UNIVERSITY OF SURREY

Lactobacilli probiotics as a potential control for avian intestinal spirochaetosis

A thesis submitted by
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“It always seems impossible until it’s done”

-unknown

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Abstract

Brachyspira are the causative agent of avian intestinal spirochaetosis, a gastrointestinal disease common in layer hens and broiler breeders. This disease costs the UK laying industry approximately £18 million per annum, resulting from reduced egg production and poor egg quality. Prevalence of *Brachyspira* is increasing, and due to the poor understanding of this pathogen, mitigation strategies have been largely unsuccessful. Therefore, preventative measures are essential.

These studies aimed to improve the understanding of *Brachyspira* pathobiology and investigate *Lactobacillus* probiotics as a suitable mitigation strategy. *Brachyspira* and *Lactobacillus* species were characterised using phenotypic and genotypic methods. Four *Lactobacillus* isolates were selected for their inhibition of *Brachyspira in vitro* and demonstrated inhibition by a number of mechanisms.

Secreted metabolites in *Lactobacillus* cell free supernatant inhibited *Brachyspira* (p value ≤ 0.05) and metabolomic studies identified the production of organic acids to be a major contributor to inhibition. Protein denaturation in cell free supernatants significantly reduced *Brachyspira* inhibition (p value ≤ 0.05), suggesting the role of bacteriocins in inhibition. Furthermore, *L. reuteri* isolates co-aggregated with *Brachyspira in vitro*, reducing pathogen viability (p value ≤ 0.05).

Pro-inflammatory responses to *Brachyspira* in HD11 avian macrophages were dominated by upregulation of IFNγ (p value ≤ 0.01) and pre-treatment of cells with *Lactobacillus* significantly reduced this response (p value ≤ 0.0001), demonstrating the ability of probiotics to alter immune responses to *Brachyspira*. *Galleria mellonella* were utilised to study *Brachyspira* virulence and probiotic intervention. *G. mellonella* exhibited a varied response to *Brachyspira*

infection and *Lactobacillus* isolates were able to protect against the mortality associated with *Brachyspira* isolates (p value ≤ 0.05).

The studies here demonstrated that *Lactobacillus* probiotics are a suitable mitigation strategy against *Brachyspira*. A number of mechanisms were identified, however future studies are required to explore these mechanisms in a more relevant *in vivo* chicken model.

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Publications, Conference papers and Awards

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List of Abbreviations

ATCC	American Type Culture Collection
ANOVA	analysis of variance
AMR	antimicrobial resistance
AIS	avian intestinal spirochaetosis
HD11	avian-like macrophage cell line
bp	base pairs
BEB	*Brachyspira* enrichment broth
BSA	*Brachyspira* selective agar
BHI	brain heart infusion
CFS	cell free supernatant
Che	chemotaxis protein
CFU	colony forming unit
MRS	De Man, Rogosa and Sharpe agar/ broth
^{o}C	degrees Celsius
DNA	deoxyribonucleic acid
FABA	fastidious anaerobic blood agar
FBS	foetal bovine serum
GRAS	generally recognised as safe
Shovill	genome assembly software
SPAdes	genome assembly software
GC content	guanine-cytosine content
H&E	haematoxylin and eosin

HIS	human intestinal spirochaetosis
IVOC	*in vitro* organ culture
IMS	industrial methylated spirits
KDa	kilodalton
LOS	lipooligosaccharide
LPS	lipopolysaccharide
Mbps	mega base pairs
mRNA	messenger ribonucleic acid
MCP	methyl-accepting chemotaxis protein
μg	micro gram
μl	micro litre
μm	micro metre
ml	millilitre
mM	millimolar
MLEE	multi locus enzyme electrophoresis
MLST	multi locus sequence typing
nm	nanometre
NGS	next generation sequencing
NMR	nuclear magnetic resonance
NA	nutrient agar
1M	one molar
OD	optical density
PM	phenotypic microarray

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIS	porcine intestinal spirochaetosis
PC	principle component
1H- NMR	proton NMR
PFGE	pulsed-field gel electrophoresis
RT-PCR	real time polymerase chain reaction
RFLP	restriction fragment length polymorphism
RT	reverse transcription
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscopy
SCFA	short chain fatty acid
SNPs	single-nucleotide polymorphism
parSNP	single-nucleotide polymorphism program
SOCS	suppressor of cytokine signalling
SAP	Surrey animal pathogen
TLR	toll like receptor
v/v	volume/volume
v/w	weight/volume
WGS	whole genome sequencing

Chapter 1: Introduction

1.1 *Brachyspira*

1.1.1 History of *Brachyspira* Taxonomy

Brachyspira are the sole genus assigned to the Brachyspiraceae family in the order Spirocheateales (Paster and Dewhirst, 2000). The order Spirocheateales contains all spirochaetes, including *Borrelia*, *Leptospira* and *Treponema* (Stanton, 2006) and there are several common properties shared by these spirochaetes. These include their helical morphology, periplasmic flagella and natural resistance to rifampicin (Paster and Dewhirst, 2000).

In the last forty years, the genus *Brachyspira* has undergone several taxonomic reclassifications. These organisms were originally known as *Treponema*, where the name *T. hyodysenteriae* was originally applied to both pathogenic, strongly haemolytic isolates and non-pathogenic, weakly haemolytic organisms isolated from swine. In 1978, Miao *et al*. (1978) discovered that human treponemes were genetically distinct from swine treponemes and that the pathogenic and non-pathogenic *T. hyodysenteriae* only shared 28% sequence homology. This subsequently led to the classification of *T. hyodysenteriae* as the aetiological agent of swine dysentery and *T. innocens* as non-pathogenic a year later (Kinyon and Harris, 1979). In 1991, Stanton *et al.* (1991) proposed the reclassification of *T. hyodysenteriae* and *T. innocens* to a new genus known as *Serpula*, when studies showed that these organisms were only distantly related to other *Treponema* species. However, the name *Serpula* was changed again in 1992, to *Serpulina,* as *Serpula* had already been used to name a genus of fungi (Stanton, 1992). Finally, Ochiai *et al*. (1997) proposed the unification of the genera *Serpulina* and *Brachyspira* owing to the similarities discovered between *S. hyodysenteriae, S. innocens,*

S. pilosicoli and *B. aalborgi.* The genus *Brachyspira* was used instead of *Serpulina* since *B. aalborgi* was proposed as a new genus is 1982 (Hovind-Hougen *et al.*, 1982), before the reclassification of *T. hyodysenteriae* to *Serpulina* in 1991.

1.1.2 The genus *Brachyspira*

Brachyspira (brachy "short" and spira "coli") are Gram negative, oxygen tolerant, anaerobic spirochetes that have been isolated from the gastrointestinal tracts of both mammals and birds, including humans, chickens, pigs, dogs and horses. These organisms have also been cultured from the faeces and blood of infected hosts, demonstrating that these bacteria have a wide host range and have the potential to cause both enteric and systemic infections.

The genus consists of seven confirmed species of *Brachyspira* as seen in Table 1.1. These include *B. aalborgi*, *B. alvinipulli, B. hyodysenteriae, B. innocens, B. intermedia, B. murdochii* and *B. pilosicoli*. There are an additional seven proposed species, including *B. canis, B. christiani, B. corvi, B. hampsonii, B. ibaraki, B. puli* and *B. suanatina*. *B. pilosicoli* is the only species to our knowledge that can infect both human and non-primate animal hosts and thus may pose a zoonotic threat.

B. alvinipulli, *B. intermedia* and *B. pilosicoli* are the species that are considered to be the causative agent of avian intestinal spirochaetosis. *B. hyodysenteriae* causes swine dysentery in pigs and *B. intermedia* causes the less severe gastrointestinal disease, porcine intestinal spirochaetosis. *B. innocens* is proposed to be non-pathogenic in both the avian and porcine hosts although it has been previously associated with reduced productivity in laying hens (Burch *et al.*, 2006). Humans of any age can be infected with *B. pilosicoli* and *B. aalborgi*, resulting in human intestinal spirochaetosis but this is frequently associated with poorly developed countries (Mikosza *et al.*, 2001).

Table 1. 1 Confirmed and proposed species of *Brachyspira* and their host range

Species	Host Range	Associated Disease	Reference
B. aalborgi	Human, primate	Intestinal Spirochaetosis	Hovind-Hougen *et al.*, 1982
B. alvinipulli	Chicken, domestic goose, dog	Intestinal Spirochaetosis	Stanton *et al.,*1998
B. hyodysenteriae	Pig, rat, mouse, chicken, goose	Swine dysentery (pigs)	Harris *et al.*, 1972
B. innocens	Pig, dog, horse, chicken	Avirulent	Kinyon and Harris, 1979
B. intermedia	Pig, chicken	Intestinal Spirochaetosis	Stanton *et al.*, 1997
B. murdochii	Pig, rat, chicken	Avirulent	Stanton *et al.*, 1997
B. pilosicoli	Pig, dog, horse, human, primate, chicken	Intestinal Spirochaetosis	Trott *et al.*, 1996
Proposed Species			
B. canis	Dog		Duhamel *et al.*, 1998
B. christiani	Human		Jensen *et al.*, 2001
B. corvi	Jackdaw, crow		Jansson *et al.,* 2008
B. hampsonii	Pig		Chander *et al.*, 2012
B. ibaraki	Human		Tachibana *et al.,* 2002
B. puli	Chicken, dog		Stephens and Hampson, 1999
B. suanatina	Pig, mallard		Råsbäck *et al.*, 2007

(Adapted from Mappley et al., 2014).

1.1.3 Phenotypic characteristics of *Brachyspira*

1.1.3.1 Cell morphology and cultural characteristics

Brachyspira are Gram negative, long, helical shaped bacteria, depicted in Figure 1.1 (Mirajkar *et al.*, 2016). Cells range in length from 2.0-11.0 μm, width from 0.20- 0.45 μm and number of flagella from 8-30 per cell as shown in Table 1.2. Additionally, the cell end can be blunt, pointed or tapered, depending on the *Brachyspira* species.

Spirochaetes possess periplasmic flagella (Figure 1.2) and the number of flagella varies between the species of *Brachyspira*, as shown in Table 1.2. The different numbers of flagella can prove useful in differential diagnosis of an isolate, using electron microscopy. *Brachyspira* species responsible for avian intestinal spirochaetosis typically have an end:middle:end flagella ratio of 8:16:8 or 5:10:5 owing to the fact that individual flagellum originate from the poles of the cell and overlap in the middle (Fellström *et al*., 1997).

Brachyspira are slow growing, fastidious organisms that typically take 3-5 days to form a thin, flat film of growth on agar. Species that are implicated in poultry disease were weakly haemolytic, characterised by a halo of weak haemolysis around areas of growth on blood agar. Other *Brachyspira* species such as *B. hyodysenteriae* are strongly haemolytic on blood agar and therefore can be distinguished from poultry isolates. Spirochaetes stain poorly with Gram stains and therefore the Warthin-Starry stain is the most appropriate method to visualise *Brachyspira* cells following culture. Additionally, cells can be visualised using dark-field microscopy, whereby the spirochaete morphology and motility are most clearly visualised. Growth of *Brachyspira in vitro* can be carried out on solid or liquid media, supplemented with blood or serum. Selective agar such as *Brachyspira* selective agar which contains 5% sheep blood, spectinomycin at a concentration of 800μg/ml and colistin and vancomycin each at a concentration of 25μg/ml supports *Brachyspira* growth (Råsbäck *et al*.,

2005). Selective media is important to inhibit growth of other organisms that could inhibit or out-grow *Brachyspira.* Cultures of *Brachyspira* can be grown more rapidly on fastidious anaerobic agar containing 10% sheep blood, but this is only recommended for pure isolates. *Brachyspira* can be grown in a number of different liquid broth media including, brain heart infusion broth (BHI) and *Brachyspira* enrichment broth, containing tryptone soya broth, 10% cysteine hydrochloride, 10% glucose and 10% serum (Råsbäck *et al*., 2005).

Brachyspira culture is carried out under anaerobic conditions, preferably in an anaerobic cabinet containing a gas composition of 10% hydrogen, 10% carbon dioxide and 80% nitrogen and growth can be observed after 3- 5 days of culture, but culture can take up to two weeks (Hampson, *personal communication*). Colonies appear as a dull flat sheet that grows over the surface of an agar plate, which is why it is difficult to isolate individual *Brachyspira* colonies. Surface growth of the species implicated in avian intestinal spirochaetosis is surrounded by weak β- haemolysis.

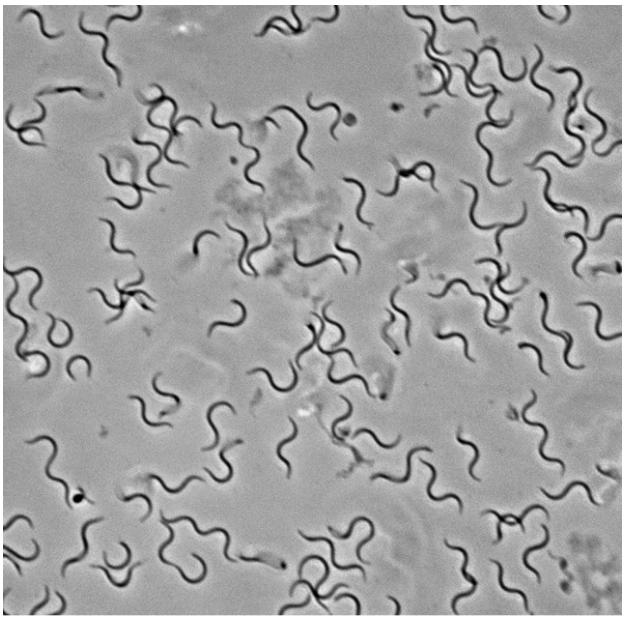


Figure 1. 1 Phase- contrast microscopy of *B. hampsonii* NSH-24 cells imaged at x100 showing the long spiral morphology of *Brachyspira* (Mirajkar *et al.*, 2016).

Table 1. 2 Size and number of flagella associated with the seven validated species of *Brachyspira.* Different species of *Brachyspira* can be distinguished using scanning electron microscopy (SEM) due to the varying lengths, widths and number of flagella possessed by each species.

***Brachyspira* species**	**Length (μm)**	**Width (μm)**	**Number of flagella**
B. aalborgi	2-6	0.2	8
B. alvinipulli	8-11	0.2-0.35	22-30
B. hyodysenteriae	7-9	0.3 -0.4	22-28
B. innocens	7-9	0.3 -0.4	20-26
B. intermedia	8-10	0.35-0.45	24-28
B. murdochii	5-7	0.23-0.3	22-26
B. pilosicoli	5-7	0.2-0.4	8-12

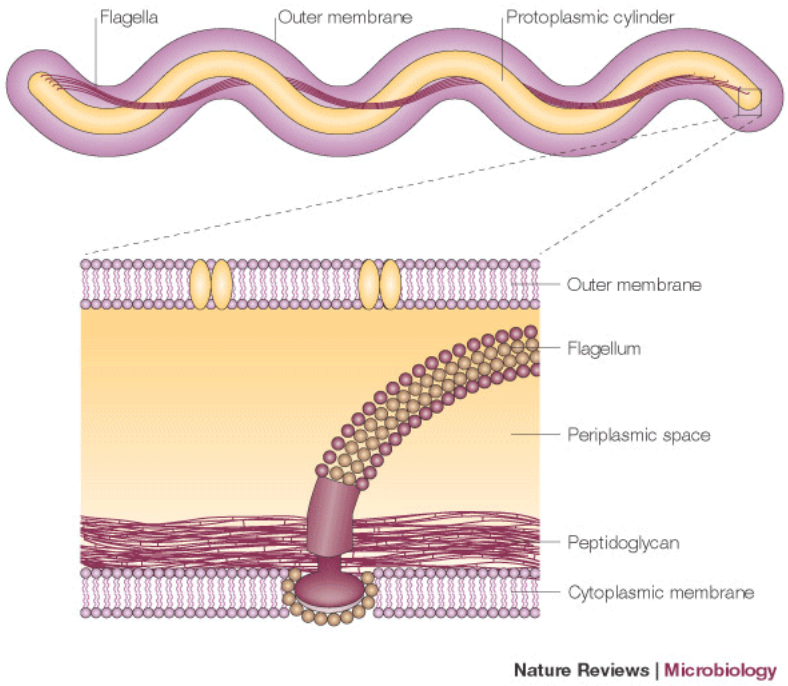


Figure 1. 2 Diagram of a spirochaete indicating that the flagella are inserted at the termini of the spirochaete cell. These bundles of flagella wind around the protoplasmic cylinder and overlap in the middle. The outer membrane ensure that the flagella remain in the periplasm. The flagella are inserted into the cytoplasmic membrane of the cell and extend into the periplasm. Spirochaete movement is facilitated by the co-ordinated rotation of the flagella. Adapted from (Rosa *et al*., 2005).

1.1.3.2 Biochemical properties of *Brachyspira*

Brachyspira produce several enzymes that can be utilised to determine the biochemical profile of each isolate in order to determine the species. Biochemical tests for *Brachyspira* include colorimetric assays for indole production, hippurate hydrolysis and the activity of α-galactosidase, α-glucosidase and β- glucosidase (Fellström & Gunnarsson, 1995; Fellström *et al*., 1999). The typical biochemical results for *Brachyspira* species are detailed in Table 1.3 whereby *Brachyspira* species are assigned groups based on their phenotypic profiles. A major drawback of these biochemical tests is that the results were initially determined using *Brachyspira* isolated from pigs, which are known to differ from avian isolates. Due to these inaccuracies, molecular methods are being used in conjunction with phenotypic methods for a more accurate identification. Additionally, as whole genome sequencing becomes more readily available, sequencing may be the gold standard for *Brachyspira* identification as PCR based methods have drawbacks that will later be discussed.

Table 1. 3 Grouping of *Brachyspira* species according to their biochemical properties (Fellström *et al*., 1999).

Group	Haemolysis	α-galactosidase	α-glucosidase	β-glucosidase	Indole production	Hippurate hydrolysis	Species indicated
I	Strong	-	±	+	±	-	*B. hyodysenteriae*
II	Weak	–	+	+	+	–	*B. intermedia*
IIIa	Weak	–	–	+	–	–	*B. murdochii*
IIIb	Weak	±	–	+	–	–	*B. innocens*
IIIc	Weak	±	+	+	–	–	*B. innocens*
IV	Weak	±	±	–	–	+	*B. pilosicoli*
ND	Weak	–	–	+	–	+	*B. alvinipulli*

1.1.3.3 Metabolic properties of *Brachyspira*

There is a significant lack of understanding of the metabolic pathways present within *Brachyspira,* however recent genome sequencing and high-throughput phenotypic testing have begun to improve the understanding of the metabolic capabilities of this organism (Mappley *et al*., 2012).

Genome sequencing has revealed that the central metabolic pathways for energy production in *Brachyspira* species include glycolysis, gluconeogenesis and the non-oxidative pentose phosphate pathway (Bellgard *et al.*, 2009; Wanchanthuek *et al.*, 2010). The end products of these metabolic pathways include acetate, butyrate, ethanol, hydrogen and carbon dioxide (Stanton and Lebo, 1988; Trott *et al.*, 1996; Stanton *et al.*, 1997; Stanton *et al.,* 1998). *Brachyspira* species have an incomplete set of genes coding for the TCA cycle, as observed with other spirochaetes such as *Borrelia burgdorferi* and *Treponema pallidum.* (Bellgard *et al.*, 2009). This therefore suggests that *Brachyspira* generate adenosine triphosphate (ATP) through the fermentation of sugars. To date, the ability of *B. pilosicoli* and *B. murdochii* to utilise the enzymes of the incomplete TCA cycle to synthesis glutamate have been noted, a metabolic pathway not shared by *B. hyodysenteriae.*

High-throughput testing of carbon metabolism for *Brachyspira* species has highlighted that many metabolic pathways are shared by *B. pilosicoli* and *B. hyodysenteriae*, but that there are key differences both between species of *Brachyspira* and within the same species (Mappley *et al*., 2012). Of 178 carbon sources tested, the utilisation of only seven were found to differ between *B. pilosicoli* and *B. hyodysenteriae* (Mappley *et al.,* 2012). However, of these seven, the ability of *B. pilosicoli* to metabolise four carbon sources differed; to fully explain this, further research is required.

1.1.3.4 Genotypic characteristics of *Brachyspira*

Brachyspira have a circular genome with an approximate size of between 2.43- 3.49Mbp, with *B. pilosicoli* generally having the smallest genome. The *Brachyspira* genome has a low GC content of between 24-28%. There is a paucity of public genome sequences data for *Brachyspira* species implicated in poultry disease with only one genome sequence available for *B. alvinipulli*, *B. intermedia* (Håfström *et al.,* 2011) and *B. innocens* and four available sequences for *B. pilosicoli* (Lin *et al*., 2013; Mappley *et al*., 2012; Wanchanthuek *et al*., 2010). *B. hyodysenteriae* is responsible for swine dysentery and therefore results in a much more severe disease than observed in poultry which may account for why twenty-five genome sequences are available (Bellgard *et al.*, 2009; Black *et al*., 2015; La *et al.,* 2016). Additionally, one *B. murdochii* (Pati *et al.*, 2010) and five *B. hampsonii* sequences are available (Genbank, NCBI).

Table 1. 4 Summary of the *Brachyspira* genome sequences and their genome size and GC content (%) available on Genbank.

***Brachyspira* species**	**Number of genomes**	**Genome size (Mbp)**	**GC content (%)**	**Reference**
B. aalborgi	1	2.5	27.0	(Mappley *et al*., 2012)
B. alvinipulli	1	3.42	26.8	(Stanton *et al.*, 1998), DOE Joint Genome Institute
B. hampsonii	5	2.93- 3.18	27.4	(Mirajkar *et al*., 2016)
B. hyodysenteriae	25	2.99-3.19	27.1	(Bellgard *et al.*, 2009; Black *et al.*, 2015; La *et al.*, 2016)
B. innocens	1	3.28	27.22	DOE Joint Genome Institute
B. intermedia	1	3.30	27.7	(Håfström *et al.,* 2011)
B. murdochii	1	3.24	27.75	(Pati *et al.*, 2010)
B. pilosicoli	4	2.56–2.89	27.44–27.90	(Lin *et al*., 2013; Mappley *et al*., 2012; Wanchanthuek *et al*., 2010)
B. suanatina	2	3.26	27.0	(Mushtaq *et al.,* 2015)

(Adapted from Hampson and Wang, 2017).

As mentioned, *B. pilosicoli* have one of the smallest genomes of all *Brachyspira* species, however the size of these genomes can vary from between 2.43 and 2.88Mbp indicating genome plasticity: *B. pilosicoli* genomes have been shown to have major genome rearrangements that coincide primarily with mobile genetic elements. Despite this variability, there were many core genome features shared with other species, such as their core metabolic pathways (Mappley *et al*., 2012; Wanchanthuek *et al*., 2010). Despite its small genome size, *B. pilosicoli* isolates encode more proteins than *B. hyodysenteriae*, this may have resulted in *B. pilosicoli* having the ability to colonise a wider host range than *B. hyodysenteriae* (Hampson & Wang, 2017).

Due to the lack of genome data for other *Brachyspira* species implicated in avian disease, further comparative genomics has not been undertaken, however, as more sequences become available, species level comparison and pangenome analysis of avian isolates will improve the understanding of *Brachyspira* and avian intestinal spirochaetosis. Furthermore, methods of genetic manipulation are required to confirm the functions of genes highlighted in bioinformatic analysis. Although research has been conducted with *Brachyspira* isolates with specific gene mutations, further tools to manipulate the genome are needed (Rosey *et al.,* 1995; Stanton *et al.,* 1999).

1.1.4 Virulence characteristics of *Brachyspira*

Brachyspira have a number of virulence factors which allow it to survive and navigate the gastrointestinal tract of the host in order to cause disease. Disease relies upon these bacteria gaining close proximity to the intestinal mucosa where they can adhere to the cells. In order to do this, *Brachyspira* must first move through the viscous mucus of the caeca and/or colon. Virulence mechanisms of *Brachyspira* are overall, poorly understood and severity of disease is dependent on several factors, including isolate of *Brachyspira*, health of the host, diet and resident intestinal microbiota.

1.1.4.1 Motility

Brachyspira are motile by periplasmic flagella and movement is facilitated by the co-ordinated rotation of the flagella which are inserted into the poles of the bacterial cell and overlap in the middle. Motility is an important virulence factor for *Brachyspira* as it is essential for host colonisation and gaining close proximity to the intestinal mucosa for attachment and invasion of enterocytes (Naresh and Hampson, 2010). Furthermore, strong motility enables

Brachyspira to evade the physical effects of mucus, which is designed to remove unattached bacteria from the gastrointestinal tract (Nakamura *et al.,* 2006; Witters & Duhamel, 1999) .

Whole genome sequencing has identified the presence of *flaA* and *flaB* genes in *Brachyspira* species and disruption of these genes in *B. hyodysenteriae* has been shown to be associated with an inability to colonise mice in a murine infection model (Kennedy *et al.,* 1997). These genes have been identified in *B. hyodysenteriae, B. murdochii* and *B. pilosicoli* (Bellgard *et al.*, 2009; Wanchanthuek *et al.*, 2010), furthermore, Mappley *et al.* (2012) identified several types of flagella associated proteins present in *B. pilosicoli* isolates, including but not limited to inner, outer and periplasmic proteins.

Periplasmic flagellar consist of three major components: the basal body, the hook and the filament, as illustrated in Figure 1.3. The basal body is comprised primarily of export proteins, the MS ring that attaches the rod assembly to the cell membrane, the C ring, the collar structure within the peptidoglycan, FilL and the motor components: MotA and MotB. The hook is primarily comprised of FlaE and the filament is composed of FlaA, the major flagellin protein and FlaB, the minor flagellin protein (Motaleb *et al.,* 2015).

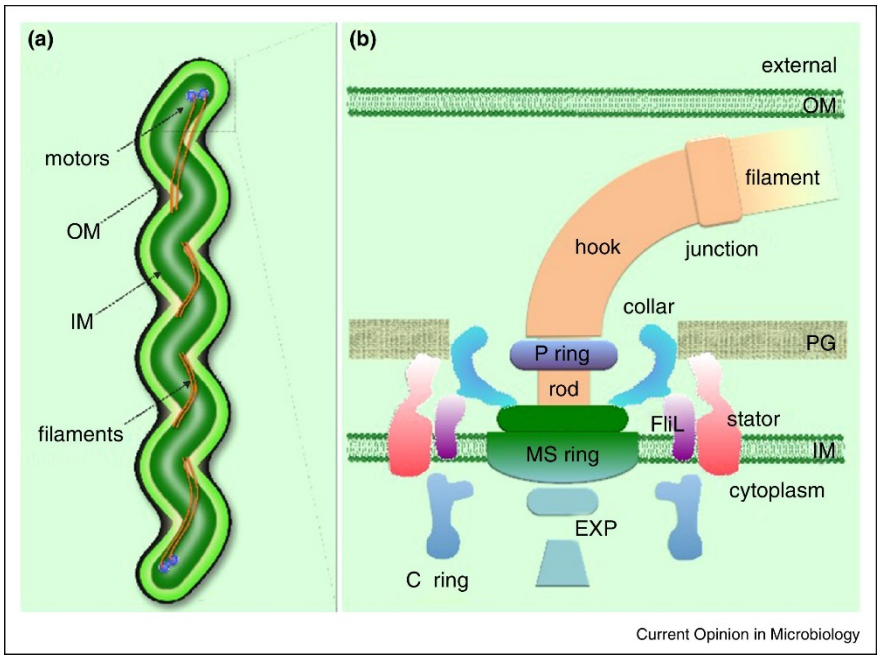


Figure 1. 3 Main components of the periplasmic flagella structure in spirochaetes. **(a)** Schematic of a spirochaete cells showing the periplasmic flagellar filaments originating from the poles of each cell, between the outer membrane (OM) and inner membrane (IM). The filaments overlap in the middle of the cell and result in the strong motility associated with spirochaetes. **(b)** Schematic of the periplasmic flagellar motor, illustrating various interacting proteins responsible for flagellar movement. PG- peptidoglycan layer, EXP- export apparatus. (Motaleb *et al*., 2015).

1.1.4.2 Chemotaxis

Chemotaxis is the movement of an organism in response to an extracellular stimulus, allowing bacteria to move towards chemoattractants. Potent chemoattractants for *Brachyspira* are mucin, fructose and L-serine and cells mediate the movement towards these chemicals using rotation of the periplasmic flagella (Kennedy and Yancey, 1996; Naresh and Hampson, 2010). The chemotaxis mechanisms have been studied extensively in *E. coli* (Parkinson, 2003), however it is proposed that similar mechanisms are utilised by *Brachyspira* as genome sequencing has revealed the presence of similar chemotaxis proteins (Che) and methyl-accepting chemotaxis proteins (MCPs) (Bellgard *et al.*, 2009; Wanchanthuek *et al.*, 2010). Chemotaxis uses a two-component histidine protein kinase dependent signal transduction pathway, comprising of both MCPs and Che proteins as illustrated in Figure 1.4. MCPs are

chemoreceptors which bind signals in the periplasmic domain. This signalling cascade begins with the histidine kinase CheA and the coupling factor CheW; CheA autophosphorylates and the phosphate group is transferred to CheY, thus activating this protein. The phosphorylated CheY proteins then binds to the FliM protein in the flagellar motors to facilitate rotation.

Although the exact pathways involved in *Brachyspira* chemotaxis are yet to be elucidated, bioinformatics has identified MCP proteins and Che proteins present in *B. hyodysenteriae* and *B. pilosicoli*. Genes for the MCP proteins, including *aer, tsr, tar, tgr* and *tap* have been identified in *B. hyodysenteriae* and genes for the Che proteins, including *cheA, cheB, cheD, cheR, cheV, cheX, cheY* and *cheZ* have been identified in *B. hyodysenteriae* and/or *B. pilosicoli*. (Bellgard *et al*., 2009; Mappley *et al*., 2012; Wanchanthuek *et al*., 2010).

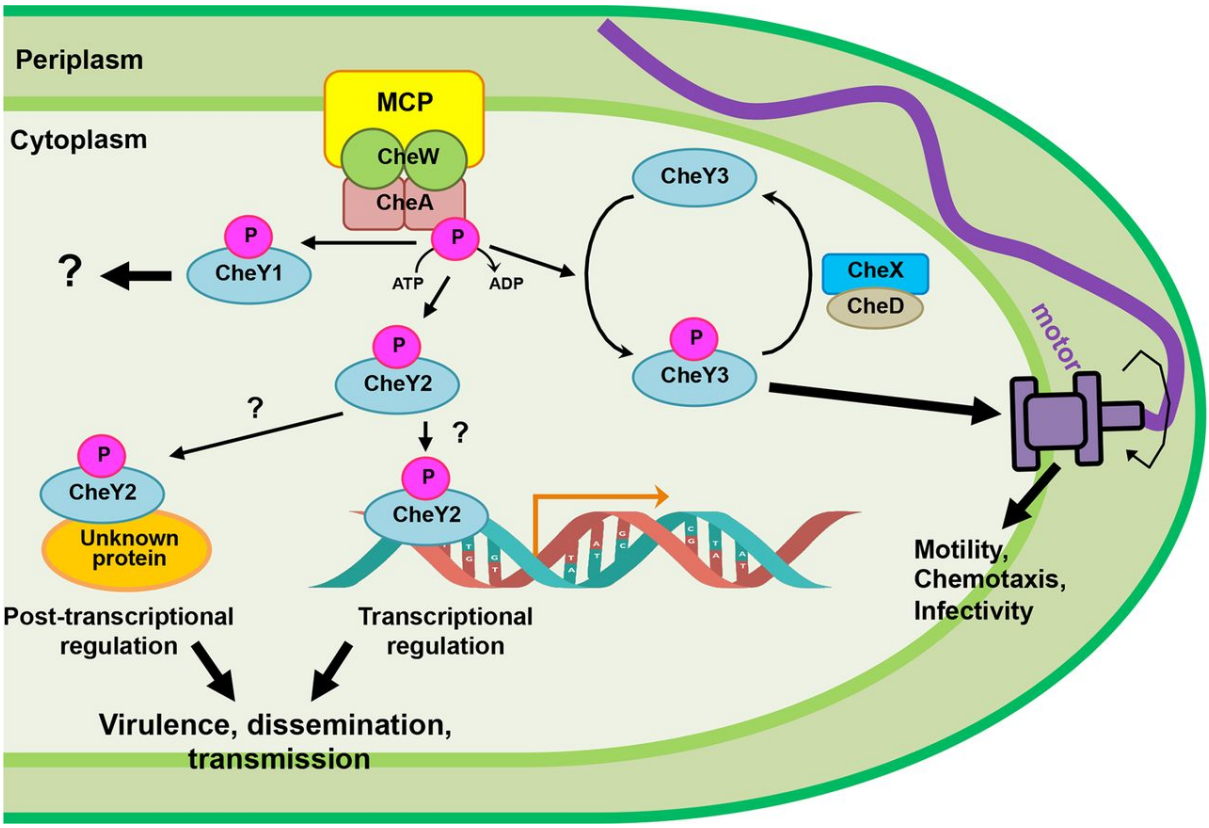


Figure 1. 4 Main components of key chemotaxis pathways in spirochaetes (Xu *et al.*, 2017). Responses to chemotactic signals in the gastrointestinal tract results in a signalling cascade from the methyl-accepting chemotaxis protein (MCP), which detects the signal, which for *Brachyspira* species may be mucin. This signal is transduced through CheW and CheA, where CheA autophosphorylates and the phosphate group is transferred to CheY. The phosphorylated CheY binds to flagellar motor proteins to initiate the movement of the periplasmic flagellar.

1.1.4.3 Adhesion and invasion

The mechanisms of adhesion and invasion by *Brachyspira* are poorly understood, however *B. pilosicoli* has been observed forming end on attachments to enterocytes to form a pseudo-brush border. *B. pilosicoli* can also invade the crypts, epithelium and the lamina propria. Thus far, it appears that only *B. aalborgi* and *B. pilosicoli* can adhere to healthy epithelial cells, whereas evidence suggests that other *Brachyspira* species adhere and invade disrupted cells (Mappley *et al.*, 2014). The attachments formed by *Brachyspira* may be responsible for disruption of the intestinal mucosa, thus resulting in poor fluid and nutrient absorption, leading to diarrhoea and poor growth performance in infected animals.

Invasion of enterocytes and between epithelial tight junctions is depicted in Figure 1.5. As with other virulence factors, this mechanism has not been well characterised, however translocation of *Brachyspira* to the blood has been noted and systemic infection in multiple organs have been observed in experimental infections of chickens with *B. pilosicoli* (Mappley *et al.*, 2013; Woodward *et al.*, 2015). In cases of human spirochaetemia, only elderly and critically ill patients have been diagnosed with *Brachyspira* in the bloodstream, therefore it may be associated with impaired immune function (Bait-Merabet *et al.,* 2008; Fournié-Amazouz *et al*., 1995).

1.1.4.4 Haemolysis

The production of haemolysins is considered a major virulence factor, particularly for *B. hyodysenteriae. Brachyspira* species known to infect poultry are weakly β- haemolytic, whereas *B. hyodysenteriae* is known to be strongly β- haemolytic. Haemolysins cause lysis of red blood cells by destroying their cell membrane and it could be suggested that they may be involved in the damage to the mucosa of the large intestine during swine dysentery. Whole genome analysis of *B. hyodysenteriae* and *B. pilosicoli* has identified eight putative haemolysin genes (Wanchanthuek *et al.*, 2010).

1.1.4.5 NADH oxidase activity

Despite their anaerobic nature, all *Brachyspira* species can tolerate oxygen due to their NADH oxidase activity. This enzyme protects the bacterial cells from oxygen toxicity and studies have shown that an inactivated *nox* gene reduces the colonisation and virulence of *B. hyodysenteriae*, but does not render them avirulent (Stanton *et al*., 1999).

1.1.4.6 Lipooligosaccharide

Lipooligosaccharides are complex carbohydrates associated with the surface of the bacterial cell wall. They are known to activate the host immune response via toll-like receptor 4 (TLR4) which is expressed on a number of immune cells, which may result in the mucosal inflammation observed during infection (John *et al.,* 2017). Furthermore, LOS forms the basis of serological testing for *B. hyodysenteriae* isolates implicated in swine dysentery and reacts strongly with the sera of infected pigs (Hampson *et al.,* 1990), suggesting that it is a prime target for the host immune response (Trott *et al.*, 2001).

A number of LOS have been described in *Brachyspira,* however, the exact mechanisms of LOS in disease is unknown. *B. pilosicoli* LOS has been shown to be serologically distinct between isolates of this species and shows no cross-reactivity with other *Brachyspira* species, which may explain why hosts may suffer from repeated infections with *Brachyspira*, and why vaccines have not been hugely successful (Lee & Hampson, 1999; Trott *et al.,* 2001).

1.2 Avian Intestinal Spirochaetosis (AIS)

1.2.1 What is avian intestinal spirochaetosis?

Avian intestinal spirochaetosis is an enteric disease resulting from the colonisation of the caeca and/or colo-rectum of poultry with spirochaetes such as *Brachyspira.* This disease primarily infects layer hens and broiler breeders over the age of 20 weeks and can range in severity of symptoms, but typically presents as a delayed onset of lay, watery brown to grey diarrhoea, a 6-10% reduction in egg production, faecally stained eggs and a reduced growth rate (Burch *et al*., 2006). More severe clinical manifestations can include typhlitis and increased mortality rates within flocks. Avian intestinal spirochaetosis has a significant impact

on the UK laying industry, exacerbated by the removal of antibiotic growth promoters from livestock feed in 2006.

The *Brachyspira* species primarily reported to cause avian intestinal spirochaetosis are *B. pilosicoli, B. intermedia* and *B. alvinipulli*; *B. innocens* is proposed to be non-pathogenic, and is often isolated alongside these pathogenic species (Burch *et al*., 2006).

1.2.2 What is the significance of avian intestinal spirochaetosis?

Since the ban of antibiotic growth promoters from livestock feed in 2006, the incidence of avian intestinal spirochaetosis has increased significantly and is becoming a growing concern (Medhanie *et al.*, 2013). This disease has a significant economic burden on the UK laying industry, with the most recent estimation stating that it costs the industry £18 million per annum. This figure was based on a national flock size of 30 millions laying hens, with 30 eggs lost per hen at a cost of 5p per egg (Mappley *et al.,* 2014). These losses estimated in the UK are likely to be similar worldwide, with studies in Australian, Italy and Iran showing similar prevalence to that observed in the UK (McLaren *et al.*, 1996; Bano *et al.*, 2008; Bassami *et al*., 2012).

1.2.3 Epidemiology of avian intestinal spirochaetosis

1.2.3.1 Host Range

Brachyspira can colonise the caeca and colo-rectum of many avian species, both domestic and wild. Of the domestic poultry, known hosts include: layer hens (Davelaar *et al.,* 1986; Dwars *et al.,* 1989; Griffiths *et al.,* 1987; Myers *et al.,* 2009; Swayne *et al*., 1992), broiler hens (Dwars *et al.*, 1990), broiler breeder hens (Stephens and Hampson, 2002) and turkeys (Mathey and Zander, 1955). Of the wild bird species, known hosts include: flamingos, rheas,

ducks, swans, (Trott *et al.*, 1996) grouse (Fantham, 1910), ducks (Råsbäck *et al*., 2007), ostriches (Stoutenburg and Swayne, 1992) and pheasants (Webb *et al.*, 1997).

1.2.3.2 Transmission and Infection

Brachyspira are transmitted through flocks via the faecal-oral route. Transmission begins when birds come into contact with vectors, such as wild birds, rodents or environmental reservoirs (Oxberry *et al.,* 1998; Boye *et al.*, 2001; Backhans *et al.,* 2010), *Brachyspira* are then shed in the faeces of infected birds and rapidly spread throughout a flock as shown in Figure 1.5 (Le Roy *et al.*, 2015). Farming practices can facilitate the spread of infection through poor biosecurity and rodent control. Furthermore, intensification of farming has led to the close proximity of poultry, facilitating the rapid spread of disease. Wet litter conditions can also promote the proliferation of bacteria and the coprophagic behaviour of chickens in these conditions can result in rapid spread of *Brachyspira*.

The infection process begins when the spirochaetes reach the caeca and colo-rectum of the bird. Once these pathogens reach the lower gastrointestinal tract, chemotaxis towards mucin and the strong motility through the mucus enables *Brachyspira* to gain close proximity to the intestinal epithelium. *B. pilosicoli,* in particular can form end on attachments to cells to form a pseudo-brush border. *Brachyspira* can also invade enterocytes and disturb tight junctions, therefore increasing the chances of translocation to the bloodstream and subsequently a systemic infection (Le Roy *et al.*, 2015). Systemic infections were reported by Mappley *et al.* (2013), whereby *Brachyspira* were isolated from the liver, spleen and reproductive tracts of experimentally infected hens.

