IMPACT OF TIAMULIN AGAINST *BRACHYSPIRA PILOSICOLI* INDUCED AVIAN INTESTINAL SPIROCHAETOSIS

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A thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy

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Date of submission: July 2016

Abstract

Colonisation of birds by *Brachyspira pilosicoli* can result in avian intestinal spyrochetosis (AIS). AIS is associated with weight loss, decrease egg production and animal death. This disease is an increased burden worldwide and results in important economic losses. Tiamulin^{TM}, an antibiotic of the pleuromutilin family is the most common and efficient way to treat AIS. However, it is badly used in farm due to absence of indications regarding the dose to be used in chickens. Furthermore, *B. pilosicoli* is becoming increasingly resistant to treatments. This work focused on the NMR-based metabonomics evaluation of AIS by studying the pathogen, the host and their reaction to Tiamulin^{TM} treatment. Work was divided up as follow:

1. *B. pilosicoli* metabolism in optimum growth condition and after Tiamulin^{TM} treatment was determined by evaluating metabolic composition of the medium throughout 120h growth using ^{1}H-NMR. Tiamulin appeared to be able to reduce *B. pilosicoli* growth by 1 log at 0.008 and 0.016 µg/ml. Highest concentrations inhibited bacterial growth. However, *B. pilosicoli* was still metabolically active up to the 0.250 µg/ml dose. These results indicate that bacteria, even if not able to divide due to antibiotic treatment, remain alive explaining re-occurrence of the disease in farms post-antibiotic treatment.

2. Host metabolism was explored using ^{1}H-NMR techniques. Metabolic composition of twelve matrixes (liver, kidney, spleen, plasma, egg, breast muscle, cortex, ileum, caecum, colon and faeces) were characterised and grouped as a metabolic atlas to be used as a database for future avian research.

3. An animal trial evaluating the impact of Tiamulin^{TM} treatment on infection and symptoms was conducted. This also allowed determination of the best dose to be used in farmyard applications. Infection was systemic and mainly associated to diarrhoea and decreased growth rate. All antibiotic doses were able to significantly reduce percentage of infected birds and infection spread in the organism while only the two highest doses re-established growth rate and increased egg production (previously unaffected by infection). Results indicate that 125 ppm of tiamulin was sufficient to efficiently treat chickens while avoiding associated economical loss.

4. Metabolic and caecal microbiota composition response to infection and antibiotic treatment were evaluated using tissues and biofluids sampled from the animal trial described in 3. Infection by *B. pilosicoli* was associated with dysbiosis and modification of energy metabolism characterised by lipolysis to maintain plasma glucose levels. Tiamulin treatment also induced dysbiosis. Even if treatment was able to cancel metabolic response to infection, Tiamulin^{TM} strongly disturbed cholesterol metabolism in a dose dependent manner. Treatment induced a decrease of the HDL/VLDL ratio and made birds age faster than untreated ones. Steroid hormonal disturbance was explored as potential cause of the cholesterol metabolism perturbation.

In conclusion, this work contributed significantly to increase *B. pilosicoli*-induced AIS general understanding. It also enlightened metabolic mechanism responsible for symptom development and finally showed that antibiotics may strongly influence metabolism.

Declaration

‘I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.’

To my parents Patrick and Florence,

In the loving memory of my great grandmother Mémé,

‘Nothing in life is to be feared, it is only to be understood.’

Marie Curie

Acknowledgment

I would like to thank my supervisors: Dr Sandrine Claus for being an amazing mentor and the supervisor I always wished to have, Prof Martin Woodward for his wise advise and thousands of ideas and Prof Roberto La Ragione for the opportunities he offered me in the PhD. I also want to thanks all of them together for always trusting me and supporting my ideas.

I acknowledge my sponsors, the University of Reading that funded the project and Novartis who allowed me to conduct the animal trial, which made this project extremely interesting.

Thanks to the staff of the University of Reading for helping me when needed in the lab and more especially Dr Radoslaw Kowalczyk for the countless hours he spent with me at the CAF trying to explaining the art of NMR. I equally thank the AHVLA staff for their help during the animal trial.

Many thanks to Dr Luke Mappley for being of real support and a great source of knowledge on *Brachyspira* and AIS during the first year of my PhD.

I am more than thankful to all my PhD and Post-Doc friends from the University of Reading and more especially Joy, Chelsea, Sophie, Ruth, Roz, Oonagh who made of these years a great experience and bought a lot of fun into my life even when work was getting hard. I would like to thank more especially Natasa for always being there for me and for her great understanding and support especially towards the end.

Thanks to Dan without whom I might not have found this project and for being my strength during the two first years.

I also want to thank my friends from back home because even if far away, they always supported and encouraged me. Marie for all those weekends in London, Roxanne for not making me feel bad to spend 9 years at uni, Lucie for her wise advise and always picking up the phone when I needed it and Celia for constantly making me laugh and for keeping me company in the library in the last bit of this long marathon.

Finally, this part of my life would not have been so pleasant without my family who I want to thank for being the best support of all since I started this PhD. Thanks to my parents and grand mother for being so patient, supportive and always pushing me to go further. Thanks to my dear sister Flore for coming so often to distract me in the UK and supporting me on every side of my life during these four years.

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List of Abbreviations:

AIS	Avian intestinal Spirochetosis
AMP	Adenosine monophosphate
APHA	Animal and Plant Health Agency
BEB	*Brachyspira* enrichment broth
CAF	Chemical Analytical Facility
CFU	Colonies forming unit
COSY	Correlation Spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill
CM	Caecal microbiota
CYP	Cytochrome P450
D	Dimension
DNA	Deoxyribonucleic acid
EFSA	European food safety authority
EU	European union
FABA	fastidious anaerobe blood agar
FAOSTAT	Food and Agriculture Organization of the United Nation
FID	Free induction decay
GABA	4-aminobutyrate
GI	Gastro Intestinal
GM	Gut microbiota
GRAS	generally regarded as safe
HDL	High density lipoprotein
HMDB	Human Metabolome Data Base
HR-MAS	High resolution magic angle spinning
HSQC	Heteronuclear Single Quantum Coherence
K	Thousand
MeOH	Methanol
MIC	minimum inhibitory concentration
NGS	Next generation sequencing
NMR	Nuclear magnetic resonance
O-PLS-DA	Orthogonal projection to latent structure discriminant analysis
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PM	Post mortum

QIIME	Quantitative insight into microbial ecology
RD	Recycle delay
RNA	Ribonucleic acid
SCFA	Short chain fatty acid
SD	Swine dysentery
T	Time
TMA	Trimethylamine
TSP	3-(tri-methylsilyl)propionate-2,2,3,3-d_4
UDPG	Uridine diphosphate glucose
US	United states
UV	Ultra violet
VLDL	Very high density lipoprotein

List of Publications:

2016

Le Roy, C. I., Mappley, L. J., La Ragione, R. M., Woodward, M. J., & Claus, S. P. (2016). NMR-based metabolic characterization of chicken tissues and biofluids: a model for avian research. *Metabolomics*, *12*(10), 157.

Frej, A. D., Clark, J., Le Roy, C. I., Lilla, S., Thomason, P. A., Otto, G. P., ... & Stephens, L. (2016). The inositol-3-phosphate synthase biosynthetic enzyme has distinct catalytic and metabolic roles. *Molecular and Cellular Biology*, *36*(10), 1464-1479.

Matysik, S., Le Roy, C. I., Liebisch, G., & Claus, S. P. (2016). Metabolomics of fecal samples: a practical consideration. *Trends in Food Science & Technology*.

2015

Le Roy, C. I., Štšepetova, J., Sepp, E., Songisepp, E., Claus, S. P., & Mikelsaar, M. (2015). New insights into the impact of *Lactobacillus* population on host-bacteria metabolic interplay. *Oncotarget*, *6*(31), 30545-30556.

Bindels, L. B., Neyrinck, A. M., Claus, S. P., Le Roy, C. I., Grangette, C., Pot, B., ... & Delzenne, N. M. (2015). Synbiotic approach restores intestinal homeostasis and prolongs survival in leukaemic mice with cachexia. *The ISME journal*.

Woodward, M. J., Mappley, L., Le Roy, C., Claus, S. P., Davies, P., Thompson, G., & La Ragione, R. M. (2015). Drinking water application of Denagard® Tiamulin for control of *Brachyspira pilosicoli* infection of laying poultry. *Research in veterinary science*, *103*, 87-95.

Le Roy, C. I., Mappley, L. J., La Ragione, R. M., Woodward, M. J., & Claus, S. P. (2015). *Brachyspira pilosicoli*-induced avian intestinal spirochaetosis. *Microbial Ecology in Health and Disease*, 26.

Vimaleswaran, K. S., Le Roy, C. I., & Claus, S. P. (2015). Foodomics for personalized nutrition: how far are we? *Current Opinion in Food Science*, *4*, 129-135.

List of selected Conferences:

2015

Poster: Impact of the antibiotic tiamulin on gut microbial ecosystem and host lipid metabolism, EMBL conference, Heidelberg (Germany) – conference travel bursary

2014

Presentation: *In vitro* investigation of *Brachyspira pilosicoli*-host metabolic interactions, SOMED and ASM congress, Chicago (USA)

2013

Presentation: Tiamulin impacts *B. pilosicoli* metabolism, Spirochaete conference, Surrey (UK), **young scientist award**

Presentation: *Brachyspira pilosicoli* infection affects glucose absorption by chicken, SOMED conference, Kosice (Slovakia), **young scientist award**

Presentation: Intestinal spirochetosis: *Brachispira pilosicoli*-induced AIS (the use of Metabonomics), **Invited speaker** au Pig veterinary Society meeting, Birmingham (UK)

Poster: *Brachyspira pilosicoli* infection affects the metabolic profile of chicken faeces, Welcome trust conference, Cambridge (UK)

Introduction

Poultry represents approximately half (49%) of meat consumption in the UK, thus profoundly impacting on the national food economy, and with increased national and international demand for food accessibility and sustainability, there is renewed focus on food production optimisation. In poultry a major contributor to losses is intestinal infection that can drastically reduce productivity and is a problem with regards to animal welfare, often with human health concerns also if the infection has zoonotic potential.

One disease that anecdotally is emerging in poultry production is avian intestinal spirochaetosis (AIS) and is caused by members of the spirochaete family, most frequently of the genus and species *Brachyspira pilosicoli*. Infection by this bacterium may arise in both broiler and layer flocks and generally the clinical signs are diarrhoea, loss of appetite, poor body condition, decreased growth rate and reduced egg production in layers. Morbidity within flocks may reach 20-25% of birds and mortality may reach 5-10%. One of the main treatments available to treat *B. pilosicoli*-induced AIS is Tiamulin^{TM}, a bacteriostatic antibiotic of the pleuromutilin family precluding protein formation by binding to the 50S region of the ribosome. To date AIS has been little studied due to complex cultural detection, although molecular methods are under development, often associated with multi-species infection and the current lack of confidence in the identification and definition of pathogenic and non-pathogenic species. Nevertheless, the increasing incidence or AIS worldwide has given impetus for new investigations aimed at improving animal welfare, reducing infection rates and better customer safety. As part of this activity,

this project is aimed at better understanding AIS as there are several knowledge gaps most notably a complete lack of understanding of the host's metabolic reaction to infection, treatment and recovery. We therefore hypothesised that infection by *B. pilosicoli* and response to treatment with Tiamulin™ will induce systemic host metabolic responses. With this closure in our current knowledge gap we will better understand the consequences of infection and have information that hopefully will enable improved outcomes to be designed. To achieve the primary goal, NMR-based metabonomics techniques were employed. Metabonomics was defined by Nicholson et al. as 'the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification'. This is an untargeted method that allows to capture the wide metabolic response of a system to environmental variations such as infection or antibiotic treatment and to thereafter formulate hypothesis.

To evaluate this, the PhD was divided in several subprojects aimed at gaining a comprehensive understanding of AIS from the pathogen level to the host. The general organisation of the PhD is described in Figure 0.1 as a workflow describing the objectives of each chapter.

Chapter I is a review presenting *B. pilosicoli*-induced AIS, its treatment and research perspective in the area.

To start understanding AIS we first focus on the pathogen itself and **Chapter II** describes *B. pilosicoli* growth and metabolism under optimum growth condition and under Tiamulin™ stress.

The host metabolism of healthy birds was described in **Chapter III** where we determined the metabolic composition of twelve biological matrices using NMR

spectrometry (liver, kidney, spleen, plasma, brain, colon, caeca, ileum, faeces, breath muscle, egg yolk and albumin

In **Chapter IV**, the experimental design of the major poultry study is detailed: here the focus was to evaluate the optimum tiamulin dose to be used in order to treat *B. pilosicoli* infected chickens and prevent associated symptoms during an animal trial.

In **Chapter V**, using the same animal trial than chapter IV and chapter III as a data base, we evaluated the impact of *B. pilosicoli* infection and Tiamulin™ on chicken systemic metabolism and caecal microbiota using ^{1}H NMR-based metabonomic and 16S rRNA NGS in order to better understand the disease and treatment effects.

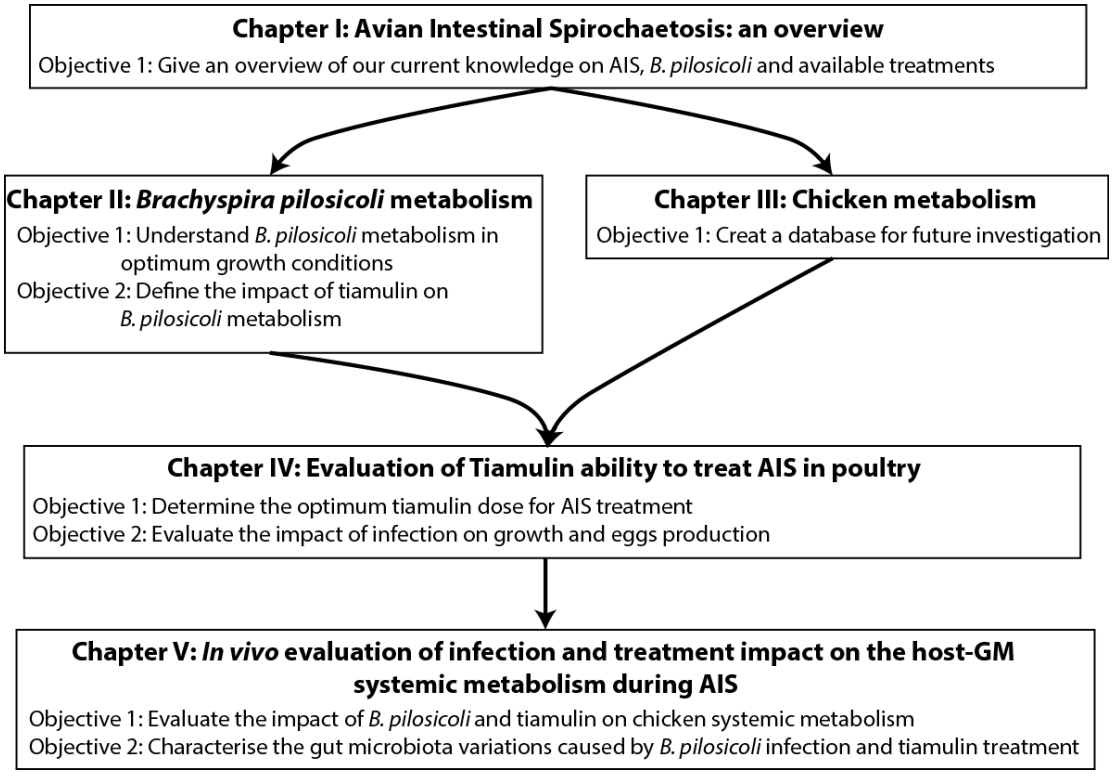
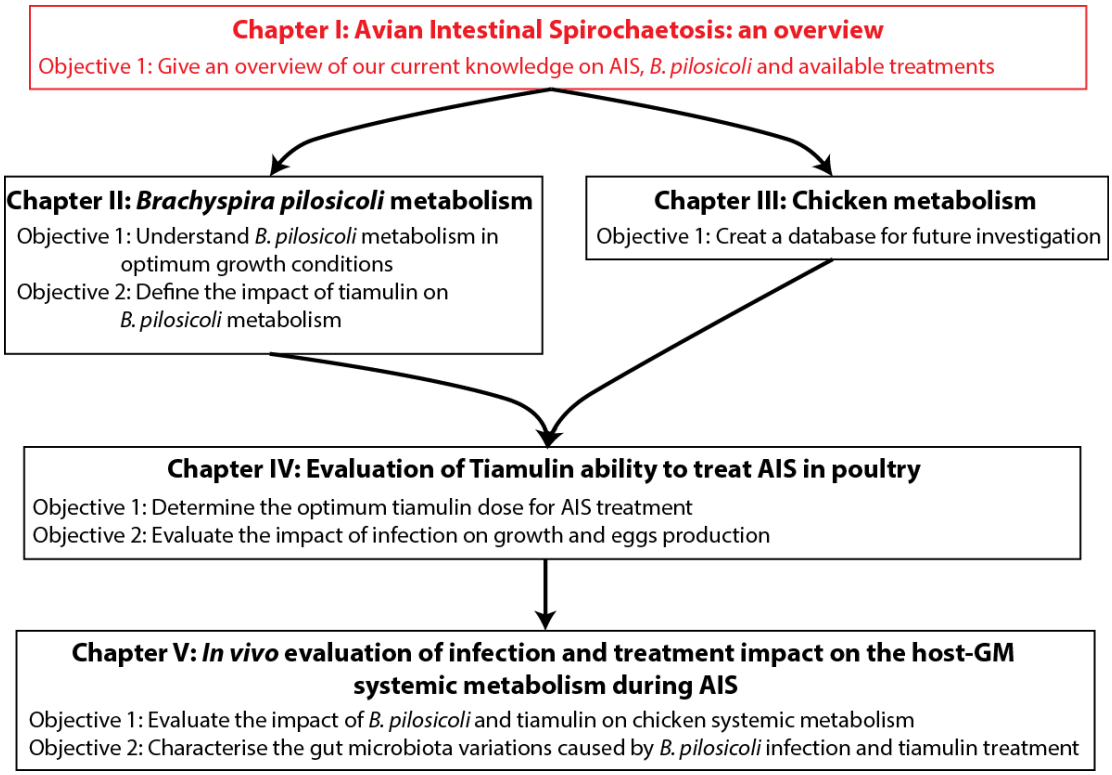


Figure 0.1: PhD workflow

Description of the bacterium, the disease and its treatments:

The first chapter was published as a review in Microbial Ecology in Health & Disease (2015). It aimed at describing the bacterium, AIS and present some of the existing treatment in order to introduce the research work of this PhD.



Chapter 1: *Brachyspira pilosicoli*-induced avian intestinal spirochaetosis

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1.1. Abstract

Avian intestinal spirochetosis (AIS) is a common disease occurring in poultry that can be caused by *Brachyspira pilosicoli*, a Gram-negative bacterium of the order Spirochaetes. During AIS, this opportunistic pathogen colonises the lower gastrointestinal (GI) tract of poultry (principally the ileum, caeca and colon), which can cause symptoms such as diarrhoea, reduced growth rate and reduced egg production and quality. Due to the large increase of bacterial resistance to antibiotic treatment, the European Union banned in 2006 the prophylactic use of antibiotics as growth promoters in livestock. Consequently, the number of outbreaks of AIS has dramatically increased in the UK resulting in significant economic losses. This review

summarises the current knowledge about AIS infection caused by *B. pilosicoli* and discusses various treatments and prevention strategies to control AIS.

1.2. Introduction

Controlled animal husbandry is essential in order to ensure safe and sustainable food production. Animal husbandry is commonly practiced in developed and some developing countries(1) as reported by the USDA and Eurostat(2, 3). The constant optimisation of breeding techniques and increased production efficiencies has reduced significantly the price of meat and dairy products over the years, providing wider access to products derived from animals in countries where they were not traditionally consumed(1). Therefore, there is a growing interest in improving breeding methods to improve animal welfare, reduce production costs and ensure higher safety and better quality for consumers. In this context, it is particularly relevant to reduce diseases of animal production, especially those that have zoonotic potential. Gastrointestinal (GI) diseases are common in production animals and their incidence has increased in large scale farming industry due to intensive farming practices that facilitate rapid spread of infection between animals(4). GI disorders in such facilities often result from the colonisation of the GI tract by pathogenic microorganisms particularly at certain times in the production cycle such as weaning (5). *Brachyspira pilosicoli* that induces intestinal spirochaetosis (IS) is an emerging pathogen causing infections in a number of species, including poultry, which is the subject of this review. Whilst *Brachyspira* spp. are found in intensive husbandry, *Brachyspira* spp. infection are particularly common in free-range and

organic farms (6, 7) due to the higher exposure of flocks to wild birds and the environment that act as infection vectors/reservoirs (7).

IS is a generic name given to largely diarrhoeal disease caused by the colonisation of the lower GI tract by Spirochaetes of the genus *Brachyspira*, and more specifically for poultry by *B. pilosicoli*(4,7), *B. alvinipulli*(8), and *B. intermedia* that are Gram negative, spiral organisms with flexible outer membrane and inter-membrane polar flagella (7-9 depending on species) possessing single circular genome comprising 4-5000 genes and a GC ratio of 27%. Pathogenic *Brachyspira spp* are presented in Table 1 with their host range and pathogenicity(9). Other *Brachyspira spp*. (not listed for brevity) are non-pathogenic but may be found in mixed infections. Also *B. hampsonii* is a newly described pathogen in several species including poultry, yet to be defined and accepted as a new species. *B. pilosicoli* is an opportunistic pathogen generally associated with swine and poultry, but has also been reported to infect other animals including dogs, horses, monkeys, turkeys, geese, and humans(10-13).

Table 1.1: List of *Brachyspira* species, their host, and pathogenicity

Species	Host	Pathogenicity	Reference
B. aalborgi	Human, primates	mild to moderate	(11)
B. alvinipulli	Chicken, goose, Red breasted, merganser, dog	mild to severe	(12)
B. hyodysenteriae	Chicken, goose, mallard, common rhea, pig, rat, mouse	severe	(13,14)
B. innocens	Chicken, pig, dog, horse	none	(15,16)
B. intermedia	Chicken, pig	mild to moderate	(10)
B. murdochii	Chicken, pig, rat	none	(10)
B. pilosicoli	Chicken, pheasant, grey partridge, feral water birds, common rhea, pig, dog, horse, primates, human	mild to moderate	(17)

Avian intestinal spirochaetosis (AIS), caused by the colonisation of the lower GI tract by bacteria of the genus *Brachyspira* in birds, generally occurs in breeder and egg laying chickens but also increasingly in broilers. The infection triggers severe diarrhoea accompanied by loss of weight, which has been associated with increased morbidity amongst flocks with 5-10% mortality if untreated with concurrent loss of egg production in layers (14-16). It often occurs by transmission of the spirochaetes *via* the fecal-oral route and can be transferred between livestock buildings by farmers(4, 17). An increasing number of recent publications have reported the presence of *Brachyspira* species in farms all over the world(20). This observation could result from several parameters such as, the 2006 EU ban of antibiotics use as prophylactic(4, 21), the modification of animal housing and finally the development of improved detection methods for this specific genus(22, 23). Thus, the impact of this disease on animal welfare and production is of high concern to the poultry industry enhancing needs for novel intervention strategies to reduce the spread of AIS.

Here, we review the current knowledge on AIS caused by *B. pilosicoli* and discuss the therapeutic and prophylactic strategies currently investigated (including antibiotics and probiotics). Vaccine development to protect against Swine Dysentery (SD), a disease caused by *Brachyspira hyodysenteriae* infection in pigs(24) is also on-going. Similarly, the development of autogenous vaccines for AIS(25), are just emerging. However, as progress regarding these interventions are still extremely limited, these will be discussed no further in this review.

1.3. An overview of the disease

Sign and symptoms

B. pilosicoli-induced AIS is generally observed in laying egg chickens over 10 weeks old in large rearing farms(4, 26). Numerous cases have been reported worldwide, especially in Europe, the US and Australia, where intensive farming offers suitable conditions for development and spread of various GI infections including those caused by *Brachyspira*.

Symptoms of infections with *B. pilosicoli* range from asymptomatic to severe, leading to mortality in chickens(4, 27). Nevertheless, the most common mild/moderate infections are generally characterised by diarrhoea, faeces with altered colour and consistency, which are frequently foamy due to increased gas production(28), so-called “cappuccino” faeces. This may progress to faeces containing mucus and blood (27). Diagnosis is generally confirmed via bacterial culture or PCR(29).

AIS infection results in a slower growth rate (28-30) and can also be associated with a delay of up to 7 weeks in the start of lay accompanied by a decrease in egg quality

(28-31). Eggs produced by infected hens are usually small, lighter in weight (i.e. 2 to 6 g less per egg)(28) and are less numerous. Poor quality shells are prone to cracks, and often contaminated by faeces (32). Yolks are generally less coloured with a decrease of 1.5 to 3 points on the Roche yolk colour fan (28), (33). Moreover, it has been suggested that infection may have long term consequences on the second generation of chickens hatched from eggs laid by infected hens(28). Indeed, it has been shown that chicks hatched from eggs laid from infected female parents presented similar symptoms (i.e. decreased weight gain, delayed lay onset, wetter and paler faeces) despite the absence of contamination(28). These results raise new hypotheses regarding potential epigenetic variations in response to *B. pilosicoli* infection.

At a microscopic level, intestinal biopsies of infected chickens displaying the symptoms described above usually reveal the presence of *B. pilosicoli* fixed to the cells of the intestinal wall(27), which is suspected to be correlated with the degree of enterocyte perturbation(27). Tissues look inflamed, often with some signs of bleeding. The intestinal wall shows evidence of a loss of microvilli(21). The loss of microvilli results in perturbation of the epithelial barrier permeability, which may contribute to the decrease in weight gain and increased amount of water in faeces. The cytoplasm of enterocytes appears damaged as indicated by abnormal vacuolation, condensation and fragmentation of the chromatin and cell sloughing(21). This is likely to result in lower nutrient absorption as indicated by increased food consumption in infected chickens(31) accompanied by increased faecal lipid content concomitant with decreased lipid levels in the general circulation(34). The same phenomenon has been observed for carotenoid

concentration, which has been found in higher quantity in faeces of infected animals, while lower in blood, and is believed to be the cause of weakened colour intensity of the yolk(28, 34).

1.4. Characteristics and mechanism of infection

Morphology:

B. pilosicoli is a bacterium of the order Spirochaetales, morphologically characterised by a corkscrew like shape(35) (Figure 1.1). It was first identified as a cause of IS in Denmark in 1982(27). *B. pilosicoli* can be found in the literature under the former name of *Serpulina pilosicoli*(27, 36). It is a Gram-negative, fastidious, aerotolerant anaerobe that can be exposed to oxygen for a few hours(37, 4). The optimum growth temperature is 38.5°C(27), but it can remain viable for sixty-six days at 4°C in water and survive up to 210 days in pig faeces mixed with soil at 10°C(37).

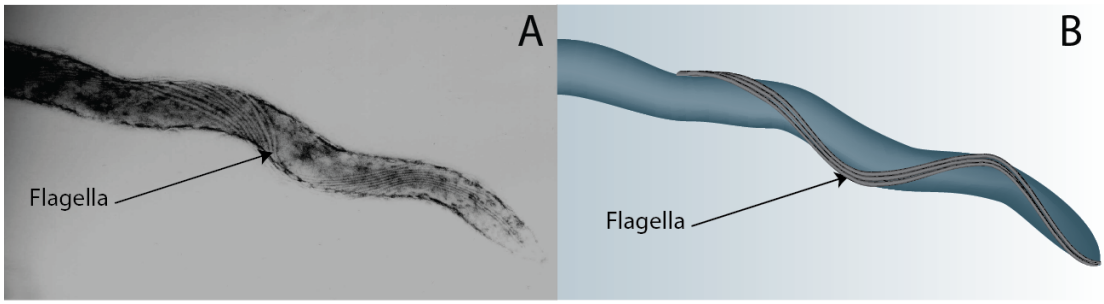


Figure 1.1: Transmission electron microscopy illustrating the flagella of Spirochaetaceae. (A) [Adapted with permission from Yano et al. (40)]. (B) Graphic representation of picture A enhancing the visualisation of the flagella.

B. pilosicoli is constituted of a central protoplasmic cylinder covered by a membrane sheet (27). The membrane sheet, also known as the outer membrane, is an important element for the integrity of the bacterium. Several studies have shown that perturbation of the membrane generally causes destruction of the flagella and of the periplasmic membrane(38). The composition of the outer membrane is not entirely known despite its high relevance to host-pathogen interactions. Yet it has been shown to be extremely labile due to its high content in sterols (cholesterol and cholestanol), which are responsible for a low resistance to osmotic stress and to low ionic buffers that trigger its destabilisation(39). Between the outer membrane and the protoplasmic cylinder is the periplasm, where the flagella of the bacteria are located. *B. pilosicoli* possesses between 8 to 10 flagella disposed equally at the poles at each end of the bacterium following the corkscrew shape of the bacterium and overlapping in the centre (27, 4) (Figure 1). This configuration is specific to the Spirochaetes and confers high motility, which constitutes an important virulence factor. The flagella works by producing helical or flat sinusoidal waves(34), which induce a clockwise or anti-clockwise movement of the bacteria and enable a non-transversal swim(41, 42). A transversal swim is also possible by the simultaneous combination of the two movements(41). Both modes of movement provide *B. pilosicoli* with the ability to swim through viscous media(40).

Infection process:

B. pilosicoli infects the lower GI tract of chickens, swine, horses, dogs, humans, and other animals(37). Upon entry *via* the oral cavity, the bacterium that survive passage through the stomach acidity reach the intestinal lumen. Using chemotaxis, the

organism migrates towards the mucus and the intestinal wall(43, 44). Indeed, *B. pilosicoli* has a high number of genes coding for chemotaxis towards the mucus in comparison to other known bacterial species, providing a significant advantage to colonise the host (45). The mucus is a viscous matrix composed of two stratums, the inner and outer layers, which form a physical barrier and protect the intestinal cells from bacterial infections by limiting their motility(43). The unique shape of *B. pilosicoli* combined to the production of specific enzymes that hydrolyse the mucus inner layer (sialidase family-like proteins), confers them the ability to swim through this medium and allow them to reach the cell wall(43, 42). These are high virulence factors associated with tissue damage(45). Another virulence factor may be the noted sensitivity of *B. pilosicoli* to the chemo-attractant serine, which is found in high concentration in the mucus secreted by goblet cells(38, 37, 46).

Once the bacterium is in contact with an intestinal cell, fixation occurs through protein-protein interactions(47), although the exact mechanism have not been fully ascertained. *B. pilosicoli* attaches vertically to the cell wall by one of its cylinder ends (47, 36) and can be found very closely packed on the cell at a density ranging from 20 to 80 bacteria per cell, forming a "false brush border"(37, 45). Attachment of the bacterium is not necessarily associated with symptoms of IS (37, 4) but an increase in bacterial concentration appears directly linked to the intensity of the symptoms(37) as previously mentioned. Adherence of *B. pilosicoli* to the cell membrane triggers a signal that results in invagination of the apical membrane and internalisation of the bacteria potentially resulting in cell apoptosis. *B. pilosicoli* can also cross the intestinal barrier by disrupting gap junctions (between cells), which in some cases may allow it to enter the blood stream(4, 48). Indeed systemic spread of *B. pilosicoli*

has been reported in one study showing evidence of colonisation of the spleen and liver (49). However, this was not commonly observed and the mechanism by which the bacterium escapes the immune system is not known yet. The infection process is summarised in Figure 1.2.

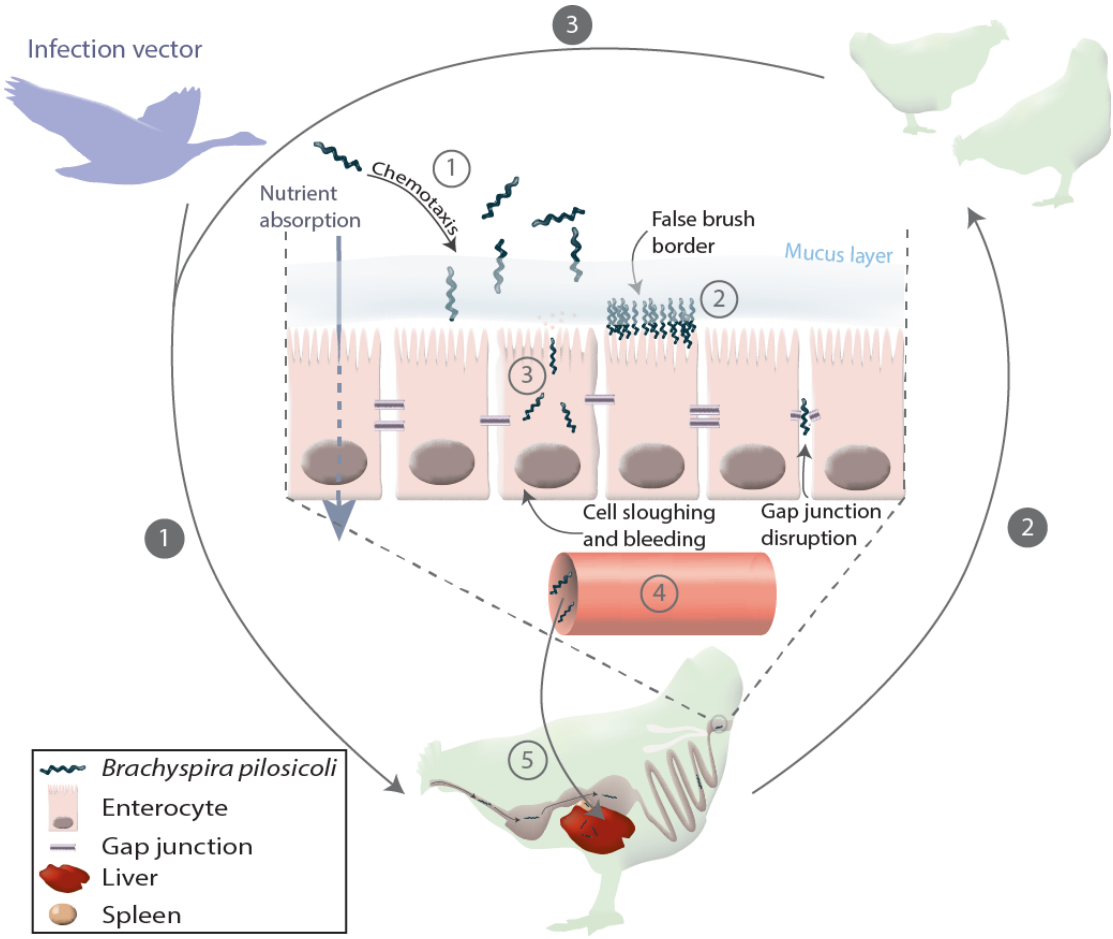
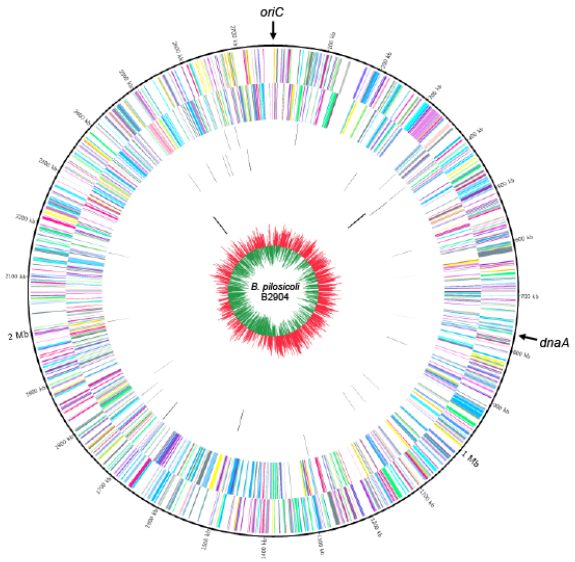


Figure 1.2: Transmission and infection process of *Brachyspira pilosicoli*. White numbers on grey circles describe the contamination process: 1, transmission of contaminated material in a farm via a vector _ wild animals, farmers, water, and other farm animals _ to a housed bird via oral route; 2, transmission of the bacterium to the rest of the flock; 3, persistence of infection between birds of a same flock. Grey numbers in white circles describe the infection process once *B. pilosicoli* has reached the lower digestive tract: 1, chemotaxis attraction of the bacteria towards the mucus and cell wall; 2, attachment of *B. pilosicoli* on the cells and formation of a 'false brush border'; 3, invasion of intestinal cells; 4, translocation to the blood stream; 5, systemic infection.

