%

N_2/6% CO_2, or at 42°C in an atmosphere of 80% H_2/20% CO_2 (Davelaar *et al*, 1986;Swayne *et al*, 1992; McLaren *et al*, 1996).

For use in phenotypic tests, spirochaetes were grown in prereduced anaerobic Trypticase Soy broth (BBL) containing 2% foetal calf serum and 1% ethanolic cholesterol, as previously described (Kunkle *et al*, 1986).

MEE. The culture of isolates, preparation of cells, and their MEE analysis all followed the procedures previously described for porcine and human intestinal spirochaetes (Lymbery *et al*, 1990; Lee *et al*, 1993; Lee and Hampson, 1994). Briefly, 500 ml of broth cultures in late log phase were harvested by centrifugation at 10,000 g for 20 mins, washed in phosphate buffered saline (pH 7.2), resuspended in 0.5 ml of sterile distilled water, and lysed by three 30 sec cycles of sonication at 50 W (Labsonic 1510). Supernatants containing the constitutive enzymes were collected following centrifugation of the lysed suspension at 20,000 g for 20 min, and stored at -70°C.

The electrophoretic mobilities of 15 constitutive enzymes were determined by electrophoresis of the thawed cell supernatants in 11.4% horizontal starch gels (Selander *et al*, 1986). Enzymes examined were acid phosphatase, alcohol dehydrogenase, adenylate kinase, alkaline phosphatase, esterase, fructose-1-6-di-phosphatase, glucose phosphate isomerase, guanine deaminase, glutamate dehydrogenase, hexokinase, mannose phosphate isomerase, nucleoside phosphorylase, L-leucyl-glycl-glycine peptidase, phosphoglucomutase and superoxide dismutase. Enzymes were localised by the addition of suitable specific substrates under appropriate buffer conditions, as described elsewhere (Lee *et al*, 1993). Differences between isolates in the electrophoretic mobility of a given enzyme were considered to reflect the presence of different alleles at the corresponding structural gene locus. Isolates were characterised by their alleles at each enzyme locus, with isolates having the same alleles at all 15 loci considered to belong to the same electrophoretic type (ET). Genetic diversity (*h*) was estimated on the basis of the relative frequencies of the different alleles at each enzyme locus, being calculated as $h=(1-?p_i^2)\ (n/n-1)$, where *p*=frequency of the *i*th allele and *n*=number of ETs (Lymbery *et al*, 1990). Genetic distance between ETs was calculated as the proportion of loci at which different alleles occurred, with the unweighted pair group method of arithmetic averages clustering strategy being

used to create a phenogram from this information (Lymbery *et al*, 1990). Figure 1 was created by combining the results of this study with previous results for porcine intestinal spirochaetes (Lee *et al*, 1993).

16S rDNA analysis. Ten avian strains were selected from the main MEE groups (group a: R-1. group b: 1380, HB60. group c 2A-20. group d: DH7926, PHB-11. group e: C-1. group f: 2726. group g: QU-1, 1772). Their 16S rRNA genes were partially copied from their DNA by amplification in polymerase chain reactions in 30 cycles, as described (Stanton *et al*, 1996). Purified products were directly sequenced, the sequences aligned, and a dendrogram constructed to demonstrate relationships between these strains and reference strains of *Serpulina* spp. and *Treponema* spp. (Figure 2).

Phenotypic characterisation. The strength of ß-haemolysis for each isolate was determined by stab-inoculating spirochaetes into Trypticase Soy agar supplemented with 5% defibrinated ovine blood, and incubating anaerobically for three days at 37°C. The strength of ß-haemolysis then was compared to that of a known strongly ß-haemolytic *S. hyodysenteriae* type strain B78^T, inoculated onto the same plate. To test for indole production, 2 ml of log-phase broth culture was extracted with 1 ml of xylene and 4 drops of Kovac's reagent were added. Development of a red/purple colour at the surface indicated a positive test. Enzyme profiles were determined using the commercial API-ZYM kit (API, Montalieu-Vercieu, France), as previously described (Hunter and Wood, 1979).

The cell dimensions, number of periplasmic flagella and shape of the cell ends for 11 representative isolates, selected from the major genetic groups identified in MEE (Figure 1), were determined by transmission electron microscopy (TEM) of negatively stained preparations. Results were compared with those previously published for B78^T and B256^T (Lee *et al*, 1993), C1 (Swayne *et al*, 1995) and 308.93 (Trampel *et al*, 1994). Growth from 1ml of log-phase broth culture was centrifuged at 10,000 rpm for 30 secs in a microcentrifuge, and the cells were resuspended in 300 µl of 0.01 M sodium phosphate buffer (pH 7.0). Fifty µl of the cell suspension was mixed with an equal volume of 3.0% phospho-tungstic acid (pH 7.0 for isolates belonging to group g in Figure 1, pH 6.0 for all others), and allowed to stand for 20 secs. Formvar-coated grids were placed in the drops for 20 secs, then removed and allowed to dry. The grids were examined in a Phillips CM100 transmission electron microscope.

2. 3. Results

MEE. The 56 chicken isolates were divided into 40 ETs (A1 through A40), which were distributed through six of the seven major genetic groups apparent on the phenogram (Figure 1). No chicken spirochaetes belonged to MEE group a (corresponding to the species *Serpulina hyodysenteriae*). The mean genetic diversity per enzyme locus for all chicken isolates included in this study was 0.587 (Table 4).

