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Brachyspira infection in laying hens

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A	adenine
adh	alcohol dehydrogenase
AIS	avian intestinal spirochaetosis
alp	alkaline phosphatase
ATCC	American type culture collection
BHI	brain heart infusion
bp	base pair
C	cytosine
CFU	colony-forming unit
d	day
DNA	deoxyribonucleic acid
EO	essential oil
est	esterase
G	guanine
gdh	glutamate dehydrogenase
glpK	glucose kinase
gyrB	gyrase subunit B
HIS	human intestinal spirochaetosis
HIV	human immunodeficiency virus
L.	Lactobacillus
MEE	multilocus enzyme electrophoresis
MIC	minimal inhibitory concentration
MLST	multilocus sequence typing
MLVA	multiple locus variable number tandem repeat analysis
NADH	reduced nicotinamide adenine dinucleotide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pgm	phosphoglucomutase
p.i.	post inoculation
PIS	porcine intestinal spirochaetosis
ppm	parts per million
qPCR	quantitative polymerase chain reaction
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
rpm	rotates per minute
rRNA	ribosomal RNA
SD	swine dysentery
SPF	specific pathogen free
ST	sequence type
T	thymine
thi	thiolase
TSA	trypticase soy agar
UK	United Kingdom
USA	United States of America
ZnB	zinc bacitracin

GENERAL INTRODUCTION

1 Genus *Brachyspira*

Brachyspira is the sole genus assigned to the Family *Brachyspiraceae*, which is classified with two other families, the *Spirochaetaceae* and the *Leptospiraceae*, in the Order *Spirochaetales*. The spirochetes, all classified in the Order *Spirochaetales*, represent a coherent monophyletic phylum within the bacteria (Paster and Dewhirst, 2000). There are seven recognized species of the genus *Brachyspira*: *B. aalborgi*, *B. hyodysenteriae* (formerly *Serpulina hyodysenteriae, Serpula hyodysenteriae* and *Treponema hyodysenteriae*), *B. pilosicoli* (formerly *Serpulina pilosicoli* and *Anguillina coli*), *B. innocens* (formerly *Serpulina innocens*, *Serpula innocens* and *Treponema innocens*), *B. intermedia*, *B. murdochii and B. alvinipulli* (last three species also formerly *Serpulina* species). Provisional species are "*B. canis*", "*B. corvi*", "*B. hampsonii*", "*B. muridarum*", "*B. muris*", "*B. pulli*", "*B. rattus*" and "*B. suanatina*". *Brachyspira* species colonize the lower intestinal tracts (caeca and colon) of animals and humans, some causing specific diseases.

Brachyspiras are Gram negative, motile, helically coiled (spiral-shaped) bacteria, with diameters ranging from 0.25-0.6 µm, lengths from 3-19 µm. Cell ends can be blunt or pointed. They are anaerobic bacteria but are aerotolerant due, at least in part, to high NADH oxidase activity. *Brachyspira* cells use soluble sugars as carbon and energy sources. Growing cells consume low concentrations of oxygen via NADH oxidase and produce acetate, butyrate, H_2 and CO_2 from glucose, some species also produce ethanol. *Brachyspira* cells share with other spirochetes common unique morphological attributes and as a result their motility is different from that of other bacteria. The helically coiled protoplasmic cylinder is bound by an inner membrane; periplasmic flagella, or endo-flagella, are located between the inner membrane and an outer membrane sheath. The endo-flagella, varying in number from species to species (8-30), are subterminally attached near each end of the cell and, winded around the spirochete cell, they extend toward the center of the cell, where they may or may not overlap. The endo-flagella function by rotating within the periplasmic space and are so involved in spirochete motility. Spirochetes can efficiently swim through highly viscous gel-like media which inhibit the motility of most other bacteria (Li et al., 2000, 2008; Paster and Dewhirst, 2000; Stanton, 2006; Nakamura et al., 2006).

The chromosome of *Brachyspira* spp. is circular with low G+C content (27.1 - 27.9%) and their 16S rRNA gene sequences are highly conserved. Whole genome sequences have only recently been made available and this for four species, *B. hyodysenteriae* strain WA1 (ATCC 49526) of porcine origin (Bellgard et al., 2009), *B. murdochii* strain 56-150^T (ATCC 51284) of porcine origin (Pati et al., 2010), *B. intermedia* strain PWS/A^T (ATCC 51140) of porcine origin (Håfström et al., 2011), and three strains of *B. pilosicoli,* strain 95/1000 (ATCC BAA-1826) of porcine origin (Wanchanthuek et al., 2010), strain B2904 of poultry origin (Mappley et al., 2012), and strain P43/6/78^T (ATCC 51139) of porcine origin (Lin et al., 2013). Of the protein-coding sequences in the genome of *B. hyodysenteriae*, a high proportion of the predicted proteins show similarities to proteins of the enteric *Escherichia coli* and *Clostridium* species. Genes may have been gradually acquired through horizontal gene transfer in the environment of the large intestine, resulting in gradually increasing the fitness of the spirochete to

survival in the colonic environment (Bellgard et al., 2009; Hampson and Ahmed, 2009). The genomes of the three sequenced *B. pilosicoli* strains have a substantial variation in size (95/1000: 2,586,443 base pairs [bp]; B2904: 2,765,477 bp; P43/6/78^T: 2,555,556 bp), and are smaller than the genomes of the other sequenced *Brachyspira* species (WA1: 3,036,634 bp; 56-150^T: 3,241,804 bp; PWS/A^T: 3,308,040 bp). Most likely *B. pilosicoli* species are members of a more specialized species that has undergone a high degree of reductive genome evolution. Such a reductive genome evolution may have allowed improved energy efficiency, and enhanced pathogenic potential. In addition to their small genomes, the sequenced *B. pilosicoli* strains lack plasmids, whereas the genomes of the other fully sequenced *Brachyspira* species have included plasmids (Mappley et al., 2012). From the analysis with multilocus sequence typing (MLST) of *B. intermedia* isolates of different geographic regions and hosts, it was concluded that *B. intermedia* seems to be extremely diverse and may rather constitute several distinct species (Phillips et al., 2010).

2 *Brachyspira* infections in mammals and humans

Brachyspira species or *Brachyspira*-like spirochetes, colonizing the intestinal tract, have been reported in several mammal species, e.g. in representatives of ruminants, horses, pigs, carnivores, rodents, marsupials, nonhuman primate species and humans. Identification or culturing of the spirochetes in mammals has not always succeeded; disease significance is not always known. Colonization in dogs and especially disease association in pigs is well documented. Wild rodents possibly are important reservoirs for farm animals. There is a difference in prevalence of human intestinal spirochaetosis (HIS) between geographic regions, where it is more common in developing countries.

Pigs

Swine Dysentery (SD) is a severe mucohaemorhagic enteric disease of pigs which has a large worldwide impact on pig production. Important losses are caused by mortality (even 50% to 90%), sub-optimal performance with higher feed conversion and reduced weight gain, and by expenses for medication. Typically growing and finishing pigs are affected. The causative agent, *B. hyodysenteriae*, colonizes the caecum, colon and rectum. Reported herd prevalences of *B. hyodysenteriae* range worldwide from 0% to near 40% (Alvarez-Ordóñez et al., 2013b). A recently described species "*Brachyspira hampsonii*", circulating among swine herds in the USA and Canada and very recently also isolated from pigs in Europe, has also been associated with mucohaemorrhagic diarrhea in swine (Chander et al., 2012; Rubin et al., 2013a; Harding et al., 2013 ; Mahu et al., 2013). Beside *B. hyodysenteriae* and "*B. hampsonii*", a third strongly haemolytic *Brachyspira* species was isolated out of pigs in Sweden and Denmark. The 16S rRNA and *nox* genes of this isolate were identical with an isolate of a Swedish mallard and the strain was provisionally designated as "*Brachyspira suanatina*". Challenge studies in pigs with the pig-isolate caused clinical signs and macroscopic changes consistent with SD. Also the mallard-isolate could colonize pigs, causing diarrhea (Råsbäck et al., 2007a).

Porcine intestinal spirochaetosis (PIS), caused by *B. pilosicoli*, is characterized by a mild to moderate typhlocolitis in pigs typically after weaning. The clinical signs include mucus-containing, usually nonbloody diarrhea, poor feed conversion and depressed growth rates. The formation of "false brush border" due to closely packed spirochetes parallel to one another and with one end attached to the colonic enterocytes is characteristic in histological examination (Trott et al., 1996a).

B. murdochii and *B. innocens* are considered as non-pathogenic in pigs, nevertheless when present in high numbers in the intestine they could lead to *Brachyspira*-associated enterocolitis (Weissenböck et al., 2005; Komarek et al., 2009; Jensen et al., 2010). Also the enteropathogenic potential of *B. intermedia* in pigs is under debate (Weissenböck et al., 2005). Rates of farms positive for weakly hemolytic *Brachyspira* species reached about 86% in a German study on 165 randomly selected fattening pig herds with rates of 80% for *B. murdochii* and *B. innocens*, almost 60% for *B. intermedia* and 10% for *B. pilosicoli* (Vögely et al., 2013).

Dogs

B. pilosicoli and "*B. canis*" are the most reported species in dogs, but some isolates were also identified as "*B. pulli*", *B. hyodysenteriae* and *B. alvinipulli*. Canine spirochetal isolates from Australia and the USA were first identified as *B. pilosicoli*, but most belonged to a new species, designated "*B. canis*" (Duhamel et al., 1998). In Australian pet shop puppies, colonized with *B. pilosicoli* and "*B. canis*", all the ''*B. canis*'' isolates came from healthy puppies, suggesting that this species is a commensal (Oxberry and Hampson, 2003). A statistically significant association between the shedding of *B. pilosicoli* and the presence of diarrhea in dogs was demonstrated in a study in Spain, were fecal shedding of *B. pilosicoli* and "*B. canis*" was diagnosed in dogs (Hidalgo et al., 2010). In a study in the Czech Republic, *B. pilosicoli* was the most commonly diagnosed species in dog feces but also a single case with *B. hyodysenteriae* was found (Sperling et al., 2010). In urban dogs in Thailand spirochetes were isolated in 12.8% (6/47) of healthy dogs and in 10.6% (11/104) in dogs with diarrhea; isolates were identified as *B. pilosicoli*, "*B. pulli*" and "*B. canis*" (Prapasarakul et al., 2011). The identification of a canine isolate of Scandinavian origin as *B. alvinipulli* can be important. Although the possibility that this dog was exposed to materials that were contaminated by birds cannot be ruled out, the presence of spirochetes that are pathogenic for birds in dogs requires further confirmation (Johansson et al., 2004).

Rodents

In a Swedish study, intestinal spirochetes were cultivated from wild rodents caught in pig and poultry units, and other locations. Besides *B. hyodysenteriae*, *B. intermedia*, *B. murdochii*, *B. innocens* and *B. pilosicoli*, three new genetic variants were found, designated as "*Brachyspira rattus*", "*Brachyspira muridarum*", and "*Brachyspira muris*". Cross-species transmission of *Brachyspira* spp. may occur between rodents and farm animals, i.e. pigs and chicken. Alternatively, rodents and farm animals may be colonized from a common environmental source (Backhans et al., 2010, 2011).

Humans

The condition 'Human intestinal spirochaetosis' (HIS) was initially defined histologically by the presence of spirochetal microorganisms attached to the apical cell membrane of the colorectal epithelium, forming a "false brush border". HIS has been associated with colitis and complaints of chronic diarrhea, rectal bleeding, abdominal pain, and weight loss (Harland and Lee, 1967; Körner and Gebbers, 2003; Smith, 2005). Recent study results suggest also a potential association between HIS and sessile serrated adenomas/polyps (Omori et al., 2014). HIS is common in poorly developed regions. In countries with high living standards it is found most frequently in homosexual males, HIV positive persons as well as in children (Körner and Gebbers, 2003; Tsinganou and Gebbers, 2010; Helbling et al., 2012). Estimated prevalence rates in developed countries vary from 1.1% to 6.9%; in developing regions it can be more than 30% and even as high as 64.3%. In groups of homosexual males in USA colonization rates as high as 20.6% to 62.5% were shown (Tsinganou and Gebbers, 2010; Westerman et al., 2012). Most identified *Brachyspira* species from humans are *B. aalborgi* and *B. pilosicoli*. It is likely that some other described species in humans, and so-called "*B. ibaraki*", "*B. christiani*" and "*B. hominis*", are not separate species but 16S-variants of *B. aalborgi* (Mikosza et al., 2004; Westerman et al., 2012, 2013b). While *B. aalborgi* is not associated with gastroenteritis in humans, there is evidence for the pathogenic potential of *B. pilosicoli* in humans (Westerman and Kusters, 2013). A review of the literature assumes that invasion of spirochetes beyond the surface epithelium may be associated with gastrointestinal symptoms which respond to antibiotic treatment, whereas individuals lacking this feature may be mostly asymptomatic (Tsinganou and Gebbers, 2010). Bloodstream infection with *B. pilosicoli* rarely occurs, and only in immunocompromised or critically ill patients (Prim et al., 2011).

3 *Brachyspira* infections in birds, other than chickens

Publications on intestinal spirochetes in birds, other than chickens, are limited. The published avian host range includes different orders. Demonstration of pathogenicity in wild birds is not obvious. Some publications report on clinical findings in domesticated species. Until today, the necrotizing typhlocolitis in common rheas, associated with *B. hyodysenteriae*, is the most serious avian intestinal spirochaetosis (AIS) entity recognized. Waterfowl could be considered as a natural reservoir for intestinal spirochetes.

Struthioniformes

At the beginning of the 1990s, intestinal spirochetes were associated with necrotizing typhlocolitis in common rheas (*Rhea americana*) from flocks across the USA. In young birds the mortality ranged from 25% to 80%. At postmortem examination a diphtheritic membrane covered ulcerated caecal mucosa. Caecal sections showed necrosis and granulomatous-to-suppurative inflammation. Strongly β-hemolytic spirochetes isolated from necrotic caeca were identified as *B. hyodysenteriae*. All isolates from non-necrotic caeca, and also some from necrotic caeca, were

weakly β-hemolytic (Sagartz et al., 1992; Stoutenburg et al., 1995; Jensen et al., 1996; Buckles et al., 1997).

Isolates of *B. hyodysenteriae* from rheas with typhlocolitis were pathogenic for neonatal common rheas after experimental infection but not for postweaning piglets (Buckles et al., 1997; Stanton et al., 1997). Strains of *B. hyodysenteriae* from rheas and pigs were shown to be genetically closely related in multilocus enzyme electrophoresis (MEE), but the genomic differences demonstrated by pulsed-field gel electrophoresis (PFGE) indicated that they were not identical (Trott et al., 1996b).

Brachyspira pilosicoli was isolated from a herd of imported common ostriches (*Struthio camelus*) in Iran, suffering from severe diarrhea and mortality (Razmyar et al., 2011).

Phoenicopteriformes

Weakly β-hemolytic spirochetes were isolated from an American flamingo (*Phoenicopterus ruber*), belonging to a zoologic collection in Ohio, USA. The bird was clinically normal (Stoutenburg et al., 1995).

Galliformes

Brachyspira infections in chickens (*Gallus gallus*) will be discussed in a separate chapter.

Caecal spirochaetosis associated with *B. pilosicoli* has been reported in captive-raised juvenile ring-necked pheasants (*Phasianus colchius*). However, the etiologic significance of the spirochete infection in the pheasants was unknown because multiple other pathogens dominated the clinicopathological manifestations. In the naturally infected pheasants formation of “false brush borders” was microscopically seen, due to attachment of the spirochetes to the caecal enterocytes, and this was also noticed in young chickens, after experimental inoculation as one-day old chicks with the pheasant isolate (96-3914A) (Webb et al., 1997). In a case-control study in UK, *Brachyspira* was isolated from pheasants as young as six weeks old but there was an increasing incidence in older birds (up to 94% in birds aged > 20 weeks). The majority of isolates were identified phenotypically as *B. innocens*. Association of isolation of *Brachyspira* spp. with abnormal caecal content or with enteritis could not be confirmed in this study (Welchman et al., 2013b).

Colonization of *Brachyspira* spp. (including *B. pilosicoli* and *B. intermedia*) in grey partridges (*Perdix perdix*), and in pheasants, was reported on Swedish game-bird farms (Jansson et al., 2001a).

In two commercial flocks of guinea fowl (*Numida meleagridis*) in Spain, *B. pilosicoli* was isolated in birds of 60 and 91 days old with enteric disorder. Because the spirochetes were isolated in association with other pathogens, the role of *B. pilosicoli* in the enteric syndrome in guinea fowl needs further investigation (Bano et al., 2013b).

Caecal spirochaetosis and typhlitis in 7.5- to 18-week-old commercial turkeys was associated with *B. pilosicoli*. Despite of absence of macroscopic changes of the caeca, histopathological examination revealed a mild typhlitis with intimate adherence of the spirochetes onto the apical brush border of caecal enterocytes (Shivaprasad and Duhamel, 2005).

Anseriformes

Free-living waterfowl, i.e. wild mallards and geese, host many species of the genus *Brachyspira*, namely *B. hyodysenteriae*, *B. intermedia*, *B. pilosicoli*, *B. alvinipulli*, *B. innocens*, *B. murdochii*, "*B. suanatina*", "*B. pulli*", "*B. hampsonii*", and isolates of unknown species affiliation, as mentioned in study-reports from Australia, Sweden, Spain and Canada (Oxberry et al., 1998; Jansson et al., 2004, 2011; Martínez-Lobo et al, 2013a, 2013b; Rubin et al., 2013b). The isolation rates of spirochetes were high. The isolation rate on farmed mallards (93%) was even higher than in wild mallards (78%) in a Swedish study, probably due to high population density on the game farms, where also wild birds could frequently fly in and out (Jansson et al., 2004). When isolates from free-living wild mallards were compared genetically and phenotypically with isolates from pigs and chickens, many isolates of the mallards were related to isolates from livestock (Jansson et al., 2011). Isolates, related to isolates from pigs, including "*B. hampsonii*", were obtained from lesser snow geese (*Chen caerulescens caerulescens*) sampled in the Canadian arctic, more than 1,500 km away from the nearest pig-producing areas, strongly supporting the conclusion that these geese are not merely transport hosts but are colonized by *Brachyspira* (Rubin et al., 2013b). Waterfowl should be considered as an asymptomatic natural reservoir and potential source of intestinal spirochetes to other animals and livestock (Oxberry et al., 1998; Jansson et al., 2011; Martínez-Lobo et al., 2013a, 2013b; Rubin et al., 2013b). It was demonstrated in a challenge model that, for instance, strongly hemolytic *Brachyspira* spp. may cross the species barrier between pigs and birds. In one week-old mallard ducklings, colonization was established with strains of "*B. suanatina*" of pig and mallard origin and with *B. hyodysenteriae* of mallard origin. None of the birds developed clinical signs; microscopically some birds had mild focal changes in the caecal mucosa (Jansson et al., 2009a). It was also demonstrated that a "*B. hampsonii"* isolate from a lesser snow goose could colonize experimentally inoculated pigs (Rubin et al., 2013b).

In a zoologic collection of birds in Ohio, USA, weakly β-hemolytic spirochetes could be cultured from different species of the Order of the Anseriformes. The birds were all clinically normal (Stoutenburg et al., 1995).

Although most studies noticed absence of clinical signs in ducks colonized with *Brachyspira* spp., typhlocolitis was associated with *B. pilosicoli* and *B. hyodysenteriae* in two Hungarian breeder duck flocks during their first egg-laying season. Clinical signs included movement difficulties, lack of appetite and depression, but diarrhea was not observed. Mortality over a 24-week period reached 18.4%. Pathological examination revealed hemorrhagic to fibrinonecrotic typhlocolitis, renal degeneration accompanied by fibrosis and mineralization, hepatic and splenic amyloidosis, and swelling of some of the metatarsal and phalangeal joints. Consistently spirochetes were demonstrated in the mucous membrane of the affected large intestine (Glávits et al., 2011).

Also in geese clinical and pathological changes were linked to *Brachyspira* colonization. Typhlocolitis in two domestic breeder goose flocks in Hungary was associated with *B. alvinipulli* and *B. hyodysenteriae*. Increased mortality of 18% to 28% during an 8- to 12-week period at the end of the first egg-laying season, in the period of moulting, was observed. Pathological examination revealed hemorrhagic to necrotic inflammation of colon and rectum and fibrinonecrotic typhlitis, and, often

fibrosis of the kidneys with secondary visceral gout. Consistently spirochetes were demonstrated in the mucous membrane of the affected large intestine (Nemes et al., 2006). Experimental infection of goslings with these *B. alvinipulli* and *B. hyodysenteriae* strains did not result in mortality or pronounced diarrhea; however, the caecal content was thinner, yellowish and frothy. *Brachyspira* cells occurred in large numbers in the lumen of the glands of the caecal mucosa, which showed mild infiltration with inflammatory cells, and spirochetes were also detectable between the epithelial cells and in the lamina propria (Ivanics et al., 2007).

Passeriformes

In a Swedish study, isolates of intestinal spirochetes were obtained from 43 of 116 sampled corvid birds of three species, i.e. jackdaws (*Corvus monedula*), hooded crows (*Corvus corone cornix*) and rooks (*Corvus frugilegus*). The spirochete isolates belonged to a single and novel species, provisionally named "*Brachyspira corvi*". Examination by light microscopy did not indicate association with enteric disease in necropsied birds (Jansson et al., 2008b). Spirochetes in jackdows were detected microscopically at the level of villar surfaces in the jejunum, glands in the ileum and also in the colonic crypts. With transmission electron microscopy, end-on attachment on jejunal enterocytes was noticed. The microvilli of colonized cells were intact but were laterally displaced (Jansson et al., 2009c).