Genetic features:

In addition to the aforementioned genetic functions, a recent publication of *B. pilosicoli* B2904 complete genome by Mappley *et al.*(45) (Figure 3) identified key genes responsible for some of *B. pilosicoli* infection- and colonisation-related processes such as: chemotaxis, mobility, adhesion and host tissue degradation. *B. pilosicoli* genome analysis also provided new insights into its metabolism. It revealed numerous genes involved in carbohydrate transport and metabolism, such as phosphoglucomutase that plays a key role in glycolysis. These genetic observations correlated to phenotypic tests using Biolog® technology (which evaluates the cell's ability to respire on a wide range of substrates) demonstrated the ability of *B. pilosicoli* to use several types of saccharides (e.g. glucose-6-phosphate) and oligosaccharides (e.g. dextrin) as primary carbon sources. Finally, another large section of the genome was allocated to amino acid synthesis and transport. Those results represent a major advancement towards understanding the interrelationship between metabolism and infection.

Figure 1.3, presents the general representation of *B. pilosicoli* B2904 complete genome feature. The six first circles are colour coded, with each colour representing a specific function of the genes. Genes' functions were assigned using Clusters of Otogenous Groups (COGs). The two first circles describe genome function using this method with forward and reverse transcription. The same process was applied for tRNA (circle 3 and 4) and rRNA (circle 5 and 6). Finally, the 7^{th} circle describes the GC skew that is linked to DNA replication and calculated using the following formula: GC skew = (G-C)/(G+C).



COG code	Colour	Gene function
A		RNA processing and modification
B		Chromatin structure and dynamics
C		Energy production and conversion
D		Cell cycle control, cell division and chromosome partitioning
E		Amino acid transport and metabolism
F		Nucleotide transport and metabolism
G		Carbohydrate transport and metabolism
H		Coenzyme transport and metabolism
I		Lipid transport and metabolism
J		Translation, ribosomal structure and biogenesis
K		Transcription
L		Replication, recombination and repair
M		Cell wall, membrane and envelope biogenesis
N		Cell motility and secretion
O		Posttranslational modification, protein turnover and chaperones
P		Inorganic ion transport and metabolism
Q		Secondary metabolite biosynthesis, transport and catabolism
T		Signal transduction mechanisms
U		Intracellular trafficking, secretion and vesicular transport
V		Defence mechanisms
W		Extracellular structures
Y		Nuclear structure wheat for cell division and chromosome partitioning
Z		Cytoskeleton
R		General function prediction only
S		Function unknown

Figure 1.3: Circos circular representation of the complete *B. pilosicoli* B2904 genome with annotated genes. The genome is orientated from the oriC and also displays the location of dnaA. Circles range from 1 (outer circle) to 7 (inner circle). Circle 1, COG-coded forward strand genes; circle 2, COG-coded reverse strand genes; circle 3, forward strand tRNA; circle 4, reverse strand tRNA; circle 5, forward strand rRNA; circle 6, reverse strand rRNA; circle 7, GC skew ((G-C)/(G_C); red indicates positive values; green indicates negative values). All genes are colour coded according to Cluster of Orthologous Group (COG) functions shown in the key table. [Adapted with permission from Mappley et al. 2012 (46)].

1.5. Impact on the food chain: a zoonotic potential?

Intestinal spirochetosis is relatively rare in humans as it occurs mostly in immunocompromised patients. In most cases, carriage by the host of the bacteria is often asymptomatic, but following the apparition of any symptom such as diarrohea and abdominal pain IS is confirmed by biopsy(50, 51). Only in some rare cases did an infection by *B. pilosicoli* cause death of a patient as a result of septicaemia(52). Such cases have only been observed in elderly and immunocompromised patients or in populations living in dense areas with poor hygiene conditions(53-55).

Despite the rare occurrence of the disease in humans, a major concern is the zoonotic potential of the bacterium(48). Indeed, it has been suggested that *B.*

pilosicoli is able to survive and be transmitted to the consumer *via* contaminated raw meat from infected chicken(56). Several studies have shown considerable genetic similarities between strains of *B. pilosicoli* infecting humans, swine and poultry, suggesting an ability to adapt to various hosts(48). In 2012, Mappley *et al*., (45) carried out a genetic comparison of three strains of *B. pilosicoli* isolated from humans, chickens and pigs, respectively. This study showed that the genotype of these three strains were very similar. However, some differences were noted in the genome size and arrangement and in some putative coding regions for carbohydrate, amino acid and nucleotide metabolism and transport(45). These data highlighted some fundamental genetic differences that are reflected in their phenotype and may have implications in host specificity and interspecies transmission(45) although this has remained untested to date. More structural rearrangements were observed in the strains isolated from chicken and human in comparison to the strain isolated from pig. Despite these variations, the functional genome comparison showed a high level of similarity in the features of the three strains except for the aforementioned transporters and enzymes(45). Additionally, genes involved in membrane fixation and in β-haemolysis were common to the three strains, which suggests a similar invasion and infection process between the bacteria(45). These genetic and phenotypic data indicate a high degree of similarity in infection processes across species and may support the potential of transmission of bacteria causing IS from farm animals to humans(48) and, therefore, is a realistic issue that requires attention. Prevention of IS spread in animal livestock is currently achieved using antibiotics.

1.6. Antibiotics: a controversial solution

Various antibiotics such as the pleuromutilins, macrolides and lincosamides are currently used to control *Brachyspira* infections in animals and have been shown to reduce associated symptoms(57, 58). The most common antibiotic used in animal husbandry is Tiamulin™, a member of the pleuromutilin family. By binding with the 50S region of the ribosome, it inhibits amino acid binding during protein synthesis(59). Tiamulin™ is used widely and has been shown to be efficient at controlling Swine Dysentery (SD), which is a severe GI disease in pigs caused by *B. hyodysenteriae*, a close relative of *B. pilosicoli*, at a dose of 7.71 µg per kg of body weight for a 5 days treatment. Nevertheless, the lack of standardised methods and techniques used to calculate the minimum inhibitory concentration (MIC) induces a large disparity in published results. Only two studies describing the impact of Tiamulin™ on *B. pilosicoli*-induced AIS in chicken have been reported: in 2002 in experimentally infected laying hens(60) and in a 2006 UK field study(61). Results suggest a positive impact of Tiamulin™ treatment in both studies with a general increase in growth rate, egg production and decrease of symptoms. Another customer concern is the possible presence of antibiotics and their metabolites in eggs although this has not been reported in the literature. One report issued by the European Medicine Agency mentioned very low antibiotic residual levels but these were not sufficient to establish a withdrawal period for eggs [Article 34(1) of Directive 2001/82/EC(62)]. Nonetheless, a withdrawal period of 24h should be applied for meat consumption [Article 34(1) of Directive 2001/82/EC(62)].

Furthermore, emerging bacterial resistance to antibiotics is another major concern (57, 63). For example, tylosin was a commonly used antibiotic to treat AIS but

resistance has recently emerged, compromising its efficiency and therefore its usage(64). Resistance factors appear as a consequence of an extensive use of antibiotics concomitant with the development of mutations in the bacteria such as on the ribosomal protein(65), which render them less susceptible. This stresses the importance of bacteriological diagnosis that should be used to determine precisely the *Brachyspira* species responsible of infection followed by antibiotic resistance test on pure culture in order to apply appropriate treatment. In response to the global rise of bacterial resistance and to protect the consumer's safety, the European commission banned the prophylactic use of antibiotics in livestock in 2006(66). Indeed, chickens grown in industrial farms used to receive prophylactic antibiotic treatments, which was also associated with increased animal fattening rate(66). Since this interdiction, infection outbreaks by *B. pilosicoli* have boomed(21, 66). Common consequences include reduced egg production, growth delay, higher food consumption and in some cases, increased mortality within infected flocks. Since 2006, the economic loss associated with AIS has been estimated to be of approximately £18 million per year in the UK (Burch, D. J. S., 2009 personal communication) pointing to the need for better prevention methods and refined treatments. Prevention of AIS outbreaks can be achieved using appropriate hygiene and biosecurity rules as demonstrated by several studies (67). *B. pilosicoli* is readily eliminated by standard farm disinfection processes(68) and the potential of vaccination against *B. pilosicoli* has been explored primarily in pigs and may be applicable poultry(69). However, treatment is commonly achieved using antibiotics such as linco-spectin and Tiamulin™ at 25mg/kg of body weight per day although this dosage regimen is derived from studies in pigs. Recently, we investigated the

optimum dose to treat laying hens and demonstrated that 250 ppm given in drinking water over three days reduce infection significantly, but the bacterium was still detectable at the end of the study (3 weeks after treatment end) (70). Notwithstanding the use of antibiotic for intervention, it remains crucial to find alternative solutions to prevent AIS to protect animal welfare and consumers.

1.7. Probiotics: a potential solution?

The gut microbiota (GM) is estimated to be composed of more than 1,000 species of bacteria(67) which are predominantly Gram-negative(71). They exert an important role for the host, as they are involved in its protection from pathogens and in the release of nutrients from the diet, which would otherwise be unavailable to the host(71). Beyond the positive impact of commensal bacteria on the digestive system and associated nutritional benefits, increasing evidence reveals a systemic impact of the GM on the host(72). Probiotics, which are defined as “live microorganisms which when administered in adequate amount confer a health benefit on the host”(73), have been developed to take advantage of this symbiosis. Protection is achieved by increasing the competition between the probiotic and pathogens for cell membrane receptors and nutrients, modulation of the immune system, improvement of the mucosal barrier permeability, secretion of toxins and lowering the pH of the GI(74, 75). Their mechanisms of action vary, depending on the probiotic but most of them remain largely misunderstood.

Only a few studies have investigated the impact of *Lactobacillus*-based probiotics on *B. pilosicoli* and most of them have been carried out *in vitro*. It has been shown that

lactic acid secreted by lactobacilli has similar effects as other acidic compounds and disinfectants on *B. pilosicoli*, whereby the bactericidal effect is mediated by destabilisation of the cellular wall, hence reducing the bacterial viability(76). Another interesting effect of lactic acid is that it induces the formation of "spherical bodies" formed by the retraction and swelling of both ends of the bacterium, which tends to create a sphere shape. At this stage the bacterium is still viable but in a dormant state(77).

Two promising *Lactobacillus* species to tackle AIS are *L. salivarius* and *L. reuteri.* They are both recognised as generally regarded as safe (GRAS) and suitable for livestock feeding(78). A recent study has shown that both lactobacilli antagonise motility, growth and cellular adherence of *B. pilosicoli*(21). *In vitro,* it appears that the presence of *L. reuteri* and *L. salivarius* reduces markedly the potential of *B. pilosicoli* to induce apoptosis of intestinal cells(21) by antagonising adhesion to the intestinal epithelium, in a process of competitive exclusion. An *in vivo* study indicated that *Lactobacillus* probiotic can prevent potential infection and associated symptoms caused by the pathogen if administered before or during challenge with *B. pilosicoli*(79), supporting its efficiency as a protective agent against AIS.

Another advantage to use probiotics in farms is their potential as animal growth promoters when used as prophylactic(80–82). In a study by Yoruk *et al*. it was demonstrated that probiotic consumption by laying hens resulted in decreased mortality and increased egg production without altering quality (81). Moreover, the consumption of *Lactobacillus*-based probiotic during the first three weeks of life was shown to increase animal growth demonstrating their potential as growth promoters in the early stages of life (82). Probiotics may also be useful to prevent infection

relapse that is often observed with AIS. Indeed by maintaining a healthy and balanced gut environment, probiotic could potentially be used in order to inhibit *B. pilosicoli* reappearance post antibiotic treatment (20).

1.8. Conclusion

B. pilosicoli-induced AIS is a growing and underestimated problem in the poultry industry. However, its occurrence and economic burden is not negligible. Antibiotics such as Tiamulin™ are still considered as a gold standard to tackle the infection, although resistance is emerging, which stimulates the need for the development of new interventions. Despite these promising novel therapies, there remains a large gap in the understanding of the pathogen itself, particularly its metabolism although some new insights were given recently by the genetic mapping of a few strains of *B. pilosicoli.* Characterising these pathways would provide a major advantage in AIS understanding in order to design more targeted treatments. Finally combination therapies that use an antibiotic followed by an appropriate probiotic may be worthy of consideration to prevent relapse by strengthening the gut microbial community.

1.9. References

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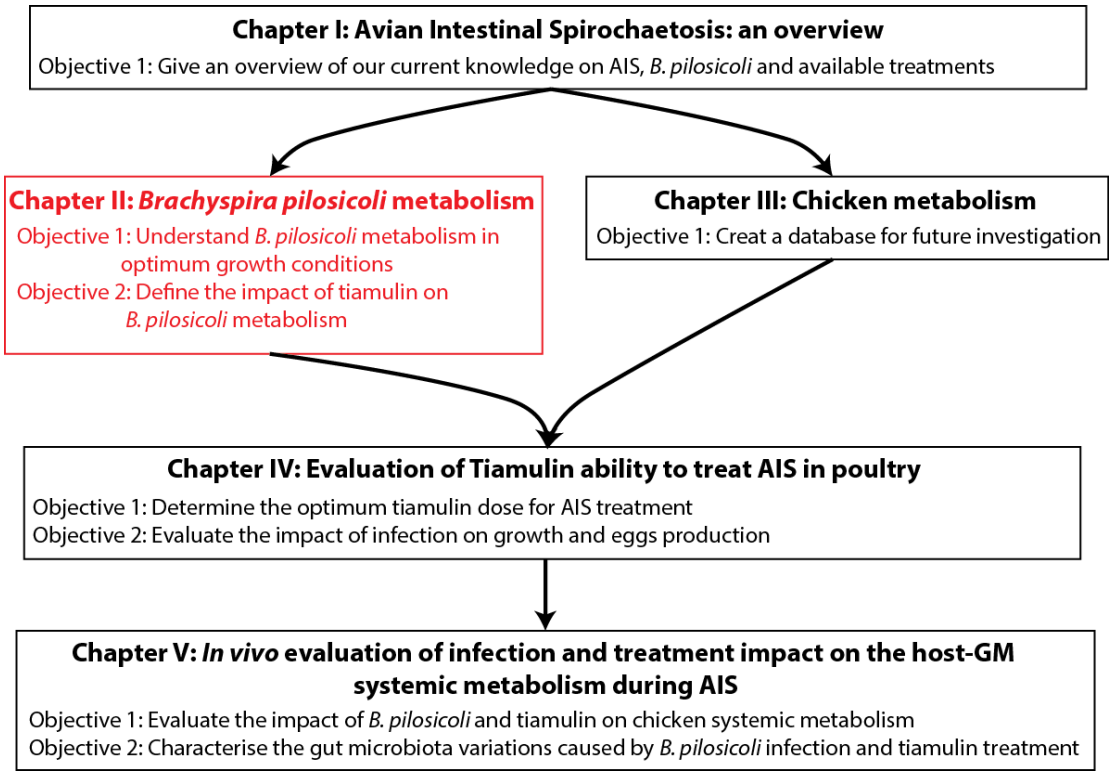
Understand *B. pilosicoli* metabolism and its response to Tiamulin™:

The first aim of this work was to evaluate *B. pilosicoli* metabolism in order to better understand the bacterium and how treatment could influence both growth and metabolism. This was done with the objective of better understanding the observations made later on *in vivo*. This work was submitted to the Anaerobe journal and is in the process of being reviewed.



Chapter 2: Metabonomics investigation of the impact of Tiamulin™ on *Brachyspira* pilosicoli growth and metabolism

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Abstract

Pathogenic *Brachyspira spp.* are responsible for an increasing number of Intestinal Spirochaetosis (IS) cases in livestock against which few approved treatments are available. Tiamulin™ is used in cases of swine dysentery caused by *B. hyodysenteriae* and more recently to treat avian intestinal spirochaetosis. In this study we evaluated the impact of Tiamulin™ at varying concentrations on the metabolism of *B. pilosicoli* by applying a ^{1}H-NMR-based metabonomics approach allowing to capture the overall bacterial metabolic response to antibiotic treatment. In simple growth curve studies, Tiamulin™ appeared to impact bacterial growth even at very low concentration (0.008 µg/ml) although metabolism was little affected after 72h post-exposure to antibiotic treatment. Indeed, variations in the metabolic composition of the medium started to appear only after 72 h when bacterial growth had ceased. Exclusively the highest dose of Tiamulin™ tested (0.250 µg/ml) induced bacterial death with a consequent major metabolic shift. This is in accordance with the known minimum inhibitory concentration (MIC) for the strain used in this study. Below this concentration, bacteria were able to recover metabolically despite a significant inhibition of their growth by the antibiotic. This demonstrated that *B. pilosicoli* was able to survive and maintain its metabolic homeostasis at concentrations below 0.25ug/ml (MIC). These findings support the need to ensure that Tiamulin™ treatment in *B. pilosicoli* infected animals achieves this minimum dose in tissues to guarantee efficacy.

Keywords: *Brachyspira pilosicoli*, metabolomics, Tiamulin™, antibiotic resistance

2.1. Introduction

Brachyspira pilosicoli is a gram-negative bacterium of the family Spirochaetes. It colonises the lower part of the intestinal track of a large range of hosts including pig, bird, human, monkey, dog and horses(Stanton & Hampson 1997; Duhamel et al. 2003; Hidalgo et al. 2010; Trott et al. 1996). Once in the intestinal lumen, the bacterium is attracted via chemotaxis to the mucin barrier(Naresh & Hampson 2010; Hopwood et al. 2007) through which it swims mediated by its unique "corkscrew" shape and rotation of its periplasmic flagella (Prapasarakul et al. 2011) aided by the secretion of mucin degrading enzymes(Naresh & Hampson 2010; Li et al. 2000). *B. pilosicoli* attaches to the enterocytes in an end on fashion and may penetrate these cells also(Falkow et al. 2006; Mappley et al. 2012; Dassanayake 2004; Nakamura et al. 2006). In poultry, colonisation by *B. pilosicoli* can lead to the development of avian intestinal spirochaetosis (AIS) the signs of which are diarrhoea, poor condition, dehydration, decreased growth rate and a drop in egg yield with faecal staining of eggs. Mortality is often significant when the disease is left untreated(Fellström & Gunnarsson 1995; Duhamel et al. n.d.; Taylor et al. 1980), a consequence that makes AIS a serious economic and welfare problem in farming. Tiamulin^{TM} is effective in treating porcine diarrhoea caused by *Brachyspira hyodystenteriae, B. hampsonii and B. pilosicoli*(Johnston et al. 2001; Wilberts et al. 2014; Burch 2008) and as a consequence, this antibiotic has recently been used in the poultry industry to control AIS(Burch & Klein 2013).

Tiamulin™ is derived from a natural pleuromutilin that binds the 50S region of the ribosome to inhibit protein synthesis(Schlünzen et al. 2004). The antibiotic blocks peptide bond formation by interfering with substrate binding(Schlünzen et al. 2004;

Poulsen 2001; Long et al. 2006; Forschungsinstitut 1974). Tiamulin™ treatment on farm is generally associated with clearance of infection and associated symptoms. However, reoccurrence of the disease can be observed post treatment indicating incomplete clearance and possibly decrease susceptibility(Sperling et al. 2011; Karlsson 2004) in response to treatment. Currently there is a lack of an internationally recognised standardised method to determine Tiamulin™ minimum inhibitory concentration (MIC) for this bacterium, which has impacts upon selection of an appropriate treatment dose. Furthermore, recent studies have indicated that *Brachyspira* may acquire resistance against Tiamulin™ and, other than blocking protein synthesis, nothing is yet known of the metabolic response of *B. pilosicoli* to Tiamulin™. We argue that evaluating this using a metabonomics approach would allow a better understanding of the bacterial response to Tiamulin™ and give insight into improving selection of effective dosing regimes.

Metabonomics allows non-targeted evaluation of the metabolic modifications occurring in a biological system in response to a stress(Nicholson & Lindon 2008), which in this study is exposure to Tiamulin™. By providing a general overview of the metabolic response, this technique allows the generation of new hypotheses and provides new insights into biological systems metabolism in response to environmental stress or genetic modification. In this study, we used an NMR-based metabonomics approach coupled with multivariate statistics to evaluate the metabolic dose-response of *B. pilosicoli* to Tiamulin™. Bacteria were exposed to gradual antibiotic doses and media was sampled along 120h growth in order to evaluate the evolution of its metabolic composition while growth was recorded. This

allowed to snapshot the metabolic response of *B. pilosicoli* to Tiamulin™ and therefore enlightened resistance process to antibiotic treatment.

2.2. Materials and methods

2.2.1. Bacterial growth and antibiotic assay

B. pilosicoli B2904 isolated from a chicken presenting clinical signs of AIS in the UK (Mappley et al. 2011) was grown from frozen stock on agar solidified Brachyspira enrichment broth (BEB) plate for four days under anaerobic conditions (94% N_2 and 6% CO_2) at 37°C. Colonies were transferred into *Brachyspira* enrichment broth media (BEB supplemented with heart infusion) for three days under similar conditions. BEB contains 12.5 g/L of calf brain, 5.0 g/L of beef heart, 10 g/L of peptone, 5.0 g/L of sodium chloride, 2.0 g/L of D(+)-Glucose and 2.5 g/L of disodium hydrogen phosphate. Brain, hart infusion and peptone are sources of carbon, nitrogen, vitamins, amino acids, and essential growth factors. The bacterial concentration was then adjusted in BEB to 1 x 10^6 CFU/ml and transferred into 24 well plates (2 ml per well) and incubated as above for 120h. Every 24 h (with a first time point at 0 h growth), the entire well content was taken and centrifuged for 2 min at 2400 g to separate growth medium from bacteria. The supernatant was kept at - 80°C for further analysis. This process was repeated at each time point in sextuplet to deliver the appropriate power for statistical analysis.

The same method was used for the Tiamulin™ assay. Bacterial cells were grown as above and bacterial pellet were resuspended in BEB with antibiotic at six different concentrations (0.008, 0.016, 0.031, 0.062, 0.125 and 0.250 µg/ml plus control).

Bacteria were then inoculated into 24 well plates as previously described and incubated for 120h. For metabolic analysis each condition (Tiamulin™ concentration) and time point (every 24h for 120h) were also repeated in sextuplet. The medium was not changed for the duration of the experiment so that antibiotic exposure was continuous.

B. pilosicoli growth was evaluated using the same experimental design as the one previously described. Bacteria were grown in a 96 well plate (0.2 ml per well) and bacterial growth was evaluated every 2 hours for 120 hours at an absorbance of 600 nm using a FLUOstar Omega (BMG LABTECH Ltd). Water was used as blank and broth media without bacteria as a negative control. Each condition (Tiamulin™ concentration) was repeated in triplicate and results are presented as an average of the log of the bacterial concentration calculated from the absorbance observed at each Tiamulin™ concentration per time point after correction with standard curve. OD measurements were converted to log of bacterial concentration after determination of the *B. pilosicoli* concentration in broth for a specific OD using counting on agar plate to calculate the concentration of viable bacteria. Knowing the bacterial concentration for a specific OD a cross multiplication was applied to determine the rest of the concentrations.

2.2.2. NMR spectroscopy

For NMR spectroscopy, 0.4 ml of medium was added to 0.2 ml of NMR phosphate buffer (made in D_2O containing 10 % water and 0.05 % sodium 3-(tri-methylsilyl)propionate-2,2,3,3-d_4 (TSP) as a ^{1}H NMR reference) and 0.5 ml of the solution was transferred into 5 mm of outer diameter NMR tubes. ^{1}H-NMR spectra

were acquired on a Bruker Avance DRX 700 MHz NMR Spectrometer (Bruker Biopsin, Rheinstetten, Germany) operating at 700.19 MHz using a standard 1-dimensional (1D) pulse sequence [recycle delay (RD)-90°-*t1*-90°-*tm*-90°-acquire free induction decay (FID)] with water suppression applied during RD of 2 s and the mixing time (*tm*) of 100 ms and a 90 pulse set at 10 µs. For each spectrum 128 scans were recorded on a total of 32K data points. A broadening line function of 0.3 Hz was used to multiply all FIDs. After acquisition, all spectra were manually phased and baseline corrected using the software MestReNova® (version 2.1.8-11880, MestreLab, Spain). Finally spectra were calibrated to the chemical shift of TSP (δ 0.00). In order to facilitate metabolite identification based on literature, a series of 2D spectra on selected samples were acquired using correlation spectroscopy (COSY) NMR spectroscopy.

2.2.3. Statistical analysis

All spectra were scaled on unit variance and mean centered prior to analysis. To evaluate metabolic variation between samples, principal component analysis (PCA) was used. Orthogonal projection to latent structure discriminant analysis (O-PLS-DA) was also performed, where ^{1}H-NMR spectra were used as a matrix of independent variables (X) and time or antibiotic concentration were used as prediction vectors (Y) To capture metabolic variations linear to time and antibiotic concentration. O-PLS DA models were generated between each Tiamulin™ concentrations at every time point independently. A heat map was generated using each of this model strength in order to visualise when Tiamulin™ impacted bacterial metabolism in comparison to control and if clusters related to dose could be observed.

2.3. Results and discussion

2.3.1. Modifications of B. pilosicoli metabolism during growth

PC1, which captured 49% of the metabolic variation, indicated that a significant metabolic shift was recorded after 96h of incubation. Distinctions between 0h, 24h and 48h were observed on the 3rd component, representing only 9% of the total variation, which suggests a modest effect on the composition of the culture medium in the first 48h. This metabolic trajectory indicates that bacterial metabolism might change depending on the growth phase (Figure 2.1A). Scores from the same time point were clustered together indicating good reproducibility of the experiment except for two samples at 96 h. This two samples occupied the same metabolic space than what was observed a 0h, indicating that media composition remained unchanged after 96 h of bacterial growth. Given the reproducibility of the results it is possible to hypothesis that *B. pilosicoli* was enable to grow is these two specific wells, explaining the stability of the metabolic composition of the media.

In the culture medium, the growth of *B. Pilosicoli* was characterised by a decrease in glucose and an increase in amino acids (phenylalanine, alanine, tyrosine, lysine, valine and methionine), fermentation products (lactate, acetate, butyrate and isovalerate), as well as other compounds involved in the regulation of cell osmosis such as *myo*-inositol and trimethylamine (TMA) as observed in the PCA results presented in Figure 2.1A and B.

Glucose was the only readily identifiable substrate that showed a reduction over time (Figure 2.1B). Decreased concentration of other substrates could not be

detected and it is possible that some may be below the detection limit of the NMR. However, it is not unreasonable to assume that glucose was the only carbon source used for bacterial anabolism and growth. *B. pilosicoli* and more especially the strain used for this experiment (B2904) is able to use a wide range of carbohydrates and hexoses as primary carbon sources but it would seem in this study that glucose was used preferentially.

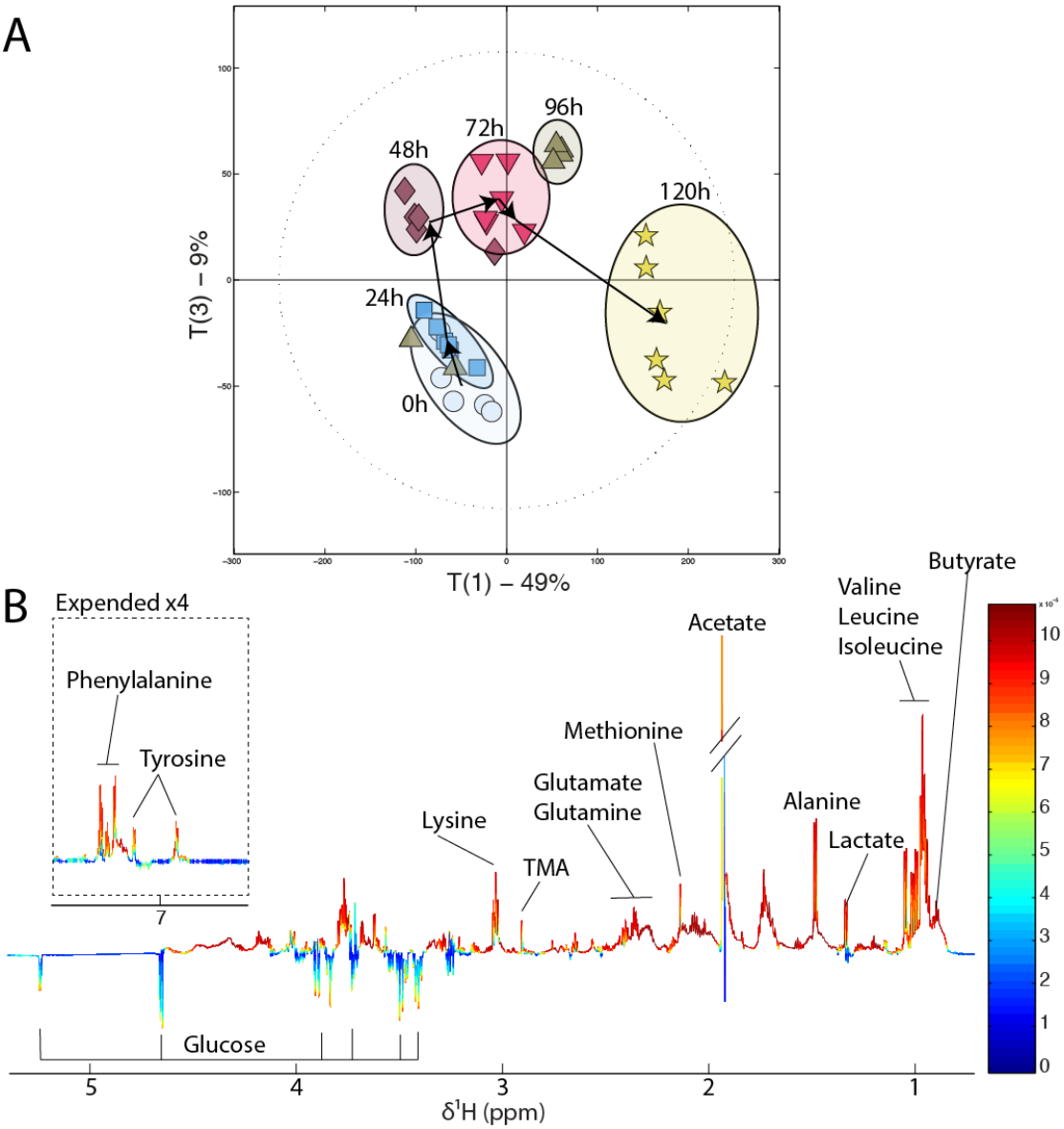

Figure 2.1: *B. pilosicoli* consumed glucose and released amino acids and fermentation products in its environment. PCA scores plot (A) and associated loadings of the first component (B). The metabolic trajectories described by the

arrows were determined by the position of the centroids calculated at each time point using the coordinate of the associated scores on the principal components.

The results obtained from *B. pilosicoli* growth in a control medium without Tiamulin™ provide new insights regarding its general metabolism. Indeed, the bacteria were able to produce lactate and acetate from glucose fermentation without secreting methanol, suggesting the use of the bifidum pathway according to the following equation: glucose → 3 acetate + 2 lactate(Gerhard 1985). However lactate was generally found in very small quantity in comparison to acetate, indicating its potential use in other metabolic reactions. Bacteria were also able to secrete butyric acid but not propionic acid. Both of these short chain fatty acids were found to be potential carbon sources for *B. pilosicoli*(Mappley et al. 2012). Bacteria also released a large number of amino acids that could be caused either by synthesis and active secretion of these amino acids, or more likely due to exogenous protein degradation. The medium contains complex proteins and *B. pilosicoli* is known to secrete complex enzymes, such as lipases that induce haemolysis, and it is reasonable to assume proteases may also be secreted since they can be produced by the bacteria(Mappley et al. 2012). This specific strain of *B. pilosicoli* was also shown to be able to use amino acids as primary carbon source[10]. However, as the bacterium favours glucose if available as primary carbon source, amino acids may only be used for protein synthesis and may become in excess in the culture medium where they accumulate.

Finally, the bacteria secreted TMA. Gut bacteria generally produce TMA from dietary L-carnitine, betaine or choline. Yet, it was not possible to detect a decrease in concentration of these compounds indicating that *B. pilosicoli* might use the three of

them independently without preferences or that the technique used was not sensitive enough to detect such variations.

2.3.2. Tiamulin™ impacts B. pilosicoli growth even at very low doses

Tiamulin™ impacted *B. pilosicoli* growth even at the lowest concentrations tested (0.008 and 0.016 µg/ml) as displayed in Figure 2.2. For these two doses, the bacterial count observed at the stationary phase was one log lower than for the control demonstrating the ability of Tiamulin™ to reduce the growth of *B. pilosicoli* at low concentrations. Up to 54 hours, growth curves of the two lowest concentrations (0.008 and 0.016µg/ml) were identical to the control (T1 and T2 on the graph) but they stopped growing shortly after and entered into the stationery phase. No bacterial growth was detected for higher Tiamulin™ concentrations (over 0.032 µg/ml) confirming its efficiency to stop bacterial proliferation. Interestingly, no gradual Tiamulin™ dose response of bacterial growth was observed. Indeed growth rates were similar for the two lowest concentrations (0.008 and 0.016 µg/ml) while higher doses induced a complete inhibition of *B. pilosicoli* growth. Decreased growth rate at such low antibiotic dose was unexpected, as previous evaluation of minimum inhibitory concentration (MIC) values for this specific strain were of 0.25 µg/ml(Pringle et al. 2012), furthermore, 10-15% of *B. pilosicoli* isolates presented MICs > 4 µg/ml(Pringle et al. 2012). Differences in the MIC values can be explained by the experimental differences. Growth curves were acquired when *B. pilosicoli* was grown in BEB media rather than on agar plates for MIC tests. The *B. pilosicoli* strain B2904 used in this study is known to have an MIC of 0.25 µg/ml(Pringle et al. 2012; Woodward et al. 2015) but showed clear inhibition of growth with concentrations

below this value. Thus, our findings confirm the previously reported observation that lower Tiamulin™ MIC values are generally found in broth compared to agar for *B. hyodysenteriae*(Rohde et al. 2004).