MEE group b (consisting of seven ETs containing porcine spirochaetes, and ETs A1 to A10) contained 16 chicken spirochaetes from WA, including the strongly ß-haemolytic HB60, as well as strain 1380 from the Netherlands and strain B230 from the UK. This group corresponded to the proposed species "*S. intermedia*", containing indole positive weakly ß-haemolytic porcine isolates (Lee *et al*, 1993).

MEE group c corresponded to the species *S. innocens*. There were only two chicken isolates in this group, both from Australia.

MEE group d is a newly identified genetic group which contained only spirochaetes isolated from chickens, and did not correspond to any of the genetic groups of porcine isolates previously identified by Lee *et al* (1993). It was the largest group, with 22 isolates from WA, one from Queensland and two from the Netherlands. It was most closely related to group b, which contained porcine and chicken isolates, and was separated from it by a genetic distance of 0.686.

MEE group e was another distinct genetic group represented by a single ET containing three isolates (C1, C2 and C4), all from a single flock in the USA. This group was previously shown to be distinct by Swayne *et al* (1995) and was most closely related to group d, separated from it by a genetic distance of 0.715.

There were only two chicken isolates in group f, one from the Netherlands (2726) and one from the USA (C5). This group apparently corresponds to the proposed new species "S. *murdochii*" (Lee and Hampson, 1994), although C5 was relatively distantly related to other isolates in the group (separated by a genetic distance of 0.630).

Group g corresponded to the new species *S. pilosicoli* (Trott *et al,* 1996), and contained three chicken isolates from the Netherlands, two from the USA and one from Queensland. The strongly ß-haemolytic isolate 13316 from the Netherlands was located in this group.

16S rDNA analysis. The 16S rDNA sequences of all ten avian strains were extremely similar to each other (<1% difference), as well as to the reference strains of *Serpulina* spp. (Figure 2). As anticipated, rhea *S. hyodysenteriae* strain R-1 was grouped with porcine strains of this species. The two chicken "*S. intermedia*" strains 1380 and HB60 (MEE group b), were grouped together, as were the two group d strains (DH7926 and PHB-11), and the two *S. pilosicoli* (group g) strains (QU-1 and 1772). The single isolates from groups c (*S. innocens*, 2A-20), e ("new", C1) and f ("*S. murdochii*", 2726) were all distinct, although again their 16S rDNA sequences were very similar.

Phenotypic characterisation. The results for the strength of ß-haemolysis on blood agar, indole production and API-ZYM profile for the isolates are presented in Table 6. Two isolates were strongly ß-haemolytic (HB60 and 13316), two isolates had an intermediate level of ß-haemolysis (E2 and A7), and all other isolates were weakly ß-haemolytic. Fifteen isolates produced indole, with 14 of these being located in group b ("*S. intermedia*") in Figure 1. The other isolate was in group d.

The morphological characteristics of representative chicken intestinal spirochaetes from the different genetic groups identified by MEE (Figure 1) are presented in Table 6. The number of periplasmic flagella per cell end was variable, even for strains belonging to the same MEE genetic group. However, in all cases these were inserted sub-terminally in a single row at each end of the cell. The chicken intestinal spirochaetes belonging to group g (*S. pilosicoli*) had features typical of those described for porcine and human strains of *S. pilosicoli* (Trott *et al*, 1996a;1996b). They had four to six periplasmic flagella at each cell end, although cells of 1772 occasionally were seen to have seven flagella. All isolates in this group had tapered cell ends, compared to the blunt ends possessed by the other *Serpulina* spp. isolates.

2.4. Discussion.

In this study, 56 intestinal spirochetes isolated from chickens in different countries were shown to be genetically heterogeneous. However, based on their grouping relative to well characterised porcine strains, all appeared to belong to the genus *Serpulina* (Stanton, 1992). This was confirmed by the close similarity in 16S rRNA gene sequence analysis amongst representative chicken spirochaetes examined.

In MEE they were divided into 40 ETs, belonging to six distinct genetic groups (Figure 1), four of which (b, c, f and g, respectively) have been considered to represent the distinct species "*S. intermedia*", *S. innocens*, "*S. murdochii*" and *Serpulina pilosicoli* (formerly"*Anguillina coli*"), previously described for porcine isolates (Lee *et al*, 1993). The other two groups, d and e, are new and only contained isolates from chickens. Again 16S rDNA gene sequence analysis confirmed that these two groups were different, although closely related (Figure 2).