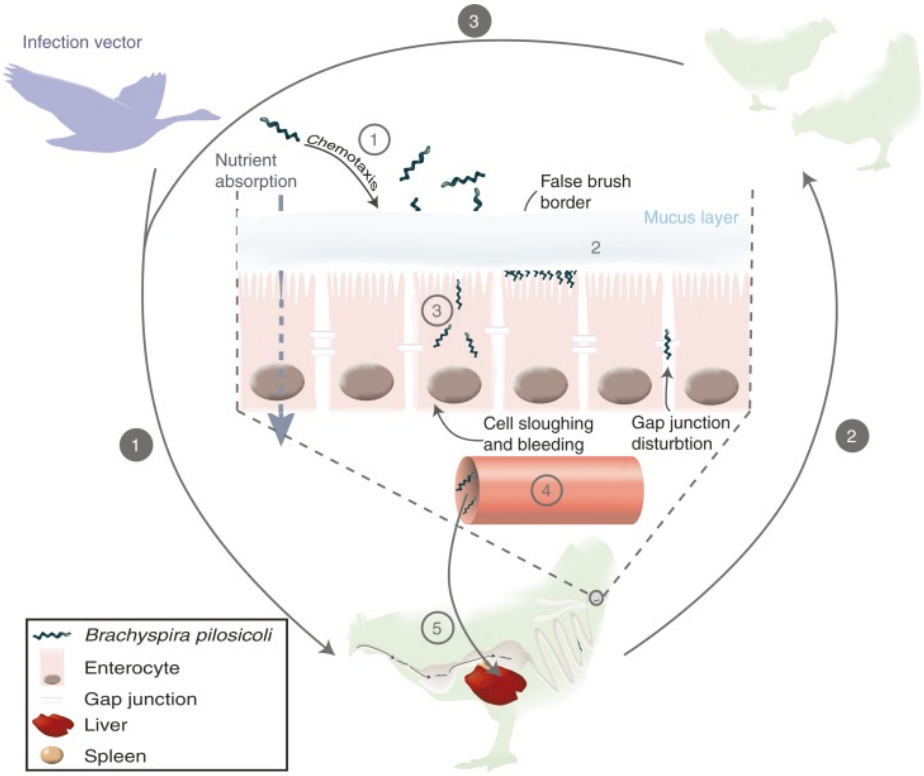


Figure 1. 5 The proposed transmission and infection process of *B. pilosicoli*. The numbers in the grey circles represent the transmission process: 1. Infection vectors such as wild bird or rodents spread *Brachyspira* to poultry, which become infected by the faecal-oral route; 2. *Brachyspira* is excreted in the faeces of infected birds which is then spread throughout the rest of the flock; 3. Persistence of infection within the flock. The numbers in white circles represent the infection process of *B. pilosicoli* once it has reached the lower gastrointestinal tract of the birds: 1. Virulence factors such as motility and chemotaxis facilitate the movement of *Brachyspira* to the mucus and subsequently the intestinal epithelium; 2. *B. pilosicoli* attach to enterocytes can form a 'false brush border'; 3. *Brachyspira* can invade the epithelial cells and disrupt the epithelial tight junctions; 4. Thus resulting in the translocation of *Brachyspira* to the blood stream; 5. Systemic infection can occur (Le Roy *et al.,* 2015).

1.2.3.3 Prevalence

Avian intestinal spirochaetosis has been well documented in Europe, North American and Australia, but is thought to be endemic worldwide (Pattinson, 2007). The prevalence of *Brachyspira* in UK laying flocks has been estimated to be as high as 100% in barn flocks, 91% in free range flocks, 86% in organic free-range flocks and up to 76% in caged flocks (Burch, *et al.,* 2009). In the study presented by Burch *et al.* (2009), *Brachyspira* infection in free-range hens was seen to occur by the age of 22 weeks, shortly after they have access to the outside.

By comparison, caged hens did not test positive for *Brachyspira* until 36 weeks of age. The infection of free-range hens at a young age is likely to be as a result of exposure to environmental reservoirs of *Brachyspira* more frequently than caged hens. *Brachyspira* have been shown to survive in soil and contaminated water supplies. At 10°C it has been shown that *B. hyodysenteriae* and *B. pilosicoli* have the potential to survive in soil for up to 210 days, this therefore makes infection control difficult in free-range flocks as *Brachyspira* infection would occur naturally from eating and scratching behaviour (Boye *et al.*, 2001). Furthermore, *Brachyspira* have also demonstrated the ability to survive for up to 66 days in contaminated water supplies (Oxberry *et al*., 1998). The abundance of the bacteria in the environment not only means that infection spreads by direct ingestion of bacteria from the environment but can also be rapidly spread on farms by vectors such as humans, dogs, rodents and wild birds. In barn and caged systems, the spread of *Brachyspira* by rodent vectors is of increasing concern. A survey in Sweden identified rodents, in particular rats, to be major carriers of *Brachyspira* on poultry farms, with 83% of rats and 33% of mice carrying the bacteria (Backhans *et al.,* 2010). Wild birds are also known to harbour intestinal spirochaetes asymptomatically, therefore the spread of pathogenic *Brachyspira* species to commercial flocks is possible. Thus, in barn and caged systems biosecurity is essential to limit the prevalence of avian intestinal spirochaetosis.

Epidemiological studies have shown that commercial poultry are most frequently infected with *B. intermedia* followed by *B. pilosicoli*, however the prevalence of *B. innocens* is much higher than the pathogenic species (Bano *et al*., 2008; Burch *et al*., 2009). *B. alvinipulli* has been rarely isolated from commercial poultry in the UK but has been isolated in poultry in the USA (Swayne *et al.*, 1992), The Netherlands (Feberwee, *et al.*, 2008) and Sweden (Jansson and Pringle, 2011).

1.2.3.4 Disease incubation period and persistence

The incubation period and persistence of avian intestinal spirochaetosis can be dependent on the dose and species of *Brachyspira* ingested and also the environment of the poultry infected, as previously discussed. Clinical symptoms can occur in experimentally infected birds as soon as five days post infection (Mappley *et al.*, 2014) but colonisation is most commonly observed after seven days post infection (Hampson *et al.,* 2002; Mappley *et al.*, 2013). However, significant colonisation and disease symptoms can persist several weeks following experimental infection, although this is not true for all experimental infections (Hampson and Jamshidi, 2002; Hampson *et al.,* 2002; Stephens and Hampson, 2002). Persistence of infection in naturally infected flocks is likely due to the close proximity of barn or caged hens and/or contact with environmental reservoirs by free-range hens (Le Roy *et al.*, 2015).

1.2.3.5 Possible zoonoses

Brachyspira species, in particular *B. pilosicoli* are of potential zoonotic concern. Although human intestinal spirochaetosis in relatively rare, it mostly occurs in immunocompromised patients and those with poor living standards (Korner and Gebbers, 2003). Epidemiological studies in humans have not been undertaken and publications are often formed of case studies where human infection has been determined by colo-rectal biopsy. These biopsies show the attachment of spirochaetes to the intestinal epithelium, noted as a false brush border (Hampson, 2018; Ngwa *et al.,* 2016). In rare cases, *B. pilosicoli* has been shown to cause bacteraemia in the elderly and immunocompromised patients (Prim *et al*., 2011; Trott *et al*., 1997).

Despite intestinal spirochaetosis being rarely observed in humans, *B. pilosicoli* has a wide host range and has been documented to infect humans, poultry and pigs. *B. pilosicoli* from these three hosts have been shown to have genetic similarity, suggesting that infected animal hosts may potentially serve as a reservoir for human disease (Hampson *et al.*, 2006; Mappley *et al.*, 2012). Furthermore, it has been shown that *B. pilosicoli* can contaminate meat of infected poultry, which may pose as a zoonotic threat to humans (Verlinden *et al.*, 2012). It is important to note, however, that broiler hens are rarely infected with *Brachyspira* and thus the infection risk comes from the meat of spent laying hens.

1.2.4 Clinical symptoms and pathology

The understanding of the clinical symptoms and pathology associated with avian intestinal spirochaetosis has developed as a results of experimental challenge of *Brachyspira* in chicks and adult hens and also from the observation of natural occurrence of disease. Although experimental challenge with *Brachyspira* does not mimic the natural environment in which commercial flocks may acquire these bacteria, in a commercial environment, milder symptoms such as diarrhoea may be undetected and thus avian intestinal spirochaetosis is often not recognised in flocks.

1.2.4.1 Sub-clinical colonisation

Colonisation with intestinal spirochaetes in the absence of clinical disease is most common in wild birds and water birds, where they are considered as commensal bacteria. Studies have shown that these birds and their habitats can be reservoirs of infection for both humans and other animals as infection with both pathogenic and non-pathogenic *Brachyspira* results in sub-clinical colonisation in these wild hosts (Oxberry *et al.,* 1998; Jansson *et al.*, 2004). Sub-clinical colonisation of commercial laying hens and broiler breeders is associated with

infection with *B. innocens* or *B. murdochii* (Stephens and Hampson, 1999, Stephens and Hampson, 2002).

1.2.4.2 Mild to moderate clinical disease

Mild to moderate clinical disease is associated with colonisation by *B. alvinipulli, B. intermedia* and *B. pilosicoli*, particularly in laying hens and broiler breeders over the age of 20 weeks. Typically, infection is associated with symptoms such as diarrhoea, increased fat content in the faeces, a 6-10% reduction in egg production, reduced growth rates and a delayed onset of lay (Davelaar *et al.*, 1986; Griffiths *et al.*, 1987; Burch *et al.*, 2006). Furthermore, eggs may have decreased nutritional value and infected broiler breeders produce weak chicks with retarded growth rates (Dwars *et al.,* 1993). Moreover, a comprehensive study carried out in the Netherlands found that broiler flocks derived from breeders infected with *Brachyspira* had poorer feed conversion and slower growth rates than the offspring of flocks where *Brachyspira* was not present. It is important to note that the broilers themselves were not colonised with spirochaetes (Smit *et al.*, 1998).

1.2.4.3 Severe clinical disease

Severe clinical symptoms are infrequently observed in commercial chicken flocks but may be exacerbated by stress (Stephens and Hampson, 2001). Severe clinical symptoms have been observed in chickens infected with *B. intermedia* and *B. pilosicoli* whereby spirochaetes have penetrated between or below caecal epithelial cells, resulting in erosion or necrosis of the mucosa (Davelaar *et al.*, 1986; Dwars *et al.*, 1990). Furthermore, the presence of spirochaetes between epithelial cells poses a risk of systemic infection as noted in studies which suggest *B. pilosicoli* can infect multiple organs in laying hens (Mappley *et al.*, 2013). Histological

examination of caeca from naturally infected birds has revealed that invasive spirochaetes and typhlitis were associated with *B. intermedia* infections (Stephens and Hampson, 2001).

1.2.4.4 Experimental infection models of avian intestinal spirochaetosis

To improve the understanding of *Brachyspira* infection and the clinical symptoms associated with each of the species implicated in poultry disease, experimental infections have been conducted. Infection with *B. alvinipulli* resulted in a dilated caeca with foamy, pale-yellow contents in both one-day old chicks and adult hens Histopathology also showed that infected birds had severe symptoms including, mild to moderately severe typhlitis, caecal villus hyperplasia and inflammation as a result of lymphocyte and/or heterophil infiltration (Swayne *et al.*, 1995). Birds naturally infected with *B. alvinipulli* also displayed the mild symptoms associated with avian intestinal spirochaetosis, in addition to some of the more severe clinical symptoms such as typhlitis and tissue necrosis (Feberwee *et al.*, 2008).

Symptoms of experimental infection with *B. intermedia* included, diarrhoea, reduced growth rates, reduced egg production and egg weights (Hampson and McLaren, 1999; Phillips *et al.*, 2004). Histopathology confirmed the presence of *B. intermedia* in the caeca and scanning electron microscopy confirmed the presence of spirochaetes in the caecal epithelium, however no associated pathology was observed. Infected birds had less fat than control birds and the caeca were more gassy and contained a higher water content (Hampson and McLaren, 1999).

Experimental challenge with *B. pilosicoli* in chicks and adult hens caused symptoms such as diarrhoea and decreased growth rates. Adult hens had increased faecal staining of egg shells and reduced egg production, in addition to foamy caecal contents. Mild pathology is often associated with experimental infection in hens including vacuolation in enterocyte cytoplasm

and crypt elongation (Dwars *et al.*, 1992; Trott *et al.,* 1995; Stephens and Hampson, 2002; Hampson and Jamshidi, 2002).

B. pilosicoli has shown a unique ability to form pseudo brush borders by forming end on attachments to enterocytes in the gastrointestinal tract of infected hosts, which can damage the microvilli and impair gastrointestinal function (Trampel *et al.,* 1994; Muniappa *et al.*, 1996). This has also been noted in human and porcine infections with *B. pilosicoli* (Mathan and Mathan, 1985; Girard *et al.,* 1995; Brito *et al.*, 1996).

1.2.5 Host immunity

The host immune mechanisms directed against *Brachyspira* are poorly understood. The innate immune response is the first line of defence against *Brachyspira* and infection has been associated with the infiltration of immune cells such as macrophages in to the lamina propria (Antonakopoulos *et al*, 1982; Padmanabhan *et al.*, 1996; Duhamel, 2001). Moreover, experimentally infected pigs and mice have been shown to develop serum antibody responses to *Brachyspira* isolates, although these adaptive immune responses have been shown to vary (Sacco *et al.,* 1997; Jamshidian *et al.*, 2004; Hampson, 2018).

Brachyspira infection can remain persistent in poultry flocks, which may be attributed to the ability of these bacteria to adhere to the intestinal epithelium which may aid in subverting the immune system or evading an immune response, but these mechanism have yet to be elucidated (Hampson, 2018). Different *Brachyspira* isolates show considerable antigenic variability in their surface lipooligosaccharide (LOS), however more work is needed to define their role in disease, including whether they may be involved in attachment or protective immunity for example.

1.2.6 Diagnosis of avian intestinal spirochaetosis

Avian intestinal spirochaetosis requires a definitive diagnosis consisting of a number of phenotypic and molecular techniques, as clinical symptoms alone are non-specific and insufficient.

Culture followed by light, phase-contrast or dark-field microscopy to confirm cell morphology and motility, can be used as a preliminary screen for *Brachyspira*. In addition, biochemical testing, can be employed to speciate isolates (Fellström and Gunnarsson, 1995), although as discussed previously the biochemical profiling of avian isolates can be unreliable (Atyeo *et al.*, 1999). Therefore, phenotypic tests that are time consuming and inconsistent, especially with mixed species infections, need to be routinely supported by molecular methods.

PCR primer sequences to identify *Brachyspira* have been designed to target either the 16S ribosomal ribonucleic acid (rRNA) gene or the NADH oxidase (*nox*) gene (Mikosza *et al.*, 2001; Phillips *et al.*, 2005; 2006). Primers were designed to identify isolates from the *Brachyspira* genus, *B. pilosicoli* primers were designed using the 16S rRNA gene and *B. intermedia* primers were designed using the *nox* gene (Mikosza *et al.*, 2001; Phillips *et al*., 2005, 2006). A major drawback of the current molecular methods for *Brachyspira* identification for avian intestinal spirochaetosis is that there are no current PCR primers to identify *B. alvinipulli* or *B. innocens*. However, with the increased availability of whole genome sequencing, designing PCR assays for less common or non-pathogenic *Brachyspira* strains will become increasingly feasible. Other molecular methods utilised to identify *Brachyspira* include restriction digestion of specific PCR products that result in species-specific banding patterns using gel electrophoresis (Barcellos *et al.*, 2000; Rohde *et al*., 2002).

Histological samples can be used to confirm the attachment of *Brachyspira* to the intestinal mucosa using the both haematoxylin and eosin staining and Warthin-Starry staining.

However, attachment of *Brachyspira* is not observed in all clinical cases, which is why combinations of diagnostic methods must be utilised. Fixed tissue can also be visualised using electron microscopy, although this is an expensive and time-consuming method of visualisation. Fluorescence *in situ* hybridisation can also be used as to support diagnosis of intestinal spirochaetosis. This method uses fluorescent oligonucleotide probes to visualise and localise *Brachyspira* associated with the intestinal mucosa in fixed tissue. This technique has been utilised in both animal and human samples and is advantageous in that it confirms the presence of *Brachyspira* and also shows where they are localised in the gastrointestinal tract (Boye *et al.*, 1998; Jensen *et al.*, 2000; Jensen *et al.,* 2001).

Finally, as whole genome sequencing techniques become more readily available, sequencing isolates will become more commonplace. This will not only improve the accuracy of *Brachyspira* diagnosis, but also aid with improving the understanding of these organisms.

1.2.7 Treatment of avian intestinal spirochaetosis

Brachyspira are susceptible to a range of antibiotics, however there are a limited number of antibiotics licensed for use in animal medicine and different countries have different legislation surrounding their use. Some of the most common antimicrobials used in the livestock industry to treat avian intestinal spirochaetosis are tiamulin, tylosin, chlortetracycline and lincomycin, with tiamulin and tylosin the only licensed antibiotics for use in the UK (Burch, 2011). Bacteriostatic antibiotics such as tiamulin and tylosin are the most effective against *Brachyspira* and target bacterial protein synthesis, although antimicrobial resistance to tylosin is increasing due to its previous use as an antibiotic growth promoter in swine feed (Hampson *et al.,* 2006; Kim *et al.*, 2016). Resistance to tylosin has been associated with mutations in the peptidyl transferase region of the 23S rRNA gene which

prevents tylosin binding (Karlsson *et al.*, 2004). Tiamulin has been used in veterinary medicine for a considerable period of time, primarily in the swine industry (Taylor, 1980). Denagard® Tiamulin (Novartis) is currently available for use in pigs and has more recently become available for use in the poultry industry for the treatment of avian intestinal spirochaetosis (Woodward *et al.*, 2015). Another benefit of tiamulin is that it has a zero-day egg withdrawal period, and therefore treatment of avian intestinal spirochaetosis does not impact adversely on the egg industry. Tiamulin resistance is also associated with point mutations in the V domain of the 23S rRNA gene and in the L3 gene. These genes are within close proximity of the peptidyl transferase binding site of tiamulin and thus mutations in these genes may affect the efficacy of tiamulin (Pringle *et al.*, 2004; Hidalgo *et al.*, 2011).

1.2.8 Interventions and prevention against avian intestinal spirochaetosis

Interventions are required to control the spread of *Brachyspira*, especially as incidence of this organism can be up to 90% in flocks and disease is hard to diagnose, as previously discussed. Despite avian intestinal spirochaetosis being easily treated with antibiotics, antimicrobial resistance is emerging and there is an increased consumer demand for antibiotic free animal products. Therefore, it is essential to implement measures to prevent disease, not only to protect animal welfare, but also address consumer demands.

1.2.8.1 Biosecurity

Efficient biosecurity on farms is essential for controlling the introduction and spread of avian intestinal spirochaetosis. Wild birds and rodent reservoirs require effective controls usually by physical containment including security fencing, bird-proof netting around openings and rodent traps (Oxberry *et al.,* 1998; Backhans *et al.,* 2010; Mappley *et al.*, 2014). Disinfectant foot-dips are utilised to prevent transmission especially as *Brachyspira* have been shown to

have a wide host range (Hampson and Jamshidi, 2002). Furthermore, chickens have been shown to be infected with *B. hyodysenteriae,* which is not known to typically infect poultry, therefore biosecurity on multi-species farms is of paramount importance (Feberwee *et al.*, 2008). Other disinfectants such as iodine, chlorine, hydrogen peroxide and quaternary ammonium disinfectants have shown efficacy against *Brachyspira* in organic matter (Phillips *et al.*, 2003; Corona-Barrera *et al.*, 2004).

1.2.8.2 Vaccines

Vaccine development against intestinal spirochaetosis has been primarily focussed to alleviate swine dysentery, however vaccines have elicited varied responses (Hampson *et al.,* 1993). Autogenous vaccines for *B. pilosicoli* and *B. intermedia* have been tested in pigs and chickens and have demonstrated the ability to elicit antibody responses, although there are no commercially available vaccines. Despite primary and secondary antibody responses, these vaccines have not been able to reduce the intestinal colonisation *Brachyspira* (Hampson *et al.*, 2000). Experimental vaccines in mice generated systemic and colonic IgG antibodies and after vaccination, mice were colonised for significantly fewer days compared to non-vaccinated controls (Movahedi and Hampson, 2010). These studies suggested the potential for oligopeptide-binding proteins as constituents for vaccines, however, further testing would be required in experimental chicken studies.

1.2.8.3 Essential oils and phytochemicals

More recently the efficacy of essential oils and phytochemicals has been assessed for inhibitory capabilities against *Brachyspira* species. Phytochemicals have a long history of use in animal nutrition as an alternative to antibiotic growth promoters; their use is generally associated with decreased bacterial load (Franz *et al.*, 2010; Grilli *et al.*, 2015). Studies have

revealed that essential oils such as thymol and cinnamaldehyde were inhibitory against *B. intermedia in vitro* and *in vivo* and that their inhibitory effects were comparable to that of other Gram negative, enteric pathogens such as *E. coli*, *Salmonella* and *Campylobacter* (Verlinden *et al.*, 2013). This study also demonstrated the potential of essential oils as an intervention for *B. intermedia* infection, with the bacterial numbers being significantly reduced compared to experimentally infected controls. Efficacy of compounds against *B. hyodysenteriae* was correlated to the carbon chain length (Maele *et al.*, 2016). Short-chain fatty acids such as lactic and formic acid were associated with high MIC values and medium chain fatty acids such as thymol and carvacrol were associated with lower MIC values (Maele *et al.*, 2016). Additionally, citrus fruit extracts such as BIOCITRO have demonstrated bactericidal and bacteriostatic activity against *B. hyodysenteriae* by damage of the cell membrane and induction of the formation of spherical bodies (de Nova *et al.*, 2017).

1.2.8.4 The introduction of probiotics to prevent avian intestinal spirochaetosis

Probiotics have been shown to be effective against *Brachyspira* both *in vitro* and *in vivo*. *L. rhamnosus* and *L. farciminis* have shown the ability to antagonise the motility of *Brachyspira* through co-aggregation. SEM analysis demonstrated that *Brachyspira* are trapped in a physical network with the *Lactobacillus* (Bernardeau *et al.*, 2009). Furthermore, the production of organic acids such as lactic acids have been shown to elicit stress responses in these pathogens (Bernardeau *et al.*, 2009). Cell free supernatant has been shown to have a lethal effect on *Brachyspira* cells which was characterised by the perforation of the bacterial cell wall. Other stress responses, such as the formation of spherical bodies form in response to toxic chemicals and changes in environmental conditions as a result of lactic acid production (Bernardeau *et al.*, 2009). It is proposed that this spherical body formation is an

important survival mechanism to protect the cells from adverse conditions, and cells have been shown to return to normal morphology when conditions improve (Wood *et al.*, 2006). Additionally, homofermentative lactic acid bacteria such as *L. salivarius*, *L. amylovorus*, *B. thermophilum* and *B. faecium* have shown inhibitory abilities against both *B. hyodysenteriae* and *B. pilosicoli*. Both live cells and cell free supernatant have elicited inhibitory effects, however heterofermentative lactic acid bacteria such as *L. reuteri* have not shown inhibitory potential against *Brachyspira* (Klose *et al.*, 2009; 2010). This is contrary to more recent research that has demonstrated inhibition of *B. pilosicoli* and *B. innocens* by *L. reuteri* and *L. salivarius* cells and their cell free supernatant *in vitro* (Mappley *et al.*, 2011). Thus, demonstrating that probiotics are isolate specific, not species specific. Inhibition of *Brachyspira* was pH dependent and low pH cell free supernatant from *Lactobacillus* resulted in significant inhibition of *Brachyspira*. Additionally, in concurrence with the studies conducted by Bernadeau *et al* (2009), the motility of *Brachyspira* was shown to be inhibited by both viable and heat-inactivated *Lactobacillus* after four and twenty-four hours of contact time. *L. reuteri* and *L. salivarius* isolates were also able to significantly reduce the adherence of *Brachyspira* to intestinal epithelium cells *in vitro* and *ex vivo* (Mappley *et al.*, 2011). In addition to these *in vitro* studies, further *in vivo* studies aimed to determine if the *L. reuteri* isolate used in Mappley *et al* (2011) would improve hen health following experimental infection with *B. pilosicoli*. The results showed decreased faecal moisture, decreased faecal staining on eggs, decreased *Brachyspira* colonisation and decreased pathology associated with disease, in addition to increased egg weights in birds pre-treated with *L. reuteri* (Mappley *et al.*, 2013).

Whilst there have been several reports of the efficacy of probiotics against *Brachyspira* and avian intestinal spirochaetosis specifically, there is a clear need to generate a greater

understanding of lactic acid bacteria such as *Lactobacillus* and their interaction with *Brachyspira* if we are to fully exploit their potential to contribute to control measures.

1.3 Lactobacilli and their potential as probiotics

1.3.1 The genus *Lactobacillus*

The *Lactobacillus* (lac "milk" and bacillum "small rod") genus is assigned to the Lactobacillaceae family, in the order Lactobacillales and class Bacilli, which belongs to the Firmicutes phylum. *Lactobacillus* species are Gram positive, rod-shaped, non- spore forming, non-motile, fermentative, facultative anaerobes. Lactobacilli can be either hetero- or homofermenters, depending on their species. Heterofermenters such as *Lactobacillus reuteri* ferment sugars such as glucose to produce lactic acid in addition to carbon dioxide, ethanol and acetic acid via the pentose phosphate pathway, whereas homofermenters such as *Lactobacillus crispatus* and *Lactobacillus salivarius* ferment glucose to produce lactic acid via the glycolysis pathway (Caplice and Fitzgerald, 1999).

These bacteria are abundant commensals in the gastrointestinal tracts of bird and mammals, in addition to being exploited for the production of fermented food products such as cheese and yogurt. *Lactobacillus* species are widely used as probiotics in human and veterinary medicine to promote good gastrointestinal health and have many known benefits.

1.3.2 What are probiotics and why are they important?

Probiotics (pro "for" and biotic "life") are defined as "live microorganisms that confer health benefits on the host when administered in adequate quantities" (FAO/WHO, 2002). Probiotic bacteria such as *Lactobacillus* are natural colonisers of both mammalian and avian gastrointestinal tracts and are generally recognised as safe (GRAS) for consumption. Probiotics work on the assumption that a healthy gastrointestinal microbiota can confer

health benefits. They are an important alternative to the use of antimicrobials, especially in livestock and have the potential to aid in the protection of the gastrointestinal tract, not only from pathogens, but also from stress related disease. It is important to note that probiotics are notoriously strain specific, thus, extensive research into specific probiotic strains against specific pathogens is required to provide sufficient evidence for their efficacy.

1.3.3 Probiotic use in poultry

Probiotics have been used extensively in the poultry industry since the 1970s. The research conducted by Rantala and Nurmi (1973) first described the use of probiotic bacteria to protect chicks from infection with *Salmonella* Infantis. This study introduced the concept of competitive exclusion, whereby two-day old chicks were pre-treated with the gastrointestinal contents from healthy, mature chickens, to protect from challenge with *Salmonella* (Rantala and Nurmi, 1973). There are also numerous studies that have demonstrated the competitive exclusion effects of probiotics against avian pathogens such as *Campylobacter jejuni*, *Salmonella* Typhimurium and *E. coli* (Weinack *et al.*, 1981; Schoeni and Wong, 1994; Revolledo *et al.,* 2006; Zhang *et al.*, 2007).

The use of probiotics in poultry aims to restore or beneficially alter the microbiota in animals, particularly those that are young, stressed or have been treated with antibiotics (Collins *et al.*, 2009). The microbiota of conventionally reared birds does not stabilise until 4-6 weeks of age (Mead, 1989) and studies have suggested that the timing of probiotic administration can be essential to their protective effects. For example, *L. reuteri* administration in the first week of life may be able to impact the abundance of *Lactobacillus* in the avian gastrointestinal tract, in addition to protecting against pathogens after 6 weeks of age (Nakphaichit *et al.*, 2011). Furthermore, intensification of poultry farming can induce stress in animals which can have a

direct effect on the gastrointestinal tract, reducing productivity and growth rates. *Lactobacillus* probiotics have demonstrated the ability to increase and diversify the intestinal microbiota following heat stress, which may contribute to promoting a healthy intestinal flora and reducing the effects of stress in poultry flocks (Lan *et al.*, 2004).

There are a number of probiotics available, some of which are well defined in terms of the probiotic strains used, and others which are undefined. Products such as Protexin Pro-Soluble® are well defined and consist of *Enterococcus faecium* NCIMB 10415, which has been shown to increase the feed conversion, growth and concentration of lactic acid bacteria in the gastrointestinal tract of birds (Samli *et al.*, 2007). Whereas products such as Aviguard® contain undefined organisms from the gastrointestinal tracts of healthy, pathogen free birds. Despite being undefined, there is sufficient evidence to suggest that this product can reduce the colonisation of pathogens such as *E. coli* and *Salmonella* through competitive exclusion (Nakamura *et al.*, 2002; Hofacre *et al.*, 2009). Typically, probiotics for use in animals are *Enterococcus*, *Bacillus* or *Saccharomyces* species, whereas *Lactobacillus* and *Bifidobacterium* species are typically consumed by humans, but are gaining more interest in the poultry industry (Simon *et al.*, 2001).

1.3.4 *Lactobacillus* probiotics in poultry

Lactobacillus are present throughout the chicken gastrointestinal tract and are often the dominant genus isolated (Stanley *et al*., 2014). Within this genus, the most abundant species of *Lactobacillus* are *L. crispatus, L. reuteri* and *L. salivarius* (Hilmi *et al.*, 2007).

Research regarding the efficacy of *Lactobacillus* probiotics for the prevention of *Brachyspira* is still very much in its infancy. However, the use of probiotics in poultry production to mitigate common pathogens such as *E. coli, Salmonella* and *Campylobacter* is well

established. Therefore, there is potential to develop suitable probiotics to prevent *Brachyspira*.

1.3.5 Guidelines for the characterisation of bacteria intended for probiotic use

There are clear guidelines set out by the European Union for the characterisation of microorganisms intended for use as animal feed additives (EFSA *et al.*, 2017). These support the use of whole genome sequencing as the gold standard for the "unambiguous" identification of organisms, in addition to screening for antimicrobial resistance, toxin and virulence factor genes.

To be considered as a suitable probiotic for animal feed supplementation, a microorganism needs to be on the qualified presumption of safety (QPS) list, which stipulates the species of microorganisms that are considered to be safe for use as animal feed additives (EFSA, 2013). Furthermore, phenotypic antimicrobial sensitivities must be determined, in addition to screening for the presence of AMR genes to ensure that isolates do not possess genes that could be transferrable to other bacteria in the gastrointestinal tract. The break-points for Gram positive bacteria are transparent and well defined in this legislation, thus ensuring the robust characterisation of bacterial isolates for use as potential feed additives. It is also recommended that the ability of isolates to produce antimicrobial metabolites is determined. This can be achieved using cell free supernatant (CFS) studies to investigate whether there are any inhibitory affects against a pathogen. If inhibition is observed, the inhibitory metabolite(s) should be identified where possible. In addition to the *in vitro* and *in silico* interrogation of bacterial isolates and their genome sequences, it is recommended that *in vivo* studies are carried out to determine the impact on the gut microbiota in the desired host and to ensure any additional additives are compatible with other additives already

administered in animal feed. The EFSA guidelines were established to provide standardised guidelines to ensure the safety of microbial products that are fed to livestock and may ultimately enter the human food chain. Furthermore, these guidelines work towards ensuring the safety of bacterial products before they are widely distributed in feed (EFSA *et al.*, 2017).

1.3.6 Proposed mechanisms of probiotic action

1.3.6.1 Competitive exclusion

Competitive exclusion is described as the exclusion of a pathogen from an ecological niche by out competition by a probiotic (Callaway *et al.*, 2008; Collins *et al.*, 2009). The term competitive exclusion was first demonstrated in 1973 (Rantala and Nurmi, 1973). Since then, probiotic bacteria have been well researched as competitive exclusion products against many common poultry pathogens such as *E. coli, Salmonella* and *Campylobacter* (Weinack *et al.*, 1981; Schoeni and Wong, 1994; Mead, 2000; Revolledo *et al.,* 2006; Zhang *et al.,* 2007). Additionally, recent studies have shown promise for probiotics as potential competitive exclusion products for *Brachyspira* (Mappley *et al.,* 2013).

In studies to investigate the potential for *Lactobacillus* probiotics to antagonise *Brachyspira,* potential *Lactobacillus* probiotics demonstrated the ability to co-aggregate with *Brachyspira* (Bernardeau *et al.*, 2009; Mappley *et al.*, 2011). This physical interaction between bacteria is proposed to antagonise the growth, motility and adherence capabilities of *Brachyspira*, thus impairing its ability to colonise the caeca and/ or large intestine, in addition to having a direct effect on motility and chemotaxis (Mappley *et al.*, 2011). Following on from this research, Mappley *et al*. (2013) were the first to demonstrate that *Lactobacillus*, namely *L. reuteri* LM1, had the ability to antagonise *B. pilosicoli in vivo*. Competitive exclusion was proposed as a mechanism of *Brachyspira* inhibition as *L. reuteri* reduced the colonisation of *B. pilosicoli* and

the associated pathology. However, it is also important to consider other inhibitory mechanisms such as production of acids and antimicrobial compounds, discussed in this chapter.

1.3.6.2 Production of organic acids

Lactobacillus produce organic acids that have been shown to induce stress responses in pathogenic bacteria and have a direct inhibitory effect. Both homo- and heterofermentative *Lactobacillus* species produce organic acids as a result of glucose fermentation and can mediate the pH of the gastrointestinal tract, in additional to having direct effects on the intestinal mucous and bacterial numbers (Skrivanová and Marounek, 2007).

Organic acids have been utilised as poultry feed and water additives for many years, as an alternative to antibiotic growth promoters (Khan and Iqbal, 2016). In the UK, formic, propionic and lactic acid are regularly used to acidify feed (Collins *et al.*, 2009) and the primary objective of this acidification is to reduce the numbers of pathogenic bacteria in the gastrointestinal tract. The addition of organic acids to poultry feed is generally considered as safe (GRAS) by the European Union and studies have suggested that this supplementation has beneficial effects directly on the host. For example, histological analysis of the small intestine has suggested that organic acids can significantly increase villus height, subsequently facilitating nutrient absorption and therefore the performance of chickens (Adil *et al.*, 2010). These histological alterations are proposed to be as a result of reducing the numbers of intestinal bacteria, thus decreasing the inflammatory responses in the intestinal mucosa, which promotes increased villus height.

Additionally, organic acids are commonly used to directly inhibit pathogenic bacteria such as *E. coli*, *Salmonella* and *Campylobacter* (Van Immerseel *et al.*, 2006; Naseri *et al.,* 2012). At a

low pH, organic acids will be undissociated and therefore lipophilic and can diffuse across the bacterial cell membrane, reducing the pH of the cell, resulting in cell death (Skrivanová and Marounek, 2007). Furthermore, organic acids have been shown to cause stress responses in *Brachyspira* species, resulting in the formation of spherical bodies and damage to the cell membrane (Bernardeau *et al.*, 2009). Lactic acid is the major metabolite produced by *Lactobacillus* isolates and has been shown to be a potent membrane permeabiliser, causing sub-lethal damage to bacterial cells (Alakomi *et al.*, 2000; Fayol-Messaoudi *et al.*, 2005).

1.3.6.3 Production of antimicrobial metabolites

In addition to pH dependent inhibition of bacteria in the gastrointestinal tract, pH independent mechanisms are proposed to effectively inhibit pathogenic bacteria. Mechanisms of inhibition include the production of bacteriocins, the production of hydrogen peroxide and the production of other secondary metabolites such as reuterin.

Bacteriocins are ribosomally synthesised, antimicrobial peptides produced by *Lactobacillus* species that were initially believed to be inhibitory against other closely related Gram positive organisms. However, there is also evidence to suggest that they are capable of inhibiting Gram negative bacteria (Cotter *et al.,* 2005; Messaoudi *et al.*, 2013). Many *Lactobacillus* isolates produce different bacteriocins, which are separated in to three main groups: Class I lantibiotics, Class II bacteriocins and Class III bacteriocins, which are now known as bacteriolysins. Class I lantibiotics contain the unusual amino acids, lanthionine and β-methyllanthionine and these proteins undergo posttranslational modification during maturation. These bacteriocins exhibit antimicrobial activity against closely related Gram positive bacteria, and rarely inhibit Gram negative bacteria (Lee and Kim, 2011). Nisin is a common Class I bacteriocin used in food preservation and forms pores in bacterial cell

membranes, resulting in cell death (Garg *et al.*, 2014). Class II bacteriocins are non-lanthionine containing bacteriocins and are more commonly produced than Class I bacteriocins. Class II bacteriocins are small (< 10KDa), heat-stable proteins that do not undergo extensive posttranslational modification (Yang *et al.*, 2014). The mechanism of action of Class II bacteriocins is similar to that of Class I, in that they permeabilise the cell membrane of the target bacterial cell, resulting in the efflux of metabolites from the target cell and subsequent cell death (Ennahar *et al.*, 2000). The final class of bacteriocins are the bacteriolysins, these are high molecular weight, heat-labile proteins which are sub-divided into two distinct groups: group A and group B. Group A are bacteriolytic proteins such as Enterolysin A, which kill the target cell through lysis of the bacterial cell wall of sensitive cells (Khan *et al.*, 2013). Group B are non-bacteriolytic proteins such as Helveticin J, that have a bactericidal effect on target cells (Joerger and Klaenhammer, 1986).

Other inhibitory metabolites produced by *Lactobacillus* species include reuterin, from some *L. reuteri* isolates and hydrogen peroxide production. Reuterin is a broad-spectrum antimicrobial produced by some isolates of *L. reuteri* and is an example of a secondary metabolite produced by *L. reuteri* in the absence of glucose, the preferred energy source. Reuterin is produced when cells metabolise glycerol as the primary carbon source and has exhibited a range of inhibitory activity against foodborne pathogens such as *E. coli* and *L. monocytogenes* (Rodríguez *et al.*, 2003). Hydrogen peroxide can be produced by some *Lactobacillus* species, namely *L. acidophilus, L. gasseri, L. johnsonii* and *L. reuteri* and is cytotoxic to a range of pathogens including *S.* Typhimurium and *E. coli in vitro* (ÜNlütürk and Turantasl, 1987; Asad *et al.*, 2004). The antimicrobial effect has been attributed to the production of reactive oxygen species from its dissociation and subsequent damage to bacterial DNA (Halliwell, 1978).

1.3.6.4 Promotion of intestinal health and immunomodulation

The gastrointestinal tract and the immune system form a complex, integrated structure that has evolved to not only digest food, but also as the first line of defence against ingested pathogenic bacteria. The natural microbiota of the gastrointestinal tract is important in forming both a physical and immunological barrier between the host and its environment, to maintain good intestinal health (Delcenserie *et al.*, 2008). A healthy gastrointestinal tract is essential for preventing disease and improving the welfare of livestock.

Due to the interactions between the gastrointestinal tract and the immune system, the immunomodulatory properties of probiotic bacteria have been proposed. Probiotic bacteria are known to influence the production of cytokines from intestinal epithelial cells and innate immune cells *in vitro* (Haller *et al.*, 2000; Zhang *et al.*, 2005; Ma *et al.*, 2011). This can be achieved through a number of mechanisms. Firstly, probiotic isolates have demonstrated the ability to activate macrophages and lymphocytes and influence phagocytosis by macrophages in a strain dependent manner, in a murine model (Perdigón *et al.*, 1988). This has the potential to improve the immune response to pathogenic bacteria by priming the immune system. Secondly, probiotic bacteria have been shown to modulate immune responses of the innate immune system by acting on pathways such as the Janus kinase/signal transducers and activators of transcription (JAK/STAT) and NFκB pathways. *Lactobacillus* isolates have demonstrated the ability to inactivate these pathways, resulting in the downregulation of important pro-inflammatory cytokines such as IFNγ and IL6 (Lee *et al.*, 2010; Llewellyn and Foey, 2017). Probiotics are also known to produce suppressor of cytokine signalling (SOCS) proteins. These proteins also inhibit the JAK/STAT pathway, in addition to TLR4 mediated cytokine responses (Dimitriou *et al.*, 2008), subsequently resulting in the downregulation of pro-inflammatory cytokines.

1.4 Project Rationale

The understanding of *Brachyspira* pathogenesis and disease may have been hampered by the absence of suitable infection models and mutagenesis strategies. While there have been a number of experimental infections using chicken models, resultant disease and pathology have been varied and do not provide sufficient data on the mechanisms of disease or the virulence of specific isolates. Furthermore, there are a number of ethical and scientific limitations of this model. For example, animal experimental models are tightly regulated and require specialist staff and research facilities which are often unavailable. Therefore, alternative models of infection need to be considered to allow for more detailed mechanistic studies, such as those presented in this thesis. Moreover, the paucity of knowledge of the *Brachyspira* genome has also resulted in the relatively poor understanding of these organisms and their pathobiology.

1.4.1 Tissue culture models

Immortalised cell lines are frequently utilised to study enteric bacterial pathogens such as *E. coli*, *Salmonella* and *Campylobacter* (Finlay and Falkow, 1990; Everest *et al.*, 1992; Jung *et al.*, 1994). The use of the HT29-16E colonic cell line has recently been developed for the study of *Brachyspira* adhesion and invasion and to determine the efficacy of probiotic interventions (Mappley *et al.*, 2011). In addition to the availability of human intestinal cells lines, avian cell lines are becoming more readily available, although to date a widely available commercial avian intestinal cell line is not available. However, the availability of the HD11 avian macrophage cell line has allowed the study of immune interactions with organisms such as *Salmonella* and *Campylobacter* to be investigated (Withanage *et al.*, 2004; Smith *et al.*, 2005; Chiang *et al.,* 2009). This cell line was derived from chicken haematopoietic cells which were

infected with avian leukaemia virus. These cells have macrophage-like properties; they express Fc receptors, are capable of phagocytosis and express macrophage cell surface antigen (Beug *et al.*, 1979).

1.4.2 *Galleria mellonella* infection model

Brachyspira virulence has not been extensively investigated due to the lack of and poor accessibility to suitable *in vivo* models and the restrictions surrounding them, as previously discussed. In order to attempt to develop a suitable *in vivo* model, the *G. mellonella* model was explored to assess the suitability for use as a model for *Brachyspira* infection. This model has been shown to be powerful and reliable for the study of bacterial virulence and treatment of a range of pathogens in the context of a functional immune system (Desbois and Coote, 2012; Betts *et al.*, 2017; Jønsson *et al.*, 2017; Mehat *et al.*, 2018). Additionally, it has received considerable interest due to its simplicity, availability and few ethical issues surrounding its use (Desbois and Coote, 2012) (Figure 1.6).