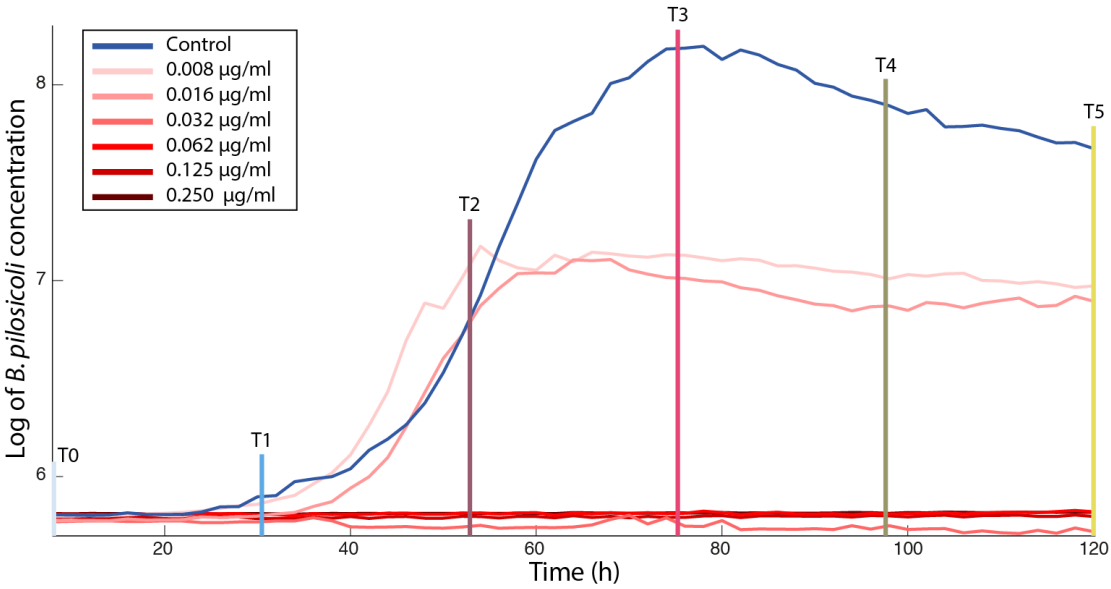

Figure 2.2: Impact of Tiamulin™ on *B. pilosicoli* growth.

2.3.3. For most concentrations, Tiamulin™ induces major metabolic shift between 72 and 96 h of growth

To gain in clarity regarding *B. pilosicoli* Tiamulin™-induced metabolic perturbation, the centroids of four chosen antibiotic concentrations (0, 0.016, 0.062 and 0.250 µg/ml) of the PCA scores (generated using all the samples) on PC1 and PC3 were plotted together (Figure 2.3A). This allowed for the evaluation of the average metabolic trajectory of the media at different antibiotic concentrations. A heat-map representing the strength of the O-PLS DA models between each Tiamulin™ concentration at all time points independently was also generated (Figure 2.3.B).

Results from Figure 2.3A shows that between 0 (T0) and 48 h (T2) the same metabolic trajectory were followed by the media, regardless of Tiamulin™

concentration, confirming that Tiamulin™ treatment does not impact bacterial metabolism in this first growth stage. This result was also assessed by the poor strength of O-PLS DA models calculated between 0 and 48 h indicating an absence of detectable metabolic variation between these conditions. Large variations in the metabolic trajectory of the media associated with antibiotic treatment occurred after 72h of growth. This result was also confirmed by the heat-map (Figure 2.3B) where the O-PLS DA model gain in "strength". These modifications of the metabolic trajectory remained until the end of the experiment (T5-120h).

This demonstrates the slow response of the bacteria to antibiotic treatment as modification of the metabolic footprint is only observed after more than 48 h of growth in presence of Tiamulin™. From these results, it seems that metabolism was stressed during the exponential phase, when bacterial division is compromised. Metabolism modification was mainly associated with increased amino acid consumption. However, as the provenance of these amino acids remains unclear this phenomenon could be explained in two different manners. Firstly, in response to antibiotic stress bacteria could use amino acids as alternative energy substrates. Secondly, *B. pilosicoli* might not be able to hydrolyse proteins present in the media because new protein synthesis, such as secreted proteases, is blocked at the ribosome. The specificity of the amino acids used indicates the first option is the most probable and that catabolite repression could be overridden to secure energy from multiple sources. This is an interesting hypothesis that needs confirmation by alternative techniques such as transcriptomics.

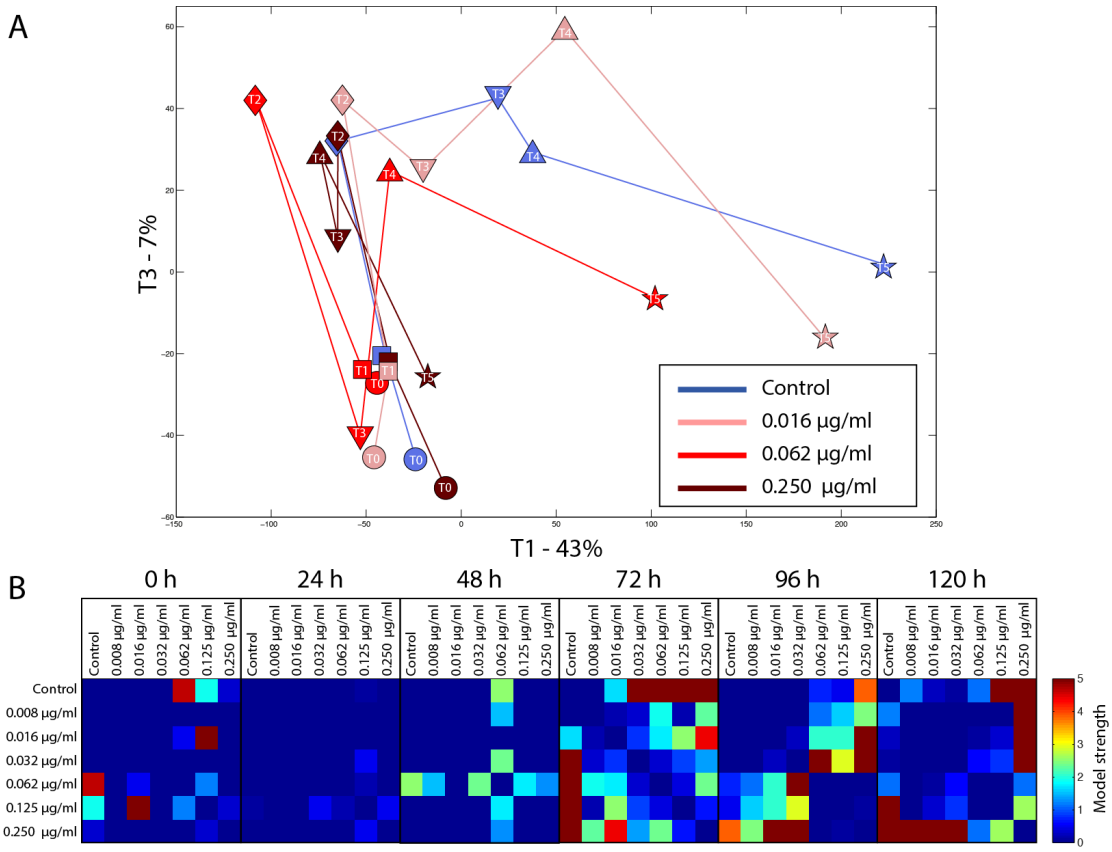

Figure 2.3: A. Tiamulin™ impacts on *B. pilosicoli* metabolism. Metabolic trajectories derived from the PCA analysis performed using all the sample population (N=288) of the study (i.e. control plus 7 Tiamulin™ dilution) on PC 1 and PC3 displaying the centroids for each time points of the control and three concentrations of Tiamulin™. B. Heat map representing the O-PLS DA model strength existing between each Tiamulin™ concentration at each time point. The values were calculated using R^2Y (the goodness of fit of the model) and Q^2Y (the goodness of prediction of the model). When Q^2Y was negative its value was brought to zero.

The metabolism of *B. pilosicoli* appears to slightly recover from all Tiamulin™ concentrations except from 0.250 µg/ml after 120h of growth. This might be due to the apparition of resistance, which is known as being a slow bacterial development process(Bock et al. 1982; Karlsson et al. 2001). The fact that *B. pilosicoli* remain viable and metabolically active without dividing despite the antibiotic treatment could partly explain the IS relapse observed in farms after Tiamulin™ intervention. Indeed it seems to arise from these results that bacteria remain viable but are not

able to divide entering therefore a dormancy stage. It is highly possible that such phenomenon occurs in the intestinal lumen, where bacteria could be inactivated in term of division but be still viable. This bacterial state might be associated with a decrease in their pathogenicity explaining the disappearance of associated symptoms. Nevertheless bacteria might remain viable but at a "dormancy" state in the intestinal lumen or animal faeces until the environment become less hostile (end of antibiotic treatment) when they can recover their pathogenic property. More details concerning the metabolic variations observed in response to each dose used are given in the followings paragraphs.

2.3.4. Tiamulin™ decrease B. pilosicoli growth without affecting its metabolism

As previously described, Tiamulin™ did not impact *B. pilosicoli* at the two lowest antibiotics doses (Figure 2.4A and B) although growth was seriously compromised (Figure 2.2). Such results indicate that modification of the metabolic trajectory is not necessarily associated with the growth phase in which the bacteria is. Moreover, bacterial concentration does not influence metabolic composition of the medium. Indeed, with 1 log more of bacteria growing in the medium, it could have been expected to observe a drastic modification of the metabolic trajectory due to increased metabolic rate. In spite of these findings, NMR-based metabonomics may not be sensitive enough to identify very subtle metabolic variations, and the use of mass spectrometry could be an interesting alternative(Romano et al. 2014).

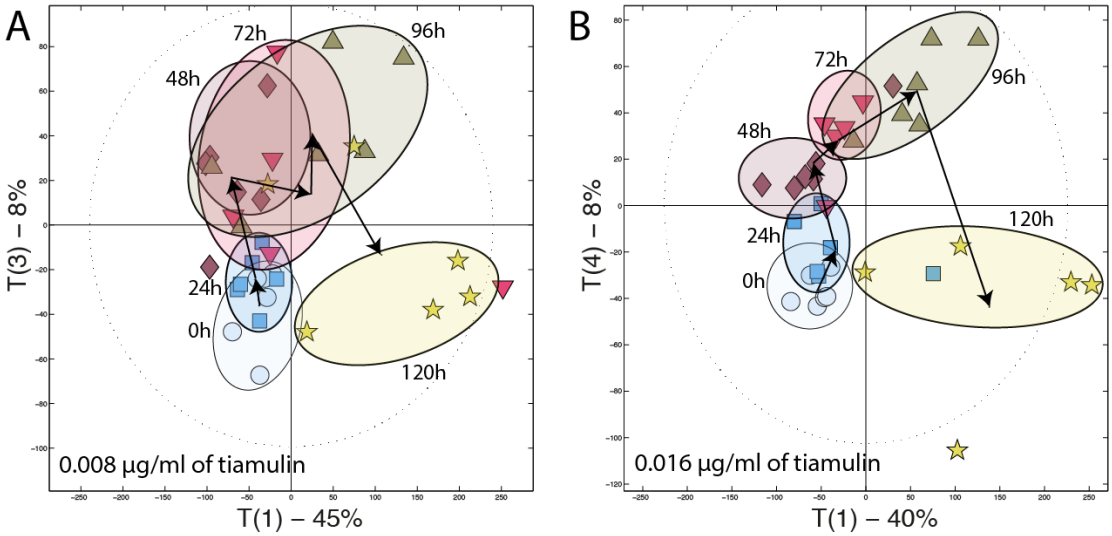

Figure 2.4: Metabolic trajectories of *B. pilosicoli* footprints in broth media for 120h at a Tiamulin™ concentration of: 0.008 µg/mL (B) and 0.016 µg/mL (C). The arrows indicate the metabolic trajectory. The metabolic trajectories described by the arrows were determined by the position of the centroids calculated at each time point using the coordinate of the associated scores on the PCs.

2.3.5. *B. pilosicoli metabolism is shifted when growth is totally inhibited by Tiamulin™*

Modifications of the metabolism of *B. pilosicoli* were observed when bacteria were exposed to 0.032 µg/ml of Tiamulin™ and above (Figure 2.5A and B). *B. pilosicoli* was still metabolically active at 0.032 µg/ml of Tiamulin™, however the metabolic trajectory was shifted in comparison to the control. Disruptions of the metabolic trajectory were associated with modifications of amino acid metabolism. A noticeable increase of tyrosine, methionine, valine, phenylalanine and lysine in to the medium from 0 to 96 h was observed. After that time, their concentration reduced, indicating consumption of these amino acids until the end of the experiment. This is the first detectable metabolic response of the bacteria in this medium to Tiamulin™ treatment.

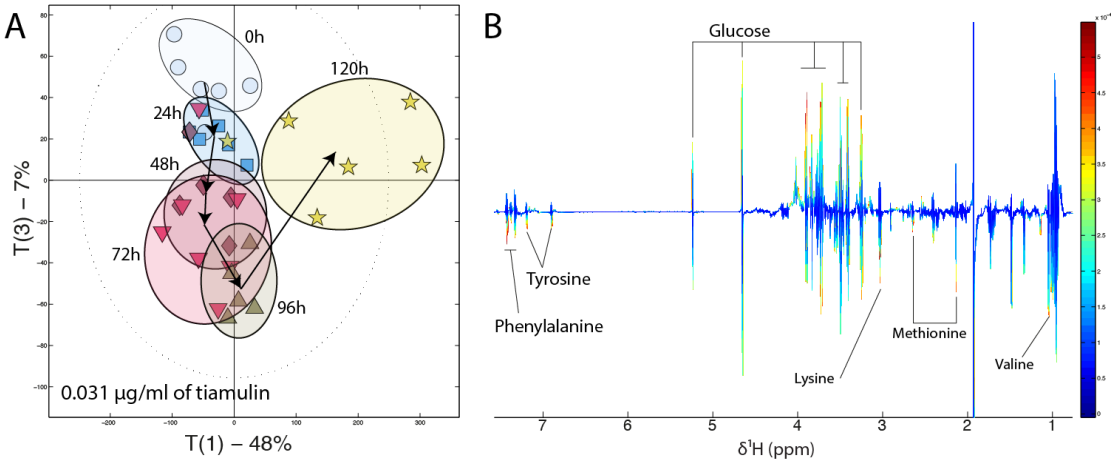

Figure 2.5: Metabolic trajectories of *B. pilosicoli* footprint in broth media for 120h at a Tiamulin™ concentration of 0.032 µg.ml on principal component 1 and 3 (A) (B). The metabolic trajectories described by the arrows were determined by the position of the centroids calculated at each time point using the coordinate of the associated scores on the PCs.

Higher doses of Tiamulin™ (0.062 and 0.125 µg/ml) induced similar responses to those observed at 0.032 µg/ml (Figure 2.6A and B) with amino acid metabolism disturbed to a greater extent.

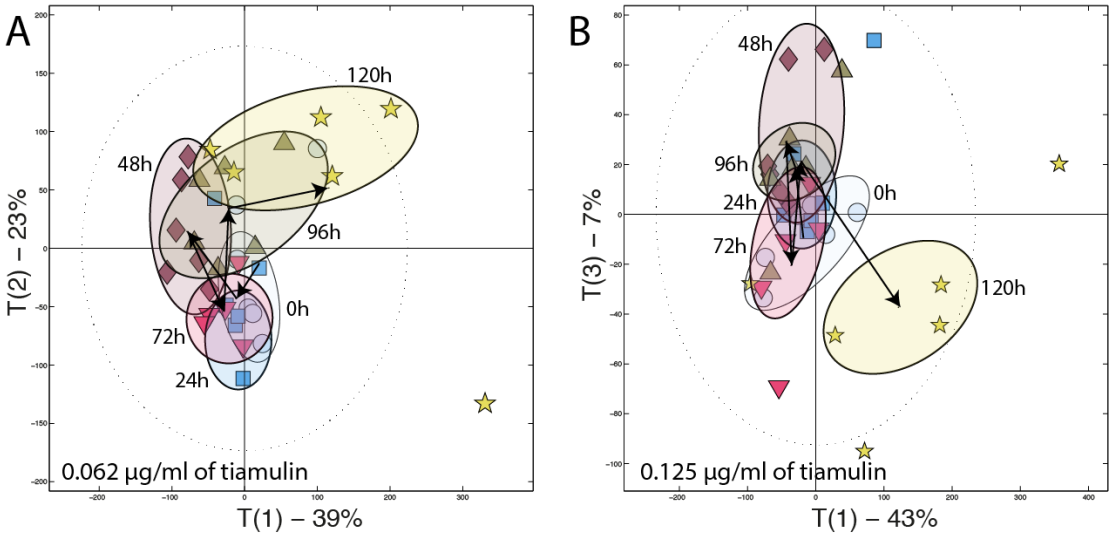

Figure 2.6: Metabolic trajectories of *B. pilosicoli* footprint in broth media for 120h at a Tiamulin™ concentration of: 0.062 µg/ml (A) and 0.125 µg/ml (B). The arrows indicate metabolic trajectory. The metabolic trajectories described by the arrows were determined by the position of the centroids calculated at each time point using the coordinate of the associated scores on the PCs.

2.3.6. Metabolic signature of death of B. pilosicoli occurs after 120h when incubated with 0.25 µg/ml of Tiamulin™

At the maximum dose tested (0.250 µg/ml) the metabolic trajectory observed for the media was drastically modified in comparison to those described previously (Figure 2.6). This time, the metabolic trajectory followed a circular shape where the scores of the samples collected after 120h of bacterial growth were clustered to the one observed at T0, indicating metabolic similarity with the initial time. Once again, amino acids were released into the medium as well as butyrate and *myo*-inositol.

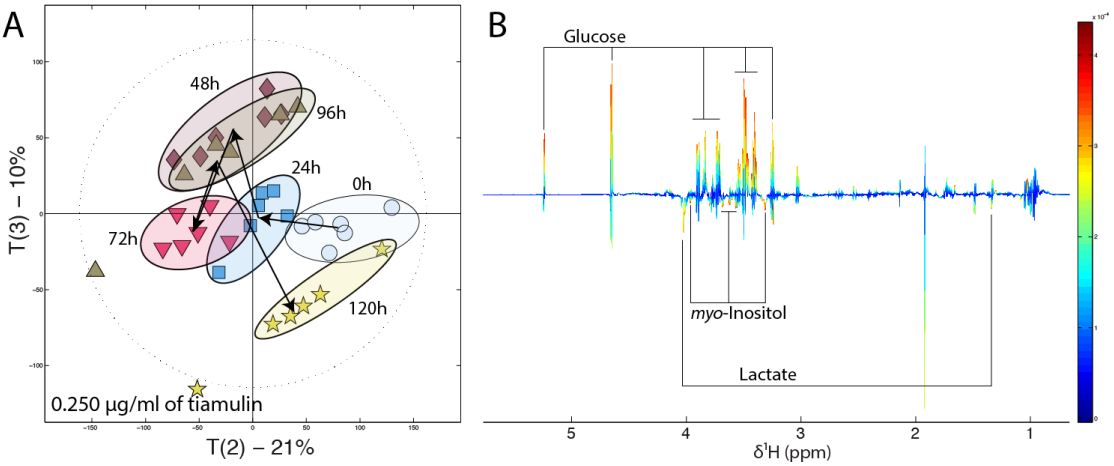

Figure 2.7: Metabolic trajectories of *B. pilosicoli* footprint in broth media for 120h at a Tiamulin™ concentration of 0.250 µg/ml. The arrows indicate the metabolic trajectory. The metabolic trajectories described by the arrows were determined by the position of the centroids calculated at each time point using the coordinate on the associated scores on the PCs.

2.4. Conclusion

This work gave a clearer understanding of *B. pilosicoli* metabolism under optimum, growth conditions, including indication regarding favoured fermentation pathways and amino acids metabolism. It supports the fact that Tiamulin™ can inhibit

efficiently bacterial growth at low concentrations. However, it was surprising to observe that Tiamulin™ could impact *B. pilosicoli* growth without influencing its metabolism. It also reveals that the bacteria try to maintain metabolic homeostasis despite an obvious stress visible on the growth curve, demonstrating that in response to xenobiotic stress, bacterial division is the first mechanism to be suspended. It appears from the results that metabolism is shifted mainly in what should be the exponential phase (a moment of intense cellular activity where bacteria divide rapidly). The metabolic shift observed for high doses of Tiamulin™ at this precise time point witness the stress encounter by the bacteria that are not able to divide properly due to antibiotic treatment. This demonstrates that Tiamulin™ present a good solution against AIS outbreaks, as it is able to significantly reduce or eradicate bacterial growth. However, the metabolic activity of *B. pilosicoli* post antibiotic treatment showed that bacteria were still alive even if growth was compromised unless high antibiotic doses were applied (0.250 µg/ml). Such findings suggest that measurement of bacterial activity might be needed to asses antibiotic efficiency.

2.5. Literature

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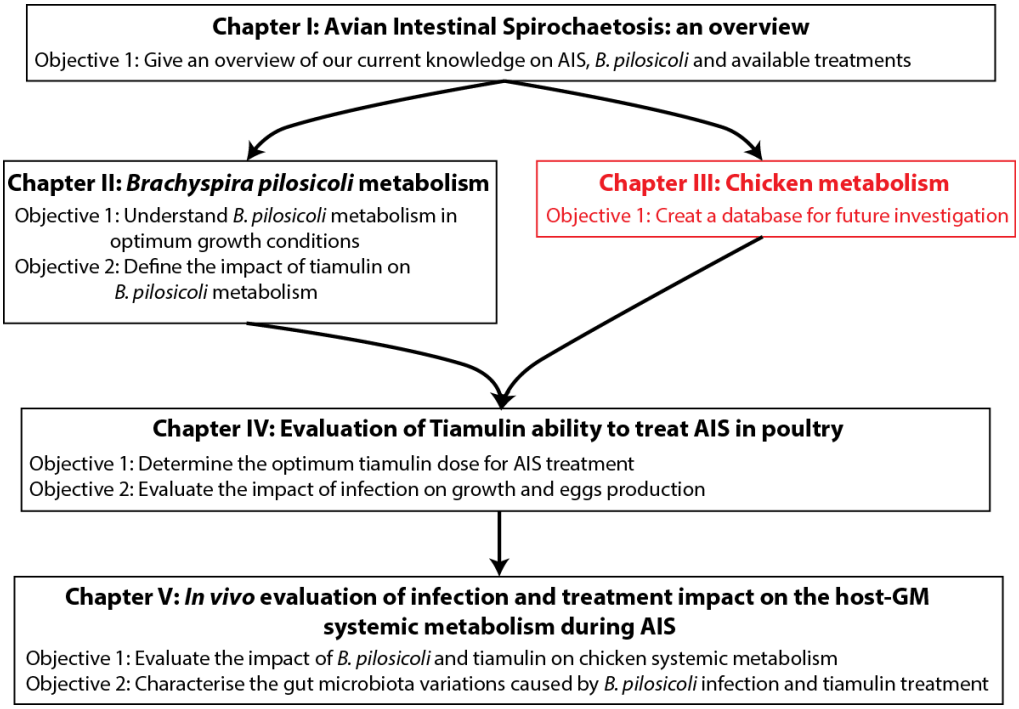
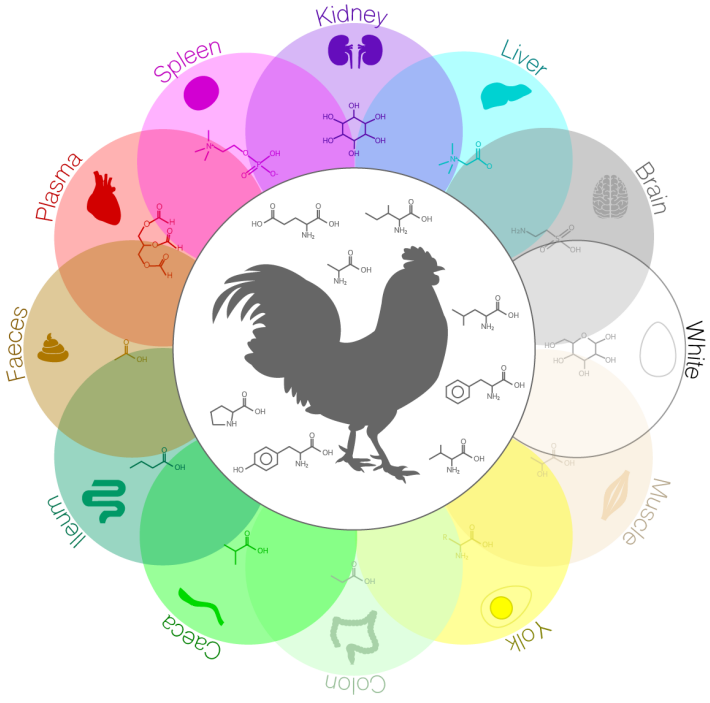
Define host metabolism:

After investigating the pathogen metabolism and its reaction to antibiotic treatment, the next step was to define the host metabolome (chicken). A metabolic atlas of chicken's tissues and biofluids proceed using high resolution NMR spectroscopy was published in the journal Metabolomics.





Chapter 3: NMR-based metabolic characterisation of chicken tissues and biofluids: a model for avian research

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Abbreviation title: Chicken metabolic atlas

Abstract

Introduction: Poultry is one of the most consumed meat in the world; nevertheless, industry is still looking for ways to improve animal welfare and productivity. It is therefore essential to understand the metabolic response of chicken to new feed formulas, various supplements, infection and treatments.

Objectives: As a basis for future research investigating the impact of diet and infections on chicken's metabolism (that should lead to improved treatment development), we established a high-resolution proton nuclear magnetic resonance (NMR)-based metabolic atlas of the healthy chicken (*Gallus gallus*).

Methods: Metabolic extractions were performed previous to 1H-NMR and 2D NMR spectra acquisition on twelve biological matrices: liver, kidney, spleen, plasma, egg yolk and white, colon, ceca, fecal water, ileum, pectoral muscle and brain of n=6 chickens. Metabolic profiles were then exhaustively characterized.

Results: Nearly 80 metabolites were identified in twelve biological matrices that were liver, kidney, spleen, plasma, egg yolk and white, colon, ceca, fecal water, ileum, pectoral muscle and brain. Biological matrices cross-comparison allowed the identification of eight-core metabolites and to determine metabolic variations between and within each section.

Conclusion: This work constitutes a database for future NMR-based metabolomic investigation in relation to avian production and health.

3.1. Introduction

The Food and Agriculture Organization of the United Nation (FAOSTAT: http://www.fao.org/home/en/), calculated that approximately 22 billion chickens were produced commercially worldwide in 2012, China being the main producer with over 5 billion birds. A major production issue in commercial systems is animal density that is favourable for rapid spread of disease. Most chicks receive a cocktail of vaccines at hatch or even *in ovo,* but remain susceptible to typical production related endemic disease and other food borne zoonosis such as *Salmonella* or *Campylobacter*(Boer and Hahné 1990; Dufrenne et al. 2001). All infections represent a large potential economic loss for the chicken industry and is one of the main cause of meat contamination by food born pathogens(Tessari et al. 2009; White et al. 1997). Vaccines and antibiotics are commonly used to tackle such infections in order to stop spread and symptoms and minimize the associated cost. With regard to antibiotic use, increasing antimicrobial resistance has been observed in animal farming and has become a major concern in recent decades, stimulating the development of alternative treatments(McEwen and Fedorka-Cray 2002; Casewell et al. 2003). Therefore, in the interest of improving animal welfare and product quality, new more specific treatments are needed. Finally in the same purpose, attention is brought towards improving animal feeding. Chicken feed generally consists of a mix of grounded grains (corn, rice, wheat) and proteins most often from soya beans. However, the grain/protein ratio is different for egg laying and meat production. There are numerous added supplements including certain amino acids, minerals and

oils. In addition feed is supplemented with vitamins, A, D3 and riboflavine and mineral salts.

Nutrimetabonomics has been developed in order to evaluate the impact of nutrition and food on host systemic metabolism(Claus and Swann 2013), this is also a useful approach to understand dietary impacts on productivity as well as host-pathogen-drug interactions. Multi-'omics' approaches help to gain better understanding of host-pathogen-drug interactions(Nicholson et al. 2004; McDermott et al. 2011). This consists in using together genomic (study of the genome)(Klug et al. 2012), transcriptomic (study of gene expression)(Bernot 2004), proteomic (studying the proteome)(Blackstock and Weir 1999) and metabonomic (studying the metabolome). Chicken genomic(Burta et al. 1995), transcriptomic(Murphy 2009) and proteomic(Doherty et al. 2004; Mann 2007; Mann and Mann 2008) data have already been published but, to date, none of them have reported a detailed analysis of the chicken metabolome. Metabonomic has been mainly developed for clinical and nutritional (Nutrimetabonomics) research(Nicholson et al. 2002; Holmes et al. 2011; Solanky et al. 2003) and allows to look at quantitative and qualitative metabolic variations caused by genetic mutation or environmental stress in a sample set(Nicholson and Wilson 2003).

This paper presents the annotated NMR metabolic profiles of twelve chicken biological matrices to serve as reference for future studies. We selected four major biological matrices for the host systemic metabolism: liver, kidney, spleen and plasma. In addition, samples from the digestive system, including: colon, caeca, ileum and faecal water were analysed. Three relevant to industrial production and

could be used to evaluate or assess product quality: egg (yolk and white) and pectoral muscle. Finally brain cortex was also analysed.

3.2. Material and methods

3.2.1. Animal husbandry and sample collection

Six 15-16 weeks of age NovoGen Brown commercial laying hens (*Gallus gallus*) were purchased from the Animal and Plant Health Agency (APHA) in Surrey. Animal husbandry conformed to animal Home Office licence (PPL 70/7249) and all procedures were performed in compliance with the Animals Scientific Procedures Act, 1986. animals were provided with food (give details of manufacturer) and water *ad libitum*. After one week of acclimatization (see food composition in supplement), animals of 15 weeks of age and weighing on average 1000 g (n=6) were sacrificed by cervical dislocation. Tissues were sampled aseptically immediately after euthanasia and snap frozen in liquid nitrogen (-176°C) and then transferred at -80°C for storage until analysis. The following tissues were sampled: liver, the end of the right lobe, the right kidney, half longitudinal cut of the spleen, the frontal right lobe of the cortex, the middle of the external surface of the left pectoral muscle. Digestive track samples were washed with PBS before freezing and faeces were collected directly by emptying the totality of the colon. One cm of proximal colon was sampled and 2 cm of the end on the left caecum were taken, 2 cm of Ileum were sampled approximately 3 cm before the caecum. Plasma was sampled by *post-mortem* cardiac puncture. Egg yolk and white (n=6) were sampled from randomly chosen

eggs laid by older animals that had just come into lay (18 week old) from the same cohort of birds on the same diet and within the same environment.

3.2.2. Sample preparation

Sample biopsies were homogenised using a bead beater (Qiagen, TissueLyser LT) at a frequency of 1/25 for 10 min for the digestive track tissue and the muscle and 3 min for the liver, the spleen, the kidney and the cortex using glass Beads. For this step, 0.1 g of tissue was homogenised in 1 mL Of a 3:1 (v/v) MeOH/H_2O solution for polar metabolite extraction. After centrifugation for 10 min at 12 000 x g, 0.9 mL of supernatant was dried in speed vacuum for 4.5h at 45°C and resuspended in 600 µL of phosphate buffer 0.2M containing 90% of D_2O and 10% of H_2O plus 0.01% of sodium 3-(tri-methylsilyl)-propionate-2,2,3,3-d4 (TSP) for NMR reference. Samples were then transferred into 5 mm NMR tubes for analysis. Egg yolk and white were prepared following the same protocol. Plasma samples were mixed at a 2:1 (v/v) ratio with phosphate saline buffer with 90% D_2O, of which, 500 µL were then transferred into 5 mm NMR tubes. Faecal samples were extracted by mixing 0.1 g of faeces in 1 mL of phosphate buffer (plus TSP) with a Bead beater for 3 min using glass beads at the frequency of 1/25. Samples were centrifuged at 12 000 x g for 10 min in a refrigerated centrifuge and supernatant was kept at 4°C overnight to let urea precipitate. After centrifugation for 5 min at 12000 x g, the supernatant was transferred into 5 mm NMR tubes.

3.2.3. NMR spectra acquisition

For all polar tissue extracts, egg yolk and faeces, ^{1}H-NMR spectra were acquired on a Bruker Avance DRX spectrometer operating at 700.19 MHz and equipped with a CryoProbe^{TM} from the same manufacturer. A standard 1-dimentional noesypr1D pulse sequence (noesypr1d – 90 degree pulse length of 7.7 µs and total acquisition time 3.34 s-) with water presaturation applied during relaxation delay (2 s) and a mixing time of 100 ms at 298K was used. Plasma and egg white ^{1}H NMR spectra were acquired using a Carr-Purcell-Meiboom-Gill (CPMG)(Meiboom and Gill 1958) pulse sequence to limit signal contribution from albumin and ovalbumin respectively. For each sample 256 scans (16 dummy scans) were recorded into 64K data points over a spectra width of 12019 Hz as for noesypr1D. 1H-1H COSY and 1H-13C HSQC were obtained for each biological matrix on one representative sample for metabolite identification purposes.

3.2.4. Data processing and analysis

Prior to Fourier transformation, an exponential window with line broadening of 0.3 Hz was applied to each 1D NMR spectrum. All spectra were phased manually and baseline corrected on MestReNova software (2013 Mestrelab Research S.L.). Spectral calibration was performed using TSP (δ 0.00) for all tissues and yolk samples, lactate (δ 1.33) for plasma and the H1 proton of α-glucose (δ 5.23) for egg white spectra. One representative spectrum was selected from each biological matrix for illustration purpose and peak assignments. For these spectra signal suppression was done at δ4.84 during FID processing to attenuate water resonance.

Signal assignment and metabolite identification was done using an in house standard database, published literature(Merrifield et al. 2011; Claus et al. 2008; Nicholson et al. 1995) and online public databases: the Human Metabolome Data Base (HMDB, http://www.hmdb.ca) and the Magnetic Resonance Data Bank (BMRB, http://www.bmrb.wisc.edu). 2D NMR plots were used to confirm metabolite assignment. Indeed, COSY 1H-1H spectra allow to see cross peaks occurring when hydrogen are attached to two neighbour carbons. Therefore, when two ^{1}H are attached to conterminous carbons, a cross peak appears on the 2D COSY spectra allowing confirmation of metabolites presence. This method cannot be used for molecules such as acetate that only present a detectable CH_3 using NMR. In such assessed presence of the metabolite was an assessed using a standard.

3.2.5. Statistical analysis

For statistical analysis, spectra were imported into MatLab (version R2013b, The MathsWorks inc.) and residual signal water region was removed (δ4.70-5.10) before normalisation (to account for variations in sample size and distribution) using a median-base probabilistic quotient method(Dieterle et al. 2006). Principal component analysis (PCA) was performed using algorithms provided by the Korrigan toolbox (Korrigan Sciences Ltd) in order to evaluate dominant sources of variation between biological matrices. Venn diagrams were also created using online Venny software (Venny 2.1 http://bioinfogp.cnb.csic.es/tools/venny/).

3.3. Results and Discussion

Systemic Metabolic characterisation of several mammals, including rodents(Claus et al. 2008; Griffin et al. 2000; Martin et al. 2007; Martin et al. 2009), pig(Merrifield et al. 2011), humans(Ndagijimana et al. 2009; Holmes et al. 1997; Nicholson et al. 1995) and horse(Escalona et al. 2014) is available but, to date, no overview of any bird metabolic phenotype has been published despite their industrial significance and source of worldwide protein for man. This work gives a summary of the metabolic composition of twelve biological matrices detectable by NMR spectrometry in order to be used for future NMR-based metabonomics research.