Three of the six MEE groups (b, e and g) contained isolates that previously have been shown to be pathogenic in experimentally infected chicks. Group b ("*S. intermedia*") contained 18 of the 56 (32 %) isolates examined, including 1380 from the Netherlands, which has been shown to be pathogenic in experimental infections of day-old broiler chickens (Dwars *et al*, 1992). Also in this group were the strongly ß-haemolytic isolate HB60, isolated from a WA layer flock with diarrhoea, and histo 6, isolated from a WA broiler breeder flock which was being culled due to poor production (McLaren *et al*, 1996). All but one of the 15 chicken spirochaetes tested in group b produced indole, and nine of 11 tested had a-glucosidase but not a-galactosidase activity (Table 3). These activities are characteristics previously reported for porcine isolates of "*S. intermedia*" (Lee *et al*, 1993), and may be useful for identifying chicken isolates belonging to this potentially pathogenic group. Group b was made up of two distinct sub-groups of ETs. The first sub-group comprised ETs A1 to A10, which contained all of the chicken spirochaetes in this group, together with ETs 30 and 31, containing porcine spirochaetes. The second sub-group comprised ETs 32 to 36, which contained only porcine isolates. This sub-grouping appeared much less pronounced in a previous study which examined only porcine strains (Lee *et al*, 1993). Further DNA:DNA reassociation studies are required to clarify the extent and significance of this sub-grouping of organisms

which otherwise have phenotypic properties consistent with those of "*S. intermedia*". Strains of this group are relatively uncommon in pigs, where they have been associated with a condition called spirochaetal colitis (Hampson and Trott, 1995), but they are comparatively common in chickens in WA, and are present in the Netherlands and the UK.

Only two isolates from chickens, both from Australia, belonged to the *S. innocens* group (group c). This species is commonly isolated from pigs (Lee *et al*, 1993), and such strains are considered to be harmless commensals (Kinyon and Harris, 1979). Porcine isolates of *S. innocens* have failed to colonise experimentally-infected chicks (Trottt *et al*, 1995), and these two isolates probably have no clinical significance.

The new genetic group d contained 25 of the 56 (45%) chicken isolates examined. All but three were from WA, with two from the Netherlands and one from Queensland. This group is not homogeneous since some ETs were separated from each other at genetic distances of up to 0.580, and thus they may not all belong to the same species. To date, the pathogenicity of isolates in this group is unknown, and hence their significance is uncertain. Most were isolated from flocks with production problems. No consistent phenotypic features were found that could be used to identify them.

Group e was represented by only three isolates, all from the USA (Figure 1). It was most closely related to groups a, b and c at a genetic distance of 0.715, and, as previously pointed out (Swayne *et al*, 1995), therefore probably is another new species of *Serpulina*. This group contained strain C1, which was isolated from a chicken in a flock with diarrhoea and has been shown to be pathogenic in day-old chicks and adult layers (Swayne *et al*, 1995). Isolates C2 and C4 came from birds in the same flock. This should therefore be considered as the second group of avian intestinal spirochaetes with known pathogenic potential. They were not represented amongst the isolates from WA or Europe, and their distribution and prevalence in the USA is not known.

Only two strains were located in the "*S. murdochii*" group (group f), C5 and 2726. However, C5, which was isolated from the same flock as the isolates in group e, was only distantly related to other members of the group. The pathogenic potential of these relatively uncommon isolates is not known, although porcine isolates of "*S. murdochii*" generally are considered to be commensals (Lee *et al*, 1993).

Six chicken strains of *S. pilosicoli* were identified (group g), these being isolated in Queensland, the USA and the Netherlands. Human and porcine strains of this species are associated with a condition termed "intestinal spirochaetosis", which is characterised by diarrhoea and end-on attachment of spirochaetes to the colonic epithelium (Trott *et al*, 1996b)). Human and porcine strains of this species have been used to experimentally infect day-old chicks, resulting in diarrhoea and a reduced growth rate (Trott *et al*, 1995). Five of the six chicken strains were ß-glucosidase negative (Table 1), as has previously been described for porcine isolates of this species (Fellstrom and Gunnarsson, 1995). As well as having ß-glucosidase activity, 13316 was unusual in being strongly ß-haemolytic. The three representative isolates in this group had typical *S. pilosicoli* morphology (Trott *et al*, 1996a; 1996b), with four to six (and for one isolate, occasionally seven) periplasmic flagella at each cell end, and tapered cell ends (Table 6). In addition, when tested in a PCR based amplification of a 16S rRNA gene sequence using a primer specific for this species (Park *et al*, 1995), all of these isolates gave a positive reaction (Atyeo *et al*, 1996). Two of these isolates, 308.93 and 42167, were isolated from a layer flock in the USA experiencing diarrhoea and a five percent drop in egg production (Trampel *et al*, 1994). Experimental infection of day-old chickens with the isolate from Queensland (QU-1) resulted in diarrhoea, with the characteristic end-on attachment of spirochetes to the caecal epithelium (Chapter 3). Therefore, *S. pilosicoli* is the third of the genetic groups identified here that has been demonstrated to have pathogenic potential in chickens. Wild birds also may be colonised by strains of this species, and therefore pose a risk of infection to chickens. For example, strain S76, which has been identified as *S. pilosicoli* by PCR and MEE (Trott *et al*, 1996c), was isolated from a chiloe wigeon in the USA. When inoculated into day-old chicks, S76 attached end-on to the cecal epithelium and induced diarrhoea (Swayne *et al*, 1993; Swayne, 1994).