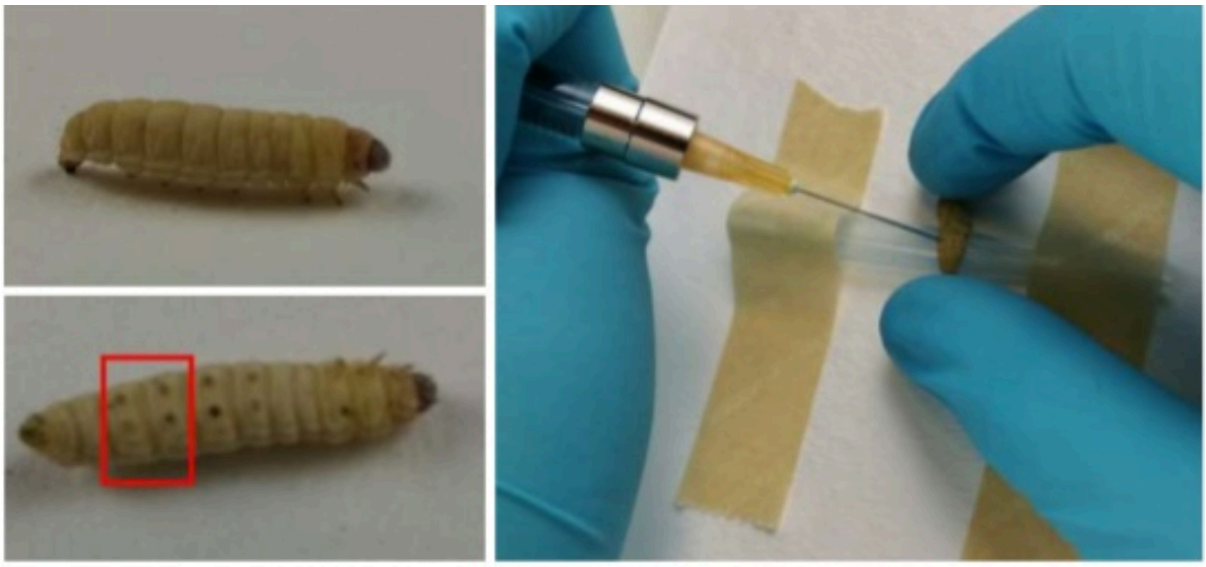


Figure 1. 6 **(A)** Healthy *G. mellonella* larvae at the instar larval stage. **(B)** Larvae are typically injected into the prolegs. For single injections, they are typically injected with a pathogen in the right proleg, for multiple injections used in these studies, one injection was into the right proleg and one into the left. **(C)** Larvae are typically injected with a Hamilton syringe in to the proleg.

G. mellonella are capable of a cellular immune response, mediated by haemocytes in the haemolymph of the larvae. These cells are phagocytic in nature and have a number of functional similarities to mammalian neutrophils, which are analogous with avian heterophils (Brooks *et al.,* 1996). This is important for *Brachyspira* infection models as this pathogen can infect both mammalian and avian hosts. Haemocytes primarily kill pathogens through respiratory burst and the production of superoxide, in addition to the production of antimicrobial peptides (Kavanagh and Reeves, 2004). An advantage of this model is that the immune response can be measured; the response to LPS and peptidoglycan activates the phenoloxidase cascade, which leads to the production of melanin. The extent of melanin production is often directly associated with bacterial load and results in a colour change in the larvae (Desbois and Coote, 2012), whereby a cream colour indicates no melanin production and dark brown indicates production, as shown in Figure 1.7. Melanin

encapsulates the invading pathogen and prevents proliferation (Söderhäll and Cerenius, 1998).

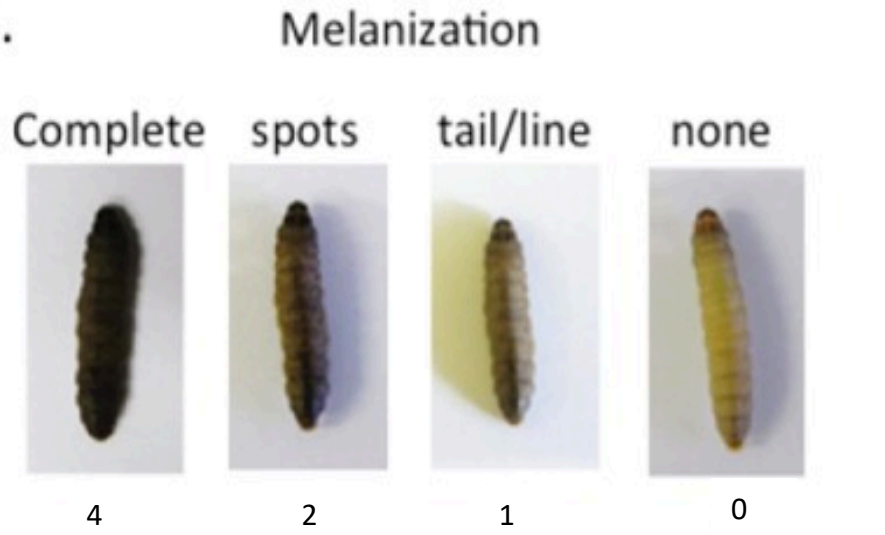


Figure 1. 7 Images of *G. mellonella* larvae showing different degrees of melanisation. A score of 0 indicates no melanisation, a score of 1 indicates melanisation at down the tail line, a score of 2 indicates spots of melanisation through the body and a score of 4 indicates complete melanisation and death. Melanisation is the synthesis and deposition of melanin to encapsulate pathogens during infection. Adapted from Tsai *et al*. (2016)

In these studies, this model represented a systemic infection and not a model of gastrointestinal infection. *Brachyspira* has been shown to cause systemic infection in both humans and in experimentally infected chickens, and therefore this is a relevant model to begin to elucidate the virulence of *Brachyspira* species *in vivo*. Although, there is an understanding that results need to be supported by follow up *in vivo* studies with a more relevant host.

1.4.3 A Metabolomics Approach

The use of nuclear magnetic resonance spectroscopy (NMR) to identify small metabolites in the cell free supernatants of *Lactobacillus* has been described in previous studies (Parolin *et al.*, 2015; Nardini *et al.*, 2016). These studies have begun to elucidate the metabolites that

may be contributing to pathogen inhibition. In the studies presented in this thesis, the metabolites produced by *Lactobacillus* were investigated in order to explore the mechanisms of *Brachyspira* inhibition that were originally identified by Mappley *et al.* (2011).

Proton NMR (^{1}H- NMR) analyses the different types of hydrogen present in a molecule and how these hydrogen atoms relate to other atoms in the molecule. It exploits the magnetic properties of atomic nuclei and works on the principle that nuclei with an odd atomic number, such as those in hydrogen, have nuclear spin. In the absence of a magnetic field, the nuclear spins of hydrogen are randomly orientated. However, when NMR applies an electromagnetic field, the nuclear spin either aligns with or against the magnetic field. The more highly populated alignment is known as the lower energy state. Electromagnetic radiation is used the 'flip' this alignment from the low energy state, to the high energy state. Removing the electromagnetic radiation allows the nuclei to return to their normal energy state, emitting a small magnetic field. This signal is collected and converted to peaks on the NMR spectrum. The basic arrangement of the NMR spectrometer is illustrated in Figure 1.8: the sample for analysis is positioned in a magnetic field and excited with radio frequency. The aligned hydrogen atoms produce a radio frequency which is used to generate an output signal, and this signal is then converted into an NMR spectra (as presented in Chapter 6). The spectra generated is a plot of the radio frequency or chemical shift against the absorption the energy applied.

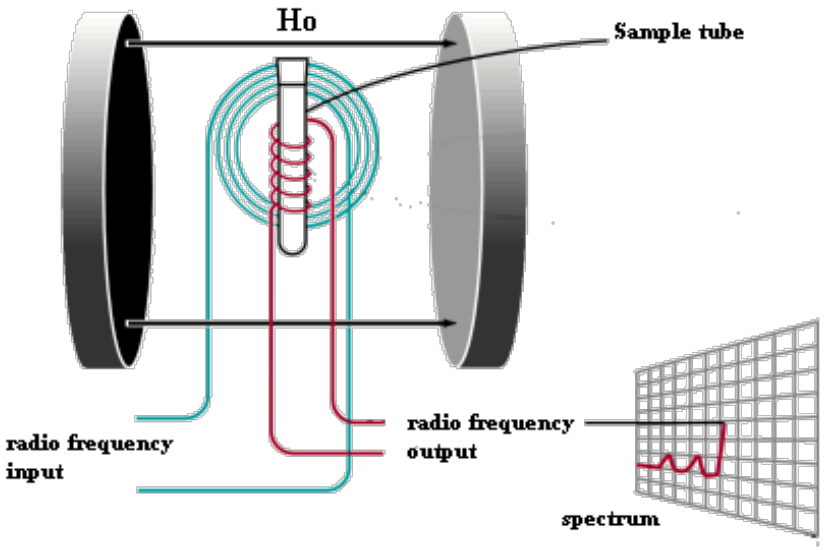


Figure 1. 8 The basic arrangement on an NMR spectrometer. The sample is placed in a magnetic field and excited with radio frequency. The alignment of hydrogen atoms with the atomic field produces a radio frequency output signal which is translated into the NMR spectra presented in Chapter 6.

1.5 Project aims and hypothesis

As discussed throughout this chapter, *Brachyspira* infection and avian intestinal spirochaetosis have a significant impact on the welfare of animals and cost the UK laying industry in excess of £18 million each year. Since the ban of antibiotic growth promoters in the EU in 2006 the prevalence of *Brachyspira* has increased and there are only limited licenced antibiotics for treatment. Therefore, it is essential that preventative measures are employed to improve animal health and reduce incidence of disease, in addition to protecting the antibiotics available to treat infection. Probiotics have demonstrated suitability as potential interventions against *Brachyspira*, however mechanisms of action have yet to be fully elucidated. Moreover, there is a paucity of knowledge surrounding the pathobiology of *Brachyspira*. Therefore, a more comprehensive understanding of the genetic and phenotypic capabilities of *Brachyspira* may be able to identify novel probiotic interventions to reduce the incidence of avian intestinal spirochaetosis.

The hypothesis of this project was that *Lactobacillus* probiotics would be a suitable mitigation strategy for avian intestinal spirochaetosis caused by *Brachyspira* species.

The main aims of this project were therefore to investigate *Lactobacillus* probiotics as an intervention against *Brachyspira* infection and to elucidate these mechanisms of inhibition. This was achieved through the following objectives:

- To characterise phenotypically and genotypically a panel of *Brachyspira* isolates, including all species involved in avian intestinal spirochaetosis (discussed in chapters 3 and 4).
- To isolate and characterise a panel of *Lactobacillus* probiotic candidates from healthy chickens in accordance with the European guidelines and determine their inhibitory capabilities against *Brachyspira* isolates (discussed in chapter 5).

- To elucidate the mechanisms attributed to the inhibition of *Brachyspira* by way of analysing the *Lactobacillus* cell free supernatant for inhibitory metabolites and investigating the effects of co-aggregation of the two bacteria in co-culture (discussed in chapter 6)
- To develop an *in vitro* tissue culture model to investigate the avian macrophage immune responses to *Brachyspira* species (discussed in chapter 7)
    - To investigate how a probiotic intervention may impact these responses.
- To develop an *in vivo Galleria mellonella* model to study the virulence of *Brachyspira* species (discussed in chapter 7).
    - To investigate how a probiotic intervention may be able to protect the *G. mellonella* following *Brachyspira* infection.

Chapter 2: Materials and Methods

2.1 Bacteriological Methodology

2.1.1 *Brachyspira* bacterial isolates and culture conditions

A panel of thirteen UK poultry *Brachyspira* isolates were assembled for use in the studies presented here. All strains were isolated from clinical cases of avian intestinal spirochaetosis (AIS). These isolates represented the four species implicated in poultry disease and are detailed in Table 2.1. For all studies, the *Brachyspira* isolates were cultured on fastidious anaerobic blood agar (FABA) (LAB M) supplemented with 5% sheep blood or *Brachyspira* selective agar (BSA), consisting of blood base agar number 2 (Oxoid), supplemented with 5% sheep blood, spectinomycin (800μg/ml), colistin (25μg/ml) and vancomycin (25μg/ml) (Sigma-Aldrich) (Råsbäck *et al.*, 2005). Where broth cultures were required *Brachyspira* were transferred from the surface of an agar plate into brain heart infusion broth (BHI), supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma- Aldrich). *Brachyspira* were cultured anaerobically in an anaerobic cabinet (Don Whitley Scientific) (10% hydrogen, 10% carbon dioxide and 80% nitrogen) at 37°C for 3-5 days.

Brachyspira stock cultures were stored at -80°C in FBS supplemented with 30% *Brachyspira* enrichment broth (BEB), consisting of tryptone soya broth (Oxoid), supplemented with 10% DL-cysteine hydrochloride (Sigma- Aldrich), 10% glucose and 10% FBS.

Table 2. 1 *Brachyspira* isolates used in this project

***Brachyspira* Species**	**Isolate Name**	**Species of Isolation**	**Source[1]**
B. alvinipulli*	ATCC 51933	Chicken	ATCC Collection (Swayne *et al.*, 1995)
B. alvinipulli	SAP 945	Chicken	University of Surrey
B. innocens*	SAP 927	Chicken	University of Surrey
B. innocens*	SAP 924	Chicken	University of Surrey
B. innocens*	SAP 943	Chicken	University of Surrey
B. intermedia	SAP 891	Chicken	University of Surrey
B. intermedia*	SAP 919	Chicken	University of Surrey
B. intermedia	SAP 866	Chicken	University of Surrey
B. pilosicoli*	B2904	Chicken	APHA (Mappley *et al*., 2012)
B. pilosicoli*	SAP 865	Chicken	University of Surrey
B. pilosicoli*	SAP 859	Chicken	University of Surrey
B. pilosicoli	SAP 903	Chicken	University of Surrey
B. pilosicoli	SAP 868	Chicken	University of Surrey

[1] Source: American Type Culture Collections, Virginia, University of Surrey, Guildford, United Kingdom, APHA, Animal and Plant Health Agency, Addlestone, United Kingdom.
* *Brachyspira* isolates fully characterised and used throughout the project.

2.1.2 *Lactobacillus* bacterial isolates and culture conditions

A panel of seventeen poultry derived *Lactobacillus* isolates were assembled for the studies presented here. The isolates are detailed in Table 2.2. All isolates were isolated from the faeces of healthy chickens at the start of the project. *Lactobacillus* isolates were grown on Man-Rogosa-Sharpe (MRS) agar and in MRS broth (Oxoid) anaerobically in an anaerobic cabinet at 37°C for 18 hours.

Lactobacillus stock cultures were stored in MRS broth supplemented with 30% (v/v) glycerol at -80°C.

Heat-inactivated *Lactobacillus* were prepared by resuspending each *Lactobacillus* isolate in 0.1M sterile phosphate buffered saline (PBS) at the required cell density, determined by optical density (OD) and heating aliquots at 95°C for 20 minutes.

Lactobacillus cell free supernatants (CFS) were prepared by centrifuging 18-hour broth cultures of each *Lactobacillus* isolate (10^9 CFU/ml) at 3000 x g for 10 minutes at ambient temperature, followed by filter-sterilisation through a 0.2µm filter (Fisher-Scientific) yielding CFS. The pH of each CFS ranged from 3.8-4.5, depending on the isolate, therefore the pH was adjusted to 3.8, 4.5 and 7.2 for inhibition assays using 1M sodium hydroxide or 1M hydrochloric acid (Sigma-Aldrich) to investigate both pH dependent and independent mechanisms of inhibition.

To further investigate the pH independent mechanism is inhibition, *Lactobacillus* CFS samples were incubated with 200µg trypsin for one hour at 37°C to denature heat-stable proteins. Additionally, CFS samples were heat treated at 120°C for 20 minutes to denature heat-labile proteins in the CFS.

Table 2. 2 *Lactobacillus* isolates presented in this project

Lactobacillus Species	**Isolate Name**	**Species of Isolation**
L. agilis	SAP 2104	Chicken
L. crispatus*	SAP 2105	Chicken
L. crispatus*	SAP 2106	Chicken
L. crispatus*	SAP 2107	Chicken
L. crispatus	SAP 2109	Chicken
L. crispatus	SAP 2110	Chicken
L. crispatus	SAP 2111	Chicken
L. mucosae	SAP 2102	Chicken
L. reuteri*	SAP 2108	Chicken
L. reuteri**	SAP 2114	Chicken
L. reuteri**	SAP 2115	Chicken
L. salivarius*	SAP 2103	Chicken
L. salivarius	SAP 2112	Chicken
L. salivarius*	SAP 2113	Chicken
L. salivarius**	SAP 2116	Chicken
L. salivarius**	SAP 2117	Chicken
L. salivarius*	SAP 2118	Chicken

* *Lactobacillus* isolates sent for whole genome sequencing.
** *Lactobacillus* isolates identified and characterised as potential probiotics.

2.1.3 Other bacterial isolates and culture conditions

E. coli K12, avian pathogenic *E. coli* isolates A1, B1 and B3 (APEC), *Salmonella* Typhimurium SAP 16 and *Enterococcus faecalis* ATCC 29212 were obtained from the Surrey Animal Pathogen (SAP) collection. Isolates were cultured on nutrient agar (NA) or nutrient broth aerobically at 37°C for 18 hours.

Stock cultures were stored in nutrient broth supplemented with 30% (v/v) glycerol at -80°C.

2.1.4 Isolation of *Lactobacillus* from chicken faeces

All *Lactobacillus* isolates were isolated from the faeces of healthy free-range laying hens in the UK. Faeces was enriched in MRS broth for 4 hours, anaerobically at 37°C, followed by culture on MRS agar in an anaerobic cabinet at 37°C for 48 hours. Single colonies of differing

morphology were selected and sub-cultured on three separate occasions on to MRS agar to ensure purity for further characterisation.

2.1.5 Identification and Characterisation of *Brachyspira*

2.1.5.1 Gram stain

A suspension of *Brachyspira* in 0.1M sterile PBS was prepared and a smear was made on a glass slide, which was then heat-fixed and 1% (w/v) crystal violet added at ambient temperature for 30 seconds. The slide was washed with water and Lugol's iodine solution added for 30 seconds, after which IMS was added for 3 seconds. The counterstain, safranin was added for 60 seconds and then the slide was washed with water and air-dried. Slides were examined under oil immersion light microscopy, where the pink Gram stain and spirochaete morphology of *Brachyspira* were determined.

2.1.5.2 Warthin- Starry silver stain

A suspension of *Brachyspira* in 0.1M sterile PBS was prepared and a smear was made onto a glass slide and heat-fixed. Slides were placed in 1% silver nitrate and incubated for one hour in a water bath at 56°C. Slides were placed in a solution of 2% silver nitrate, gelatin and hydroquinone until staining appeared golden brown (1-3 minutes). Slides rinsed with warm water and dehydrated using 100% ethanol before coverslips were mounted.

Slides were examined under light microscopy to determine spirochaete morphology.

2.1.5.3 α-Glucosidase, β-glucosidase and α-galactosidase activity tests

A suspension of *Brachyspira* in 0.1M sterile PBS (≥ McFarland 4.0) was prepared by transferring surface growth from FABA agar using a sterile swab. Three aliquots of the suspension were transferred into separate universal tubes and an α- glucosidase (p-

nitrophenyl-α-D-glucopyranoside), β-glucosidase (p-nitrophenyl-β-Dglucopyranoside) or α-galactosidase (p-nitrophenyl-α-D-galactopyranoside) diatab (Rosco Diagnostics) was added to each. The suspensions were incubated anaerobically at 37˚C for 16 hours. Results were recorded whereby a yellow colour change was regarded as positive and no colour change as negative for the respective enzyme activity.

2.1.5.4 Hippurate test

A suspension of *Brachyspira* in 1% (w/v) sodium hippurate solution (Sigma-Aldrich) (≥ McFarland 2.0) was prepared by transferring surface growth from FABA agar using a sterile swab. The suspensions were incubated anaerobically at 37˚C for 24 hours, after which 150 µl API NIN (ninhydrin) reagent (BioMérieux) was added. Following 10-minute incubation at ambient temperature, results were recorded whereby a blue-purple colour change was regarded as positive and a clear-orange colour change as negative for the ability to hydrolyse sodium hippurate to glycine and sodium benzoate.

2.1.5.5 Indole test

A suspension of *Brachyspira* in BHI + 10% FBS (≥ McFarland 4.0) was prepared by transferring surface growth from FABA agar using a sterile swab. The inoculated BHI broth was incubated anaerobically, at 37˚C for 24 hours, after which 150 µl API JAMES (Kovac's) reagent (BioMérieux) was added. Following a 10-minute incubation at ambient temperature, results were recorded whereby the formation of a pink-red pellicle was regarded as positive and a yellow pellicle as negative for the ability to cleave indole from tryptophan.

2.1.5.6 Antimicrobial sensitivity testing of *Brachyspira* isolates

Antimicrobial sensitivity testing of all *Brachyspira* isolates were performed using the broth microdilution method.

An antimicrobial doubling series for gentamicin, tylosin and chlortetracycline (Sigma-Aldrich) ranged from 0.125- 128 µg/ml, for lincomycin (Sigma-Aldrich) 0.0625- 64 µg/ml and for tiamulin (Sigma-Aldrich) 0.008- 0.25 µg/ml. Antimicrobial 10× stock solutions were prepared in accordance to the manufacturer's instructions and diluted to double the desired working concentration for each antimicrobial, these were filter sterilised using a 0.2µm filter. Antimicrobials were double diluted into BHI broth + 10% FBS (Karlsson *et al.*, 1999) to support the growth of *Brachyspira,* 100µl of *Brachyspira* cell suspension at a concentration of 10^6 CFU/ml was added to each well of a 96 well plate and incubated anaerobically at 37°C for 3-4 days. The MIC was recorded as the lowest antimicrobial concentration to inhibit the growth of *Brachyspira.* Each assay was repeated in triplicate with three biological repeats.

2.1.5.7 *Brachyspira* growth curves

Brain heart infusion (BHI) broth supplemented with 10% FBS was prepared by transferring surface growth from FABA agar using a sterile swab to yield a concentration of 10^6 CFU/ml. 100µl of each broth was transferred into a 96 well plate. Plates were incubated in an anaerobic cabinet for 5 days at 37°C. Optical density (OD_{620}) readings were taken every 10 hours for the duration of experiment using a Tecan Spark 10 plate reader (Tecan). Bacteria were enumerated using a Helber counting chamber under dark field microscopy (x40) every 24 hours. Each assay was repeated in triplicate with five biological repeats.

2.1.5.8 Biolog Phenotypic Microarray™

The metabolic capabilities of *Brachyspira* were analysed using Biolog Phenotypic Microarray™ (PM) technology which screened for the utilisation of 190 carbon sources (PM1 and PM2) by each isolate. All PM1 and PM2 plates and reagents were supplied by Biolog and experiments were conducted according to the manufacturer's instructions. In brief, all plates and reagents were pre-incubated in an anaerobic cabinet 24 hours before use. Using a sterile swab, the surface growth of *Brachyspira* from FABA was inoculated in to 7 ml of Biolog inoculating fluid (IF-0 x1) to achieve a transmittance of 40% using a Biolog turbidimeter. 6ml of this inoculum was diluted into 18 ml of inoculating fluid containing Dye mix D (Biolog), potassium ferricyanide (Sigma-Aldrich) and menadione sodium bisulfite (Sigma- Aldrich). Bacterial suspensions were pipetted into the PM1 and PM2 96 well plates at a volume of 100µl/ well and incubated in an anaerobic cabinet at 37°C for 72 hours.

A major modification was made to the way the PM1 and PM2 plates were read due to technical issues with the OmniLog plate reader. All plates were read using a Tecan Spark 10 plate reader (Tecan) at 540nm wavelength to measure the reduction of the tetrazolium dye (yellow) to formazan (purple), indicative of *Brachyspira* respiration at 37°C, every 24 hours for 72 hours. It is important to note that the dye reduction is indicative to cellular respiration, which occurs independent of cell growth. Each experiment was conducted with three biological repeats.

PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 m-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 m-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

Figure 2. 1 Plate layout for PM1 Biolog plate. Each of the 96-wells contains a different carbon source available to determine bacterial respiration capabilities. Well A1 does not contain a carbon sources and is therefore a negative control for growth.

PM2A MicroPlate™ Carbon Sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 i-Erythritol	B11 D-Fucose	B12 3-0-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 a-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 a-Keto-Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanedione	H12 3-Hydroxy 2-Butanone

Figure 2. 2 Plate layout for PM2 Biolog plate. Each of the 96-wells contains a different carbon source available to determine bacterial respiration capabilities. Well A1 does not contain a carbon sources and is therefore a negative control for growth.

2.1.6 Identification and Characterisation of *Lactobacillus* as Potential Probiotics

2.1.6.1 Gram stain

Details of the Gram stain protocol are provided in section 2.1.5.1, where *Lactobacillus* were determined by the purple Gram Stain and bacillus morphology.

2.1.6.2 Catalase test

A drop of 3% (w/v) hydrogen peroxide solution (Sigma-Aldrich) was placed on to a glass slide and a small amount of *Lactobacillus*, transferred from MRS agar was added. Results were recorded immediately with the production of gaseous bubbles regarded as catalase positive and the absence of gaseous bubbles as catalase negative. Catalase-positive *E. coli* K12 was used as a positive control.

2.1.6.3 Growth curves

Lactobacillus isolates were inoculated into MRS broth from the surface growth on MRS agar at a concentration of 10^5 CFU/ml. Isolates were grown anaerobically at 37°C on a shaking platform for 24 hours, optical density (OD) readings were taken at an absorbance of 600nm every 2 hours for 10 hours and again at 24 hours. Isolates were plated onto MRS agar, using the Miles-Misra technique every 2 hours to enumerate the bacteria. Assays were performed with three technical and three biological repeats.

2.1.6.4 Antimicrobial sensitivity testing of *Lactobacillus*

Antimicrobial sensitivity testing of all *Lactobacillus* isolates was performed using the broth microdilution method. This method is recommended in the EFSA guidelines which outline the guidelines for antimicrobial resistance testing in potential animal feed additives (European Food Safety Authority, 2012).

The antimicrobial doubling series for ampicillin, chloramphenicol, clindamycin, erythromycin and gentamicin (Fisher Scientific) ranged from 0.06-128 µg/ml and for kanamycin, streptomycin, tetracycline and vancomycin (Fisher Scientific) ranged from 1-256 µg/ml. Antimicrobial 10x stock solutions were prepared in accordance to the manufacturer's instructions and diluted to double the desired working concentration for each antimicrobial, these were filter sterilised using a 0.2µm filter. Antimicrobials were double diluted into Lactic acid bacteria susceptibility medium (LSM), this medium is composed of 90% iso-sensitest broth and 10% MRS broth which supports the growth of *Lactobacillus* and has no effect on the potency of the antimicrobials tested (Klare *et al.*, 2005). 100µl of each cell suspension at a concentration of 10^5 CFU/ml was added and the 96 well plates were incubated anaerobically at 37°C for 24 hours. The MIC was recorded as the lowest antimicrobial concentration to inhibit the growth of *Lactobacillus.* Each assay was repeated in triplicate with three biological repeats.

Enterococcus faecalis ATCC 2912 was used as a control strain and results were compared to published break-points to confirm the accuracy of the MIC results.

2.1.6.5 Acid tolerance assay

The four selected *Lactobacillus* isolates, *L. reuteri* SAP 2114 and 2115 and *L. salivarius* SAP 2116 and 2117 were tested for tolerance to low pH. 0.1M sterile PBS was adjusted to pH 1.9 using 1M hydrochloric acid (Sigma-Aldrich). *Lactobacillus* isolates (10^8 CFU/ml) were resuspended in PBS at pH 1.9 and pH 7.2 and incubated anaerobically at 37°C for 3 hours. *Lactobacillus* were plated on to MRS agar using the Miles-Misra technique at 0 hours and again after 3 hours of incubation under both pH conditions. MRS plates were incubated

anaerobically at 37°C for 18 hours, after which lactobacilli were enumerated. Assays were performed with three technical and three biological repeats.

2.1.6.6 Bile salt tolerance assay

The tolerance of *Lactobacillus* to oxgall (containing bovine bile salts) and two poultry bile salts taurocholic acid and sodium taurochenodeoycholate (Sigma-Aldrich) was determined for the four selected potential probiotic candidates *L. reuteri* SAP 2114 and SAP 2115 and *L. salivarius* SAP 2116 and SAP 2117. *Lactobacillus* isolates (10^5 CFU/ml) were inoculated into MRS broth containing 1% or 0.3% (w/v) oxgall, taurocholic acid and sodium taurochenodeoycholate, control broths contained MRS alone. A concentration of 0.3% bile salts was considered by Gilliland *et al*. (1984) to be the critical concentration, high enough to screen for resistant isolates. For each condition, 200µl of broth mixture was transferred into a 96 well plate and incubated anaerobically at 37°C in a Tecan Spark 10 plate reader (Tecan). Optical density (OD) readings were taken at an absorbance of 600nm, every 15 minutes for 24 hours. After 24 hours, each condition was plated on to MRS agar using the Miles-Misra technique to enumerate the bacteria following exposure to bile salts and subsequently compared to control broths. Assays were performed with three technical and three biological repeats.

Isolates were initially classified in accordance to previously published methods (Chateau *et al.,* 1994) whereby *Lactobacillus* growth in the presence of bile salts was compared to the control broth which contained no bile salts. The difference in the time taken for the absorbance to increase by an OD_{600} of 0.3 was used to determine tolerance to bile salts. A delay in growth of less than 15 minutes between the test and control determined an isolate to be resistant to bile salts, between 15 and 40 minutes determined an isolate to be tolerant, between 40 and minutes determined an isolate to be weakly tolerant and greater than 60

minutes determined an isolate to be sensitive to bile salts. However, this method only accounted for the lag phase of growth and it could be observed that some of the bile salts impacted the log growth phase, therefore this was considered when analysing the data.

2.1.6.7 Growth and inhibition assay of *Brachyspira*

In a 96 well plate, *Lactobacillus* CFS adjusted to pH 3.8, pH 4.5 and pH 7.2 at 10% (v/v) was added to *Brachyspira* culture in BHI + 10% FBS (10^6 CFU/ml) and incubated anaerobically at 37°C for 120 hours. Optical density (OD 620) readings were taken at 10-hour intervals for the duration of the experiment. BHI + 10% FBS without *Brachyspira* was added to the 96 well plate to standardise the OD readings. Control broths were prepared by adjusting MRS to pH 3.8, pH 4.5 and pH 7.2. All assays were performed with five biological repeats each with three technical repeats.

To establish if the *Brachyspira* inhibitory components were proteinaceous in nature, the effect of the proteolytic enzyme trypsin on the CFS inhibition of *Brachyspira* was investigated. Treated CFSs were added to *Brachyspira* cultures in BHI at 10% (v/v) and incubated anaerobically at 37°C. Optical density readings were taken as detailed about. Control broths were prepared by trypsin or heat-treating MRS broth. All assays were performed with five biological repeats each with three technical repeats.

2.1.6.8 Nuclear magnetic resonance of *Lactobacillus* CFS

Cell free supernatants from all *Lactobacillus* isolates were prepared by combining 400µl of CFS with 200µl of phosphate buffer. The samples were vortexed and centrifuged at 10,000x g for 10 minutes at ambient temperature. 550µl of each supernatant was transferred into a 5mm NMR tube. For each sample a ^{1}H NMR spectra was acquired using a Bruker 800 spectrometer. Each experiment was repeated in triplicate. Spectra were digitised and

analysed using MatLab 2017 and metabolite analysis was performed using Chenomx software.

2.1.6.8.1 Phosphate buffer recipe

28.86 g sodium phosphate dibasic (Na_2HPO_4)

5.25 g sodium phosphate monobasic (NaH_2PO_4)

0.172 g (Sodium 3(Trimethylsilyl) propionate-d4) TSP (1 mM)

0.193 g sodium azide (NaN_3)

1L D_2O

2.1.6.9 Agar viability assay of *Brachyspira*

Agar viability assays were performed using the 'spot test' as previously described (Bernadeau *et al*., 2009). *L. reuteri* SAP 2114 and SAP 2115 and *L. salivarius* SAP 2116 and SAP 2117, viable and heat-inactivated, were resuspended in 0.1M sterile PBS (10^9 CFU/ml) and incubated with *Brachyspira* cell suspensions in 0.1M sterile PBS (10^9 CFU/ml) in a 96 well plate at 37°C, anaerobically for 4 hours. Following incubation, 5µl of each cell suspension was spotted onto *Brachyspira* selective agar and incubated at 37°C, anaerobically for 5 days. The extent of viability and presence of haemolysis were quantified on the 5^{th} day by measuring the zone of *Brachyspira* growth and compared to control suspension of *Brachyspira* incubated with PBS alone. Assays were performed in triplicate with three biological repeats.

2.2 Molecular Methodology

2.2.1 Identifying *Brachyspira* using PCR

Established *Brachyspira* genus and species-specific PCRs were used for the initial identification of *Brachyspira* isolates. Primer sequences and PCR product sizes are presented in Table 2.3. The genus specific PCRs were based on the 16S rRNA gene and used to confirm that all the isolates were from the *Brachyspira* genus (Phillips *et al*., 2005). PCRs designed for the identification of *B. intermedia* and *B. pilosicoli* were based on two well conserved genes in the genus, the NADH oxidase (*nox*) and 16S rRNA genes, respectively (Mikosza *et al.*, 2001; Phillips *et al*, 2006).

PCR was used to amplify target DNA sequences detailed in Table 2.3. A 20µl reaction mixture was prepared consisting of GoTaq®MasterMix (Promega), the forward and reverse primers (20pmol/µl) (Sigma-Aldrich), DNA template (20-50ng/µl) and DNAse free water.

PCR amplifications were performed using a Techne thermocycler as follows: 95°C for 5 minutes to denature the DNA, followed by 30 cycles of denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds and extension at 72°C for 60 seconds, followed by a final DNA extension at 72°C for 7 minutes. Samples were then cooled to 4°C.

Table 2. 3 Primer sequences for *Brachyspira* genus and species-specific PCRs. Target gene, primer name, primer sequence and PCR product size are detailed below.

Target species	Target gene	Primer name	Primer sequence (5'-3')	Size	Reference
***Brachyspira* genus**	16S rRNA	Br16S-F Br16S- R	TGAGTAACACGTAGGTAATC GCTAACGACTTCAGGTAAAAC	1309	(Phillips *et al.,* 2005)
Brachyspira intermedia	*nox*	Int1-F Int1-R	AGAGTTTGATGATAATTATGAC ATAAACATCAGGATCTTTGC	567	(Phillips *et al*., 2006)
Brachyspira pilosicoli	16S rRNA	Acoli-F Acoli-R	AGAGGAAAGTTTTTTCGCTT CCCCTACAATATCCAAGACT	439	(Mikosza *et al.*, 2001)

Brachyspira genus-specific primers targeted the amplification of the 16S rRNA gene which produced an amplicon of 1309 bp (Phillips *et al*., 2005). *B. pilosicoli* species- specific primers targeted the 16S rRNA gene in *B. pilosicoli* which produced an amplicon of 439 bp and *B. intermedia* species-specific primers targeted the NADH oxidase (*nox*) gene in *B. intermedia* which produced an amplicon of 567 bp (Mikosza *et al.*, 2001; Phillips *et al.*, 2006). Gel electrophoresis of the PCR products was performed, and the species of each isolate was inferred using the amplicon sizes stated above and summarised in Table 3.1.

2.2.2 Gel electrophoresis

Agarose (Sigma- Aldrich) was melted in 1x Tris-Borate- EDTA (TBE) (Sigma-Aldrich) at 1% (w/v), SYBR™ safe gel stain was added to the agarose (1: 10,000) (Fisher Scientific) and set into a gel casting tray using a comb to form the loading wells. The gel was placed in Sub- Cell tank (Bio-Rad) and submerged in 1x TBE buffer. 5µl of each PCR product was loaded alongside a 1 Kb molecular marker ladder (Promega). Gels were electrophoresed at 100 volts (V) for

approximately 45-minutes. Following this, the gels were visualised using the Odyssey® FC imaging system.

2.2.3 Identifying *Brachyspira* using whole genome sequencing

2.2.3.1 Genomic DNA extraction

DNA was extracted using the GenElute™ bacterial genomic DNA kit (Sigma-Aldrich) as per the manufacturer's instructions. Briefly, a heavy suspension of *Brachyspira* in sterile water was prepared and centrifuged at 16,000 x g for 2 minutes at ambient temperature. Pelleted cells were resuspended in 180µl of lysis solution and 20µl of RNase A and incubated at ambient temperature for 2 minutes. 20µl of Proteinase K was added to the sample and incubated at 55°C for 60 minutes, following this 200µl of lysis solution C was added to the sample and vortexed for 15 seconds and then incubated at 55°C for a further 60 minutes.

500µl of column preparation solution was added to each GenElute Miniprep binding column to maximise the binding of DNA to the membrane and centrifuged at 12,000 x g or 1 minute. 200µl of ethanol (95-100%) was added to the lysate and vortexed for 5-10 seconds, the lysate was transferred to the binding column and centrifuged at ≥ 6500 x g at ambient temperature for 1 minute. Columns were washed twice with 500µl of wash solution 1 and centrifuged at ≥ 6500 x g at ambient temperature for 1 minute. DNA was eluted in 70µl DNase free water, incubated at ambient temperature for 5 minutes and then centrifuged at ≥ 6500 x g at ambient temperature for 1 minute.

The concentration and purity of the DNA was assessed using a BioDrop spectrophotometer. DNA was then stored at -20°C before being sent for whole genome sequencing.

2.2.3.2 Whole genome sequencing

Isolates selected for whole genome sequencing are detailed in Table 2.1. Isolates were cultured from -80°C stocks and sub-cultured for 48-72 hours. DNA was extracted from these isolates and quantified as detailed in section 2.2.3.1. DNA samples were sequenced on an Illumina MiSeq platform conducted at the APHA, Weybridge, UK. 2x 150bp paired end sequence reads were processed and assembled into contiguous sequences using the Shovill pipeline (https://github.com/tseemann/shovill). This pipeline uses SPAdes, however is a more rapid genome assembler. SPAdes is a *de novo* genome assembler for Illumina whole genome sequences data for bacteria and is a major improvement on previous assemblers such as Velvet (Bankevich *et al.*, 2012)

2.2.3.2.1 Illumina sequencing

Illumina sequencing is split in to four key parts: the sample preparation, cluster generation, sequencing and data analysis. In brief, the sample preparation involves breaking the DNA into fragments, adapters are added to the ends of the DNA fragments and through reduced cycle amplification additional motifs are introduced to the fragments, including the sequencing binding site and regions complimentary to the flow cell oligonucleotides. Cluster generation occurs on the flow cell, which is a glass slide containing different lanes. Each lane is coated with oligonucleotides. Each fragment of DNA is isothermally amplified on the flow cell. Firstly, the DNA fragment hybridised to the oligonucleotide that is complementary to the adapter region on the fragment. Once hybridised, a DNA polymerase creates a complementary strand to create a double stranded molecule. This molecule is then denatured, and the original template is washed away. Stands are then clonally amplified by bridge amplification whereby the remaining single strand folds over and the adapter region on the other end of the DNA

fragment hybridised to a commentary oligonucleotide on the flow cell surface. DNA polymerase then creates a complementary strand, forming a double stranded bridge. This bridge is denatured, resulting in two single stranded copies of the molecule bound to the flow cell. This process is repeated, resulting in clonal replication of all of the DNA fragments in the sample. After clonal amplification, the reverse strands are cleaved and washed away, leaving only the forward strands. The 3' ends of these strands are blocked to prevent any unwanted priming. Sequencing beings with the extension of the first sequencing primer to produce the first read and then with each subsequent cycle, additional fluorescently tagged nucleotides are added the nucleotide chain based on the sequences of the template. Each nucleotide emits a fluorescent signal and the wavelength and signal intensity determines the base added to the DNA strand. The number of cycles determines the length of the read. This process is repeated for the forward and reverse strands of DNA and continues for all DNA fragments, thus producing millions of reads. The final step is the data analysis. The described process produces millions of reads which represent all of the fragments of DNA initially created. Reads with similar sequences are clustered together, with forward and reverse reads being paired, thus creating contiguous sequencings which can be aligned to a reference genome.

2.2.3.3 Sequence analysis

Bioinformatic analysis was performed on all *Brachyspira* isolates with the help from Dr Arnoud van Vliet, University of Surrey. The total dataset contained the 8 *Brachyspira* genomes sequences in this project, 13 *B. pilosicoli* genomes that were sequenced for a collaborative project and all of the publicly available genomes downloaded from Genbank. The assembly statistics (number of contigs, genome size) were obtained by using Quast v2 (Gurevich *et al.*, 2013). Genome sequences were annotated using Prokka v1.12 (Seemann, 2014).

Brachyspira genomes, were phylogenetically clustered using core genome single nucleotide polymorphisms (SNPs) using parSNP (Treangen *et al.*, 2014) using the "-x" switch, which uses PhiPack to remove regions of recombination, and the "-a 13" switch to increase sensitivity (van Vliet, 2017). Figtree was used for the visualisation of phylogenetic tree (http://tree.bio.ed.ac.uk/software/figtree/).

Pan-genome analysis of all *Brachyspira* isolates was carried out using Roary v.3.12.0 with an 80% minimum percentage identity for BLASTP (Page *et al.*, 2015). Roary outputs gave a matrix of presence/absence of genes, enabling comparisons between isolates or species to be inferred. The Roary_plots.py script (Sanger Centre) was used to visualise the pangenome with the phylogenetic trees. Additionally, an ABRicate database was created with the methyl-accepting chemotaxis protein gene sequences of interest, which allowed for the screen of all *Brachyspira* genomes for the presence of these genes, with a 75% percentage identity (https://github.com/tseemann/abricate).

2.2.4 Identifying *Lactobacillus*

Lactobacillus isolates were identified using 16S rRNA sequencing for a rapid identification in the initial characterisation of a panel of 60 isolates. After further characterisation and determination of *Brachyspira* inhibition, 11 isolates were sent for whole genome sequencing by Illumina sequencing as described in section 2.2.3.2.1.

2.2.5 Identifying *Lactobacillus* with 16S rRNA sequencing

The 16S rRNA gene was amplified by PCR from the *Lactobacillus* DNA template using the primers 8F (5´-AGT TTG ATC CTG GCT CAG-3´) and 1492R (5´-ACG GCT ACC TTG TTA CGA CTT -3´). The 1537bp PCR product was confirmed by gel electrophoresis as described in section 2.2.2 and purified prior to sequencing. Sequencing was performed by Sanger sequencing.