Representative ^{1}H-NMR spectra of the twelve biological matrices investigated in this study are presented in Figures 3.1, 3.2, 3.3 and 3.4 to offer an overview of the chicken metabolome. Organs and biofluids related to: the general metabolism (liver, kidney, plasma and spleen –Figure 3.1-), product destined to consumption (egg yolk and white and muscle –Figure 3.2-), the frontal cortex (Figure 3.3) and the lower digestive track (colon, caeca ileum and faeces –Figure 3.4-). The numerical key for annotation is presented in Table 1 and complementary information provided by 2D spectroscopy for peak assignment is given in Figures 3.5 and 3.6.

3.3.1. Matrix characterisation

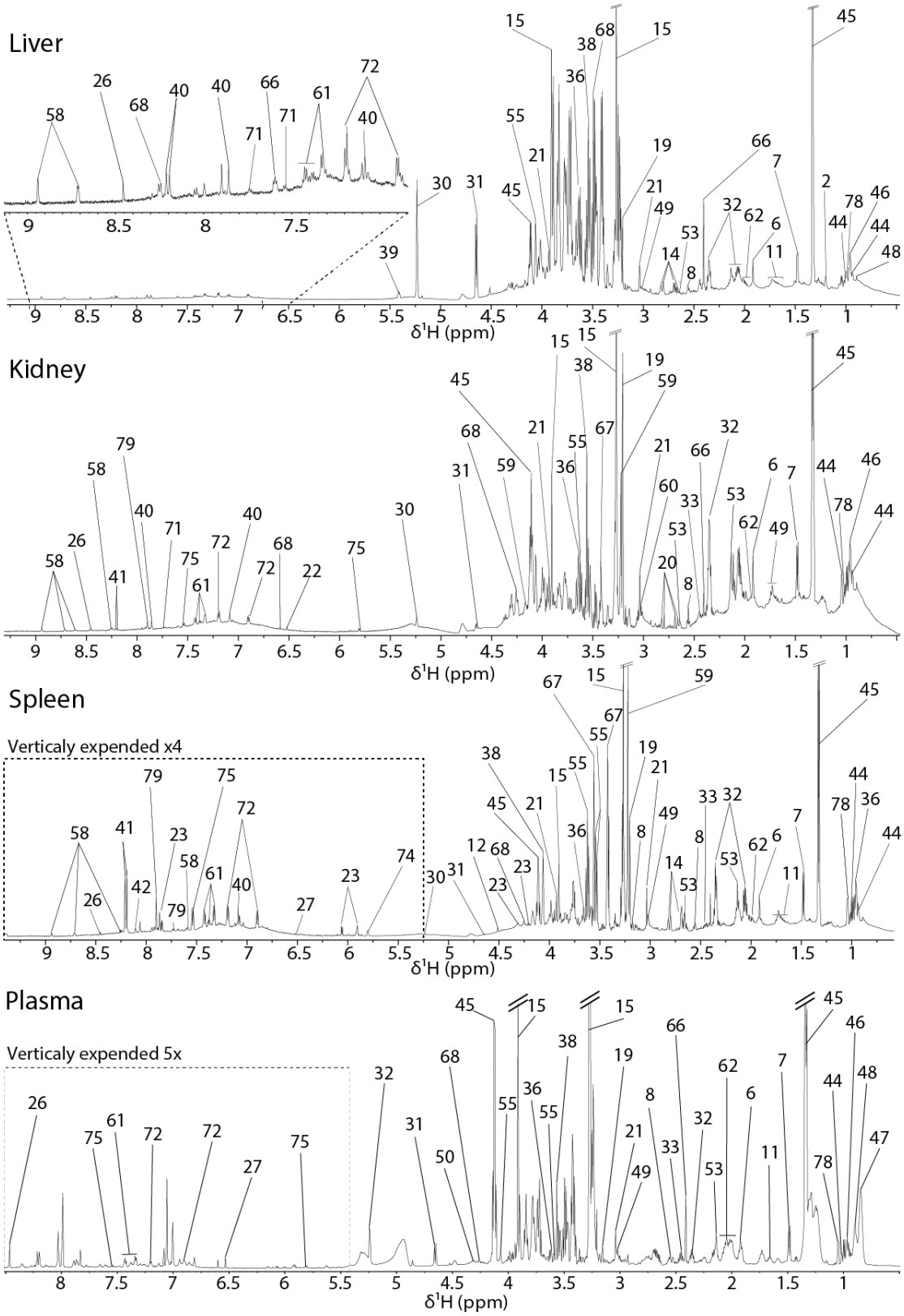

Figure 3.1: Partially assigned 700 MHz 1D NMR spectra of chicken liver, kidney, spleen and plasma. Numerical key described in Table 3.1.

Hepatic metabolic profile (figure 3.1) was caracterised by high levels of betaine, lactate and glucose. This was the only biological matrix where it was possible to detect glutathione (in its oxidised form since the total pool of glutathione becomes oxidised during tissue extraction), in very small quantities, in contrast to what is commonly found in mammalian hepatic metabolic profiles(Martin et al. 2007; Waters et al. 2002; Duarte et al. 2005; Claus et al. 2008).

Similarly, kidney metabolic profiles were rich in lactate, which is consistent with the important role of the kidney in energy metabolism. In addition, betaine and creatine were found in very high concentrations. Betaine is an important osmolyte in the kidney and its concentration generally increases in case of water privation. In birds the most important kidney osmolytes are *myo*-inositol, betaine, glycerophosphorylcholine, and taurine(Lien et al. 1993) that were all detected using ^{1}H-NMR.

The metabolic profile of the spleen was characterized by high levels of betaine, *myo*-inositol and phosphocholine. This was one of the few matrices that did not possess any unique metabolic feature, as all the metabolites detectable by NMR spectrometry were shared with liver, kidney and plasma. This similarity may be explained by the high vascularization of this tissue. In particular, it shared with plasma high lactate and betaine. Unique to plasma metabolic fingerprints were large resonances from lipoproteins, mainly HDL and VLDL. It was also possible to see high lactate, glucose and betaine levels. The metabolic profile was similar to liver, kidney

and spleen, but it was the only matrix where it was possible to identify malate, involved in the citric acid cycle.

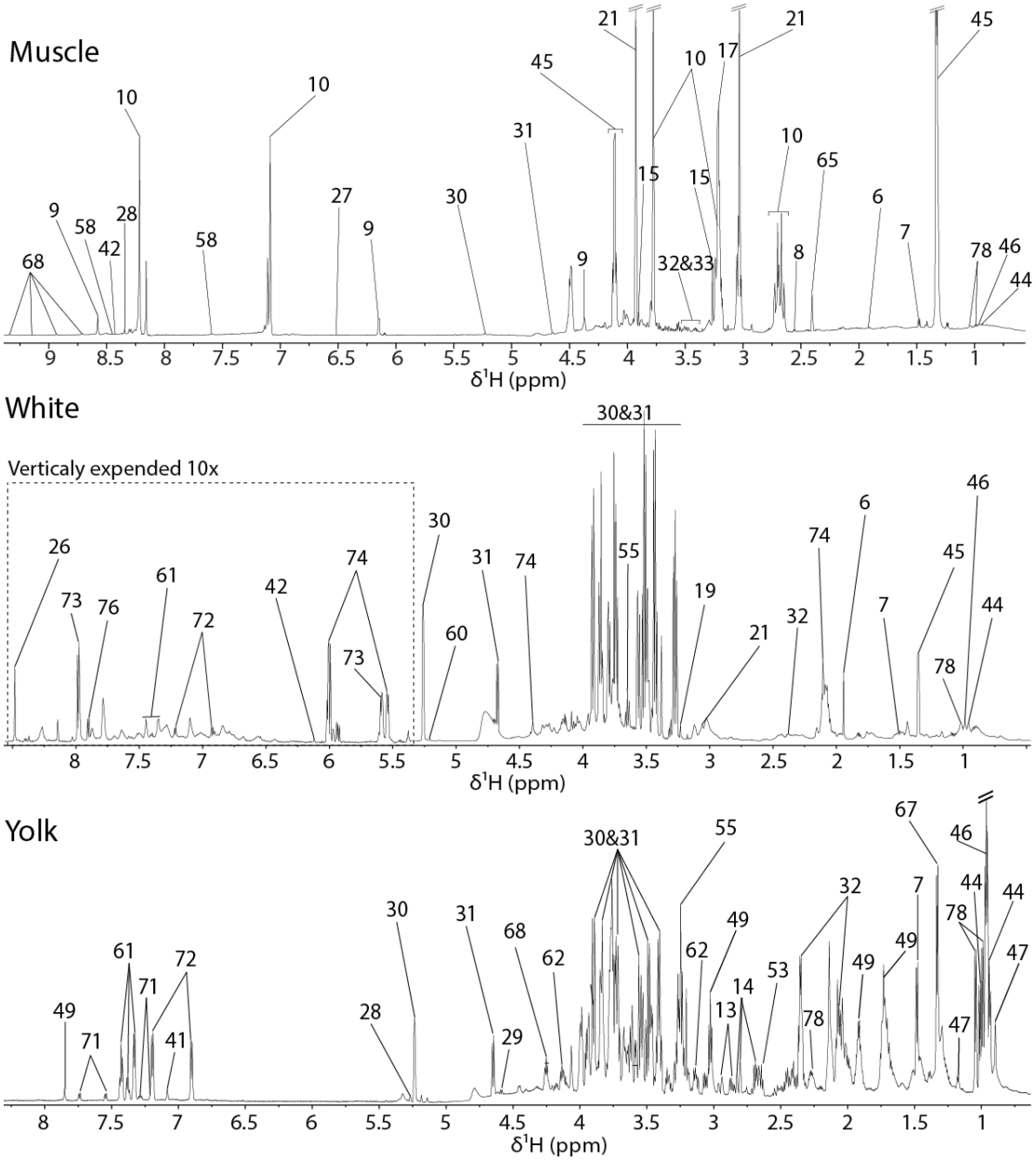

Figure 3.2: Partially assigned 700 MHz 1D NMR spectra of chicken muscle, egg white and yolk. Numerical key described in Table 3.1. In the figure, white egg white and egg yolk

The pectoral muscle presented the most distinctive metabolic features in respect to the other tissue type samples, with only twenty-three identifiable metabolites. Three

metabolites were in noticeably high concentration with: anserine, creatine and lactate. We only detected AMP in muscle. Due to its pKa close to 7 anserine is a very good buffer that maintain muscle pH neutrality(Boldyrev et al. 2013). The ability of anserine to maintain a certain pH in the muscle is known to increase the rate of glycolysis(Davey 1960). It is also a well-known antioxidant(Kohen et al. 1988), playing an important role during muscle contraction.

The metabolic profile of egg white had high glucose content and presented only twenty-three detectable metabolites. This was not surprising knowing that egg white is relatively poor in micronutrient and is mainly constituted of water (88%), protein (10%) and less that 1% of carbohydrates(Reserves 2007). Egg nutritive values for embryo development are mainly attributed to these proteins(Reserves 2007). It was also the only matrix where we could detect glucose derived molecules, such as uridine diphosphate glucose (UDPG) involved in embryo retina development(Dreyfus et al. 1975) and UDP-N acetyl glucosamine (UDP-GlcNAC) as previously described by Donovan *et al*(Donovan et al. 1967) that can be associated to muscle expansion(Ullrich et al. 1981). UDPG is involved in polysaccharide synthesis and UDP-GlcNAC is related to glycosaminoglycan, proteoglycan and glycolipid anabolism but nothing has been published yet on this matter.

In contrast, Yolk polar phase metabolic profile was featured by by amino acids and carbohydrate such as glucose and galactose. All amino acids essential for protein synthesis but cysteine (that can be generated from methionine or serine) were detectable in the yolk as well as residual lipids that constitute 66% of yolk dry

matter(Reserves 2007). No particularly distinctive metabolites were observed in the yolk.

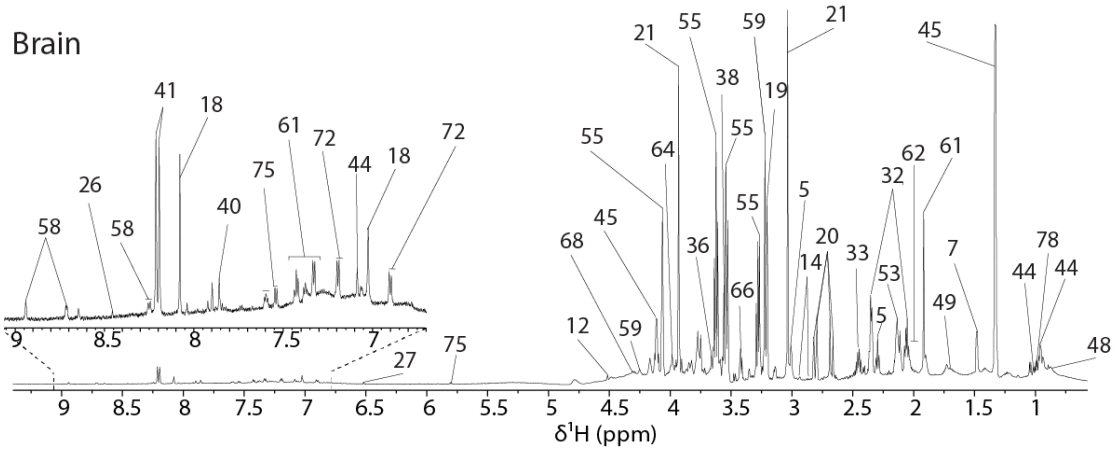

Figure 3.3: representative 700 MHz proton NMR spectrum of chicken brain cortex. The Numerical key is described in Table 3.1.

The metabolic profile of the brain cortex presented a high content in *myo*-inositol, creatine, glutamate, taurine and 4-aminobutyrate (GABA). Carnosine was also detected, which is a known brain antioxidant(Kohen et al. 1988). Surprisingly in contrast with muscle, it was not possible to detect anserine, which has been reported to be present in birds central nervous system(Biffo et al. 1990).

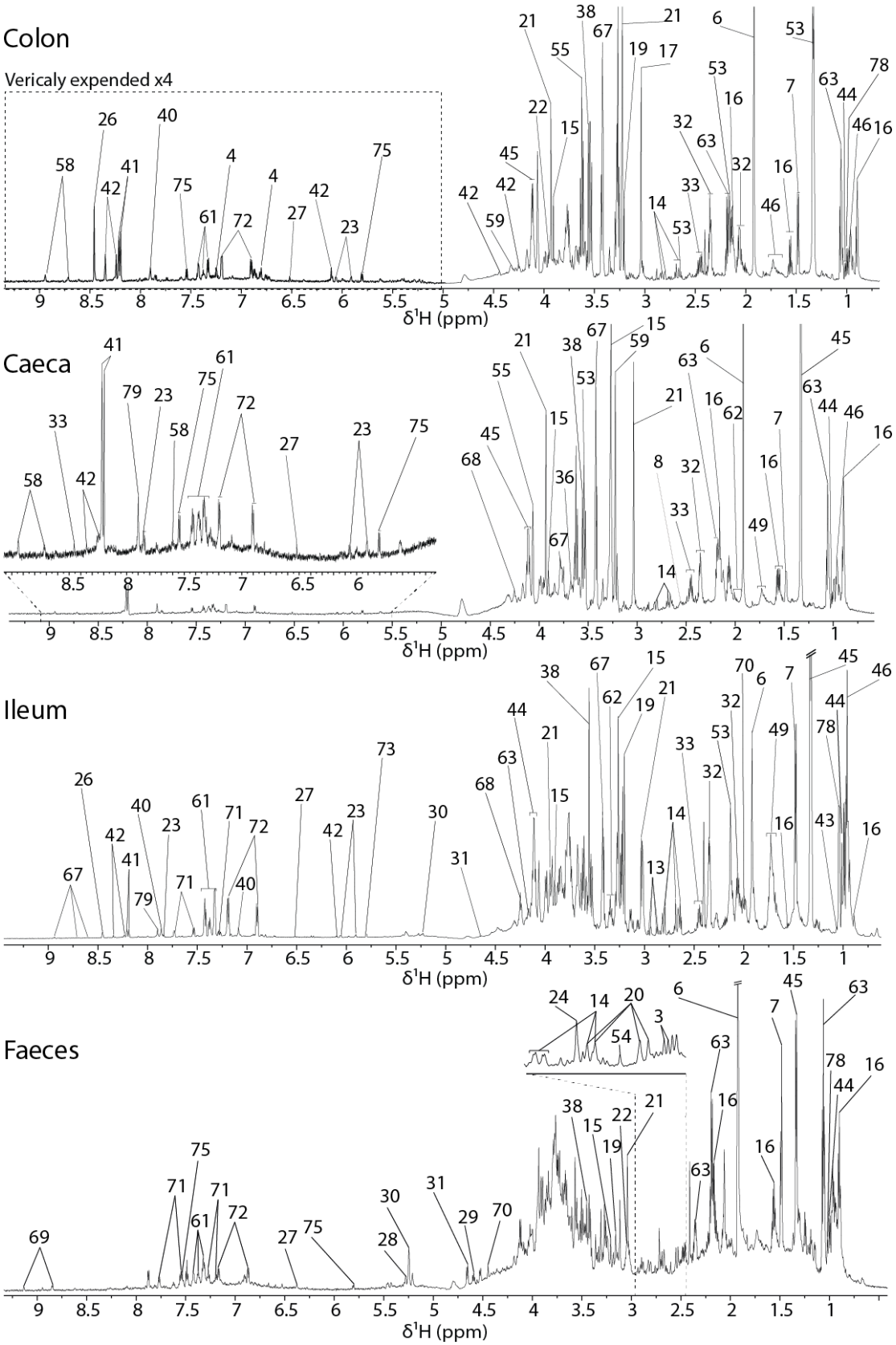

Figure 3.4: Partially assigned 700 MHz 1D NMR spectra of chicken colon, caecum, ileum and faeces. The Numerical key is described in Table 3.1.

The metabolic profiles of gastrointestinal segments were characterised by the presence of amino acids and SCFAs. Distinctive features of the ileum was the presence of glucose. Furthermore, the aromatic region was rich in phenylalanine and tyrosine in comparison to colon and caeca. This tissue did not present any unique metabolic feature. The metabolic profile of the caeca contained short chain fatty acids and amino acids composition. It was also possible to detect isobutyrate a product of amino acid degradation by gut bacteria. A very high level of o-phosphocholine related to immunologic responses(Wiens et al. 2003) to pneumococcal infection was observed in this tissue. The metabolic profile of the colon was high in short chain fatty acids (acetate, propionate and butyrate) and amino acids (alanine, aspartate, glutamate, glutamine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine and valine). It was the only tissue where we detected 3-hydroxyphenylacetate. Unlike previously published results for rodents(Claus et al. 2008), glucose resonances were not visible in the colon, despite its presence in faeces. Colon was the digestive system related matrix presenting the poorest metabolic diversity with thirty-six detectable metabolites. Finally, in birds, faeces also contain urine since digestive and urinary systems share the same portal (the cloaca). Therefore, it was not surprising to observe forty-three metabolites, of which only ten of them pertained exclusively to faeces: 2-hydroxybutyrate, 3-hydroxyisobutyrate, arabinose, benzoate, dimethylamine, methylamine, N-acteylglucosamine, N-acetyltyrosine and trigoneline.

Table 3.1: ^{1}H assignment for identified metabolites and tissue/biofluid. Legend: L, liver; K, kidney; S, pleen; B, cortex; M, pectoral muscle; Ce, ceca; Co, colon; I, ileum; F, feces; P, plasma; W, egg white; Y, egg yolk.

	Metabolite	Assignement	Matrix
1	2-Hydroxybutyrate	CH_3 0.90 t, CH_2 1.70 m, CH 4.0 dd	F
2	3-Hydroxybutyrate	CH_3 1,19 d, 1/2CH_2 2.30 dd, 1/2CH_2 2.39 dd, CH 4.14 m	L
3	3-Hydroxyisobutyrate	CH_3 1.05 d, CH 2.48 m, 1/2CH_2 3.53 dd, 1/2CH_2 3.70 dd	F
4	3-Hydroxyphenylacetate	CH_2COOH 3.47 s, C4H 6.78 m, C6H 6.80 m, C2H 6.85 m, C3H 7.24 t	Co
5	4-Aminobutyrate	βCH_2 1.88 m, αCH_2 2.29 t, γCH_2 3.01 t	B
6	Acetate	CH_3 1.92 s	L, K, S, B, M, Ce, Co, I ,F, P, W
7	Alanine	βCH_3 1.46 d, αCH 3.78 q	L, K, S, B, M, Ce, Co, I ,F, P, Y, W
8	β-Alanine	CH_2COOH 2.56 t, N-CH_2 3.19 t	L, K, S, M, Ce, I, P
9	AMP	P-CH_2 4.01 m, C1H 4.36 m, C2H 4.50 q, C3H 4.79 t, C4H 6.12 d, C8H 8.25 s, C5H 8.58 s	M
10	Anserine	βCH_2 2.68 m, 1/2δCH_2 3.03 dd, 1/2δCH_2 3.21 dd, αCH_2 3.22 m, CH_3 3.76 s, γCH_2 4.48 m, CH 7.07 s, N-CH 8.20 s	M
11	Arginine	γCH_2 1.66 m, βCH_2 1.91 m, δCH_2 3.27 t, αCH 3.77 t	L, S, P, Y, W
12	Ascorbate	CH_2 3.73 ddd, CH 4.01 d, C5 4.51 d	S, B, P
13	Asparagine	1/2βCH_2 2.86 dd, 1/2βCH_2 2.96 dd, αCH 4.00 dd	L, S, B, Ce, I, Y
14	Aspartate	1/2βCH_2 2.68 dd, 1/2βCH_2 2.82 dd, αCH 3.91 dd	L, S, Ce, Co, I, F, P, Y
15	Betaine	N-(CH_3)3 3.37 s, CH_2 3.93 s	L, K, S, B, M, Ce, Co, I, F, P, Y
16	Butyrate	CH_3 0.88 t, βCH_2 1.55 m, αCH_2 2.15 t	Ce, Co, I, F
17	Carnitine	αCH_2 2.43 m, N-(CH_3)$_3$ 3.21 s, γCH_2 3.42 m, βCH 4.56 m	B
18	Carnosine	βCH_2 2.67 m, 1/2δCH_2 3.03 dd, 1/2δCH_2 3.16 dd, αCH_2 3.22 m, γCH_2 4.46 m, CH 7.08 s, N-CH s	B, M
19	Choline	N-(CH_3)$_3$ 3.22 s, βCH_2 3.53 dd, αCH_2 4.06 t	L, K, S, B, Ce, Co, I, F, P, Y, W
20	Citrate	1/2γCH_2 2.55 d, 1/2γCH_2 2.70 d	K, B, I, F, Y
21	Creatine	N-CH_3 3.03 s, N-CH_2 3.94 s	L, K, S, B, M, Ce, Co, I ,F, P, W
22	Creatinine	N-CH_3 3.05 s, N-CH_2 4.06 s	K, Ce, Co, I, F, P
23	Cysteine	βCH_2 3.03 dd, αCH_2 3.97 t	S, Ce, Co, I, P
24	Dimethylamine	CH_3 2.72 s	F
25	Ethanolamine	CH_2NH_2 3.13 t, CH_2COH 3.83 t	B, I
26	Formate	HCOOH 8.46 s	L, K, S, B, Ce, Co, I, F, P, W
27	Fumarate	HCOOH 6.51 s	K, S, B, M, Ce, Co, I, P, Y
28	α-Galactose	C6H 3.74 m, C2H 3.80 m, C3H 3.84 m, C4H 3.98 m, C5H 4.07m, C1H 5.26 d	F, Y
29	β-Galactose	C2H 3.48 m, C3H 3.63 m, C5H 3.69 m, C6H2 3.74 m, C4H 3.92 m, C1H 4.57 d	F, Y
30	α-Glucose	C4H 3.42 m, C2H 3.54 m, CH3 3.72 m, 1/2C6H2 3.73 m, 1/2C6H2 3.77 m, C5H 3.87m, C1H 5.23 d	L, K, S, M, F, P, Y, W
31	β-Glucose	C2H 3.25 m, C4H 3.49 m, C5H 3.49 m, C3H 3.50 m, 1/2C6H2 3.88 m, 1/2C6H2 3.91 m, C1H 4.66 d	L, K, S, M, F, P, Y, W
32	Glutamate	βCH_2 2.02 m, γCH_2 2.34 m, αCH 3.76 dd	L, K, S, B, M, Ce, Co, I ,F, P, Y, W
33	Glutamine	βCH_2 2.15 m, γCH_2 2.44 m, αCH 3.77 t	L, K, S, B, M, Ce, Co, I ,F, P, Y
34	Glutarate	CH_2 1.78 m, 2HCOOH 2.17 t	B
35	Glutathione	CH_2 2.17 m, CH_2 2.53 m, S-CH_2 2.95 dd, N-CH 3.83 m, CH 4.56 q	L
36	Glycerol	1/2CH_2 3.58 m, 1/2CH_2 3.62 m, CH 3.77 t	L, K, S, B, M, Ce, P, W
37	Glycerophosphocholine	N-(CH_3)$_3$ 3.22 s, NCH_2 3.68 m, OCH_2 4.32 m	L, K
38	Glycine	αCH_2 3.55 s	L, K, S, B, M, Ce, Co, I ,F, P, Y
39	Glycogen	C2H 3.63 dd, C4H 3.66 dd, C5H 3.83 q, C6H 3.87 d, C3H 3.98 d, C1H 5.41 m	L
40	Histidine	1/2CH_2 3.16 dd, 1/2CH_2 3.23 dd, CH 3.98 dd, CH 7.09 s, CH 7.90 s	L, K, S, B, Ce, Co, I, P, Y
41	Hypoxanthine	CH 8.18 s, CH 8.21 s	L, K, S, B, Ce, Co, I, P
42	Inosine	1/2CH_2 3.83 dd, 1/2CH_2 3.91 dd, C1H 4.27 dd, C2H 4.43 dd, C3H 4.76 t, C4H 6.09 d, NH-CH 8.23 s, N-CH 8.34 s	M, Ce, Co, I
43	Isobutyrate	(CH_3)$_2$ 1.05 d, CH 2.38 m	Ce
44	Isoleucine	γCH_3 0.94 t, δCH_3 1.02 d, 1/2γCH_2 1.26 m, 1/2γCH_2 1.47 ddd, βCH 2.01 m, αCH 3.65 d	L, K, S, B, M, Ce, Co, I ,F, P, Y, W
45	Lactate	βCH_3 1.33 d, αCH 4.12 q	L, K, S, B, Ce, Co, I, F, P, W
46	Leucine	δCH_3 0.93 d, βCH_2 0.94 d, γCH 1.71 m, αCH 3.73 m	L, K, S, B, Ce, Co, I, F, P, Y, W
47	Lipoproteins (HDL)	C***H***$_3$(CH_2)$_n$ 0.84 t, (CH_2)n 1.25 m, C***H***$_2$-C=C 2.04 m, C***H***$_2$-C-O 2.24 m, =CH-C***H***$_2$-CH= 2.75 m, CH=CHC***H***$_2$ 5.32 m	L ,B, F, P, Y

48	Lipoproteins (VLDL)	C***H***$_3$CH$_2$CH$_2$C= 0.87 t, C***H***$_2$CH$_2$CH$_2$CO 1.29 m, C***H***$_2$CH$_2$O 1.57 m, C***H***$_2$-C=C 2.04 m, C***H***$_2$-C-O 2.24 m, =CH-C***H***$_2$-CH= 2.75 m, CH=CHC***H***$_2$ 5.32 m	L, B, F, P, Y
49	Lysine	γCH$_2$ 1.46 m, δCH$_2$ 1.71 m, βCH$_2$ 1.84 m, εCH$_2$ 3.01 t	L, K, S, B, I, F, Y
50	Malate	1/2HCOOH 2.38 dd, 1/2HCOOH 2.66 dd, H-CH 4.30 dd	P
51	α-Mannose	C5H 3.37 m, C4H 3.56 m, C3H 3.65 m, C6H 3.73 m, C2H 3.92 m, C1H 5.17 d	W
52	β-Mannose	C4H 3.65 m, C5H 3.80 m, C3H 3.84, C6H 3.88, C2H 3.92 m, C1H 4.89 d	W
53	Methionine	δCH$_3$ 2.13 s, βCH 2.14 m, γCH$_2$ 2.60 t, αCH 3.78 t	L, K, S, B, Ce, Co, I, F, P, Y
54	Methylamine	CH$_3$ 3.29 s	F
55	*myo*-Inositol	C5H 3.29 t, C1H C3H 3.53 dd, C4H C5H 3.63 t, C2H 4.06 t	L, K, S, B, Ce, Co, I, P, Y, W
56	N-Acetylglucosamine	CH3 1.98 s, C3H 3.44&3.76 t, C5H 3.45&3.84 m, C4H 3.48&3.53 t, C2H 3.66&3.86 m, C6H 3.77 m & 3.87 dd, C1H β 4.71 α 5.19 d, NH 8.10 d	F
57	N-acetyltyrosine	CH$_3$ 1.92 s, 1/2βCH$_2$ 2.83 dd, 1/2βCH$_2$ 3.08 dd, αCH 4.37 m, C3H C5H 6.84 m, C2H C4H 7.14 m, NH 7.75 d	F
58	Nicotinurate	CH$_2$ 3.99 s, H5 7.60 dd, H4 8.25 d, H6 8.71 d, H2 8.94 s	L, K, S, B, M, Ce, Co, I
59	O-Phosphocholine	N-(CH$_3$)$_3$ 3.21 s, CH$_2$ 3.58 m, O-CH$_2$ 4.16 m	L, K, S, B, Ce, Co, I, Y
60	Ornithine	1/2γCH$_2$ 1.72 m, 1/2γCH$_2$ 1.82 m, βCH$_2$ 1.93 m, δCH$_2$ 3.04 t, αCH 3.77 t	K, Y
61	Phenylalanine	1/2βCH$_2$ 3.12 dd, 1/2βCH$_2$ 3.26 dd, C3H C5H 7.33 m, C4H 7.35 m, C3H C6H 7.40 m	L, K, S, B, Ce, Co, I, F, P, Y, W
62	Proline	γCH$_2$ 2.03 m, 1/2βCH$_2$ 2.03 m, 1/2βCH$_2$ 3.35 m, 1/2δCH$_2$ 3.38 m, 1/2δCH$_2$ 3.41 m, αCH 4.41 dd	L, K, S, B, Ce, Co, I, F, P, Y, W
63	Propionate	CH$_3$ 1.04 t, CH$_2$ 2.17 q	Ce, Co, F
64	Serine	αCH 3.85 dd, 1/2βCH$_2$ 3.95 dd, 1/2βCH$_2$ 3.95 dd	K, S, B, Ce, I, Y
65	*scyllo*-inositol	CH 3.35 s	K
66	Succinate	CH$_2$ 2.04 s	L, K, S, M, Ce, Co, I, F, P
67	Taurine	N-CH$_2$ 3.26 t, S-CH$_2$ 3.43 t	L, K, S, B, Ce, Co, I, P
68	Threonine	γCH$_3$ 1.32 d, αCH 3.60 d, βCH 4.25 m	L, K, S, B, Ce, I F, P, Y
69	Trigonelline	CH$_3$ 4.43 s, C4H 8.07 m, C3H C5H 8.91 m, C1H 9.11 s	F
70	Trimethylamine *N*-oxide	N-(CH$_3$)$_3$ 3.27 s	L, K, B, Ce, Co, I, F, P
71	Tryptophan	1/2βCH$_2$ 3.31 dd, 1/2βCH$_2$ 3.49 dd, αCH 4.06 dd, C5H 7.21 t, C6H 7.29 t, C1H 7.33 s, C3H 7.55 d, C4H 7.74 d	L, K, S, Ce, Co, I, F, Y
72	Tyrosine	1/2CH$_2$ 3.04 dd, 1/2CH$_2$ 3.18 dd, N-CH 3.94 dd, C3H C5H 6.89 m, C2H C6H 7.18 m	L, K, S, B, Ce, Co, I, F, P, Y, W
73	UDP-glucose	C4H 3.47 t, C2H 3.54 m, C3H 3.77 t, 1/2C6H 3.77 dd 1/2C6H 3.85 dd, C5H 3.88 m, 1/2CH$_2$ 4.19 m, 1/2CH$_2$ 4.24 m, O-CH 4.28 m, C'3H 4.36 dd, C'2H 4.37 dd, C1H 5.97 d, O-CH-N 5.97 d, N-CH 7.94 d	W
74	UDP-*N*-acetyl glucose	CH$_3$ 2.07 s, C4H 3.55 t, C3H 3.80 t, 1/2C6H 3.81 dd, 1/2C6H 3.86 dd, C5H 3.91 m, C2H 3.98 m, 1/2CH$_2$ 4.18 m, 1/2CH$_2$ 4.23 m, O-CH 4.28 m, C'3H 4.35 dd, C'2H 4.36 dd, C1H 5.51 dd, CH 5.95 d, O-CH-N 5.97 d, N-CH 7.94 d, NH 8.35 d	W
75	Uracil	C5H 5.80 d, C6H 7.54 d	L, K, S, B, Ce, Co, P
76	Uridine	1/2CH$_2$ 3.81 dd, 1/2CH$_2$ 3.92 dd, C4H 4.12 dt, C3H 4.24 dd, C2H 4.36 dd, C1H 5.88 d, C5H 5.92 m, C6H 7.88 d	W, S
77	Valerate	CH$_3$ 0.88 t, γCH$_2$ 1.29 m, βCH$_2$ 1.51 m, αCH$_2$ 2.17 t	Ce, F
78	Valine	γCH$_3$ 0.98 d, γ'CH$_3$ 1.04 d, βCH 2.27 m, αCH 3.62 d	L, K, S, B, Ce, Co, I, F, P, Y, W
79	Xanthine	CH 7.92 s	K, S, B, Ce, Co, I

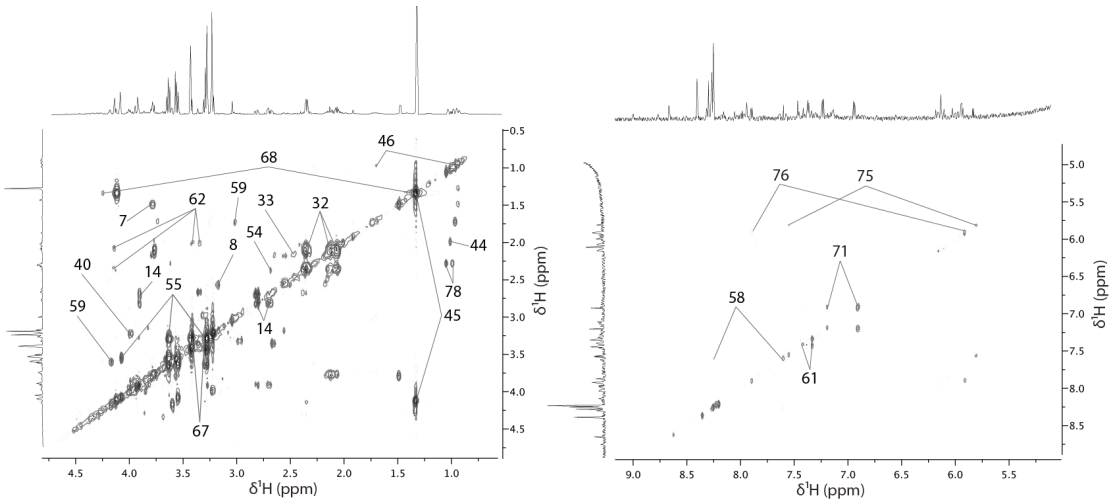

Figure 3.5: 700 MHz ^{1}H-^{1}H COSY NMR spectra of spleen, key indicated by Table 3.1

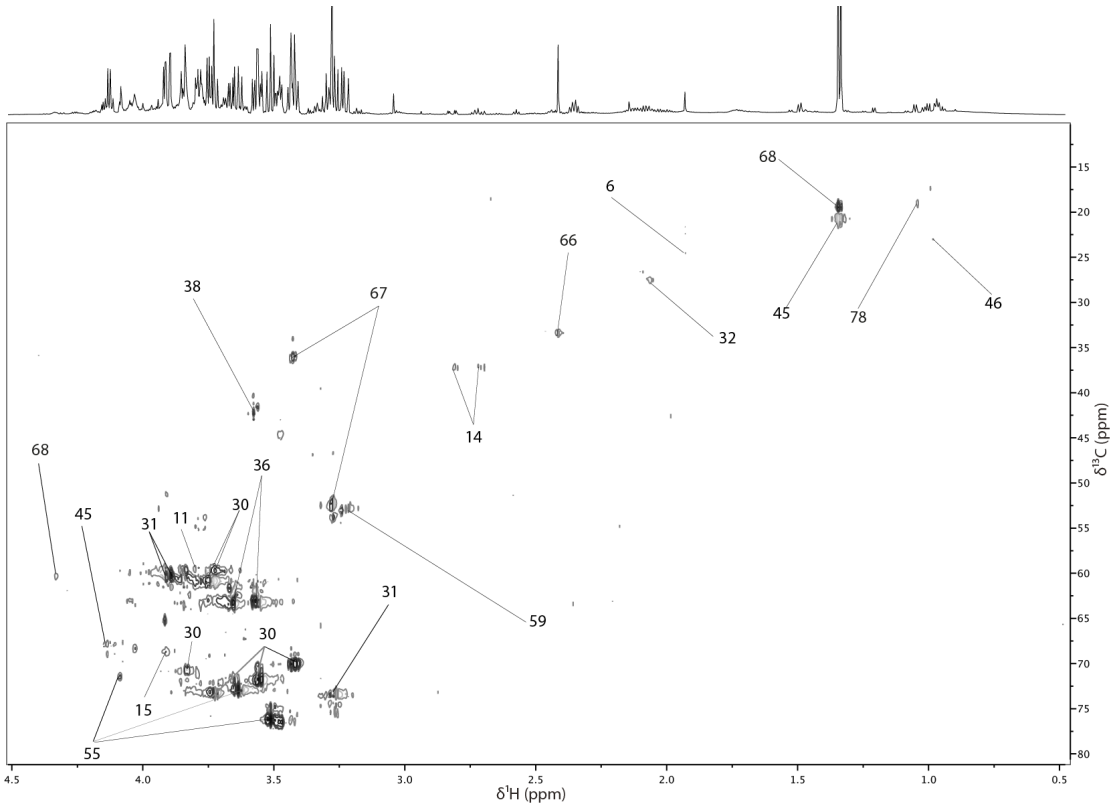

Figure 3.6: 700 MHz ^{1}H-^{13}C HSQC NMR spectra of Liver, key indicated by Table 3.1

3.3.2. Matrix cross comparison

Cross tissues comparison of detectable metabolites was performed using a Venn diagram (Figure 3.7 and Table 3.2) and revealed the high metabolic variability existing between the twelve biological matrices investigated in this study. Only eight-core metabolites were found out of a total of seventy-nine detected molecules. Detected core metabolites were all amino acids: alanine, glutamate, isoleucine, leucine, phenylalanine, proline, tyrosine and valine and can be considered ubiquitous stable metabolites. Matrices related to general metabolic processes (liver, kidney, spleen and plasma) shared twenty-eight metabolites related to energy and protein metabolism. Biological matrices related to the Digestive system(colon, caeca, ileum and faeces) shared 23 core metabolites associated with microbial activity, energy metabolism and protein degradation.