In summary, intestinal spirochaetes colonising chickens are a diverse group of bacteria, some of which may be representative of at least two new species within the genus *Serpulina*. However, DNA:DNA reassociation studies are necessary to confirm the taxonomic position of these strains, and to name the new species. Clearly, chickens also may be colonised by other intestinal spirochaetes that are not cultivable under the conditions used, which were based on those developed for porcine spirochaetes of the genus *Serpulina*. Of the genetic groups identified, three contained isolates that appear to be

pathogenic for chickens: 1380, HB60 and histo 6 in group b ("*S. intermedia*"), C1 in group e (new, unnamed group), and 308.93, 42167 and QU-1 in group f (*S. pilosicoli*). Isolates of "*S. intermedia*" can routinely be identified by their weak ß-haemolysis on blood agar and their production of indole, whilst a PCR is available to identify isolates of *S. pilosicoli* (Park *et al,* 1995). Distinguishing phenotypic characteristics have not yet been identified for isolates in group e. This study has provided a basis for understanding the diversity and disease associations amongst a range of intestinal spirochaetes infecting chickens. It should now be possible to develop diagnostic techniques to detect and identify the pathogenic species, to study their epidemiology, and to investigate the pathogenic mechanisms involved in infections with these microorganisms.

FIGURE 1. Phenogram of genetic distance amongst 40 ETs containing intestinal spirochetes isolated from chickens, outlined in bold, and comparison with ETs containing porcine intestinal spirochetes. Group a corresponds to the species *Serpulina hyodysenteriae*, group b to the proposed species "*S. intermedia*", group c to the species *S. innocens*, groups d and e are newly identified, unnamed groups, group f corresponds to the proposed species "*S. murdochii*", and group g to the species *S. pilosicoli*.

FIGURE 2. 16S rRNA dendrogram indicating the phylogenetic relationships among intestinal spirochaetes isolated from chickens, and reference strains of *Serpulina* spp. and *Treponema* spp. The scale bar indicates a 5% difference in nucleotide sequences. distance between two organisms is the sum of the horizontal branch lengths. Strain names of spirochaetes from birds are underlined, and their MEE groups and species names are detailed in Table 3 and in the text.

TABLE 3. Electrophoretic type, origin, disease association, strength of ß-haemolysis on blood agar, indole production and API-ZYM profile of the 56 avian intestinal spirochaetes examined.

te	n[b]	ase ciation[c]	emolysis[d]	e[e]	ZYM[f]
					12.10.1
					4.2.1
					4.2.1
	erlands				4.10.1
					13.10.1
					4.10.1
0-9					14.10.1
6					4.10.1
G 33					
					4.10.1
)					4.10.1
)					4.10.1
5					
G 34					
)					
354.03	nsland				12.3.0
/					
					13.11.1
Heal	erlands				15.11.1
11					13.11.1
14					
966.2	nsland				15.11.1
8					

3		
		13.11.1
3		13.3.1
G 35		
		13.15.1
)26	erlands	4.13.1
		12.3.1
5		
9		12.3.1
	(Ohio)	4.3.1
	(Ohio)	4.3.1
	(Ohio)	
	(Ohio)	12.7.1
	erlands	2.6.1
	erlands	12.3.0
7	(Iowa)	4.3.0
)3	(Iowa)	4.3.0
	erlands	12.3.0
6	erlands	13.11.8
	nsland	12.3.0

a ET, electrophoretic type in multilocus enzyme electrophoresis (Figure 1). ETs A1-A10, MEE group b; ETs A11-A12, group c; ETs A13-A32, group d; ET A33, group e; ETs A34-A35, group f; ETs A36-A40, group g.

b WA,Western Australia. UK, United Kingdom. USA, United States of America.

c Signs of disease in flock from which the strain was isolated. W, wet litter. L, low production. D, diarrhoea. N, no disease signs. NA, no information on disease signs available.

d S, strong ß-haemolysis. I, intermediate ß-haemolysis. W, weak ß-haemolysis.

e +, indole produced. -, indole not produced. ND, not determined.

f Biochemical profiles with the API-ZYM test kit (Hunter and Wood, 1979). Profiles ending with 10.1 have a-glucosidase but not a-galactosidase activity; those ending with 3.0 lack ß-glucosidase activity.

TABLE 4. Allele frequencies and genetic diversities at 15 enzyme loci in 40 ETs of chicken intestinal spirochaetes.

Enzyme	Frequency of indicated allele									Gd
locus*	0	1	2	3	4	5	6	7	8	
GDA	0.125	0.225	0.025		0.250	0.225	0.050	0.050	0.050	0.833
AK			0.175	0.300	0.225	0.075	0.225			0.792
MPI		0.075	0.325	0.150	0.325	0.075	0.050			0.772
PGI		0.350	0.225	0.250	0.125			0.025	0.025	0.767
HEX		0.125	0.325	0.275	0.250	0.025				0.759
ALP		0.150	0.250	0.275	0.325					0.753
EST				0.025	0.500	0.300	0.050	0.125		0.658
GDH		0.125	0.250	0.500	0.125					0.673
PGM		0.100	0.575	0.250	0.075					0.606
LGG	0.075	0.250	0.625	0.050						0.553
ADH		0.050	0.700	0.200		0.050				0.477
NP		0.050	0.025	0.750	0.125		0.050			0.427
ACP	0.775	0.050	0.075				0.075	0.025		0.395
FDP			0.025	0.150	0.800	0.025				0.345
SOD			1.000							0.000
Mean genetic										0.587

Gd, genetic diversity.