Homology searches to determine the species of each isolate were performed using Basic Local Alignment Search Tool (BLAST) to compare sequences to the GenBank, NCBI online database.

2.2.5.1 Purification of PCR products

PCR products were purified before being sent for sequencing using the QIAquick® PCR purification kit (Qiagen) as per the manufacturer's instructions. Briefly, five volumes of buffer PB were added to one volume of the PCR product and transferred into a QIAquick spin column. The column was centrifuged at 17,900 x g for 1 minute at ambient temperature, the flow-through was discarded. The column was washed with buffer PE and centrifuged at 17,900 x g for 1 minute, an additional centrifugation step was performed to remove residual buffer. The DNA was eluted in 30µl of DNase free water and incubated for 5 minutes at ambient temperature and centrifuged at 17,900 x g for 1 minute. DNA was analysed for concentration and purity on a NanoDrop™ spectrophotometer and stored at -20°C.

2.2.6 Identifying *Lactobacillus* with whole genome sequencing

2.2.6.1 Genomic DNA extraction

Genomic DNA was extracted from *Lactobacillus* as described in section 2.2.3.1, with the addition of steps at the start of the protocol for complete lysis of Gram positive cells.

A heavy suspension of *Lactobacillus* was prepared in sterile water and centrifuged at 16,000 x g for 2 minutes at ambient temperature, the water was removed, and the cell pellet was resuspended in 200µl of lysozyme solution (prepared according the manufacturer's instructions) and incubated at 37°C for 1 hour. Following incubation, 20µl of RNase A was added to the sample and incubated at ambient temperature for 2 minutes. 20µl of proteinase K was added to the sample, followed by 200µl of lysis solution C and vortexed for 15 seconds. The sample was incubated at 55°C for 20 minutes to ensure cell lysis.

The remainder of the protocol is described in section 2.2.3.1.

2.2.6.2 Whole genome sequencing

Isolates selected for whole genome sequencing are detailed in Table 2.2. Isolates were cultured from -80°C stocks and sub-cultured for 18 hours. DNA was extracted from these isolates and quantified as detailed in section 2.2.3.1 DNA samples were sequenced on an Illumina MiSeq or HiSeq 2500 platform conducted at the Microbes NG, University of Birmingham, UK. 2x250bp paired end sequence reads were processed and assembled into contiguous sequences using the SPAdes 3.10 genome assembler (Bankevich *et al.*, 2012).

2.2.6.3 Sequence analysis

Bioinformatic analysis was performed on all *Lactobacillus* isolates, the total dataset contained ten *Lactobacillus* genomes. The assembly statistics (number of contigs, genome size) were obtained by using Quast v2 (Gurevich *et al.*, 2013). Genome sequences were annotated using Prokka v1.11b (Seemann, 2014).

Lactobacillus genomes, were phylogenetically clustered using core genome single nucleotide polymorphisms (SNPs) using parSNP (Treangen *et al.*, 2014) and Figtree was used for the visualisation of phylogenetic tree (http://tree.bio.ed.ac.uk/software/figtree/).

The genome sequences of all *Lactobacillus* isolates were screened for the presence of antimicrobial resistance genes using ABRicate (https://github.com/tseemann/abricate), this allowed for mass screening of contigs for antimicrobial resistance genes using several available databases, Resfinder, the comprehensive antibiotic resistance database (CARD) and Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT).

Lactobacillus genome sequences were also screened for the presence of bacteriocin and secondary metabolite gene clusters. Bacteriocin mining using BAGEL4 was used to identify

potential genes involved in bacteriocin production (http://bagel.molgenrug.nl) (de Jong *et al.*, 2006; van Heel *et al.*, 2013). Potential secondary metabolite gene clusters were identified using anti-SMASH (https://antismash.secondarymetabolites.org/#!/start) (Blin *et al.*, 2017).

2.2.7 RNA preparation for HD11 avian macrophages

2.2.7.1 RNA extraction

RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) as per the manufacturer's instructions. Briefly, samples were thawed on ice, the lysate was transferred to a gDNA eliminator column and centrifuged at 10,000 x g for 30 seconds at ambient temperature. 350µl of 70% ethanol was added to the lysate and mixed before it was transferred to an RNeasy spin column and centrifuged at 10,000 x g for 15 seconds at ambient temperature. All flow-through was discarded and 700µl of Buffer RW1 was added to the spin column and centrifuged at 10,000 x g for 15 seconds at ambient temperature. The column was washed twice with 500µl of Buffer RPE and centrifuged at 10,000 x g for 2 minutes to dry the column and remove all ethanol contamination. RNA was then eluted in 30µl of RNase free water and the concentration and purity of the RNA was assessed using a BioDrop spectrophotometer. RNA was stored at -80°C prior to RT-PCR.

2.2.7.2 DNase treatment and cDNA synthesis

The RNA was treated with DNase to remove any contaminating DNA. The DNA-free Kit (Ambion) was used according to the manufacture's guidelines. In brief, 3µl of DNase I buffer and 1µl of rDNase I were added to 30µl of RNA and incubated at 37°C for 30 minutes. 3µl of DNase inactivation reagent was subsequently added to the RNA and incubated at room temperature for 2 minutes, mixing occasionally. The RNA mix was centrifuged at 10,000 x g

at room temperature for 2 minutes and the RNA transferred into a clean, nuclease-free tube for subsequent reverse transcription.

cDNA synthesis was carried out using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) in accordance to the manufacturer's guidelines. In brief, a reverse transcription mastermix was made, consisting of RT buffer, dNTP mix, RT random primers, reverse transcriptase and nuclease-free water. 10µl of mastermix was combined with 10µl of each RNA sample and sealed in a 96-well plate. cDNA synthesis was performed using the BioRad CFX96 Touch system with the following thermal cycler conditions: 25°C for 10 minutes, 37°C for 120 minutes to enable reverse transcription and 85°C to denature the reverse transcriptase. Samples were then cooled to 4°C and stored at -20°C before use.

2.2.7.3 Quantitative RT-PCR

RNA expression was determined by RT-PCR using a BioRad CFX96 Touch system. Primers and probes for 28S, IL1β, IL6, IL8, IL10 and IFNγ are described in Table 2.4. RT-PCR was performed with SsoAdvanced universal probes supermix (BioRad) with the following cycles: 95°C for 2 minutes, then 50 cycles of 95°C for 10 seconds and 60°C for 1 minute. Data were visualised and analysed using the BioRad CFX Manager 3.1. All RT-PCR experiments were repeated in triplicate with three biological repeats.

Table 2. 4 Cytokine primer and probe sequences used for RT-PCR

Target	Probe Sequence	Forward Primer Sequence	Reverse Primer Sequence
28S [a]	5′-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′	5′-GGCGAAGCCAGAGGAAACT-3′	5′-GACGACCGATTTGCACGTC-3′
IL-1β [a]	5′-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3	5′-GCTCTACATGTCGTGTGTGATGAG-3′	5′-TGTCGATGTCCCGCATGA-3′
IL- 6 [a]	5′-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′	5′-GCTCGCCGGCTTCGA-3′	5′-GGTAGGCTGAAAGGCGAACAG-3′
IL-8 [b]	5_-(FAM)-GCCCTCCTCCTGGTTTCAG-(TAMRA)-3_	5′-TGGCACCGCAGCTCATT-3	5′-TCTTTACCAGCGTCCTACCTTGCGACA-3′
IFN- γ [a]	5′-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3′	5′-GTGAAGAAGGTGAAAGATATCATGGA-3′	5′-GCTTTGCGCTGGATTCTCA-3′
IL-10 [b]	5′-(FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)-3′	5′-CATGCTGCTGGGCCTGAA-3′	5′-CGTCTCCTTGATCTGCTTGATG-3′

a. Primer sequences obtained from (Kaiser *et al.*, 2000).
b. Primer sequences obtained from (Smith *et al.*, 2005).

2.3 *In vitro* Phenotypic Methodology

2.3.1 Culture of HD11 avian macrophage cells

HD11 avian macrophage cells (Beug *et al.*, 1979) were selected to investigate the immune responses elicited by *Brachyspira* on avian innate immune cells *in vitro*.

HD11 stock cultures were thawed from liquid nitrogen (-196°C) at 37°C in a water bath and were reconstituted in RPMI 1640 supplemented with L- glutamine 1x and sodium bicarbonate (Sigma-Aldrich). The media was further supplemented with 2.5% chicken serum (Sigma-Aldrich), 2.5% FBS (Sigma-Aldrich), 10% tryptose phosphate broth (Sigma-Aldrich) and 1% penicillin-streptomycin (Sigma-Aldrich). Initially, HD11 cells were seeded into a T25 tissue culture flask and incubated at 37°C in the presence of 5% carbon dioxide, until a 80% confluent monolayer was present. To propagate the HD11 cells, they were trypsinised with 0.5% trypsin-EDTA (Sigma-Aldrich) and resuspended in supplemented RPMI and seeded into T75 tissue culture flasks. Prior to experiments, HD11 cells were trypsinised, resuspended in the supplemented RPMI and seeded into 24-well plates at a concentration of $1x10^5$ cells/ml and grown to confluency for 48 hours.

HD11 stock cultures were stored in the supplemented RPMI plus 10% DMSO and 70% FBS and placed liquid nitrogen (-196°C).

2.3.2 HD11 cytokine release in response to *Brachyspira* infection

HD11 cytokine responses have been previously characterised for other pathogenic gastrointestinal bacteria such as *Salmonella* and *Campylobacter.* Therefore, cytokine response assays were performed as previously described (Kaiser *et al.*, 2000; Smith *et al.*, 2005). Briefly, *Brachyspira* inocula were prepared by transferring the surface growth from a

FABA plate into 0.1M sterile PBS to yield a concentration of 5 x 10^8 CFU/ml, which was resuspended in tissue culture media to a concentration of approximately 5 x 10^7 CFU/ml. Confluent HD11 cells in 24 well plates were in washed twice with pre-warmed 0.1M sterile PBS to remove media containing antibiotics. Antibiotic-free media was added to each well and designated wells were infected with either *B. pilosicoli* SAP 859*, B. alvinipulli* ATCC 51933*, B. intermedia* SAP 919 or *B. innocens* SAP 924 and incubated at 37°C in the presence of 5% carbon dioxide for 3 hours.

HD11 cells were lysed using 350µl buffer RTL + β- mercaptoethanol and the lysates were frozen at -80°C prior to RNA extraction. All assays were repeated in duplicate with three biological repeats. *Salmonella* Typhimurium SL1344 (SAP 16) was used as a positive control and PBS was used as a negative control.

2.3.3 HD11 Cytokine release in response to *Brachyspira* infection following *Lactobacillus* pre-exposure

To investigate the possible immunomodulatory properties of *Lactobacillus,* HD11 cells were infected with *Lactobacillus* prior to infection with *Brachyspira*.

Lactobacillus inocula were prepared by transferring the surface growth from an MRS plate into 0.1M sterile PBS to yield a concentration of 5 x 10^8 CFU/ml, which was resuspended in tissue culture media to a concentration of approximately 5 x 10^7 CFU/ml. Confluent HD11 cells in 24 well plates were in washed twice with 0.1M sterile PBS to remove media containing antibiotics. Antibiotic-free media was added to each well and designated wells were infected with either *L. reuteri* SAP 2115 or *L. salivarius* SAP 2117 and incubated at 37°C in the presence of 5% carbon dioxide for 30 minutes prior to *Brachyspira* infection. The remaining methodology is detailed in section 2.3.2. All assays were repeated in duplicated with three biological repeats.

2.3.4 Survival of *Brachyspira* in the HD11 infection model

2.3.4.1 Gentamicin protection assay

The gentamicin protection assay was performed in order to determine if *Brachyspira* survived intracellularly. After 4-hours of incubation the media was removed and the HD11 cells were washed three times with PBS. 100µl of this spent media was plated on to FABA to determine whether *Brachyspira* would survive for the duration of the experiment in sub-optimal conditions.

HD11 cells were resuspended in media supplemented with gentamicin at a concentration of 100µg/ml and incubated at 37°C with an atmosphere of 5% CO_2 for 2 hours to kill any extra-cellular bacteria. Media was removed, and cells were washed 3 times with PBS before being lysed with 1% Triton X-100. The lysate was plated on to FABA and incubated anaerobically at 37°C for 5 days. These assay were repeated in triplicate with three biological repeats.

2.4 *In vivo* Methodology

2.4.1 Development of a *Galleria mellonella* virulence assay for *Brachyspira*

Galleria mellonella larvae were purchased from Wiggly Wigglers, larvae were stored on wood chips in the dark at 17°C to prevent pupation. All larvae were inspected prior to use in experiments to ensure they were healthy, indicated by a pale-yellow colour. Experiments with *G. mellonella* were performed as described by Ramarao *et al*. (2012), with some modifications.

2.4.1.1 Determining a suitable *Brachyspira* concentration for *G. mellonella* infection

The optimal *Brachyspira* inoculum for use in the *G. mellonella* virulence assay was initially determined to inform subsequent experiments. *Brachyspira* inocula were prepared by

transferring the surface growth from a FABA to 0.1M sterile PBS to yield concentrations of 10^7, 10^6, and 10^5 CFU/larvae. *G. mellonella* were injected into the front left proleg with 10µl of each concentration of *Brachyspira* inoculum. Control larvae were injected with PBS to control for any lethal effects from the injection process. The injections were performed with a Hamilton 1705 syringe (Fisher-Scientific) with 10 larvae infected per experimental condition, repeated in triplicate.

Larvae were incubated for 120 hours at 37°C in Petri dishes lined with Whatman™ filter paper (Fisher Scientific) with mortality scores recorded every 24 hours.

2.4.1.2 Infection of *G. mellonella* with *Brachyspira*

The optimal inocula for all *Brachyspira* isolates, 10^7 CFU/ larvae was prepared in 0.1M sterile PBS as described above in section 2.4.1.1. *G. mellonella* larvae were injected with 10µl of these inocula into the top left proleg. Control larvae were injected with PBS to control for any lethal effects of the injection process and non-injected controls were used to monitor the pupation of the larvae. Ten larvae were infected with each isolate of *Brachyspira* with five biological repeats. Larvae were incubated for 120 hours at 37°C in Petri dishes lined with Whatman™ filter paper (Fisher Scientific) with mortality and morbidity scores taken every 24 hours.

The mortality scores scored each larvae as either being dead or alive. The morbidity scoring system scored each larvae depending on the degree of melanisation with a score of 0 indicating no melanisation, a score of 1 indicating melanisation down the tail line, a score of 2 indicating spotting and dark pigment across the body and a score of 4 indicating complete melanisation and death. Examples can be seen in Figure 1.7 (Tsai *et al.,* 2016).

2.4.1.3 *G. mellonella* histopathology

G. mellonella were fixed in 10% (v/v) neutral buffered formalin for 14 days. For light microscopy, both transverse and longitudinal sections were cut and stained with several different staining methods. Haematoxylin and eosin (H&E) staining was used to observe pathology associated with *Brachyspira* infection, silver staining was used to specifically visualise *Brachyspira* bacteria in the *G. mellonella* and Gram staining was used to stain for any other bacteria that may have been present within the *G. mellonella.* Three larvae were fixed per timepoint and three sections in each orientation were mounted on to each slide.

Table 2. 5 The criteria used for *Galleria mellonella* histopathology scoring.

Score	Bacteria in gut (cocci)	Melanin/inflammatory nodules	Clusters of haemocytes	Morphological descriptor
0	negligible	none	none	Normal
1	very small colonies within the layers close to the gut lumen	presence of few very small melanocytic bodies	very few groups of up to 3 hemocytes under the cuticle	Minimal
2A	small number of colonies, small to medium size within the layers close to the gut lumen and minimally invading the intestinal wall	presence of small numbers of small to medium size inflammatory nodules/melanocytic bodies normally within the adipose bodies or the hemolymph	small number of clusters of hemocytes composed of 3-10 cells under the cuticle	Mild
2B	moderate number of colonies, small to medium size within the layers close to the gut lumen and minimally invading the intestinal wall	presence of moderate numbers of small to medium size inflammatory nodules/melanocytic bodies (generally more pigmented) normally within the adipose bodies or the hemolymph	moderate numbers of clusters of hemocytes composed of 3-10 cells under the cuticle	Moderate
3	abundant large colonies, transmural	abundant melanocytic bodies throught the body, normally colaescing within different adipose bodies and hemolypmh	abundant clusters of hemocytes composed of 3-10 melanocyetic cells under the cuticle or larger than 10 cells	Severe

2.4.1.4 Giemsa staining of *G. mellonella* haemocytes

Giemsa stain is a differential stain that can be used to differentiate bacterial cells from haemocytes. In this study, the Giemsa stain was used to differentiate *Brachyspira* cells from *G. mellonella* haemocytes. *G. mellonella* tails were cut and the haemolymph was collected into a sterile tube. 10µl was smeared in to a glass slide and fixed in pure methanol for 5-7 minutes. The slides were air dried and then immersed in Giemsa stain (Sigma-Aldrich) for 60 minutes. The slides were rinsed with deionised water, air dried and evaluated using light microscopy (x40). Haemocytes stain blue/purple and bacterial cells stain pink and thus can be differentiated.

2.4.1.5 Culture of *Brachyspira* from *G. mellonella* haemolymph

G. mellonella tails were cut with sterile scalpels and the haemolymph from three larvae was pooled into a sterile tube at 0, 2, 4, 6, 24, 48, 72, 96 and 120 hours post infection with *Brachyspira.* 50µl of haemolymph was plated in to *Brachyspira* selective agar (BSA) and incubated anaerobically at 37°C for 5-7 days to assess the survival of *Brachyspira in vivo.*

2.4.2 Development of a *G. mellonella* model to assess the protective activity of *Lactobacillus* against *Brachyspira*

2.4.2.1 Determining suitable *Lactobacillus* cell and CFS concentrations for *G. mellonella* infection

The virulence of four probiotic candidates, *L. reuteri* SAP 2114, *L. reuteri* SAP 2115, *L. salivarius* SAP 2116 and *L. salivarius* SAP 2117 were predetermined by injecting larvae with 10µl of each isolate at concentrations of 10^2, 10^3, 10^4 and 10^5 CFU/ larvae into the top left proleg. *Lactobacillus* inocula were prepared by transferring the surface growth of each isolate from MRS agar into 0.1M sterile PBS to achieve the above concentrations. Control larvae were

injected with PBS to control for any lethal effects from the injection process. Ten larvae were infected with each *Lactobacillus* isolate, repeated in triplicate.

Larvae were incubated for 120 hours at 37°C in Petri dishes lined with Whatman™ filter paper (Fisher Scientific) with mortality and morbidity scores were taken every 24 hours as detailed in section 2.4.1.2.

The effect of the *Lactobacillus* CFS from *L. reuteri* SAP 2114, *L. reuteri* SAP 2115, *L. salivarius* SAP 2116 and *L. salivarius* SAP 2117 was also predetermined by injecting larvae with 10µl of neat CFS and a 1:10 dilution of the CFS into the top left proleg. Control larvae were injected with MRS broth to control for any lethal effects cause by the broth. Ten larvae were infected with each *Lactobacillus* CFS, repeated in triplicate.

Larvae were incubated for 120 hours at 37°C in Petri dishes lined with Whatman™ filter paper (Fisher Scientific) with mortality and morbidity scores taken every 24 hours as detailed in section 2.4.1.2.

2.4.2.2 *G. mellonella* probiotic protection model

To assess the ability of the four probiotic candidates to protect against *Brachyspira*, separate groups of *G. mellonella* were injected with 10µl of each *Lactobacillus* isolate at a concentration of 10^4 CFU/larvae or the 1:10 dilution of *Lactobacillus* CFS into the top left proleg and incubated for one hour at 37°C in petri dishes lined with Whatmann filter paper. After incubation, pre-treated *G. mellonella* groups were injected with 10µl of each *Brachyspira* isolate at a concentration of 10^7 CFU/ larvae into the top right proleg. Control larvae were injected with PBS or MRS as non-pre-treated controls. Sixteen larvae were pre-treated with *Lactobacillus* and infected with *Brachyspira*, repeated five times.

Larvae were incubated for a further 120 hours at 37°C in petri dishes lined with Whatman™ filter paper with mortality and morbidity scored taken every 24 hours as detailed in section 2.4.1.2.

2.5 Data analysis and statistics

Data from all experiments presented were tabulated using either Microsoft Excel 2017 or Graphpad Prism 7, the means, standard deviations and standard errors were calculated in these programs.

The data for experiments presented in sections 2.1.6.5, 2.1.6.6 and 2.1.6.7 were analysed for statistical significance using a one-way ANOVA with either a Sidak's or Dunnett's multiple comparisons post-test with a 95% confidence interval. A one-way ANOVA was used as each experiment consisted of three or more experimental groups, including controls. The Sidak's test was used when comparing selected sets of means and the Dunnett's test was used to compare every mean to the mean of a control.

Data for experiments presented in section 2.1.6.8 were visualised and analysed using MatLab 2017 and scripts provided by Dr Caroline Le Roy (Korrigan Sciences Limited., 2010-2011). Principle component analysis (PCA) was employed to investigate the variation between the NMR data collected from multiple isolates of *Lactobacillus*. PCA transforms a large number of possibly correlated variables into a smaller number of uncorrected variables called principle components in order to simplify complex data and investigate the variability in the dataset. PCA is an unsupervised analysis and therefore finds patterns in data without knowing the samples used (Lever *et al*., 2017). Colourplots were generated from the principle components in order to identify the metabolites responsible for the variance.

Gene expression studies with HD11 avian macrophages presented in section 2.3 were initially analysed using BioRad CFX Manager 3.1. This software normalised the gene expression to the 28S house-keeping gene (Δ Cq) and calculated the relative fold gene expression ($\Delta\Delta$ Cq) for all of the samples. These calculations are as follows:

ΔCq = Cq (gene of interest) - Cq (housekeeping gene)

$\Delta\Delta$ Cq = ΔCq (treated sample) - ΔCq (control sample)

These values were then collated in Graphpad Prism 7 to generate graphs of relative expression. Statistical significance was determined using a one-way ANOVA with a Tukey post-test to compare all means with one another to fully investigate how gene expression differed between cells treated with multiple isolates of both *Brachyspira* and *Lactobacillus* in multiple combinations.

Galleria mellonella survival assays were analysed using Graphpad Prism 7 which generated Kaplan-Meier survival curves. Analysis of survival curves was performed using the log rank test.

In all experiments a p value of ≤ 0.05 indicated statistical significance. Where further categorisation of p values was required asterisks (*) were noted as follows: p value ≤ 0.05 *, p value ≤ 0.005 **, p value ≤ 0.0050 *** and p value ≤ 0.0001 ****.

Chapter 3: The characterisation of *Brachyspira* species implicated in avian intestinal spirochaetosis

3.1 Introduction

Brachyspira species are the causative agent of avian intestinal spirochaetosis (AIS) which causes disease in laying hens and broiler breeders. The species primarily considered to be pathogenic to poultry are *Brachyspira alvinipulli* (Stanton *et al.,* 1998), *Brachyspira intermedia* (Stanton *et al.*, 1997) and *Brachyspira pilosicoli* (Stephens and Hampson, 2001) with *Brachyspira innocens* (Stanton *et al.*, 1997) considered to be non-pathogenic in the avian host (Stephens and Hampson, 2001). *Brachyspira* infections in poultry commonly manifest as diarrhoeal disease and result in reduced egg production (Davelaar *et al.*, 1986; Dwars *et al,* 1993).

Since the ban of antibiotic growth promoters as feed supplements in 2006, (European Commission, 2005) the incidence of *Brachyspira* has increased and is estimated to infect up to 90% of free range hens and 76% of caged hens (Burch, 2010), costing the UK laying industry an estimated £18 million per annum (Mappley *et al*., 2014). Despite a number of attempts to mitigate the disease, including creation of autogenous vaccines and improving biosecurity (Phillips *et al.,* 2003; Corona-Barrera *et al.*, 2004; Amin *et al.*, 2009), the incidence of *Brachyspira* continues to rise. Therefore, suggesting that an improved understanding of this organism is required to ensure adequate control strategies can be implemented.

Until recently, identification of *Brachyspira* species has been heavily reliant on the biochemical properties of each species. However, the metabolic capabilities of *Brachyspira* are poorly understood and previous development of confirmatory biochemical tests utilised porcine isolates, which were later shown to be somewhat distinct from poultry isolates (Atyeo *et al.*, 1999). In recent years, molecular techniques, such as PCR have resulted in improved

detection and identification of *Brachyspira* isolates (Mikosza *et al.*, 2001; Phillips *et al.,* 2005, 2006). Furthermore, whole genome sequencing is not only becoming increasingly important for the improved understanding of *Brachyspira* and its pathobiology, but also proves useful as a diagnostic tool for confirming species identification.

Therefore, this chapter focused on techniques including, biochemical testing, antimicrobial resistance testing, PCR and whole genome sequencing to phenotypically and genotypically characterise *Brachyspira* isolates from chickens with avian intestinal spirochaetosis. This enabled the assembly of a collection of well characterised *Brachyspira* isolates that could be studied in experimental models to investigate the pathobiology of this organism and elucidate the mechanisms by which control strategies, such as the use of probiotics may mitigate disease caused by this organism.

Therefore, the aims and objectives of this chapter were:

- To use a combination of phenotypic and genotypic characterisation techniques to identify *Brachyspira* isolates from species implicated in poultry disease, namely *B. alvinipulli*, *B. intermedia, B. innocens* and *B. pilosicoli*.
- To investigate the metabolic diversity of avian *Brachyspira* isolates using Biolog PM™ technology.
- To determine the growth kinetics of a panel of avian *Brachyspira* isolates.
- To determine the antimicrobial resistance profiles of the panel of avian *Brachyspira* isolates in light of emerging resistance to commonly used antibiotics.

3.2 Results

3.2.1 Phenotypic characterisation of avian *Brachyspira* isolates

3.2.1.1 *Brachyspira* staining and biochemistry

All *Brachyspira* isolates described here were isolated previously at the APHA from the faeces of poultry with suspected avian intestinal spirochaetosis in the United Kingdom. A control strain of *B. alvinipulli,* isolated from chicken caeca in Ohio, USA, was purchased from the American Type Culture Collection (ATCC) to ensure all species implicated in poultry disease were utilised in these studies.

Gram stains and Warthin-Starry stains were performed on each isolate of *Brachyspira*, followed by examination using light microscopy to determine the morphology of the cells. The Gram stain revealed Gram negative, helical spirochaetes and the Warthin-Starry stain, which was specific for spirochaetes, revealed a positive brown stain with spirochaete morphology, as shown in Figure 3.1. Dark-field microscopy was employed as a further confirmatory test for *Brachyspira* species, whereby the cells were observed for their 'cork-screw like" motility, an important characteristic for spirochaetes.

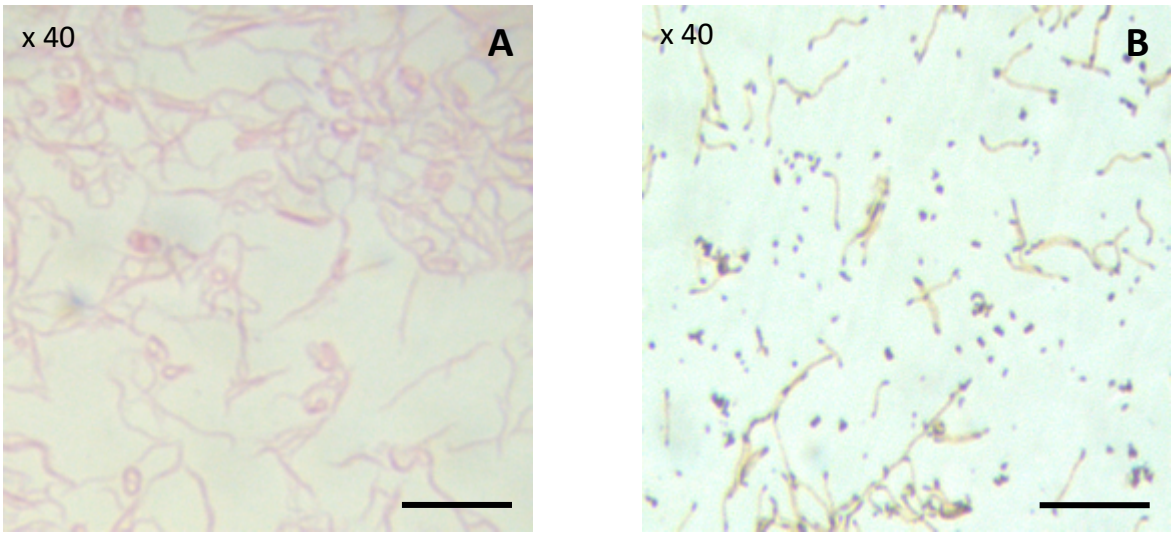


Figure 3. 1 Gram and Warthin- Starry stained images of *Brachyspira pilosicoli* SAP 865. These staining techniques were carried out to ensure that each isolate was *Brachyspira*. (A) The Gram stain illustrates Gram negative spirochaetes (scale bar 5μm). (B) The Warthin-Starry stain illustrates spirochaetes strained brown (scale bar 5μm). These images are characteristic of *Brachyspira* species.

Biochemical tests were employed to preliminarily speciate all *Brachyspira* isolates (Table 3.1). It was evident from the data presented in Table 3.1, that phenotypic characterisation by means of biochemical testing was insufficient for the identification of all *Brachyspira* isolates as approximately 40% (5/13) of the isolates were unable to be identified (expected results are shown in Table 1.3). Therefore, genotypic methods of characterisation by means of PCR and whole genome sequencing were employed to accurately identify all *Brachyspira* isolates. These additional tests highlighted that biochemical testing alone, was only correct for only 46% (6/13) of the isolates.

The isolates selected for further phenotypic study were as follows: *B. pilosicoli* B2904, *B. pilosicoli* SAP 858, *B. pilosicoli* SAP 865, *B. intermedia* SAP 919, *B. innocens* SAP 924, *B. innocens* SAP 927, *B. innocens* SAP 943 and the *B. alvinipulli* ATCC 51933 type strain. These isolates were selected because they could be definitively identified using a combination of phenotypic and genotypic tests. The *Brachyspira* isolates that could not be identified using a

combination of biochemistry and PCR were not sent for whole genome sequencing because they were likely to be mixed species and the aim of this work was to characterise at least one isolate of each species implicated in avian intestinal spirochaetosis.

Table 3. 1 A summary of the PCR, biochemistry and whole-genome sequencing results for all *Brachyspira* isolates used in these studies. Both phenotypic and genotypic methods of characterisation are shown here to illustrate the difficulties when trying to identify the species of each isolate of *Brachyspira*. These data show the preliminary identification of each isolate before the start of the project and the series of PCRs and biochemical tests carried out in order to identify each isolate. Ultimately, whole genome sequencing was used to ensure the correct identification of several isolates due to the varied PCR and biochemistry results. The isolates marked with an asterisk (*) were genome sequenced and were used in future studies.

Preliminary Identification	Isolate Name	PCR 16S rRNA primer for *Brachyspira* genus	PCR *nox* gene for *B. intermedia*	PCR 16s rRNA gene for *B. pilosioli*	PCR Identification	Biochemistry Results					Biochemistry Idenification	Group	Whole Genome Sequencing	Final Identification
						a-glucosidase	a-galactosidase	B-glucosidase	Indole	Hippurate				
B. pilosicoli	B2904	+	-	+	*B. pilosicoli*	-	-	-	-	+	*B. pilosicoli*	IV	*B. pilosicoli*	*B. pilosicoli**
B. pilosicoli	SAP 859	+	-	+	*B. pilosicoli*	-	-	-	-	+	*B. pilosicoli*	IV	*B. pilosicoli*	*B. pilosicoli**
B. alvinipulli	SAP 865	+	-	-	unknown	-	-	+	-	+	*B. alvinipulli*	unknown	*B. pilosicoli*	*B. pilosicoli**
B. alvinipulli	SAP 866	+	+	-	*B. intermedia*	-	+	+	-	-	unknown	IIIb	n/a	unknown
B. alvinipulli	SAP 943	+	-	-	unknown	-	-	+	-	-	unknown	IIIa	*B. innocens*	*B. innocens**
B. alvinipulli	SAP 945	+	-	-	unknown	-	+	+	-	-	unknown	IIIb	n/a	n/a
B. intermedia	SAP 919	+	+	-	*B. intermedia*	+	-	+	+	-	*B. intermedia*	II	*B. intermedia*	*B. intermedia**
B. intermedia	SAP 891	+	+	-	*B. intermedia*	+	-	+	+	-	*B. intermedia*	II	*B. intermedia* (mixed)	*B. intermedia*
B. innocens	SAP 903	+	-	+	*B. pilosicoli*	+	+	-	-	-	unknown	unknown	n/a	unknown
B. innocens	SAP 927	+	-	-	unknown	-	+	+	-	-	*B. innocens*	IIIb	*B. innocens*	*B. innocens**
B. innocens	SAP 943	+	-	-	unknown	-	+	+	-	-	*B. innocens*	IIIb	*B. innocens*	*B. innocens**
B. innocens	SAP 868	+	-	+	*B. pilosicoli*	-	+	-	-	-	unknown	unknown	n/a	n/a
B. alvinipulli	ATCC 51933	+	-	-	unknown	-	-	+	-	+	*B. alvinipulli*	ND	*B. alvinipulli*	*B. alvinipulli**

3.2.1.2 Growth kinetics of *Brachyspira*

Using the *Brachyspira* isolates detailed in the section 3.2.1.1, a growth curve protocol was developed to characterise the growth kinetics of the eight chosen isolates to inform future studies presented in Chapters 5, 6 and 7.

Brachyspira isolates were slow growing as illustrated in Figure 3.2. The log phase of growth began after approximately 25 hours of anaerobic culture at 37°C and the stationary phase was reached between 80-100 hours of culture for the all isolates except *B. pilosicoli* SAP 865 and *B. innocens* SAP 927. These isolates demonstrated overall poor growth in broth as seen in Figures 3.2 C and H. The ability of *Brachyspira* to grow in brain heart infusion (BHI) broth under the given conditions varied not only between species, but also within a species, which demonstrated the varying growth patterns between all isolates tested. *B. intermedia* SAP 919 grew the most successfully in broth, whereas *B. alvinipulli* ATCC 51933 had relatively poor growth. The three isolates of both *B. pilosicoli* and *B. innocens* showed a range of growth phenotypes in broth. *B. pilosicoli* B2904 and *B. innocens* SAP 924 exhibited high growth in broth, indicated by an optical density (OD_{600}) that exceeded 0.3 at the end of the exponential growth phase. *B. pilosicoli* SAP 859 and *B. innocens* SAP 943 exhibited intermediate growth in broth, indicated by an OD_{600} between 0.25 and 0.3 at the end of the exponential growth phase. *B. pilosicoli* SAP 865 and *B. innocens* SAP 927 exhibited poor growth in broth, indicated by an OD_{600} of less than 0.2 at the end of the exponential growth phase. Overall, each isolate exhibited variability in growth independent of species.

The results from these growth curves informed the selection of *B. pilosicoli* and *B. innocens* isolates for experiments in Chapters 5, 6 and 7. *B. pilosicoli* SAP 859 and *B. innocens* SAP 924 were selected because these isolates could be grown successfully in broth, ensuring sufficient bacterial numbers could be achieved for subsequent assays. Additionally, *B. intermedia* SAP

919 and *B. alvinipulli* ATCC 51933 were selected to ensure that all of the *Brachyspira* species implicated in poultry disease were utilised in these studies. *B. pilosicoli* B2904 was used as a control due to its known ability to grow under laboratory conditions (Mappley *et al.*, 2011, Mappley *et al.,* 2013).

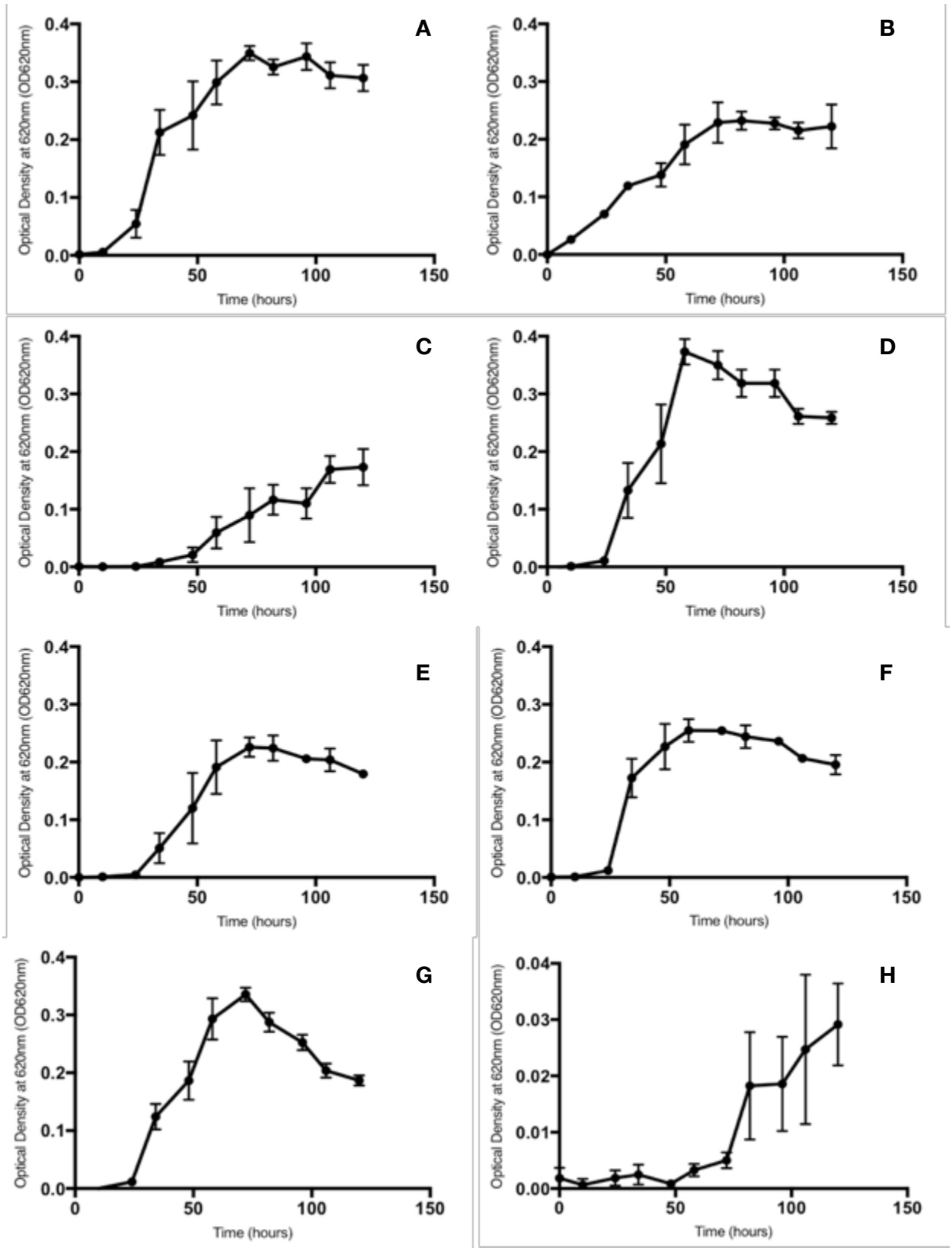


Figure 3. 2 *Brachyspira* growth curves **A:** *B. pilosicoli* B2904 **B:** *B. pilosicoli* SAP 859 **C:** *B. pilosicoli* SAP 865 **D:** *B. intermedia* SAP 919 **E:** *B. alvinipulli* ATCC 51933 **F:** *B. innocens* SAP 943 **G:** *B. innocens* SAP 924 **H:** *B. innocens* SAP 927 in BHI broth + 10% serum. Growth was monitored over 120 hours of anaerobic incubation at 37°C. Optical density readings were taken at 620nm wavelength every ˜10 hours.These data were averages of five biological replicates, each with three technical replicates. The standard error of the mean is also shown.

3.2.1.3 Antimicrobial sensitivity testing of *Brachyspira* isolates

The antimicrobial resistance profiles of the *Brachyspira* isolates against four clinically relevant antibiotics (tylosin, chlortetracycline, lincomycin and tiamulin) were conducted (Table 3.3). Seven of the eight of *Brachyspira* isolates tested were susceptible to chlortetracycline, lincomycin, tiamulin and tylosin, with *B. alvinipulli* ATCC 51933 showing decreased sensitivity to chlortetracycline. There is an absence of recognised clinical breakpoints for *Brachyspira*, thus breakpoints used in these studies were inferred from previous studies (Duhamel *et al.*, 1998; Brooke *et al.,* 2003; Burch, 2005; Hampson *et al.,* 2006; Pringle *et al.*, 2012; Hellman *et al.*, 2014). Sensitivity to each antibiotic was therefore determined by an MIC of < 4 mg/L for chlortetracycline, < 50 mg/L for lincomycin $<$ 0.25 mg/L of tiamulin and < 16 mg/L for tylosin.

Table 3. 2 Minimum inhibitory concentrations (MICs) of the antibiotics chlortetracycline, gentamicin, lincomycin, tiamulin and tylosin determined by broth microdilution against eight *Brachyspira* isolates. The experiment was performed with three biological replicates, each with three technical replicates.

***Brachyspira* Species**	**Isolate Name**	**MIC μg/ml Chlortetracycline**	**Lincomycin**	**Tiamulin**	**Tylosin**
B. alvinipulli	ATCC 51933	4	0.5	0.0625	4
B. innocens	SAP 927	0.125	< 0.0625	< 0.016	0.5
B. innocens	SAP 924	0.125	< 0.0625	< 0.016	1
B. innocens	SAP 943	0.125	< 0.0625	< 0.016	0.5
B. intermedia	SAP 919	1	< 0.0625	< 0.016	1
B. pilosicoli	B2904	0.25	0.125	0.125	2
B. pilosicoli	SAP 865	2	0.5	0.0625	4
B. pilosicoli	SAP 859	0.125	32	0.125	< 0.016

3.2.1.4 Metabolic profiling of *Brachyspira* isolates using Biolog Phenotype MircoArray™ technology

Biolog Phenotypic Microarray™ (PM) technology was implemented to further phenotypically characterise the panel of eight *Brachyspira* isolates used in these studies, furthermore it allowed metabolic comparisons to be made between avian *Brachyspira* isolates. The use of the Biolog technology determined the ability of each *Brachyspira* isolate to metabolise 190 different carbon sources on PM1 and PM2 plates (Figures 2.1 and 2.2).