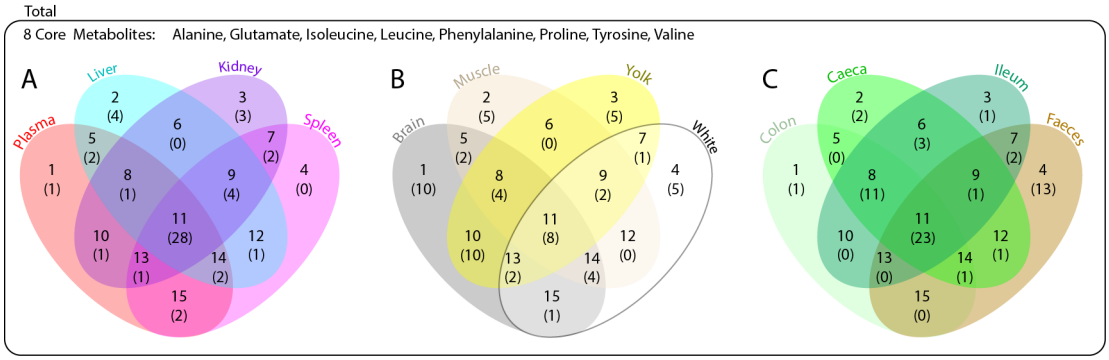

Figure 3.7: Venn diagram representing metabolic similarities between the 12 studied chicken matrixes. A: chicken general metabolism: plasma, Liver, Kidney, Spleen. B: Muscle, egg yolk, egg white and brain cortex. C Digestive system: Colon, Caecum, Ileum, Faeces. Each umber represents a zone of intersection, the numbers in brackets indicate the number of metabolites shared in the specified zone, details of the metabolites are displayed in Table 3.2. The Venn diagram comes first in the discussion; you must be consistent.

Table 3.2: Summary of the metabolites found in each zone of the Venn diagram presented in Figure 3.7.

	Zone	N	Metabolites
A	1	1	Malate
	2	4	3-Hydroxybutyrate, Glutathione, Glycerophosphocholine, Glycogen
	3	3	Citrate, Ornithine, scyllo-inositol
	5	2	Lipoprotein HDl and VLDL
	7	2	Serine, Xanthine
	8	1	Trimethylamine N-oxide
	9	4	Lysine, Nicotinurate, O-Phosphocholine, Tryptophan
	10	1	Creatinine
	11	28	Acetate, Alanine, β-Alanine, Betaine, Choline, Creatine, Formate, α and β-Glucose, Glutamate, Glutamine, Glycerol, Glycine, Histidine, Hypoxanthine, Isoleucine, Lactate, Leucine, Methionine, myo-inositol, Phenylalanine, Proline, Succinate, Taurine, Threonine, Tyrosine, Uracil, Valine
	12	1	Asparagine
	13	1	Fumarate
	14	2	Arginine,Aspartate
	15	2	Ascorbate, Cysteine
B	1	10	4-Aminobutyrate, Ascorbate, Carnitine, Ethanolamine, Glutarate, Hypoxanthine, Taurine, Trimethylamine N-oxide, Uracil, Xanthine
	2	5	β-Alanine, AMP, Anserine, Inosine, Succinate
	3	5	Aspartate, α-Galactose, β-Galactose, Ornithine, Tryptophan
	4	5	α-Manose, β-Manose, UDP-glucose, UDP-N-acetyl glucose, Uridine
	5	2	Carnosine, Nicotinurate
	7	1	Arginine
	8	4	Betaine, Fumarate, Glutamine, Glycine
	9	2	α-Glucose, β-Glucose
	10	10	Asparagine, Citrate, Histidine, Lipoproteins (HDL), Lipoproteins (VLDL), Lysine, Methionine, O-Phosphocholine, Serine, Threonine
	11	8	Alanine, Glutamate, Isoleucine, Leucine, Phenylalanine, Proline, Tyrosine, Valine
	13	2	Choline, myo-Inositol
	14	4	Acetate, Creatine, Glycerol, Lactate
	15	1	Formate
C	1	1	3-Hydroxyphenylacetate
	2	2	Glycerol, Isobutyrate
	3	1	Ethanolamine
	4	13	2-Hydroxybutyrate, 3-Hydroxyisobutyrate, Dimethylamine, α-Galactose, β-Galactose, α-Glucose, β-Glucose, Lipoproteins (HDL), Lipoproteins (VLDL), Methylamine, N-Acetylglucosamine, N-acetyltyrosine, Trigonelline
	6	3	β-Alanine, Asparagine, Serine
	7	2	Citrate, Lysine
	8	11	Cysteine, Fumarate, Histidine, Hypoxanthine, Inosine, mho-inositol, Nicotinurate, O-Phosphocholine, Taurine, Uracil, Xanthine
	9	1	Threonine
	11	23	Acetate, Alanine, Aspartate, Betaine, Butyrate, Choline, Creatine, Creatinine, Formate, Glutamate, Glutamine, Glycine, Isoleucine, Lactate, Leucine, Methionine, Phenylalanine Proline, Succinate, Trimethylamine N-oxide, Tryptophan, Tyrosine, Valine
	12	1	Valerate
	14	1	Propionate

The largest source of metabolic variation between the twelve biological matrices was visualised using PCA (Figure 3.8). The scores of liver, kidney and spleen samples were clustered together on the three first principal components representing 77 % of the total variance (PC1, PC2 and PC3, Figure 3.8). Surprisingly, this was also observed for muscle and brain cortex tissues. Metabolic profiles of samples derived from the

Digestive system were also grouped together but presented the highest variability between samples of the same matrix. These were the samples driving separation on the first component , which was associated with increased levels in short chain fatty acidsproduced by gut microbial activity). Finally, plasma, egg yolk and egg white were clustered together on PC2 due to their high glucose content. Yolk and plasma metabolic profiles also clustered together because they shared high lipid levels.. Interestingly, Egg-derived samples were the most metabolically homogenous, with the least inter-individual variability indicating that their metabolism is tightly regulated.

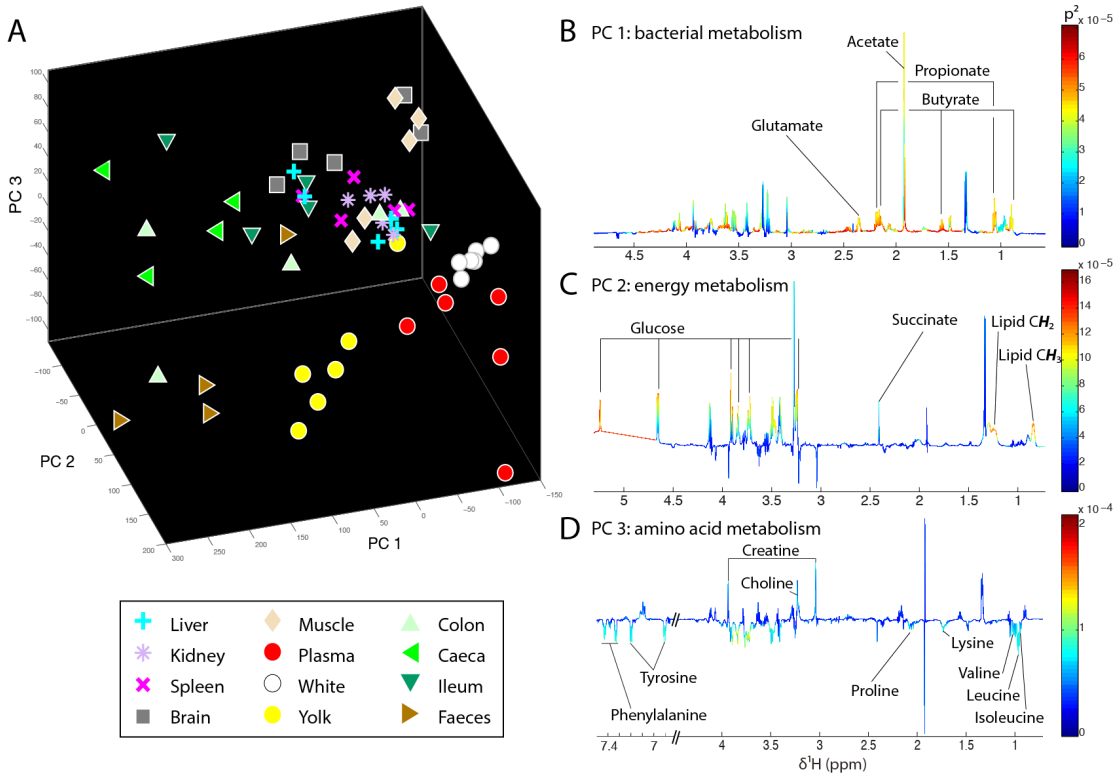

Figure 3.8: Metabolic variability between the twelve described chicken matrices. A. 3 dimensional PCA scores plot calculated using all ^{1}H-NMR spectra used for the study (n=72). B. Loading corresponding to the metabolic variations observed on PC1, mainly related to microbial metabolism. C. Loadings representing the metabolic variations observed on PC2 associated to energy metabolism. D. Loadings of PC3 related to amino acids metabolism.

The metabolic profiles of colon, caecum, ileum and faecal water shared many similar metabolic patterns. 23-shared metabolites related to microbial catabolism of polysaccharides (acetate, butyrate) and protein degradation (amino acids). Propionate, another important product of polysaccharide, fermentation was not found in the ileum but all other digestive matrices indicating that propionate fermentation does not occur in this part of the digestive system. It was not possible to separate caeca and colon using pairwise comparison indicating their metabolic similarity. On the contrary it was possible to distinguish the ileum due to lower SCFAs concentration showing that gut microbiota at this level of the GI track is less active. The same observation was done in mice where more SCFAs were found in the lower part of the GI track due to high microbial colonization(Martin et al. 2009). This metabolic characteristic clearly separated them on the PCA plot from the other matrices. Faecal water was the biofluid presenting the highest quantity of identifiable metabolites, of which ten were uniquely found in this matrix probably as a result of the complexity of the food provided (see material supplement) and high microbial activity. These ten metabolites were mostly SCFAs, possibly related to gut microbiota activity as well as methyl donors including methylamines. The high similarity level existing between the GI track metabolic profiles and the faecal water shows the great level of exchange existing between the GI lumen and the enterocytes. Birds were fed with un-medicated layer pellets (Dodson and Horrell) that mainly contain wheat rich in complex carbohydrate, vegetable oil and soya as a protein source (for more information see supplement material).

Highly metabolically active tissues, liver, kidney and spleen, appear to be very similar although they serve different purposes (i.e. spleen is more involved in immune control) as presented on the PCA plot. However, due to the high number of studied matrices and their high variability, this model lacks sensitivity to separate the three tissues which present a high number of similarity qualitatively and quantitatively but also some notable differences regarding glucose and creatine levels that were detectable using pairwise comparisons.

Egg metabolic profiles were dominated by energy metabolites (saccharides) and amino acids for both yolk and white matrices. Yolk was also extremely rich in cholesterol and lipids, which are essential to cell membrane formation(Yeagle 1989; Spector and Yorek 1985) but are also sources of energy. These results confirm the high nutritive value of chicken eggs due to their initial purpose for fetal development.

The metabolic profile of muscle has only been described in mice for cardiac muscle(Griffin et al. 2001), which in its structure and function is different to striated skeletal muscle. Despite their differences, both muscle metabolic profiles appear to be characterized by lactate, which is the main product of glucose anaerobic fermentation by muscle during exercise(Brooks 1986), creatine, that is known to be principally present in muscular fiber due to its important energy input by ATP release during muscular contraction(Bessman and Geiger 1981; Casey et al. 1990), and taurine, also involved in contractility(Pierno et al. 1998).

In comparison to previous metabolic profiles of mammals such as mice, pigs and humans, these profiles show high qualitative but not necessarily quantitative similarity in liver, kidney, ileum, colon and plasma metabolic profiles. This shows that

despite the level of genetic and evolutionary differences existing between birds and mammals, there core metabolic functions remain very similar. The main difference previously mentioned between chicken and mammals metabolic profiles were observed in the liver regarding the glutathione level that was noticeably lower in birds. Glutathione is involved in cell protection due to its antioxidant properties(Meister 1983). This difference had been already reported in quail(Gregus et al. 1983), suggesting a major shift in system detoxification by the liver between mammals and birds. Indeed, several publications relate the higher susceptibility of birds to toxic substances and the higher bioaccumulation in comparison to mammals(Walker 1983) indicating a modification of detoxification metabolism during evolution.

3.4. Conclusion

This study presents a large overview of chicken metabolic profiles in various tissues and biofluids that could be used as a database for future NMR-based metabonomic analysis in avian industry. Such analysis could focus on metabolic impacts of GI infection and treatment on host metabolism but also on diet and growth condition impact on consumption product quality, and production yields (i.e. meat and egg). These data integrated with the other omics approaches will contribute to the understanding of host response to environmental changes, infection and treatment that should lead to improved animal welfare.

3.5. References

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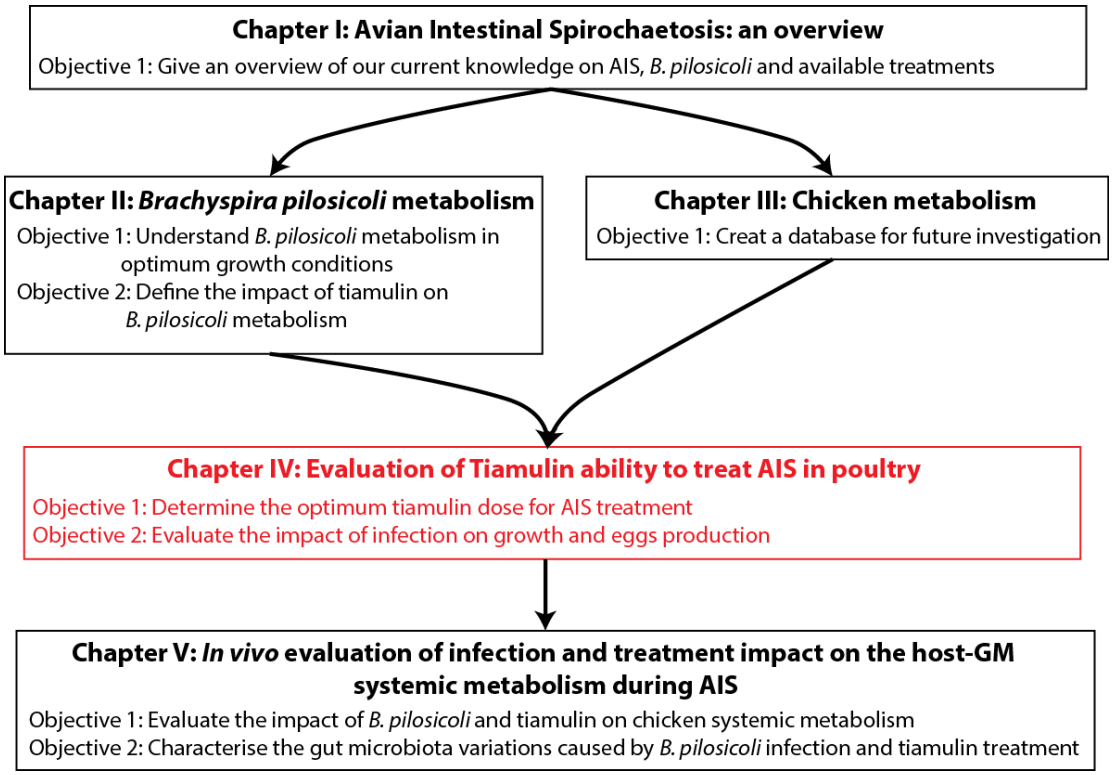
Tiamulin™ use to treat *B. pilosicoli*-induced AIS:

After evaluating both pathogen and host metabolism, the next step was to evaluate the impact of infection and antibiotic treatment *in vivo*. An animal trial funded by Novartis was conducted in order to determine the optimum Tiamulin™ dose to be used to treat *B. pilosicoli* infection. This work was published in 2015 in Research in Veterinary Science.



Chapter 4: Drinking water application of Denagard® Tiamulin for control of *Brachyspira pilosicoli* infection of laying poultry

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Abstract

Avian intestinal spirochaetosis (AIS) caused by *Brachyspira* spp., and notably *Brachyspira pilosicoli*, is common in layer flocks and reportedly of increasing incidence in broilers and broiler breeders. Disease manifests as diarrhoea, increased feed consumption, reduced growth rates and occasional mortality in broilers and these signs are shown in layers also associated with a delayed onset of lay, reduced egg weights, faecal staining of eggshells and non-productive ovaries. Treatment with

Denagard® Tiamulin has been used to protect against *B. pilosicoli* colonisation, persistence and clinical presentation of AIS in commercial layers, but to date there has been no definitive study validating efficacy. Here, we used a poultry model of *B. pilosicoli* infection of layers to compare the impact of three doses of Denagard® Tiamulin. Four groups of thirty 17 week old commercial pre-lay birds were all challenged with *B. pilosicoli* strain B2904with three oral doses two days apart. All birds were colonised within 2 days after the final oral challenge and mild onset of clinical signs were observed thereafter. A fifth group that was unchallenged and untreated was also included for comparison as healthy birds. Five days after the final oral *Brachyspira* challenge three groups were given Denagard® Tiamulin in drinking water made up following the manufacturer's recommendations with doses verified as 58.7 ppm, 113 ppm and 225 ppm. Weight gain body condition and the level of diarrhoea of birds infected with *B. pilosicoli* were improved and shedding of the organism reduced significantly ($p = 0.001$) following treatment with Denagard® Tiamulin irrespective of dose given. The level and duration of colonisation of organs of birds infected with *B. pilosicoli* was also reduced. Confirming previous findings we showed that the ileum, caeca, colon, and both liver and spleen were colonized and here we demonstrated that treatment with Denagard® Tiamulin resulted in significant reduction in the numbers of *Brachyspira* found in each of these sites and dramatic reduction in faecal shedding ($p < 0.001$) to approaching zero as assessed by culture of cloacal swabs. Although the number of eggs produced per bird and the level of eggshell staining appeared unaffected, egg weights of treated birds were greater than those of untreated birds for a period of approximately two weeks

following treatment. These data conclusively demonstrate the effectiveness of Denagard® Tiamulin in reducing *B. pilosicoli* infection in laying hens.

4.1. Introduction

Avian intestinal spirochaetosis (AIS) has been recognised as a disease in poultry since 1985 (Davelaar et al., 1985) and the condition arises from the colonisation of the GI tract of the birds by the anaerobic spirochaete, *Brachyspira* (Swayne and McLaren, 1997; Stephens and Hampson, 2001). Currently, three species of this genus are considered pathogenic in poultry and have been shown to induce AIS in experimentally challenged chickens; *Brachyspira alvinipulli* (Swayne et al., 1995; Stanton et al., 1998), *Brachyspira intermedia* (Hampson and McLaren, 1999) and *Brachyspira pilosicoli* (Stephens and Hampson, 2002). *B. pilosicoli* has a wide host range, also causing intestinal spirochaetosis in pigs (PIS) (Trott et al., 1996) and humans (HIS) (Tsinganou and Gebbers, 2010), with a potential for zoonosis (Hampson et al., 2006a, 2006b). Of these, *B. pilosicoli* infection seems to be the most prevalent and increasing in incidence worldwide although infection with the other *Brachyspira* species notably *B. intermedia* and *B. alvinipulli*, and infection with multiple *Brachyspira* species is noted (Medhanie et al., 2013). AIS is common in layer flocks (Stephens and Hampson, 1999) although an emerging issue in broilers and broiler breeders (Medhanie et al., 2013). Disease manifests as diarrhoea, increased feed consumption, reduced growth rates and 10% or greater mortality in broilers and these signs are shown in layers also associated with a delayed onset of lay, reduced egg weights, faecal staining of eggshells and non-productive ovaries

(Davelaar et al., 1986; Griffiths et al., 1987; Swayne et al., 1992). AIS is a production problem and an economic burden in commercial flocks (Burch, pers. commun.: Anon, 2013: 6th Int Spirochaete Conference, Surrey) and currently treatment consists of the application of antibiotics such as ampicillin, lincomycin–spectinomycin in combination and the pleuromutilins such as Tiamulin, although relapse after treatment is common and resistance has been observed also (Hampson et al., 2006b; Pringle et al., 2012). A characteristic of *B. pilosicoli* colonisation is its ability to form end on attachments to the intestinal epithelial surface and invade (Jensen et al., 2000, 2001). In poultry, *B. pilosicoli* organisms were found to form a dense fringe, penetrating between enterocytes and associated with reactive and mild inflammatory responses such as crypt hyperplasia and increased numbers of goblet cells (Feberwee et al., 2008). It has been suggested that the apparent increase in AIS is possibly due to the 2006 EU ban on the use of antibiotics as growth promoters in livestock. However, there is a lack of data to confirm this suggestion as there are insufficient comprehensive longitudinal epidemiological studies pre and post-ban and other factors that have yet to be elucidated may contribute also. Thus, there is a need for validated controls for this emerging disease to ensure the productivity of the industry. Tiamulin™, is a bacteriostatic agent belonging to the group of pleuromutilin antibiotics. Pleuromutilins are di-terpene compounds derived originally from the basidiophyte fungi. The earliest versions were shown to have antibacterial activity and were used initially against Mycoplasma infections, with success. Subsequent chemical modifications gave rise to Tiamulin™ that was demonstrated to have 20–50 fold higher antibacterial activity than the progenitor compounds (Shang et al., 2013). Tiamulin™ has a wide spectrum of activity, but is

particularly effective against *Brachyspira*, Lawsonia and the Mycoplasmas. This category of antibiotics is not used in human medicine. The pleuromutilin antibiotics target the 50S subunit of the ribosome, to interact with the peptidyl-transferase to inhibit protein synthesis. Concern has been expressed at recent, but still rare occurrence of resistance, the mechanism of which has been identified as mutation in the rRNA (A445G) that is the support structure of the ribosome and a change in protein L3 (Asn149Asp) where Tiamulin™ binds (Poulsen et al., 2001). Until recently, the literature suggests that poultry models for *Brachyspira* infection studies are variable in outcome. However, the work of Mappley et al. (2013) has resulted in a more reproducible model by the simple expedient of pre-dosing with sodium bicarbonate to neutralise the crop prior to oral dosing with challenge *B. pilosicoli*. With this model, it is possible to assess reproducibly the impact of the use of Tiamulin™ in current and variant treatment regimens against AIS. Tiamulin™ has been used in veterinary medicine for a considerable period and deployed notably for use in the pig sector (Taylor, 1980; Wilberts et al., 2014). Denagard® Tiamulin (Novartis) is already marketed for use in pigs and more recently chicken at a concentration of 250 ppm (parts per million) for the reduction of the severity of disease caused by Mycoplasma spp., Lawsonia and *B. hyodysteriae* in pigs and to control gastro-intestinal infections, notably AIS in chickens. This study specifically seeks to evaluate the ability of different concentrations of Denagard® Tiamulin to protect against *B. pilosicoli* colonisation, persistence and clinical presentation of AIS in commercial layers. The current level of usage is recommended at 250 ppm for control of Mycoplasma respiratory infections and this study aims to assess impact at

this and reduced levels of 125 and 67.5 ppm against *B. pilosicoli* infections of laying chickens.

4.2. Materials and methods

4.2.1. Bacterial strain and culture

B pilosicoli strain B2904 was used as the challenge strain and is fully characterised to genome sequence level (Mappley et al., 2011). *B. pilosicoli* stored in 70% *Brachyspira* Enrichment Broth (BEB: Oxoid) + 30% foetal calf serum at −80°C and was inoculated into fresh pre-warmed BEB. Anaerobic growth was for five days at 37°C with gentle shaking. Inocula were prepared by recovery by centrifugation and resuspension in diluent (0.1 M Phosphate Buffered Saline, pH 7.2) of strain B2904 grown in BEB. The number of organisms was enumerated in a Helber cell counting chamber and adjusted to give a dose comprising $5x10^9$ cells in 1 ml that was delivered to the chickens immediately on preparation. Detection of strain B2904 from cloacal swabs was by plating onto *Brachyspira* selection agar (FABA fastidious anaerobe blood agar supplemented with whole horse blood) (Rasbeck et al., 2005) and incubation for up to eight days at 37°C, anaerobically. For enumeration of the bacterium from tissue, 1 mg of tissues was homogenised (VDI 12 homogeniser, VWR International Ltd., Lutterworth, UK) into 9 ml of PBS 0.1 M. Samples (20 µl) were plated following serial dilution (10^{-1} to 10^{-7}) onto the same *Brachyspira* selection agar (Mappley et al., 2013) and incubated as above. To ensure sterility between samples rotor blades of the homogeniser were vortexed in sterile water to remove debris and then stood in alcohol for 1 min. Prior to the start of the study, the sensitivity of strain B2904 to

Denagard® Tiamulin was confirmed in vitro by minimum inhibitory concentration (MIC) by multipoint inoculation onto a BEB agar supplemented with a dilution series of Tiamulin™ following standard BSAC recommended procedures.

4.2.2. PCR

To prepare samples for PCR testing, a 5 µl sterile loop was used to pick individual colonies from selective agar plates or take approximately 5 µl of material from faecal and environmental samples and thoroughly mixed in 100 µl sterile de-ionised water placed in a sterile microcentrifuge tube to obtain a homogeneous suspension. This was then placed into a thermocycler and heated at 95°°C for ten minutes. The micro-centrifuge tube was then placed on ice for one minute. It was centrifuged at 2400 g for one minute at 1°C, and stored at −20°C ± 5°C until used. The PCR reactions comprised in a final volume of 20 µl in a PCR tube 10 µl HotStartTaq® DNA polymerase MasterMix (Qiagen), 6 µl sterile water, 2 µl prepared sample and 1 µl of each Forward and Reverse primers (20 pmol/µl). The primers used were: *Brachyspira* genus-specific PCR (Phillips et al., 2005):

Forward: 5'-TGAGTAACACGTAGGTAATC-3'

Reverse: 5'-GCTAACGACTTCAGGTAAAAC-3'

B. pilosicoli-species specific PCR (Mikosza et al., 2001):

Forward: 5'-AGAGGAAAGTTTTTTCGCTTC-3'

Reverse: 5'-CCCCTACAATATCCAAGACT-3'.

The PCR tube containing the reaction mixture was placed in a GeneAmp® PCR system 9700 thermal cycler and the PCR conditions were 95°C for 15 min, followed by 30 cycles 1 min at 55°C, 1 min 72°C and 1 min at 95°C followed by a final DNA

extension stage of 7 min at 72°C. On completion, the tubes were cooled to 4°C and then 5 µl of the PCR reaction was electrophoresed on a 1% (w/v) agarose gel and visualised for the presence of amplicons under UV light. The remaining PCR reaction was stored at −20°C ± 5°C.

4.2.3. Animals

The study reported here used the experimental model developed by Mappley et al. (2013) using the same facilities at where the Mappley studies had been performed. Study animals were NovoGen Brown commercial layers sourced from a commercial supplier (Tom Barron Ltd., UK). Prior to delivery, the birds had received a pullet vaccination programme that did not include vaccination against *Brachyspira* spp. nor any antibiotic treatment. A total of 160 sixteen week-old birds were delivered to the (day 10) and randomly split into 5 groups of 32 birds each that were housed in separate rooms. The five groups were to be used in the following treatments:

- Group A: Untreated, uninfected controls
- Group B: Untreated, infected controls
- Group C: Infected + Tiamulin at ~62.5 ppm
- Group D: Infected + Tiamulin at ~125 ppm
- Group E: Infected + Tiamulin at ~250 ppm.

Birds were housed according to Home Office guidelines and all procedures were performed in compliance with the Animals Scientific Procedures Act, 1986. Feed and water were given ad libitum. Feed was unmedicated layer pellets (Dodson and Horrell) and water was from the mains supply. The weight of feed and water consumed was recorded daily. One day after arrival (day 9) each bird was winged

tagged with a uniquely identifiable number. The birds were permitted to acclimatize to their new environment and feed regime without further procedure until two days prior to the study commencing (day 2) when each bird was individually weighed and the group size reduced to 30 birds small birds weighing below1 kg. In addition, from each bird and each pen cloacal, freshly voided faeces and environmental swabs were taken and plated as described in bacterial strain and culture section to ensure absence of *Brachyspira* prior to experimentation. 2.4. Challenge and Denagard® Tiamulin treatment Prior to receiving the *B. pilosicoli* challenge or sham dose, the birds were first dosed by oral gavage with 2ml of 10% (w/v) sodium bicarbonate solution between 12 min and no more than 46 min prior to administration of the challenge dose to neutralise the crop acid as used previously (Mappley et al., 2013; Carroll et al., 2004; Randall et al., 2006). All birds in groups B to E were challenged by oral gavage with 1 ml of *B. pilosicoli* B2904 suspension (5 x 10^9 CFU/ml). Group A received a sham dose of 1 ml of sterile 0.1 M Phosphate Buffered Saline (pH 7.2). Dosing was performed on days 0, 2 and 4 when the birds were 17 weeks of age. Denagard® Tiamulin (lot number P768329UK) was administered following the procedures recommended by the manufacturer at the appropriate concentration: group C, 62.5 ppm: group D, at 125 ppm: group E, at 250 ppm. The Investigational Veterinary Product (IVP) was supplied as a 12.5% solution (125 mg/ml). On each of days 13 to 17, this was added to the drinking water for groups C, D and E at a rate of 0.5 ml per litre, 1 ml per litre and 2 ml per litre respectively to produce final concentrations in the drinking water of ~62.5, ~125 and ~250 ppm. Each day freshly treated water was prepared in 10 l aspirators that were used instead of mains water until empty. An approximate 50 ml sample of each batch of freshly prepared IVP was

taken and shipped to Stewardship Analytics Laboratory for analysis of the final concentration administered. On day 17, each room was thoroughly washed and disinfected (1% Virkon) to prevent reinfection from the environment.

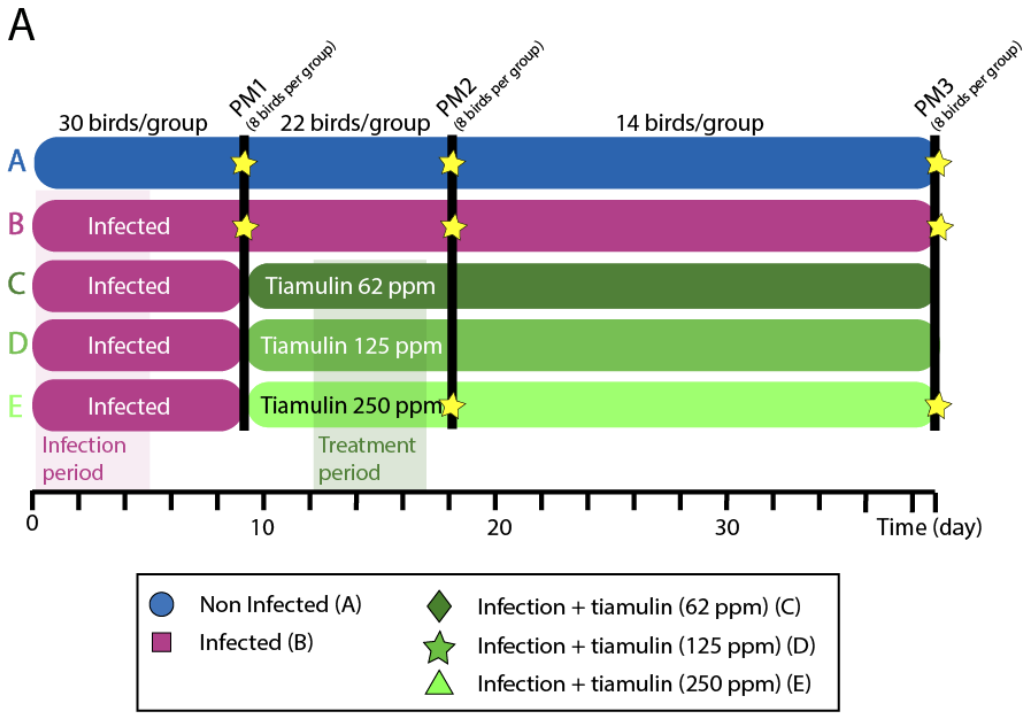

Figure 4.1: Experimental plan.