TABLE 5. Morphological characteristics of chicken intestinal spirochaetes selected as representatives of six genetic groups identified by multilocus enzyme electrophoresis, and comparison with the porcine type strains of *Serpulina hyodysenteriae* (B78^T) and *S. innocens* (B256^T)

Strain	MEE group[a]	No. of periplasmic flagella	Cell ends	Length (μm)	Diameter (μm)	Wavelength (μm)[b]
Serpulina hyodysenteriae B78^T	a	7-10	blunt	8.3-9.8	0.23-0.45	ND
1380	b	9-14	blunt	8.69-18.0	0.41-0.50	2.88-4.38
A7	b	10-13	blunt	10.88-13.83	0.32-0.43	3.58-5.06
histo 6	b	11-14	blunt	7.55-13.94	0.41-0.43	2.44-3.00
2A-10	b	8-12	blunt	7.27-12.00	0.25-0.50	2.80-3.64
HB60	b	11-15	blunt	7.87-11.22	0.17-0.43	2.33-3.62
S.innocens B256^T	c	7-10	blunt	7.4-14.1	0.31-0.40	ND
2A-20	c	8-15	blunt	8.75-12.38	0.19-0.35	2.19-3.38
PHB-11	d	8-12	blunt	11.08-26.62	0.27-0.39	2.56-4.38
PHB-9	d	5-7	blunt	10.69-17.46	0.32-0.39	2.54-4.08
C1	e	8	blunt	8.76±0.78	0.32±0.02	3.31±0.52
2726	f	9-13	blunt	8.44-11.31	0.29-0.47	3.96-6.23
308.93	g	5	tapered	3.4-7.5	0.3-0.4	2.2-3.0
1772	g	5-7	tapered	9.08-11.00	0.23-0.37	3.00-4.00
QU-1	g	4-6	tapered	8.25-17.28	0.23-0.56	2.47-4.08

[a] Genetic group in multilocus enzyme electrophoresis (Figure 1). [b] ND, not determined

CHAPTER THREE

PATHOGENICITY TESTING OF INTESTINAL SPIROCHAETES FROM AUSTRALIAN POULTRY IN DAY-OLD CHICKS AND ADULT LAYERS

3.1. Introduction.

Several intestinal spirochaetal isolates from chickens have been used in experimental infections of day-old chickens, resulting in diarrhoea and reduced weight gain (Davelaar *et al*, 1986; Dwars *et al*, 1992; Swayne *et al*, 1995). From the results of chapter two, the isolates used by these other researchers have now been identified as belonging to MEE groups b ("*S. intermedia"*) and group e (unnamed group). No isolates from the latter group have yet been identified in Australia. Laying hens experimentally infected with intestinal spirochaetes from these two groups that are pathogenic in chicks also developed diarrhoea, and showed reduced egg production and reduced egg weight (Dwars *et al*, 1993; Swayne *et al*, 1995). Another spirochaete, identified in the current study as *S. pilosicoli* (MEE group g), has been shown to induce diarrhoea and reduced egg production in naturally-infected flocks in the USA (Trampel *et al*, 1994).

The purpose of the work described in this chapter was to determine whether Australian strains from the groups identified previously in overseas studies as being pathogenic, and which were present in Australia (ie "*S. intermedia*" and *S. pilosicoli*) were pathogenic. Strains from MEE group d (unnamed group) also were tested because members of this group were the most commonly encountered type of intestinal spirochaete in the prevalence survey in WA. Isolates selected from these three groups initially were tested in day-old commercial broiler chicks, because this is an easy and convenient model to test pathogenicity in the target species. A single isolate of "*S. intermedia*" from a WA flock with problems of wet litter and poor production also was tested in young birds approaching lay, to determine its effects on egg production.

3.2. Materials and methods.

Spirochaetes. Three isolates from MEE group b ("*S. intermedia*": HB60; A7; 2A-10), three from unnamed group d (PHB-11; 60-9; PHB-9) and one from group g (*S. pilosicoli*; QU-1) were used to infect chicks, whilst HB60 was used

to infect the layers. Each isolate was grown to mid-log phase in Kunkle's broth medium (Kunkle *et al*, 1996), to a density of approximately 10^8 cells/ml.

Infection of chicks. Day-old chicks used were commercial broiler chickens supplied by Inghams Pty Ltd, WA**.** Groups of 20 chicks were infected, except for strains 2A-10 and 155-5 where only ten birds were used (Table 6). Each bird was dosed with 2ml of broth culture by crop tube, on three consecutive days. The birds were housed in sterile Zeitz-filtered boxes and fed sterile chick mash and water ad libitum. They monitored daily for signs of ill-health, weighed at weekly intervals, and faecal samples were cultured for spirochaetes every 3-4 days. When three weeks of age, the birds were anaesthetised with diazepam, xylazine and ketamine, and killed by cardiac puncture after all samples were obtained. Sections of the caecae were fixed for light and electron microscopic examination using Bouin's and chilled half strength Karnovsky's fixatives. Swabs from the caecal wall were cultured for intestinal spirochaetes using selective TSA plates.

Infection of layers. Two groups of commercial layer pullets, each comprising ten 14-week-old birds, were housed in individual cages in separate rooms of an isolation animal house. One group was inoculated with 5ml of active broth culture of strain HB60 (5 X 10^8 cells) by crop tube daily for three days, whilst the other group received the same amount of sterile broth culture. The birds were fed a commercial layer diet, and weighed and faecal swabs taken at two week intervals over 16 weeks. Faeces were cultured for spirochaetes, and a percent faecal dry matter content measured by drying a weighed sample to constant weight. Eggs were collected and weighed daily, once laying commenced at 20 weeks of age.