The heat maps in Figures 3.3 and 3.4 illustrated the utilisation of different carbon sources on PM1 and PM2 plates. The data presented were the means of three biological repeats.

Furthermore, all data were normalised to the A1 well on each PM plate, which contained no carbon source, to assess the baseline respiration of the cells in the absence of a carbon

source. Isolates that exhibited high utilisation of carbon sources were shown in dark purple (Figures 3.3 and 3.4), corresponding to an optical density at 540nm of greater than 0.4, isolates that exhibited moderate utilisation of carbon sources are shown in purple, corresponding to an OD_{540} of between 0.2-0.39, isolates that exhibited low utilisation of carbon sources are shown in pale purple, corresponding to an OD_{540} of between 0.1-0.2 and isolates that were unable to utilise carbon sources are shown in white, corresponding to an OD_{540} of less than 0.1.

Figure 3. 3 Heatmap of the differences in the utilisation of carbon sources for 8 isolates of *Brachyspira* using the Biolog Phenotypic Microarray™ (PM) technology. This figure summarises the respiration of all 8 isolates: *B alvinipulli* ATCC 51933, B*. pilosicoli* B2904, *B. pilosicoli* SAP 859, *B. pilosicoli* SAP 865, *B. intermedia* SAP 919, *B. innocens* SAP 924, *B. innocens* 927 and *B. innocens* SAP 943, on the carbon sources on PM1 plates. Plates were incubated anaerobically for 48 hours, after which the optical density readings were taken at 540nm wavelength. The levels of respiration are colour-coded whereby dark purple indicates high levels of respiration on a carbon source, purple indicates moderate levels of respiration on a carbon source, pale purple indicates low levels of respiration on a carbon source and white indicates no respiration on a carbon source. Assays were performed with three biological replicates and the mean values are presented in this figure.

Figure 3. 4 Heatmap of the differences in the utilisation of carbon sources for 8 isolates of *Brachyspira* using the Biolog Phenotypic Microarray™ (PM) technology. This figure summarises the respiration of all 8 isolates: *B alvinipulli* ATCC 51933, B. *pilosicoli* B2904, *B. pilosicoli* SAP 859, *B. pilosicoli* SAP 865, *B. intermedia* SAP 919, *B. innocens* SAP 924, *B. innocens* 927 and *B. innocens* SAP 943, on the carbon sources on PM2 plates. Plates were incubated anaerobically for 48 hours, after which the optical density readings were taken at 540nm wavelength. The levels of respiration are colour-coded whereby dark purple indicates high levels of respiration on a carbon source, purple indicates moderate levels of respiration on a carbon source, pale purple indicates low levels of respiration on a carbon source and white indicates no respiration on a carbon source. Assays were performed with three biological replicates and the mean values are presented in this figure.

Approximately 20% (39/190) of the carbon source utilisation was shared by all eight *Brachyspira* isolates and approximately 56% (107/190) of the carbon sources were not used by any isolate, as shown in Table 3.4 and Appendix I. Therefore, thirty-nine carbon sources featured in the core *Brachyspira* metabolic pathways, these included carbon sources such as glucose, glucose-6-phosphate, fructose-6-phosphate, fructose and galactose.

The *Brachyspira* isolates utilised an extensive number of sugars for the generation of energy but did not utilise amino acids such as L- proline, D-serine and L- glutamine and short chain fatty acids such as formic and propionic acids. L- lactic acid was, however, solely metabolised by *B. alvinipulli* ATCC 51933.

Each species utilised between 57-66 different carbon sources. *B. pilosicoli* demonstrated the ability to respire on the most carbon sources, 35% (66/190), whereas *B. alvinipulli* utilised 31% (59/190) of the total carbon sources available and *B. intermedia* and *B. innocens* utilised 30% (57/190). Notably, the metabolic data collected from *B. alvinipulli* ATCC 51933 and *B. pilosicoli* B2904 in these studies largely matched that of a previous studies (Stanton *et al.,* 1998; Mappley *et al.*, 2012).

Table 3. 3 Carbon sources utilised by eight *Brachyspira* isolates. This table shows the total number of carbon sources utilised by all eight isolates, the total number of carbon sources not used by these isolates, the number of carbon sources common to all isolates and the number of carbon sources utilised by each of the four species of *Brachyspira*, *B. alvinipulli, B. innocens, B. intermedia* and *B. pilosicoli*. These data represent the utilisation of each carbon source after 48 hours of anaerobic culture at 37°C using optical density at 540nm wavelength to capture the reduction of the tetrazolium dye and subsequent colour change, indicative of cellular respiration.

Total carbon sources utilised by *Brachyspira* species (%)						
Total used	**Total not used**	**Shared by all**	***B. alvinipulli***	***B. innocens***	***B. intermedia***	***B. pilosicoli***
44%	56%	20%	31%	30%	30%	35%
(83/190)	(107/190)	(39/190)	(59/190)	(57/190)	(57/190)	(66/190)

The ability to utilise, and the extent of utilisation of different carbon sources differed both between different species and between isolates within the same species. It is important to note that only one *B. alvinipulli* and one *B. intermedia* isolate were used in these studies and therefore these data may not be representative of these species.

These data have identified potential metabolic targets that could be incorporated in to differential biochemical tests to improve the accuracy of the biochemical tests developed by Fellström and Gunnarsson (1995). For example, β- glucosidase activity has previously been used as the only biochemical method to differentiate *B. alvinipulli* and *B. pilosicoli,* using this Biolog data it was evident that *B. alvinipulli* uniquely utilised glycerol, lactic acid, D-glutamic acid, 1,2- propanediol, α-keto-glutaric acid, Tween 80, glycolic acid and thymidine (Table 3.5), potentially identifying carbon sources unique to *B. alvinipulli* which could be implemented to improve the differentiation from *B. pilosicoli*, although further validation would be required. Biochemical testing was unable to correctly identify any *B. alvinipulli* isolates (Table 3.1) and therefore improvements to these techniques are required.

Table 3. 4 Differential carbon source utilisation for each species of *Brachyspira*. This table shows the number of unique carbon sources utilised by the different species of *Brachyspira*. These data represent the utilisation of each carbon source after 48 hours of anaerobic culture at 37°C using optical density at 540nm wavelength to capture the reduction of the tetrazolium dye and subsequent colour change, indicative of cellular respiration.

Number of carbon sources unique to each *Brachyspira* species			
B. alvinipulli [a]	***B. innocens***	***B. intermedia*** [a]	***B. pilosicoli***
8	2	2	5
Glycerol	α- methyl-D- mannoside [b]	Amygdalin	Inosine [c]
Lactic acid	β- methyl-D- xyloside [b]	β-hydroxy butyric acid	L- alanine [d]
D- glutamic acid			2-aminoethanol [e]
1,2-propanediol			Inulin [d]
α-keto-glutaric acid			Mannan [d]
Tween 80			
Glycolic acid			
Thymidine			

[a] There was only one isolate per species and thus this may not be representative of the species
[b] Carbon sources utilised by only SAP 924
[c] Carbon source utilised by only B2904
[d] Carbon sources utilised by only SAP 865
[e] Carbon source utilised by only SAP 859

Metabolic variation was observed between different isolates within the *B. pilosicoli* species. Although in total, *B. pilosicoli* isolates metabolised sixty-six carbon sources, twenty of these differed between the three isolates tested, with SAP 865 able to respire on ten different carbon sources compared to that of SAP 859 and B2904, as shown in Table 3.5. *B. pilosicoli* B2904 and SAP 859 were more phenotypically alike and shared the ability to respire on α-acetyl neuraminic acid, β- methyl-D- galactoside and butyric acid and only differed in the ability to respire on adenosine, inosine, 2-aminoethanol, laminarin, α-keto-butyric acid and cellobiose. Therefore, it was not possible to attribute specific carbon source utilisation to *B. pilosicoli* to inform metabolic targets for species identification. This metabolic variation was also noted by Mappley *et al.* (2012) and emphasised the need to test more *Brachyspira*

isolates to build comprehensive biochemical profiles of each species, with the intention of improving the differential diagnosis of *Brachyspira* isolates.

Table 3. 5 Metabolic variation between three isolates of *Brachyspira pilosicoli*. This table shows the different carbon sources utilised by each *B. pilosicoli* isolate and the carbon sources shared by two out of three *B. pilosicoli* isolates to demonstrate the metabolic variation between isolates after 48 hours of anaerobic culture at 37°C using optical density at 540nm wavelength to capture the reduction of the tetrazolium dye and subsequent colour change, indicative of cellular respiration.

Carbon sources variation in *B. pilosicoli* isolates				
B2904 only	**SAP 859 only**	**SAP 865 only**	**B2904 and SAP 859 only**	**SAP 859 and SAP 865 only**
Adenosine	2-aminoethanol	D- trehalose	α- acetyl neuraminic acid	Cellobiose
Inosine	Laminarin	D-L- α glycerol phosphate	β- methyl-D-galactoside	
	α-keto-butyric acid	Maltose	Butyric acid	
		β- methyl-D-glucoside	L- ornithine	
		Maltotriose		
		L- alanine		
		Methyl-pyruvate		
		Inulin		
		Mannan		
		Salicin		

3.2.2 Genotypic characterisation and identification of *Brachyspira* isolates

3.2.2.1 PCR

As previously demonstrated, phenotypic characterisation of *Brachyspira* was insufficient for the identification all isolates, therefore genotypic characterisation methods were also employed. PCR was performed as a preliminary screen to confirm isolates were of the *Brachyspira* genus and to identify isolates belonging to either the *B. pilosicoli* or *B. intermedia* species.

PCR correctly identified 3 of the 13 isolates that formed the original panel of *Brachyspira* isolates (Table 3.1). A major drawback of this characterisation method was that it was only

able to identify *B. pilosicoli* and *B. intermedia*. There were no specific primers sequences available for the detection for *B. alvinipulli* or *B. innocens* and therefore these isolates could not be detected using PCR in these studies. Primer sequences have been developed to identify the non-pathogenic *Brachyspira* species, *B. innocens* and *B. murdochii*, but cannot distinguish between them (Atyeo *et al.*, 1999) which made them unsuitable for these studies. Furthermore, due to the poor understanding of *B. alvinipulli* and the paucity of genomic data, primer design has not previously been attempted. This confirmed the need to use whole genome sequencing for species identification.

Using the data summarised in Table 3.1, seven *Brachyspira* isolates were selected for whole-genome sequencing (excluding *B. alvinipulli* ATCC 51933 as the genome sequence was available), these isolates were those that were most likely to have been identified using biochemistry, PCR or a combination of both as this increased the likelihood of obtaining isolates from all four of the species implicated in poultry infection. The isolates sent for whole-genome sequencing are listed in Table 3.1 and were B2904 and SAP 859, which were presumptive *B. pilosicoli*, SAP 865 and SAP 943 which were presumptive *B. alvinipulli*, SAP 891 and SAP 919 which were presumptive *B. intermedia* and SAP 924 and SAP 927 which were presumptive *B. innocens.* The genome sequence for *B. alvinipulli* ATCC 51933 was already publicly available on Genbank.

3.2.2.2 Whole genome sequencing and phylogenetics

An overview of the whole genome sequence data processing and analysis can be seen in Figure 3.5. In brief, raw sequence reads were assembled *de novo* using the Shovill pipeline, a genome assembler for Illumina whole genome sequence data (https://github.com/tseemann/shovill). The quality of these assemblies was assessed using

Quast v2 (Gurevich *et al.*, 2013), a summary of these data are presented in Table 3.7. All of the sequences presented in this table were of sufficient quality for annotation and phylogenetic analysis, as indicated by the high N50 values and low L50 values which described the quality of assembled contigs.

The *Brachyspira* genomes ranged in size from 2.54 - 3.49 Mbps, with a GC content ranging from 26.9-28.1%. *B. pilosicoli* isolates had the smallest genome size, ranging from 2.57- 2.75 Mbps, *B. alvinipulli* and *B. intermedia* had the largest genome size at 3.42 and 3.49 Mbps, respectively and *B. innocens* had a genome size of ranging from 3.15- 3.20 Mbps (this will be further discussed in Chapter 4). The number of coding sequences varied with the genome size, with *B. pilosicoli* having the lowest number and *B. alvinipulli* and *B. intermedia* having the highest numbers. Overall, the GC content (%) and the number of tRNAs (38 or 39) were consistent across all eight *Brachyspira* isolates.

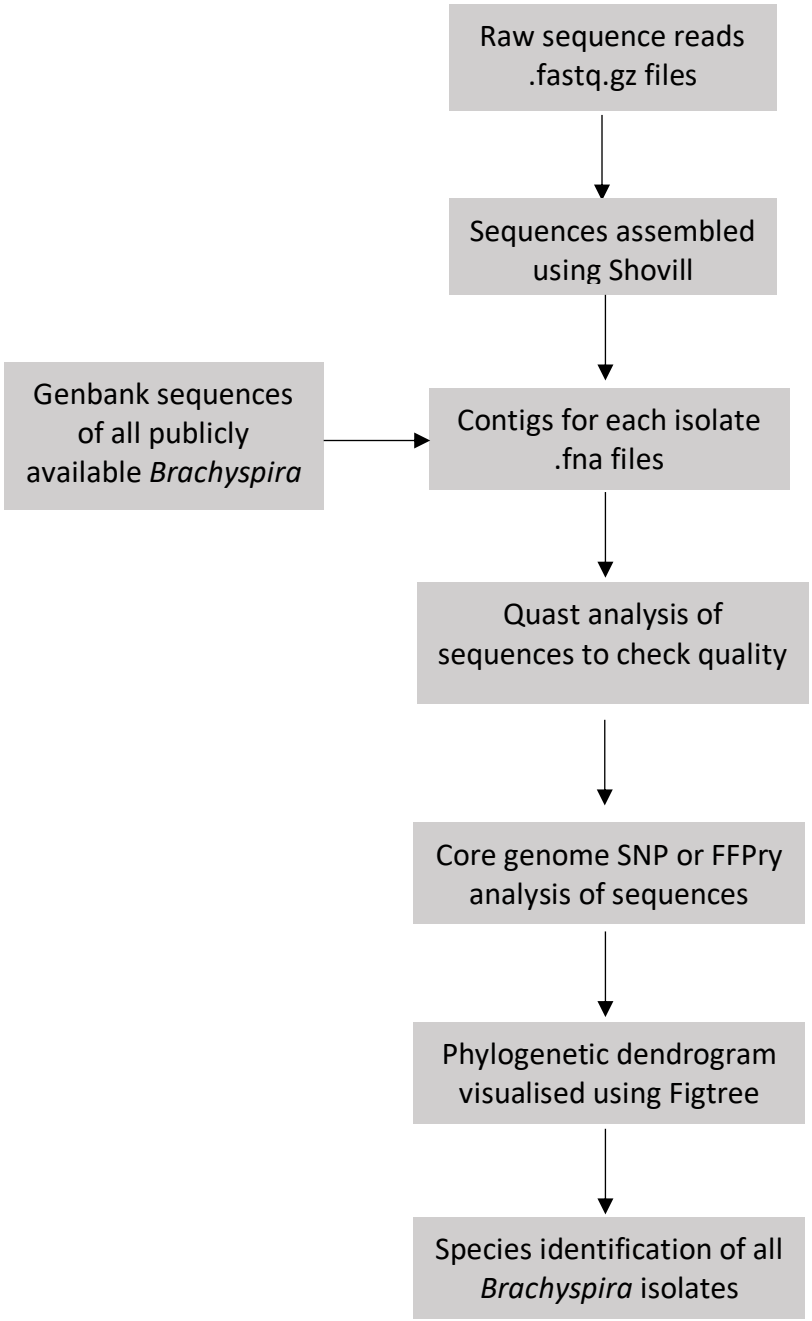


Figure 3. 5 Summary of whole genome sequence data processing and analysis. Raw sequence reads from each *Brachyspira* isolate were assembled *de novo* using Shovill and all available Genbank sequences were downloaded. Assembly statistics were generated for all *Brachyspira* sequences using Quast to ensure the sequences were of sufficient quality for further analysis. Sequences of good quality were annotated using Prokka, followed by core genome SNP analysis. Phylogenetic dendrograms of these data were generated using Figtree, which allowed the accurate speciation of each *Brachyspira* isolates.

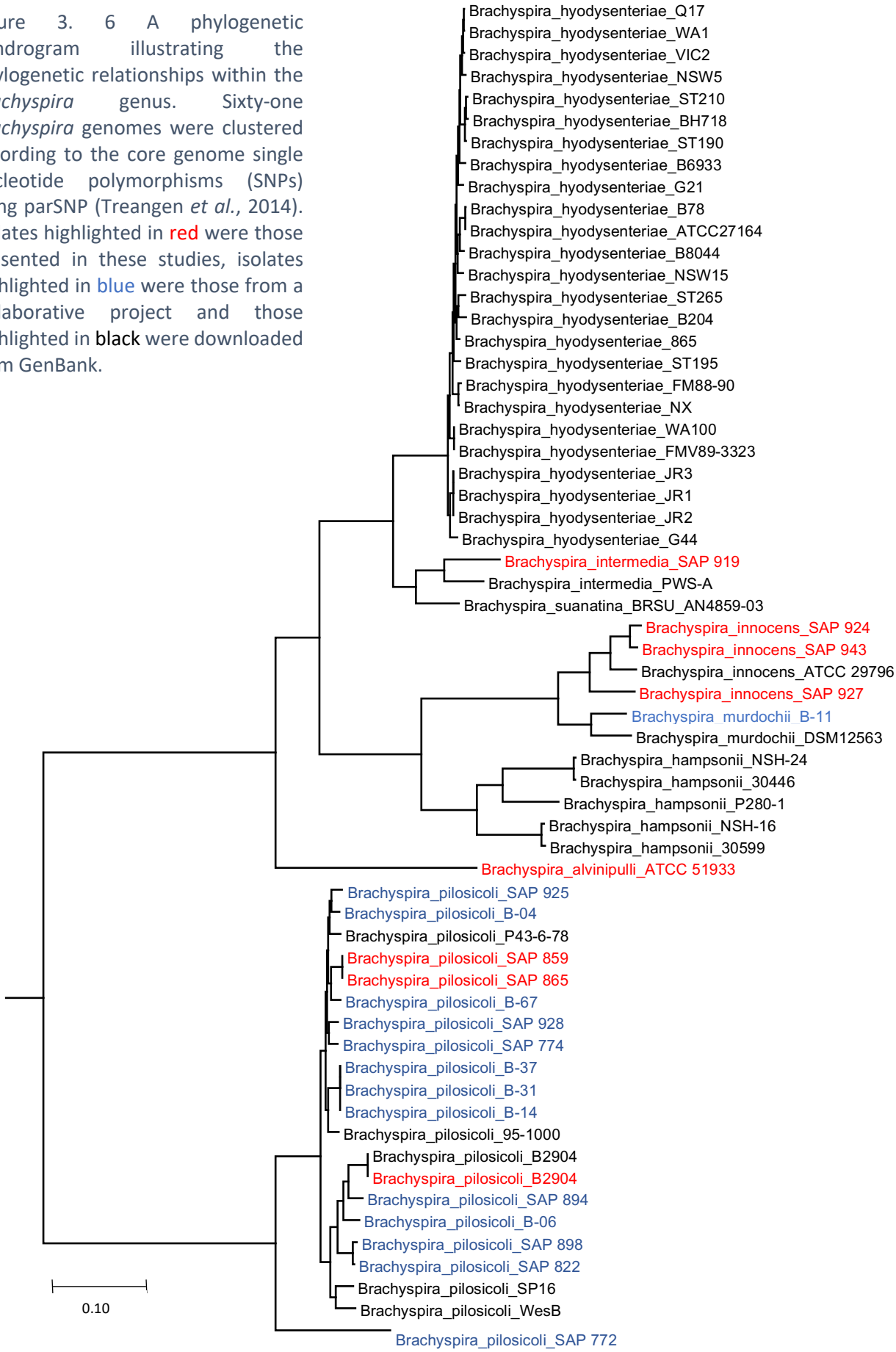
Table 3. 6 The size of the genomes in base pairs (bp), GC content (%), number of coding sequences, number of RNAs, number of contigs, largest contig, the N50 value and the L50 value for each of the *Brachyspira* genomes. Data for the assembly statistics were generated from Prokka and the data for the assembly quality were generated from Quast.

***Brachyspira* Isolate**	**Size (bp)**	**GC content (%)**	**Number of coding sequences**	**Number of tRNAs**	**Number of Contigs**	**Largest Contig**	**N50***	**L50****
***B. alvinipulli* ATCC 51933**	3420763	26.9	3209	39	22	582949	342358	4
***B. innocens* SAP 924**	3150198	27.8	2725	39	95	379134	66364	13
***B. innocens* SAP 927**	3203920	28.1	2816	39	139	171035	51776	20
***B. innocens* SAP 943**	3200075	27.8	2811	39	72	361958	102553	11
***B. intermedia* SAP 919**	3493658	27.4	3005	38	144	217871	80677	15
***B. pilosicoli* B2904**	2765477	27.0	2658	38	1	2765477	2765477	1
***B. pilosicoli* SAP 859**	2542553	28.1	2288	38	13	637327	409203	3
***B. pilosicoli* SAP 865**	2545332	28.1	2252	38	22	698261	637106	2

* N50 value is defined as the minimum contig length required to cover 50% of the genome, i.e. half of the contigs are equal to or larger than the N50 contig size. The larger the N50 value, the higher the quality of the assembly.
** L50 value is defined as the number of contigs required to account for 50% of the genome. The lower the L50 value, the higher the quality of the assembly.

To observe the genetic diversity amongst *Brachyspira* isolates and to speciate each of the isolates selected for these studies, phylogenetics were employed using parSNP to analyse the core genome single nucleotide polymorphisms (SNPs) of all available *Brachyspira* genomes. This included forty genomes downloaded from Genbank, thirteen *B. pilosicoli* and one *B. murdochii* genomes sequenced in a collaborative project (Figure 3.6) and seven genomes sequenced during these studies. *B. pilosicoli* B2904 was sequenced in these studies to ensure that the isolate was identical to the isolate used by Mappley *et al*. (2012) as it was used as a control strain. *B. alvinipulli* ATCC 51933 was used in phenotypic assays and genome analysis but was not sent for sequencing as the genome sequence was publicly available. The phylogenetic dendrogram, which can be seen in Figure 3.6, illustrated distinct clusters of well characterised *Brachyspira* species, namely *B. hyodysenteriae* and *B. pilosicoli*. Clusters of *B. intermedia* and *B. suanatina*, *B. hampsonii* and the non-pathogenic species *B. innocens* and *B. murdochii* could also be identified despite the small numbers of available genome sequences.

Figure 3. 6 A phylogenetic dendrogram illustrating the phylogenetic relationships within the *Brachyspira* genus. Sixty-one *Brachyspira* genomes were clustered according to the core genome single nucleotide polymorphisms (SNPs) using parSNP (Treangen *et al.*, 2014). Isolates highlighted in red were those presented in these studies, isolates highlighted in blue were those from a collaborative project and those highlighted in black were downloaded from GenBank.



Following phylogenetic analysis, it was possible to confidently determine the species of each of the novel *Brachyspira* isolates used in these studies. To summarise: B2904, SAP 859 and SAP 865 were identified as *B. pilosicoli*, SAP 919 was identified as *B. intermedia* and SAP 924, 927 and 943 were identified as *B. innocens*. These isolates were selected for further studies presented in Chapters 5, 6 and 7, in addition to *B. alvinipulli* ATCC 51933 to ensure all species of *Brachyspira* implicated in avian intestinal spirochaetosis were considered. Presumptive *B. intermedia* SAP 891 was determined to be a mixed culture which contain both low GC contigs, characteristic of *Brachyspira*, and high GC contigs. Therefore, this isolate was excluded from any further analysis.

3.3 Discussion

These studies focussed on phenotypically and genotypically characterising a panel of eight avian *Brachyspira* isolates for subsequent study into the pathobiology of this organism, and how *Lactobacillus* probiotics may be able to mitigate avian intestinal spirochaetosis caused by *Brachyspira* species. It was essential to select and characterise isolates that represented all species implicated in avian intestinal spirochaetosis, including *B. alvinipulli, B. intermedia*, *B. pilosicoli* and *B. innocens.* Although *B. innocens* is currently identified as non-pathogenic, it has been associated with significantly reduced egg production in free range flocks and was therefore utilised in these studies (Burch *et al.*, 2009).

A combination of Gram staining, Warthin-Starry staining and dark field microscopy identified the morphology and phenotype of the cells: Gram negative staining and their characteristic "corkscrew-like motility" (Naresh and Hampson, 2010). Until recently biochemistry was the primary method used to speciate *Brachyspira* isolates (Fellström and Gunnarsson, 1995; Hommez *et al.*, 1998). These methods were only able to identify 46% of the *Brachyspira* isolates. Similar results were reported by Feberwee *et al* (2008), where less than 50% of isolates were identifiable with biochemistry alone. The biochemical profiling methods developed by Fellström and Gunnarsson (1995) grouped *Brachyspira* species in order to differentiate them. *B. intermedia* isolates were placed in group II because they were indole positive. *B. pilosicoli* isolates were placed in group IV because they were hippurate positive and indole negative. Importantly, *B. pilosicoli* and *B. alvinipulli* isolates could only be distinguished in the ability to metabolise β-glucosidase, where *B. pilosicoli* was negative and *B. alvinipulli* was positive, although *B. alvinipulli* has not been officially grouped biochemically. *B. innocens* isolates were placed in group III, which is sub-sectioned into IIIa, IIIb and IIIc. Groups IIIb and IIIc contained *B. innocens,* but vary in their α-glucosidase activity, indicating

metabolic variation within the *B. innocens* species. Traditionally, *B. innocens* could be distinguished from *B. murdochii*, in group IIIa by the ability to metabolise α-galactosidase (Hommez *et al.*, 1998), however biochemically, *B. innocens* SAP 943 was classified as group IIIa. Biochemical testing developed by Fellström and Gunnarsson (1995) was determined using porcine isolates which have been shown, in some cases, to be distinct from avian isolates (Atyeo *et al.*, 1999). This may, in part contribute to the fact that some of the avian *Brachyspira* isolates used here appear to have 'atypical' metabolic capabilities.

Understanding the growth kinetics of each *Brachyspira* isolate was essential to begin to consider mitigation strategies to improve poultry welfare. *Brachyspira* are fastidious, slow growing organisms as seen in Figure 3.2; this considered, probiotics could be a suitable mitigation strategy as they are fast growing, less fastidious organisms. Many *Lactobacillus* species grow to high densities in 16-24 hours and *Brachyspira* reaches log phase after approximately 25 hours of growth, therefore it may be possible that *Lactobacillus* could exert their inhibitory effects against *Brachyspira* to prevent exponential growth.

Antimicrobial resistance is a growing concern with many bacterial pathogens including *Brachyspira*. The emergence of antimicrobial resistance in *Brachyspira* has been noted in several studies, with the highest resistance observed to macrolide antibiotics such as tylosin and lincosamide antibiotics such as lincomycin. Resistance to tiamulin is also increasing but still remains one of the most effective antibiotics used to treat *Brachyspira* infections (Karlsson *et al.*, 2004; Burch, 2005; Hampson *et al.,* 2006; Pringle *et al.,* 2006).

The antibiotic resistance profiles of eight *Brachyspira* isolates presented in Table 3.2 showed that the majority of isolates were sensitive to all antibiotics tested, with *B. pilosicoli* SAP 865 and *B. alvinipulli* ATCC 51933 showing decreased sensitivity to chlortetracycline. It is important to note that clinical breakpoints are yet to be formally defined for *Brachyspira*

isolates and thus it is not possible to officially specify that an isolate as clinically sensitive or resistant to an antibiotic (Mirajkar *et al*., 2016). Many break-point values for common antimicrobials such as tiamulin, tylosin and lincomycin have been proposed as a way of bridging this gap in knowledge, with break-point determination based on both agar dilution and broth microdilution methods (Duhamel *et al.*, 1998; Burch, 2005; Pringle *et al.*, 2012; Hellman *et al.*, 2014). The general consensus for the broth microdilution method was that an MIC of $<$ 0.25 mg/L deemed a *Brachyspira* isolate sensitive to tiamulin, an MIC of $<$ 16 mg/L deemed a *Brachyspira* isolate sensitive to tylosin and an MIC of $<$ 50 mg/L deemed a *Brachyspira* isolates sensitive to lincomycin (Burch, 2005; Pringle *et al.*, 2012; Hellman *et al.*, 2014). These values have therefore been used in the studies presented here to determine the sensitives of all eight isolates to the antibiotics tested. The only available proposed break-points for chlortetracycline were determined using the agar dilution method, whereby an MIC of $<$ 4 mg/L deemed a *Brachyspira* isolate sensitive to chlortetracycline (Brooke *et al*., 2003; Hampson *et al*., 2006). On average, the MIC values from the broth microdilution method were reported as one dilution lower than that of the agar dilution method, and therefore an MIC of $<$ 2 mg/L was used to interpret the sensitivity of each *Brachyspira* isolate to chlortetracycline (Rohde *et al.*, 2004).

Although *Brachyspira* are sensitive to a number of antimicrobials, the bacteriostatic antibiotics tiamulin and tylosin are the most effective and widely used, with their mode of action targeting bacterial protein synthesis. Widespread resistance to tylosin has been identified which may be partially attributed to the use of tylosin as an antibiotic growth promoter in the swine industry (Kim *et al.*, 2016). Resistance to tylosin has been linked to two mutations on the 23S rRNA gene whereby a transversion from an A to a T in position 2058 or

a transversion from an A to a C or a transition from an A to a G in position 2059 are associated with tylosin resistance (Karlsson *et al.*, 2004).

Many *Brachyspira* isolates are still susceptible to tiamulin, which correlates with the results presented in this study. Epidemiological studies have estimated that 10-15% of isolates tested were identified as resistant between 1990 and 2010 (Pringle *et al.*, 2012) and more recent studies have also estimated that approximately 9% of *Brachyspira* isolates tested were resistant to tiamulin (Mirajkar *et al.,* 2016). Resistance to tiamulin has been associated with point mutations in the domain V of the 23S rRNA gene and the ribosomal protein L3 gene. These genes are in close proximity to the peptidyl transferase centre which is the binding site of tiamulin thus, mutations in this binding site and in neighbouring proteins may affect the efficacy of tiamulin (Pringle *et al.*, 2004; Hidalgo *et al.*, 2011). Considering the decreasing susceptibly to commonly used antibiotics and the removal of antibiotic growth promoters, it is imperative that control strategies are implemented to promote intestinal health and prevent disease. The use of probiotics may be able to decrease the need for antibiotic use in poultry, thus decreasing the selective pressure on pathogens such as *Brachyspira*, in addition to potentially promoting a healthy intestinal microflora and improving overall health and welfare of poultry.

There is a significant lack of understanding of *Brachyspira* metabolism, only recently have high throughput methods been used to investigate the carbon metabolism of *Brachyspira* isolates using Biolog technology (Mappley *et al.*, 2012). Previous characterisation of *Brachyspira* isolates has been conducted by supplementing media with sugars such as fructose, maltose, mannose and sucrose, to name a few, in order to determine the metabolic capabilities of different species. These methods were established in the late 1980s and 1990s and were limited to a relatively small number of sugars (Stanton and Lebo, 1988; Trott *et al.*, 1996;

Stanton *et al*., 1998). More recently, with the availability of whole genome sequences, metabolic modelling has been used to infer the metabolic capabilities of *Brachyspira* but little phenotypic testing has been reported (Wanchanthuek *et al.*, 2010). Therefore, the use of the Biolog Phenotypic Microarray™ technology has the ability to screen many more carbon sources, which could not only help to improve the identification of *Brachyspira* isolates but could also improve the understanding the metabolic capabilities of these organisms.

Biolog PM™ technology was used as a further phenotypic characterisation method for the panel of eight *Brachyspira* isolates presented in these studies. It was apparent that more comprehensive phenotypic testing with a much greater number of *Brachyspira* isolates is required to identify carbon sources unique to each species with the aim of improving current biochemical testing methods.

The results of this study indicated that all *Brachyspira* isolates utilised a number of carbon sources involved in glycolysis which therefore plays a major role in energy production for this organism, these included glucose, glucose-6-phosphate, fructose-6-phosphate, fructose and galactose. Metabolic modelling, following whole genome sequencing, has also indicated that glycolysis is the backbone of energy productive for *Brachyspira* species (Wanchanthuek *et al.*, 2010). Glucose-6-phosphate and fructose-6-phosphate are also important constituents of the pentose phosphate pathway which is important for biosynthetic production of fatty acids and nucleotides. *B. pilosicoli, B. murdochii* and *B. hyodysenteriae* were found to have a complete set of genes for the pentose phosphate pathway including genes such as ribulose-5-phosphate epimerase and isomerase, to convert glucose-6-phosphate to ribulose-5-phosphate, a major constituent of this pathway (Bellgard *et al.*, 2009; Wanchanthuek *et al.*, 2010). *Lactobacillus* species are also able to metabolise many of these carbon sources using similar pathways, therefore this could be evidence that potential probiotic isolates could

directly compete with *Brachyspira* for available nutrients (Saulnier *et al.*, 2011). *Brachyspira* isolates were unable to metabolise many of the intermediates involved in the tricarboxylic acid cycle (TCA) such as malic acid, fumaric acid and succinic acid, as a result of having an incomplete set of genes for this metabolic pathway. Thus, suggesting that ATP is generated through the fermentation of sugars. This can be observed with other spirochaetes such as *Borrelia burgdorferi* (Lackum and Stevenson, 2005) and *Treponema pallidum* (Bellgard *et al.*, 2009).

B. pilosicoli isolates metabolised more carbon sources than other *Brachyspira* isolates on the PM1 and PM2 plates, and this more extensive metabolic capacity may contribute to the ability to infect a wide host range, including pigs, poultry, humans, horses and dogs (Trott *et al.*, 1996). This versatility may have contributed to the success of *B. pilosicoli* as an intestinal pathogen.

It was not possible to attribute unique carbon source utilisation to a specific species of *Brachyspira* for a several reasons. Firstly, only one isolate of *B. alvinipulli* and *B. intermedia* was used thus these data may not be representative of each species due to the notable metabolic variations described in other species (Mappley, 2012). Additionally *B. innocens* and *B. piloscioli* isolates exhibited variation within each species. For example, inosine was utilised soley by *B. pilosicoli* B2904 in these studies. When this was compared to data from Mappley *et al* (2012) it was observed that *B. pilosicoli* 95/1000 and WesB could also utilise inosine, whereas *B. pilosicoli* SAP 859 and 865 did not. Of these five *B. pilosicoli* isolates only SAP 865 was able to utilise inulin, trehalose, β- methyl-D-glucosidase, methyl pyruvate and mannan, suggesting that SAP 865 may be an atypical isolate of *B. piloscioli*. However, more isolates would need to be tested in future studies to generate typical phenotypes for each *Brachyspira* species. Similarly carbon sources that were specific to *B. innocens* were only representative

of one isolate and in the absence of data from more isolates, it was not possible to select carbon sources that would be suitable targets for differential diagnoses of *Brachyspira.*

The Biolog data in these studies largely agreed with the data published by Mappley *et al* (2012), however there were discrepancies with 47 carbon sources. This could be attributed to the modifications of methods used in these studies whereby Biolog plates were prepared and incubated in an anaerobic cabinet for 48 hours instead of being incubated as specified by Biolog. According to the manufacturer, anaerobic gas can auto-reduce the tetrazolium dye after 48 hours of incubation, and therefore the duration of this experiment was limited. Mappley *et al* (2012) incubated plates for 120 hours, which may have resulted in the respiration on more carbon sources by *Brachyspira*. Initial descriptions of *B. pilosicoli* (Trott *et al.*, 1996), *B. intermedia* (Stanton *et al.*, 1997), *B. innocens* (Ochiai *et al.,* 1997) and *B. alvinipulli* (Stanton *et al*., 1998) characterised metabolic profiles for a relatively small number of carbon sources. These also largely match the data presented in these studies, however it was apparent that once multiple isolates from each species were characterised, more carbon sources could be utilised than originally identified. For example, *B. intermedia, B. innocens* and *B. pilosicoli* were reported as not being able to metabolise L-rhamnose, however all isolates in the studies presented here were able to utilise this carbon source, this is further evidence to suggest that there are metabolic variations between *Brachyspira* isolates within the same species, as previously observed.

With this in mind, future work could focus in applying this phenotypic assay to a wider range of *Brachyspira* isolates in order to build metabolic profiles for each species implicated in avian intestinal spirochaetosis. The use of this rapid, high throughput method may then have the ability to improve current diagnostic methods, especially for avian isolates which are overall, less well characterised than porcine isolates.

Genotypic characterisation of *Brachyspira* isolates was used to correctly identify all isolates. It was evident from Table 3.1 that biochemistry results alone, were highly variable and did not always correspond to the species identified by PCR. It has been noted that in addition to phenotypic variations between avian and porcine isolates, there are also genotypic variations whereby species-specific primers designed for *B. intermedia* and *B. pilosicoli* were able to identify 100% of porcine isolates, but only 40% and 85% of avian isolates, respectively (Atyeo *et al.*, 1999). The species-specific PCR primers used in these studies correctly identified three of the four *B. pilosicoli* isolates tested and both of the *B. intermedia* isolates tested, although following whole genome sequencing one of these was identified as mixed with *B. innocens*, highlighting the need for further investigation into the design of *B. innocens* species-specific primers. This may also highlight the need to review *B. pilosicoli* PCR primers in future studies as they were initially developed using human *Brachyspira* isolates (Park *et al.*, 1995; Mikosza *et al.*, 2001) and thus the diversity observed between porcine and avian isolates may also apply with human isolates. Additionally, the need for PCR primers to identify all *Brachyspira* isolates implicated in poultry disease is apparent. However, due to the paucity of information about the *B. alvinipulli* species and the absence of genome data, this is not possible. Differential PCR based methods have been developed to distinguish avian *Brachyspira* species using the *nox* gene instead of the 16S or 23S rRNA genes because, although well conserved in the *Brachyspira* genus with approximately 86% sequence identity among isolates, they are less well conserved than the 16S or 23S rRNA genes which show a maximum sequence divergence of 2% and 3% respectively, therefore there is a greater likelihood of species specificity (Atyeo *et al.*, 1999). These PCRs have been developed to distinguish pathogenic species of *Brachyspira* from non-pathogenic species and therefore can detect the presence of *B. innocens* and *B. murdochii* but cannot distinguish between them.

Considering the difficulty in identifying the panel of *Brachyspira* isolates presented in Table 3.1, seven isolates were sent for whole genome sequencing for definitive identification to inform isolate section for further studies presented in Chapters 5, 6 and 7. Using phylogenetics it was shown that both isolates SAP 865 and SAP 943 were not *B. alvinipulli*, but belonged to *B. pilosicoli* and *B. innocens*, respectively, further highlighting the need to fully characterise *B. alvinipulli*.

The genome sequence of each *Brachyspira* isolates detailed in Table 3.6 showed that each isolate had a genome size of between 2.54- 3.49 Mbp with a GC content (%) of between 26.9-28.1%. These data were representative of the *Brachyspira* genus, with *B. pilosicoli* having the smallest, but most variable genome size when compared to any other species (Wanchanthuek *et al.*, 2010; Håfström *et al.*, 2011; Mappley *et al.*, 2012; Lin *et al.*, 2013; Black *et al.*, 2015). In studies presented here, the *B. pilosicoli* genome size ranged from 2.54- 2.76 Mbp but are known to be as large as 2.88 Mbp in the example of *B. pilosicoli* WesB (Mappley *et al.*, 2012), however *B. hyodysenteriae* have a more stable genome size ranging from 3.01- 3.1 Mbp, with the majority of isolates approximately 3.03 Mbp (Black *et al.*, 2015). This may be as a result of *B. pilosicoli* having undergone increased genome evolution as a result of the ability to infect a wide host range, whereas pigs are the primary host for *B. hyodysenteriae*, although there have been reports of this organism colonising of poultry in close proximity to pig units, in addition to be isolated from rheas in the USA (Jensen *et al*., 1996; Thomson *et al.*, 2007).

In summary, this chapter described the identification and characterisation of eight *Brachyspira* isolates, including all species implicated in avian intestinal spirochaetosis. A combination of phenotypic and genotypic methods were utilised to ensure the accurate identification of isolates, including biochemical testing, PCR and whole genome sequencing.

Combining these techniques was imperative to the accurate identification of all *Brachyspira* isolates, which were used in subsequent studies presented in this thesis.

Chapter 4: Comparative genomics of *Brachyspira* species implicated in intestinal spirochaetosis

4.1 Introduction

There is a paucity of data relating to both the physiology and genomics of *Brachyspira* species. This lack of knowledge may be, in part due to the small number of isolates that have undergone whole genome sequencing. Furthermore, there are *Brachyspira* species, such as *B. aalborgi,* that have yet to have their genome sequences determined. There have been very few comparative genomics studies conducted on the *Brachyspira* genus, however as whole genome sequencing is becoming more economic and readily accessible, the understanding of *Brachyspira* biology and evolution will no doubt improve significantly.

Previous comparative genomics studies have focussed on comparing *Brachyspira* isolates within one species, in particular *B. hyodysenteriae* (Black *et al.*, 2015) and *B. pilosicoli* (Mappley *et al.*, 2012), as these are the most well characterised *Brachyspira* species. These studies have found that *B. hyodysenteriae* is a relatively conserved species and highly adapted to cause disease in pigs (Bellgard *et al.*, 2009; Black *et al.*, 2015), whereas *B. pilosicoli* is a diverse species, capable of causing disease in a wide host range (Mappley *et al.*, 2012). Additionally, a study that compared one isolate of *B. hyodysenteriae, B. pilosicoli* and *B. murdochii* indicated the diversity between these species, although the interpretation of these data are limited as analysis was conducted with single isolates from each species (Wanchanthuek *et al.*, 2010). Finally, more recently genome features from other *Brachyspira* species, such as *B. intermedia* and *B. innocens* have been investigated, however pangenome analysis has been limited to a small number of publicly available sequences and therefore, comparisons must be treated as preliminary (Hampson and Wang, 2017).