Charadriiformes

An intestinal spirochete, probably representing a new species within the genus *Brachyspira*, was isolated out of a cloacal swab from a snowy sheathbill (*Chionis alba*) in Antarctica. It was the first report of an intestinal spirochete from an animal living in a polar region (Jansson et al., 2009b).

4 *Brachyspira* infections in chickens

Avian intestinal spirochaetosis is widespread among flocks of adult laying hens and breeder hens. The main *Brachyspira* species involved in clinical cases are *B. intermedia* and *B. pilosicoli*, which are probably worldwide distributed in chickens, while *B. alvinipulli* being less frequently reported.

The economic significance of AIS is mainly related to reduced egg production and the production of lower value eggs (eggs for processing) because of fecal staining of egg shells. In 2006 the annual losses in the UK, due to AIS caused by *B. intermedia* and *B. pilosicoli*, were estimated at £14 million (€17.5 million) or 1.5% of the UK commercial laying industry production (Burch et al., 2006; Hampson and Swayne, 2008). Offspring from broiler breeder flocks with clinical signs of AIS have increased feed consumption with subsequent losses to the broiler industry. The economic impact of increased feed consumption in offsprings from parents with AIS was estimated at a yearly loss of approximately €13,600 for a broiler farm of 50,000 birds, running at six turnaround periods per year (Smit et al., 1998). The breeder flock with clinical signs of AIS is faced with additional losses, due to decreased egg production and increased food intake, estimated at approximately €14,600 per flock annually (Smit et al., 1998).

4.1 Epidemiology

High *Brachyspira* colonization rates in layers have been reported in field studies from many countries, almost globally distributed. Different *Brachyspira* species have been reported, i.e. *B. intermedia*, *B. pilosicoli*, *B. alvinipulli*, *B. hyodysenteriae*, *B. innocens*, *B. murdochii*, and *"B. pulli"*, occurring in layers and broiler breeders. Several risk factors for colonization with *Brachyspira* species have been identified, linked to the housing system and management of the farm, or to feed components. Cross-contaminations between flocks and contaminations from rodents and wild birds, possible reservoirs of spirochetes, are important in the transmission of AIS on a poultry farm.

4.1.1 Prevalence

Colonization of chickens by *Brachyspira* spp. has been reported from Australia, Europe (Belgium, Czech Republic, Finland, Germany, Hungary, Italy, The Netherlands, Poland, Sweden, the United Kingdom, former Yugoslavia), the Americas (Iowa-USA, Ohio-USA, Pennsylvania-USA, Ontario-Canada, Mexico, Colombia), Middle East (Iran), and Southeast Asia (Malaysia) (Davelaar et al., 1986; Griffiths et al., 1987; Dwars et al., 1989; Swayne et al., 1992; Trampel et al., 1994; McLaren et al., 1996; Smit et al., 1998; Stephens and Hampson, 1999; Jansson et al., 2001b, 2008 ; Kizerwetter-Świda et al., 2005; Phillips et al., 2005; Burch et al., 2006, 2009; Thomson et al., 2007; Corona-Barrera et al., 2007; Razmyar et al., 2007, 2011; Skrzypczak et al., 2007; Feberwee et al., 2008; Bano et al., 2008, 2013a; Alvarez et al., 2009; Ivanics et al., 2009; Jamshidi et al., 2009; Mat Amin et al., 2009; Myers et al., 2009; Šperling and Čížek, 2013, Medhanie et al., 2013a).

The prevalence of *Brachyspira* species among layer flocks is globally high and reported as 35%-54% in Australia (McLaren et al., 1996; Stephens and Hampson, 1999), 90% in flocks over 40 weeks of age in Pennsylvania (Myers et al., 2009), 24.3%-63.5% in Ontario (Medhanie et al., 2013a), 20.6% in Colombia (Alvarez et al., 2009), 40% in Sweden (Jansson et al., 2008a), 69%-92% in the UK (Thomson et al., 2007; Burch et al., 2009), 42%-71% in Italy (Bano et al., 2008, 2013a), 47% in the Czech Republic (Šperling and Čížek, 2013), and 17% in Iran (Jamshidi et al., 2009). High *Brachyspira* prevalence of 26%-53% was reported among broiler breeder flocks in Australia (McLaren et al., 1996; Stephens and Hampson, 1999). Reported within-flock prevalence of *Brachyspira* species ranged from 10% to 100% (Stephens and Hampson, 1999; Bano et al., 2008; Myers et al., 2009).

Brachyspira intermedia and *B. pilosicoli* are possibly endemic worldwide. These two spirochetes are, together with *B. alvinipulli*, currently considered as the most pathogenic *Brachyspira* species for poultry. *Brachyspira intermedia* and *B. pilosicoli* have both been reported in North America, Australia, Europe and Southeast Asia. *Brachyspira alvinipulli*, in 1992 reported in a field case in Ohio associated with pasty vents and dirty eggshells in layers (Swayne et al., 1992), has been only recently identified outside the USA and is present at a low level in Europe (Thomson et al., 2007; Feberwee et al., 2008; Jansson et al., 2008a, Bano et al, 2013a). The prevalence of infection was higher for *B. intermedia* than for *B. pilosicoli* in Western Australia, Pennsylvania, The Netherlands, Sweden, Czech Republic, Malaysia and also higher or almost equal in the UK and Italy (McLaren et al., 1996; Thomson et al., 2007; Bano et al., 2008, 2013a; Feberwee et al., 2008; Jansson et al., 2008a; Burch et al., 2009; Mat Amin et al., 2009; Myers et al., 2009; Šperling and Čížek; 2013). In contrast, the

prevalence of *B. pilosicoli* was higher than that of *B. intermedia* in Eastern Australia (Stephens and Hampson, 1999). In surveys in Columbia, Iran and Ontario, only *B. pilosicoli* was identified (Alvarez et al., 2009; Razmyar et al., 2011; Medhanie et al., 2013b).

Brachyspira hyodysenteriae was identified at low level in layers in The Netherlands and the UK and was also reported in chickens in Malaysia and in breeders in Hungary (Feberwee et al., 2008; Thomson et al., 2007; Burch et al., 2009; Ivanics et al., 2009; Mat Amin et al., 2009). Presence of *B. innocens* and *B. murdochii* was commonly reported in Australia, Pennsylvania, and Europe (McLaren et al., 1996; Stephens and Hampson, 1999; Phillips et al., 2005; Thomson et al., 2007; Feberwee et al., 2008; Jansson et al., 2008a; Bano et al., 2008, 2013a; Burch et al., 2009; Mat Amin et al., 2009; Šperling and Čížek, 2013). Colonization with "*B. pulli*" was reported in Australia, The Netherlands, Sweden and Malaysia (McLaren et al., 1996; Phillips et al., 2005; Feberwee et al., 2008; Jansson et al., 2008a; Mat Amin et al., 2009).

Co-infection with different *Brachyspira* spp. was detected commonly both at flock and bird level (Stephens and Hampson, 1999; Kizerwetter-Świda et al., 2005; Phillips et al., 2005; Thomson et al., 2007; Feberwee et al., 2008; Jansson et al., 2008a; Bano et al., 2008; Meyers et al., 2009; Ivanics et al., 2009).

4.1.2 Risk factors

A significant positive association between age of the chickens and *Brachyspira* colonization has been demonstrated (Stephens and Hampson, 1999; Philips and Hampson, 2005; Bano et al., 2008, 2013a; Burch et al., 2009; Mat Amin et al., 2009; Medhanie et al., 2013a). The higher odds of *Brachyspira* species for flocks from multi-age farms compared to single-age farms suggest inter-flock transmission within a farm (Medhanie et al., 2013a).

The higher risk of rearing on litter, in contrast to cages, for being colonized with *Brachyspira* species and association with poor performance, was already demonstrated in an early work in the UK (Griffiths et al., 1987). This could not be confirmed in a field survey of 1988 in The Netherlands where no significant difference was found in colonization between housing in cages or on litter (Dwars et al., 1989). However, in more recent studies in The Netherlands, UK, Sweden, and Malaysia, hens in open housing systems, free range or organic were at higher risk for colonization than hens in other housing systems {eg, in a Swedish study, organic laying hen flocks were at higher risk (RR = 2.3; 95% CI 1.5-3.6) for being colonized by *Brachyspira* spp. than laying hens in indoor housing systems (Jansson et al., 2008a)} (Wagenaar et al., 2003; Jansson et al., 2001b, 2008a; Burch et al., 2009; Mat Amin et al., 2009). In a UK field survey, below target egg production was significantly associated in caged flocks with *B. intermedia*, and in free-range flocks with *B. innocens*, usually considered as a non-pathogenic species (Burch et al., 2009).

Deep pits in barns of laying hens is a higher risk factor for intestinal spirochetes colonization than conveyor belts, however there was no difference between the two waste disposal systems regarding the presence of the two pathogenic species, i.e. *B. intermedia* and *B. pilosicoli* (Bano et al., 2008). In the Ontario-study, the odds of *Brachyspira* species for flocks housed in A-frame cages with manure curtains were significantly higher than for flocks housed in stacked cages; and, for unclear

reasons, the odds of *Brachyspira* spp. among flocks housed in newer barns were 10 times higher than flocks housed in older barns (Medhanie et al., 2013a).

Flock size seems not to be a risk factor for colonization with intestinal spirochetes (Bano et al., 2008, 2013a).

Pigs and chickens harbor many identical species of the genus *Brachyspira* and chickens can be colonized experimentally with different *Brachyspira* species from porcine origin. Analysis of *B. intermedia* isolates from poultry and pig origin with MLST suggested that cross-species transmission may occur (Phillips et al., 2010). Yet there are, as far as we known, no published studies on the risk that presence of pigs on a poultry farm can represent for spirochetal colonization of the chickens, except one study where there was no evidence of a higher risk (Dwars et al., 1989).

Laying hen flocks infected with *B. intermedia* should not be given a wheat-based diet. A wheat-based diet and even the wheat variety used in the diet, in contrast to diets based on barley and/or sorghum, enhanced colonization with *B. intermedia* (strain HB60; strain reference, see Table 1) in experimental trials in layers, and this apparently through diet-related alterations in the intestinal microenvironment. Addition of dietary enzymes (xylanase, α-amylase and β-gluconase based) had inconsistent and non-significant effects on altering the susceptibility of hens to colonization by *B. intermedia* in these trials (Phillips et al., 2004a, 2004b). Still, in a previous trial the extent of colonization by *B. intermedia* (strain BH60) was significantly reduced when an enzyme complement, containing predominantly xylanase and protease, was added to the wheat-based diet of challenged layers (Hampson et al., 2002b). The opposite effect of the addition of zinc bacitracin (ZnB) to the feed on the colonization of *B. intermedia* (inhibited) in contrast to *B. pilosicoli* (enhanced) in layers, will be discussed later (see: '4.5.1 Therapeutics').

4.1.3 Transmission routes

Viability of the pathogenic spirochetes outside the animal body is an important factor for disease spreading. Presence of organic material and low temperature prolonged the survival time of *Brachyspira* species in water. A survival time for up to 70 days in lake water held at 4°C was demonstrated for *B. pilosicoli* strains from pig- and human origin; the survival was reduced to 4 days at 25°C (Oxberry et al., 1998). In laboratory microcosms, the survival times for *Brachyspira* strains from pig origin were extended when manure was added to soil and when the temperature was decreased. Survival time was about half as long for the *B. hyodysenteriae* strain than for the *B. pilosicoli* strain which reached 120 days of survival in pure soil at 10°C and 210 days in soil mixed with 10% porcine feces or in pure porcine feces (Boye et al., 2001). Survival times for avian strains (*B. intermedia* HB60, *B. pilosicoli* CPSp1; strain references, see Table 1) in chicken caecal feces under laboratory conditions were much shorter than those reported for pig strains and varied from 41-84 hours at 4°C, and 17-74 hours at 25°C (Phillips et al., 2003). The acidic and relatively dry composition of chicken feces were proposed as possible explanations responsible for the short survival times; also the possibly inferior protective condition for the spirochetes in experimentally seeded feces than in the feces from naturally infected chickens, was questioned (Phillips et al., 2003).

Avian intestinal spirochetes are inactivated in less than one minute by several common disinfectants (quaternary ammonium, iodine as an iodophor, chlorine from a chlorine-release agent, glutaraldehyde and hydrogen peroxide), which was demonstrated in an *in vitro* experiment (Phillips et al., 2003). Regarding the reported short survival time of avian *Brachyspira* species in chicken caecal feces, it was postulated that 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 fecal matter (Phillips et al., 2003). It would be unlikely in these conditions that infection occurs in new batches of birds from spirochetes surviving in the shed from a previous batch of birds. It is suggested that on multi-age production farms, although the original source of infection is unknown, mechanical spread of fresh feces from older hens can be the source of infection in young new flocks (Phillips et al., 2003, 2005).

Wild birds, rodents and dogs are possible reservoirs for many different *Brachyspira* species and potential sources of AIS on poultry farms. In a Swedish study (Backhans et al., 2011), *Brachyspira* isolates collected from rodents, pigs and chickens on the same farms were analyzed by random amplified polymorphic DNA (RAPD) and PFGE. Identical isolates of *B. pilosicoli*, *B. intermedia*, *B. murdochii* and *B. innocens* from pigs and rodents and of *B. murdochii* from laying hens and rodents were found. The same RAPD type of *B. murdochii* was detected in laying hens sampled in 2005 and then in house mice in 2006, despite intense rodent control between the two sampling occasions. These results indicate cross-species transmission at farm level and that *Brachyspira* genotypes may remain present on the farm over time. An environmental reservoir must be available, such as rodents, other wild animals, water or soil, to ensure survival of *Brachyspira* spp. despite all-in all-out management (Backhans et al., 2011).

4.2 Pathology

4.2.1 Disease association

In general, AIS is present in chickens from the onset of lay and is associated with intestinal diseases (chronic diarrhea, wet feces and litter, pasty vents) and production losses (delayed and decreased egg production, drop in egg production, fecal staining of eggshells, reduced eggshell quality, pale-colored egg-yolks, increased feed conversion, retarded growth rate, decrease in hatchability, increased mortality). The association of colonization with *Brachyspira* species and the occurrence of signs of AIS was significantly demonstrated in several field studies (Dwars et al., 1989; McLaren et al., 1996; Stephens and Hampson, 1999; Jansson et al., 2001b; Burch et al., 2009; Bano et al., 2008, 2013a; Razmyar et al., 2011; Medhanie et al., 2013a). Nevertheless, in some field studies (in Sweden, the USA and Malaysia), no significant differences were found between *Brachyspira* positive and *Brachyspira* negative flocks with regard to clinical parameters like wet litter, fecal staining of eggshells or egg production (Jansson et al., 2008a; Myers et al., 2009; Mat Amin et al., 2009). Suggested possible reasons for the lack of a causal relationship between *Brachyspira* colonization and signs of AIS included difficulties in defining the clinical problem (due to the use of less-objective recordings of some parameters - for instance wet manure - or due to the use of data only related to the sample-day instead of long term data - for instance egg production), the possible presence of

confounding factors such as the presence of other pathogenic microorganisms, the absence of quantitative methods for measuring the colonization rates in individual fecal samples or birds, the possible strain-difference in virulence of a *Brachyspira* species, and the limited number of flocks in the study (Jansson et al., 2008a; Myers et al., 2009, Mat Amin et al., 2009). In Italy, colonization with *B. intermedia* and/or *B. pilosicoli* was significantly associated with the presence of reduced egg production but not with the presence of enteric disorders like increased fecal water content and/or pasty vents (Bano et al., 2008, 2013a). Statistical analysis of data concerning performance and *Brachyspira* infection in layer flocks in the UK supported previous findings of the pathogenicity of *B. intermedia* and *B. pilosicoli*, and showed moreover that the closest association between *Brachyspira* species and 'disease' was for *B. intermedia*. Interestingly, this analysis demonstrated also an apparent association between the presumed 'non-pathogenic' *B. innocens* and poor egg production (Welchman et al., 2013a).

Natural colonization with *Brachyspira* spp. has not been demonstrated in broiler flocks (Stephens and Hampson, 1999; Jansson et al., 2001b; Razmyar et al., 2011). *Brachyspira* colonization in broiler breeders can have a negative effect on their offspring. In a study in The Netherlands with data from 136 broiler flocks and 8 breeder flocks, it was demonstrated that the broiler flocks from affected breeders with clinical signs of AIS had a higher feed conversion, an increased number of weak chicks, slower growth and poorer feed digestion than the offspring of flocks where spirochetes were not present (Smit et al., 1998).

4.2.2 *Brachyspira* strains from poultry-origin

In challenge trials in laying hens with *Brachyspira* strains from poultry origin, the pathogenic potential of the species *B. intermedia*, *B. pilosicoli* and *B. alvinipulli* was demonstrated. In general, gross changes of the caeca and microscopic lesions of typhlitis were rather mild to moderate but the presence of pathology was not always consistent. The possible pathogenicity in layers of "*B. pulli*" and *B. hyodysenteriae*, and even of *B. innocens* and *B. murdochii* remains unclear due to a lack of experimental infections in layers with isolates from poultry-origin. Challenge studies in chickens with *Brachyspira* strains from poultry origin are summarized in Table 1.

Table 1. Challenge studies in chickens with *Brachyspira* strains from poultry-origin

Brachyspira species	Challenge strain reference	Challenged birds	Study reference
Brachyspira intermedia			
	Strain 1380: recovered from Dutch chickens showing diarrhea (Dwars et al., 1990)		
		20-week-old SPF layer hens	Dwars et al., 1990
		20-week-old SPF layer hens	Dwars et al., 1991
		one-day-old broilers	Dwars et al., 1992
		14-week-old broiler parents	Dwars et al., 1993
	Strain HB60: isolated from an Australian laying hen with diarrhea (McLaren et al., 1996, 1997)		
		14-week-old conventional layer hens	Hampson and McLaren, 1999
		19-week-old conventional layer hens	Hampson et al., 2002a
		22-week-old conventional layer hens	Hampson et al., 2002b
		20-week-old conventional layer hens	Phillips et al., 2004a
		21-week-old conventional layer hens	Phillips et al., 2004b
		26-week-old conventional layer hens	Amin et al., 2009
	Unnamed strains		
		one-day-old SPF broiler chickens	Hampson and McLaren, 1997
Brachyspira pilosicoli			
	Strain CPSp1: isolated from a broiler breeder in Queensland, Australia (Stephens and Hampson, 2002a)		
		17-week-old conventional broiler breeders	Stephens and Hampson, 2002a
		22-week-old conventional broiler breeders	Stephens and Hampson, 2002b
		22-week-old conventional layer hens	Jamshidi and Hampson, 2002
		17-week-old conventional layer hens	Mappley et al, 2013b
	Strain B2904: isolated from a chicken exhibiting clinical symptoms of AIS in the UK (Mappley et al., 2011)		
		18-week-old conventional layer hens	Mappley et al., 2013a
		17-week-old conventional layer hens	Mappley et al., 2013b
	Unnamed isolate recovered from a flock in Queensland		
		one-day-old SPF broiler chickens	Hampson and McLaren, 1997
Brachyspira alvinipulli			
	Strain 91-1207/C1: isolated from a conventional layer hen with diarrhea in Ohio, USA (Swayne et al., 1992)		
		one-day-old and 14-month-old SPF layer hens	Swayne et al., 1995
"Brachyspira pulli"			
	Unnamed West Australian strains (McLaren et al., 1996)		
		one-day-old SPF broiler chickens	Hampson and McLaren, 1997
Brachyspira innocens			
	Strain CPSi1: isolated from a broiler breeder hen in Queensland, Australia (Stephens and Hampson, 2002a)		
		17-week-old conventional broiler breeders	Stephens and Hampson, 2002a

Brachyspira intermedia

A limited number of *Brachyspira intermedia* strains from poultry origin were used in challenge studies in layer hens (Dwars et al., 1990, 1991; Hampson and McLaren, 1999; Hampson et al., 2002a, 2002b; Phillips et al., 2004a, 2004b; Amin et al., 2009). Clinical signs were mostly restricted to abnormal feces. In some studies no significant effect on fecal moisture content was noticed while others report only a transient increase in fecal water content or only an increase of the amount of crude fat in the fecal dry matter. Different studies report development of diarrhea and caecal droppings becoming lighter in color, slimy, wet and frothy. Although one study mentioned that infected birds tended to be lighter, most studies report no significant effect on body weight. Reductions in mean number of eggs laid and mean egg weights were significant in studies with a long follow up for 16 to 18 weeks p.i., while this was not significant in studies with a short follow up for only 4 to 6 weeks. Postmortem, no or no consistent gross lesions could be observed; only distension or ballooning of the

caeca with yellowish-light brown, frothy, slimy, gassy and wet contents was regularly seen. Less abdominal fat was noticed in one study.

In several cases, no pathological changes could be found microscopically in the caeca. In others, only mild inflammatory changes were observed, with congestion and slightly increased numbers of mixed inflammatory cells in the lamina propria. Many spirochetes were observed, covering the caecal mucosal surface and filling up the crypts lumina. Scanning electron microscopy revealed the spirochetes overlaying and only loosely associated with the caecal epithelium. Occasionally spirochetes were seen intra-epithelial and in the lamina propria just under the epithelium. In localized areas, mostly on the tips of the villi and in the deeper parts of the crypts, the epithelium was separated from the underlying lamina, opening into subepithelial cavities, that were crowded with spirochetes ("gaplike lesions"). Occasionally, massive erosion or desquamation of epithelium heavily infected by spirochetes occurred. No clear signs of inflammation or other involvement of connective and lymphatic tissues were seen.