3.3. Results

The mean weights of the infected and uninfected control chicks over the three week period post-infection are shown in Table 7. Chicks infected with "*S. intermedia*" strains HB60 and A7 were significantly ($p<0.02$) lighter than uninfected controls on weeks one and three post-infection. Chicks infected with *S. pilosicoli* strain QU-1 were significantly lighter than the control birds on weeks one and two post-infection. No other differences were significant. No birds infected with *S. innocens* strain 155-5 became colonised, but all birds in the other three infected groups were culture positive throughout. All birds infected with "*S. intermedia*" strains and *S. pilosicoli* developed obvious

diarrhoea within 7-9 days of inoculation, whilst those colonised by strains from MEE group d only developed diarrhoea after 12-13 days. At post-mortem examination, no consistent gross or microscopic changes were found in birds infected with the various strains. The birds infected with *S. pilosicoli* strain QU-1 had spirochaetes attached by one cell end to the caecal mucosa (Figure 3), whilst birds infected with "*S. intermedia*" and group d strains had unattached mats of spirochaetes in the lumen and crypts of the caecae (Figure 4).

Laying birds. Intestinal spirochaete strain HB60 was re-isolated from nine of the infected birds at irregular periods post-infection, and was isolated from the caecae of all nine at slaughter. One bird was not colonised at any time, and so results for this bird were excluded from the analysis. None of the control birds were infected with spirochaetes, but one failed to lay, and so also was excluded from the analysis of results.

The average weight of the nine birds in the infected group was less than the controls from week two post-infection (pi) onwards, but this difference was only significant on week 14 pi, when the mean weight of the control group was 1.709 kg compared to 1.514 kg for the infected group ($p < 0.035$) (Table 8).

Infection with "*S. intermedia*" strain HB60 resulted in a decrease in the percent faecal dry matter content from a group mean of 22.87 % in control birds over the experimental period to 19.86 % in infected birds (Table 9). This difference of faecal water content (mean 2.94% overall) was statistically highly significant ($p<0.000$). Infected birds were observed to have wetter and less well-formed faeces than the control birds.

Over an eight week period when lay had commenced (7-15 weeks post-infection) the nine control birds produced an average of 0.670 eggs per day compared to 0.503 per day for the infected birds (Table 10). This difference (0.167 eggs/day) was statistically significant ($p<0.05$). Over the period the average weight of the eggs produced by the control birds was 45.36 g compared to 44.56 g for the infected birds (Table 10). Again this difference of 0.8 g/egg was significant ($p<0.001$).

At post-mortem examination the infected birds were observed to have less abdominal fat and their caecae contained more gas and fluidy content than the control birds. No consistent gross or histological differences could be found in the caecal wall in birds in the two groups, although by scanning electron

microscopy spirochaetes were observed overlaying and loosely associated with the caecal epithelium only in birds from the infected group.

3.4. Discussion.

Infection of day-old chicks with different strains of intestinal spirochaetes from three genetic groups present in Australia resulted in colonisation and diarrhoea. From the work described in chapter 2, two of the groups ("*S. intermedia*" and *S. pilosicoli*) have been shown to be capable of inducing disease in overseas studies. In the current study, infection with isolates from these two groups induced diarrhoea earlier than did infection with isolates from the other common unnamed Australian group of isolates (group d). This would be consistent with them being more pathogenic than isolates from group d. Similarly, significant effects of this colonisation on body weight were found only in birds infected with two of the "*S. intermedia*" strains (these being lighter on weeks one and three), and in birds infected with *S. pilosicoli* (these being lighter on weeks one and two). The differences in duration of growth depression suggests that infection with *S pilosicoli* has less long term effects than "*S. intermedia*". It was recognised that the results were limited by the relatively small group sizes used (for example there were only 10 birds infected with "*S. intermedia*" strain 2A-10, and weight differences were not significant), and further larger studies involving more birds are required. Despite effects on growth rate and the occurrence of diarrhoea, there was a lack of specific histological changes in the caecae of birds in any group. The porcine strain of *S. innocens* failed to colonise any birds, and it might be argued that colonisation of young chicks with any strain of intestinal spirochaete is likely to induce digestive disorders and result in diarrhoea. Colonisation with *S. pilosicoli* resulted in end-on attachment of the spirochaetes to the caecal enterocytes, a feature seen in *S. pilosicoli* infection in pigs and other species (Hampson and Trott, 1995), and in natural infections of layer birds (Trampel *et al*, 1994).