The studies presented in this chapter follow on from the results presented in Chapter 3, where whole genome sequencing was used to confirm the species of *Brachyspira* isolates used in subsequent studies. A comparative genomics approach was used to compare *Brachyspira* species implicated in both avian, human and porcine disease. These comparisons build upon published data by using more sequences from *B. pilosicoli*, *B. innocens* and *B. intermedia* (Bellgard *et al.*, 2009; Wanchanthuek *et al.*, 2010; Mappley *et al.,* 2014; Hampson and Wang, 2017). Additionally, key virulence genes such as methyl-accepting chemotaxis protein (MCP) genes and chemotaxis genes (*che*) were investigated to determine any key differences between *Brachyspira* species which may give insight into the host range or pathogenicity.

Therefore, the aims and objectives of this chapter were:

- To determine the genome sequences of a panel of *Brachyspira* species implicated in avian intestinal spirochaetosis.
- To use a pangenome approach to compare all available *Brachyspira* whole genome sequences, including those determined in this study. Comparative genomics was completed by splitting species in to three comparable groups: "*B. hyodysenteriae*", "*B. pilosicoli*" and "Other" (comprising of all other *Brachyspira* species).
- To use the output from the comparative studies to select target genes of interest to highlight similarities/ differences between *Brachyspira* species.

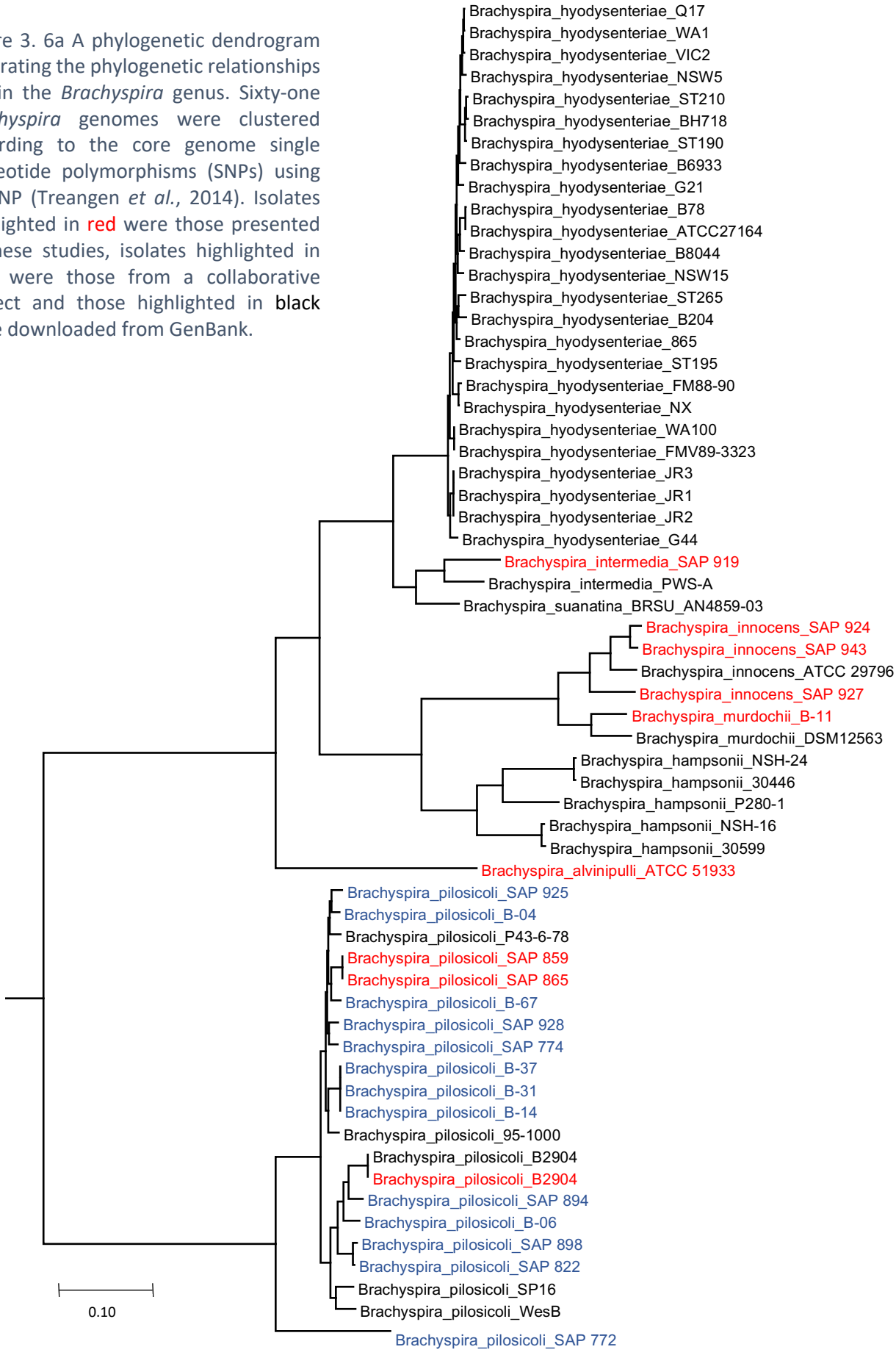
4.2 Results

4.2.1 Genome size comparisons between *Brachyspira* species

The phylogenetic tree presented in Chapter 3 (also shown below) demonstrated evolutionary divergence between *B. hyodysenteriae* and *B. pilosicoli*, with other species such as *B. alvinipulli*, *B. innocens*, *B. intermedia* and *B. murdochii* positioned between them. As mentioned, the phylogenetic relationships between species of the *Brachyspira* genus have not been extensively investigated, most likely due to the lack of available genome sequences. More recently, Hampson and Wang (2017) took a similar approach to the studies presented here and compared all publicly available *Brachyspira* genome sequences. However, in the studies presented here nineteen *B. pilosicoli* genomes were included, compared to three in previously published studies (Mappley *et al.*, 2012). Furthermore, novel *B. innocens, B. intermedia* and *B. murdochii* sequences were included.

For the purpose of these analyses, fifty-six genomes from the analysis presented in Chapter 3 were split in to three groups: "*B. hyodysenteriae*", "*B. pilosicoli*" and "Other", the latter containing the genome sequences of all other available *Brachyspira* species. This grouping was necessary to ensure that *Brachyspira* species with only one or two available genome sequences were not excluded from analysis at this early stage in the studies. It must be noted that four genomes were removed from this analysis due to poor sequence quality as determined by Quast (three *B. hampsonii* and one *B. pilosicoli*), additionally, the *B. pilosicoli* B2904 genome was present twice, and one version was removed (Appendix II)

Figure 3. 6a A phylogenetic dendrogram illustrating the phylogenetic relationships within the *Brachyspira* genus. Sixty-one *Brachyspira* genomes were clustered according to the core genome single nucleotide polymorphisms (SNPs) using parSNP (Treangen *et al.*, 2014). Isolates highlighted in red were those presented in these studies, isolates highlighted in blue were those from a collaborative project and those highlighted in black were downloaded from GenBank.



Comparisons of *Brachyspira* genome size and number of coding sequences showed that these varied considerably between different *Brachyspira* species, as shown in Table 4.1 and Figure 4.1. From the genome information presented in Table 4.1 it can be seen that *B. intermedia* has the largest genome size, between 3.33 and 3.49 Mbps, although it is important to note that there were only two available genomes for comparison. Notably, *B. pilosicoli* had on average the smallest genome size, but also represented the largest range in genome sizes, ranging from 2.43 to 2.75 Mbps, with an average of 2.64 Mbps. *B. hyodysenteriae* had an average genome size of 3.06 Mbps with a range between 2.99 and 3.19 Mbps.

Table 4. 1 Available *Brachyspira* whole genome sequences used in the pangenome analysis are presented here. These sequences were either obtained from GenBank, NCBI or sequenced for the purpose of the studies presented here. The number of genomes per species are detailed, in addition to the assembly status and the source of the sequences.

Species	Number of genomes	Genome Size (Mbp) (standard deviation)	Assembly status	Source
B. alvinipulli	1	3.42	Complete	NCBI
B. hampsonii *	2	3.16-3.18 (0.08)	4 complete, 1 scaffold	NCBI
B. hyodysenteriae	25	2.99-3.19 (0.05)	3 complete, 18 scaffold, 4 contigs	NCBI
B. innocens	4	3.15-3.28 (0.05)	Contigs	1 NCBI, 3 this study
B. intermedia	2	3.33-3.49 (0.13)	1 complete, 1 contigs	1 NCBI, 1 this study
B. murdochii	2	3.14-3.24 (0.07)	1 complete, 1 contigs	NCBI
B. pilosicoli **	19	2.43-2.76 (0.09)	3 complete, 1 scaffold, 17 contigs	4 NCBI, 17 this study
B. suanatina	1	3.25	Contigs	NCBI

* three *B. hampsonii* sequences were removed from the pangenome analysis due to poor quality genome sequences (30446, 30599 and NSH-24)
** two *B. pilosicoli* sequences were removed from the pangenome analysis because: (a) there were duplicate B2904 genomes and (b) the sequence for WesB was poor quality.

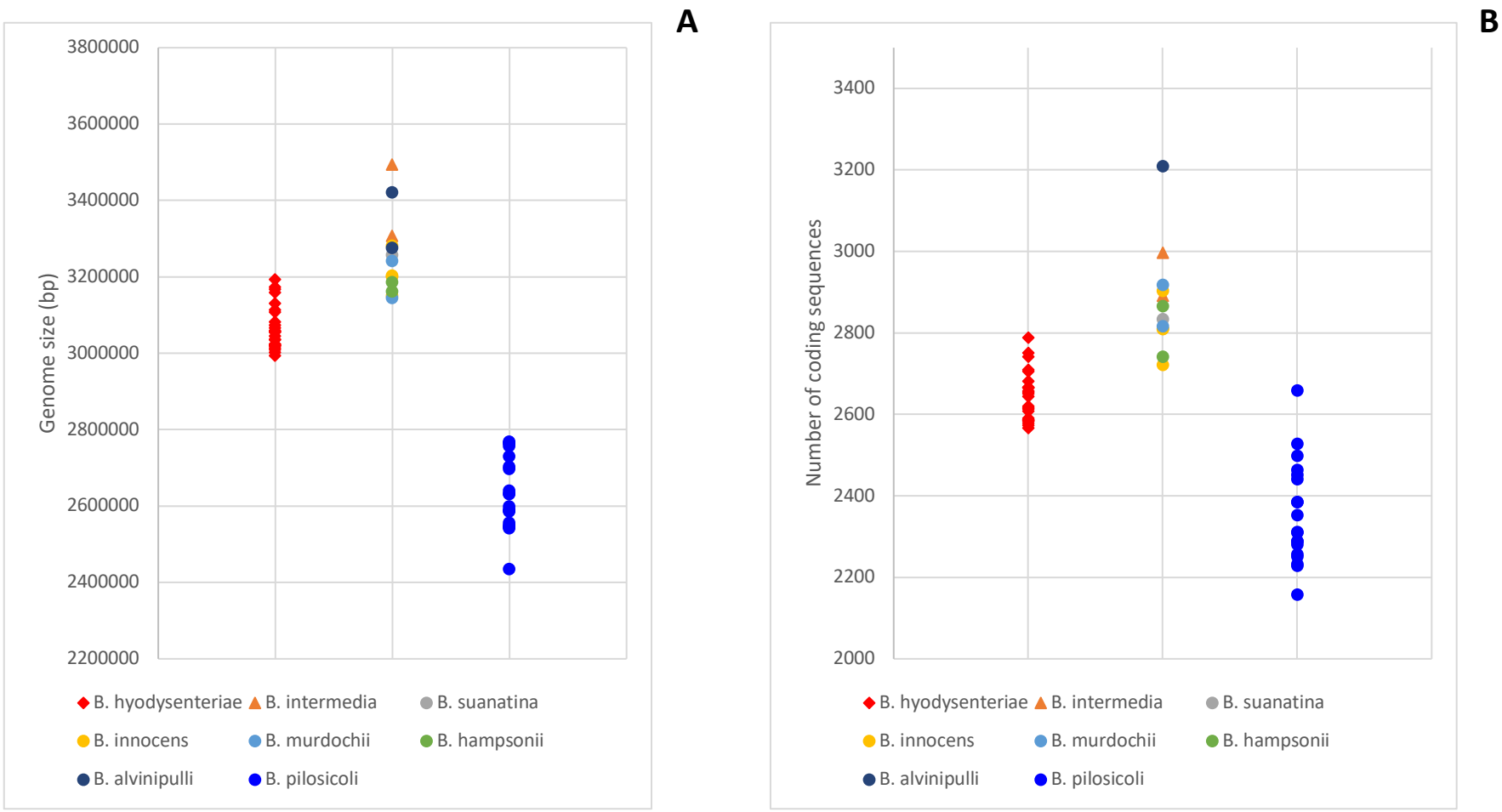


Figure 4. 1 Comparisons of the genome size (A) and the number of coding sequences (B) between different species in the *Brachyspira* genus. The size of *Brachyspira* genomes range from approximately 2.43- 3.49 Mbps depending on the species, with *B. pilosicoli* having overall the smallest genomes and *B. alvinipulli* and *B. intermedia* appearing to have some of the largest. The number of coding sequences is consistent with genome size, with *B. pilosicoli* having some of the lowest numbers of coding sequences and *B. alvinipulli* and *B. intermedia* having some of the highest. The genomes are separated into groups on the x axis, the first group showing *B. hyodysenteriae*, and second group showing the "Other" group of *Brachyspira* species and the third group showing *B. pilosicoli* sequences.

4.2.2 Pangenome analysis of *Brachyspira* species

A pangenome approach was used to compare all fifty-six *Brachyspira* genomes detailed in Table 4.1. All newly sequenced genomes were assembled using Shovill (https://github.com/tseemann/shovill), annotated using Prokka (Seemann, 2014) and subjected to Roary analysis (Page *et al.*, 2015). A Quast analysis was completed for all available genomes to ensure they were of suitable quality for the pangenome analysis using the criteria discussed in Chapter 3 (Gurevich *et al.*, 2013). The workflow is detailed in Figure 4.2, which builds upon the results presented in Chapter 3. Roary is a pangenome pipeline that allows for the comparison of annotated genomes. Coding sequences are converted into protein sequences and all comparisons are performed with BLASTP with a defined percentage sequence identity. A sequence identity cut off of 80% was used, as demonstrated in Figure 4.3. To identify the optimal cut off, the number of unique BLAST hits were plotted against the percentage BLAST cut off. The rationale behind selecting a suitable cut off was to identify the point at which the number of unique hits plateaued. This was at approximately 90%, however because multiple species of *Brachyspira* were being compared, an 80% cut off was used to ensure that comparisons were not overly stringent.

The Roary analysis produced a presence/absence gene matrix for each annotated gene in all selected *Brachyspira* isolates. This analysis was conducted with paralog clustering enabled, which ensured that paralogous genes were not split into separate groups, thus correcting for high allelic variation within the genomes (van Vliet, 2017).

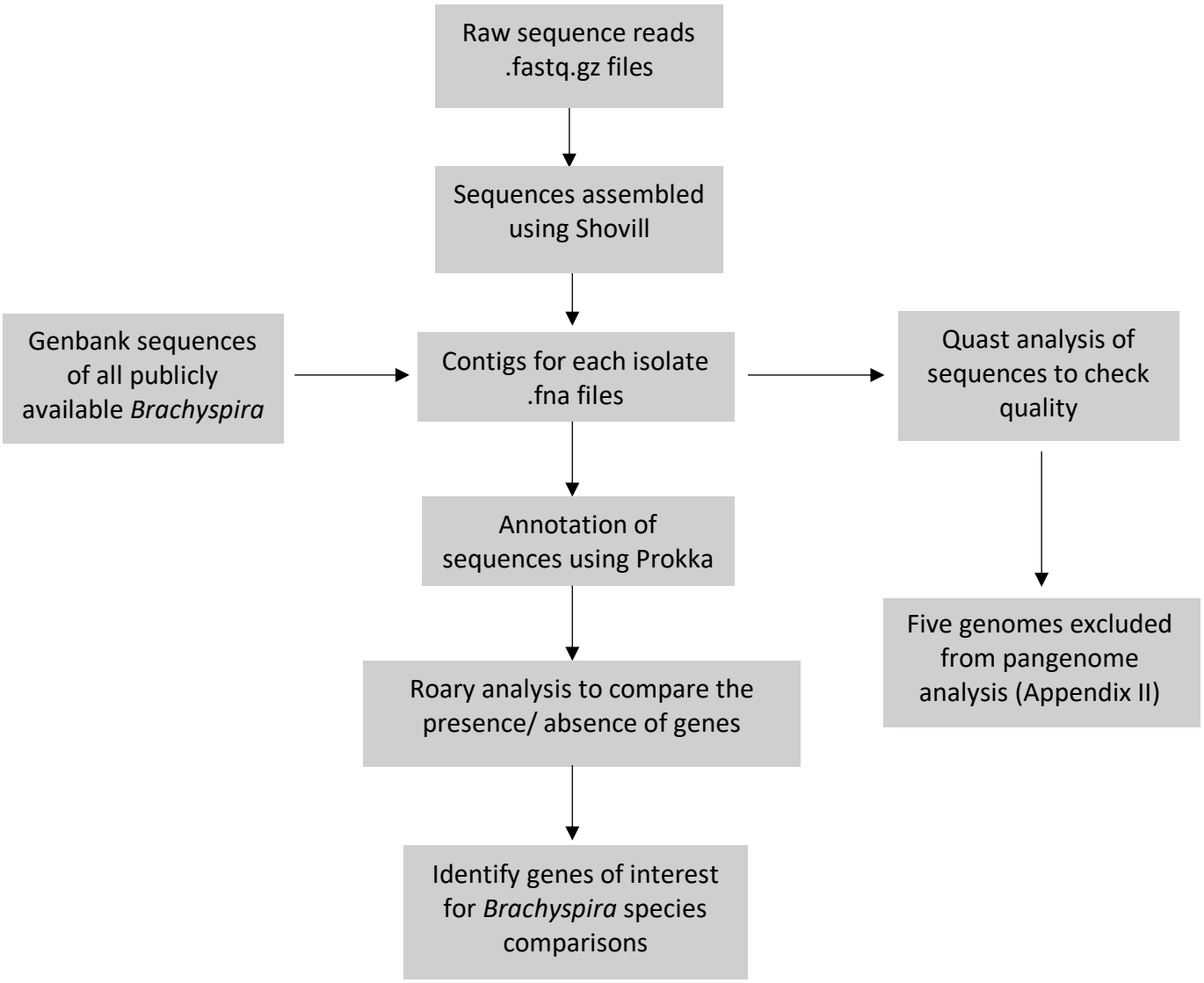


Figure 4. 2 Summary of sequence data processing and analysis for the comparative genomics. Raw sequence reads from each *Brachyspira* isolate used in these studies, were assembles *de novo* using Shovill. Publicly available *Brachyspira* sequences were also obtained from Genbank and assembly statistics were generated for all fifty-six genome sequences using Quast to ensure the sequences were of sufficient quality for further analysis. Sequences of sufficient quality were annotated using Prokka, followed by Roary analysis to determine the presence/absences of each of the genes annotated by Prokka. An 80% sequence identity cut-off was used. The genes of interest selected for comparative analysis were motility and chemotaxis genes as they are key virulence factors for *Brachyspira* and showed interesting distribution between species.

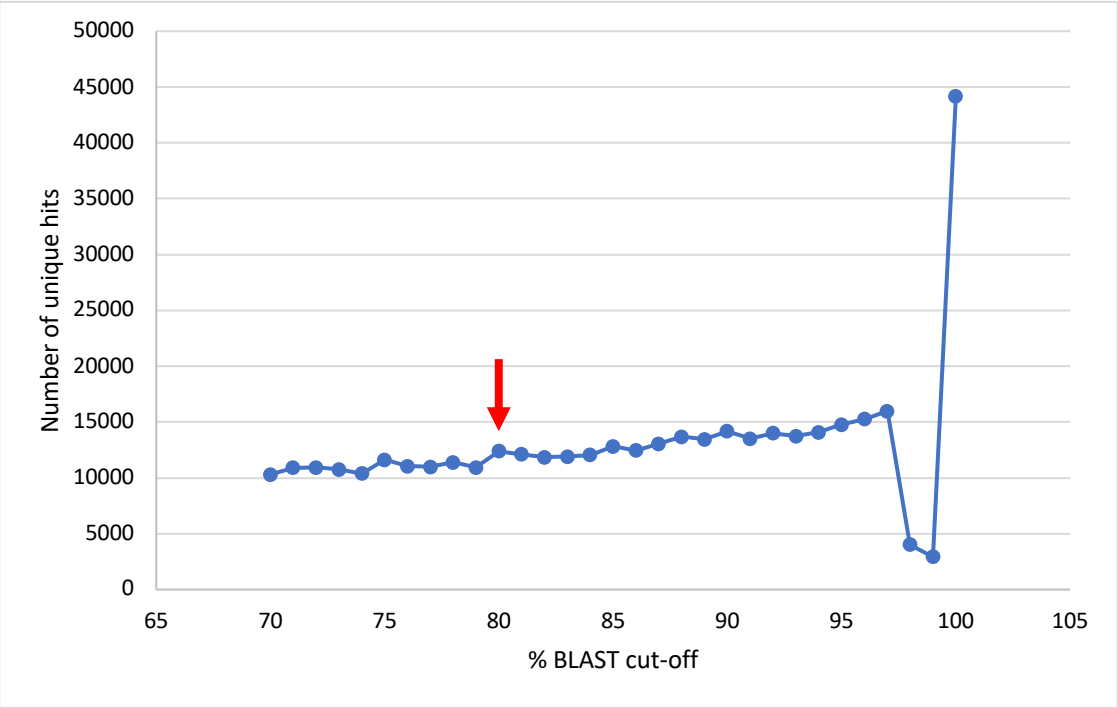


Figure 4. 3 The effect of the BLAST identity cut off on the number of unique hits within the dataset. The cut-off is usually set where the graph begins to tail off, which for *Brachyspira* is approximately 90%. However, because comparisons were being made between species in the genus, a cut-off of 80% was chosen, as indicated by the red arrow.

The pangenome analysis, comparing all available *Brachyspira* genome sequences revealed that the core genome of the *Brachyspira* genus consisted of 1089 genes. There was evidence to suggest the divergence of *B. pilosicoli* from other species of *Brachyspira*; analysis of the accessory genome identified 1423 genes that were unique to *B. pilosicoli*, only thirty-five genes shared with *B. hyodysenteriae* and 210 genes shared with other species in the genus, as shown in Figure 4.4. In contrast, *B. hyodysenteriae* had 306 unique genes and shared 1329 genes with the other species of *Brachyspira.* The group of "Other" *Brachyspira* had 1502 unique genes, however this group represented six different species of *Brachyspira* (*B. alvinipulli*, *B. innocens*, *B. intermedia*, *B. murdochii*, *B. hampsonii* and *B. suanatina*), thus highlighting that *B. pilosicoli* isolates are distinct within the *Brachyspira* genus, demonstrated by the 1423 unique genes.

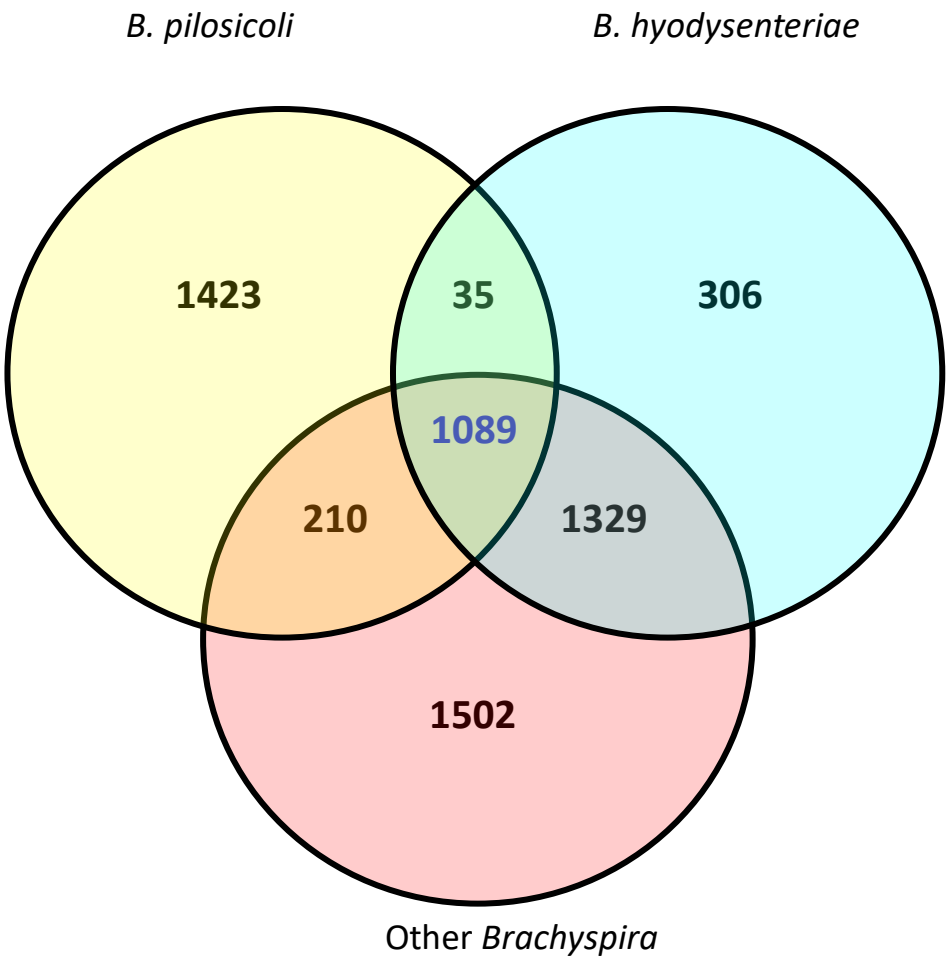


Figure 4. 4 A Venn diagram of the unique and shared genes between the three groups of *Brachyspira* used in these studies: *B. pilosicoli*, *B. hyodysenteriae* and "Other", which contained *B. alvinipulli*, *B. intermedia, B. hampsonii, B. murdochii, B. suanatina* and *B. innocens.* Each circle represents the number of genes in each group and the overlapping regions represent the number of genes shared by the respective species.

The *Brachyspira* pangenome can also be visualised as a presence/ absence matrix generated by Roary, as shown in Figure 4.5. These data clearly show that *B. pilosicoli* was genetically distinct from all other species of *Brachyspira* that have currently been sequenced. As illustrated in Figures 4.4 and 4.5, *B. hyodysenteriae* and the "Other" species of *Brachyspira* share a significant proportion of genes, although *B. hyodysenteriae* has its own, smaller set of unique genes. Conversely, *B. pilosicoli* only shares a small number of genes with the "Other" *Brachyspira* species and *B. hyodysenteriae* yet has a large proportion of unique genes.

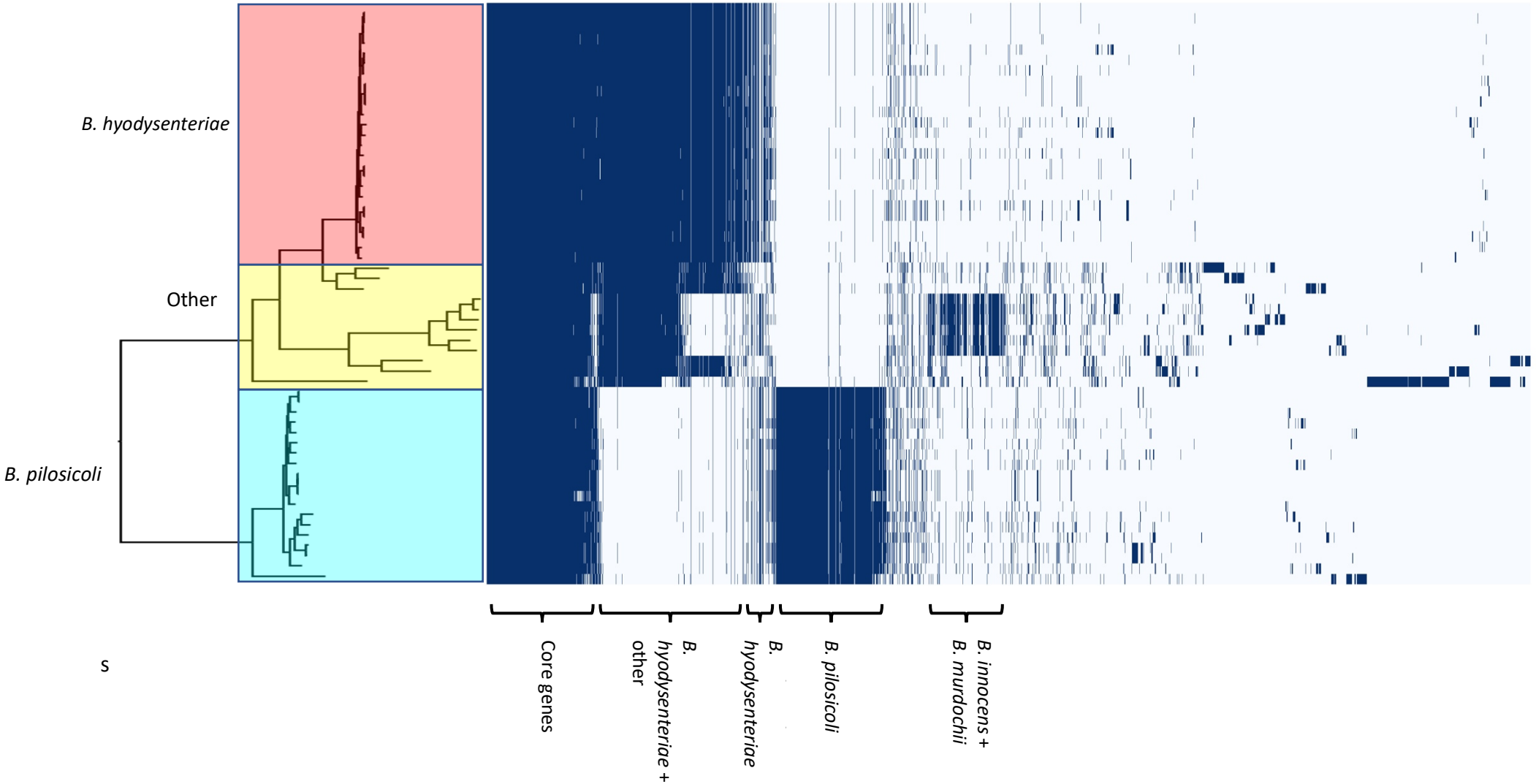


Figure 4. 5 Identification of genes present in the *Brachyspira* pangenome. The genomes were ordered according to the SNP phylogenetic tree, with dark blue indicating the presence of a gene and white indicating the absence of a gene. Genes were firstly ordered according to the core genes, followed by the genes shared between *B. hyodysenteriae* (red) and the "Other" *Brachyspira* species (yellow), genes present in only *B. hyodysenteriae* isolates and genes present only in *B. pilosicoli* isolates (blue). A small set of genes also appeared to be specific to the non-pathogenic species *B. innocens* and *B. intermedia*. The overview of genes was identified using Roary, based on an 80% BLAST identity cut-off, with paralog clustering enabled. These data highlight that *B. pilosicoli* is evolutionarily divergent from other species of *Brachyspira* and that it has its own unique accessory genome, sharing very few genes with other species of *Brachyspira. B. hyodysenteriae* and the "Other" *Brachyspira* species are more closely related to one another than to *B. pilosicoli*.

As a result of the diversity observed within *B. pilosicoli* isolates, analysis was conducted to investigate the pangenome of this species, and that of *B. hyodysenteriae* as a comparison. *B. pilosicoli* and *B. hyodysenteriae* had a similar pangenome size, with *B. pilosicoli* consisting of 3705 genes and *B. hyodysenteriae* consisting of 3401 genes, as illustrated in Figure 4.6. However, *B. pilosicoli* core genes (95-100% of genomes contained these genes) represented only 47% of the pangenome, with 1749 genes, whereas *B. hyodysenteriae* core genes represented 70% of the pangenome, with 2385 genes. *B. pilosicoli* also have a larger proportion of cloud genes (15-95% of genomes contained these genes) and shell genes (less than 15% of genomes contained these genes) compared to *B. hyodysenteriae*, with 944 cloud genes compared to 632 genes, respectively and 1012 shell genes compared to 384 genes, respectively.

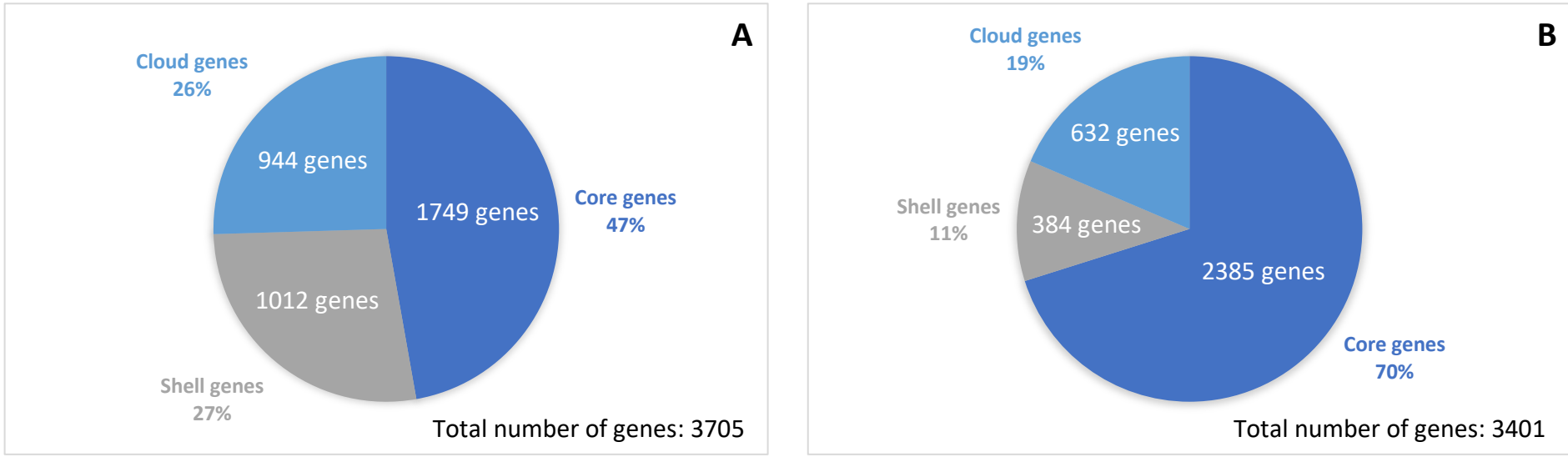


Figure 4. 6 Pangenome analysis of **(A)** *B. pilosicoli* and **(B)** *B. hyodysenteriae*. The genes were identified using Roary with an 80% identity cut-off, with paralog clustering enabled. The size of the pangenomes were similar when comparing *B. pilosicoli* and *B. hyodysenteriae*, however the core genome (genes present in 95-100% of the genome sequences) was distinctly different between the two species. *B. pilosicoli* had a much larger accessory genome, consisting of cloud genes (genes present in 15-95% of the genome sequences) and shell genes (genes present in less than 15% of the genome sequences), when compared to *B. hyodysenteriae.*

4.2.3 Distribution of chemotaxis genes in *Brachyspira* species

An initial analysis of the annotation of the genes showing differential distribution in the three groups suggested an overrepresentation of genes associated with motility and chemotaxis. Therefore, these genes were further investigated using a comparative genomics approach.

Motility and chemotaxis are key virulence factors for *Brachyspira* and contribute to the pathogenicity associated with this genus, as discussed in Chapter 1. Therefore, the hypothesis was that proteins such as methyl-accepting chemotaxis proteins (MCP) and chemotaxis proteins (Che), involved in directed motility and chemotaxis towards specific chemical stimuli differ between species of *Brachyspira*, which may make an important contribution to the ability to infect different hosts and host tissues.

The different MCP and Che proteins annotated within the genome sequences of isolates selected for analysis are shown in Figure 4.7. There were a high number of MCP genes present within the *Brachyspira* pangenome compared to other gastrointestinal pathogens such as *E. coli* (which has four well characterised MCPs) and *Campylobacter* (which has at least ten MCPs) (Liu and Parales, 2008; Li *et al.*, 2014). Approximately fifteen MCP genes were found uniquely in *B. pilosicoli* and approximately forty different MCP proteins were present within the *B. hyodysenteriae* pangenome, with many of these were shared with other species of *Brachyspira*, but not with *B. pilosicoli*. Therefore, *B. pilosicoli* appeared to have its own set of unique MCP genes which made this species distinct from other *Brachyspira* species. Additionally, Figure 4.7 suggested that there may be some MCPs that were predominantly present in *B. innocens* isolates, however more genome sequences would need to be analysed in order to strengthen these data as this was based on only four genome sequences.

In contrast, the chemotaxis protein machinery appeared to be well conserved between all *Brachyspira* species whereby all of the key *che* genes, for example *cheA*, *cheY* and *cheX*, were

present in multiple copies and were mostly shared between species, although *B. pilosicoli* was missing some copies of genes.

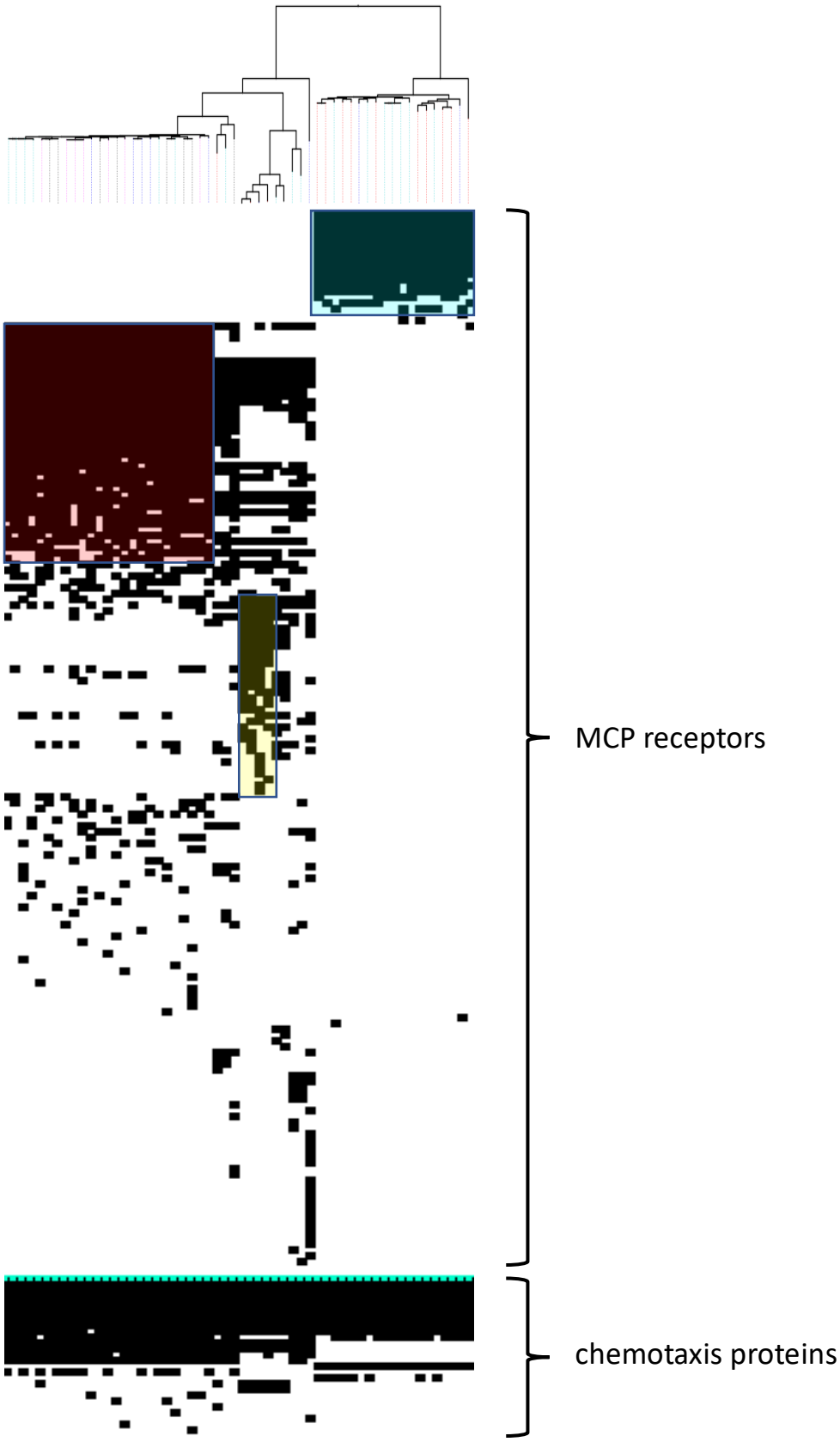


Figure 4. 7 Distribution of methyl-accepting proteins (MCPs) and chemotaxis proteins. The genes encoding these proteins were identified from the Roary analysis with an 80% cut-off identity with paralogue clustering enabled. The red highlights the genes present in *B. hyodysenteriae*, the blue highlights the genes present in *B. pilosicoli* and the yellow highlights the genes present in *B. innocens* (although a small number of genomes were used). There were distinct groups of MCPs unique to *B. pilosicoli*, however *B. hyodysenteriae* appeared to share some of the same MCPs with other species of *Brachyspira.* The chemotaxis proteins were similar within the genus, with *B. pilosicoli* and the "Other" species of *Brachyspira* appearing to have few copies of genes compared to *B. hyodysenteriae.*

As a result of discovering the distinct sets of MCPs in the genus a more detailed investigation of DNA sequences was conducted. *B. pilosicoli* B2904 and *B. hyodysenteriae* WA1 genomes were downloaded from Genbank as representative genomes and a database of all of the MCP genes was created. *B. pilosicoli* B2904 contained fifteen MCP genes and *B. hyodysenteriae* WA1 contained thirty-nine genes. This database was created in ABRicate (https://github.com/tseemann/abricate) and all available *B. pilosicoli* and *B. hyodysenteriae* genome sequences were screened for the fifty-four MCP genes in that database. In the interest of comparing pathogenic species with non-pathogenic species of *Brachyspira* to investigate if MCPs may relate to pathogenicity, *B. innocens* and *B. murdochii* sequences were also screened for the presence of these MCP genes as this may potentially provide an insight in to genes that may contribute to pathogenicity.