In a challenge study in broiler breeders a decrease in egg production and egg weight, and pale egg yolks with decreased carotenoid content were noticed. Progeny hatched from eggs from infected parents showed a reduced body weight gain, a tendency to develop rickets, and in blood plasma low concentrations of carotenoids and elevated alkaline phosphatase activity. No spirochaetes were detected in these chicks (Dwars et al., 1993).

Brachyspira pilosicoli

Gross and histologic pathology in chickens due to colonization with *B. pilosicoli* strains from poultry origin was described in a limited number of challenge studies (Hampson and McLaren, 1997; Stephens and Hampson, 2002a, 2002b; Jamshidi and Hampson, 2002; Mappley et al., 2013a, 2013b), and in a few field observations (Trampel et al., 1994; Feberwee et al., 2008). Comparable to *B. intermedia*, clinical signs after challenge in layers and breeders with *B. pilosicoli* strains from poultry-origin were more than once not significant or transient. However, in several studies, there was an increase in fecal moisture content with fecal staining of the eggshells, a reduced body weight gain, a delayed onset of egg production with one or two weeks and a reduction in egg production and in mean egg weight.

In some challenge studies, there were no gross or histological lesions in the caeca or other organs apart from a more fluid, gassy, frothy and paler caecal content. Histological changes in the caeca consisted of distended crypts and secondary lymphoid follicle proliferation, increased numbers of goblet cells, crypt abscesses and dilated crypts containing spirochetes, cellular debris and inflammatory cells. The epithelium surrounding crypts displayed attenuation, degradation and necrosis alongside crypt hyperplasia. Sometimes subepithelial hemorrhages were present. Infiltration, mild to accumulated, of lymphocytes, macrophages and heterophils was observed in the lamina propria. In none of the challenge studies in layers or breeders there was evidence of end-on attachment of spirochetes to the caecal epithelium. There are only a few descriptions in the literature of *B. pilosicoli* strains from poultry origin colonizing the chickens caecal enterocytes with the characteristic end-on attachment, forming a dense layer over the epithelium. This "false brush border" formation was mentioned in a case report in layers in the USA, a field survey in layers in The Netherlands and a

challenge study in day-old SPF broiler chickens with an Australian strain (Trampel et al., 1994; Hampson and McLaren, 1997; Feberwee et al., 2008). Spirochaetes were also observed penetrating between enterocytes, sometimes to the level of the lamina propria.

In recent challenge studies with Australian and UK strains of *B. pilosicoli*, spirochetes were isolated in low numbers from some birds, not only from caeca and colon but also from ileum, liver, spleen and/or oviduct. Histopathological changes in the liver and spleen were consistent with systemic spread of the spirochete but direct visualization of the etiological agent, which was not performed in these studies, is necessary to determine the specificity of the changes (Mappley et al., 2013a, 2013b).

Brachyspira alvinipulli

Only a few publications describe pathological changes in chickens colonized with *B. alvinipulli*. In a case report in laying hens in Ohio-USA, pasty vents, dirty eggshells, and green, pasty feces in colons and caeca were seen. Histologically, mild lymphocytic typhlitis with mild heterophil exocytosis, mild epithelial hyperplasia, and occasional epithelial necrosis were identified. Numerous spirochetes were identified in the caecal lumina and crypts (Swayne et al., 1992). Induction of diarrhea and mild to moderately severe typhlitis was confirmed in challenge studies in one-day-old SPF chicks and 14-month-old SPF laying hens with the Ohio-field strain. There was no influence on weight gain. Rarely, spirochetes were also seen penetrating between or beneath the caecal epithelial cells (Swayne et al., 1995). In a field study in The Netherlands on laying flocks with diarrhea and reduced production, focal necrosis was seen in the caeca with presence in the lumen of necrotic material containing *B. alvinipulli* spirochetes (Feberwee et al., 2008).

"Brachyspira pulli"

The pathogenic potential of *"B. pulli"* for laying hens has not yet been fully determined. Only in a single challenge study in one-day-old SPF broiler chickens with an Australian poultry field strain, diarrhea developed after 12-13 days and spirochetes were present in large numbers in the caecal crypts and lumen (Hampson and McLaren, 1997).

Brachyspira innocens

In a challenge study in broiler breeders with an Australian poultry field strain, no negative effect on fecal water content or on body weight gain and egg production was noticed. No gross or histological changes developed (Stephens and Hampson, 2002a). This seems to confirm the non-pathogenic status of *B. innocens* for chickens.

4.2.3 *Brachyspira* strains from non-poultry-origin

In the last two decades of the previous millennium, infection in young chicks (one day old) was used as an experimental model for the study of the enteropathogenicity of *B. hyodysenteriae* in the context of SD. Also for investigating the pathogenicity of other *Brachyspira* species, and this from different hosts, infection of one day old chicks was used as animal model. Only a few experiments were performed in adult layers in the context of zoonotic investigation or for development of diagnostic tools.

Brachyspira hyodysenteriae

One day old chicks - conventional broiler chicks, conventional layer chicks, or SPF layer chicks - were inoculated with *B. hyodysenteriae* strain B78^T (ATCC 27164), strain B204^R (ATCC 31212), strain WA15 or strain SA3, all from porcine origin (Sueyoshi et al., 1986, 1987; Sueyoshi and Adachi, 1990; Trott et al., 1995; Trott and Hampson, 1998, Prapasarakul et al., 2011). The severity of gross pathology and microscopic lesions was strain dependent. A reduced mean bodyweight could be noticed. Atrophic and elastic caeca were observed, filled with white watery fluid. Sometimes caeca were congested. Also thickening of the caecal mucosa and a caecal core, grayish, hard, and rod shaped could be observed. The caecal core consisted of eroded cells, mucus and spirochetes. Microscopically regressive changes were noticed, consisting in desquamation or severe erosion of superficial columnar epithelial cells. Progressive changes consisted of hyperplasia of mucosal epithelial cells, including goblet cells, and elongation of the crypts. Heterophilic and lymphocytic infiltrations in the lamina propria, edema and sometimes hemorrhagic foci were observed in the lamina propria. Numerous spirochetes were visible on the surface of damaged enterocytes, in the crypts, and between the luminal epithelial cells and lamina propria. Using electron-microscopic examination, membrane-bound vesicles in the epithelial cells were observed, microvilli were scarce and irregular, junctional complexes between cuboidal cells unclear and the intercellular spaces were widened (Sueyoshi and Adachi, 1990; Trott and Hampson, 1998).

Brachyspira pilosicoli

In one day old conventional or one day old SPF laying hen chicks that were inoculated with *B. pilosicoli* strains from human origin, canine origin, rhesus monkey origin, pig origin, or pheasant origin, the formation of a “false brush border” was noticed microscopically. Variation both with regard to the degree of spirochete attachment and the resulting development of lesions was observed between *B. pilosicoli* strains as well as between individual chicks infected with the same strain (Trott et al., 1995; Muniappa et al., 1996, 1997, 1998; Webb et al., 1997; Trott and Hampson, 1998; Prapasarakul et al., 2011). Using transmission electron microscopy, individual spirochetes were seen to be invaginated into the cellular membrane of the host, but no direct penetration into the cytoplasm was evident. Large numbers of vacuoles in the apical cytoplasm and lateral separation between adjacent columnar epithelial cells was noticed (Trott et al., 1995). Spirochetes were seen in the goblet cells in challenge studies in one day old conventional laying hen chicks with a Thai canine *B. pilosicoli* strain and with the *B. pilosicoli* ATCC51139 strain (porcine origin) (Prapasarakul et al., 2011). In one day old broiler chicks, inoculated with a human derived *B. pilosicoli* strain, gap-like lesions and focal erosion were produced; spirochetes were found sometimes between enterocytes, or formed subepithelial accumulations (Dwars et al., 1992).

After experimental infection of conventional laying pullets of 38 weeks with a human derived *B. pilosicoli* strain (strain HIV3AB2), a persistent and significant increase in fecal water content was noticed and birds were culture positive for *B. pilosicoli*. Neither specific pathological changes were found post-mortem, nor the formation of a “false brush border”, but the spirochetes were present between the intestinal villi (Jamshidi and Hampson, 2003, 2007). The absence of end-on attachment of the spirochetes to the luminal surface of the caecal enterocytes may have been due to the presence

of an extensive micro flora in laying hens in contrast to one day old chicks (Jamshidi and Hampson, 2007).

Brachyspira intermedia, Brachyspira innocens, Brachyspira murdochii, Brachyspira aalborgi, "Brachyspira pulli", "Brachyspira canis"

In a challenge study of one day old SPF laying hen chicks with a porcine *B. intermedia* strain (strain 889), some chicks became colonized and diarrhea and wet litter were observed. Weight gain was not significantly reduced. Caeca were macroscopically normal; histological examination showed only mild changes. The spirochetes were confined to the crypts, but occasionally, individual cells were seen within the lamina propria (Trott and Hampson, 1998).

In several challenge studies, with *B. innocens* strains from porcine and canine origin, with a porcine *B. murdochii* strain, and with a *B. aalborgi* strain, all strains failed to colonize one day old SPF or one day old conventional laying hen chicks (Trott et al., 1995; Muniappa et al., 1996, 1997; Hampson and McLaren, 1997; Trott and Hampson, 1998). Only in a single reported experimental challenge study in one day old conventional laying hens, the porcine *B. innocens* strain B256 (ATCC 29796) colonized some chicks, with presence of spirochetes in the caecal crypts and moderately extensive inflammatory cell infiltration of the lamina propria. The caecal contents were macroscopically normal, none of the chicks showed signs of illness and body weight gain was not influenced (Prapasarakul et al., 2011). In the same study, colonization was also realized with the Thai canine isolates "*B. pulli*" (strain AT1.8.8) and "*B. canis*" (strain BT7.4.12). Birds showed no signs of illness but body weight gain was significantly lower and caeca contained green watery/mucoid exudate. Inflammatory cell infiltration, crypt cell proliferation and villus shortening were present with large numbers of spirochetes in the crypts and the epithelial surface, and in case of the "*B. pulli*" infected chicks, also in the goblet cells and in the lamina propria. The most extensive changes were seen in the chicks infected with "*B. pulli*", possibly reflecting the fact that this species is adapted to chickens (Prapasarakul et al., 2011).

4.3 Pathogenesis

The pathogenesis of intestinal spirochaetosis is poorly understood. Of various attributes that were identified in *Brachyspira* cells, supposed "lifestyle" virulence factors for initial surviving in and colonization off the anaerobic environment of the lower intestinal tract, like utilization of substrates, motility and chemotaxis, are likely shared by commensal and pathogenic *Brachyspira* species. Nevertheless, differences in such lifestyle factors can probably cause differences in behavior and play a role in pathogenicity. More "essential" virulence factors are involved in lesion production and/or causing disease. The potential of proposed virulence factors, i.e. the hemolytic activity, the toxic activity of the lipooligosaccharides in the cell envelope, the chemotactic response and the attraction to mucine, has been mainly studied in SD and PIS caused by *B. hyodysenteriae* and *B. pilosicoli* (Hampson and Swayne, 2008).

During natural and in experimental infections with *B. pilosicoli* in different host species, this spirochete was able to form palisades by attaching with one cell end to the enterocytes, invaginating

into the cell surface without penetrating the cell membrane. The basis of the polar attachment of *B. pilosicoli* cells to the enterocytes is not clearly understood (Trott et al., 2001). *In vitro* studies demonstrated that *B. pilosicoli* strains vary with regard to their ability to attach to cultured Caco-2 cells. The attached strains induced pathological changes like actin accumulation at the cell junctions, loss of tight junction integrity and apoptosis. The colonized monolayers of Caco-2 cells demonstrated an up-regulation of interleukin-1β and interleukin-8 expression (Naresh et al., 2009). *In vitro* studies demonstrated also attraction of *B. pilosicoli* to mucin, with a considerable difference between strains (Naresh and Hampson, 2010). Exposure to norepinephrine, which may be present in elevated levels in stressed animals, enhanced *B. pilosicoli* growth *in vitro*, attraction to mucin and attachment to Caco-2 cells (Naresh and Hampson, 2011). In a recent study, *B. pilosicoli* was shown to attach by one cell end to chicken and goose erythrocytes *in vitro* and to aggregate them. This activity has the potential to contribute to disease severity in hosts that develop a spirochetemia (Naresh and Hampson, 2013).

A number of genes with potential roles in the pathogenesis and virulence of *B. hyodysenteriae* and *B. pilosicoli* were identified in the recently completely sequenced genomes of these species, i.e. core lipooligosaccharide biosynthesis genes, genes associated with chemotaxis and motility, genes for surface associated proteins, lipoproteins and ankyrin-like protein (likely important for adhesion and interaction with the host cell), genes for hemolysin, phospholipase and protease production (likely involved in host tissue degradation), and phage and other mobile genetic elements (Bellgard et al., 2009; Wanchanthuek et al., 2010; Mappley et al., 2012).

4.4 Diagnosis

Anaerobic culture of fresh (caecal) feces or cloacal swabs on selective agar plates is a standard procedure for AIS diagnosis. The identification of the isolates is often obtained by biochemical analysis (phenotyping) and species-specific PCR tests for *B. intermedia* and *B. pilosicoli.* Frequently also a combined PCR for *B. innocens*/*B. murdochii* is used.

Most isolates of poultry intestinal spirochetes species are weakly hemolytic. So therefore bacterial colonies must be collected from selective agar plates at random, which is in contrast to the situation in pigs where highly hemolytic growth is more suggestive for enteropathogenicity (Jansson, 2013). Co-infection with several species and/or several genotypes of a species is common within individual flocks and also within individual birds. Combined with the swarming growth pattern of the spirochetes on agar plates, co-colonization hampers isolation of pure cultures. Repeated subculture or serial dilution is necessary to obtain pure cultures for phenotyping, sequence analysis and 'finger-printing' techniques, and also for antimicrobial susceptibility testing (Jansson et al., 2008a; Jansson and Pringle, 2011; Jansson, 2013). To produce uncontaminated spirochete isolates, a simpler and more cost-efficient culture technique than serial dilution has been described. It is based on the intrinsic spirochetal feature of motility that allows only spirochetes to pass through filters (0.45 µm pore size) (Jansson et al., 2011).

Phenotypical identification is routinely based on the strength of hemolysis on blood agar and on a limited number of biochemical reactivities. A phenotypic profile pattern can be obtained, indicated by a combination of five digits, representing hemolysis, spot-indole, hippurate, α-galactosidase and β-glucosidase (Backhans et al., 2010). A summary of the phenotypic profile patterns of *Brachyspira* species is given in Table 2. Variations in biochemical reactions render phenotyping for identification of isolates unreliable.

Table 2. Phenotypic profile patterns of *Brachyspira* species

Brachyspira species	Hemolysis and biochemical reactions β-hemolysis / spot-indole / hippurate hydrolysis / α-galactosidase / β-glucosidase (numeral*)				
B. hyodysenteriae	2/1	1/0	0	0	1
"B. suanatina"	2	1	0	0	1
"B. hampsonii"	2	0	0	0	1/0
B. intermedia	1	1/0	0	0	1
"B. canis"	1	0	0	0	1
B. murdochii	1	0	0	0	1
B. innocens	1	0	0	1	1
"B. pulli"	1	0	0	1	1
B. pilosicoli	1	0/1	1/0	1/0	0/1
B. alvinipulli	1	0	1	1/0	1
B. aalborgi	1	0	0	0	0
"B. corvi"	1	0	0	1/0	0/1
"B. rattus"	1/0	0	0	0	1
"B. muridarum"	0/1	0	0	0	0
"B. muris"	0/1	0	0	0	0

* The numeral 0 indicates no hemolysis or a negative reaction, 1 indicates weak hemolysis or a positive reaction, and 2, strong hemolysis (Backhans et al., 2010).
Reaction results adapted from: Swayne et al., 1995; Stanton et al., 1998; Fellström et al., 1999; Stephens et al., 2005; Råsbäck et al., 2007a; Jansson et al., 2008a, 2008b; Chander et al., 2012; Mahu et al., 2013.

Species identification can be performed with species-specific PCR technology but is in general limited to the species *B. intermedia* (mainly based on *nox* gene sequences), *B. pilosicoli* (mainly based on 16S rRNA gene sequences) and *B. hyodysenteriae* (mainly based on *nox* or *tylA* sequences) (La et al., 2003; Phillips et al., 2005; Jansson et al., 2008). Species-specific primers for the poultry pathogenic species *B. alvinipulli* have until now not been published. Therefore, isolate identification can be performed with sequence analysis of 16S rRNA or NADH oxidase (*nox*) genes. Of these, the *nox* gene is now more widely used since the 16S rRNA gene has close sequence similarities between some species (Atyeo et al., 1999; Hampson, 2013). Multilocus enzyme electrophoresis and restriction fragment length polymorphism (RFLP) analysis of 16S rRNA or *nox* sequences have also been used for identification of poultry *Brachyspira* species in field surveys (McLaren et al., 1997; Bano et al., 2008; Šperling and Čížek, 2013). Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been applied on *Brachyspira* strains and is promising to become a reliable identification method for *Brachyspira* strains at the species level, overcoming the problems encountered with biochemical and genetic-based methods (Calderado et al., 2013).

A PCR test can also be applied directly on DNA extracted from fecal samples or from organs for diagnosis of AIS. A genus-specific PCR (mainly based on 16S rRNA gene sequences) followed by species-specific PCR tests are used, with the limitation that *B. alvinipulli,* and the presumed for poultry non-pathogenic species, can't be identified. Duplex, multiplex and qPCR tests have been described for use in AIS detection (Phillips et al., 2006; Song and Hampson, 2009). Limitations may occur; e.g. some strains of *B. intermedia* can be misidentified with the *nox*-based species-specific PCR due to the high genetic heterogenicity of the species (Atyeo et al., 1999; Jansson et al., 2008a, 2011).

Immunodiagnostic methods have been reported in some research studies on AIS but are in general not in use for AIS in routine diagnostic labs due to a lack of commercialy available reagents with sufficient species-specificity for identification. Direct and indirect fluorescent antibody tests were performed on caecal mucosal scrapings or content smears in some field surveys and case reports (Davelaar et al., 1986; Dwars et al., 1989; Smit et al., 1998; Feberwee et al., 2008), immunohistochemical staining was used in studies on *Brachyspira* colonization in chickens, turkeys, pheasants, ducks and geese (Webb et al., 1997; Shivaprasad and Duhamel, 2005; Nemes et al., 2006; Ivanics et al., 2007; Feberwee et al., 2008; Thuma et al., 2011; Prapasarakul et al, 2011; Glávits et al., 2011).

Only a single publication deals with serology for diagnosis of AIS in chickens (Smit el al., 1998), but a serological test with sufficient sensitivity and specificity is currently not available.

For studying the genetic relationship between isolates and molecular epidemiology, different techniques have been used, i.e. MEE, PFGE, multiple locus variable number tandem repeat analysis (MLVA) and MLST (Atyeo et al., 1996; Trott et al., 1996b; Suriyaarachchi et al., 2000; Townsend et al., 2005; Phillips et al., 2005; Stephens et al., 2005; Hampson et al, 2006b; Råsbäck et al., 2007b; Bano et al., 2008; Backhans et al., 2011; Neo et al., 2013a, 2013b). Multilocus sequence data have been made available at PubMLST where global catalogues of strains can be developed, publicly available for comparative studies (Råsbäck et al., 2007b; Hampson, 2013).

4.5 Therapy

Until date only few publications report on antimicrobial treatment of AIS, and with variable results. The use of antimicrobials in laying hens is also limited due to long withdrawal times for eggs for human consumption and the lack of appropriately licensed products. Acquired resistance among *Brachyspira* strains from poultry origin against classic antimicrobials is a growing cause of concern.

4.5.1 Therapeutics

In an *in vitro* susceptibility test of the *B. intermedia* strain HB60 (strain reference, see Table 1) to ZnB, performed in a broth dilution assay in concentrations of ZnB from 1 to 512 µg/ml, no inhibition of growth was observed (Hampson et al., 2002b). However, in experimental trials in layers with in-feed ZnB at concentrations of 50 ppm and 100 ppm, an inhibition of *B. intermedia* (strain HB60) colonization was noticed (Hampson et al., 2002a, 2002b). In contrast, 50 ppm ZnB in-feed enhanced the colonization of *B. pilosicoli* strain CPSp1 (strain reference, see Table 1) in experimental studies in

layers and broiler breeders (Jamshidi and Hampson, 2002; Stephens and Hampson, 2002b). ZnB is a bactericidal polypeptide antibiotic that influences the overall composition of the intestinal microflora (Engberg et al., 2000). An indirect effect of ZnB on other components of the intestinal microflora which in turn interact with the spirochetes, stimulating or inhibiting spirochete growth, is a possible explanation but the different outcome between *B. intermedia* and *B. pilosicoli* requires further investigation (Hampson et al., 2002b; Jamshidi and Hampson, 2002).

In several experimental- and field studies in layers and broiler breeders, treatments with different antimicrobials resulted in alleviation of symptoms, an improvement in performance, and clearing of spirochetes, but frequently spirochetal recolonization was noticed after a certain withdrawal period. Posologies of antimicrobials, used for curative treatments of AIS in chickens, are summarized in Table 3.