4.2.5. Observation and sampling

All birds were cloacally swabbed, weighed and condition scored of day 2 and thereafter every three days for the duration of the study. In addition, at each visit to the study rooms, (i) environmental swabs were taken, (ii) eggs were collected, weighed and scored for faecal eggshell staining and (iii) freshly voided faecal samples were collected at random from each pen, scored for moisture and consistency and subjected to culture and *B. pilosicoli* specific PCR. Eight birds that were selected randomly using Graphpad Quickcalc software from each group for the three post-

mortem examination dates. Birds were selected based on random selection of tag numbers at the beginning of the study. On days 9, 18 and 38 were euthanased by sedation using Rompun/Ketamine mixture as an intramuscular injection followed by an intravenous injection of Pentobarbitone. Death was confirmed by cervical dislocation. Immediately prior to post-mortem examination, each bird was sprayed with 70% ethanol and the abdominal and thoracic cavities opened aseptically. Duplicate samples (approximately 1 g each) of the caecal contents, caeca, liver, spleen, ileum and colon were taken using a separate set of sterile instruments for each bird. Spleen and liver were sampled prior to intestinal tissues with care taken to ensure no superficial contamination following the methods of Mappley et al. (2013). One of each pair of samples was placed untreated in a Universal containing 9 ml of 0.1 M PBS for bacteriology. The other sample was placed in a Duran containing 10% neutral buffered formalin for subsequent histopathological examination in case gross pathological lesions were observed but as no gross pathology was seen in this study no sample processing or visualisation of tissues undertaken. Caecal content samples were placed in a Universal and snap frozen and stored at −20°C. By the end of the study on day 38 there were six birds remaining in each study group. These were not examined.

4.2.6. Clinical scoring

Before pooling, faecal samples were scored as normal, firm (dark solid with white urate splash) [0], soft [1], watery/frothy [2] or watery with blood [3]. Once pooled, shavings and gross debris were removed and three replicate samples of between 1 and 3 g from each pool were weighed. The three replicates were dried overnight in a

hot air oven set at 65°C ± 3°C in order to enable faecal moisture content calculation. Condition scores were normal full breast/no clinical symptoms [0], slightly thin breast/pasty vent (mild)/loss of colour from comb (mild) [1], moderately thin breast/pasty vent (moderate)/loss of colour from comb (moderate) [2] or severely thin breast/pasty vent (severe)/loss of colour from comb (severe) [3]. Egg staining was scored as clean [0], light staining (10%) [1], moderate staining (10–29%) [2], heavy staining (30–69%) [3] or severe staining (70%) [4].

4.2.7. Data analysis and statistics

The presence of Brachyspira spp. in the faeces detected by culture was summarised in contingency tables and groups were compared with respect to cure rates by using Fisher's exact tests, separately for the various assessment days. Mann–Whitney U tests and Kruskal–Wallis tests were applied for the comparison of groups with respect to egg production parameters (number of eggs per bird and egg weight) and with respect to faecal scores, faecal eggshell staining and condition scores, separately for the various assessment days or assessment periods. Repeated measurement analyses of variance were applied on the growth performance of birds with or without change from baseline and study groups were compared thereof if normal distribution assumptions are satisfied (Shapiro–Wilk test). Descriptive statistics (arithmetic and geometric mean, median, minimum, maximum, standard deviation and coefficient of variation CV%) were calculated for any continuous parameter, separately for the various assessment days. Categorical data like the presence of pathogenic *Brachyspira* spp. or any scoring data will be summarised in frequency tables. The statistical unit was the experimental group if central values of

groups are compared. In other cases, the statistical unit was the individual animal. The level of significance is $\alpha = 0.05$, all tests were performed two-sided. The statistical analyses described above were done using the STATA statistical package (STATA12.0/IC for Windows (2011), StataCorp LP, 4905 Lakeway Drive, College Station, TX, 77845, USA.) employing the following commands: summarise = summary statistics, swilk = Shapiro–Wilk test, and ranksum = Mann–Whitney test. Kwallis = Kruskal–Wallis test ANOVA = analyses of variance and tabulate = contingency tables.

4.3. Results

4.3.1. Denagard® Tiamulin dose achieved

The desired dose was 62.5, 125 and 250 ppm in the drinking water of each of the test groups C, D and E, respectively. To test the actual dose given, triplicate samples from the drinkers from each of the study groups were taken and sent for third party analysis on each of the five days of administration, days 13–17. The mean concentrations achieved were 58.7, 113 and 227 ppm in the drinking water for each of groups C, D and E, respectively. The recommended dose of Denagard®Tiamulin in chickens is 25 mg per kg bodyweight and in this study 8.13, 15.90 and 36.19 mg per kg bodyweight was achieved for groups C, D and E, respectively. Water consumption was measured for each room every day. Knowing the number of birds and their average weight, it was possible to determine the average volume of water drank per day per kg of chicken and thereafter to determine drug consumption. Conversion

from ppm to concentration in mg/L of water was done knowing that 1 ppm is equivalent to 1 mg/L.

4.3.2. B. pilosicoli infection reduced growth performance of birds

All the birds in each of the study groups were weighed on each sampling day and the mean weight gain per group was calculated and plotted (Figure 4.1). Group A (uninfected–untreated) showed the normal anticipated growth performance whereas group B (infected–untreated) showed depressed performance due to the burden of infection. Indeed, between 1 and 12 days and 13–17 days (post-infection) the average weight was 50 g and 190 g less than group A ($p = 0.001$). Groups D and E that were infected and treated with the higher doses of Denagard® Tiamulin, 125 and 250 µg/mL respectively, gave statistically significantly improved weight gain compared to the infected–untreated group B ($p < 0.001$: Figure 4.1a) and group C that was infected and received the lowest dose of Denagard® Tiamulin (62.5 µg ml−1) ($p < 0.001$: Figure 4.1a) using pairwise t-test comparison. Both groups D and E that had the higher concentrations of Denagard® Tiamulin showed higher overall weight gain than group C that was statistically significant ($p < 0.001$: Figure 4.1b). Both group D and E reached similar weight gain than the control group by the end of the study indicating that only the two highest antibiotic doses were able to re-establish growth delayed associated with infection.

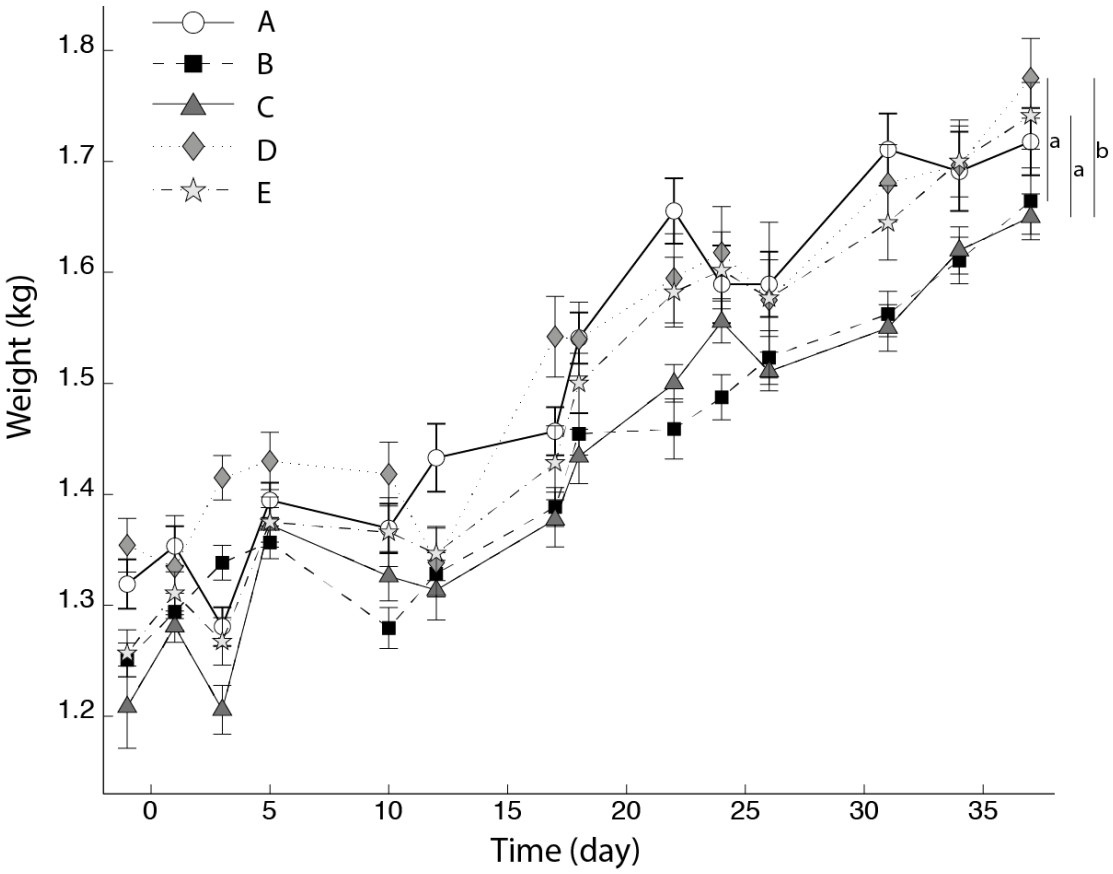

Figure 4.2: Mean bodyweight gain for each treatment group. A=unchallenged, untreated control, B=challenged, untreated control, C=challenged, treated [~65 ppm], D=challenged, treated [~125 ppm] and E challenged, treated [~250 ppm].

4.3.3. Feed and water consumption

There was reduced consumption of feed over the study period in groups C, D and E than either the negative (uninfected– untreated) or positive (infected–untreated) control whereas water intake showed a steady rise over the period of the study for all groups. There were no statistically significant differences between any of the groups between feed intake and water consumption (data not shown). Over the entire study the feed conversation ratios were 4.22, 6.47. 8.56. 7.4 and 6.01 for each of groups A–E respectively which is substantially below industry standard and

infection impacted negatively upon FCR that was not reversed by Denagard® Tiamulin treatment.

4.3.4. Condition of birds during treatment

All the groups that were dosed with *B. pilosicoli* irrespective of whether they received Denagard® Tiamulin treatment (groups B, C, D and E) showed noticeable reduction in condition with the poorest condition score achieved on or around day 10. Group A, unchallenged and untreated, remained in excellent condition throughout the study with all birds achieving a score of 0. Group B, challenged and untreated, gave the highest mean condition score of 0.86 from day 10 to day 38, significantly poorer than the other 4 groups ($p = 0.001$). Mean condition scores for groups C, D and E (challenged and treated) from day 10 to day 38 were 0.23, 0.41 and 0.39 respectively, but these differences were not significant.

4.3.5. Isolation of B. pilosicoli from birds and the environment

As anticipated all groups were negative for *B. pilosicoli* by cloacal swab before challenge and treatment and group A remained negative throughout the entire study. After challenge and prior to antibiotic treatment there was no significant difference ($p=0.4183$) in the number of birds per group yielding positive isolations by cloacal swab for the infected groups B, C, D and E. Once Denagard® Tiamulin treatment started on day 13, there was a significant reduction in the number of isolations from the infected–treated birds of groups C, D and E when compared to group B (infected–untreated). However, there were no significant differences between Denagard® Tiamulin treatment groups during treatment. The data are

presented in Figure 4.2. In the post-treatment period from day 18 onward, the difference between untreated and treated birds was still observed ($p < 0.001$). However, group E that had the highest dose of 227 ppm Denagard® Tiamulin showed a significantly ($p < 0.05$) lower number of isolations than either groups C or D that received 113 ppm and 58.7 ppm, respectively. There was no significant difference ($p = 0.502$) between groups C and D. Confirmation of presumptive isolates was by PCR as described in materials and methods. To assess the environmental burden of *B. pilosicoli* infection, pooled freshly voided faecal samples were taken from birds in groups B, C, D and E. The results mirrored the results of the cloacal swabs with only group B giving samples that were consistently positive by culture for the duration of the experiment up to day 38. Samples from groups C, D and E were positive from day 5, after the second oral inoculation until day 19 and were negative at all time-points thereafter (data not shown). None of the environmental swabs taken throughout the study were *Brachyspira* culture positive suggesting extreme sensitivity of these strict anaerobes to oxygen. PCR was also performed on the environmental samples and gave sporadic positives from all infected groups during dosing and immediately thereafter. Only group B gave sporadic PCR positives for the duration of the study (data not shown).

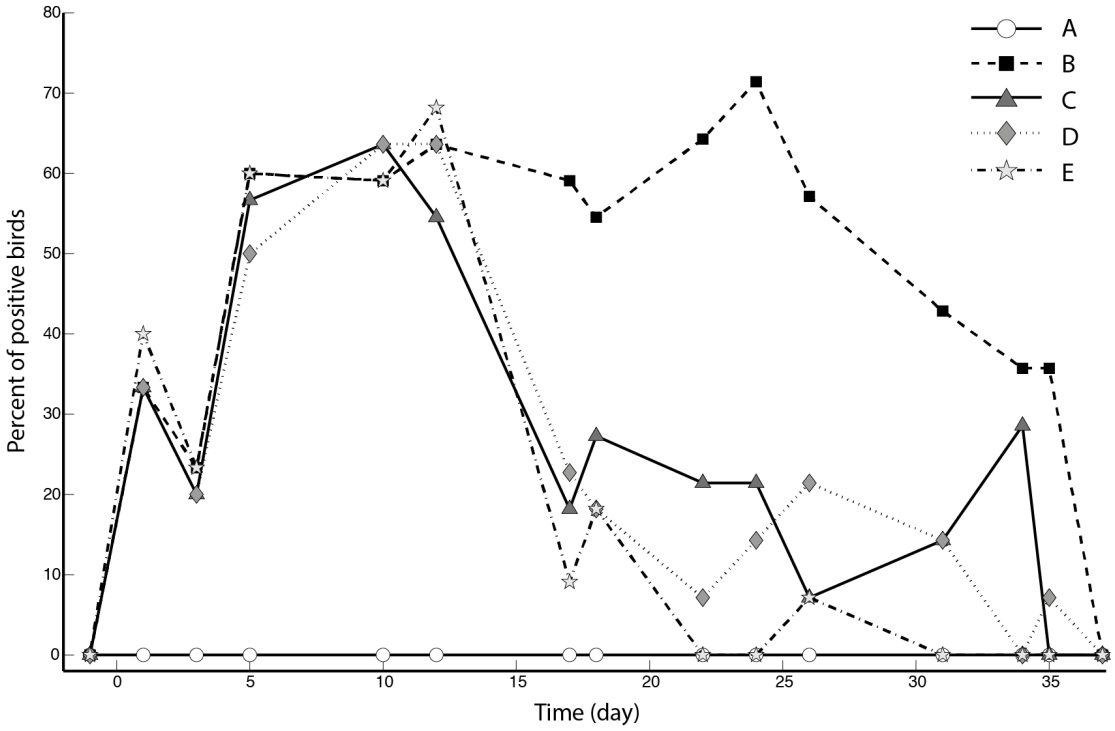

Figure 4.3: Number of positive cloacal swabs per group on each sampling day. Denagard® Tiamulin was administered in the drinking water from days 13–17 and samples marked *were those taken during Denagard® Tiamulin administration. The number of birds per group shows the time points at which 8 birds were taken at random for post-mortem examination.

4.3.6. Faecal moisture content and consistency scoring

The moisture content of faeces samples was determined and a score for consistency at the time of collection made also (Figure 4.3). From the entire study only one consistency score above 2 was observed and this was from group B (infected–untreated) taken on day 15. The faeces score for group A remained consistently low throughout the study. Prior to Denagard® Tiamulin treatment, the scores for all the infected groups (B to E) were similar. However, the scores of the treated groups (C to E) reduced noticeably once treatment started, although they did not reduce to the level of the uninfected birds but this differences were not statistically significant.

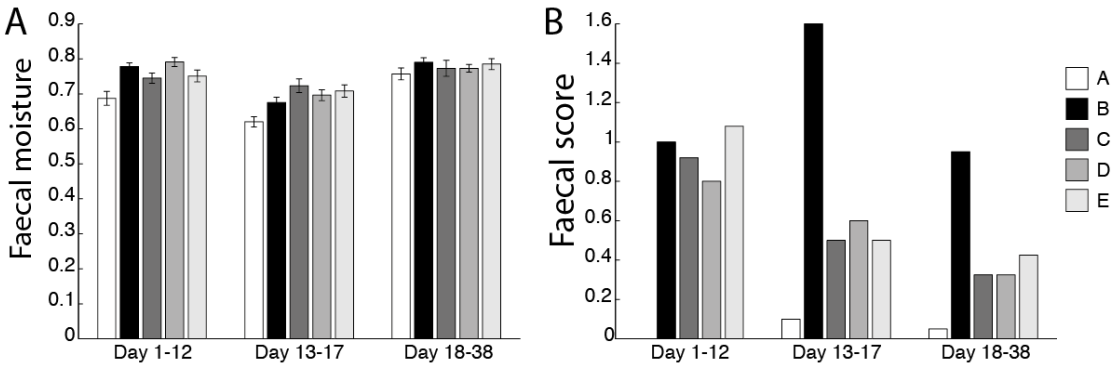

Figure 4.4: Mean faecal moisture content and consistency scores per group.

4.3.7. B. pilosicoli tissue colonisation

The isolation rates and mean colony counts from all the homogenized tissue samples at the three post-mortem examination dates are summarised below (Figure 4.4). The identity of the bacteria showing 'putative' *Brachyspira* colonial morphology was confirmed by PCR at the genus and species level for 189 randomly selected colonies from these studies that all confirmed the presence of *B. pilosicoli*. All organs from Group A birds were negative at all three post-mortem examination dates. The recent findings of Mappley et al. (2013) showed that *Brachyspira* infections in birds has a systemic phase to colonise deep tissues and in this study liver and spleen samples were analysed for the presence of *B. pilosicoli* by culture and confirmatory PCR taking due precaution to ensure no superficial contamination as described in methods and Mappley et al. (2013). Over the three post mortem samplings taken on days 9, 18 and 38, a total of 24 animals from each of the infected groups was analysed, eight birds per group per time. The positive control group B (infected–untreated) had 14 liver and 13 spleen samples positive for *B. pilosicoli*, whereas groups C, D and E, (infected–treated) had 9, 4 and 6, liver and 13, 10 and 9 spleen samples positive respectively after antibiotic treatment. Only group B gave 3 liver

and spleen samples positive at day 38. Three tissues from the gastrointestinal (GI) tract were analysed and these were the ileum, caecum and colon as per the schedule described in methods. The positive control group B (infected–untreated) had 13 positive ileal samples, whereas for groups C, D and E, (infected–treated) the number of samples positive for *B. pilosicoli* was 9, 5 and 6 respectively. The group B positive control (infected–untreated) gave positive samples at day 38 with numbers of the pathogen in the order of 10^3 CFU/g. Interestingly, one bird from the group E test group (infected–treated) also gave positive samples at the third sampling at day 38 within the order of 10^2 CFU/g of the pathogen. The caecum and the colon are regarded as sites of primary colonisation by *B. pilosicoli* and these too were sampled as per the schedule described above. The positive control group B (infected–untreated) had 22 positive caecal samples, whereas for groups C, D and E, (infected–treated) the number of samples positive for *B. pilosicoli* was 16, 15 and 14, respectively. The group B positive control (infected–untreated) gave positive samples at the third sampling at day 38 with high numbers of the pathogen in the order of 10^6 CFU/g. Interestingly, the groups C and E test groups (infected–treated) also gave 2 positive samples each at the third sampling at day 38 within the order of 10^3–10^4 CFU/g of the pathogen whereas the birds from group D were negative. The positive control group B (infected–untreated) had 20 positive caecal samples, whereas for groups C, D and E, (infected–treated) the number of samples positive for *B. pilosicoli* was 14, 12 and 11, respectively. The group B positive control (infected–untreated) gave positive samples at the third sampling at day 38 with high numbers of the pathogen in the order of 10^6 CFU/g. Interestingly, the groups C and E test groups (infected–treated) also gave one positive sample each at the third sampling

at day 38 within the order of 10^3–10^4 CFU/g high numbers of the pathogen whereas the birds from group D were negative.

Comparison of the number of tissues positive and the mean counts were not significantly different between any of the four groups, B–E, at day 9. This indicated that the infection model was effective, as each group was colonised to a similar extent in each of the tissues examined prior to antibiotic treatment. Thereafter, for all tissues and time points group B (infected–untreated) showed higher rates of colonisation than any of the three antibiotic treatment groups, C–E. Pairwise t-tests for each group and time point for *B. pilosicoli* concentration were made in order to asses if Tiamulin treatment was able to significantly reduce colonisation in all tissues. Pairwise t-tests were also used between all infected and treated groups in order to identify dose dependent responses. Results showed that at day 18 and 38, level of colonisation by *B. pilosicoli* in all tissues was significantly higher in group B compared to the other groups C–E indicating that tiamulin was able to significantly reduce colonisation regardless of the dose used. Comparison between each of the antibiotic treated groups showed no significant differences between any tissues on days 18 showing that all tiamulin doses used resulted in similar reduction of colonisation. However, on day 38 a non-significant ($p= 0.087$) decrease in infection was shown for caecal samples when comparing either group C and E with group D.

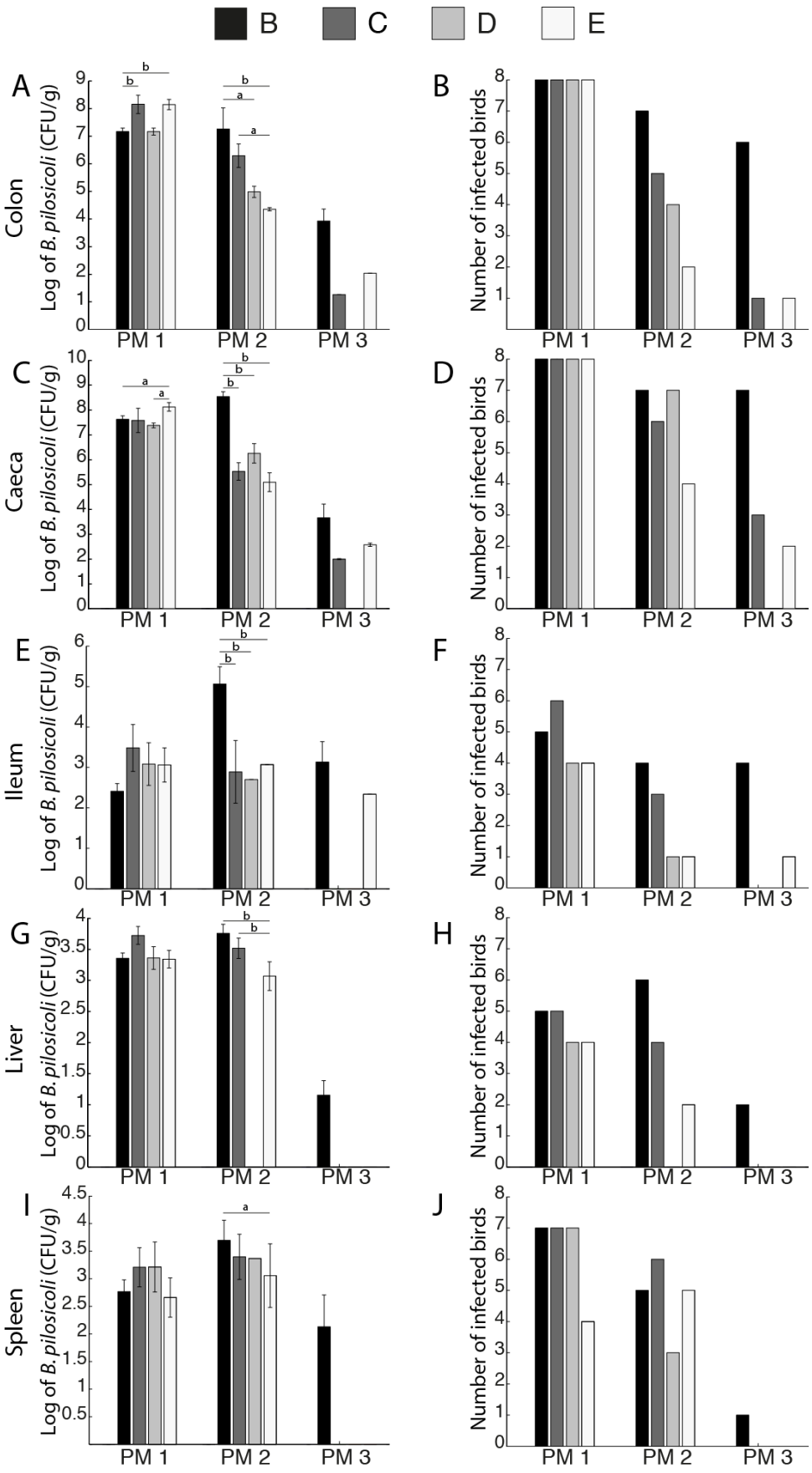
B
C
D
E
A
Colon
Log of B. pilosicoli (CFU/g)
B
Number of infected birds
C
Caeca
Log of B. pilosicoli (CFU/g)
D
Number of infected birds
E
Ileum
Log of B. pilosicoli (CFU/g)
F
Number of infected birds
G
Liver
Log of B. pilosicoli (CFU/g)
H
Number of infected birds
I
Spleen
Log of B. pilosicoli (CFU/g)
J
Number of infected birds
PM 1
PM 2
PM 3

Figure 4.5: Number of birds positive and counts of *B. pilosicoli* in tissues from infected birds. The number of birds subjected to post-mortem examination at each time point was 8. The mean count of *B. pilosicoli* isolates is given from the tissues collected from positive birds only. Student t-test between pairs of data sets were performed and are marked on the figure where the p values are a=or b 0.01 and b=or b 0.05. PM1 = period of dosing with *B. pilosicoli*, PM2 = period of antibiotic treatment and PM3 = period after antibiotic treatment.

4.3.8. Egg productivity

The data regarding egg productivity is given in Table 4.1. There did not appear to be any significant delay in the onset of egg production due to infection with *B. pilosicoli*. In fact, the uninfected–untreated control group A was the last to come into lay although, given the sporadic nature of laying in all groups initially, this observation should not be given any weight. As the number of birds in each test group changed through the study, the data shown in Table 4.1 are based on mean values per group. In terms of numbers of eggs laid, there was no significant difference (p = 0.85) in egg production between any groups at any time during the study. There was no evidence that the egg staining score of the infected– untreated group was significantly worse than the treated groups. However, there were significant differences (p=0.015) between the scores for the three treated groups, with group C giving the lowest score and group D the highest. The statistical analysis of the egg weight data was affected by the non-normality of the data, making ANOVA unsuitable. A Kruskall–Wallis equality of population's analysis of the means of the groups for the entire period from day 18 onwards showed no significant difference (p= 0.172) between groups. However, the mean egg weights of group B (infected– untreated group) were less than the negative control group A (uninfected–untreated) for the entire study period as were each of the treated groups for approximately 14 days after

treatment. However, by the end of the study, the egg weights of all five groups were very similar. Interestingly, the egg weights of group C that was treated with the lowest concentration of Denagard® Tiamulin showed the highest weights of the treated groups but this trend was not significantly different.

Table 4.1: Egg productivity data.

Interval (Study day)	Measure	A	B	C	D	E
13-17	Mean number of eggs per bird	0	0	0	0.04	0.06
	Mean egg staining scores per bird	0	0	0	1.1	1
	Mean egg weight per bird	0	0	0	41.48	41.9
18-24	Mean number of eggs per bird	0.15	0.17	0.27	0.11	0.31
	Mean egg staining scores per bird	0.21	0.73	0.82	1.55	1.33
	Mean egg weight per bird	46.07	42.74	47.9	42.86	45.51
24-38	Mean number of eggs per bird	0.51	0.61	0.66	0.57	0.69
	Mean egg staining scores per bird	0.74	1.04	0.9	1.38	1.03
	Mean egg weight per bird	50.79	47.33	50.37	47.97	48.24

4.4. Discussion

B. pilosicoli has been identified as one of three anaerobic spirochaetes that are considered to be pathogenic in poultry and responsible for the disease AIS, the other two being *B. intermedia* and *B. alvinipulli* (Swayne et al., 1995; Stanton et al., 1998; Hampson and McLaren, 1999; Stephens and Hampson, 2002). AIS has been associated with delayed onset of lay, reduced egg weights, diarrhoea, faecal staining of eggshells, reduced growth rates, increased feed consumption and non productive ovaries (Davelaar et al., 1985). However, symptoms can be mild and relatively non-specific and, in this study, infection with *B. pilosicoli* alone induced some, but not all of these symptoms. Symptoms when induced were generally mild but the most striking and statistically significant impact was the reduction of growth rates of the

infected birds. Onset of lay was not affected, but egg weights were reduced although with the numbers collected the differences were not statistically significant. Some diarrhoea was observed but this was not particularly severe and there was no significant staining of eggshells. This may suggest the model developed by Mappley et al. (2013) may not produce full symptoms or the strain used was not severely virulent. Of importance, this model did induce reduced growth rates and colonisation of deep tissues as well as the gastro-intestinal tract. All three concentrations of Denagard® Tiamulin had the same effect of highly statistically significant reductions in colonisation and faecal shedding that resulted in recovery of growth.

B. pilosicoli strain B2904 was fully susceptible to Denagard® Tiamulin with an MIC of 0.125 μg/ml that was determined by the methods of Pringle et al. (2012) prior to use in the model. Given this strain was a recent field isolate and was retained with few passages, it was reasonable to assume that this was a suitable candidate for the studies reflecting the likely outcome of other sensitive field isolates and, furthermore, the genome sequence of this strain (Mappley et al., 2012) indicated there was no evidence of mutation leading to resistance. It is regrettable that the study did not encompass an analysis of emergence of resistance, if any, especially as the sensitivities of *B. pilosicoli* of poultry origin remain little studied compared with that in pig production where it has been noted that resistance has emerged (Hampson et al., 2006b). At the recent International Spirochaete Conference held in the UK in 2013 (Anon, 2013), reports indicated MIC50, MIC90 and MIC range values for *B. pilosicoli* of poultry origin to be 0.62, 0.25 and 0.0075–4.0 μg/ml respectively

(Burch and Klein, 2013a). Furthermore, when Denagard® Tiamulin was given to chickens at 250 ppm in drinking water for 5 days, the concentration achieved in caecal contents was 0.69 µg/ml (Burch and Klein, 2013a). It was shown that this caecal concentration was successful in eliminating *B. pilosicoli* with an MIC of 0.125 µg/ml (Burch and Klein, 2013b). The study here focused on microbiological factors in the main and the opportunity to add pharmacokinetic studies would have been useful.

The impact of Denagard® Tiamulin on the recovery of the infectious agent is perhaps the most important data regarding control of the infectious agent. The data are compelling in that the statistical comparisons of the number of cloacal positive swabs after dosing with *B. pilosicoli* and prior to Denagard® Tiamulin treatment showed that all four groups were colonised with similar frequency as each other with *B. pilosicoli* B2904. Thus, treatments with Denagard® Tiamulin are directly comparable between each of the groups. All three concentrations of Denagard® Tiamulin had a profound impact in reducing the number of cloacal swabs that were positive for viable *B. pilosicoli*. Given that colonisation is measured by the extent of excretion of viable *B. pilosicoli*, it is not unreasonable to assume that the number of positive swabs is potentially a close approximate measurement of the state of colonisation of the gastro-intestinal tract in each study group of birds. From the swabbing data, therefore, we can conclude that Denagard®Tiamulin reduced colonisation from 60% positive swabs to approaching zero during the course of Denagard® Tiamulin treatment. The data indicate that there is a concentration dependent effect in that the highest concentration of Denagard® Tiamulin (227 ppm)

resulted in the greatest reduction followed by 113 ppm and then 58.7 ppm. The number of positive swabs from the group B infected–untreated control remained high and did not drop below 30% positivity for the duration of the study. These data are highly significant statistically and do correlate well with the bacteriological data obtained from tissues examined following post-mortem examination.

It is interesting that irrespective of the concentration of Denagard® Tiamulin, the three treatment regimens caused a dramatic decline in positive swabs during treatment. Beyond treatment there continued sporadic shedding of *B. pilosicoli*. There was a direct correlation between the number of cloacal swabs positive and the concentration of Denagard® Tiamulin and this lends support to using ~225 ppm as an appropriate dosing regimen for control of AIS caused by *B. pilosicoli*, certainly for strains with similar physiology to *B. pilosicoli* strain B2904. It is not possible to comment on treatment across a wide variety of isolates nor other *Brachyspira* species but if it is assumed B2904 is typical of all *Brachyspira* that infect poultry, it may be possible to infer that ~225 ppm is an appropriate dose. This dose is supported by data of Burch and Klein (2013a,b).

Pooled freshly voided faeces were tested for the presence of *B. pilosicoli* and the organism was detected as anticipated in the vast majority of samples from the positive control (infected–untreated) group B, very infrequently from the groups C, D and E (infected–treated) and not at all from the negative control group A (uninfected–untreated). This indicates the environmental burden of this pathogen is reduced by Denagard® Tiamulin treatment. Also, residual antibiotic in faeces may continue to have a suppressive effect on survival of *B. pilosicoli* which being a

fastidious anaerobe is less likely to survive for extended periods in the environment. Also it is not clear whether residual Denagard® Tiamulin in the faeces suppressed recovery of viable organisms when plating samples for detection by culture. Caution need be expressed however as this study did not show complete elimination of the pathogen from the birds at day 38 suggesting either re-infection from the environment or some latency within with bird. Perhaps additional treatments such as disinfection could be considered along with antibiotic treatment. This is worthy of further analysis as this may aid in reduction of in-house bird to bird spread of the infectious agent. Nevertheless, these data strongly indicate that the environmental burden from freshly voided faeces is low and this will contribute positively to reduced transmission.