As a result of uncertainties of the relevance of the chick model in assessing pathogenicity of isolates potentially inducing reduced production in layer birds (where natural infection has been most commonly reported), a small group of commercial birds approaching lay were infected with a WA isolate of "*S. intemedia*" (HB60). Nine of ten birds were successfully colonised, and whilst there was little significant effect on body weight (in part due to small group sizes), colonisation was associated with a significant increase in faecal moisture, with reduced egg weights, and with reduced egg production. A mean

increase in faecal moisture of 2.94% was sufficient to make the faeces much more fluid, and would certainly cause wet litter problems (staining of egg shells, mechanical problems woth removal of excretia, attraction of flies etc). Average daily losses were 0.167 eggs/bird over an eight week period, with the eggs produced on average being 0.8 g lighter than those from uninfected birds held under the same conditions. Although it is recognised that very few birds were used in this experiment, and that only one spirochaetal strain was tested., it is tempting to try to extrapolate these losses over the whole cycle of production, and to calculated national average losses based on proportion of infected flocks. Overall 35 % of layer flocks were infected with spirochaetes in the WA survey, and 16/55 (29%) of WA isolates tested were "*S. intermedia*". Hence as there are approximately 146 layer flocks in WA, 15 (10%) may be infected with "*S. intermedia*". This would represent around 70,000 layers in the State, assuming that spirochaetes are infecting flocks of all sizes equally. If even only 20% of birds in these flocks are suffering losses equivalent to those observed experimentally, annual daily State losses would amount to 14,000 X 0.167 eggs (2,338), and 14,000 X 0.8 grams (11,200 g) of weight over the eggs produced. Yearly figures would be multiplied by 365. Assuming WA production is around 10% of national production, annual national egg losses would be around 8,533,700. Assuming an average wholesale price of 20 cents an egg, total annual losses would be valued at $1,706,740. This is a very approximate figure, which makes many assumptions, and, for example does not take into account other losses associated with reduced egg weights, or other production difficulties associated with wet litter. Furthermore, it does not consider potential losses associated with spirochaetes other than "*S. intermedia*", which made up 70% of total isolates identified. Clearly these should be tested for their effects on egg production, since, for example, *S. pilosicoli* infections have been reported to reduce egg production by 5% in infected flocks in the USA (Trampel *et al*, 1994).

Further more large scale and detailed work is now required to confirm these findings under experimental and field conditions, and to develop improved means to diagnose and control these most significant infections.

TABLE 6. Strains of intestinal spirochaetes and number of day-old chicks used.

Strain	Species	Number
Control	uninfected	23
HB60	"*S. intermedia*"	20
A7		20
2A-10		10
PHB-9	MEE group d	20
PHB-11		20
60-5		20
QU-1	*S. pilosicoli*	20
155-5	*S. innocens*	10

TABLE 7. Mean ± SD weights in grams of control and infected chicks post infection (pi).

Strain	Day 1 pi	Day 7 pi	Day 14 pi	Day 21 pi
Control	44.05±4.11	103.2±29.8	219.9±34.4	378.5±65.6
HB60	47.38±3.83	85.68±12.9*	216.6±33.4	337.9±43.3*
A7	46.75±4.68	81.15±10.4*	213.17±23.2	342.3±39.6*
2A-10	37.62±2.57	104.2±23.8	227.2±15.35	370.8±37.3
PHB-9	43.21±3.45	142.5±28.5	228.0±46.1	412.1±67
PHB-11	45.74±4.18	98.36±10.3	237.6±26.8	420.2±44.7
60-5	43.82±3.34	149.6±20.5	240.8±34.3	427.8±51.8
QU-1	45.39±4.44	82.81±11.4*	190.7±35.2*	384.13±59.9

*Weights are significantly less ($p<0.02$) than those of uninfected control birds of the same age.

TABLE 8. Weight in grams of adult layers at weekly intervals post-infection with "*S. intermedia*" strain HB60

Week	Control (±SD)	Infected (±SD)
0	1141.4±121.1	1214.7±62.8
2	1403.3±112.0	1349.1±80.7
4	1497.4±137.3	1431.3±92.2
6	1625.7±153.4	1547.1±103.0
8	1690.5±192.6	1597.0±100.2
10	1701.7±247.5	1605.5±127.0
12	1650.2±255.6	1546.1±140.8
14	1709.2±205.6	1514.0±177.3
16	1701.7±229.9	1553.0±154.3

TABLE 9. Mean percent faecal dry weights in adult layers

Weeks P.I.	Uninfected	HB60
Week 2	23.80±2.03	20.82±3.93
Week 4	24.98±2.53	20.61±2.85
Week 6	20.58±3.32	18.72±2.23
Week 8	24.19±3.23	19.71±4.04
Week 10	21.36±0.99	21.03±2.27
Week 12	22.43±3.10	17.57±4.79
Week 14	22.28±3.45	21.02±4.43
Week 16	22.19±2.10	19.55±4.68

TABLE 10. Weekly egg production and mean egg weights in grams in weeks 7-15 post infection (pi)

	Mean egg production (eggs/hen/week)		Mean egg weights (SD)	
Week (p.i.)	Controls	Infected	Controls	Infected
7	1.6	2.0	38.83 (4.58)	39.28 (1.82)
8	3.6	2.3	40.14 (3.87)	40.22 (2.25)
9	5.4	4.1	41.44 (2.96)	42.82 (3.85)
10	4.3	3.4	45.18 (5.21)	43.65 (3.38)
11	5.4	4.2	46.33 (4.81)	44.39 (3.54)
12	5.1	4.1	45.42 (5.76)	44.89 (3.62)
13	4.7	4.1	46.46 (3.00)	44.97 (3.28)
14	5.8	3.2	48.08 (4.40)	46.24 (3.25)
15	6.3	4.1	48.80 (4.23)	46.67 (2.78)

FIGURE 3. “*S. intermedia*” strain HB60 invading caecal crypts in an infected chick.