Table 3. Posologies of antimicrobials used for curative treatments of AIS in chickens

Chicken-type	Antimicrobial	Administration and posology	Reference
		Drinking water	
Broiler breeders	Lincomycin	20 mg/kg bw*/day for 5 days	Stephens and Hampson, 2002b
	Lincomycin-Spectinomycin	Linco-Spectin® 50 mg/bird°/day for 7 days	Stephens and Hampson, 1999
	Tiamulin	25 mg/kg bw/day for 5 days	Stephens and Hampson, 1999, 2002b
	Oxytetracycline	60 mg/kg bw/day for 4 days	Stephens and Hampson, 1999
	Ronidazole▪	120 ppm$ Ridzol S® for 6 days	Smit et al., 1998
Layers	Tiamulin	12.5 mg/kg bw/day for 5 days	Burch et al., 2006
	Tiamulin	25 mg/kg bw/day for 5 days	Hampson et al., 2002a
	Tiamulin	250 ppm for 5 days	Burch and Klein, 2013b
		In-feed	
Layers	Dimetridazole▪	125 ppm for 10 days	Griffiths et al., 1987

*bw = body weight
°broiler breeders of 40 weeks of age
$based presumably on active compound content
▪banned in food-producing animals

In a free-range flock, with recurring infections of *B. intermedia* and egg production drops 3-4 weeks following treatment with tiamulin, a preventive medication programme of tiamulin at 4 mg/kg bodyweight for 2 days/week for 5 weeks stabilized production for a further 5 weeks (Burch, 2007).

The reasons for recurring infections can be searched in sources for re-infection from other not treated or incompletely treated birds and contaminated environment (e.g. litter), or due to a proliferation of low numbers of residual organisms (Hampson et al., 2002a). In an infection trial with *B. pilosicoli* strain CPSp1 in broiler breeders housed in isolation unable to physically contact each other, the observation that birds did not become re-infected after tiamulin or lincomycin treatment suggests that the source of re-infection on a farm is most likely other incompletely treated birds rather than an endogenous residual infection or the immediate environment in the case of caged birds (Stephens and Hampson, 2002b).

4.5.2 Resistance to antimicrobials

Antimicrobial susceptibility tests

In contrast with data on SD, until date only a few publications deal with antimicrobial susceptibility testing of *Brachyspira* spp. from chickens and other birds (Trampel et al., 1999; Hampson et al., 2006a; Nemes et al., 2006; Alvarez et al., 2009; Jansson and Pringle, 2011; Burch and Klein, 2013a). In Table 4, the minimal inhibitory concentration (MIC) ranges of different antimicrobial agents for *Brachyspira* isolates from chickens are summarized as reported in the literature. Aquired resistance against common antimicrobials, e.g. tylosin, lincomycin and ampicillin, exists among intestinal spirochetes from laying hens.

There are very little published studies regarding the pharmacokinetics of antimicrobials in the intestines of chickens, which hampers the clinical breakpoint setting for *Brachyspira* species in chickens. In a pharmacokinetic trial with broiler chickens weighing approximately 2.3kg, birds were treated for 6 days with either 500 ppm tiamulin in the feed or 250 ppm in the drinking water, giving doses of approximately 30mg/kg bodyweight per day. The mean tiamulin caecal contents concentrations were 0.54 µg/g (feed medication) and 0.69 µg/g (drinking water medication). These concentrations were above the “wild-type” cutoff value of 0.5 µg/ml generated from sensibility tests with avian UK field isolates of *B. intermedia*, *B. pilosicoli* and *B. innocens* (Burch and Klein, 2013a).

Genetic basis for resistance in* Brachyspira *species

The genetic basis for resistance has been identified in *B. hyodysenteriae* isolates from pigs and in *B. pilosicoli* isolates of porcine, human and recently mallard-origin, but not yet in *Brachyspira* isolates from chickens.

Macrolide and lincosamine resistance is caused by a point mutation in the 23S rRNA gene of *B. hyodysenteriae* (an A→G transition or A→T transition mutation in position 2058, *E. coli* numbering) and of *B. pilosicoli* (an A→T transition mutation in positions 2058, and an A→G transition or A→C transition mutation in position 2059) (Karlsson et al., 1999, 2004; Hidalgo et al., 2011b).

Mutations identified in ribosomal protein L3 and 23S rRNA in the peptidyl transferase region are associated with reduced tiamulin and valnemulin susceptibility in porcine *B. hyodysenteriae* field isolates (Pringle et al., 2004; Hidalgo et al., 2011b; Hillen et al., 2014). Resistance is caused by alteration of the drug binding site. The presence of additional resistance determinants in clinical field isolates that do not affect tiamulin binding to the ribosome indicates that other tiamulin resistance mechanisms function in *Brachyspira* spp. (Pringle et al., 2004).

A point mutation in the 16S rRNA gene (G→C in position 1058, *E. coli* numbering) has been associated with low level resistance for tetracycline and was found in Swedish and German porcine isolates of *B. hyodysenteriae* with increased doxycycline MICs (Pringle et al., 2007).

Resistance to beta-lactam antibiotics mediated by β-lactamase activity has been reported in a resistant human *B. pilosicoli* strain from France, and a novel class D β-lactamase (OXA-63) hydrolyzing oxacillin, benzylpenicillin, and ampicillin was described. The $bla_{\text{OXA-63}}$ gene was chromosomally located and not part of a transposon or of an integron (Meziane-Cherif et al., 2008). Ampicillin and oxacillin resistance in human and porcine *B. pilosicoli* strains from Australia was

associated with the production of OXA-136 and OXA-137, two new variants of the class D *β*-lactamase OXA-63 (Mortimer-Jones et al., 2008). Another new variant of a class D β-lactamase gene, designated OXA-192, has recently been identified in a *B. pilosicoli* isolate of mallard origin in Sweden, the first report of a β-lactamase gene of *Brachyspira* spp. found in an avian host (Jansson and Pringle, 2011).

Discernible mutations, both spontaneous and UV induced, in the *B. hyodysenteriae gyrB* gene result in coumermycin A_1 resistance. The basis for coumermycin resistance in some other *B. hyodysenteriae* strains remains unknown, since the *gyrB* sequences of these strains are identical to that of wild-type strain B204. Mutations in genes other than *gyrB* have been associated with coumermycin resistance in other bacteria (Stanton et al., 2001).

4.5.3 Natural antimicrobial compounds

The *in vivo* inhibitory effects against pathogenic bacteria of major importance makes plant extracts and their components, such as essential oils, and organic acids the subject for further research as alternatives to antibiotics in the control of animal infectious diseases. Until date, in contrast to other pathogens, e.g. *Campylobacter* spp. and *Salmonella* spp., little is known about the *in vitro* and *in vivo* action of these natural antimicrobials against *Brachyspira* spp. and intestinal spirochaetosis, and only in *Brachyspira* strains of pig origin.

In vitro activity against isolates of *B. pilosicoli* and/or *B. hyodysenteriae* of pig origin was demonstrated for two garlic derivates, propyl propyl thiosulfinate and propyl propyl thiosulfonate, an extract of citric seeds and an extract obtained from citrus fruits (Hidalgo et al., 2011; Lobova and Cizek, 2004; Alvarez-Ordóñez et al., 2013a). In an *in vivo* model in mice, oral prophylactic treatment with a methanolic extract of the *Hypoxis hemerocallidea* corn (African potato) ameliorated *B. hyodysenteriae*-induced typhlocolitis, suggesting that it might provide preventive benefits for typhlocolitis in other animal species too (Liu et al., 2010).

Table 4. *In vitro* activities (MIC ranges) of 8 antimicrobial agents for isolates of *Brachyspira* spp. of chicken origin

Isolates			Antimicrobial							
			Tylosin			Tiamulin			Valnemulin	
Species	Origin	N*	MIC ranges[#]	MIC50[#]	MIC90[#]	MIC ranges	MIC50	MIC90	MIC ranges	Reference
B. intermedia	Australia	25	<4 - >100	<4	>100	<0,1 - 4	1	4		Hampson et al., 2006a
	Sweden	7	≤2 - 8			≤0,063 - 0,5			≤0,031 - 0,25	Jansson and Pringle, 2011
	UK	25				0,015 - 8,0	0,125	0,5		Burch and Klein, 2013a
B. pilosicoli	USA	2	10			0,1				Trampel et al., 1999
	Australia	17	<4 - >100	20	>100	<0,1 - 1	<0,1	1		Hampson et al., 2006a
	Colombia	12	5			≤0,1				Alvarez et al., 2009
	UK	25				0,0075 - 4,0	0,062	0,25		Burch and Klein, 2013a
B. alvinipulli	USA	2	0,1 - 10			0,01				Trampel et al., 1999
	Sweden	3	≤2			≤0,063 - 0,125			≤0,031 - 0,063	Jansson and Pringle, 2011
B. innocens	Sweden	8	≤2 - 4			≤0,063 - 0,25			≤0,031 - 0,125	Jansson and Pringle, 2011
	UK	25				0,015 - 8,0	0,062	2,0		Burch and Klein, 2013a
B. murdochii	Sweden	5	≤2 - 8			≤0,063 - 0,25			≤0,031 - 0,125	Jansson and Pringle, 2011
"B. pulli"	Sweden	3	≤2 - 4			0,125 - 0,25			0,063 - 0,25	Jansson and Pringle, 2011

*N = number of isolates analysed
[#]MIC = minimal inhibitory concentrations; in mg/l

Table 4. - Continued - *In vitro* activities (MIC ranges) of 8 antimicrobial agents for isolates of *Brachyspira* spp. of chicken origin

Isolates			Antimicrobial							
			Lincomycin			Ampicillin			Doxycycline	
Species	Origin	N*	MIC ranges[#]	MIC50[#]	MIC90[#]	MIC ranges	MIC50	MIC90	MIC ranges	Reference
B. intermedia	Australia	25	<1 - 50	<1	50	<1 - 50	<1	<1		Hampson et al., 2006a
	Sweden	7	≤0,5 - 1			1 - 2			0,25	Jansson and Pringle, 2011
B. pilosicoli	USA	2	25							Trampel et al., 1999
	Australia	17	<1 - 50	10	50	<1 - >100	10	>100		Hampson et al., 2006a
	Colombia	12	1							Alvarez et al., 2009
B. alvinipulli	USA	2	12,5							Trampel et al., 1999
	Sweden	3	≤0,5			≤0,5 - >32			≤0,125 - 0,25	Jansson and Pringle, 2011
B. innocens	Sweden	8	≤0,5			≤0,5 - 1			≤0,125 - 0,5	Jansson and Pringle, 2011
B. murdochii	Sweden	5	≤0,5 - 1			≤0,5			≤0,125 - 0,25	Jansson and Pringle, 2011
"B. pulli"	Sweden	3	≤0,5 - 1			1 - >32			≤0,125 - 0,25	Jansson and Pringle, 2011

*N = number of isolates analysed
[#]MIC = minimal inhibitory concentrations; in mg/l

Table 4. - Continue - *In vitro* activities (MIC ranges) of 8 antimicrobial agents for isolates of *Brachyspira* spp. of chicken origin

Isolates			Antimicrobial						
			Tetracycline			Metronidazole			
Species	Origin	N*	MIC ranges[#]	MIC50[#]	MIC90[#]	MIC ranges	MIC50	MIC90	Reference
B. intermedia	Australia	25	<1 - 5	<1	5	<0,1 - 1	1	1	Hampson et al., 2006a
B. pilosicoli	Australia	17	<1 - 20	<1	5	<0,1 - 1	1	1	Hampson et al., 2006a

*N = number of isolates analysed
[#]MIC = minimal inhibitory concentrations; in mg/l

4.6 Prophylaxis

Several risk factors for colonization with *Brachyspira* species have been identified, linked to the housing system and management of the farm, or to feed components (see before: 4.1.2 Risk factors). Some of these factors can be adjusted by the farmer's management, but some are inherent to the production type or process (e.g. organic, age). Cross-contamination between flocks can be tackled with good hygiene practice. Rodents and wild birds, possible reservoirs of spirochetes, represent a source of AIS on the poultry farm.

4.6.1 Probiotics

In recent studies, antagonistic effects of probiotic bacterial strains on poultry *B. pilosicoli* isolates and reduction of symptoms of AIS have been demonstrated. In *in vitro* studies, lactobacilli strains (*Lactobacillus reuteri* LM1 and *L. salivarius* LM2) antagonized the growth, motility and adherence of *B. pilosicoli* strain B2904 (strain reference, see Table 1) (Mappley et al., 2011). In an *in vivo* study oral treatment of chickens with *L. reuteri* LM1 reduced *B. pilosicoli*-induced pathology, i.e. reduction in spirochetal colonization, decrease of histopathological changes, higher bird weights, lower fecal moisture contents, improved egg weight and improved fecal staining score of the eggshells. It is possible that the antagonistic effect on *B. pilosicoli* is largely responsible for these ameliorations; however some effects such as weight gain, may relate to a direct effect from probiotic supplementation. The rapid growth and robust nature of lactobacilli compared with the slow-growing, fastidious *Brachyspira* make these species ideal probiotic candidates for intervention against AIS, possibly by niche competition, passive co-aggregation and/or acidification (Mappley et al., 2013a).

Also *B. pilosicoli* and/or *B. hyodysenteriae* strains of pig origin were in *in vitro* tests antagonized in growth by *L. amylovorus*, *L. rhamnosus*, *L. farciminis*, *L. salivarius*, *Bifidobacterium thermophilum*, *Enterococcus faecium* and *Bacillus subtilis*. Some properties of the probiotics were demonstrated, like coagreggation properties of *Lactobacillus* strains with inhibition of the motility of *Brachyspira*. Further studies will be required to determine the nature of the effects, e.g. direct niche competition, secretion of organic substances, metabolites and/or antibacterial compounds such as bacteriocins. Experimental challenge trials are needed to evaluate whether these bacteria exert their antagonistic effects on SD also *in vivo* (Bernardeau et al., 2009; Klose et al., 2010a, 2010b).

4.6.2 Vaccination

There are no vaccines against AIS available for use in poultry. Only a single report deals with *Brachyspira* vaccination in chickens: vaccination with an autogenous bacterin failed to prevent colonization by *B. intermedia* in experimentally infected laying chickens (Amin et al., 2009). Also for pigs, no vaccine is currently available which provides adequate protection against SD (Alvarez-Ordóñez et al., 2013b).

5 Zoonotic potential of *Brachyspira*

A lack of host-specificity is predominantly noticed in *B. pilosicoli*. Colonization with *B. pilosicoli* has been described in a lot of different mammalian species (ruminants, horses, pigs, carnivores, rodents, marsupials, and nonhuman primate species), different avian species, and humans. In experimental infection studies, it has been demonstrated that *B. pilosicoli* may cross the animal species barrier. Spirochetal colonization was demonstrated in chickens after experimental infection with *B. pilosicoli* strains from different origin, including human-, pig-, dog-, rhesus monkey- and pheasant-origin (Dwars et al., 1992; Trott et al., 1995; Muniappa et al., 1996, 1997, 1998; Webb et al., 1997; Trott and Hampson, 1998; Jamshidi and Hampson, 2003, 2007; Prapasarakul et al., 2011).

Genetic analysis of *B. pilosicoli* strains from diverse geographic origin and from different host species, demonstrated a general lack of clustering of strains based on host species or geographic origin. This was demonstrated with MEE and PFGE (Trott et al., 1998; Hampson et al., 2006b), MLVA and MLST (Neo et al., 2013a, 2013b). The occurrence of a cluster of MLST profiles of Australian *B. pilosicoli* isolates originating from pigs, chickens and a human being, suggests the likelihood of relatively recent transmission of members of this clonal group between species (Neo et al., 2013b). Also the occurrence of *B. pilosicoli* strains of humans and dogs with the same PFGE types or highly similar RFLP pattern, strongly supports the possibility of cross-species and zoonotic transmission (Koopman et al., 1993; Trott et al., 1998). Analysis with MLST confirmed that *B. pilosicoli* has a strongly recombinant population structure in contrast to the more highly clonal population structures of the related pathogenic species *B. hyodysenteriae* and *B. intermedia*. Analysis of the complete genome maps of four *B. pilosicoli* strains also showed an extensive rearrangement of MLST loci on their genome across the four strains (Neo et al., 2013b). The greater genomic plasticity of the recombinant *B. pilosicoli* may help to explain why it can colonize the large intestines of a wider range of hosts compared to other *Brachyspira* species (Neo et al., 2013b).

Zoonotic transfer of *B. pilosicoli* isolates likely occurs after exposure to infected mammals or birds, their feces, or contaminated water (Hampson et al., 2006b). Water sources, contaminated by *B. pilosicoli* from animals (e.g. wild birds, rodents, dogs), were identified as risk factors for HIS (Oxberry et al., 1998; Munshi et al., 2004; Margawani et al., 2004). The contact with colonized pet animals as possible contamination route for humans, and especially children, needs further investigation. Nevertheless, it was documented that pet shop puppies in Western Australia were commonly infected with these intestinal spirochetes (Oxberry and Hampson, 2003). Also intestinal spirochetes in guinea pigs, associated with typhlitis, were PCR-identified as *B. pilosicoli* (Vanrobaeys et al., 1998, Hélie et al., 2000).

Eggs stored dry at room temperature are likely not to be a potential source of *B. pilosicoli* infection for humans. After experimental colonization of laying hens with a human derived *B. pilosicoli* strain, egg surface and egg contents remained culture-negative for *Brachyspira* species. Due to the dry environment of the egg surface at room temperature and the unfavorable aerobic condition, the survival of *B. pilosicoli* was dramatically reduced (Jamshidi and Hampson, 2005). Transfer to humans can also be hypothesized to occur through the consumption of meat from infected chickens or chicken carcasses contaminated during processing (Smith, 2005), or through consumption of cross-

contaminated raw food (e.g. vegetables, salads). However, no data for the contamination of chicken meat with *Brachyspira* spp. are available.

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SCIENTIFIC AIMS

Avian intestinal spirochaetosis (AIS) is a disease complex in poultry characterized by colonization of the caeca with anaerobic intestinal spirochetes of the genus *Brachyspira* and has been reported worldwide mainly in laying hens and broiler breeder hens. In layers, AIS is associated with reduced egg production, chronic diarrhea, and fecal staining of eggshells. Colonization tends to be chronic in laying hen flocks, and antimicrobial treatment has met with limited success at long-term control. Only few data on antimicrobial susceptibility testing of *Brachyspira* spp. from chickens are available and little is known about acquired resistance against different antimicrobial drugs, hampering the treatment of AIS. Therefore, the first aim of this thesis was to determine the antimicrobial resistance pattern of recent field isolates of *Brachyspira* species from laying hens.

The number of antimicrobials available for use in laying hens producing eggs for human consumption is limited. Also the amounts of antibiotics used in veterinary medicine should be minimized due to, from a public health perspective, growing concerns of antimicrobial resistance. Therefore, the second aim was to examine whether alternatives for antimicrobial drugs, such as essential oils, can represent an alternative in the control of AIS.

Brachyspira pilosicoli is associated with chronic enteritis in humans, and may also cause disease in other mammalian species such as pigs and dogs. Some avian *B. pilosicoli* strains are closely related to human *B. pilosicoli* strains and exchange between man and birds is likely. The transfer to humans can be hypothesized to occur through contact with feces of infected animals or through the consumption of undercooked meat from infected chickens or carcasses contaminated during processing, or through consumption of cross-contaminated raw food (e.g. vegetables, salads). However, no data with regard to the contamination of chicken meat with *Brachyspira* spp. are available. Therefore, the third aim was to investigate whether viable *Brachyspira* species could be recovered from spent laying hen carcasses sold in supermarkets, and to determine the diversity of strains of *Brachyspira* on the carcasses.

EXPERIMENTAL STUDY 1

Antimicrobial susceptibility pattern of *Brachyspira intermedia* isolates from European layers

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ABSTRACT

A broth microdilution method was used to determine the antimicrobial susceptibility of 20 *Brachyspira intermedia* isolates obtained from different layer flocks in Belgium and The Netherlands between 2008 and 2010. The antimicrobial agents used were tylosin, tilmicosin, tiamulin, valnemulin, doxycycline, and lincomycin. The minimal inhibitory concentration (MIC) distribution patterns of tylosin, tilmicosin, lincomycin, and doxycycline were bimodal, demonstrating acquired resistance against doxycycline in three strains, against the macrolides in two strains, and against lincomycin in one strain. The MICs of tiamulin and valnemulin showed a monomodal distribution, but with tailing toward the higher MIC values, possibly suggesting low-level acquired resistance in six isolates. Sequencing revealed a G1058C mutation in the 16S rRNA gene in all doxycycline resistant strains. The strain resistant to tylosin, tilmicosin, and lincomycin had an A2058T mutation in the 23S rRNA gene.

INTRODUCTION

Avian intestinal spirochaetosis (AIS) is a disease complex characterized by colonization of the caeca by anaerobic intestinal spirochetes of the genus *Brachyspira*. It mainly affects laying hens and broiler breeder hens. AIS has been reported in Europe, Australia, and the United States (Hampson and Swayne, 2008; Stephens and Hampson, 2001). It is associated with reduced egg production, reduced average egg weights, delayed onset of egg laying, growth retardation, wet droppings, higher lipid content in feces, pasty vents, chronic diarrhea, and fecal staining of eggshells (Davelaar et al., 1986; Dwars et al., 1992, 1993; Griffiths et al., 1987; Swayne et al., 1992). Of the currently considered pathogenic species for poultry, *Brachyspira intermedia* and *Brachyspira pilosicoli* have been reported for AIS in layers in Australia, Europe, and the United States and *Brachyspira alvinipulli* has been only recently identified outside the United States at a low level in Europe (Feberwee et al., 2008; Hampson and Swayne, 2008; Myers et al., 2009).