The results of this study indicated that cloacal swabbing is a good indicator of GI colonisation that could be use as a quick diagnostic for *Brachyspira* infection. However, *post-mortem* examination of intestinal tissues is still the best method to assess the absence of colonisation by *B. pilosicoli* due to the bacteria ability to attach to the intestinal cell wall. Indeed, when reviewing the tissue culture data there is a clear difference in potential interpretation. In earlier work in the author's laboratory on *Salmonella* infections in chickens it was established that cloacal swabbing could only be used as a rapid means of determining likely shedding into the environment of the organism (Cooper et al., 1994a, 1994b, 1995; Allen-Vercoe et al., 1998; Allen-Vercoe and Woodward, 1999) rather than an accurate estimate of presence in the GI tract or actual colonisation. The data generated in this study reinforces this. Here, the data indicate differences between the data derived by swabbing and that from

tissue analysis where there is a higher burden of *B. pilosicoli* in tissues than from swabs. It is therefore important to recognise the differences in the outcome generated by the methods used. *B. pilosicoli* probably does not colonise cloacal tissues, and here we mean colonisation in the true sense of being intimately attached to the epithelium. However, analysis of tissues further up the GI tract did enumerate bacteria and this was uniformly higher than that obtained by cloacal swabbing. We suggest that swabbing enumerates transient bacteria being shed from sites of probable intimate colonisation further up the GI tract. This reduction is significant as it shows the antibiotic is having an effect. Denagard® Tiamulin treatment did not eliminate *B. pilosicoli* possibly because of some protective effect of the intimate attachment but the data do provide very clear evidence that Denagard® Tiamulin reduced the burden of *B. pilosicoli* in a dose dependent way in the GI tract. Whilst we have inferred intimate epithelial association of *B. pilosicoli* based on previous findings (Mappley et al., 2013) we did not undertake confirmatory histopathology in this study.

Denagard®Tiamulin treatment lead to a dose dependent and notable reduction of infection by day 18. However, the treatment did not completely eliminate the infection as *B. pilosicoli* were present in GI tract tissues (ileum, caeca and/or colon) in between 12.5 and 25% of birds analysed at post-mortem examination on day 38, some twenty days after dosing. It is not possible to determine whether this is the 'tail' of the elimination process [caused by immunity, on-going effects of the antibiotic or gut microbiota exclusion] or re-infection from the environment. As discussed above, given the failure to detect viable *B. pilosicoli* from the pooled

faeces from the treated groups, it is reasonable to assume the organism does not survive well in the environment and is less of a threat for re-infection. Thus, it is possible *B. pilosicoli* was not completely eliminated by Denagard® Tiamulin treatment. Perhaps a longitudinal series of post-mortem examinations beyond day 38 should be undertaken.

Another significant finding regarding infection and its reduction by Denagard®Tiamulin treatment was the fact that no liver or spleen samples taken from each of the three treatment groups were infected at the close of the experiment, day 38. The positive control group B (infected–untreated) was positive for 3/8 and 1/8 liver and spleen samples, respectively. Also the bacteriological burden for these organs was in the region of 103 CFU/g. The discussion above raises concern regarding recurrence of infection and the possibility that the environment or the GI tract that is not cleared may be a source of re-infection. Of importance here is that the deep tissues that could be a site for long-term quiescent colonisation are completely cleared by Denagard® Tiamulin. However, other potential sites of long-term colonisation were not analysed. Other spirochaetes such as the *Leptospires* are known to colonise kidney, joints and even ocular fluid and perhaps these may be included in future studies. However, of importance was that Denagard® Tiamulin treatments at all concentrations were highly effective at eliminating *B. pilosicoli* colonisation of liver and spleen that is likely to impact on reducing recurrence of disease after treatment. Also, it would have been of considerable value to assess the innate and induced immune responses as induction

by infection should mediate a protective effect and reduce the likelihood of reoccurrence of infection.

The mean weight gain was clearly suppressed in group B but it is interesting to note that the mean weight gain of each of the three infected and Denagard®Tiamulin treated groups (C, D and E) were as good if not better than of the uninfected–untreated control. Indeed the mean weight gain of group E that was infected and treated with the highest concentration of Denagard® Tiamulin was the highest of all study groups. This outcome is under further investigation. Feed and water intake showed no significant differences between any groups. This suggests that the impact of infection and treatment did not unduly reduce appetence. However, of importance was the impact on feed conversion for the group B positive control (infected–untreated) that clearly showed suppression in overall weight gain. The infected–treated groups C, D and E, irrespective of the Denagard® Tiamulin dose, clearly recovered performance to the same or above that of the negative (uninfected–untreated) group A. Whilst, perhaps, not of such significance to layers, this finding may be of considerable interest to broiler productivity and this needs investigation.

The production of eggs and their quality was assessed, but the findings were equivocal. There were no differences in numbers of eggs produced between any of the five study groups. Staining was not excessive in any study group and given the scoring is subjective it is unclear why group C (infected–treated 58.7 ppm) had the lowest egg staining score and group D (infected–treated 113 ppm) had the highest

egg staining score. These findings are surprising given that positive control group B (infected–untreated) produced soft/watery faeces more and for longer than any other group. The findings therefore are counterintuitive.

In summary, this study has demonstrated that Denagard® Tiamulin at three doses was effective at reducing *B. pilosicoli* infection of chickens in the animal model used. Whilst the symptoms induced in the positive control were mild the treatment with Denagard® Tiamulin clearly reduced the burden of infection in deep tissues, reduced shedding, improved weight gain and improved egg weight. These parameters are of economic importance to producers and suggest Denagard® Tiamulin is a viable treatment of AIS.

4.5. Reference

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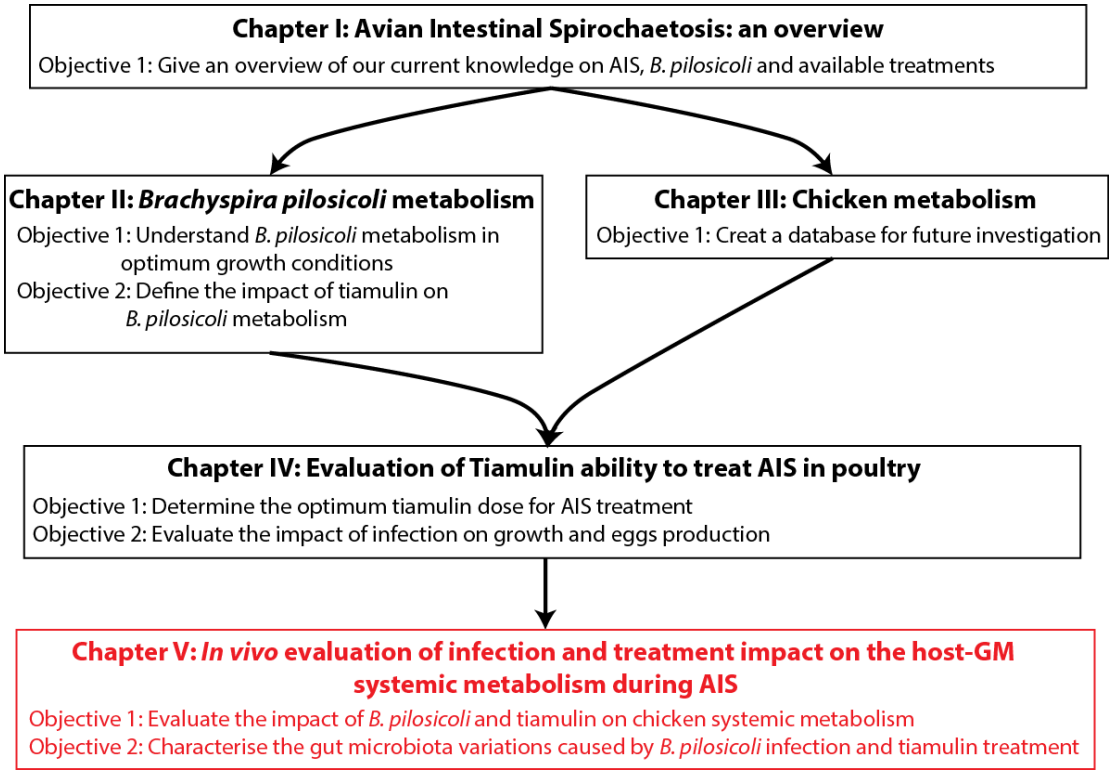
Impact of infection and tiamulin treatment on host metabolism:

The final step of this work was to evaluate the impact of both: infection and antibiotic treatment on host systemic metabolism and and caecal microbiota populaition. Biopsy samples from *post mortum* evalution of the previous study were analysed using high resolution ^{1}H NMR and caecal microbiota was characterised with 16SRNa next generation sequencing. Manuscript will be submitted shortly to the ISME journal.



Chapter 5: Infection by *Brachispira pilosicoli* and antibiotic treatment reorient profoundly the caecal microbiota composition and modify host energy metabolism

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Abstract

Infection of the digestive track by gastro-intestinal pathogens results in the development of symptoms ranging from mild diarrhea to more severe clinical signs such as blood loss, severe dehydration and potentially death. Antibiotics are often used to tackle this type of disease by reducing the numbers of the specific pathogen but as a consequence also disturbing the 'normal' gut microbiota. The metabolic impact of both symptom development and recovery of the host gut microbiota by antibiotic treatment are yet not fully understood. In this exemplar study, we evaluated the impact of infection of a chicken model by the gastro-intestinal pathogen *Brachyspira pilosicoli* and its resolution by antibiotic treatment with tiamulin. Using high-resolution ^{1}H nuclear magnetic resonance (NMR) systemic metabolism was studied whilst 16S next generation sequencing (NGS) was used to assess the composition of the caecal microbiota. Infection induced a modification of systemic host energy metabolism characterized by the utilization of glycerol as a glucose precursor explaining in part diarrhea associated dehydration and animal weight loss. An unexpected finding related to antibiotic treatment was the triggering of an increased VLD/HDL ratio in the host. The caecal microbiota showed a significant shift upon infection and there was strong reduction of CM diversity after antibiotic treatment. In this study, we demonstrated how infection and antibiotic treatment could both impact host systemic metabolism in line with CM composition causing phenotypic and health modification. Thus, these results provide a better understanding of symptom development post bacterial infection.

Subject category: Microbe-microbe and microbe-host interactions

Keywords: microbiota, metabolism, antibiotic, chicken, energy

5.1. Introduction

Gut microbiota (GM) composition is known to strongly influence host health by a wide range of mechanisms ranging from control of immune functions(Hooper et al. 2012), metabolic homeostasis(Ley et al. 2006; Cani & Delzenne 2009) and including drug metabolism(Claus et al. 2011). Even if generally stable within a species, the GM composition can be strongly impacted by exposure to environmental stress (Spor et al. 2011) (nutrition, xenobiotic and infection). Modification of this ecosystem can affect host health due to the symbiotic relationship existing between the host and its gut flora(Spor et al. 2011). For instance, infection of the digestive track by bacteria can be asymptomatic but also induce severe health damage depending on severity of infection and pathogenicity of the specific bacterial pathogen. Furthermore, infection is generally associated with bacterial dysbiosis of the digestive track(Antharam et al. 2013) but the impact of such modification on host metabolism and symptom development such as weight loss is still poorly understood. Reduction of symptoms is generally observed post antibiotic treatment due to reduction in the pathogenic bacteria and the decline of the sequel of their infection. However, antibiotic use is also related to loss of the GM diversity that has been in several cases linked to further host metabolic weakening(Cox et al. 2014).

Avian intestinal spirochaetosis (AIS) is caused by the colonization of bird's lower digestive track by the pathogen *Brachyspira pilosicoli*(Le Roy et al. 2015; Mappley et al. 2014). The bacterium attaches to the cell wall and generally triggers diarrhea associated with decreased growth rate and egg production. The most used treatment to tackle this disease is Tiamulin™, an antibiotic of the pleuromutilin

family that inhibits protein synthesis by binding to the 50S region of the ribosome(Poulsen 2001; Pringle et al. 2012). Only a few studies have shown its efficiency in chickens despite it intensive use to treat avian flocks in industry(Stephens & Hampson 2002; Burch et al. 2006). To date this disease and its treatment have been little studied and remain poorly understood. Indeed, the causality of symptoms such as weight loss and decreased egg production are still partly unexplained. Furthermore the mechanism by which Tiamulin™ treatment can reduce symptoms but also increase growth rate and egg production are not known. Understanding such factors would be of great interest in order to understand metabolic mechanisms triggering symptom development during infection.

In one of our recent studies (Woodward et al. 2015), we evaluated the efficiency of three Tiamulin™ doses to treat laying hens orally challenged with *B. pilosicoli* B2904. This study revealed that infection was associated with decreased growth rate and that birds treated with Tiamulin™ were recovering from infection regardless of the dose used while weight gain was only observed for the two highest doses. We evaluated that this study could be used as a model to understand systemic metabolic response to digestive track infection and antibiotic treatment using NMR-based metabonomics. Metabonomics was defined in 1999 by Nicholson et al(Nicholson et al. 1999) as the "quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification". This is a recent biological field that allows a non-targeted evaluation of metabolic fluctuation occurring in biological system by coupling analytical methods such as NMR spectroscopy and multivariate statistics. To date this technique has been used to evaluate the impact of infection(Wang et al. 2004),

antibiotic treatment(Yap et al. 2008; Romick-Rosendale et al. 2009), diet changes(Claus & Swann 2013) or gut microbiota composition(Claus et al. 2011) on host systemic metabolism allowing a wider comprehension of how these factors can potentially influence host homeostasis. In addition the evolution of caecal microbiota composition in response to infection and treatment was followed using 16S next generation sequencing, an essential step, as the gut microbiota are inextricably linked to host's metabolic responses. Both analyses provided new insight into the impact of infection and antibiotic treatment on host health, explaining physiological response to both bacterial and chemical exposure.

5.2. Materials and methods

5.2.1. Animal study and experimental design

The experimental plan followed for the study was described previously by Woodward et al(Woodward et al. 2015). All samples preparation and data analysis regarding general impact of infection and Tiamulin™ treatment on birds' infection, growth, condition, eggs production, water and food consumption are also explained in the same paper. However, for reading clarity, the experimental plan presenting the various groups and samples collection point are summarized in Figure 5.1. Five groups of 30 16-17 weeks old NovoGen Brown commercial layers sourced from a commercial supplier (Tom Barron Ltd, UK) were housed in separate rooms at APHA (Addelstone, Surrey, UK). The five groups were used in the following treatments: Group A: Untreated, uninfected controls; Group B: Untreated, infected controls;

Group C: Infected + Tiamulin™ at 62.5ppm; Group D: Infected + Tiamulin™ at 125ppm; Group E: Infected + Tiamulin™ at 250ppm.

After crop neutralization, birds were challenged by oral gavage with *B. pilosicoli* for five days every two days. One week after the end of the challenge, group C, D and E received Tiamulin™ in drinking water for five days. Birds were then kept for observation for three more weeks. Feed was un-medicated layer pellets (Dodson and Horrell) and water was given from the mains supply, both were given *ad libitum*. Birds were housed according to Home Office guidelines (Home office license -PPL 70/7249-) and all procedures were performed in compliance with the Animals Scientific Procedures Act, 1986.

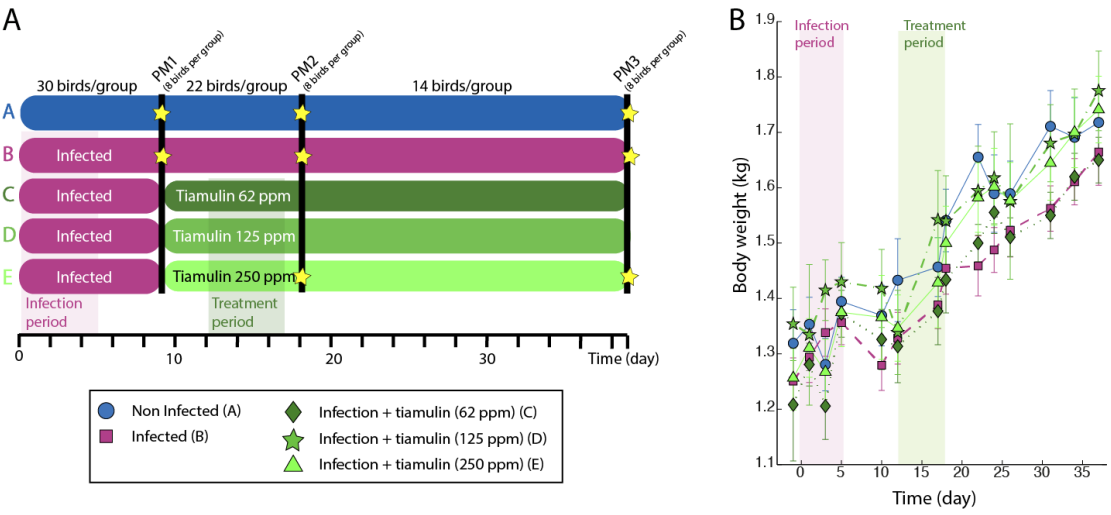

Figure 5.1: Experimental plan (A) and birds weight (B).

5.2.2. Sample collection from animal study

Biopsy, plasma and faecal samples were collected during *post-mortem* examination at three time points: the day after the end of the infection process (PM1), the day after the end of the antibiotic treatment (PM2) and at the end of the study (PM3)

(Figure1A). For each group and time point eight birds were selected randomly and killed humanely. Blood was sampled first by direct puncture by needle with syringe of the heart and serum was frost -80°C. Tissue biopsy samples and faecal samples (approx. 1g) were snap frozen in liquid nitrogen and then stored at -80°C.

5.2.3. Sample preparation for NMR

Kidney, pancreas, spleen and liver polar metabolite extraction was done by homogenizing 0.1 g of biopsy samples in 1ml of 3:1 (v/v) methanol/H_2O solution using a tissue lyser. After centrifugation (10 min at 12 000 x g), supernatants were dried in a speed vacuum (eppendorf) and resuspended in 600 µl of phosphate buffer (0.2M) containing 90% of D_2O and 10% of H_2O plus 0.01% of sodium 3-(tri-methylsilyl)-propionate-2,3-d4 (TSP used as internal standard). Samples (0.5 ml) were then transferred to 5 mm NMR tubes for acquisition. Plasma samples were mixed to phosphate saline buffer with 90% D2O at a 2:1 (v/v) ratio, 0.5 ml were then transferred to 5 mm NMR tubes. 0.0150g of liver biopsy were added with phosphate buffer in spinner for solid state NMR spectroscopy.

4.2.4. NMR spectroscopy

For tissues ^{1}H-NMR spectra were acquired on a 700 MHz Bruker Advance Spectrometer using a standard noesypr1D pulse program with water presaturation (relaxation delay of 2 s and 100 ms of mixing time). Plasma 1D NMR spectra were acquired using a Carr-Purcell-Meiboom-Gill (CPMG) pulse. Liver biopsies were acquired on 500 MHz Bruker Advance Spectrometer using a HR MAS prob. Spectra

were acquired using a standard noesypr1D pulse as well as CPMG. For all matrixes, 2D NMR experiments were run on chosen samples to help metabolites identification. Spectra were acquired with using 256 scans with 16 dummy scans (DS). All spectra were recorded as 64k data points (15 ppm).

5.2.5. Next generation sequencing 16S rRNA

DNA from faecal samples were extracted using PowerSoil® DNA Isolation Kit (*MO BIO Laboratories, Inc)*. To ensure DNA samples quality, PCR of the universal V4 region of the 16S rRNA was performed post extraction (cycling conditions: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min; followed by 72 °C for 8 min) and concentration was assessed using a Nano drop. PCR primers were the following:

U515F: 5'-GTGYCAGCMGCCGCGGTA

U927R: 5'-CCCGYCAATTCMTTTRAGT

The V4 and V5 region of the 16S rRNA region was then sequenced on the GS FLX Titanium platform according to the manufacturer's instructions (Roche Diagnostics) and in accordance by the method described previously by Ellis *et al*. (Ellis et al, 2013).

The data were processed using the Quantitative Insights Into Microbial Ecology software package (QIIME v1.3.0) implemented in Biolinux 6. Taxonomy was assigned according to the RDP classifier and the relative abundance of taxa at multiple levels of resolution (phylum, order, family, etc) was determined for each sample. Jackknifed beta-diversity was calculated using the unweighted UniFrac metric a re-sampling size of 250. Other statistical analysis such as PCA was performed using MatLab.

5.2.6. Statistical analysis

For metabonomics analysis, after exponential window with line broadening of 0.3 Hz and Fourier transformation, spectra were individually phased and base line corrected on the software MestReNova. Spectra were then transferred to Matlab (the Mathwork ® 2013a) where they were calibrated on TSP (δ 0.00) for all tissue extract, lactate (δ 1.33) for plasma and the H^1 proton of α-glucose (δ 5.23) for liver biopsy. Spectra were normalized for each matrixes individually using a probabilistic quotient method. Metabolic variation between samples was evaluated in a first place using principal component analysis (PCA). This step was also used to remove potential outliers. When group clusters of interest were spotted, orthogonal projection to latent structure discriminant analysis (O-PLS DA) was used to evaluate metabolic variation between groups using NMR spectrum as a matric of independent variables and infection or treatment as a prediction vector. Metabolites identification was done based on previously published chicken metabolic atlas.

Alpha diversity represents the species biodiversity in a specific in habitat. The determination of the alpha diversity in this study was calculated as the mean of species observed from 10 reads of 16 rRNA NGS.

5.3. Results

5.3.1. Infection induces systemic metabolic response of the host

Systemic metabolic response to infection by *B. pilosicoli* was observed directly after the end of the challenge period (day 6). Infection was associated with a modification of kidney, liver, spleen and plasma metabolome (Figure 5.2A, B, C and D). Livers of

infected birds were richer in glycerol, lactate, choline, succinate and acetate (Figure 5.2A). In the spleen, infection resulted in decreased O-phosphocholine, glutamine and AMP and increased glycerol, uracil, citidine and leucine (Figure 2B). In kidney, infection induced an increase glycerol, uracil and xanthine content, concomitant with a decrease in inosine (Figure 5.2C). Finally, increased betaine and glycerol concentration were also associated to infection in plasma (Figure 5.2D). Two weeks after the end of infection period (PM2), kidney, liver and spleen of infected birds recovered their metabolic homeostasis as, no metabolic variations were observed in response to infection. However, the glucose level dramatically dropped in plasma of infected birds (Figure 2E). Finally, just after infection (PM1), the content of the colon of infected birds was richer in polysaccharides and amino acids (Figure 5.3).

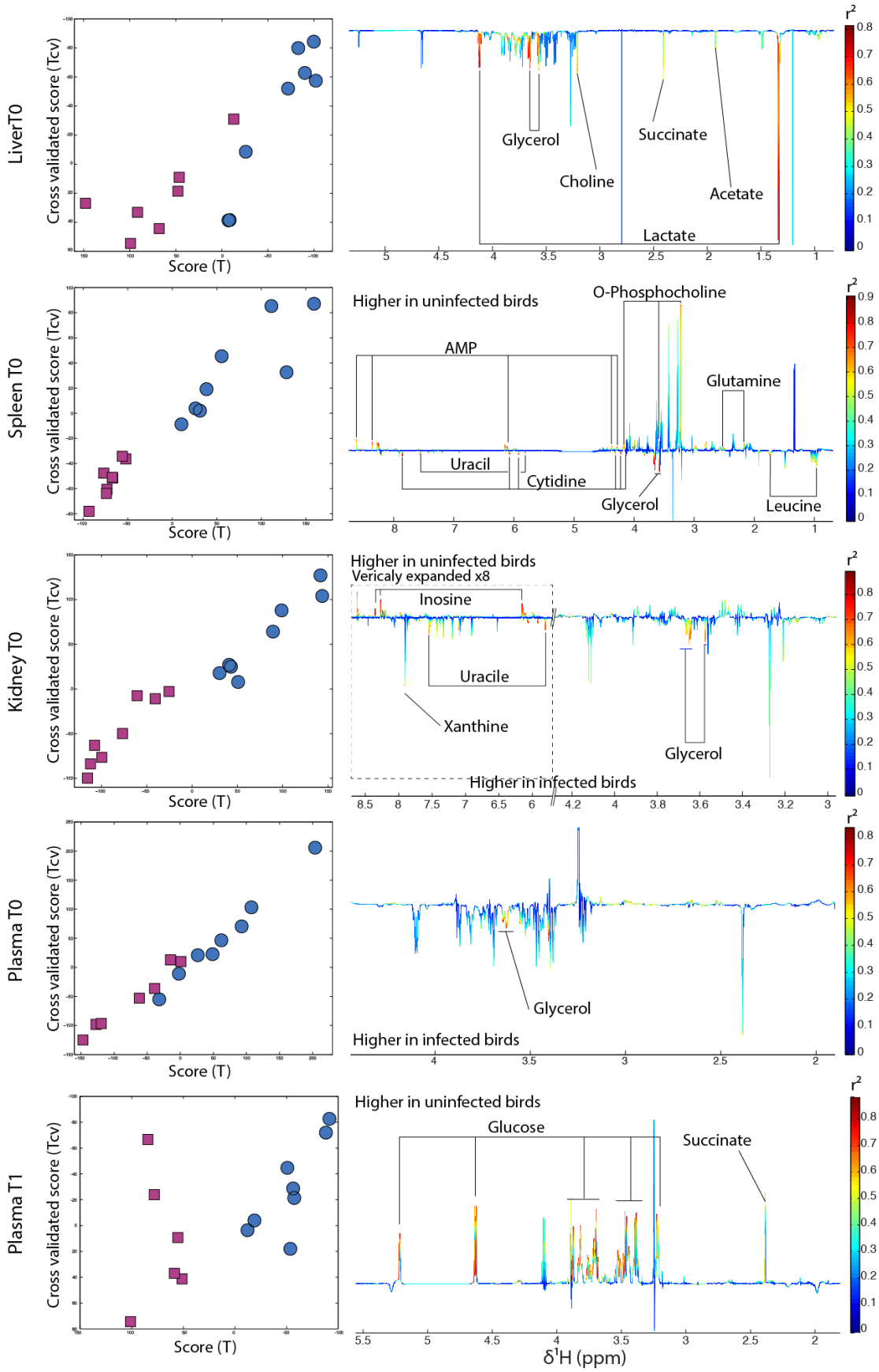

Figure 5.2: *B. pilosicoli* infection is associated with major systemic metabolism modification. (A) Plot of the scores against the cross-validated scores of infected

birds (red square) and uninfected birds (blue circle) and coefficient plot of the discrimination between infected birds (top) and healthy birds (bottom) of the O-PLS-DA model calculated using 1D-NMR spectra of birds' liver at T0 as a matrix of independent variables and infection as a predictor. (B) Same for the spleen. (C) same for the kidney. (D) same for the plasma. (E) same for the plasma at T1.

No other metabolic variation in response to infection was observed in other tissues. Surprisingly, metabolism of gut tissues (colon and caeca) was not affected by *B. pilosicoli* despite infection locality.

By the end of the study it was not possible to metabolically differentiate infected from uninfected birds using metabonomics techniques in any of the previously sited tissue or biofluid.

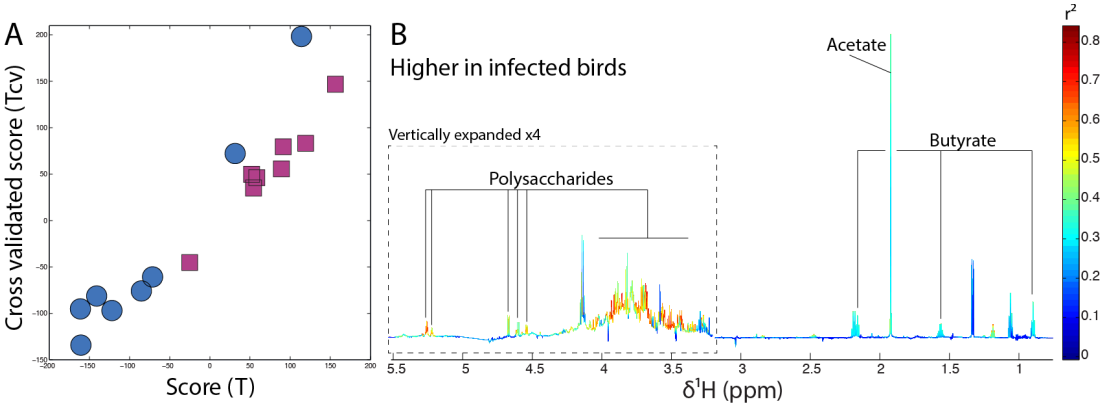

Figure 5.3: Infection modifies GM metabolic activity and polysaccharide intestinal lumen content. (A) OPLS-DA scores against cross-validated scores calculated using faecal water spectra of group A and B at PM2 and infection as a predictor. (B) Loading plot associated to the OPLS-DA model described in A.

5.3.2. Tiamulin™ treatment attenuate metabolic response to infection

We next investigated if antibiotic treatment with Tiamulin™ was associated with any metabolic response of the host to infection. At PM2, a higher plasma level of betaine was observed in response to infection (*p*-value<0.01 –Figure 5.4-). However, birds

infected but treated with Tiamulin™ presented similar plasma level of betaine than the control but interestingly the response observed was not dose dependent.

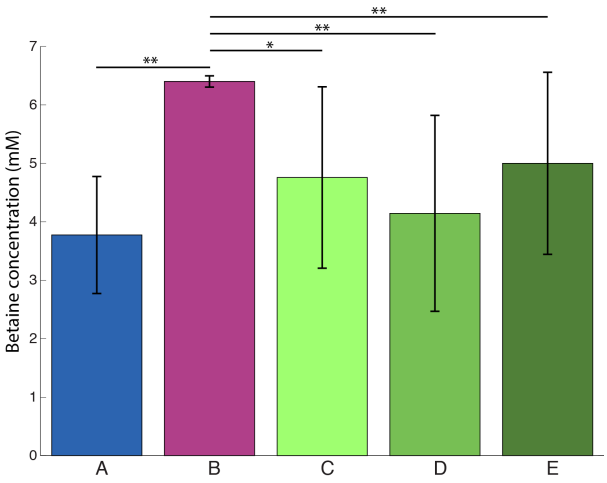

Figure 5.4: Plasma level of betaine at PM2 for all groups. .*, *p*-value<0.05; **, *p*-value<0.01.

In the previous section, it was described that infection induced a glucose level drop in chicken plasma at PM2. Evaluation of average glucose concentration per group at PM2 (Figure 5.5) revealed that Tiamulin™ treatment induced an increase in plasma glucose level. However, the glucose level in plasma of treated birds was still lower than in the control group. Interestingly, the plasma glucose level was inversely proportional to treatment dose.

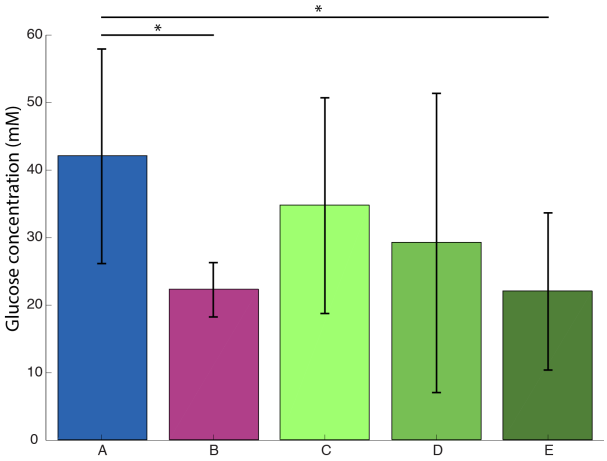

Figure 5.0.5: Glucose plasma level at PM2 for all groups. *, *p*-value<0.05; **, *p*-value<0.01.

5.3.3. Tiamulin™ treatment is responsible for a major shift in lipid metabolism

The PCA score plot displaying the general impact of treatment on plasma metabolic profile using NMR-based metabonomics at PM2 (Figure 5.6A) revealed a clear separation between the scores for the birds treated with antibiotic and for un-treated on principal component 1 (PC1). Plasma metabolic profiles of chickens treated with antibiotic were characterized by increased very low-density lipoprotein (VLDL) and decreased high-density lipoprotein (HDL) level (Figure 5.6A, B and C). Analysis of the same dataset with supervised analysis (O-PLS DA), using antibiotic dose as a predictor (Figure 5.7) revealed that the lipoprotein response to treatment was dose dependent.

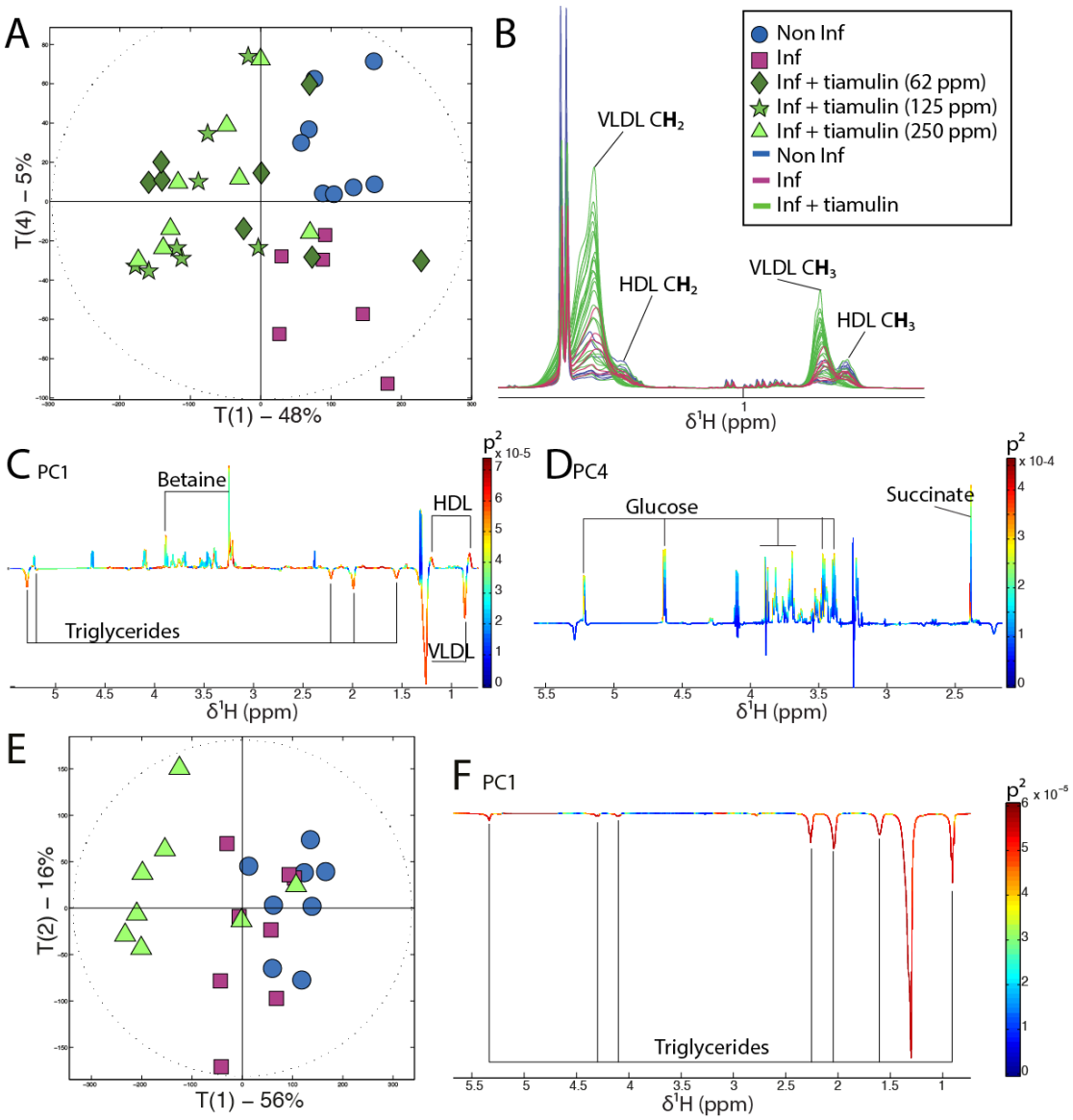

Figure 5.0.6: Tiamulin™ induces plasma metabolic variations. (A) PCA score plot on the first (T1 48%) and the fourth (T4 5%) principal component derived from the model calculated using the 1d-NMR spectra of birds' plasma at T2. (B) Color-coded plot of the plasma 1D-NMR spectra of control birds (blue), infected and non-treated birds (pink) and treated birds (green). (C) Plot of the principal component 1 (PC1) loadings, molecules pointing up positively correlated with PC1, molecule pointing down negatively correlated with PC1. (D) Plot of the principal component 4 (PC4) loadings, molecules pointing up positively correlated with PC4, molecule pointing down negatively correlated with PC4. (E) PCA scores plot derived from the model calculated using the HR-MAS NMR spectra acquired from liver biopsy. (F) Plot of the loadings of principal component 1 (PC1) of the PCA model presented in E.