FIGURE 4. *S. pilosicoli* strain QU-1 attached by one cell end to the caecal epithelium in an infected chick.

CHAPTER FOUR

IMPLICATIONS AND RECOMMENDATIONS

This study has demonstrated that intestinal spirochaetes colonise egg and broiler breeder flocks in WA at a high frequency (35.1 - 53.3 % of flocks tested respectively). It therefore seems highly likely that these organisms are equally as common in flocks in other Australian states.These spirochaetes, and isolates from diseased poultry in Europe and the USA, were shown to be genetically heterogeneous, being representatives of six groups that are all likely to be separate species in the genus *Serpulina*. Isolates shown to be pathogenic in other countries were shown to belong to three of these groups. Representatives from one of these groups ("*S. intermedia*") were common amongst the spirochaetes recovered in WA. An isolate from another pathogenic group (*S. pilosicoli*) was received from a Queensland flock, whilst no isolates from the third (unnamed) pathogenic group were recovered in Australia. Australian isolates from the two pathogenic groups induced diarrhoea and reduced growth rates in day-old chicks, whilst the single isolate of "*S. intermedia*" that was tested in layer birds induced wet litter, and reduced egg production. Another common unnamed group induced diarrhoea in chicks, although it appeared to be less virulent than the other two groups.

The main implication from this study is that infection with "*S. intermedia*", and to a lesser with *S. pilosicoli,* is common and is likely to be causing large and hitherto unaccounted losses in potential egg production in Australia, as well as contributing to production difficulties associated with wet litter. The fact that this is occurring without it being specifically recognised or diagnosed is particularly important as no appropriate measures are being taken to control the infections, and to reduce associated losses. Annual losses of egg production caused by "*S. intermedia*" alone were estimated at around one and three quarter million dollars, if results from experimental infections were extrapolated based on the prevalence of infection in the field.

It is highly recommended that studies into these organisms in Australia continue. First it should be confirmed that infection is equally as common in other States as in WA, and that similar *Serpulina* spp. are involved. Second, the pathogenic potential of isolates from all the various groups should be investigated in experimental infections of layer birds. Third, improved methods should be developed to detect and identify spirochaetes from the main and

most common pathogenic groups. Fourth, the antimicrobial drug sensitivities of Australian isolates from the main pathogenic groups should be established by in vitro testing. Fifth, effective drugs should be tested for their efficacy in vitro by testing in groups of experimentally-infected birds. Sixth, effective drug regimens should be tested in the field on flocks that are known to be infected. Seventh, other potential means of control, particularly the use of highly digestible diets, or diets treated with exogenous enzymes, which protect against swine dysentery, should be trialled in experimental and field conditions. Eigth, efforts should be made to publicise the findings of the current study amongst Australian egg producers, and providers of diagnostic services to the Industry.

CHAPTER FIVE

TECHNICAL SUMMARY

Anaerobic selective-agar culture of faecal samples from birds in 37 layer and 30 broiler breeder flocks in WA demonstrated that 13 (35.1%) of the former and 16 (53.3%) of the latter were infected with intestinal spirochaetes. A significant association was found between the presence of spirochaetes in a flock and wet litter and production problems. Genetic and phenotypic analysis of representative isolates, and isolates obtained from Europe and the USA demonstrated that the bacteria could be divided into six genetic groups (equated with distinct species). Isolates from four of these groups were encountered in the WA survey, whilst an isolate from another group (*S. pilosicoli*) was received from Queensland. Pathogenic isolates fom overseas studies were shown to be "*S. intermedia*" (from Europe), an unnamed group e (from the USA), and *S. pilosicoli* (from the USA). "*S. intermedia*" isolates were common in WA (29% of isolates), whilst the unnamed pathogenic group e was not encountered. Another unnamed group d was common in WA (40% of isolates), and also included two isolates from the Netherlands and one from Queensland. The other groups all contained relatively few isolates.

Pathogenicity tested focused on the two groups present in Australia which have been shown to be pathogenic in overseas studies, and group d, which was the most common group in the WA survey. A porcine isolate of *S. innocens* failed to colonise control birds. Chicken isolates from all three groups induced diarrhoea in chicks infected at day-old, but diarrhoea started earlier in the birds colonised with "*S. intermedia*" and *S. pilosicoli* than with the group d isolates, and only the latter two species induced reduced weight gain, implying that these are more pathogenic. Infection of a small number (10) of birds approaching lay led to a subsequent increase in faecal water content (by 2.94 %) and a reduction in egg weight and average daily egg production (by 0.8 g and 0.167 eggs/day respectively). Crude extrapolations from these results, based on established flock prevalences of infection, suggested a national annual loss of 8,533,700 eggs with a wholesale value of $1,706,740, attributable just to infection with "*S. intermedia*". Further experimental infections and field studies are required to confirm the magnitude of these losses, to give better national estimates of losses, and to determine potential losses (if any) associated with infection with other species of intestinal spirochaete in Australia. Further work is also required to develop improved means to diagnose

and control the infections in the field, and to make producers aware of these problems. Problems in the broiler breeder industry also must be addressed.

CHAPTER SIX.

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