To date, only a few publications deal with antimicrobial susceptibility testing of *Brachyspira* spp. from chickens. Trampel et al. (1999) tested two *B. pilosicoli* and two *B. alvinipulli* isolates from chickens in the United States. All four isolates were highly susceptible to lincomycin, carbadox, and tiamulin. Variable minimal inhibitory concentrations (MICs) were seen for chlortetracycline, oxytetracycline, tylosin, bacitracin, erythromycin, neomycin, and penicillin, suggesting that these antimicrobials may be effective against some isolates but not against others. The MIC of streptomycin was consistently high. Hampson et al. (2006) tested 25 *B. intermedia* isolates from chickens (24 Australian isolates and 1 isolate from The Netherlands) and 17 *B. pilosicoli* isolates from chickens (13 Australian isolates, 3 isolates from The Netherlands, and 1 US isolate). Based on available breakpoint values for *Brachyspira hyodysenteriae* or other Gram-negative enteric veterinary pathogens, isolates of both species were considered to be generally susceptible to tiamulin, lincomycin, metronidazole, and tetracycline, although one or more strains had elevated MIC ranges for tiamulin, lincomycin, tetracycline, and ampicillin. About half of the *B. intermedia* and *B. pilosicoli* isolates had increased MIC ranges for tylosin.

Only a few molecular mechanisms for antimicrobial resistance in *Brachyspira* spp. have been described. A point mutation at position 2058 in the 23S rRNA gene in *B. hyodysenteriae* from pigs and at position 2058 or 2059 in the 23S rRNA gene in *B. pilosicoli* was described as the genetic basis of macrolide and lincosamide resistance (Karlsson et al., 1999, 2004). A point mutation G1058C in the 16S rRNA gene of *B. hyodysenteriae* was described as the genetic background for decreased susceptibility to doxycycline (Pringle et al., 2007).

The aim of this study was to determine the antimicrobial resistance pattern of recent layer field isolates of *B. intermedia* from Belgium and The Netherlands. The molecular mechanism of acquired resistance was also studied.

MATERIALS AND METHODS

Bacterial isolates

Twenty isolates of *B. intermedia* were obtained from the caeca of chickens of 20 different laying flocks in Belgium (n=10) and The Netherlands (n=10) during the period 2008–2010. Laying hens were collected from flocks with symptoms of wet litter or diarrhea, and/or complaints of fecal staining of the eggs, and/or poor egg production. For primary isolation, samples of cecal content were cultured on trypticase soy agar (TSA) supplemented with 5% defibrinated sheep blood, 0.1% yeast extract, spectinomycin (400 µg/ml), vancomycin (25 µg/ml), and colistin (25 µg/ml) (Jenkinson and Wingar, 1981) and incubated for 4 days at 39°C in an anaerobic atmosphere (84% N2, 8% H2, and 8% CO2). Subculturing was done on TSA plates to obtain pure cultures, which were harvested in a peptone–glycerol medium and stored at -80°C. Identification and typing of the isolates were done using *Brachyspira* genus-specific and species-specific polymerase chain reactions (Jansson et al., 2008; Phillips et al., 2005). The type strains *B. intermedia* PWS/A^T (ATCC 51140) and *B. hyodysenteriae* B78^T (ATCC 27164) were included as control strains for standardization of antimicrobial susceptibility testing (Pringle et al., 2006).

Antimicrobial susceptibility testing

Six antimicrobial agents were used (range of final concentrations in mg/L of culture medium): tylosin (0.016–128), tilmicosin (0.016–128), tiamulin (0.031–4), valnemulin (0.016–2), doxycycline (0.063–8), and lincomycin (0.016–128) (Sigma-Aldrich, Seelze, Germany). The compounds were dissolved and diluted according to the Clinical and Laboratory Standards Institute (CLSI) recommendations to make stock solutions (CLSI, 2007).

The broth dilution method as described by Karlsson and Franklin (2000) was used, with some modifications. In the present study, fresh antimicrobial solutions were used instead of precoated multiwell plates. Briefly, 100 µl of twofold serial dilutions of the antimicrobial agents in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) supplemented with 10% fetal calf serum was transferred to wells of 48-well culture plates (Greiner Bio-One, Frickenhausen, Germany). Wells containing 100 µl BHI broth supplemented with 10% fetal calf serum but no antimicrobials were used as growth controls. The panels were prepared and stored at 4°C for less than 24 hr before use.

For preparation of the inocula, bacteria harvested from 4 days incubated TSA plates were suspended in BHI broth supplemented with 10% fetal calf serum. The procedure described by Karlsson and Franklin (2000) was followed to obtain final inoculum concentrations of $1x10^6$ to $5x10^6$ CFU/ml. Each well in the panels was filled with 0.4 ml of this inoculum. The panels were incubated in an anaerobic atmosphere (84% N_2, 8% H_2, and 8% CO_2; Hypoxic Workstation, Ruskinn Technology, South Wales, UK) for 4 days on a rotary shaker (60–80 rpm) at 37°C. The MIC was read as the lowest concentration of the antimicrobial agent that prevented visible growth after 4 days of incubation. All growth control wells were checked for pure growth by examining aliquots under a phase-contrast microscope.

Molecular mechanism of resistance

Amplification of a 388-bp fragment of the 23S rDNA (position 1858–2244 according to the *Escherichia coli* 23S rRNA gene sequence) was accomplished with primers, as described by Karlsson et al. (1999). Amplification of a 644-bp fragment of the 16S rDNA (position 800–1443 according to the *E. coli* 16S rRNA gene sequence) was accomplished with the previously described primers 5'-GTAGTCCACGCCGTAAACG-3' (Johansson et al., 2004) and 5'-GCTAACGACTTCAGGTAAAAC-3' (Phillips et al., 2005). The PCR was performed to amplify the fragments by using the following conditions: 15min at 95°C, 30 cycles of 30 sec denaturation at 94°C, 30 sec of annealing at 55°C, and 1 min of elongation at 72°C, followed by a final extension of 2min at 72°C. The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (AB Applied Biosystems) and the aforementioned primers. The purified PCR products were sequenced using an ABI Prism™3100 Genetic Analyzer. Contigs were created using the ContigExpress program, included in the Vector NTI Advance™10 (Invitrogen). Sequence alignment was done with the ClustalW2-Multiple Sequence Alignment program (EMBL-EBI).

RESULTS

Antimicrobial susceptibility testing

The MIC results are presented in Table 1.

For the field isolates, the MIC distribution patterns of tylosin, tilmicosin, lincomycin, and doxycycline were bimodal, demonstrating acquired resistance against doxycycline in three strains (MIC 2 mg/L), against tylosin and tilmicosin in two strains (MIC≥128 mg/L), and against lincomycin in one strain (MIC 8 mg/L). The lincomycin-resistant isolate was also resistant to the macrolides. The MICs of tiamulin and valnemulin showed a monomodal distribution, but with tailing toward the higher MIC values.

Table 1. Distribution of minimal inhibitory concentrations (MICs) of six antimicrobials on 20 *Brachyspira intermedia* isolates from laying hens in Belgium and The Netherlands

Antimicrobial agent	Number of isolates with MIC (mg/l) of													
	≤0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	≥128
Tilmicosin				1	4	5	6	2°	*					**2**
Tylosin						3	10	5		°	*			**2**
Tiamulin		5°*	1	6	2	**4**	**1**	**1**						
Valnemulin	5°*	4	5	**2**	**4**									
Doxycycline			1°	6	9*	1		**3**						
Lincomycin				3*	2°	6	8			**1**				

B. intermedia isolates considered to have acquired resistance according to the microbiological criterion are represented in bold.
° MIC for *B. hyodysenteriae* B78^T (ATCC 27164)
* MIC for *B. intermedia* PWS/A^T (ATCC 51140)

Molecular mechanism of resistance

The three doxycycline-resistant strains showed a G1058C mutation in the 16S rRNA gene, whereas all doxycycline susceptible isolates had the wild-type sequence. The strain that was resistant to tylosin, tilmicosin, and lincomycin had an A2058T mutation in the 23S rRNA gene. No mutations were found at position 2059. The type strains *B. intermedia* PWS/A^T (ATCC 51140) and *B. hyodysenteriae* B78^T (ATCC 27164) showed the wild-type sequences for both fragments.

DISCUSSION

There is no generally accepted or standardized method for antimicrobial susceptibility testing of *Brachyspira* species. Although agar dilution is a commonly used method for susceptibility testing of *B. hyodysenteriae* isolates from pigs, a broth dilution method has been described by Karlsson and others (2000, 2002, 2003). The appearance of hemolysis is used to indicate growth of *Brachyspira* spp. in the agar dilution method (Kitai et al., 1979). As the weak β-hemolysis of *B. intermedia* is much less visible on agar than the strong hemolysis of *B. hyodysenteriae*, we found a broth dilution method more suitable for the present study. In the present study, the MIC results of tylosin, tiamulin, valnemulin, doxycycline, and lincomycin for the type strain *B. hyodysenteriae* B78^T (ATCC 27164) fit into the quality-control ranges suggested by Pringle et al. (2006). The MIC results of tylosin and tiamulin for type strain *B. intermedia* PWS/A^T (ATCC 51140) were comparable to those previously reported (Karlsson et al., 2003).

Most of the investigated *B. intermedia* isolates had very low MICs for tylosin and tilmicosin, but two strains showed acquired resistance to tylosin and tilmicosin. One of these two strains additionally showed resistance to lincomycin, whereas all other strains had low MICs for lincomycin. Karlsson et al. (1999, 2004) described a single base mutation at position 2058 or 2059 in the 23S rRNA gene as a genetic basis of macrolide and lincosamide resistance in *B. hyodysenteriae* and *B. pilosicoli* strains of pigs. In the present study, the A2058T point mutation in the 23S rRNA gene was found only in the strain that was resistant to both macrolides and lincomycin. The other macrolide-resistant strain had the wild-type sequence at positions 2058–2059. The genetic background of strains with acquired resistance to only macrolides is not known in *Brachyspira* spp.

A G1058C mutation in the 16S rRNA gene in *B. hyodysenteriae* has been described as a genetic background for decreased susceptibility to doxycycline (Pringle et al., 2007). All three *B. intermedia* strains resistant to doxycycline had this G1058C mutation, whereas it was absent in all susceptible strains.

Several authors from different countries reported on pleuromutilin resistance of *B. hyodysenteriae* and *B. pilosicoli* from pigs with a parallel decreased susceptibility to tiamulin and valnemulin (Gresham et al., 1998; Hidalgo et al., 2009; Karlsson et al., 2001, 2002; Lobová et al., 2004; Molnár, 1996; Pringle et al., 2006; Rhode et al., 2004; Vyt and Hommez, 2006). In this study, the MICs of tiamulin and valnemulin showed a monomodal distribution, but with tailing toward the higher MIC values, possibly suggesting a low-level acquired resistance to both pleuromutilins in 6

B. intermedia isolates. Analysis of a larger collection of *B. intermedia* field strains is necessary to more efficiently determine pleuromutilin wild-type distributions of this microorganism, which may allow defining epidemiological cutoff values.

There are no accepted clinical breakpoints for *Brachyspira* species in chickens (CLSI, 2007). In the present study, the epidemiological cutoff value or microbiological criterion was used for interpretation of MIC results (Turnidge and Paterson, 2007). Using this criterion, the interpretation was obvious for tilmicosin, tylosin, lincomycin, and doxycycline, because MIC distributions of these antimicrobials were bimodal, indicating acquired resistance in isolates in the higher range of MIC values. For the pleuromutilins, MICs rather showed an extended frequency distribution range wherein the division between isolates with or without acquired resistance was more difficult to establish. The microbiological criterion refers to direct *in vitro* interactions between the antimicrobial agents and the *B. intermedia* isolates and is not necessarily linked to therapeutic success. However, for lincomycin and doxycycline, MIC values were at least 8 times higher for isolates with acquired resistance and were up to 100 times higher for the macrolides. The likelihood that chickens infected with these isolates will respond less well to treatment with these antimicrobials is high.

In conclusion, acquired resistance to tilmicosin, tylosin, lincomycin, and doxycycline was demonstrated in *B. intermedia* field strains from Belgian and Dutch layers. An A2058T mutation in the 23S rRNA gene was found as the genetic base for macrolide and lincosamide resistance and a G1058C mutation in the 16S rRNA gene as the genetic base for doxycycline resistance.

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EXPERIMENTAL STUDY 2

In vitro sensitivity of poultry *Brachyspira intermedia* isolates to essential oil components and *in vivo* reduction of *Brachyspira intermedia* in rearing pullets with cinnamaldehyde feed supplementation

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ABSTRACT

Caecal enteritis due to *Brachyspira* infections tends to be chronic in laying hens. Limited availability of antimicrobial drugs for use in laying hens emphasizes the need for alternative control measures. A broth microdilution method was used to determine the antimicrobial susceptibility of 20 *Brachyspira intermedia* field isolates from laying hen flocks to components of essential oils (EO). Minimal inhibitory concentration (MIC) distributions, obtained for 8 EO components, were all monomodal. Cinnamaldehyde had the lowest MIC values (40 to 80 mg/L), followed by nerolidol, capsaicin, carvacrol, and thymol (80 to 320 mg/L), eugenol (160 to 640 mg/L), and linalool (320 to 1,280 mg/l). The MIC ranges of piperine were mostly above the test range of 1,280 mg/l. In an *in vivo* experiment, coated *trans*-cinnamaldehyde was supplemented to the feed of rearing pullets. A completely randomized experimental design with 4 treatments and 3 replicates each (replicate = group of seven 1-d-old laying hen chickens) was applied. The negative and positive controls received a conventional feed during the whole trial. The positive controls were orally inoculated on 3 consecutive days (d 22, 23, and 24) with 1 ml of 1.0×10^8 CFU/ml of a *B. intermedia* field isolate. Two treatment groups (preventive and curative), identically inoculated, received the coated *trans*-cinnamaldehyde-supplemented feed (500 mg/kg of *trans*-cinnamaldehyde), the preventive group from d 1, the curative from d 25. On d 32, caeca were collected for bacteriologic *Brachyspira* enumeration. The mean enumeration of *Brachyspira* cells was decreased ($P < 0.05$) in the curative treated group versus the positive control group. The *in vitro* results of the present study demonstrate the potential of EO components as antimicrobials against poultry *Brachyspira* isolates, including isolates with acquired resistance for classic antimicrobial drugs. Reduction of *Brachyspira* colonization in young pullets was obtained, in a curative way, in an *in vivo* study using feed supplemented with coated *trans*-cinnamaldehyde. Further studies are necessary to investigate the mode of action of the coated *trans*cinnamaldehyde in reducing *Brachyspira* colonization of the caeca.

INTRODUCTION

Avian intestinal spirochaetosis (AIS) is a disease complex characterized by colonization of the caeca with anaerobic intestinal spirochetes of the genus *Brachyspira*. Avian intestinal spirochaetosis has been reported in Europe, Australia, and the United States and affects mainly laying hens and broiler breeder hens. The currently considered pathogenic species for poultry are *Brachyspira intermedia*, *Brachyspira pilosicoli*, and *Brachyspira alvinipulli* (Hampson and Swayne, 2008), with *B. intermedia* being the most frequently isolated one in European laying hen flocks (Bano et al., 2008; Feberwee et al., 2008; Jansson et al., 2008; Burch et al., 2009). In laying hens, AIS is associated with reduced egg production, chronic diarrhea, and fecal staining of eggshells (Swayne et al., 1992; Feberwee et al., 2008). Colonization tends to be chronic in laying hen flocks, and antimicrobial treatment has met with limited success at long-term control (Stephens and Hampson, 1999). Decreased susceptibility or acquired resistance against different antimicrobial drugs in poultry *Brachyspira* isolates has been documented (Jansson and Pringle, 2011; Verlinden et al., 2011). The use of antimicrobials in laying hens is also limited due to long withdrawal times for eggs for human consumption and the lack of appropriate licensed products.

Essential oils (EO) are steam-volatile or organic-solvent extracts of plants. Besides their currently flavoring and appetizing use, their antioxidative effect and effect on digestive physiology and gut microbiology has been demonstrated and used in ruminant, pig, and poultry nutrition (Lee et al., 2004; Franz et al., 2009). During the last 2 decades, the ban of antibiotic growth promoters in livestock has increased the interest in EO and other secondary plant metabolites as growth and health promoters (Greathead, 2003; Franz et al., 2009). In poultry nutrition, a positive effect of EO on growth performance in broilers has been observed in a limited number of controlled studies and has recently been reviewed by several authors (Windisch et al., 2008; Brenes and Roura, 2010; Wallace et al., 2010). A positive effect of EO on performance in laying hens was mostly the result of improved egg quality (Al-Harthi et al., 2009; Bozkurt et al., 2012; Özek, 2012). *In vitro* tests demonstrated strong antimicrobial activity of many EO against various microorganisms (Lang and Buchbauer, 2012). Thus, EO might be interesting alternatives to the use of antimicrobials to control AIS in laying hens. To date, as far as we know, no data have been published concerning the susceptibility of *Brachyspira* species to EO.

The first objective of the present study was to assess the *in vitro* susceptibility of poultry *B. intermedia* isolates to different components of EO. A selection was made from components of EO blends in use in poultry nutrition and documented in the literature with *in vivo* experiments. A selection of *B. intermedia* isolates was chosen based on their differences in susceptibility against classic antimicrobial drugs. A second objective was to test *in vivo* if an in-feed supplemented EO compound, with documented antimicrobial activity, could reduce the colonization of *B. intermedia* in the chicken caeca.

MATERIALS AND METHODS

Bacterial Isolates

Twenty isolates of *B. intermedia*, obtained from laying hens of 20 different flocks in Belgium (n = 10) and The Netherlands (n = 10), during the period 2008 to 2010, were used in *in vitro* antimicrobial susceptibility tests. The isolates had different antimicrobial resistant profiles, including isolates with acquired resistance against tylosin, tilmicosin, lincomycin, or doxycycline. Sampling, culturing, identification, and antimicrobial resistant profiles of the isolates have been described elsewhere (Verlinden et al., 2011). The isolates were stored in peptone-glycerol medium at −80°C. The type strains *B. intermedia* PWS/A^T (ATCC 51140) and *B. hyodysenteriae* B78^T (ATCC 27164) were included as control strains in the *in vitro* antimicrobial susceptibility test. For preparation of the inocula for the antimicrobial susceptibility test, bacteria harvested from 4-d incubated trypticase soy agar (TSA) plates, supplemented with 5% defibrinated sheep blood and 0.1% yeast extract, were suspended in brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) supplemented with 10% fetal calf serum. The procedure described by Karlsson and Franklin (2000) was followed to obtain final inoculum concentrations of 1×10^6 to 5×10^6 CFU/ml.

Brachyspira intermedia strain MV10/1121, one of the field isolates of above, was used as inoculum in an *in vivo* trial. This strain was isolated from laying hens on an industrial farm with chronic problems with AIS. The genetic sequence profile of this strain, obtained after multi-locus sequence typing (MLST) is available on the *Brachyspira* MLST website (http://pubmlst.org/brachyspira/). For preparation of the inocula for the *in vivo* trial, bacteria harvested from 4-d-incubated TSA plates, supplemented with 5% defibrinated sheep blood and 0.1% yeast extract, were suspended and reincubated in BHI broth supplemented with 10% fetal calf serum. After 40 h of anaerobic incubation (84% N_2, 8% H_2, and 8% CO_2; Hypoxic Workstation, Ruskinn Technology, South Wales, UK) on a rotary shaker (80 to 100 rpm) at 37°C, the concentration was measured spectrophotometrically and adjusted to a final inoculum concentrations of 1×10^8 CFU/ml (Karlsson and Franklin, 2000).

Compounds of EO

For the *in vitro* antimicrobial susceptibility tests, the monoterpenes carvacrol, thymol, and linalool, the sesquiterpene nerolidol, and the phenylpropanoids cinnamaldehyde, eugenol, piperine, and capsaicin were obtained from Sigma-Aldrich (Steinheim, Germany). Stock solutions were prepared with absolute ethanol. Further dilutions were made in BHI broth supplemented with 10% fetal calf serum so that the final concentration of ethanol did not exceed 2.5% vol/vol in the presence of bacteria.

For an *in vivo* experiment, coated *trans*-cinnamaldehyde (kindly provided by Tim Goossens, Nutri-ad International, Dendermonde, Belgium), containing 30% wt/wt pure *trans*-cinnamaldehyde and 70% wt/wt coating material, was mixed in a conventional feed for rearing pullets, so that a concentration of 500 mg/kg of pure *trans*-cinnamaldehyde was obtained.