The analysis presented in Figure 4.8, highlighted, once again how distinct the presence/absence of MCP genes was between different species. For example, *B. pilosicoli* did not share any MCP genes with either *B. hyodysenteriae*, *B. innocens* or *B. murdochii*. Interestingly, the fifteen MCP genes are shared between all *B. pilosicoli* in the analysis, except *B. pilosicoli* SAP 772, which lacked two of the fifteen MCP genes, which matched its position as an outgroup within *B. pilosicoli* in the phylogenetic tree shown in (Figure 3.6). Not all of the *B. hyodysenteriae* isolates contained all thirty-nine MCP genes that were present in *B. hyodysenteriae* WA1, however between thirty-four and thirty-eight genes were found in the *B. hyodysenteriae* isolates analysed. Interestingly, *B. innocens* and *B. murdochii* shared between thirteen and sixteen of these genes with *B. hyodysenteriae* but none with *B. pilosicoli*, despite occupying some of the same hosts. The MCP genes identified in the *B. innocens* and *B. murdochii* genome analysis highlighted that MCP genes are shared by these

non-pathogenic species, but also by *B. hyodysenteriae,* therefore may not directly result in pathogenicity.

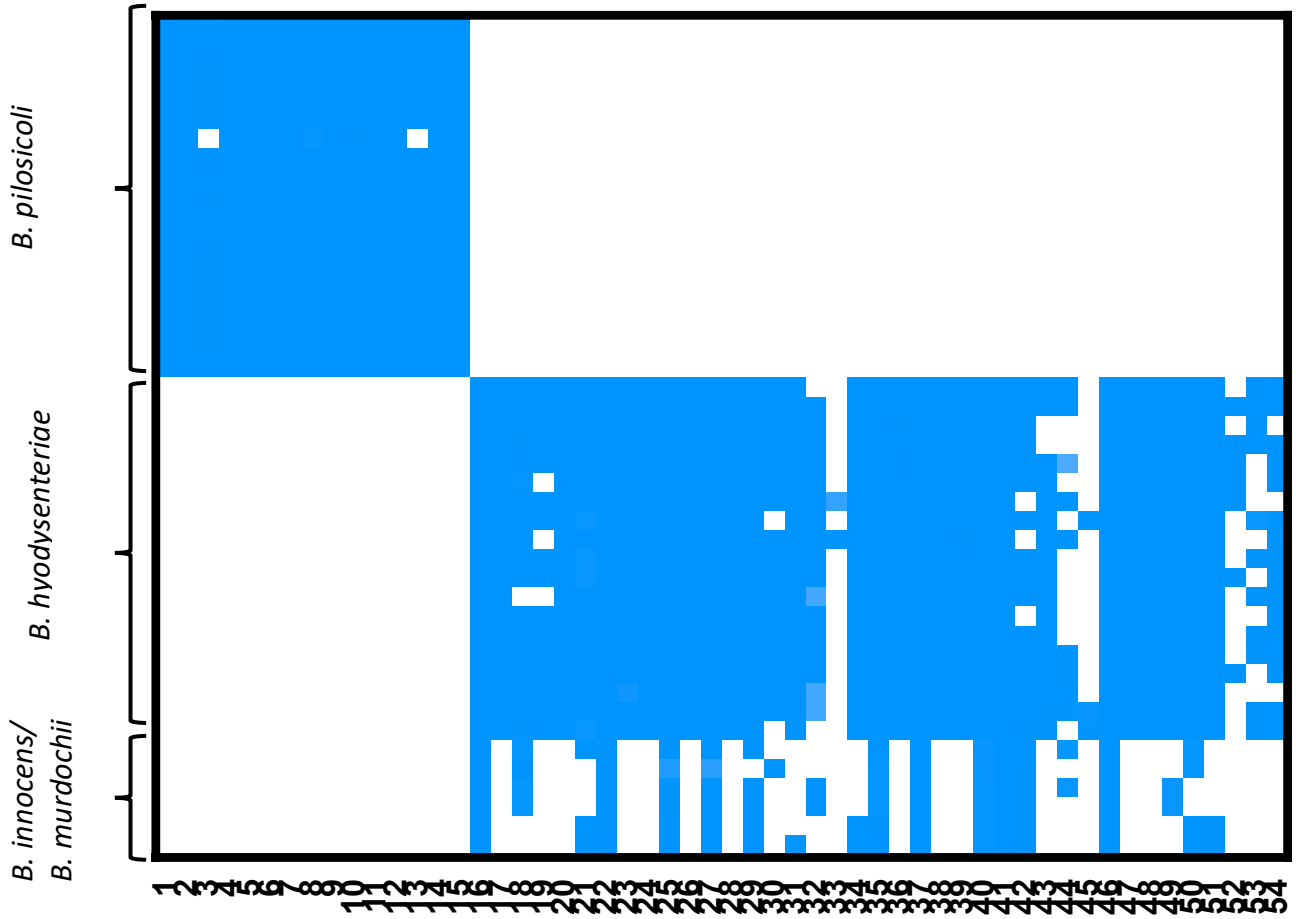


Figure 4. 8 Distribution of the genes encoding methyl-accepting proteins present in *B. pilosicoli*, *B. hyodysenteriae*, *B. innocens* and *B. murdochii* genome sequences. A list of MCP genes was obtained from the complete genome sequences of *B. pilosicoli* B2904 and *B. hyodysenteriae* WA1, which were used as example genomes because they were complete and previous analysis suggested that they contained a high proportion of MCP genes identified during the Roary analysis. An ABRicate database was generated and the genes present in the *B. pilosicoli*, *B. hyodysenteriae*, *B. innocens* and *B. murdochii* were screened against this database using a 75% cut-off. Blue indicates presence of a gene and white indicates absence of a gene. These data demonstrated once again that *B. pilosicoli* had distinct MCP genes from other species of *Brachyspira* and that non-pathogenic species of *Brachyspira* shared some MCP genes with the pathogenic *B. hyodysenteriae* but that the presence of certain MCP genes was unique to the non-pathogenic isolates and could easily be distinguished from *B. hyodysenteriae.*

To explore the sequence similarity between the different MCPs in *B. pilosicoli* and *B. hyodysenteriae,* the sequences were translated into protein sequences and aligned using COBALT (Constraint-based Multiple Alignment Tool) (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi) to investigate areas of sequences homology. The sequences of *B. pilosicoli* MCPs appeared to be highly conserved between alignment position 805 and 871, as shown in red in Figure 4.9. On analysis of this region using the Conserved Domains tool on NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) it appeared that this region was a signalling domain thought to be responsible for transducing a signal to CheA. The areas highlighted in blue are less well conserved sequences, of which there are several, but protein function could not be assigned to these regions using the Conserved Domains database indicating that the MCPs present in *B. pilosicoli* are distinct from one another. Similarly, *B. hyodysenteriae* MCP sequences showed a small region of conserved sequence at the approximate alignment positions of 790 and 803, which were also shared with the sequences of *B. pilosicoli* MCPs (Figure 4.10), although protein function could not be assigned. These data demonstrated that the *Brachyspira* MCPs do have some sequences homology, but that they are also distinct both within a species and between different species in the genus.

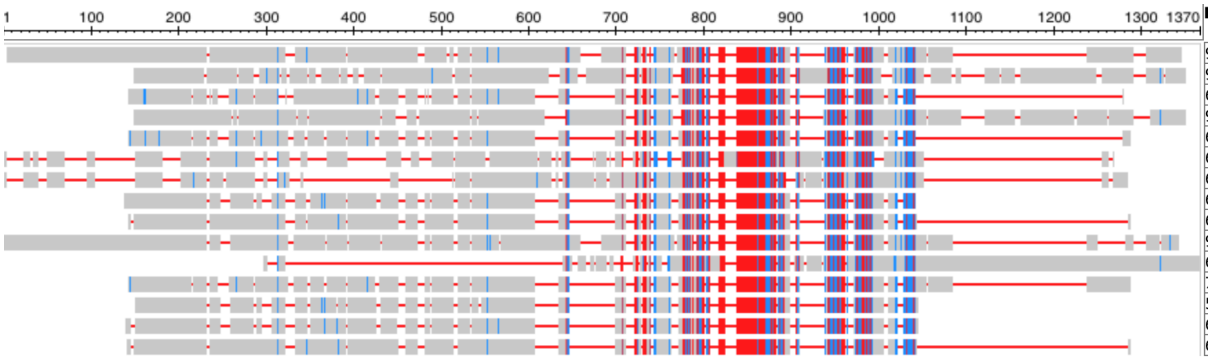


Figure 4. 9 Alignment of fifteen *B. pilosicoli* methyl-accepting protein sequences. The alignment was performed using COBALT whereby red indicated areas of highly conserved residues, blue indicated areas of less conserved residues and grey indicated regions with no homology or gaps in the protein sequence. The horizontal scale represents the amino acid alignment position. *B. pilosicoli* MCPs showed significant homology in residues between alignment position 805 and 871, however there were less homologous residues which make these MCPs distinct from one another.

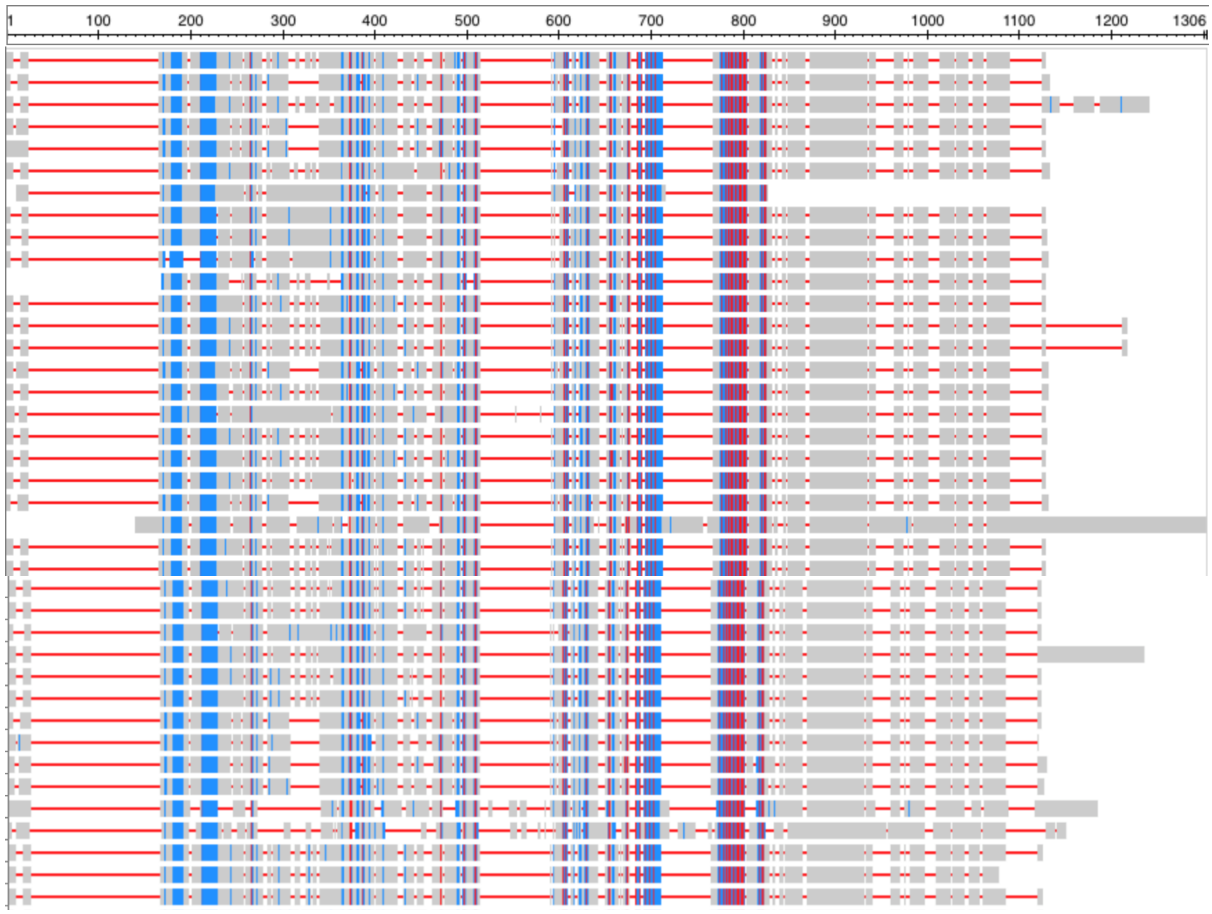


Figure 4. 10 Alignment of thirty-nine *B. hyodysenteriae* methyl-accepting protein sequences. The alignment was performed using COBALT whereby the red indicated areas of highly conserved residues, blue indicated areas of less conserved residues and grey indicated regions of no homology or gaps in the protein sequence. The horizontal scale represents the amino acid alignment position. *B. hyodysenteriae* MCPs showed significant homology in residues between alignment position 790 and 803, however had more regions with low homology, suggesting there was diversity in the MCPs.

4.3 Discussion

The studies presented in this chapter have used a comparative genomics approach to compare all available *Brachyspira* whole genome sequences, including those available on Genbank and those generated in this thesis. The data presented builds on other comparative genomics studies that make genetic comparisons within one species of *Brachyspira* (Bellgard *et al.*, 2009; Mappley *et al.*, 2012) or interspecies comparisons with a small number of different *Brachyspira* species (Wanchanthuek *et al.*, 2010; Håfström *et al.,* 2011). The strength of the comparisons made in this chapter arises from having fifteen novel *B. pilosicoli* genome sequences available for analysis. Furthermore, the studies presented here have provided additional genome sequences for *B. intermedia*, *B. innocens* and *B. murdochii*. This overall contributed significantly more data to the limited data currently available in the public domain.

Genome sizes of *Brachyspira* isolates varied depending on the species of *Brachyspira*. *B. pilosicoli* had the smallest genome size and yet is able to infect the largest host range. This had previously been proposed to be as a result of *B. pilosicoli* undergoing a high degree of reductive evolution (Håfström *et al.* 2011; Mappley *et al.*, 2012) and thus it has been suggested that *B. pilosicoli* may be an older pathogen than other *Brachyspira* species such as *B. hyodysenteriae* (Håfström *et al.,* 2011). Reductive genome evolution may have reduced the gene redundancy observed in species such as *B. hyodysenteriae*, furthermore the selection of different gene sets may have resulted in the ability to more effectively colonise the desired niche. For example, *B. pilosicoli* and *B. aalborgi* have the unique ability to form end on attachments to enterocytes which may enhance the colonisation and pathogen survival in the large intestine. Furthermore, other spirochaete species such as *Leptospira* have undergone net loss of genes during evolutionary divergence to become intermediate and

pathogenic species (Xu *et al.*, 2016). The genetic divergence of *B. pilosicoli* may have been as result of a similar process, thus suggesting that gene loss is a result of spirochaetes adapting to their ecological niche and may have contributed to the reduced genome size of *B. pilosicoli*. In addition to a reduction in genome size, the selection of differing gene sets is likely to have occurred to contribute to the genetic diversity of *B. pilosicoli*, as exemplified by the unique set of MCP genes.

It is likely that *Brachyspira* species originally infected multiple hosts, but that *B. hyodysenteriae* later specialised to colonise the porcine host. Despite *B. hyodysenteriae* having a larger genome size compared to *B. pilosicoli*, it is smaller than other *Brachyspira* species capable of infecting multiple hosts, such as *B. alvinipulli* and *B. intermedia*. This could be as a result of host specialisation and subsequent loss of genes that were not required for the colonisation of the porcine gastrointestinal tract.

A pangenome analysis of *Brachyspira* species revealed that the core genome contained approximately 1089 genes; a previous study, comparing *B. pilosicoli* 95/1000, *B. murdochii* 56-160^{T} and *B. hyodysenteriae* WA1 reported a core genome of 1087 genes, thus validating the data presented here (Wanchanthuek *et al.*, 2010). Interestingly, in these studies, *B. pilosicoli* shared relatively few genes with *B. hyodysenteriae* (35 genes) and the "Other" *Brachyspira* species (210 genes), further supporting the evolutionary divergence of this species. Another study suggested that *B. pilosicoli* and *B. hyodysenteriae* shared approximately 592 genes of their accessory genome (Wanchanthuek *et al.*, 2010). However, this was limited to the comparison of one genome from each species thus giving an inaccurate representation and demonstrating the importance of analysing a larger number of genome sequences for accurate interpretation of pangenome analyses. In the data presented here, *B. hyodysenteriae* and the "Other" species of *Brachyspira* shared 1329 genes, suggesting that

these species are more closely related to one another than *B. pilosicoli*. This concurred with previous reports that *B. hyodysenteriae* was more closely related to *B. intermedia* and *B. murdochii* (Håfström *et al.,* 2011).

On closer analysis of the individual *B. pilosicoli* and *B. hyodysenteriae* pangenomes, it was evident that *B. hyodysenteriae* had a larger core genome, with 70% of the pangenome consisting of core genes. This is likely to be associated with this species being host restricted and infecting only the porcine host (Harris *et al.*, 1972; Hampson *et al.,* 2006). *B. pilosicoli* had a smaller core genome, with 47% of the pangenome consisting of core genes and 53% consisting of accessory genes. This may be as a result of infecting a wider host range and therefore being exposed to a wider gene pool which could be exchanged with *B. pilosicoli* through horizontal gene transfer or bacteriophage infection for example. Furthermore, a large accessory genome may aid in adaptation and colonisation of different hosts.

B. pilosicoli had previously been reported to have a core genome of 2132 gene, based on the analysis of three genome sequences (Mappley *et al.*, 2012). The studies presented here indicated a slightly smaller core genome of 1749 genes, from the analysis of nineteen genomes. This relatively small proportion of core genes, in addition to allowing the infection of multiple hosts, may also be responsible for the varied disease status and pathology associated with both natural and experimental infections with *B. pilosicoli* (Mappley *et al.,* 2014).

Comparisons of the *Brachyspira* genome sequences revealed significant differences in the genes responsible for coding methyl-accepting proteins (MCPs), however chemotaxis proteins (Che) were generally well conserved. Thus, suggesting that the MCPs responsible for detecting different chemotactic signals differ between species. However, the process of directed motility using chemotaxis machinery (Che proteins) is similar in all species within the

genus. MCPs are essential for *Brachyspira* responses to chemotactic signals to facilitate directed movement of the bacterial cells. In order to colonise the large intestine, *Brachyspira* must gain close proximity to the intestinal epithelium, facilitated by chemotaxis to compounds such as mucins (Naresh and Hampson, 2010). There were distinct differences in both the number of MCPs and also the distribution between different species of *Brachyspira*. *B. pilosicoli* had a small number of MCPs (approximately fifteen) compared to *B. hyodysenteriae* (between forty and fifty). As previously mentioned, *B. pilosicoli* have the ability to attach to the enterocytes to form a pseudo-brush border, whereas *B. hyodysenteriae* colonise the crypts of the enterocytes, but also the lumen of the colon, which may result in having to detect an increased number of chemotactic signals, hence the large number of MCPs. Interestingly, *B. pilosicoli* did not share any MCPs with any other species of *Brachyspira* investigated. Further studies with *B. aalborgi* may be able to investigate whether these MCPs are specific to *B. pilosicoli* or if they are potentially specific to *Brachyspira* species that can attach to epithelial cells, or indeed those that can infect humans. Conversely, *B. hyodysenteriae* shared a number of MCPs with the "Other" *Brachyspira* species, in addition to having a unique set of MCPs. This suggests further evidence for *B. pilosicoli* being more evolutionarily divergent for other species in the genus and highlights some key differences in key *Brachyspira* virulence factors (Bellgard *et al.*, 2009; Wanchanthuek *et al.*, 2010). Previous comparisons of *Brachyspira* species revealed that *B. pilosicoli* genes code for McpA and McpB, but not for McpC, despite McpC being detected in the genomes of other *Brachyspira* species. McpA is responsible for taxis towards glucose, α methyl glucose, McpB is responsible for taxis towards asparagine, aspartate, glutamine and histidine and McpC is responsible to taxis towards cysteine, proline, threonine, glycine, serine, lysine, valine and arginine. This may

result in *B. pilosicoli* not being chemotactic to amino acids such as serine which are known to be potent chemoattractants for other species such as *B. hyodysenteriae* (Muller *et al.*, 1997). The large number of MCPs present in the *B. hyodysenteriae* pangenome analysis may be associated with the need to transduce multiple chemotactic signals in the ever-changing environment of the colonic lumen. Furthermore, a large number of genes could suggest redundancy in the system, whereby *Brachyspira* and other spirochaetes can rapidly adapt to their changing environment. Other spirochaetes such as *T. pallidum* and *B. burgdorferi* also possess multiple MCPs, suggesting it is commonplace amongst spirochaetes (Bellgard *et al.*, 2009).

A comparison was made between *B. pilosicoli, B. hyodysenteriae* and the non-pathogenic species *B. innocens* and *B. murdochii* in order to compare the presence/absence of MCP genes. This may begin to elucidate if there is an association between MCP genes and pathogenicity. It was evident that although *B. innocens* and *B. murdochii* share some MCP genes with *B. hyodysenteriae*, many present in this pathogen were absent in the non-pathogenic species. This could suggest that MCPs are related to pathogenicity, with certain MCPs facilitating chemotactic responses to allow *B. hyodysenteriae* to effectively colonise its ecological niche and subsequently cause disease. It could be likely that this chemotactic response is not present in non-pathogenic species of *Brachyspira* due to the absence of specific MCPs, which prevents disease following infection. In previous studies, *B. hyodysenteriae* have been shown to be chemotactic towards a variety of mucin components, likely to be due to the large number of genes encoding MCPs (Kennedy and Yancey, 1996), however no such studies have been conducted with non-pathogenic species of *Brachyspira*. Therefore, the next logical step to further investigating if MCPs contribute to the

pathogenicity of different *Brachyspira* species would be to investigate chemotaxis of *B. innocens* and *B. murdochii in vitro*.

Overall, this chapter provides further evidence that *B. pilosicoli* is evolutionarily distinct from other *Brachyspira* species. The process of reductive evolution may have resulted in a smaller genome size following the ability to infect a wide range of hosts. This may have resulted in the loss of redundant genes and the retaining of only genes essential for survival and pathogenesis. Pangenome analysis suggested that *B. pilosicoli* shared few genes with other species of *Brachyspira*, suggesting early evolutionary divergence from other species in the genus. *B. hyodysenteriae* are more closely related to the other species of *Brachyspira* and shared similar MCPs which were distinct from *B. pilosicoli*. Additionally, these studies provided evidence that MCPs contribute to pathogenicity as non-pathogenic species shared some, but not all MCPs with *B. hyodysenteriae*, suggesting that these MCPs may be essential for chemotaxis in these species, but may not be directly related to pathogenicity. These studies were a starting point for the investigation of chemotaxis genes in *Brachyspira* species, however further *in vitro* and *in silico* studies will be required to fully elucidate their roles in the pathobiology of *Brachyspira*.

Chapter 5: The characterisation of *Lactobacillus* species as potential probiotic candidates for the control of avian intestinal spirochaetosis

5.1 Introduction

Since the ban of antibiotic growth promoters in livestock in the EU in 2006, the necessity for alternatives has become of paramount importance, *Lactobacillus* probiotics are one such alternative. Probiotics are defined as "live micro-organisms that confer health benefits to the host when administered in adequate quantities" (FAO/WHO, 2002) and there is increasing evidence that probiotics can promote good gut health based on the principle that a healthy intestinal microbiota protects against disease (Rantala and Nurmi, 1973; Fuller, 1989; Collins *et al.*, 2009).

It is well documented that probiotic bacteria can inhibit a range of poultry pathogens such as *Campylobacter jejuni, Salmonella* and *E. coli* (La Ragione and Woodward, 2003; Resta-Lenert and Barrett, 2003; Wolfenden *et al.*, 2007; Santini *et al.*, 2010; Olnood *et al.*, 2015). More recently, studies have demonstrated the potential for *Lactobacillus* isolates to antagonise *Brachyspira* both *in vitro* and *in vivo* (Mappley *et al.*, 2011; Mappley *et al.,* 2013), however the exact mechanisms of action have yet to be elucidated.

Therefore, this chapter aimed to isolate and characterise a number of avian *Lactobacillus* isolates as potential probiotics for the control of avian intestinal spirochaetosis. A range of appropriate tools were utilised to characterise these isolates in accordance to The European Food Safety Authority guidelines (EFSA *et al.*, 2017). These included antimicrobial resistance testing, bile and acid tolerance, 16S rRNA sequencing and whole genome sequencing to phenotypically and genotypically characterise the panel of potential probiotics. This ensured that any probiotics met the safety criteria for use in animal nutrition. Furthermore, these *Lactobacillus* isolates were tested to ascertain the inhibitory potential against a panel of

Brachyspira isolates, with the intention of selecting potential probiotic candidates for further investigation in Chapters 6 and 7.

Therefore, the aims and objectives of this chapter were:

- To isolate a panel of *Lactobacillus* isolates from the faeces of healthy, free-range chickens.
- To create phenotypic and genotypic profiles of each isolate in accordance to the EFSA guidelines to ensure selected probiotic candidates would be suitable as commercial animal feed supplements.
- To determine the inhibitory ability of each *Lactobacillus* isolate against the panel of eight *Brachyspira* isolates characterised in Chapter 3.
- To select an appropriate number of probiotic candidates suitable for further study.

5.2 Results

5.2.1 Isolation and identification of potential probiotics

A total of fifty lactic acid bacteria were isolated from healthy free-range laying hens. These fifty isolates were initially subjected to Gram stains, catalase tests and each isolate was cultured on MRS agar under both aerobic and anaerobic conditions, and in broth at 37°C to ensure sufficient growth for subsequent experiments. Of the fifty isolates tested, seventeen were catalase negative, Gram positive rods with a strong growth phenotype under both aerobic and anaerobic conditions, on both agar and in broth. Therefore, these seventeen isolates, shown in (Table 5.1) were further characterised as potential probiotics because of their presumptive *Lactobacillus* identification and their robustness and versatility as indicated by the growth studies.

5.2.2 Genotypic characterisation of *Lactobacillus* isolates

These seventeen presumptive *Lactobacillus* isolates were initially speciated using 16S rRNA sequencing (Table 5.1). It was noted that the majority of isolates identified were either *L. crispatus*, *L. reuteri* or *L. salivarius*; the most abundant *Lactobacillus* isolates in the chicken gastrointestinal tract (Abbas Hilmi *et al.*, 2007). Following the identification of these isolates one isolate, *L. agilis* SAP 2014 was removed from further study as it was not an approved species for animal feed supplements according to the EFSA guidelines (European Food Safety Authority, 2015).

Table 5. 1 A summary of the biochemistry, 16S rRNA sequencing and whole genome sequencing results for all *Lactobacillus* isolates characterised in these studies. Isolates were further characterised in accordance to probiotic guidelines. At this early stage of characterisation, eleven isolates met these criteria and were sent for whole genome sequencing to confirm their species and investigate potential for antimicrobial resistance and virulence genes and predict bacteriocin gene clusters.

***Lactobacillus* Species**	**Isolate Name**	**Catalase**	**16S rRNA sequencing identification**	**Whole Genome Sequencing identification**
L. agilis	SAP 2104	-	*L. agilis*	-
L. crispatus	SAP 2105	-	*L. crispatus*	*L. crispatus*
L. crispatus	SAP 2106	-	*L. crispatus*	*L. crispatus*
L. crispatus	SAP 2107	-	*L. crispatus*	*L. crispatus*
L. crispatus	SAP 2109	-	*L. crispatus*	-
L. crispatus	SAP 2110	-	*L. crispatus*	-
L. crispatus	SAP 2111	-	*L. crispatus*	-
L. mucosae	SAP 2102	-	*L. mucosae*	-
L. reuteri	SAP 2108	-	*L. reuteri*	*L. reuteri*
L. reuteri	SAP 2114	-	*L. reuteri*	*L. reuteri*
L. reuteri	SAP 2115	-	*L. reuteri*	*L. reuteri*
L. salivarius	SAP 2103	-	*L. salivarius*	*L. salivarius*
L. salivarius	SAP 2112	-	*L. salivarius*	-
L. salivarius	SAP 2113	-	*L. salivarius*	*L. salivarius*
L. salivarius	SAP 2116	-	*L. salivarius*	*L. salivarius*
L. salivarius	SAP 2117	-	*L. salivarius*	*L. salivarius*
L. salivarius	SAP 2118	-	*L. salivarius*	Poor sequence

5.2.2.1 Whole genome sequencing for characterisation of potential probiotics

The most recent edition of the EFSA guidelines stipulated that whole genome sequencing should be used for the 'unequivocal taxonomic identification of the strain, as well as for characterisation of the strain regarding their potential "functional traits of concern" (EFSA *et al.*, 2017). Therefore, whole genome sequencing was employed to confirm the identity of each *Lactobacillus* isolate and interrogate the genomes for possible traits of concern. Five isolates *L. crispatus* SAP 2109, 2110 2111, *L. mucosae* SAP 2102 and *L. salivarius* SAP 2112 were not sent for sequencing due to poor quality and yield of DNA following multiple attempts at DNA extraction. Hence, these five isolates were subsequently removed from further studies. The eleven remaining isolates were sent for whole genome sequencing using Illimina MiSeq or HighSeq technology, conducted at MicrobesNG, University of Birmingham. A summary of the sequence data is shown in Table 5.2.

The whole genome sequences were processed using the same methods illustrated in Figure 3.5. The only difference was that the 2x250bp paired end sequence reads were processed and assembled into contiguous sequences using the SPAdes 3.10 genome assembler (Bankevich *et al.*, 2012). Following assembly, Quast v2 analysis (Gurevich *et al.*, 2013) was used to ensure the sequences were of sufficient quality for further analysis, as shown in Table 5.2. All of these sequences, except for *L. salivarius* SAP 2118 (which was subsequently removed) were of sufficient quality to be annotated using Prokka, subsequent phylogenetic analysis was conducted using the core genome SNPs to speciate each of the potential probiotic candidates presented in Table 5.1. It was worth noting that the results from the 16S rRNA sequencing were concurrent with the identification of all isolates using whole genome sequencing.

Lactobacillus genomes ranged from 2.0-2.2 Mbp in size, with a GC content ranging from 32.5-38.6%, the *L. reuteri* isolates had the smallest genomes and the highest GC content. The number of coding sequences and RNAs were similar amongst all isolates and are representative of the *L. crispatus, L. reuteri* and *L. salivarius* species (Table 5.2) (Frese *et al*., 2011; Lee *et al.,* 2017; Ojala et al., 2014).

Table 5. 2 The size of the genomes in base pairs (bp), GC content (%), number of coding sequences, number of RNAs, number of contigs, largest contig, the N50 value and the L50 value for each of the *Lactobacillus* genomes. Data for the assembly statistics were generated from Prokka and the data for the assembly quality were generated from Quast.

***Lactobacillus* Isolate**	**Size (bp)**	**GC content (%)**	**Number of coding sequences**	**Number of RNAs**	**Number of Contigs**	**Largest Contig**	**N50***	**L50****
***L. crispatus* SAP 2105**	2110158	32.62	2042	86	36	345434	137660	5
***L. crispatus* SAP 2106**	2023727	36.83	2025	73	36	554180	131824	4
***L. crispatus* SAP 2107**	2228254	36.68	2227	73	40	554181	140039	5
***L. reuteri* SAP 2108**	2042049	38.57	2014	74	81	144585	68674	11
***L. reuteri* SAP 2114**	2040448	38.57	2008	73	73	121930	68674	11
***L. reuteri* SAP 2115**	2054410	38.58	2020	89	72	122758	68766	11
***L. salivarius* SAP 2103**	2110631	32.62	2042	86	38	400788	137103	5
***L. salivarius* SAP 2113**	2108064	32.59	2047	80	37	359028	157700	5
***L. salivarius* SAP 2116**	2113188	32.63	2043	81	51	359220	137658	5
***L. salivarius* SAP 2117**	2111031	32.61	2045	80	35	358986	137658	5

* N50 value is defined as the minimum contig length required to cover 50% of the genome, i.e. half of the contigs are equal to or larger than the N50 contig size. The larger the N50 value, the higher the quality of the assembly.
** L50 value is defined as the number of contigs required to account for 50% of the genome. The lower the L50 value, the higher the quality of the assembly.

Following assembly and annotation, additional screening of the *Lactobacillus* genomes was undertaken to identify any potential virulence factors, plasmids and antimicrobial resistance genes that may have been present in these genomes. This was undertaken using ABRicate software which used a combination of databases, including CARD, ARG-ANNOT, Resfinder, the virulence factor database and Plasmidfinder to identify any of these traits that may make them unsuitable as probiotics, as seen in Table 5.3 (https://github.com/tseemann/abricate). Of the ten *Lactobacillus* isolates, 50% carried the *tetM* and *ermB* genes, which may confer resistance to tetracycline and macrolide antibiotics such as erythromycin and 20% of the isolates carried the *tetW* gene, which may also confer resistance to tetracycline. 30% of the isolates, all of which were *L. reuteri,* carried no resistance genes. None of the isolates characterised harboured any potential known virulence genes or plasmids which may have deemed them unsuitable for probiotic use. Overall, it was clear that *L. reuteri* isolates were more suitable as potential probiotic candidates due to absence of any antimicrobial resistance genes, plasmids or virulence factors. All *L. salivarius* isolates demonstrated the presence of genes conferring resistance to tetracycline and macrolide antibiotics, which needed to be tested in order to confirm the phenotype.

Additional online tools such as Bagel 4 (http://bagel4.molgenrug.nl/) and Anti-SMASH (https://antismash.secondarymetabolites.org/#!/start) were utilised to predict potential bacteriocin and secondary metabolite gene clusters present in the genome sequences. The secretion of these inhibitory metabolites may contribute to the inhibition of intestinal pathogens such as *Brachyspira* and thus enhance the probiotic action of the *Lactobacillus* isolates. These tools were used in combination to enable the identification of all known potential bacteriocin gene clusters and protein sequences, these were then screened using BLAST, to confirm their identification.

Table 5. 3 Summary of results generated by ABRicate to investigate the potential antimicrobial resistance genes, virulence genes and plasmids present in all of the *Lactobacillus* genomes. These data show that 70% of the *Lactobacillus* isolate harbour antimicrobial resistance genes that confer resistance to tetracycline or macrolide antibiotics, or a combination of both. The *L. reuteri* isolates did not appear to have any antimicrobial resistance genes, potentially making them more suitable probiotics.

Species	Isolate Name	Predicted antimicrobial resistance genes [a]	Predicted antibiotic resistance	Virulence genes [b]	Plasmids [c]
L. salivarius	SAP 2103	*tetM, ermB*	Tetracycline, Macrolide	None	None
L. crispatus	SAP 2105	*tetM, ermB*	Tetracycline, Macrolide	None	None
L. crispatus	SAP 2106	*tetW*	Tetracycline	None	None
L. crispatus	SAP 2107	*tetW*	Tetracycline	None	None
L. reuteri	SAP 2108	None	None	None	None
L. salivarius	SAP 2113	*tetM, ermB*	Tetracycline, Macrolide	None	None
L. reuteri	SAP 2114	None	None	None	None
L. reuteri	SAP 2115	None	None	None	None
L. salivarius	SAP 2116	*tetM, ermB*	Tetracycline, Macrolide	None	None
L. salivarius	SAP 2117	*tetM, ermB*	Tetracycline, Macrolide	None	None

a AMR genes were predicted using ABRicate which used the CARD, ARG-ANNOT and Resfinder databases to predict AMR genes.
b Virulence genes were predicted using ABRicate which used the Virulence Factor Database (VFBD) to known predict virulence factors.
c Plasmids were predicted using ABRicate which used the PlasmidFinder database to predict known plasmids.

Anti-SMASH produced limited results, as shown in Table 5.4. This database was only able to detect the presence of potential salivaricin gene clusters in *L. salivarius* isolates. Salivaricin is a class I bacteriocin and is classified as a lantibiotic with bactericidal properties (Barbour *et al.*, 2016). Class I bacteriocins are small, often heat stable peptides that contain the non-proteogenic amino acids lanthionine and β-methyllanthionine, which form covalent bridges between amino acids resulting in internal ring motifs.

Table 5. 4 Summary of the secondary metabolite gene clusters predicted by Anti-SMASH. This database was able to predict the presence of salivaricin gene clusters in *L. salivarius* genome sequences but did not predict any other gene clusters in other species of *Lactobacillus*.

Species	Isolate Name	Predicted secondary metabolite gene clusters
L. salivarius	SAP 2103	Salivaricin
L. crispatus	SAP 2105	None
L. crispatus	SAP 2106	None
L. crispatus	SAP 2107	None
L. reuteri	SAP 2108	None
L. salivarius	SAP 2113	Salivaricin
L. reuteri	SAP 2114	None
L. reuteri	SAP 2115	None
L. salivarius	SAP 2116	Salivaricin
L. salivarius	SAP 2117	Salivaricin

Bagel 4 identified the presence of predicted enterolysin A and helveticin J bacteriocin gene clusters in addition to an unnamed class II bacteriocin, as shown in Table 5.5. Putative gene clusters for enterolysin A were present in all of the *Lactobacillus* isolates as previously observed by Chung *et al* (2018) and helveticin J gene clusters were identified in all *L. crispatus* isolates. Enterolysin A and helveticin J are class III bacteriocins, these are large (> 30kDa), heat

labile proteins that are subdivided into bacteriolytic enzymes such as enterolysin A which cause lysis of cell walls or non-lytic proteins such as helveticin J (Yang *et al.*, 2014).

Table 5. 5 Summary of the bacteriocin gene clusters predicted by Bagel 4. This database was able to predict class IIc bacteriocins, these bacteriocins are small (5-10 kDa), heat stable peptides which are not subject to extensive post translational modifications. It was also able to detect class III bacteriocins, these are larger (> 30kDa), heat stable proteins which are split into two groups. Group A are bacteriolytic proteins such as enterolysin A and Group B are non-lytic such as helveticin J.

Species	Isolate Name	Predicted bacteriocin gene clusters
L. salivarius	SAP 2103	Enterolysin A Class III Bacteriocin Class II
L. crispatus	SAP 2105	Enterolysin A Class III Bacteriocin Class II
L. crispatus	SAP 2106	Enterolysin A Class III Helveticin J
L. crispatus	SAP 2107	Enterolysin A Class III Helveticin J
L. reuteri	SAP 2108	Enterolysin A Class III
L. salivarius	SAP 2113	Enterolysin A Class III Bacteriocin Class II
L. reuteri	SAP 2114	Enterolysin A Class III
L. reuteri	SAP 2115	Enterolysin A Class III
L. salivarius	SAP 2116	Enterolysin A Class III Bacteriocin Class II
L. salivarius	SAP 2117	Enterolysin A Class III Bacteriocin Class II

Anti-SMASH and Bagel 4 were used together to ensure that all possible putative gene clusters were identified from the *Lactobacillus* genome sequences. It was noted during these analyses that the core protein sequences for salivaricin (produced by Anti-SMASH) and bacteriocin class II (produced by Bagel 4) were 100% homologous (Appendix III), and thus these may be the same genes with a different annotation, as shown in Figure 5.1. Interestingly, Anti-SMASH identified the putative bacteriocin gene as salivaricin (a class I bacteriocin) but Bagel 4

identified it as a class II bacteriocin, indicating the need for more complete bacteriocin databases.