Since liver is the central regulating organ for cholesterol and lipid metabolism, metabolic profiles of liver biopsy were generated using HR-MAS NMR spectroscopy.

This analysis revealed that the liver of birds treated with Tiamulin™ were richer in lipoproteins than non-treated birds (Figure5.6C and F) confirming the impact of Tiamulin™ on central cholesterol metabolism.

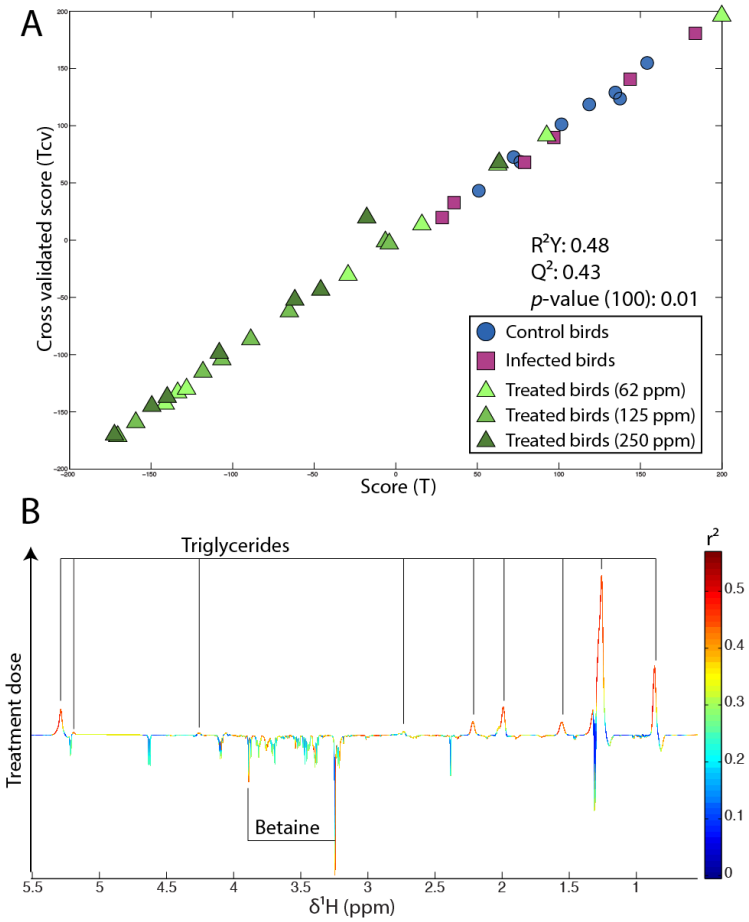

Figure 5.7: Linear plasma response to Tiamulin™ treatment dose. (A) Plot of the scores against the cross-validated scores of the O-PLS-DA model calculated using ^{1}H-NMR spectra of birds at PM2 as a matrix of independent variables and Tiamulin™ dose as a predictor. B. Associated loadings plot.

5.3.4. Tiamulin™ accelerate metabolic aging

When looking at the impact of Tiamulin™ on chicken plasma metabolic profile on the overall study (all groups PM1, 2 and 3), it appeared that age was also a strong source of metabolic variation (Figure 5.8). Age was associated with decreased HDL, glucose,

succinate and lactate level, while VLDL level increased (Figure 5.8). Analysis of the scores (Figure 5.8B) revealed that Tiamulin™ treated birds were metabolically similar to older birds (PM3) at PM2 and that to the contrary, untreated birds had similar metabolic profile to birds from the younger age group (PM1).

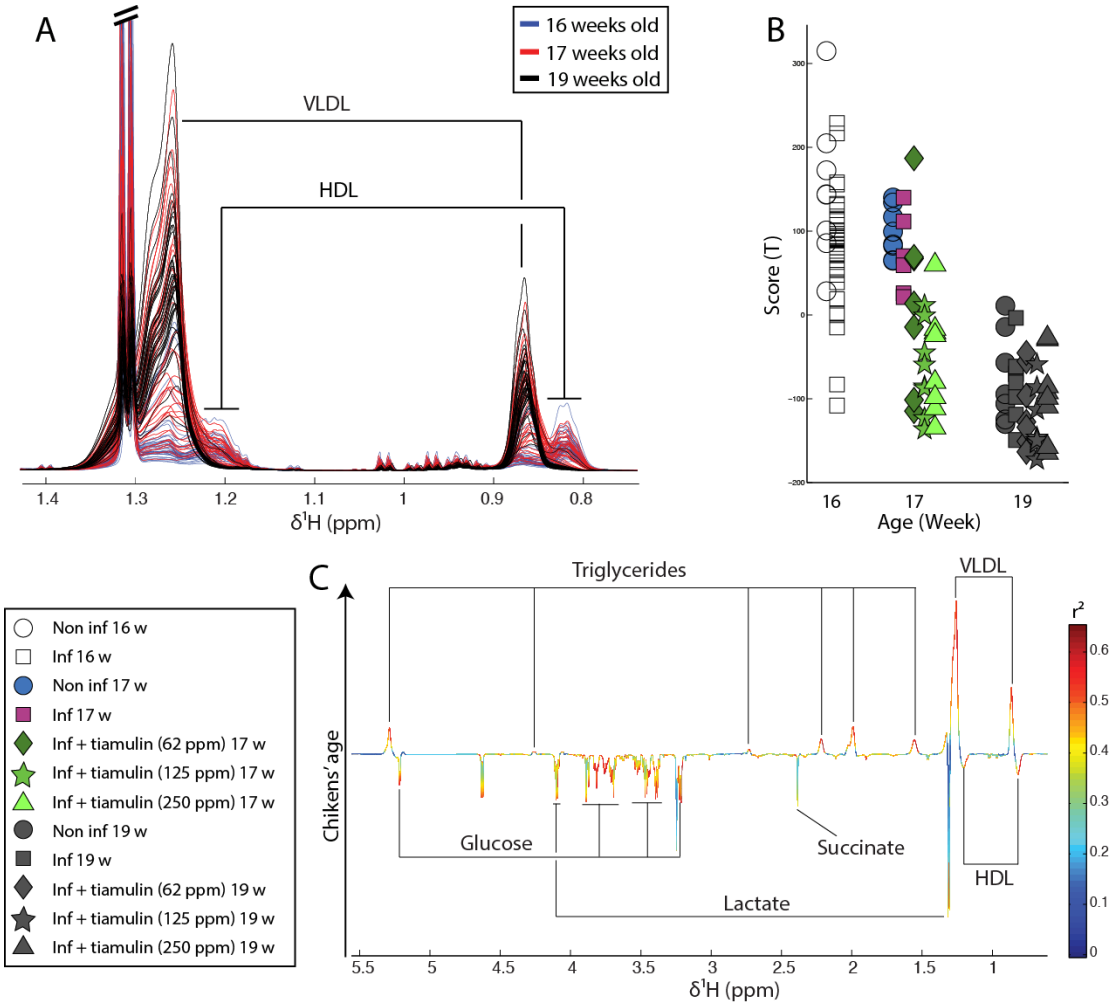

Figure 5.8: Age is related to increased VLDL and decrease HDL and glucose level. (A) Plot of the Colour-coded plot of the plasma 1D-NMR spectra of 16 weeks old birds (blue), 17 weeks old birds (red) and 19 weeks old birds (black). (B) Plot of the scores of the O-PLS-DA model calculated using ^{1}H-NMR spectra of birds at all time point as a matrix of independent variables and the birds' age as a predictor. (C) O-PLS-DA coefficient plot related to the birds age.

5.3.5. Infection and Tiamulin™ shifted CM composition

The composition of the CM population in response to infection and antibiotic treatment was evaluated using next generation 16S sequencing. The CM population was extremely stable through time in the control group as shown by PCA score plots (Figure 5.9A to C) and pie charts (Figure 5.9E).

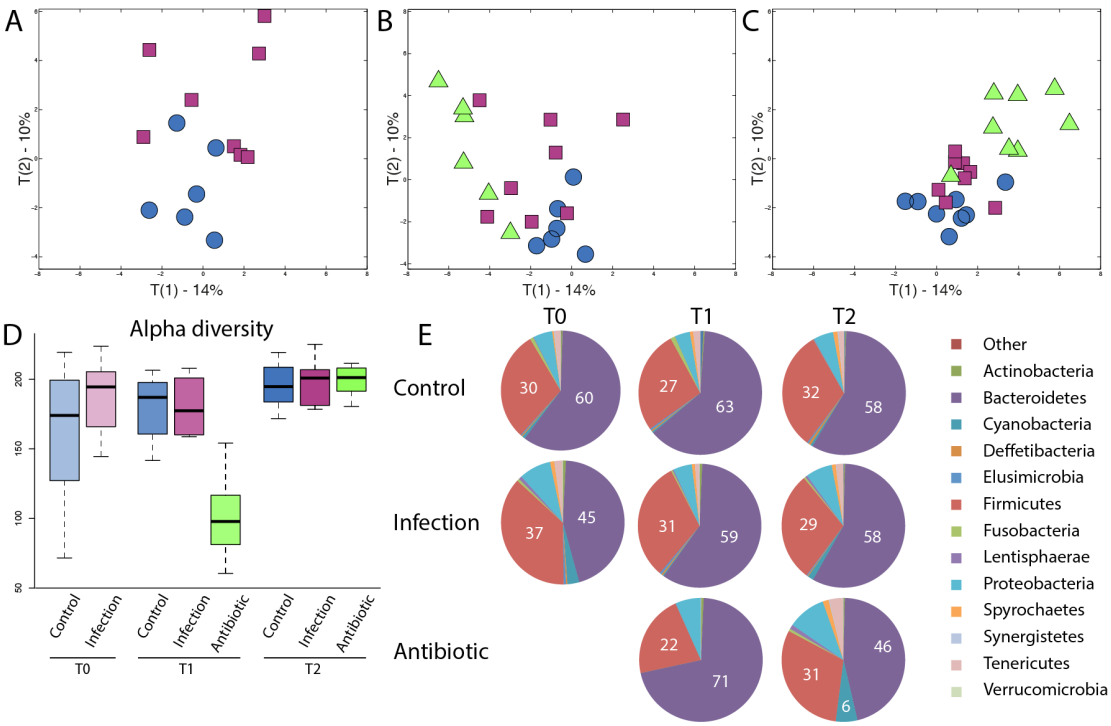

Figure 5.9: Tiamulin™ treatment enhances a profound alteration of gut microbial diversity and population. (A) PCA score plots calculated using the bacterial relative percentage of abundance of OTU at a family level for all birds but displaying only the scores (n=8) of control (blue circle) and infected birds (pink square) post infection (T0). (B) Same PCA score plot than A but displaying only the scores (n=8) of control (blue circle), infected birds (pink square) and treated birds (green triangles) post treatment (T1). (C) Same PCA score plot than A and B but displaying only the scores of control (blue circle), infected birds (pink square) and treated birds (green triangles) three weeks post treatment (T2). (D) Alpha diversity calculated for control, infected and treated birds at each *post mortem*. (E) Pie chart presenting the bacterial relative abundance at a phylum level for each group (control, infected and treated) for the three time points chosen in this study.

Infection was associated with a modification of the commensal caecal microbiota in comparison to control (Figure 5.9A and B), but community balance was reestablished at the end of the study (Figure 5.9C). This modification of the CM was mainly associated with an increase in Lactobacillales, Burkholderiales and Campylobacterales two orders of the Proteobacteria phylum (Figure 5.10).

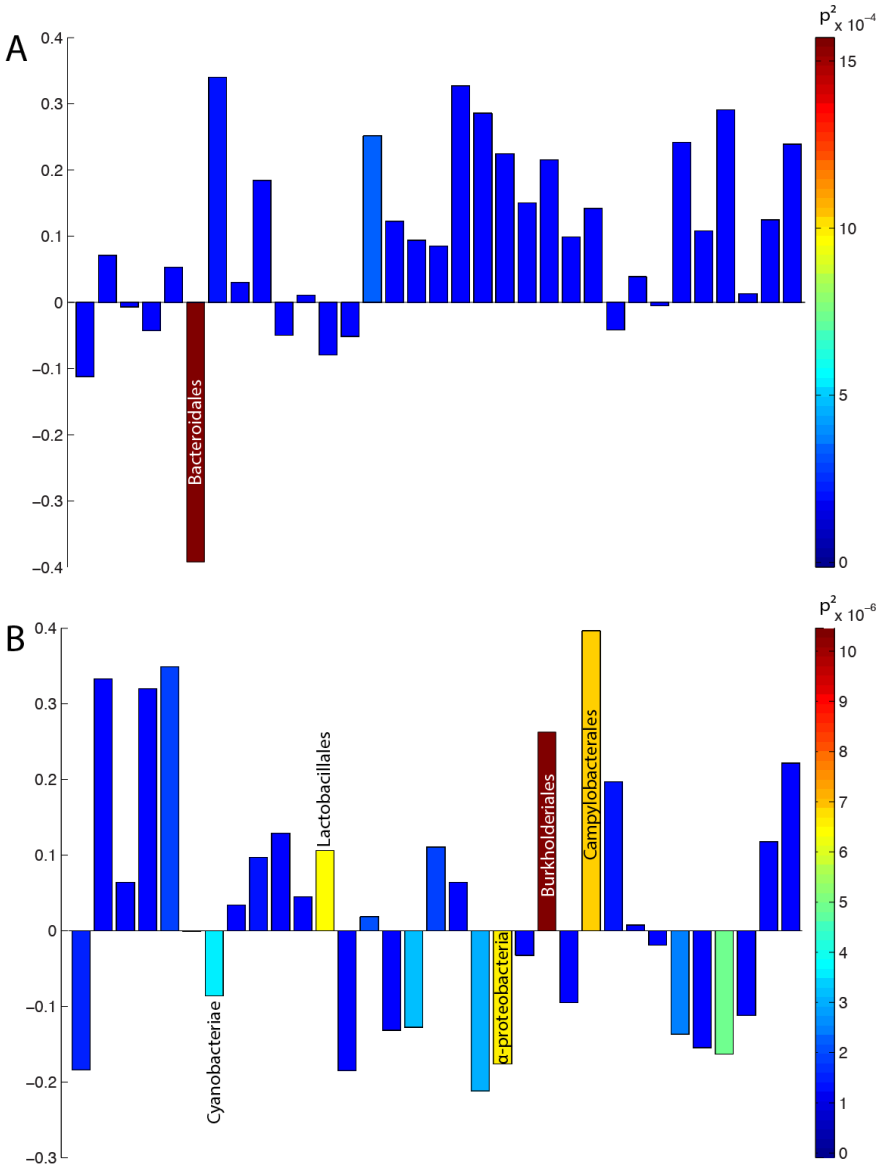

Figure 5.10: Loadings of the corresponding to the 16S PCA scores plot in Figure 5.9 calculating using the OTUs. A, loadings of PC1. B, Loadings of PC2.

After Tiamulin™ treatment the Spirochaetes class (to which belong *B. pilosicoli*) was no longer detectable (Figure 5.11). Yet, this bacterial class had reemerged three weeks after the end of the antibiotic treatment (Woodward et al, 2015). Furthermore, their relative percentage of abundance was higher that in both non-treated groups.

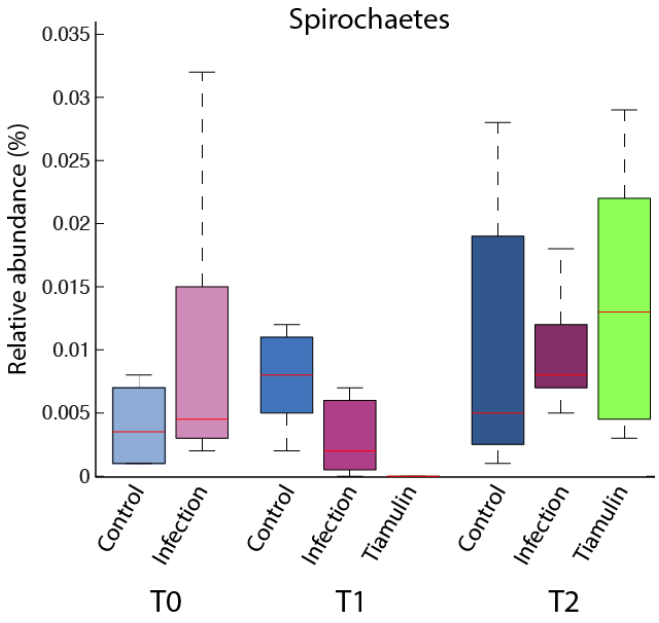

Figure 5.11: Relative abundance in percentage of the Spirochaetes OTU for each treatment group along the study.

Antibiotic treatment was also associated with a dramatic decrease of the bacterial biodiversity in comparison to the two other groups (Figure 5.9D). This loss of alpha diversity was observed straight after the end of antibiotic treatment. However, three weeks after the end of antibiotic treatment caecum microbiota had recovered its level of diversity. Tiamulin™ resulted in a major shift in CM community visible on PCA score plot (Figure 5.9B) and pie charts (Figure 5.9E). This was driven by a

decrease percentage in the relative abundance of Firmicutes (from 30% to 22%) and a drastic increase of the Bacteroidetes Phylum (from 60 to 71 %). The Firmicutes/Bacteroidetes ratio was changed from approximately ½ to 1/3. In the three weeks post antibiotic treatment, CM evolved in term of diversity and composition. However, individuals were not able to regain their normal CM composition (Figure 5.9B and C).

5.4. Discussion

Still relatively little is known of the relationship between the gut microbiota during intestinal disease and recovery after antibiotic treatment and the host metabolism, a knowledge gap that stimulated this study. Gastro-intestinal infections can trigger gut microbiota dysbiosis and are generally associated with symptoms ranging from mild to severe. Compromised growth rate in production animals is often noted. Gut microbiota composition is recognized for having an important role to play in host growth and severe dysbiosis can therefore be responsible for abnormal development (Subramanian et al. 2014; Claus 2013). In this study, we hypothesized that significant decrease growth rate associated with infection were triggered by caecal microbiota dysbiosis resulting in host metabolic response and that it was possible to correct using antibiotic treatment. The model selected for this study was *B. pilosicoli* infection of egg laying chickens that showed significantly decreased growth rate amongst other sequel (Woodward et al. 2015). Infection also induced a strong CM response characterized by an increase in some Proteobacteria many of which are considered as potential pathogens and generally associated with increase

diarrhea risk(Saulnier et al. 2011; Kerckhoffs et al. 2011). These bacteria are able to degrade proteins present in the intestinal lumen partially explaining why its fecal content was richer in amino acids post infection. Intestinal dysbiosis characterized by Proteobactetia richness has been mainly associated with metabolic syndrome(Tremaroli & Bäckhed 2012).

In our study, infection and bacterial dysbiosis was followed by profound host systemic changes. The range of the tissues affected by infection (liver, spleen, kidney and plasma) indicates a systemic metabolic response of the organism to *B. pilosicoli* colonization and dysbiosis. Interestingly increased glycerol levels were noticeable in all compartment cited above. Systemic glycerol increase is a marker of lipolysis in adipose tissues where triglycerides are lysed into lipids and glycerol by lipase enzyme(Moussard 2012). Glycerol is then released in the general circulation to be used a glucose precursor in the liver or/and the kidney. Such a mechanism is generally activated by prolonged low plasma glucose level. GI infection can trigger low glucose absorption due to gut barrier disruption. *B. pilosicoli* is known to strongly affect the intestinal wall(Mappley et al. 2011) and can therefore initiate such impairment of glucose absorption. Indeed in this study as well as a previous one (Le Roy et al. 2013) we observed increased glucose and carbohydrate content in faeces of infected birds indicating their lower absorption level. The polysaccharide increase could also be associated with the ability of *B. pilosicoli* to degrade mucin(Naresh & Hampson 2010; Mappley et al. 2012). Indeed it has been described that this pathogen can degrade the mucin layer thus polysaccharide might be released within the lumen(Bäumler & Sperandio 2016). Furthermore the concomitant increase in butyrate and acetate observed with infection attest a higher

fermentation of these polysaccharides and therefore a modification of the GM metabolic activity. Plasma glucose concentration is highly controlled and regulated since its level needs to be maintained to sustain brain and muscular activity. To sustain the glucose level alternative pathways reducing fat storage such as the one described above are activated. Thus, the use of glycerol as an energy precursor in response to infection could be directly linked to the decreased growth rate observed in chickens colonized by *B. pilosicoli* as described previously (Mappley et al. 2013) and in our study(Woodward et al. 2015). However, the drop in plasma glucose level observed at PM2 and the reestablishment of the glycerol level, suggests that this alternative metabolic pathway cannot sustain energy demand for a large lapse of time. The total reestablishment of host metabolic homeostasis was reached at the end of the study (PM3), coinciding with a net decrease in percentage of infected birds in all groups(Woodward et al. 2015) but also a stabilization of the CM indicating that both factors are most probably associated with the host metabolic response observed.

Antibiotics are known to attenuate symptoms developed during infection. In the paper published previously by Woodward et al. it was indeed observed that Tiamulin™ was able to decrease infection and associated symptoms(Woodward et al. 2015). Indeed, decreased growth rate in response to infection was canceled by the two highest antibiotic doses. In this study, Tiamulin™ was also able to attenuate infection-induced metabolic response such as the betaine increase and glucose drop in plasma. It is not possible to know if the observations made were due to reduced viability with associated loss of pathogenic function of *B. pilosicoli* induced by the antimicrobial properties of the Tiamulin™ (i.e. gut barrier disruption), by the

antibiotic itself and unrelated to status of *B. pilosicoli* or, if other mechanisms were involved. However, we observed in our previous study(Woodward et al. 2015) that at PM2, infection was reduced equally by antibiotic treatment irrespective of the dose administrated which, could explain why no dose dependence was observed in the betaine response. We hypothesize that increased betaine level in response to infection was related to the central osmoprotectant role of this molecule(Felitsky et al. 2004). Betaine has been used previously as food suplement for chicken due to its ability to protect the gut barrier against pathogens such as *Coccidia* (Craig 2004). Indeed, *B. pilosicoli* by invading the cells induces swelling and disturbance of the osmotic balance(Mappley et al. 2011; Mappley et al. 2014). Increased amount of betaine could therefore be transported from other tissue towards the gut barrier *via* general circulation explaining its increased level in plasma.

Antibiotics are chemical molecules that also interplay directly with the host. It has been reported that Tiamulin™ interacts with cytochrome P450 3A (CYP3A) family (present in the liver for drug clearance) forming a complex that results in the inactivation of the cytochrome *in vitro* and *in vivo* (Witkamp et al. 1996; De Groene et al. 1995; Zweers-Zeilmaker et al. 1999; Rátz et al. 1997). CYP3A is also involved in steroid hormone clearance (progesterone, estrogen and testosterone). It was shown is several studies that a decrease in CYP3A activity generally resulted in increased plasma steroid hormone concentrations(Natsuhori et al. 1997; Bertilsson et al. 1998)'(Lemley et al. 2008). Finally it was also established that an increased level of progesterone in the plasma results in a concomitant increase in VLDL and decrease in HDL and glucose level in the general circulation (Kushwaha et al. 1991; Judge et al. 1983; Sacks & Walsh 1990) as observed in this study. From our results and data

found in the literature, it is possible to suggest that Tiamulin™[TM] induces a hormonal disturbance resulting in a cholesterol metabolism switch. Furthermore, antibiotic treatment was conducted in a very specific hormonal period: puberty, in order to observe the impact of treatment on delayed laying time induced by *B. pilosicoli* infection (Woodward et al. 2015). Surprisingly, infection was not associated to delayed of onset of lay as previously observed (Taylor et al. 1993; Taylor et al. 2010). However, the two treated groups that received the highest antibiotic doses (D and E) started laying earlier than the two untreated groups and the group treated with the lowest Tiamulin[TM] dose(Woodward et al. 2015). As onset of lay is regulated by hormonal changes triggered by progesterone and estrogen, this suggests that Tiamulin[TM] might affect steroid metabolism. Lastly, we observed that Tiamulin[TM] induced an increase in bird's metabolic age that is normally induced by changes in hormonal status linked to puberty. This last result strongly support the potential steroid metabolic regulation by Tiamulin[TM].

However, such cholesterol metabolic response can be tightly linked to composition of the gut microbiota. The host-GM metabolic interplay has been widely investigated with many studies observing that obesity or energetic metabolism homeostasis was strongly associated with gut microbiota composition(Musso et al. 2011; Tremaroli & Bäckhed 2012; Everard & Cani 2013; Larsen et al. 2010). Furthermore, it has been demonstrated that use of antibiotic before puberty in humans and mice can induce increased risk for 'metabolic disease' due to modification of the gut microbiota (Cho et al. 2012; Cox et al. 2014; Trasande et al. 2013). Interestingly diminution of the ratio Firmicutes/Bacteroidetes has been reported for being related to a lean phenotype with decreased 'metabolic disease' risk triggered by modification of

cholesterol metabolism(Ley et al. 2006; Ley et al. 2005; Turnbaugh et al. 2006). This is, however, contrary to what was observed with treated birds in our study that suggested cholesterol metabolic modification detected was in response to antibiotic treatment rather than associated with caecal microbiota transformation. Never the less individual bacteria phyla can also be responsible for modification of cholesterol metabolism. Indeed Lactic acid bacteria are known to be able to catalyze cholesterol(Pereira & Gibson 2002) and their use as feed supplement in broiler resulted in decreased plasmatic cholesterol concentration(Jin et al. 1998). In short, further experimentation is needed to tease this aspect apart. The Home Office license under which this study was performed did not permit the use of antibiotic alone in healthy birds so the role of Tiamulin™ as the may only be inferred at this stage. Tiamulin™ is associated with transient dysbiosis but, it is possible to suggest that the method use to study the CM in not sufficiently powerful to allow the identification of a specific bacterial genus associated with the cholesterol metabolic response of the host.

In conclusion, this work demonstrates the strong implication that a perturbation of the normal caecal microbiota can have on host systemic metabolism and later on its phenotype. In this study, we demonstrated that infection was associated with caecal microbiota dysbiosis associated with decreased nutrient absorption and host energy metabolic disorder that resulted in significant decreased growth rate. This work gave a clearer understanding of the metabolic adaptation of the host to intestinal infection by a pathogen to maintain sufficient energy supplies for survival but still resulting in impaired weight gain. On the other hand, antibiotic treatment by Tiamulin™ appears to reduce infection and associated symptoms while modifying

cholesterol metabolism. It is strongly supposed given our results and previously published work that host metabolic response to antibiotic treatment resulted from a modification of steroid metabolism. However, no conclusion could be made regarding the implication of the strong modification of the caecal on this increased bird's metabolic aging.

Acknowledgment

The authors want to thanks, the staff of APHA and Dr Luke J Mappley for taking care of the birds during the study and assisting sample collection. We also want to thanks Dr Radoslaw Michal Kowalczyk from the Chemical Analytical Facility (CAF) of the University of Reading for his help during the 2D NMR spectra acquisition.

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Chapter 6: General discussion

This work aimed at better understanding *B. pilosicoli*-induced AIS and its treatment by the most consumed antibiotic in industry (Tiamulin^{TM}) using a NMR-base metabonomics approach. Several questions regarding the pathogen, the host, the disease and its treatment were raised in this project:

1. How does *B. pilosicoli* respond metabolically to Tiamulin^{TM} exposure (Chapter II)?

2. What are the metabolic characteristics of the host and the pathogen (Chapter II and III)?

3. Can Tiamulin^{TM} reduce *B. pilosicoli*-induced symptoms and what is the optimum dose to be used in chicken (Chapter IV)?

4. Does *B. pilosicoli* infection induce a host metabolic systemic response and does it affect the caecal microbiota (Chapter V)?

5. How does Tiamulin^{TM} impact host metabolism and caecal microbiota composition after infection by *B. pilosicoli* (Chapter V)?

Metabonomics is an untargeted approach based on the analysis of complex metabolic profiles to evaluate multiparametric responses of a living system to an external stress. It allowed us to follow a top-down approach to AIS understanding and revealed some interesting mechanisms about the response of the host to infection and antibiotic treatment as well as regarding microbial response to Tiamulin^{TM}. This powerful approach highlighted modifications of metabolic profiles and allowed to generate new hypotheses about affected metabolic pathways that need to be further validated. An immediate next step would be to evaluate the gene

regulation and expression of these pathways of interest using molecular tools such as targeted RT-PCR or high throughput transcriptomics.

6.1. Infection, dysbiosis and metabolism

GI infection and associated dysbiosis have been related to reduced growth rate and weight loss in humans and many other animal species. Reduced growth rate is often explained by the alteration of the gut barrier function resulting in poor nutrient absorption. Intestinal barrier disruption by *B. pilosicoli* has previously been documented, however, our work is the first one revealing that intestinal lumen content was richer in hexose in response to infection validating the hypothesis that nutrient absorption was compromised by pathogen colonisation. This work also revealed for the first time that infection by *B. pilosicoli* was followed by dysbiosis and more specifically an increase in the Proteobacteria phylum often observed post bacterial infection. This same study described the systemic host metabolic response associated with reduced growth rate triggered by infection. This was characterized by a systemic increase of glycerol post infection. Glycerol is a marker of lipolysis used to maintain glucose plasma levels when energy supply becomes insufficient. Thus, we hypothesised that the decreased growth rate observed in infected birds was directly linked to reduced nutrient absorption triggering increased lipolysis to maintain glycaemia level. Nevertheless, to confirm this hypothesis, it would be necessary to measure the expression of the lipase enzymes in adipose tissue and the expression of genes involved in gluconeogenesis from glycerol within the liver and the kidney. This analysis could not be done in this study since no adipose tissues

were sampled and that liver and kidney were not correctly stored for preservation of mRNA.

To evaluate if chicken energy disturbance is due to infection by *B. pilosicoli* or dysbiosis, it would be interesting to use axenic birds to colonise them with CM of previously infected chicken to assess if the composition of the CM alone can cause the responses described in the previous paragraph.

6.2. Antibiotic resistance

The antibiotics market is now almost reaching $ 45 billion per year. This market is expected to keep rising mainly in response to increasing cattle production that requires high quantities of antibiotic for prophylactic use or to treat infections. Even if antibiotics are still the best way to fight against bacterial infections, concerns have arisen due to increased resistance that may strongly impact on antibiotic efficiency. In our study we investigated several aspect of this issue by trying to evaluate the metabolic response of *B. pilosicoli* to antibiotic treatment and also assessing the minimum dose to be used in chicken to treat AIS and associated symptoms. In Chapter II, we showed that Tiamulin^{TM} was able to inhibit bacterial growth at very low concentrations (0.032-0.125 µg/ml) although bacteria were still metabolically active. Bacterial metabolism was altered with higher antibiotic doses and the highest Tiamulin^{TM} concentration (0.250 µg/mL) inactivated bacterial metabolism. This strongly supports that we must be very careful when using MIC values for bacteriostatic molecules such as Tiamulin^{TM} since our results showed that even if bacterial growth was prevented, they were still metabolically active and therefore a

potential threat. Furthermore, after incubation with the antibiotic, it would be interesting to re-suspend the bacteria in fresh media without the antibiotic to evaluate their growth recovery. Finally the same experiment could be reproduced with different antibiotics and other *Brachyspira* species to determine if similar results would be obtained.

The animal trial revealed that the two highest Tiamulin^{TM} doses used during the study were able to significantly reduce infection and associated symptoms (Chapter IV) indicating that 125 ppm might be sufficient to be used in farms. However, by the end of the study even if no more symptoms were observed and that metabolic homeostasis was reached, chickens were still colonized with the bacterium (Chapter IV and V). This indicates that Tiamulin^{TM} was efficient to prevent colonization only for a short period of time but that *B. pilosicoli* was therefore able to recolonize. This confirmed the concerns that arose from Chapter II where bacteria seem to enter a dormancy state in presence of Tiamulin^{TM} but are still alive. This reinforces the necessity to evaluate the recovery ability of *B. pilosicoli* post-antibiotic clearance *in vitro*.

Tiamulin^{TM} induced a systemic metabolic response of the host. We first demonstrated that some of the metabolic response to infection, such as the betaine drop, was rescued by the use of antibiotic, suggesting that the treatment was able to reduce the impact of infection at both macroscopic and metabolic levels. We also observed that Tiamulin^{TM} treatment was responsible for a switch in lipoprotein metabolism. The Antibiotic treatment was also associated to a modification of the CM with a strong loss of biodiversity. Interestingly, three weeks after antibiotic treatment, the birds were still not able to recover to their normal CM ecosystem.

Poor GM diversity or abnormal composition can increase the risk of infection. Thus, interventions such as probiotic intake or shared housing with untreated birds should be explored in order to restore the integrity of the CM ecosystem post-antibiotic treatment. Moreover, we suggested that the metabolic response observed in birds plasma post antibiotic treatment was not due to caecal dysbiosis but to other mechanisms discussed further below. Nevertheless, in order to validate that CM had no impact on this metabolic response, a faecal transplant experiment should be conducted in germ-free animals to measure the metabolism of chickens colonized by faecal CM from treated and non-treated birds.

Finally, considering the current literature, it can be hypothesized that the metabolic shift observed after Tiamulin™ treatment can be partially due to a decreased activity of CYP3A that triggers a disruption of steroids hormone clearance. However, this theory needs to be tested. To do so, an animal trial should be conducted using a control and an antibiotic treated group, where CYP3A expression and activity as well as steroid hormones levels would be measured. I suggest that the time of experiment should be consistent with the animal trial that was conducted in this study (i.e. starting just before puberty).

6.3. General conclusion and future work

This original work significantly contributed to the general understanding of AIS and one of its main pathogen *B. pilosicoli*. It also generated new knowledge regarding host-GM metabolic interactions and finally enlightened the need for a better understanding of the action of antibiotics in a context of emerging antimicrobial resistances.

This work provided new insights into the biological mechanisms underpinning the disturbance of host energy metabolic homeostasis during infection by a pathogen followed by a decreased growth that further generates economical loss. By understanding these mechanisms it is therefore possible to imagine ways of preventing post-infection co-morbidities occurring in humans and animals during/post infection. It is important to keep in mind that the work presented here was based on a metabonomics approach, which is a hypothesis generating technique. Therefore, the results observed here should serve as a base for future investigations. Indeed, the metabolome is the end result of a complex process involving gene and protein expression and their regulation that all interact in interconnected metabolic pathways that need to be identified for these proposed hypotheses to be validated.

Finally, this study raises questions about the actual mechanisms stimulated by growth promoting antibiotics. To date the accepted mode of action is that the prophylactic use of low dose antibiotics results in the reduction of the number of gut microbes that improves feed efficiency. However, few studies have investigated this in detail and our results suggest that other mechanisms might be involved. Therefore, I suggest that more studies using similar analytical approaches as the ones conducted in this project should be done to explore the metabolic impact of growth promoting antibiotics and lasting consequences.