Antimicrobial Susceptibility Testing

The broth dilution method as described in detail elsewhere was followed (Verlinden et al., 2011). Briefly, 2-fold serial dilutions of the compounds were made in BHI broth supplemented with 10% fetal calf serum so that a range of final concentrations in milligrams per liter of culture medium was obtained from 10 to 1,280. A 2-fold serial dilution of BHI broth supplemented with 10% fetal calf serum and ethanol (2.5% vol/vol ethanol in the first dilution in the presence of bacteria) was used as growth control for each inoculum. The serial dilution panels were prepared immediately before use in 48-well culture plates (Greiner Bio-One, Frickenhausen, Germany); 100 µl of each dilution was transferred to the wells. Four hundred microliters of the inocula with concentrations of 1×10^6 to 5×10^6 CFU/ml was added to the panels, and incubation was performed on a rotary shaker at 37°C in an anaerobic atmosphere (84% N_2, 8% H_2, and 8% CO_2; Hypoxic Workstation). The minimal inhibitory concentration (MIC) was read as the lowest concentration of the antimicrobial agent that prevented visible growth after 4 d of incubation.

In Vivo Trial with Supplementation of Coated Trans-Cinnamaldehyde

Eighty-four *Brachyspira*-free day-of-hatch laying hen chickens (Lohmann Brown), purchased from a local hatchery (De Biest, Kruishoutem, Belgium), were housed on wood shavings in 12 separated pens with 7 birds in each pen. Feed and drinking water (provided with a nipple system) were provided ad libitum. The temperature was gradually decreased from 35°C on d 1 to 21°C on d 22. Chickens were weighted on d 1, 21, and 32 (end of the trial).

A completely randomized experimental design with 4 treatments and 3 replicates each (a group of 7 birds was a replicate) was applied. The negative control birds received a conventional feed for rearing pullets during the whole trial and were orally sham-inoculated with BHI broth supplemented with 10% fetal calf serum. The untreated *Brachyspira*-positive control birds received the same conventional feed during the whole trial and were orally inoculated 3 times, on 3 consecutive days (d 22, 23, and 24), with 1-ml inoculum of approximately 1.0×10^8 CFU/ml of the *B. intermedia* strain MV10/1121. Two treatment groups (preventive and curative) were inoculated identically to the positive control birds and were fed the conventional feed supplemented with coated *trans*-cinnamaldehyde (final *trans*-cinnamaldehyde concentration in the feed of 500 mg/kg), the preventive group from d 1, and the curative group from d 25. At 32 d of age, all chickens were euthanized, necropsied, and the caeca with their contents were collected for *B. intermedia* enumeration.

Caeca with contents were cut into little fragments, weighed, and diluted 1:9 wt/vol in BHI broth. After homogenization, a 10-fold dilution series was made in BHI broth. Of each dilution, 100 µl was spread on TSA plates supplemented with 5% defibrinated sheep blood, 0.1% yeast extract, spectinomycin (400 µg/ml), vancomycin (25 µg/ml), and colistin (25 µg/ml) (Jenkinson and Wingar, 1981). Colonies were counted after 6 d of incubation at 38°C under anaerobic conditions. Individual birds were designated *Brachyspira*-positive when *Brachyspira* cells were demonstrated in the bacteriologic enumeration method with a detection limit of 100 CFU/g of caeca.

Statistical Analysis

Data of the *in vivo* trial were analyzed by IBM SPSS Statistics 19 software for Windows (IBM Corporation, Armonk, NY). A 2-factor nested ANOVA was carried out to compare the means of the BW of the chickens of all treatment groups; *P*-values below 0.05 were considered significantly different. *Brachyspira* counts were first transformed to log base 10 before statistical analysis. For all samples in which no growth of *Brachyspira* colonies was noticed, a log10 score of 1 was assigned, assuming possible presence under the detection limit of 100 CFU/g. A nonparametric ANOVA (Kruskal-Wallis) was carried out to compare the means of log10 transformed counts in chicken caecal samples of all inoculated treatment groups. Significant differences (*P*-values <0.05) were assessed by post-hoc Bonferroni-corrected Mann-Whitney U tests.

RESULTS AND DISCUSSION

The MIC results are presented in Table 1. The MIC of the tested compounds all showed a monomodal distribution for the 20 *B. intermedia* field isolates. The MIC for the type strains *B. intermedia* PWS/A^T (ATCC 51140) and *B. hyodysenteriae* B78^T (ATCC 27164) were in the same ranges as the field strains. Cinnamaldehyde had the lowest MIC values (40 to 80 mg/L), followed by nerolidol, capsaicin, carvacrol, and thymol (80 to 320 mg/L). Eugenol (160 to 640 mg/L) and linalool (320 to 1,280 mg/L) showed higher MIC ranges, whereas doses of piperine were mostly above the test range of 1,280 mg/L.

Table 1. Distribution of minimal inhibitory concentrations (MICs) of eight essential oil components on 20 *Brachyspira intermedia* isolates from laying hens in Belgium and The Netherlands

	Number of isolates with MIC (mg/L) of							
Component	≤20	40	80	160	320	640	1280	>1280
Carvacrol			6	5°*	9			
Thymol				10°*	10			
Linalool					2°*	7	11	
Nerolidol			1	19°*				
Cinnamaldehyde		2*	18°					
Eugenol				1	9°*	10		
Piperine							2	18°*
Capsaicin				20°*				

° MIC for *B. hyodysenteriae* B78^T (ATCC 27164)
* MIC for *B. intermedia* PWS/A^T (ATCC 51140)

Publications on MIC results for EO components are limited; most studies comment on MIC results for blends of EO. The MIC results for carvacrol, thymol, linalool, cinnamaldehyde, and eugenol were in the same ranges for the *Brachyspira* isolates, as described in the literature for *Escherichia coli*,

Salmonella Typhimurium, and *Campylobacter jejuni* strains, other representatives of gram-negative bacteria (Cosentino et al., 1999; Si et al., 2006; Zhou et al., 2007; Hermans et al., 2011). The MIC results of piperine suggest rather weak antimicrobial activity against the *Brachyspira* isolates. Nevertheless, piperine can, as an active component in blends of EO, be of value for antimicrobial use because of other physiological effects [e.g., its modulating effect on the bioavailability of other phytochemicals (Srinivasan, 2007)].

Most of the tested components were successfully used in EO blends in *in vivo* experiments in poultry to ameliorate performance and to reduce opportunistic pathogens or foodborne pathogens. Blends of EO, with combinations of carvacrol, thymol, eugenol, curcumin, piperine, cinnamaldehyde, or capsaicin, resulted in *in vivo* experiments in broilers in a reduction of numbers of *Clostridium perfringens*, *Escherichia coli*, or *Salmonella enterica* (Jamroz et al., 2003; Mitsch et al., 2004; Orndorff et al., 2005; Amerah et al., 2012; Kollanoor-Johny et al., 2012). A prophylactic effect of capsaicin on experimental *Salmonella* Enteritidis challenge was shown in young Leghorn chicks and in layers in production (Tellez et al., 1993; Vicente et al., 2007).

The investigated *B. intermedia* isolates of layers had different susceptibilities for classic antimicrobial drugs (Verlinden et al., 2011). No differences were seen between the isolates regarding their susceptibility to the EO components; the susceptibility patterns were all monomodal, suggesting the wild-type distribution. This could open perspectives in using EO as an alternative for the classic antimicrobial drugs for control of AIS.

Cinnamaldehyde had the lowest MIC ranges in the present study and is largely in use in blends of EO in animal nutrition. Cinnamaldehyde has been identified between 23 EO compounds as one of the most active antimicrobial against 4 major foodborne pathogens (Friedman et al., 2002). For the reasons above, cinnamaldehyde was chosen for an *in vivo* trial to investigate if the EO component was also able to reduce *B. intermedia* colonization in the poultry intestine *in vivo*. Results of the *in vivo* study are shown in Table 2. No *Brachyspira* was recovered from the caecal samples of the negative control birds. The individual enumeration of *B. intermedia* cells in the caeca of *Brachyspira*-positive birds varied from 2.0 log10 to 9.6 log10 CFU/g of cecum. The mean enumeration of *Brachyspira* cells was significantly different ($P < 0.05$) between the positive control group and the curative treated group. In contrast, there was no significant difference demonstrated for this parameter between the preventive treated group and the other groups. Body weight was not significantly different ($P > 0.05$) between the 4 treatments (data not shown).

Coated *trans*-cinnamaldehyde was added to the feed, with a final concentration of pure *trans*-cinnamaldehyde of 500 mg/kg, in the opinion that a concentration above the MIC of 80 mg/L would be released in the gut after the passage through the crop and stomach. The used concentration of 500 mg/kg is 10-fold or more greater than concentrations mentioned in the literature concerning performance trials. This rather high concentration as a feed additive had no negative effect on weight gain, which was also reported by others, even with cinnamaldehyde in higher concentrations (Kollanoor-Johny et al., 2012).

Table 2. Bacteriologic enumeration of cecal *Brachyspira intermedia* in inoculated rearing pullets fed two diets differentially treated with coated *trans*-cinnamaldehyde.

	Treatment[1]											
	Positive control group				Preventive group				Curative group			
Number of positive chickens (3 replicates)[2]	10/21	(3/7	4/7	3/7)	6/21	(2/7	3/7	1/7)	1/21	(0/7	0/7	1/7)
Mean enumeration in log10 cfu/g (3 replicates)[3]	3.85[a]	(3.79	4.96	2.79)	2.69[ab]	(2.64	4.30	1.14)	1.29[b]	(1.00	1.00	1.88)

[a,b]Means within a row without a common superscript differ ($P < 0.05$).
[1]Treatment: Feed with 500 ppm *trans*-cinnamaldehyde; Preventive = fed all the study period long; Curative = fed only after *B. intermedia* inoculation.
[2]Positive chicken: when *Brachyspira* cells were demonstrated in the bacteriologic enumeration method with a detection limit of 100 cfu/g ceca.
[3]Mean of the results of bacteriologic quantification of *B. intermedia* in the ceca; birds under the detection limit of 2 log10 were given a log10 score of 1.

The *in vivo* way of action of EO, including cinnamaldehyde, in reducing pathogens that are mainly colonizing the chicken cecum is not yet clearly known. Different effects on the intestinal microbiota, and on physiologic parameters of the gut wall, have been designated to cinnamaldehyde, pure or in blends of EO. In jejunal simulations of the pig gastrointestinal tract, the use of *trans*-cinnamaldehyde, in a dose of 100 mg/L, could result in a shift in the microbial ecology in favor of lactic acid-producing bacteria and reducing the number of coliform bacteria (Michiels et al., 2009). In a study with broilers, a blend of EO consisting of 15 g/tonne of thymol and 5 g/tonne of cinnamaldehyde affected the caecal microbiota; in particular, increases in the proportions of *Lactobacillus* and *Escherichia coli* were observed, and also major effects on caecal metabolites were measured, with for instance an increase in the proportion of caecal butyrate (Tiihonen et al., 2010). In an experiment in broilers fed diets with plant extracts, changes in the depth of the jejunum crypt and height of villi were noticed, and a thicker mucus layer on the wall of the jejunum suggests villi-related protective properties of the use of a carvacrol, cinnamaldehyde, and capsaicin mixture (Jamroz et al., 2006).

The metabolization of cinnamaldehyde in the chicken intestine is not completely known. It was demonstrated in piglets that *trans*-cinnamaldehyde showed a pronounced degradation in the intestinal tract with a disappearance in the caecal content (Michiels et al., 2008). In the present study, adaptation and shifts in the microbial flora with metabolization of cinnamaldehyde could be possible explanations for the failure in the preventive group in significantly reducing *Brachyspira* cells. Nonetheless, it is only speculative to designate the possible modes of action of cinnamaldehyde that are involved in the reduction of *Brachyspira* in the present study. Nevertheless, the *in vivo* study demonstrated the ability of coated *trans*-cinnamaldehyde to reduce *B. intermedia* colonization in the caeca of young rearing pullets, which opens perspectives for alternative control of AIS.

In conclusion, the *in vitro* results of the present study demonstrate the potential of EO components as antimicrobials against poultry *Brachyspira* isolates, including isolates with acquired resistance for classic antimicrobial drugs. Reduction of *Brachyspira* colonization in young pullets was obtained, in a curative way, in an *in vivo* study using feed supplemented with coated *trans*-cinnamaldehyde. Further studies are necessary to investigate the mode of action of the coated *trans*-cinnamaldehyde in reducing *Brachyspira* colonization of the caeca.

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EXPERIMENTAL STUDY 3

Occurrence of viable *Brachyspira* spp. on carcasses of spent laying hens from supermarkets

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ABSTRACT

Brachyspira spp. are frequent inhabitants of the chicken's intestine and some have been associated with enteric disease in humans. We studied contamination with *Brachyspira* spp. of carcasses of spent laying hens as a possible source of infections for humans and animals that may eat this meat. Eleven batches of hen carcasses, for a total of 110 carcasses, were bought in Belgian supermarkets during 2009-2010. Carcass rinse samples were examined for the presence of *Brachyspira*. *Brachyspira* spp. were cultured from some carcass in all batches. Besides presumably non-pathogenic species such as *Brachyspira murdochii* and *Brachyspira innocens*, the poultry pathogen *Brachyspira intermedia* and the poultry and suspected human pathogen *Brachyspira pilosicoli* were identified in 7/11 and 1/11 carcass batches, respectively, at high numbers, as shown using quantitative polymerase chain reactions. Multilocus sequence typing (MLST) demonstrated the presence of 2 and 13 MLST types of *B. pilosicoli* and *B. intermedia*, respectively, with all strains belonging to novel MLST types. The findings show that carcasses of spent laying hens are commonly contaminated with high numbers of *Brachyspira* spp., including the suspected zoonotic agent *B. pilosicoli*.

INTRODUCTION

Avian intestinal spirochaetosis (AIS) is the cause of intestinal problems in laying hens. The illness is characterized by a pronounced colonization of the caecum with pathogenic *Brachyspira* species. Pathogenic species in laying hens and broiler breeders are *Brachyspira intermedia*, *Brachyspira pilosicoli* and *Brachyspira alvinipulli* (Hampson and Swayne, 2008). Other *Brachyspira* spp., however, are considered part of the normal microbiota (Hampson and Stanton, 1997).

B. pilosicoli has zoonotic potential. It is associated with chronic enteritis in humans (Hampson and Stanton, 1997; Trott et al., 1997; Margawani et al., 2004), and may also cause disease in other species such as dogs (Koopman et al., 1993; Trott et al., 1997; Hidalgo et al., 2010). In humans *B. pilosicoli* is common in poorly developed regions. In countries with high living standards it is found most frequently in homosexual males and HIV positive persons (Körner and Gebbers, 2003). Some avian *B. pilosicoli* strains are closely related to human *B. pilosicoli* strains and transmission between man and bird is likely (Hampson et al., 2006). The transfer to humans can be hypothesized to occur through contact with feces of infected poultry or through the consumption of meat from infected chickens or chicken carcasses contaminated during processing (Smith, 2005). However, no data for the contamination of chicken meat with *Brachyspira* spp. are available.

There are no reports of broiler chickens being naturally infected with intestinal spirochetes while in flocks of laying hens *Brachyspira* colonization tends to be chronic with morbidity increasing with age (Stephens and Hampson, 1999; Bano et al., 2008). Spent hen carcasses may thus be contaminated more likely than those of broilers. Spent laying hens are typically more than 70 weeks old and have gone through one or two cycles of egg production before they are slaughtered.

The purpose of the present study was to investigate whether *Brachyspira* species could be recovered from spent laying hen carcasses sold in supermarkets, and to determine the species loads and diversity of strains of *Brachyspira* on the carcasses.

MATERIALS AND METHODS

Sampling and bacteriologic analysis

On 11 different days, from October 2009 until February 2010, a total of 110 refrigerated spent laying hen carcasses were bought in different supermarkets in Belgium. On each sampling day, a batch of carcasses, with each carcass in an aerobic packaging and with all carcasses identified with a similar label including the same expiry date, was bought. The number of carcasses in each batch varied from 5 to 20. Six batches of carcasses were examined 5 days before the expiry date mentioned on the label, 2 batches 4 days before, 2 batches 3 days before and one batch on the expiry date. Carcass rinse samples were obtained as described before (Cox et al., 1981) with some modifications. Briefly, each carcass was placed in a sterile plastic bag and washed with 175 ml of phosphate-buffered saline. One-half of the diluent was poured into the body cavity and the other half was poured over the outside of the carcass. The surface of the carcass was hand massaged for 1 min and subsequently the carcass was shaken vigorously with a rocking reciprocal motion for 2 min. DNA was

extracted from the rinse samples for quantitative polymerase chain reaction (qPCR) (see below, 'Quantification of *Brachyspira* organisms on spent laying hen carcasses') and rinse samples were cultured. For bacteriologic culture, 100 µl of the carcass rinse and 200 µl of a hundred-fold dilution of the carcass rinse, were spread on plates of trypticase soy agar (TSA) supplemented with 5% defibrinated sheep blood, 0.1% yeast extract, spectinomycin (400 µg/ml), vancomycin (25 µg/ml) and colistin (25 µg/ml) (Jenkinson and Wingar, 1981). After incubation for 4-7 days at 39 °C in an anaerobic atmosphere (84% N_2, 8% H_2 and 8% CO_2), suspected *Brachyspira* colonies were picked from the plates and serially subcultured on supplemented TSA agar plates until pure cultures were obtained. Cultures were harvested into a peptone-glycerol medium and stored at -80 °C. One isolate for each *Brachyspira*-positive carcass was further identified.

Identification of Brachyspira isolates

Identification was done using biochemical tests to obtain a phenotypic profile pattern, and genetically with *Brachyspira* genus-specific and species-specific PCR. The phenotypic profile patterns were indicated by a combination of five digits, in the following order: hemolysis, spot-indole, hippurate, α-galactosidase and β-glucosidase. The numeral 0 indicates no hemolysis or a negative reaction, 1 indicates weak hemolysis or a positive reaction, and 2, strong hemolysis (Backhans et al., 2010). Phenotypes of *Brachyspira* species have been described by others (Swayne et al., 1995; Stanton et al., 1998; Fellström et al., 1999; Jansson et al., 2008a, 2008b).

To confirm that the isolates belonged to the genus *Brachyspira* and to confirm the identity of the *B. pilosicoli* and *B. intermedia* isolates, PCRs were carried out with DNA from all isolates, as described by others (Jansson et al., 2008a; Phillips et al., 2005). For chromosomal DNA preparation, strains were grown in 1 ml Brain Heart Infusion broth (Merck, Darmstadt, Germany) supplemented with 10% fetal calf serum, for 2 days at 39 °C, in an anaerobic atmosphere. The broth was centrifuged for 5 min at 3300 *g*; the pellet was suspended in 500 µl ultra-pure water and heated for 5 min at 95 °C; and the supernatant was collected after centrifuging for 5 min at 15,700 *g*. PCR tests were done with primers designed to amplify a genus-specific 1309-bp portion of the 16S rRNA gene (Phillips et al., 2005), a species-specific NADH oxidase (*nox*) gene sequence of 567 bp for *B. intermedia* (Jansson et al., 2008a) or a species-specific 823-bp region of the 16S rRNA gene for *B. pilosicoli* (Phillips et al., 2005).

Quantification of Brachyspira organisms on spent laying hen carcasses

To determine the loads of *B. intermedia* and *B. pilosicoli* organisms on the carcasses contaminated with culturable *Brachyspira* cells, qPCR was carried out on the carcass rinse samples that were positive for these *Brachyspira* species after bacteriologic culture. The qPCR method described by Song and Hampson (2009) was used. For DNA extraction, the carcass rinse samples were centrifuged for 15 min at 2700 *g*. Each pellet was resuspended in 2 ml of phosphate buffered saline solution (Oxoid, Basingstoke, UK) and centrifuged again for 10 min at 5500 *g*. The pellets were suspended in 500 µl PrepMan reagent (Applied Biosystems, Foster City, CA, USA) for lysis during incubation for 10 min at 100 °C followed by centrifuging for 3 min at 15,700 *g*.

Strain typing of B. intermedia and B. pilosicoli isolates

Multilocus sequence typing (MLST) on the isolates identified as *B. intermedia* and *B. pilosicoli* was performed according to the protocol described by Råsbäck et al. (2007) with some modifications. Briefly, primer sets for seven loci, namely genes encoding alcohol dehydrogenase (*adh*), alkaline phosphatase (*alp*), esterase (*est*), glutamate dehydrogenase (*gdh*), glucose kinase (*glpK*), phosphoglucomutase (*pgm*) and thiolase (*thi*). For *adh*, only the primer pairs ADH-F206 and ADH-R757 were used and for *alp* only ALP-F354 and ALP-R1262. For those isolates that were not amplified under the described conditions, the annealing temperature was lowered from 50 °C to 43 °C. The PCR products were purified with the MSB®Spin PCRapace kit according to the manufacturer's instructions (Invitek, Berlin, Germany). The PCR to amplify the fragments was performed using the same primers and under the following conditions: 15 min at 95 °C, 30 cycles of 30 s denaturation at 94 °C, 30 s of annealing at 55 °C and 1 min of elongation at 72 °C, followed by a final extension of 2 min at 72 °C. The PCR products were purified with the DyeEx®2.0 Spin kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The purified PCR products were sequenced using an ABI Prism™3100 Genetic Analyzer. For analyses, the aligned loci sequences were trimmed to conform to the nucleotide positions and allele base pair length as described in the *Brachyspira* MLST and *B. intermedia* MLST websites [http://pubmlst.org/*Brachyspira*/and http://pubmlst.org/b*intermedia*/]. MLST results were analyzed using Bionumerics Software (Applied Maths, Kortrijk, Belgium).

RESULTS

Isolation and identification of Brachyspira

All 11 batches and 65 (59%) of the 110 carcasses were positive for *Brachyspira*. The frequency of positive carcasses in the same batch varied from 30% to 100% (Table 1). The genus specific PCR confirmed that all 65 isolates belonged to the genus *Brachyspira*. All 65 isolates were weakly β-hemolytic. Using the species-specific PCR tests, 35 isolates from 7 batches were identified as *B. intermedia* and 10 isolates from 1 batch were identified as *B. pilosicoli*. The other 20 isolates, which were not further genetically identified, showed 3 different phenotypic profile patterns, namely those of *Brachyspira murdochii* 10001 (n = 17), *Brachyspira innocens* and '*Brachyspira pulli*' 10011 (n = 2), and '*Brachyspira corvi*' 10010 (n = 1). With 22 of the 65 positive samples, the supplemented TSA plates with the hundred-fold dilutions of the carcass rinse fluids carried more than one *Brachyspira* colony, indicating numbers in the rinse fluid of ≥ 10^3 CFU/ml.

Quantitative real time polymerase chain reaction

Genomic equivalence values for *B. intermedia* varied from 7.8 x 10^1 to 3.8 x 10^4/ml of carcass rinse, and for *B. pilosicoli* from 1.4 x 10^4 to 1.7 x 10^5/ml of carcass rinse. The range of genomic equivalence values/ml rinse fluid for each batch is shown in Table 1.

Table 1. Isolation, quantification and identification of *Brachyspira* spp. from batches of carcasses of spent laying hens bought in supermarkets

Batch	Number of carcasses / batch	Culture [a]			qPCR [d]	
		Number of carcasses positive for *Brachyspira* spp.	Identification of isolates [b]		Range of genomic equivalence values / ml carcass rinse	
			Number of *B. intermedia* isolates [c]	Number of *B. pilosicoli* isolates [c]	*B. intermedia*	*B. pilosicoli*
1	10	3	3	0	$2.4.10^{3}$-$3.8.10^{4}$	
2	10	10	0	10		$1.4.10^{4}$-$1.7.10^{5}$
3	20	6	4	0	$1.0.10^{2}$-$7.0.10^{2}$	
4	15	6	0	0		
5	20	13	13	0	$1.2.10^{3}$-$2.3.10^{4}$	
6	5	4	4	0	$1.7.10^{3}$-$1.6.10^{4}$	
7	5	5	5	0	$1.6.10^{3}$-$2.3.10^{3}$	
8	5	3	1	0	$9.7.10^{2}$	
9	5	5	0	0		
10	5	5	0	0		
11	10	5	5	0	$7.8.10^{1}$-$2.4.10^{2}$	

[a] Isolates were recovered from carcass rinse samples.
[b] Identification tests of one isolate from each positive carcass.
[c] Identification of DNA from isolates by species-specific polymerase chain reactions (PCR).
[d] Quantitative PCR (qPCR) was carried out on the carcass rinse samples where *B. intermedia* or *B. pilosicoli* was identified after culture.

Table 2. Multilocus sequence typing (MLST) data for *Brachyspira intermedia* and *B. pilosicoli* isolates recovered from carcasses of spent laying hens

Species	Batch identification	Number of isolates	Number of isolates with same ST	ST [a]	Allele no [a]						
					adh	*alp*	*est*	*gdh*	*glpK*	*pgm*	*thi*
B. intermedia	1	3	3	N1	n1	/ [b]	n1	n1	n1	n1	n1
	3	4	2	N2	n1	n1	n1	n2	n2	n2	n2
			1	N3	1	n2	n2	1	/	n3	n3
			1	N4	/	n3	n3	/	n3	n4	n4
	5	13	11	N5	n2	n1	n4	n3	n4	n5	n5
			2	N6	1	n4	n5	5	13	n6	n6
	6	4	4	N7	n3	n5	n6	n4	n5	n7	n7
	7	5	4	N8	1	n6	2	2	3	n8	n8
			1	N9	1	n2	n7	1	n6	n3	n3
	8	1	1	N10	n3	n7	n8	n5	n7	n9	n9
	11	5	3	N11	3	1	n9	n6	n8	14	n10
			1	N12	3	n8	n9	n6	n8	14	n10
			1	N13	1	n8	n10	n6	14	14	n10
B. pilosicoli	2	10	7	N14	n4	n9	n11	n7	n9	n10	n11
			3	N15	n5	23	n12	n8	n10	n11	n12

[a] Sequence types (STs) according to the PubMLST website, as listed in the *Brachyspira* MLST and *Brachyspira intermedia* MLST databases; known STs and alleles types are indicated with their identification number in the database; new STs or new alleles types are preceded by “N” or “n”.
[b] No successful sequencing.

Multilocus sequence typing

A total of 35 *B. intermedia* and 10 *B. pilosicoli* isolates were analyzed for genetic relatedness using MLST. In five *B. intermedia* isolates, all 7 loci could not be successfully sequenced, namely 3 isolates from the same batch at the *alp* locus, 1 isolate at the *glp* locus and 1 isolate at the two loci *adh* and *gdh*. In total, fifteen different sequence types (STs) were identified (13 for *B. intermedia* and 2 for *B. pilosicoli*), of which none was previously included in the PubMLST database. These new STs include, for *B. intermedia*, 12 previously known alleles and 54 novel alleles, and for *B. pilosicoli*, 1 previously known allele and 13 novel alleles (Table 2). The ST distribution was highly diverse. No ST was found in more than one batch. Within a batch, 1 to 3 different STs were found.

DISCUSSION

Of the tested spent laying hen carcasses, 59% contained living *Brachyspira* cells. Of the potentially poultry-pathogenic species, *B. intermedia* was the most common with isolation from 7 out of the 11 batches of carcasses. In one batch *B. pilosicoli* was cultured from all ten carcasses tested. *B. alvinipulli*, also a poultry-pathogenic species, was not isolated. Phenotypic profiles of species presumably non-pathogenic for poultry were found, namely *B. murdochii*, *B. innocens* or '*B. pulli*' and '*B. corvi*' but these species were not further genetically identified in this study because of their apparent lack of pathogenic relevance. More than one *Brachyspira* species were found in 3 batches. The finding of a range of *Brachyspira* species in this screening is in agreement with recent publications on investigations of European laying hens where mixes of different *Brachyspira* species were common in many flocks (Feberwee et al., 2008; Jansson et al., 2008a; Bano et al., 2008). Since *Brachyspira* species are believed to be limited to the intestinal tract of the bird, carcass contamination probably occurs through fecal contamination during slaughtering, as is the case with *Campylobacter* (Herman et al., 2003).

The MLST of *B. intermedia* isolates revealed a genetically diverse *B. intermedia* population. The marked genetic diversity of this species has been demonstrated before, using MLST (Phillips et al., 2010).

In one batch *B. pilosicoli* was cultured from all ten carcasses. *B. pilosicoli* is a potentially zoonotic agent and thus unhygienic handling of the spent hen carcasses in the kitchen and consumption of undercooked meat from them could lead to infection of susceptible human individuals. Feeding such meat to animals could cause disease in other species. The use-by date for all sampled batches was at least 5 days after slaughter. *B. intermedia* was isolated from a batch of carcasses examined on the expiry date. This indicates that the survival time of *B. intermedia* strains on packed hen carcasses is at least 5 days. Phillips et al. (2003) found a maximum survival time of 84 h for *B. intermedia* in chicken caecal feces at 4 °C. Certain environment factors must be more favorable for the survival of *B. intermedia* in packed hen carcasses than in chicken feces. One of them may be the higher water activity of carcass surfaces, due to the packaging.

Our findings demonstrated that *Brachyspira* contamination, with different species including the potentially zoonotic agent *B. pilosicoli*, was common on carcasses of spent laying hens. *B. intermedia* was the most prevalent species and MLST demonstrated a wide genetic diversity of this species. The packaging of carcasses apparently provides an environment favorable for *Brachyspira* survival for up to five days.

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GENERAL DISCUSSION

Brachyspira intermedia is the most prevalent spirochetal species associated with AIS in western European countries and with high colonization rates in layers. Yet, only few publications report on antimicrobial treatment of AIS, even with conflicting results. Data on antimicrobial susceptibility testing of *Brachyspira* spp. from chickens are scarce and the current lack of standardization hampers the comparison of antimicrobial susceptibility testing results. The aim of *Study 1* was to determine the antimicrobial resistance pattern of field isolates of *B. intermedia* from Belgian and Dutch laying hens. The methodology of antimicrobial susceptibility testing and the criteria for interpretation of the MIC results had to be selected in the setup of this *in vitro* trial because of the lack of a generally accepted or internationally standardized method for testing the antimicrobial susceptibility of *Brachyspira* species (CLSI, 2012). A broth dilution method with homemade antibiotic panels was used in *Study 1*, adapted from a previously described method (Karlsson et al., 2002). Broth dilution was the preferred method above agar dilution mainly due to difficulties in visualization of the hemolysis of the weakly β-hemolytic *Brachyspira* strains on agar plates. Standardization of the methods used for antimicrobial susceptibility testing of these fastidious anaerobic spirochetes would be helpful in comparing MIC data of different labs. At present, antibiotic panels (VetMICBrachy, SVA, Uppsala, Sweden) to perform these broth dilution tests are commercially available – however not yet recognized by the Clinical and Laboratory Standards Institute – which may promote the performance of MIC tests in diagnostic labs and the quality agreement between diagnostic labs. The latter can be demonstrated by international ring tests, as performed in the past (Råsbäck et al., 2005).
At the present date, there are no accepted clinical breakpoints for *Brachyspira* species in chickens (CLSI, 2012) which hampers the antibiotic choice by the veterinary clinician. In *Study 1*, the epidemiological cutoff value or microbiological criterion was used for the interpretation of MIC results (Turnidge and Paterson, 2007). Since data on antimicrobial susceptibility of poultry *Brachyspira* spp. are scarce, more results of sensitivity tests would be helpful in determining the susceptible 'wild-type' distributions of *B. intermedia* for these antimicrobials. This may allow to define more precisely the epidemiological cutoff values. For clinical breakpoint setting, there is need for more knowledge of the antimicrobials' pharmacokinetic and pharmacodynamics parameters, together with studies on the clinical efficacy of these antimicrobials in *Brachyspira* infected chickens (Turnidge and Paterson, 2007). In line with other studies (Hampson et al., 2006; Alvarez et al., 2009; Jansson and Pringle, 2011; Burch and Klein, 2013), the results of *Study 1* demonstrated a decreased susceptibility and acquired resistance to several commonly used antimicrobials (e.g. tetracycline, doxycycline, tylosin, tilmicosin, lincomycin and ampicillin) among intestinal spirochetes from laying hens.

During the last two decades the antibiotic resistance level of several pathogenic and commensal bacteria has increased (Aarestrup, 2005; Garcia-Migura et al., 2014). From a public health perspective, the selection and dissemination of resistant bacteria from animals has to be controlled. To achieve this, the use of antimicrobial growth promoters in livestock was banned in the European Union. Further actions are required to reduce the amounts of antibiotics used in veterinary medicine (van den Bogaard and Stobberingh, 2000). The use of antimicrobials in laying hens is also limited due to long

withdrawal times for eggs for human consumption and due to the lack of licensed products. More monitoring data on the antimicrobial susceptibility of poultry *Brachyspira* isolates are needed to provide information on the extent to which resistance occurs and how it changes over time. So, the need for a strategic use of selected available antibiotics to guarantee their long-term efficiency, can be evaluated.

Due to the awareness for the increasing antibiotic resistance level, there is a growing interest in natural antimicrobial compounds such as prebiotics and probiotics for controlling infectious diseases of the digestive tract in livestock. However, the way of action of these alternatives in controlling gut pathogens is poorly understood. The effect of probiotics can result from immune modulation, from acting directly on other microorganisms, or from affecting microbial products, host products or food ingredients (Oelschlaeger, 2010). However, how *L. reuteri* – until date the only probiotic tested *in vivo* for AIS control – reduced colonization of *B. pilosicoli* in an experimental *in vivo* trial in layers remains unclear. *Lactobacillus reuteri* was hypothesized to act directly on the colonization ability of the spirochetes, a hypothesis based on *in vitro* tests (Mappley et al., 2011, 2013). In addition, it was suggested by the authors that nutritional modification by prebiotics may enhance probiotic cell numbers - including those naturally present - and induce the same effect as supplementation of the probiotic (Mappley et al., 2013). This could be one of the possible effects of the cinnamaldehyde, supplemented to the feed in *Study 2*. Whether the beneficial effect of the cinnamaldehyde supplementation was the result of direct action on the *Brachyspira* cells and/or due to such induction of alterations of the intestinal microbiota needs further investigations. Altering the colonization and the pathogenicity of *Brachyspira* species by other components of the intestinal microbiota has been demonstrated in mammals. Indeed, in pigs and mice, other organisms of the intestinal microbiota have been shown to have a synergistic or inhibitory effect on spirochetes (Whipp et al., 1979; Suenaga and Yamazaki, 1986). Facilitation or inhibition of these synergistic bacteria could have an indirect effect on the colonization and pathogenicity of *B. hyodysenteriae*. Composition-changes of the diet can possibly indirectly predispose or protect against swine dysentery by altering the intestinal microbiota. For instance, feeding diets low in soluble non-starch polysaccharides, oligosaccharides and/or resistant starch reduced the clinical expression of swine dysentery (Pluske et al. 1998). Probably also in chickens, composition-changes of the diet alter certain components of the intestinal microflora which act synergistically or inhibit the colonization of *Brachyspira* species. Indeed, altering the endogenous microflora was the supposed way of action of feed components or feed additives on AIS in experiments in layers, i.e. the influence of the addition of zinc bacitracin on the colonization with *Brachyspira* (Jamshidi and Hampson, 2002) and the enhanced colonization with *B. intermedia* due to a wheat-based diet in contrast to diets based on barley and/or sorghum (Phillips et al., 2004a, 2004b). These interactions between other components of the intestinal microflora and the spirochetes may help to explain some of the variation in the degree of colonization and clinical signs observed in infected birds in the field (Jamshidi and Hampson, 2002).

To obtain a concentration above the MIC value, a concentration of *trans*-cinnamaldehyde of 500 mg/kg feed was used in the *in vivo* trial of *Study 2*. Although this high concentration, which is a 10-fold or even higher concentration than mentioned in the literature concerning performance trials in chickens, had no negative effect on performance, precaution could be recommended. In spite of the fact that a considerable number of EO components are approved flavorings for food and are used in the fields of medicine, paramedicine and aromatherapy, some research data show negative effects like irritation and toxicity, allergic contact dermatitis and spasmolytic or spasmogenic properties in humans (Burt, 2004). Essential oils are used in animal nutrition because of beneficial effects: improvement of feed characteristics, improvement of digestion and performance and improved characteristics of animal products (Franz et al., 2009). Until date, only a few publications deal with negative impacts of the use of EO compounds in poultry. Acute oral toxicity of the different tested EO compounds in *Study 2* is in general rather low regarding the high lethal dose 50% values in rodents (e.g. for cinnamaldehyde: 1160-2220 mg/kg body weight) (Mosciano, 1991; Saito and Yamamoto, 1996; Yang et al., 2005; Michiels, 2009; Randhawa et al., 2011). It is not expected that these EO compounds will pose major toxicological risks, but this has not been confirmed in chickens. The rather high concentration of cinnamaldehyde (0.5 g/kg feed) having no effect on weight gain as found in *Study 2* was also reported in a study in 20-day old broiler chickens where even higher concentrations of cinnamaldehyde were used (5 and 7.5 g/kg feed) (Kollanoor-Johny et al., 2012). Importantly, however, in the same study, body weight and feed consumption were significantly lower in birds with eugenol supplementation at concentrations of 7.5 and 10 g/kg feed. The authors stated that the aroma or flavor induced by eugenol could have reduced the likeability of the feed for the chickens, thereby leading to decreased body weights in the eugenol-supplemented birds. More data are welcome regarding the toxicity of EO in poultry and their possible negative impact on performance.

There is evidence for the pathogenic potential of *B. pilosicoli* in humans, causing human intestinal spirochaetosis, which is associated with colitis and complaints of chronic diarrhea, rectal bleeding, abdominal pain, and weight loss (Westerman and Kusters, 2013). *Brachyspira pilosicoli* may indeed cross the animal species barrier and has zoonotic potential (Koopman et al., 1993; Trott et al., 1998; Neo et al., 2013). The transfer of the suspected zoonotic agent *B. pilosicoli* to humans can be hypothesized to occur through contact with feces of infected poultry or through the consumption of meat from infected chickens or chicken carcasses contaminated during processing (Smith, 2005). The results of *Study 3* show that carcasses of spent laying hens are commonly contaminated with high numbers of *Brachyspira* spp., including *B. pilosicoli*. It cannot be excluded that consumption of undercooked meat from these carcasses or, more likely, consumption of cross-contaminated salads (e.g. raw vegetables, fruits) due to unhygienic handling of the spent hen carcasses in the kitchen, could lead to infection of susceptible human individuals.

Cross-species transmission can create an environmental reservoir present on the farm (or in backyard chickens). Animals like dogs, rodents and wild birds can be infected through contact with feces of infected hens but also by eating from contaminated carcasses. In a Swedish study, identical

strains of *Brachyspira* spp. were found over time - on the same farm - in rodents and farm animals. It was suggested that an environmental reservoir must be present, such as rodents, other (wild) animals, water or soil, to ensure persistence of *Brachyspira* spp. despite all-in all-out management (Backhans et al., 2011). After cleaning and disinfection of the poultry stables, environmental reservoirs are well known risk factors for the maintenance of infections with other pathogens. For instance presence of rodents and dogs was a risk factor for persistent *Salmonella* infections in French broiler houses and in Belgian layer farms, respectively (Rose et al., 2000; Dewaele et al., 2012). In *Study 3* the survival time of *Brachyspira* spp. on the carcasses was found to be longer than reported until now for *Brachyspira* cells in chicken caecal feces (Phillips et al., 2003). Possible longer survival times of poultry *Brachyspira* species, under favorable conditions, promote the maintenance of environmental reservoirs.

Different *B. intermedia* genotypes can be found within the same layer flock and between various layer farms, as demonstrated using PFGE, MEE or MLST (Phillips et al., 2005, 2010; Stephens et al., 2005). The genotyping of the *Brachyspira* isolates was studied in *Study 3* using the MLST method in which the nucleotide sequence of internal fragments from multiple conserved genes with core metabolic functions ("housekeeping genes") are analyzed. This is a robust, consistent and portable technique, used for several other pathogens (Maiden et al., 1998). Data can be stored in a central database (PubMLST) allowing the development of catalogues of strains. Via the internet this database is made openly available to produce a powerful resource for global epidemiology (Råsbäck et al., 2007; Hampson, 2013). A pronounced genetic diversity of *B. intermedia* isolates within and between the batches of spent laying hen carcasses was demonstrated in *Study 3*. Although the flock-origin of the carcasses was not known, the genetic diversity of the *B. intermedia* isolates, within and between the batches, may reflect that of the spirochetes in the laying hen flocks before slaughtering. Differences in sequence type of *B. intermedia* isolates between spent laying hen flocks was demonstrated in a preliminary study (unpublished results). In this study, only one isolate per flock was sequenced, consequently the possible presence of different genotypes in the same flock could not be demonstrated. Presumably, these different strains vary in their biological properties, potentially including their virulence and antimicrobial susceptibility pattern. This genetic diversity within a *Brachyspira* species on a laying farm could play a role in the clinical outcome of infection (Myers et al., 2009). Unfortunately there are no tools yet for identifying pathotypes due to the lack of knowledge concerning the pathogenesis of AIS. Also the lack of knowledge on possible co-factors, e.g. other components of the intestinal microbiota, hampers the identification of factors that influence the expression of virulence of a *Brachyspira* strain.

In conclusion, examination of carcasses of spent laying hens sold in supermarkets, demonstrated marked contamination with high numbers of viable *Brachyspira* spp., including the suspected zoonotic agent *B. pilosicoli*. In these carcasses, and in spent laying hen flocks, *B. intermedia* was the most prevalent species and possessed a wide genetic diversity. Presumably, these different strains vary in their biological properties, including their virulence. The lack of tools for

genetic manipulation in *Brachyspira* species has hindered the study on the pathogenesis. Advances in this molecular field will facilitate the identification of virulence factors which is important to understand the pathogenesis and to develop control strategies. The study on the pathogenesis of AIS can aim to develop a molecular diagnostic tool to identify pathotypes among the different species and genotypes of *Brachyspira* from chickens.

Acquired resistance to different antimicrobial drugs (tilmicosin, tylosin, lincomycin, and doxycycline) was demonstrated in *B. intermedia* field strains from Belgian and Dutch layers and the genetic base for macrolide and lincosamide resistance as well as for doxycycline resistance was identified. Monitoring the antimicrobial susceptibility of poultry *Brachyspira* isolates will provide information on the extent of resistance and support a strategic use of selected antibiotics, in view of a long-term efficiency. Results of *in vitro* tests demonstrated the potential of essential oil components as an aid in the control of *Brachyspira* infections in poultry, including infections with *B. intermedia* isolates showing acquired resistance against the usual antimicrobial drugs. Reduction of *Brachyspira* colonization in young pullets was obtained, in a curative way, in an *in vivo* study using feed supplemented with coated *trans*-cinnamaldehyde. The molecular study of *Brachyspira* strains, together with *in vitro* and *in vivo* tests, can be helpful to understand the interaction of *Brachyspira* cells with other components of the intestinal microbiota. This may provide a better insight in the way of action of alternative control measures (e.g. prebiotics, probiotics, natural antimicrobial compounds). Knowledge on the pharmacokinetic of prebiotics and natural antimicrobial compounds has to be completed. This could identify the site (e.g. small intestine, caecum) of modulation of the microbiota. Finally, further *in vivo* experiments and field studies are necessary to evaluate the potential and efficacy of these alternative measures in the control of AIS.

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SUMMARY

Intestinal spirochaetosis results from the colonization of the colon, rectum and/or caeca of humans and various mammalian and avian species with bacteria belonging to the genus *Brachyspira*. In general, Avian intestinal spirochaetosis (AIS) is present in laying hens from the onset of lay and is associated with intestinal diseases (chronic diarrhea, wet feces and litter, pasty vents) and production losses (delayed and decreased egg production, fecal staining of eggshells, reduced eggshell quality, pale-colored egg-yolks, increased feed conversion, retarded growth rate). High *Brachyspira* colonization rates in laying hens have been reported in field studies from many countries across the world. *Brachyspira intermedia* and *B. pilosicoli* are possibly endemic in poultry flocks worldwide. These two spirochetes are, together with *B. alvinipulli*, currently considered as the most pathogenic *Brachyspira* species for poultry. Other *Brachyspira* species reported in layers and broiler breeders are *B. hyodysenteriae*, *B. innocens*, *B. murdochii*, and *"B. pulli"*. Waterfowl are considered to be a natural reservoir for intestinal spirochetes. Environmental reservoirs such as rodents, wild birds, dogs, water or soil, may be present on poultry farms, to ensure persistence of *Brachyspira* species.
Colonization tends to be chronic in laying hen flocks, and antimicrobial treatment has met with limited success at long-term control. Only few data on antimicrobial susceptibility testing of *Brachyspira* spp. from chickens are available and little is known about acquired resistance against different antimicrobial drugs, hampering the treatment of AIS. The number of antimicrobials available for use in laying hens producing eggs for human consumption is limited. Also the amounts of antibiotics used in veterinary medicine should be minimized, among other things to cope with the growing concerns of antimicrobial resistance from a public health perspective. *Brachyspira pilosicoli* is associated with chronic enteritis in humans, and may also cause disease in other mammalian species such as pigs and dogs. Some avian *B. pilosicoli* strains are closely related to human *B. pilosicoli* strains and exchange between man and birds is likely. The transfer to humans can be hypothesized to occur through contact with feces of infected animals or through the consumption of undercooked meat from infected chickens or carcasses contaminated during processing, or through consumption of cross-contaminated food (e.g. vegetables, salads). However, no data with regard to the contamination of chicken meat with *Brachyspira* spp. are available.

The aims of this thesis were to investigate the susceptibility of *Brachyspira* field strains from laying hens to usual antimicrobial drugs and to explore an alternative control strategy for AIS. A third aim was to investigate the contamination of chicken meat with *Brachyspira* spp. in view of the zoonotic potential of *B. pilosicoli*.

In a first study, the antimicrobial resistance pattern of recent field isolates of *Brachyspira* species from laying hens was determined. The methodology of antimicrobial susceptibility testing and the criteria for interpretation of the minimal inhibitory concentration (MIC) results had to be selected in the setup of this *in vitro* trial because of the lack of a generally accepted or internationally standardized method for testing the antimicrobial susceptibility of *Brachyspira* species. A broth microdilution method with homemade antibiotic panels was used to determine the antimicrobial susceptibility of

20 *Brachyspira intermedia* isolates obtained from different layer flocks in Belgium and The Netherlands between 2008 and 2010. The antimicrobial agents used were tylosin, tilmicosin, tiamulin, valnemulin, doxycycline, and lincomycin. The MIC distribution patterns of tylosin, tilmicosin, lincomycin, and doxycycline were bimodal, demonstrating acquired resistance against doxycycline in three strains (MIC 2 mg/L), against tylosin and tilmicosin in two strains (MIC≥128 mg/L), and against lincomycin in one strain (MIC 8 mg/L). The lincomycin-resistant isolate was also resistant to the macrolides. The MICs of tiamulin and valnemulin showed a monomodal distribution, but with tailing toward the higher MIC values, possibly suggesting low-level acquired resistance in six isolates. To study the molecular mechanism of acquired resistance, fragments of the 23S rDNA and of the 16S rDNA were amplified and sequenced, revealing a G1058C mutation in the 16S rRNA gene in all doxycycline resistant strains. The strain resistant to tylosin, tilmicosin, and lincomycin had an A2058T mutation in the 23S rRNA gene.

In a second study, we examined whether alternatives for antimicrobial drugs, such as essential oils, could represent an alternative in the control of AIS. A broth microdilution method was used to determine the antimicrobial susceptibility of 20 *B. intermedia* field isolates from laying hen flocks to components of essential oils (EO). The *B. intermedia* isolates were selected based on their differences in susceptibility against commonly used antimicrobial drugs. A selection of 8 EO components was made from components of EO blends in use in poultry nutrition. The MIC distributions, obtained for the 8 EO components, were all monomodal. Cinnamaldehyde had the lowest MIC values (40 to 80 mg/L), followed by nerolidol, capsaicin, carvacrol, and thymol (80 to 320 mg/L), eugenol (160 to 640 mg/L), and linalool (320 to 1,280 mg/l). The MIC ranges of piperine were mostly above the test range of 1,280 mg/L. These *in vitro* results demonstrate the potential of EO components as antimicrobials against poultry *Brachyspira* isolates, including isolates with acquired resistance against antimicrobial drugs. In an *in vivo* experiment, coated *trans*-cinnamaldehyde was supplemented to the feed of rearing pullets. A randomized experimental design with 4 treatments and 3 replicates each (replicate = group of seven 1-day-old laying hen chickens) was applied. The negative and positive controls received a conventional feed during the whole trial. The positive controls were orally inoculated on 3 consecutive days (day 22, 23, and 24) with a *B. intermedia* field isolate. Two treatment groups (preventive and curative), identically inoculated, received the coated *trans*-cinnamaldehyde-supplemented feed (500 mg/kg of *trans*-cinnamaldehyde), the preventive group from day 1 onwards, the curative from day 25 onwards. On day 32, caeca were collected for *Brachyspira* enumeration. The mean number of *Brachyspira* cells was decreased ($P < 0.05$) in the curative treated group versus the untreated positive control group. This *in vivo* study showed a reduction of *Brachyspira* colonization in young pullets, in a curative way, using feed supplemented with coated *trans*-cinnamaldehyde. Further studies are necessary to investigate the mode of action of the coated *trans*-cinnamaldehyde in reducing *Brachyspira* colonization of the caeca.

In a third study, we investigated whether viable *Brachyspira* species could be recovered from spent laying hen carcasses sold in supermarkets, and the species loads and diversity of strains of

Brachyspira on the carcasses was determined. Eleven batches of hen carcasses, for a total of 110 carcasses, were bought in Belgian supermarkets during 2009-2010. Carcass rinse samples were examined for the presence of *Brachyspira*. *Brachyspira* spp. were cultured from carcasses in all batches. Identification was done using biochemical tests to obtain a phenotypic profile pattern, and genetically with *Brachyspira* genus-specific and species-specific PCR. Besides presumably non-pathogenic species such as *B. murdochii* and *B. innocens*, the poultry pathogen *B. intermedia* and the poultry and suspected human pathogen *B. pilosicoli* were identified in 7/11 and 1/11 carcass batches, respectively, at high numbers, as shown using quantitative polymerase chain reactions. Multilocus sequence typing (MLST) demonstrated the presence of 2 and 13 MLST types of *B. pilosicoli* and *B. intermedia*, respectively, with all strains belonging to novel MLST types. The findings show that carcasses of spent laying hens are commonly contaminated with high numbers of *Brachyspira* spp., including the suspected zoonotic agent *B. pilosicoli*. In these carcasses, and in spent laying hen flocks, *B. intermedia* was the most prevalent species and possessed a wide genetic diversity. Additionally in this study, the survival time of *Brachyspira* spp. on the carcasses was found to be longer than reported until now for *Brachyspira* cells in chicken caecal feces. Possible longer survival times of poultry *Brachyspira* species, under favorable conditions, promote the maintenance of environmental reservoirs.

To conclude, this thesis provides insight in the susceptibility of *Brachyspira* field strains from Belgian and Dutch layers for different antimicrobials and identified the genetic base for acquired resistance against some commonly used antimicrobial drugs. The potential of essential oils as an alternative for antibiotics in the control of AIS was demonstrated in *in vitro* and *in vivo* experiments. Contamination of chicken meat with several viable *Brachyspira* species, including the suspected zoonotic *B. pilosicoli*, was demonstrated on carcasses of spent laying hens sold in supermarkets.

SAMENVATTING

Intestinale spirochaetosis wordt veroorzaakt door bacteriën van het genus *Brachyspira* die het colon, rectum en/of de caeca koloniseren van mensen en van meerdere zoogdier- en vogelsoorten. Aviaire intestinale spirochaetosis (AIS) komt bij legkippen meestal pas voor vanaf het begin van de leg en gaat gepaard met klachten ten gevolge van darmproblemen (chronische diarree, met mest bevuilde cloacale streek, te nat bodemstrooisel) en klachten van productieverlies (uitgestelde en verlaagde eiproductie, met mest bevuilde eieren, verminderde eischaalkwaliteit, verbleking van de eidooier, verhoogde voederconversie, vertraagde toename van het lichaamsgewicht). In veldstudies werd, bijna wereldwijd verspreid, een hoge *Brachyspira* kolonisatiegraad gerapporteerd bij legkippen. *Brachyspira intermedia* en *B. pilosicoli* komen bij deze dieren waarschijnlijk endemisch voor. Deze twee *Brachyspira* species worden, samen met *B. alvinipulli*, beschouwd als de pathogene species voor kippen. Ook andere species zoals *B. hyodysenteriae*, *B. innocens*, *B. murdochii* en "*B. pulli*" kunnen de caeca van legkippen en reproductiekippen koloniseren. Watervogels worden beschouwd als het natuurlijk reservoir voor intestinale spirocheten. Wilde vogels, knaagdieren, honden en waterplassen kunnen als omgevingsreservoir een rol spelen in het onderhouden van AIS op een pluimveebedrijf.
AIS blijkt bij legkippen een chronisch verloop te kennen. Een behandeling met antibiotica levert zelden een lange-termijn-resultaat op. Er zijn maar weinig gegevens beschikbaar over de antimicrobiële gevoeligheid van *Brachyspira* spp. geïsoleerd uit kippen. Het gamma antimicrobiële middelen dat mag gebruikt worden bij kippen die eieren produceren voor humane consumptie is zeer beperkt. Daarenboven dient het gebruik van antibiotica in de diergeneeskunde zo veel mogelijk beperkt te worden, onder andere omdat het kan bijdragen tot spreiding van antimicrobiële resistentie bij bacteriën van mensen.
Brachyspira pilosicoli wordt geassocieerd met chronische enteritis bij de mens en veroorzaakt ook ziekte bij andere zoogdieren zoals het varken en de hond. Sommige *B. pilosicoli* stammen van aviaire oorsprong zijn nauw verwant met humane *B. pilosicoli* stammen en uitwisseling tussen mens en vogel is waarschijnlijk. Hypothetisch kan de overdracht naar de mens gebeuren door contact met feces van besmette dieren of door de consumptie van onvoldoende verhit vlees (karkassen van besmette kippen of kruisbesmetting van karkassen tijdens de verwerking) of door de consumptie van bijvoorbeeld salades besmet door kruiscontaminatie tijdens de bereiding. Bij de aanvang van dit doctoraatsonderzoek waren er echter geen gegevens beschikbaar over de mogelijke besmettingsgraad van kippenvlees met *Brachyspira* kiemen.

Doelstellingen van deze thesis waren enerzijds het testen van de gevoeligheid van *Brachyspira* isolaten van leghennen voor de gebruikelijke antibiotica en anderzijds het onderzoeken van een alternatieve strategie voor de controle van AIS. Als derde doelstelling werd nagegaan of kippenvlees mogelijk gecontamineerd kan zijn met *Brachyspira* kiemen, en in het bijzonder met de mogelijk zoönotische *B. pilosicoli*.

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In een eerste studie werd de antimicrobiële gevoeligheid bepaald van recente *Brachyspira* isolaten afkomstig uit leghennenbedrijven. Er is geen internationaal geaccepteerde of

standaardmethode voorhanden voor het uitvoeren van gevoeligheidstesten van *Brachyspira* species. Daarom moest de *in vitro* methodologie van de gevoeligheidstest eerst op punt gesteld worden. Ook dienden de criteria vastgelegd te worden voor de interpretatie van de bekomen minimale inhibitorische concentraties (MIC) van de verschillende antibiotica. Twintig *B. intermedia* stammen, die geïsoleerd werden tussen 2008 en 2010 uit leghennen van 20 Belgische en Nederlandse tomen, werden hiervoor geselecteerd. Hun gevoeligheid voor tylosine, tilmicosine, tiamuline, valnemuline, doxycycline en lincomycine werd met een bouillon-microdilutiemethode getest. Het verdelingspatroon van de MIC waarden van tylosine, alsook van tilmicosine, lincomycine en doxycycline, was bimodaal en toonde verworven resistentie tegen doxycycline aan bij drie isolaten (MIC 2 mg/L), tegen tylosine en tilmicosine bij twee isolaten (MIC≥128 mg/L) en tegen lincomycine bij één isolaat (MIC 8 mg/L). Dit laatste isolaat was ook resistent tegen de macroliden tylosine en tilmicosine. De MIC waarden van tiamuline en valnemuline volgden een monomodaal verdelingspatroon dat echter uitliep naar de hogere MIC waarden hetgeen verminderde gevoeligheid suggereerde bij zes isolaten. Delen van het 23S rDNA en van het 16S rDNA werden geamplificeerd en gesequeneerd om mogelijke moleculaire mechanismen voor de verworven resistentie te achterhalen. Hierbij werd een G1058C mutatie aangetoond in het 16S rRNA gen bij de drie doxycycline resistente isolaten en een A2058T mutatie in het 23S rRNA gen bij het isolaat dat resistentie vertoonde tegen tylosine, tilmicosine en lincomycine.

In een tweede studie onderzochten we of essentiële oliën (EO) kunnen gebruikt worden als alternatief voor antibiotica in de controle van AIS. De EO componenten cinnamaldehyde, nerolidol, capsaïcine, carvacrol, thymol, eugenol, linalool en piperine werden geselecteerd op basis van hun reeds beschreven gebruik in pluimveevoeding. Twintig *B. intermedia* isolaten met verschillende gevoeligheid voor de gebruikelijke antibiotica (met ook verworven resistentie) werden *in vitro* met een bouillon-microdilutiemethode getest op hun gevoeligheid voor de bovenvermelde acht EO componenten. Al de bekomen MIC distributiepatronen waren monomodaal. Cinnamaldehyde had de laagste MIC waarden (40 tot 80 mg/L), gevolgd door nerolidol, capsaïcine, carvacrol en thymol (80 tot 320 mg/L), eugenol (160 tot 640 mg/L) en linalool (320 tot 1,280 mg/l). De MIC waarden van piperine overschreden meestal de geteste grens van 1280 mg/L. Deze *in vitro* resultaten illustreren het potentieel van EO componenten als antimicrobieel middel tegen *Brachyspira* isolaten, zelfs tegen isolaten met een verworven resistentie tegen de gebruikelijke antibiotica. Het effect *in vivo* van cinnamaldehyde, onder gecoate vorm toegevoegd aan het voeder, werd verder onderzocht in een behandelingsproef met leghenkuikens. Vier behandelingen werden getest, elk in 3 herhalingen (één herhaling = groep van zeven eendagskuikens). De negatieve en positieve controle groepen kregen een conventioneel voeder gedurende de ganse proefopzet. De positieve controledieren werden gedurende drie opeenvolgende dagen (dag 22, 23 en 24) oraal geïnoculeerd met een *B. intermedia* veldisolaat. Twee behandelingsgroepen (preventieve en curatieve groep) werden op dezelfde manier geïnoculeerd en kregen voeder toegediend met gecoat *trans*cinnamaldehyde aan een dosis van 500 mg *trans*cinnamaldehyde per kg voeder. De preventieve groep kreeg dit voeder vanaf de eerste dag, de curatieve groep vanaf dag 25. Op de 32^{e} dag werd het aantal *Brachyspira* kiemen aanwezig in de caeca bepaald. Het gemiddeld aantal *Brachyspira* kiemen was verminderd ($P < 0.05$) in de curatief

behandelde groep ten opzichte van de onbehandelde positieve controle groep. In dit *in vivo* experiment kon dus aangetoond worden dat er een reductie bekomen werd van de *Brachyspira* kolonisatie in jonge opfokhennen door toevoeging van gecoate *trans*cinnamaldehyde aan het voeder. Volgens welke werkingsmechanismen het gecoate *trans*cinnamaldehyde deze reductie tot stand bracht, moet nog verder onderzocht te worden.

In een derde studie onderzochten we of er nog levensvatbare *Brachyspira* kiemen konden gevonden worden op pluimveevlees, in het bijzonder op karkassen van soepkippen. Elf loten soepkippen (in totaal 110 karkassen) werden in 2009 en 2010 gekocht in Belgische grootwarenhuizen. Spoelvloeistof van de karkassen werd onderzocht op het voorkomen van kweekbare *Brachyspira* kiemen. Uit alle 11 loten konden *Brachyspira* kiemen gekweekt worden. Identificatie werd uitgevoerd op basis van het biochemisch profiel van de verschillende species en genetisch met genus-specifieke en species-specifieke PCR testen. *Brachyspira intermedia* en de vermoedelijk zoönotische species *B. pilosicoli* werden respectievelijk in 7/11 en 1/11 van de loten geïdentificeerd en bleken, door onderzoek met kwantitatieve PCR testen, in hoge aantallen aanwezig te zijn op de karkassen. Ook de vermoedelijk niet-pathogene species *B. murdochii* en *B. innocens* werden geïdentificeerd. In de multi locus sequence typing (MLST) analyse van de *B. pilosicoli* en *B. intermedia* isolaten werden respectievelijk 2 en 13 verschillende MLST types aangetoond. Uit dit onderzoek blijkt dat karkassen van soepkippen algemeen gecontamineerd zijn met hoge aantallen *Brachyspira* species, de potentieel zoönotische species *B. pilosicoli* inbegrepen. *Brachyspira intermedia* was de meest aanwezige species en is gekenmerkt door een grote genetische diversiteit. Aanvullend werd in deze studie vastgesteld dat de overlevingstijd van *Brachyspira* spp. op de karkassen langer was dan tot dusver gerapporteerd voor *Brachyspira* kiemen in caecale kippenfeces. De mogelijke langere overlevingstijd van *Brachyspira* kiemen, in voor de kiemen gunstige milieuomstandigheden, kan bijdragen in het onderhouden van omgevingsreservoirs op pluimveebedrijven.

De resultaten van deze thesis geven een inzicht in de gevoeligheid voor verschillende antimicrobiële middelen van *Brachyspira* veldisolaten, afkomstig van Belgische en Nederlandse leghennen. De genetische basis voor verworven resistentie tegen sommige gebruikelijke antibiotica werd geïdentificeerd. Met *in vitro* en *in vivo* experimenten werd aangetoond dat essentiële oliën mogelijk een alternatief bieden voor antibiotica in de controle van AIS. Uit onderzoek van karkassen van soepkippen uit grootwarenhuizen werd aangetoond dat kippenvlees kan gecontamineerd zijn met meerdere levensvatbare *Brachyspira* species, de mogelijk zoönotische *B. pilosicoli* inbegrepen.

CURRICULUM VITAE

Marc Verlinden werd in Lier geboren op 6 februari 1955 en volgde er zijn middelbare studies, richting Latijn-wiskunde, aan het Koninklijk Atheneum. Na de kandidatuursjaren doorlopen te hebben aan het Rijksuniversitair Centrum Antwerpen, behaalde hij in 1980 het diploma Doctor in de diergeneeskunde aan de Rijksuniversiteit Gent.

Na drie jaar gewerkt te hebben als praktiserend dierenarts 'grote en kleine huisdieren' (praktijk te Hombeek - Mechelen) trad hij eind 1983 in dienst bij het Provinciale Verbond voor Dierenziektebestrijding te Lier als dierenarts voor het pluimvee. Uitwerken van de georganiseerde pluimveeziektebestrijding en diagnostiek van ziekteproblemen op pluimveebedrijven behoorden tot de kerntaken. Na de fusies van de provinciale verbonden tot Dierengezondheidszorg Vlaanderen (DGZ-Vlaanderen) werd hij hoofd van de afdeling pluimveegezondheidszorg van 2000 tot 2007. In 2008 vervoegde hij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten aan de Faculteit Diergeneeskunde, Universiteit Gent, als assistent aan de afdeling voor pluimvee, bijzondere gezelschapsdieren, wildlevende dieren en proefdieren. Tijdens dit assistentschap deed hij ook onderzoek over *Brachyspira* infecties bij kippen onder begeleiding van Prof. dr. An Martel, Prof. dr. Frank Pasmans en Prof. dr. Freddy Haesebrouck. Eind 2013 werd hij diplomate van de European College of Poultry Veterinary Science. Sinds januari 2014 is hij kliniekhoofd "industrieel pluimvee" bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten.

Zijn wetenschappelijk onderzoek leidde tot meerdere publicaties in internationale tijdschriften. Hij nam tevens deel aan verschillende nationale en internationale congressen.

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