All *Lactobacillus* isolates tested had putative bacteriocin gene clusters present in the genome. The *L. reuteri* isolates only had one predicted cluster for enterolysin A, whereas the *L. salivarius* and *L. crispatus* genomes contained clusters for salivaricin, the class II bacteriocin and helveticin J, respectively. Further studies presented in Chapter 6 explored the inhibition of metabolites in the *Lactobacillus* cell free supernatant to attempt to determine the role of these bacteriocins against *Brachyspira.*

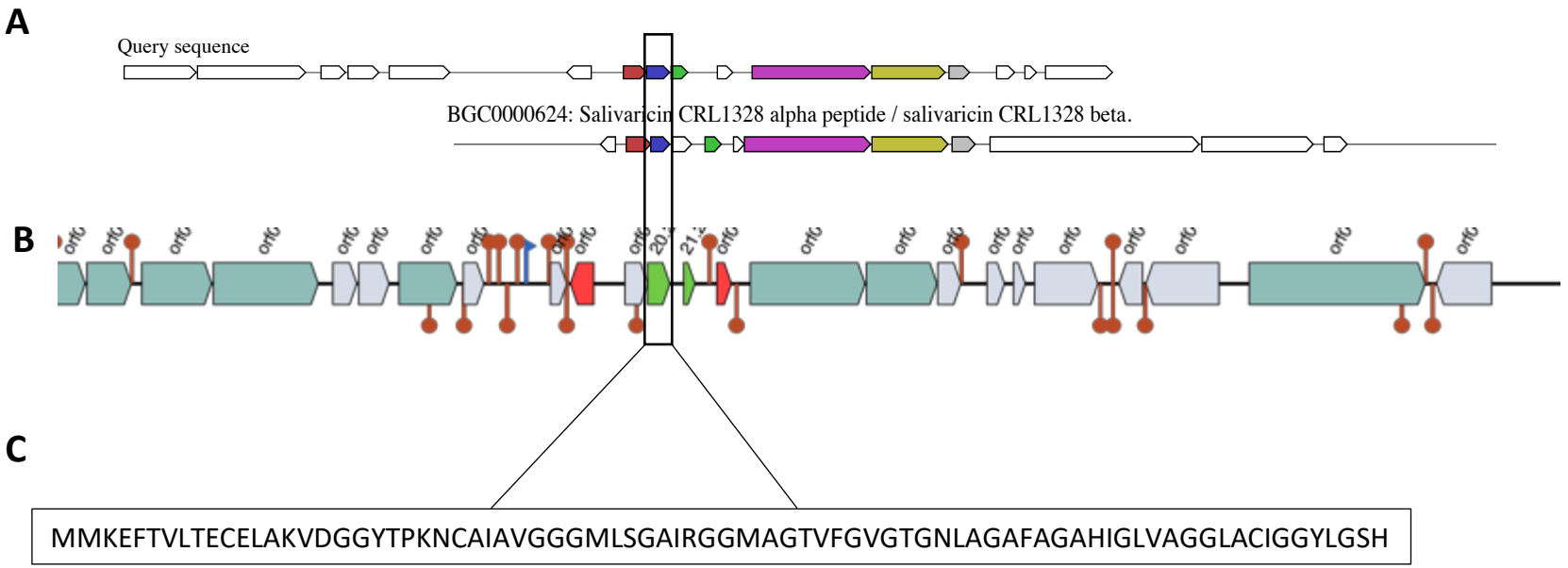


Figure 5. 1 Aligned outputs from Anti-SMASH and Bagel 4 to show a predicted bacteriocin gene clusters for *L. salivarius* SAP 2116. (A) Shows the output from Anti-SMASH, the query sequence is aligned to the known Salivaricin gene cluster which shows that 50% of the genes are similar. (B) Shows the output from Bagel 4 which identified a potential gene cluster for a type II bacteriocin. It is evident from this figure that both Anti-SMASH and Bagel 4 can identify some similar putative gene clusters, however they are annotated differently, which is why it is beneficial to use both pipelines. (C) Shows the protein sequence of the protein identified as the core peptide of the bacteriocin gene cluster. This sequence was highlighted by both Anti-SMASH and Bagel 4 and identified as bacteriocin from *L. salivarius* using BLAST but did not elude to the identification of this bacteriocin.

5.2.2.2 Phylogenetics

Phylogenetics using the whole genome sequences confirmed the identity of the panel of *Lactobacillus* isolates. All sequences from the three species highly abundant in the chicken gastrointestinal tract, *L. crispatus, L. reuteri* and *L. salivarius*, were downloaded from Genbank. Following this, parSNP analysis was employed to analyse the core genome single nucleotide polymorphisms (SNPs) of all available *Lactobacillus* sequences to create the phylogenetic dendrograms seen in Figures 5.2, 5.3 and 5.4.

The phylogenetic dendrograms clearly showed that each of the *Lactobacillus* isolates presented in these studies, belonged to the correct species as identified by the 16S rRNA sequencing. The data showed high levels of genetic similarity within each species from the isolates characterised here, this was as a result of isolates being isolated from one flock of hens. However, these were later tested for their inhibition against *Brachyspira* and each exhibited a different phenotype, thus suggesting that they were not identical isolates.

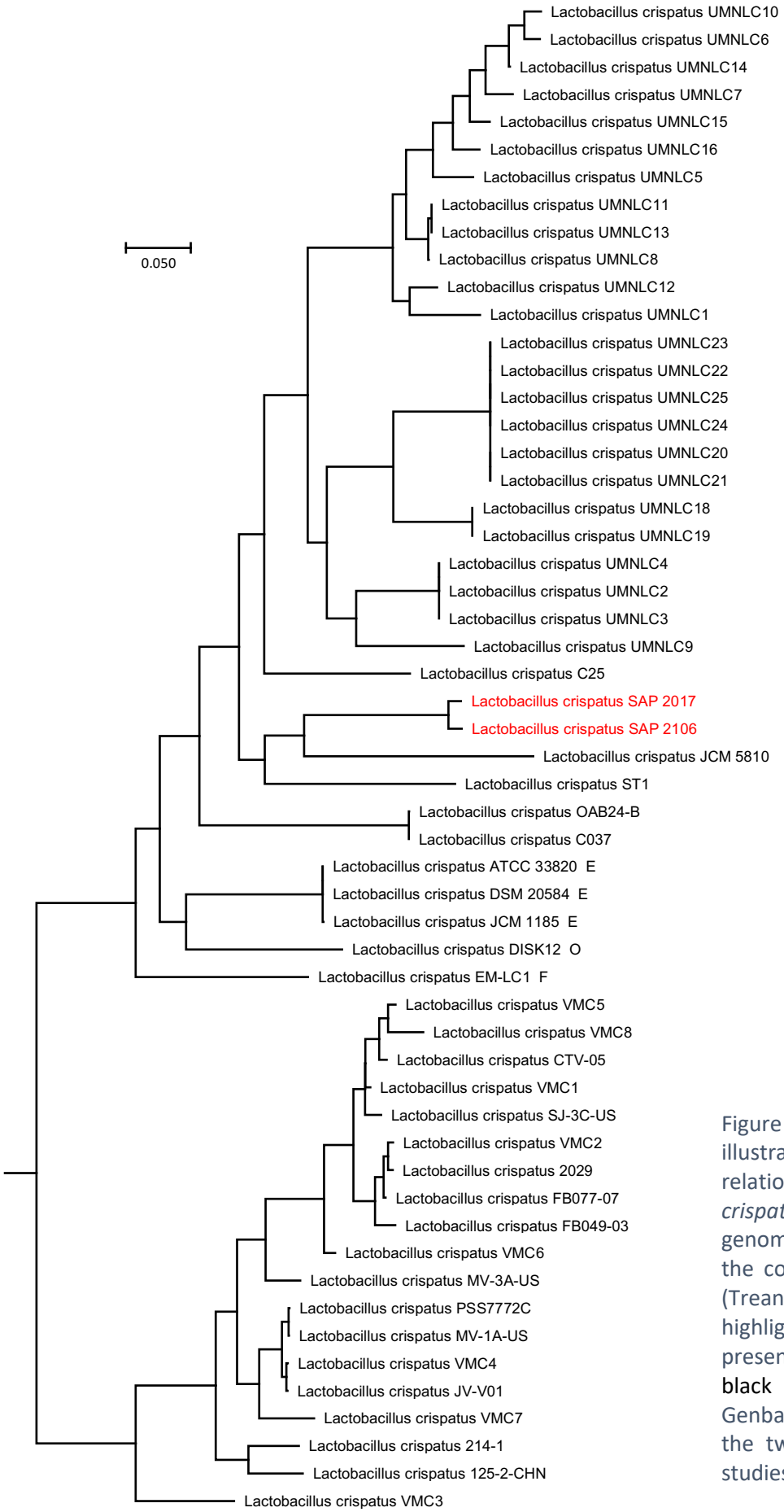


Figure 5. 2 A phylogenetic dendrogram illustrating the phylogenetic relationships within the *Lactobacillus crispatus* species. Fifty-five *L. crispatus* genomes were clustered according to the core genome SNPs using parSNP (Treangen *et al.*, 2014). Isolates highlighted in red were those presented in these studies, isolates in black were downloaded from Genbank. This dendrogram shows that the two isolates presented in these studies were *L. crispatus.*

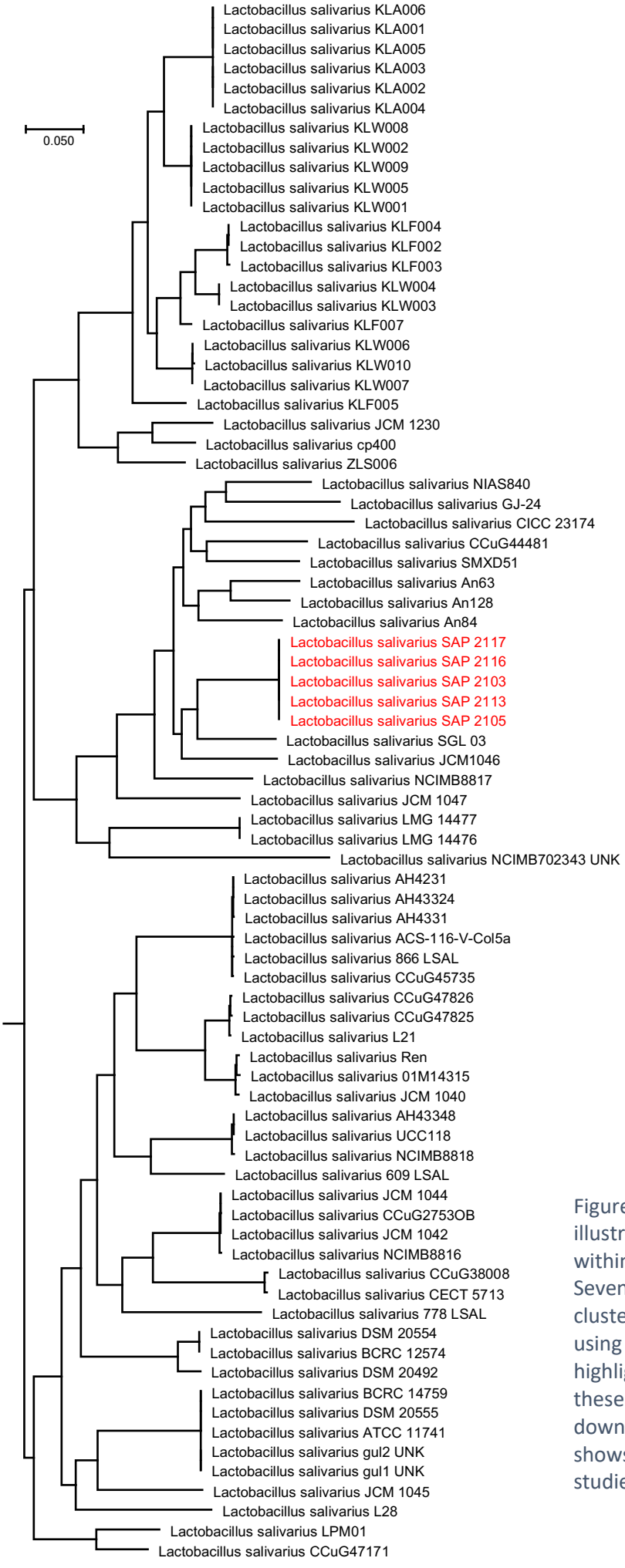


Figure 5. 3 A phylogenetic dendrogram illustrating the phylogenetic relationships within the *Lactobacillus salivarius* species. Seventy-nine *L. salivarius* genomes were clustered according to the core genome SNPs using parSNP (Treangen *et al.*, 2014). Isolates highlighted in red were those presented in these studies, isolates in black were downloaded from Genbank. This dendrogram shows that the five isolates presented in these studies were *L. salivarius.*

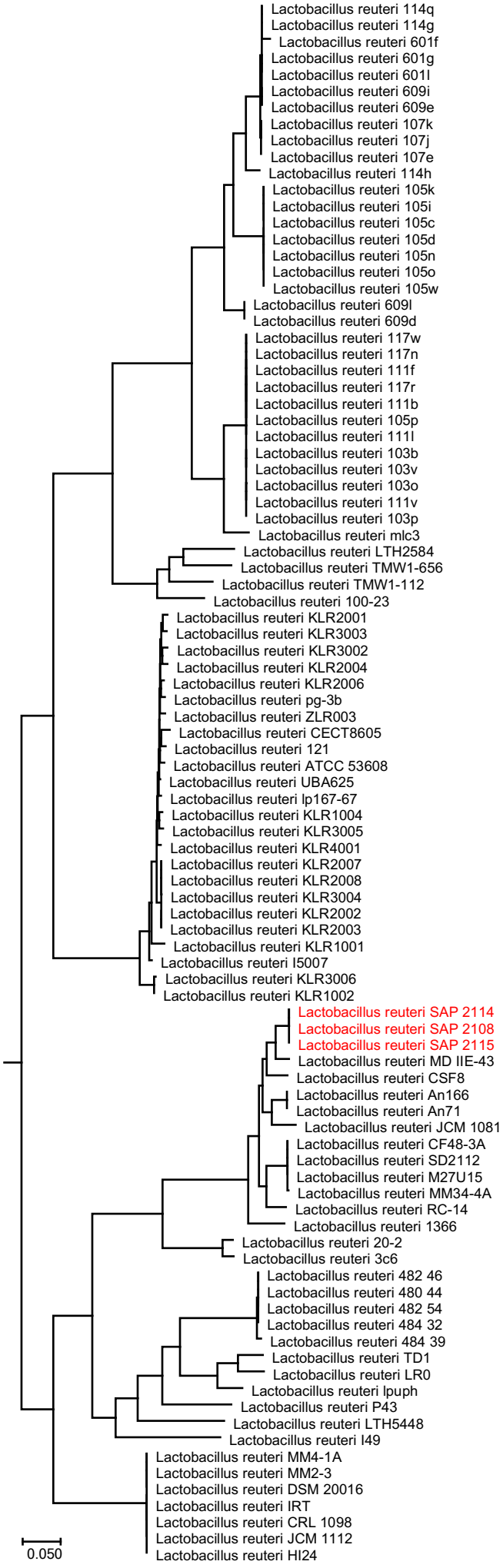


Figure 5. 4 A phylogenetic dendrogram illustrating the phylogenetic relationships within the *Lactobacillus reuteri* species. Ninety-five *L. reuteri* genomes were clustered according to the core genome SNPs using parSNP (Treangen *et al.*, 2014). Isolates highlighted in red were those presented in these studies, isolates in black were downloaded from Genbank. This dendrogram shows that the three isolates presented in these studies were *L. reuteri.*

5.2.3 Screening *Lactobacillus* isolates as potential probiotics

In addition to confirming the identification of *Lactobacillus* isolates using phenotypic and genotypic tests as described previously, candidate probiotics needed to be screened for their inhibition against *Brachyspira* in order to ascertain their potential success as an intervention for avian intestinal spirochaetosis. The most inhibitory *Lactobacillus* isolates underwent further characterisation to determine antimicrobial resistance profiles in line with EFSA guidelines, in addition to confirming resistance phenotypes informed by whole genome sequencing. Further bile salt and acid tolerance tests were conducted to predict the likelihood of isolate survival through the avian gastrointestinal tract.

5.2.3.1 Initial screening *Lactobacillus* isolates for inhibitory effects against *Brachyspira*

The panel of ten *Lactobacillus* isolates that met the preliminary selection criteria and for which a genome sequences was now available (Table 5.1) were screened for their inhibitory activity against five of the *Brachyspira* isolates which had the ability to grown sufficiently in broth. The CFS of each *Lactobacillus* isolate used in these studies was obtained as described in Chapter 2. The pH of each CFS after culture ranged from pH 3.8 to 4.5, depending on the *Lactobacillus* isolate, therefore the pH of each CFS was adjusted to pH 3.8 and 4.5 to investigate the upper and lower pH within this range. To explore inhibition at a neutral pH the CFS was also adjusted to pH 7.2 and MRS broth controls were pH adjusted to either pH 3.8, 4.5 or 7.2.

The inhibition of *Brachyspira* was dependent on the isolate of *Lactobacillus* used and it was important to note that there was not one *Lactobacillus* isolate that was inhibitory against all *Brachyspira* isolates. There was evidence of both pH-dependent and pH-independent mechanisms of inhibition, observable in Table 5.6. These mechanisms will be explored further

in Chapter 6; however, it was observed that there was a significant difference between the growth of *Brachyspira* when the broth was supplemented with MRS at pH 3.8 compared to MRS at pH 7.2 thus indicating this pathogen's sensitivity to acid ($p \leq 0.05$).

The pH-independent mechanism was explored here in order to determine which *Lactobacillus* isolates had the greatest inhibitory potential against *Brachyspira,* thus the p values in Table 5.6 correspond to the differences of the CFS and its pH matched control. Of the three different pH values tested, CFS at pH 3.8 had the greatest inhibitory effect against *Brachyspira*. *B. pilosicoli* SAP 865 was the most tolerant to the *Lactobacillus* CFS at pH 3.8 and growth was significantly reduced (p value $\leq$ 0.05) by only two of the ten *Lactobacillus* CFSs tested. *B. pilosicoli* B2904 and *B. intermedia* SAP 919 were the most susceptible to the different CFSs, with between five and six different *Lactobacillus* isolates significantly (p value $\leq$ 0.05) reducing growth. *B. innocens* SAP 493 was also significantly susceptible to four different CFSs at pH 3.8 (p value $\leq$ 0.05). Overall this indicated that *Brachyspira* isolates were susceptible to low pH *Lactobacillus* CFS. There were very few observable differences between the growth of *Brachyspira* supplemented with CFS at pH 4.5 and the pH matched control, with only *B. pilosicoli* SAP 859 and *B. innocens* SAP 943 showing significant decreases in growth. At pH 7.2, however, there appeared to be an increase in the number of *Lactobacillus* isolates capable of significantly reducing the growth of some isolates of *Brachyspira*. *B. pilosicoli* B2904 was more susceptible to the pH 7.2 CFS with six *Lactobacillus* isolates significantly reducing growth (p value$\leq$ 0.01). *B. pilosicoli* SAP 865 and *B. innocens* SAP 943 were inhibited by approximately the same number of CFSs, however the *Lactobacillus* isolates responsible for this inhibition were different. On the other hand, *B. intermedia* SAP 919 and *B. pilosicoli* SAP 859 were inhibited by fewer *Lactobacillus* isolates at a neutral pH.

Of the ten *Lactobacillus* isolates tested, four were selected as potential probiotics for further exploration into the mechanisms of inhibition against *Brachyspira in vitro* and *in vivo*, described in Chapters 6 and 7. The isolates selected were *L. reuteri* SAP 2114 and 2115 and *L. salivarius* SAP 2116 and 2117. The primary criteria for selecting these isolates was their inhibitory activity against the *Brachyspira* isolates tested, as shown in Table 5.6. These four isolates were overall the most inhibitory against *Brachyspira* at the different pH values. *L. reuteri* SAP 2114 significantly inhibited *B. pilosicoli* B2904 ($p \leq 0.001$) and SAP 859, *B. intermedia* SAP 919 ($p \leq 0.05$) at pH 3.8 and *B. pilosicoli* SAP 859 ($p \leq 0.0001$) and *B. innocens* SAP 934 ($p \leq 0.001$) at pH 4.5. *L. reuteri* SAP 2115 demonstrated the greatest inhibitory effects against *Brachyspira,* significantly inhibiting *B. pilosicoli* B2904 ($p \leq 0.001$) and SAP 859 ($p \leq 0.05$), *B. intermedia* SAP 919 ($p \leq 0.05$) and *B. innocens* SAP 943 ($p \leq 0.005$) at pH 3.8, *B. pilosicoli* SAP 859 ($p \leq 0.005$) at pH 4.5 and *B. pilosicoli* B2904 ($p \leq 0.005$), *B. intermedia* SAP 919 ($p \leq 0.05$) and *B. innocens* SAP 943 ($p \leq 0.05$) at pH 7.2. *L. salivarius* SAP 2116 significantly inhibited *B. pilosicoli* B2904 ($p \leq 0.0001$) and *B. innocens* SAP 943 ($p \leq 0.05$) at pH 3.8 and *B. pilosicoli* SAP 859 ($p \leq 0.005$) at pH 4.5. Although this *Lactobacillus* isolate did not inhibit *Brachyspira* as significantly as other isolates, it was one of the four that inhibited *Brachyspira* at pH 4.5 and was therefore selected for further characterisation. *L. salivarius* SAP 2117 demonstrated the greatest inhibitory ability against *Brachyspira* compared to all other *L. salivarius* isolates tested and significantly inhibited *B. pilosicoli* B2904 ($p \leq 0.005$) and SAP 859 ($p \leq 0.05$) and *B. innocens* SAP 943 ($p \leq 0.005$) at pH 3.8, *B. pilosicoli* SAP 859 ($p \leq 0.005$) at pH 4.5 and *B. pilosicoli* B2904 ($p \leq 0.005$) and SAP 859 ($p \leq 0.05$) at pH 7.2.

Following the selection of the most inhibitory *Lactobacillus* isolates against *Brachyspira*, it was important to consider the EFSA guidelines (EFSA *et al.*, 2017) and investigate the antimicrobial resistance profiles of these four isolates. Screening of the *L. reuteri* whole genome sequences

for potential antimicrobial resistance (AMR) genes revealed that these isolates did not harbour any of the AMR genes, however the *L. salivarius* isolates potentially harboured *tetM* and *ermB* which potentially conferred resistance to tetracycline and erythromycin. Therefore, MIC testing was employed to determine if the AMR genotype conferred phenotypic resistance to these antibiotics.

Table 5. 6 Initial screening of *Lactobacillus* cell free supernatant (CFS) for inhibition against *Brachyspira.* The area under the curve was calculated for each of the *Brachyspira* growth curves supplemented with different CFS, using GraphPad Prism 7. Significant inhibition of *Brachyspira* growth are highlighted in green whereby the p value was ≤ 0.05. Significant increases in *Brachyspira* growth are highlighted in orange. The area under the curve values were compared to the pH matched MRS control using a one- way ANOVA to determine significant inhibition of *Brachyspira* independent of pH. A pH dependent mechanism of inhibition was also noted when comparing growth of *Brachyspira* at pH 3.8 and 7.2. The p values presented were used to determine the inhibitory capability of each *Lactobacillus* CFS against each *Brachyspira* isolate. It was evident that this inhibition was dependent on the isolate of *Brachyspira*, the isolate of *Lactobacillus* and the pH of the CFS, therefore only the most inhibitory *Lactobacillus* isolates were selected for further study. These isolates were *L. reuteri* SAP 2114 and 2115 and *L. salivarius* 2116 and 2117. These experiments were performed with five biological replicates, each with three technical replicates.

		P- Value ≤ 0.05				
		B. pilosicoli B2904	*B. pilosicoli* SAP 859	*B. pilosicoli* SAP 865	*B. intermedia* SAP 919	*B. innocens* SAP 943
	MRS 3.8 vs MRS 7.2	0.0001	0.0272	0.3387	0.0042	0.0001
pH 3.8	MRS vs. SAP 2103 CFS	0.9999	0.166	0.0349	0.0008	0.0714
	MRS vs. SAP 2105 CFS	0.9996	0.166	0.9931	0.0137	0.7573
	MRS vs. SAP 2106 CFS	0.9996	0.166	0.9996	0.0008	0.7573
	MRS vs. SAP 2107 CFS	0.0011	0.0004	0.0172	0.6399	0.9991
	MRS vs. SAP 2108 CFS	0.9996	0.166	0.5742	0.6399	0.0039
	MRS vs. SAP 2113 CFS	0.1922	0.0004	0.6474	0.0008	0.7573
	MRS vs. SAP 2114 CFS	0.0001	0.2607	0.7107	0.05	0.1267
	MRS vs. SAP 2115 CFS	0.0011	0.0265	0.999	0.0289	0.004
	MRS vs. SAP 2116 CFS	0.0001	0.1135	0.999	0.986	0.0188
	MRS vs. SAP 2117 CFS	0.0055	0.0071	0.999	0.7272	0.0042
pH 4.5	MRS vs. SAP 2103 CFS	0.9853	0.116	0.6035	0.9995	0.7506
	MRS vs. SAP 2105 CFS	0.9997	0.4531	0.9999	0.9944	0.9999
	MRS vs. SAP 2106 CFS	0.1048	0.572	0.6035	0.1563	0.9999
	MRS vs. SAP 2107 CFS	0.8237	0.4531	0.6035	0.9991	0.9999
	MRS vs. SAP 2108 CFS	0.8034	0.9999	0.9999	0.9996	0.6693
	MRS vs. SAP 2113 CFS	0.9988	0.4531	0.9999	0.9943	0.9999
	MRS vs. SAP 2114 CFS	0.9996	0.0001	0.6035	0.7067	0.0009
	MRS vs. SAP 2115 CFS	0.5	0.0026	0.9999	0.9997	0.9999
	MRS vs. SAP 2116 CFS	0.782	0.003	0.7043	0.0999	0.9999
	MRS vs. SAP 2117 CFS	0.9873	0.0026	0.6035	0.9994	0.0999
pH 7.2	MRS vs. SAP 2103 CFS	0.0001	0.2132	0.8748	0.9999	0.9999
	MRS vs. SAP 2105 CFS	0.1948	0.9999	0.9224	0.1322	0.0476
	MRS vs. SAP 2106 CFS	0.0063	0.9179	0.0001	0.0791	0.0186
	MRS vs. SAP 2107 CFS	0.9999	0.2696	0.4356	0.1727	0.0636
	MRS vs. SAP 2108 CFS	0.0016	0.9987	0.9997	0.0014	0.0008
	MRS vs. SAP 2113 CFS	0.0016	0.011	0.4356	0.5984	0.7985
	MRS vs. SAP 2114 CFS	0.1617	0.999	0.4356	0.9932	0.9999
	MRS vs. SAP 2115 CFS	0.0016	0.6864	0.4356	0.0262	0.05
	MRS vs. SAP 2116 CFS	0.99	0.9597	0.456	0.999	0.8865
	MRS vs. SAP 2117 CFS	0.0016	0.05	0.4356	0.8587	0.9731

5.2.3.2 Antimicrobial sensitivity testing of *Lactobacillus*

The European Food Safety Authority (EFSA) (European Food Safety Authority, 2012) stipulates that in order for an organism to be considered as a potential feed additive, the antimicrobial resistance profiles for ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin need to be determined in order to assess the risk of transference of AMR genes. Notably, some *Lactobacillus* isolates are innately resistant to vancomycin and thus there is no risk of transmitting this resistance. The MICs for the four selected potential probiotic candidates, *L. reuteri* SAP 2114, *L. reuteri* SAP 2115, *L. salivarius* SAP 2116 and *L. salivarius* SAP 2117 were tested using the broth microdilution method and the results are summarised in Table 5.7. MIC values were interpreted according to the EFSA guidelines, using the FEEDAP official MIC cut-off values detailed in brackets in Figure 5.7 (EFSA *et al.*, 2017).

L. reuteri SAP 2114 and 2115 were the most suitable probiotic candidates because firstly, all of the MIC values obtained were at or below the cut-off concentrations stipulated by the EFSA guidelines, and secondly, they did not harbour potential antimicrobial resistance genes. The *L. salivarius* isolates SAP 2116 and 2117 had MIC values higher than stated in the guidelines for erythromycin, kanamycin, streptomycin and tetracycline. These isolates were therefore less suitable as probiotic candidates, however remained in further studies to characterise the mechanisms of inhibition due to their inhibitory activity against *Brachyspira* as detailed in Table 5.6

Table 5. 7 Minimum inhibitory concentration (MIC) of the antibiotics ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin determined by broth microdilution against the four selected *Lactobacillus* isolates, *L. reuteri* SAP 2114 and 2115 and *L. salivarius* SAP 2116 and 2117. *E. faecalis* NCTC 12697 was used as a control strain to ensure correct antibiotic concentrations were used (Andrews, 2001). The experiment was performed with three biological replicates, each with three technical replicates. The accepted break-points for *L. reuteri, L. salivarius* and *E. faecalis* are presented in brackets underneath the recorded MIC.

Lactobacillus	**Strain**	**MIC μg/ml**								
Species	**Name**	Ampicillin	Chloramphenicol	Clindamycin	Erythromycin	Gentamicin	Kanamycin	Streptomycin	Tetracycline	Vancomycin
L. reuteri	SAP 2114	2 (2)	4 (4)	< 0.0062 (1)	0.25 (1)	2 (8)	32 (64)	32 (64)	32 (16)	R (n.r)
L. reuteri	SAP 2115	1 (2)	4 (4)	< 0.0062 (1)	0.125 (1)	1 (8)	16 (64)	16 (64)	4 (16)	R (n.r)
L. salivarius	SAP 2116	2 (4)	4 (4)	0.25 (1)	8 (1)	16 (16)	256 (64)	128 (64)	128 (8)	R (n.r)
L. salivarius	SAP 2117	2 (4)	4 (4)	0.125 (1)	4 (1)	16 (16)	256 (64)	128 (64)	128 (8)	R (n.r)
E. faecalis	NCTC 12697	1 (1)	4 (4)	8 (8)	4 (4)	8 (8)	128 (1024)	256 (1024)	8 (16)	2 (2)

5.2.3.3 Acid tolerance

In order to promote successful passage through the gastrointestinal tract, any potential probiotics must be tolerant to acid. The gizzard is the most acidic section of the avian gastrointestinal tract, with a pH between 1.9 and 4.5 (Sivihus, 2014). Therefore, it was important to ascertain the survival of each of the selected *Lactobacillus* isolates at pH 1.9 to ensure that they could theoretically survive passage through the gizzard.

At pH 1.9 a significant reduction ($p \leq 0.05$) in numbers of all four *Lactobacillus* isolates were observed after 3 hours of incubation (Jin *et al.*, 1998) (Table 5.8). The numbers of both *L. salivarius* isolates were below the limit of detection after incubation at pH 1.9. However, a ~1-1.5 log reduction in bacterial survival was observed for both *L. reuteri* isolates which indicated that these isolates were tolerant to acidic conditions, despite the reduction in cell numbers. No significant differences in numbers of *Lactobacillus* were observed with isolates incubated for 3 hours in PBS at pH 7.2.

Table 5. 8 Survival of four potential probiotic strains, *L. reuteri* SAP 2114 and 2115 and *L. salivarius* SAP 2116 and 2117, in acidic conditions following three hours of incubation in PBS at pH 1.9 and 7.2. The data represent the mean of three biological replicates, each with three technical replicates, a significant reduction in CFU/ml is indicated by an Asterix (*) (p value < 0.05). Standard deviation is shown in brackets.

Lactobacillus Species	**pH 1.9 (CFU/ml)**		**pH 7.2 (CFU/ml)**	
	0 hours	3 hours	0 hours	3 hours
***L. reuteri* SAP 2114**	1.65×10^8 (7.64×10^6)	1.28×10^7 * (9.43×10^6)	1.70×10^8 (8.01×10^6)	1.10×10^8 (6.08×10^7)
***L. reuteri* SAP 2115**	1.08×10^8 (6.00×10^7)	6.78×10^6 * (2.90×10^6)	1.28×10^8 (5.67×10^6)	1.63×10^8 (6.05×10^7)
***L. salivarius* SAP 2116**	3.3×10^7 (7.01×10^6)	0 * (0)	3.6×10^7 (6.72×10^6)	2.52×10^7 (5.48×10^6)
***L. salivarius* SAP 2117**	3.27×10^7 (3.27×10^7)	0 * (0)	3.0×10^7 (2.89×10^6)	2.98×10^7 (9.50×10^6)

5.2.3.4 Bile salt tolerance

The four probiotic candidates were also assessed for their ability to tolerate oxgall (bovine bile) and two constituents of poultry bile, taurocholic acid and sodium taurochenodeoxycholate (Yeh and Hwang, 2001). Despite these probiotics being intended for use in poultry, the sensitivity to oxgall was tested to assess the feasibility for use in other livestock species such as cattle.

Overall, the *L. reuteri* isolates were more tolerant to the bile salts, compared to *L. salivarius*. Using the method developed by Chateau *et al.* (1994), it could be observed that *L. reuteri* SAP 2114 and SAP 2115 were resistant to 0.3% taurocholic acid as the difference in lag time compared to the control was zero hours, as seen in Table 5.9. Interestingly, the growth of *L. reuteri* SAP 2115 was improved by 1% taurocholic acid whereby the lag time was one hour shorter compared to control. The log phase of growth was also an important consideration when analysing these data. For example, Figure 5.5A suggested that despite the difference in the lag phase of 1% sodium taurochenodeoxycholate being two hours when compared to control (therefore sensitive to this bile salt in accordance to Chateau *et al.* (1994)), it was evident that this bile salt improved the growth of *L. reuteri* SAP 2114 significantly (p value $\leq$ 0.001). Similarly, 1% taurocholic acid and 0.3% sodium taurochenodeoxycholate significantly increased the overall growth (p value $\leq$ 0.01) of *L. reuteri* SAP 2114 and 2115 (Figure 5.5A and B). Therefore, it was important to consider both the lag and log phase when analysing these data to determine tolerance to bile salts, especially for the more tolerant *Lactobacillus* isolates. *L. salivarius* isolates were significantly less tolerant to bile salts, where oxgall and taurochenodeoxycholate inhibited the growth of SAP 2116 (Figure 5.5C) and SAP 2117 (Figure 5.5D) at both 1% and 0.3% (p value $\leq$ 0.001). *L. salivarius* SAP 2116 was sensitive and 2117 was tolerant to 0.3% taurocholic acid indicated by Table 5.9 and the methods described by

Chateau *et al.* (1994), however it was observed from Figure 5.5C and D that, although there was a significant decrease in *Lactobacillus* growth (p value ≤ 0.001), these isolates still grew sufficiently to be weakly tolerant to the bile salt.

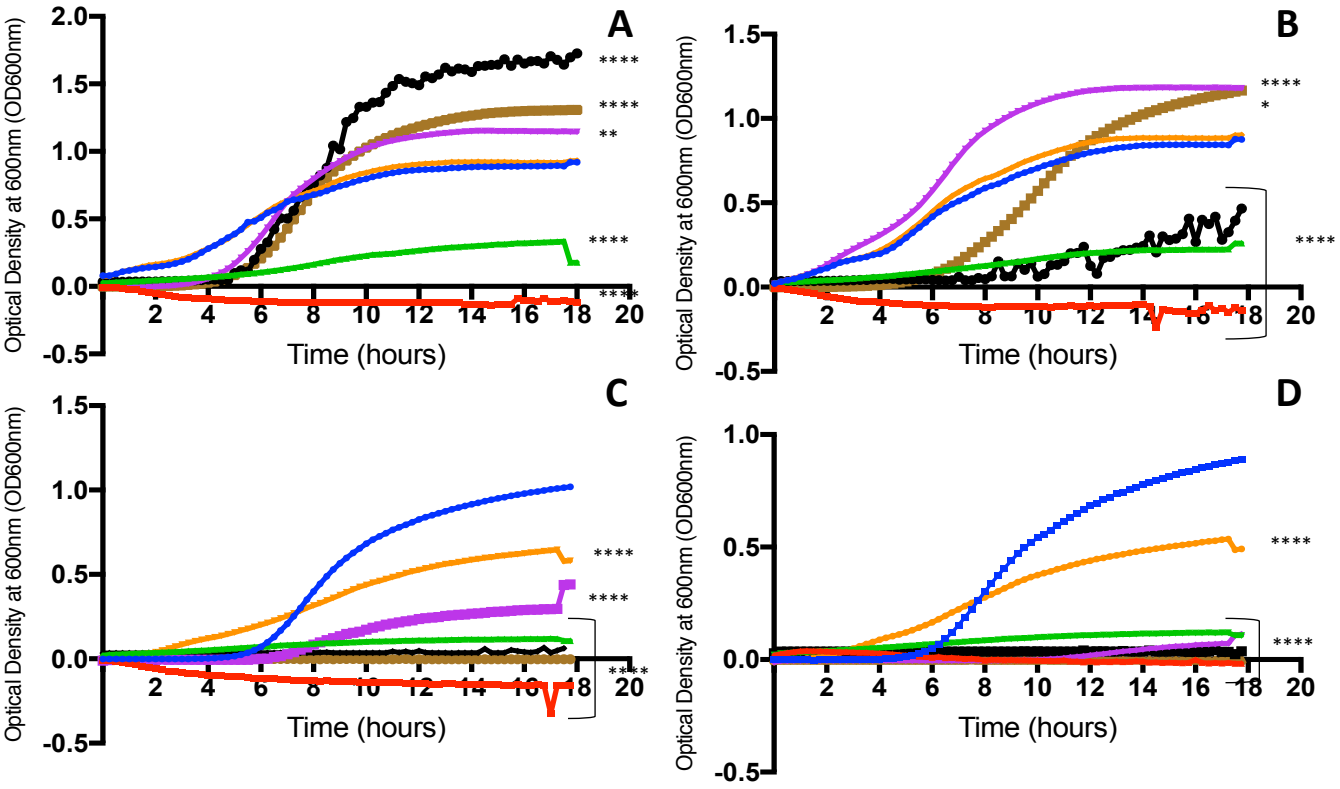


Figure 5. 5 Tolerance of (A) *L. reuteri* SAP 2114, (B) *L. reuteri* SAP 2115, (C) *L. salivarius* SAP 2116 and (D) *L. salivarius* SAP 2117 to poultry and bovine bile salts. Taurocholic acid was tested at 1% (purple) and 0.3% (orange) (w/v) in MRS, sodium taurochenodeoxycholate was tested at 1% (black) and 0.3% (brown) (w/v) in MRS and oxgall was tested at 1% (red) and 0.3% (green) (w/v) in MRS. The growth of each *Lactobacillus* isolate under these experimental conditions was compared to growth in MRS alone (blue) using area under the curve analysis, followed by a one-way ANOVA with Dunnett's multiple comparison (*, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001). *L. reuteri* isolates were more tolerant to the poultry bile salts compared to *L. salivarius* and it is evident that none of the isolates were tolerate to oxgall at either 1% or 0.3% (w/v). Growth was measured for 18 hours using optical density (OD_{600}) and the data presented were means of three biological replicates, each with three technical replicates.

Table 5. 9 Lag time of *L. reuteri* SAP 2114 and 2115 and *L. salivarius* 2116 and 2117 as a measure of tolerance to the poultry bile salts taurocholic acid and sodium taurochenodeoxycholate and the bovine bile salt oxgall. MRS media was supplemented with 1% or 0.3% (w/v) of each bile salt and growth was measured by optical density for 18 hours. The lag time was defined as the time taken for each culture to reach an OD_{600} of 0.3 (Chateau *et al.*, 1994). Data are presented as means with standard deviation of three biological replicates, each with three technical replicates. The difference in lag time between the media supplemented with bile salts and the control were calculated to determine the tolerance of each Lactobacillus isolate. Tolerance was defined Chateau wehereby a delay in growth of less than 15 minutes between the test and control determined an isolate to be resistant to bile salts, between 15 and 40 minutes determined an isolate to be tolerant, between 40 and minutes determined an isolate to be weakly tolerant and greater than 60 minutes determined an isolate to be sensitive to bile salts.

Bile Salt Product	**Lag time (hours)**							
	***L. reuteri* SAP 2114**	**Difference in lag time compared to control**	***L. reuteri* SAP 2115**	**Difference in lag time compared to control**	***L. salivarius* SAP 2116**	**Difference in lag time compared to control**	***L. salivarius* SAP 2117**	**Difference in lag time compared to control**
Control	4.25 ± 0.25		5.0 ± 1		5.5 ± 0.25		8 ± 1	
1% oxgall	-	-	-	-	-	-	-	-
0.3% oxgall	14.14 ± 2	9.89	-	-	-	-	-	-
1% taurocholic acid	5.75 ± 0.5	1.5	4.0 ± 2	-1	17.25 ± 3	11.75	-	-
0.3% taurocholic acid	4.25 ± 0.5	0	5.0 ± 1	0	7.75 ± 2	2.25	8.5 ± 1.5	0.5
1% sodium taurochenodeoxycholate	6.25 ± 0.25	2	15.50 ± 1.5	10.50	-	-	-	-
0.3% sodium taurochenodeoxycholate	6.50 ± 0.25	2.25	8.25 ± 1	3.35	-	-	-	-

- 0.3 OD_{600} not reached

5.3 Discussion

The studies presented here focussed on the isolation and characterisation of *Lactobacillus* isolates as potential poultry probiotics capable of inhibiting *Brachyspira*, the causative agent of avian intestinal spirochaetosis.

A panel of *Lactobacillus* isolates were isolated from the faeces of healthy, free-range hens. The isolates were preliminarily characterised using staining, biochemical testing and 16S rRNA sequencing to ensure they fulfilled the basic *Lactobacillus* probiotic criteria defined by EFSA (EFSA *et al.*, 2017). These guidelines have been implemented to ensure the safety of potential probiotics that are designed as animal feed supplements. Furthermore, *Lactobacillus* species categorised under these guidelines have excellent safety records, and detrimental effects produced following ingestion are rare (Gueimonde *et al.*, 2013).

Although there are undefined probiotic products on the market for the poultry industry, such as Aviguard® (Collins *et al.*, 2009), to identify probiotic products that target *Brachyspira*, the bacterial isolate needs to be well characterised and have proven efficacy against this pathogen. Another consideration for a potential probiotic is that it can effectively colonise the gastrointestinal tract of the target host and not cause disease (Klaenhammer and Kullen, 1999). This is why all potential probiotics in these studies were isolated from the faeces of healthy chickens, as this suggested that the isolates could colonise the gastrointestinal tract without causing disease. However, *in vitro* testing such as cytotoxicity in gastrointestinal cell lines and *in vivo* testing in chickens would be required to confirm this.

More recently, as whole genome sequencing has become more readily available, EFSA have stipulated that whole genome sequencing is to be used for the 'unequivocal taxonomic identification' of each potential probiotic isolate intended for animal consumption, therefore the selected *Lactobacillus* isolates were sent for whole genome sequencing. In terms of

probiotic characterisation, genome sequencing is the gold standard and has many advantages especially in the identification of an organism and the ability to screen for negative gene traits. The sequencing used in these studies confirmed the species of each isolate and allowed for preliminary comparisons with other published genome sequences to ensure these isolate were representative of the species within the *Lactobacillus* genus (Yun *et al.*, 2009; Frese *et al.*, 2011; Ojala *et al.*, 2014; Lee *et al.*, 2017). Additionally, screening for negative gene traits such as antimicrobial resistance genes, virulence genes and plasmids was highly advantageous as the detection of these putative genes led to targeted phenotypic assays, such as MICs. These assays determined if the presence of an AMR gene conferred phenotypic resistance *in vitro*.

ABRicate software identified the presence of three antimicrobial resistance genes, *tetM, tetW* and *ermB* as shown in Table 5.3. All *L. salivarius* isolates harboured both *tetM* and *ermB*, indicating resistance to tetracycline and erythromycin. *L. crispatus* isolates harboured all three genes and *L. reuteri* lacked any of these antimicrobial resistance genes, thus *L. reuteri* isolates were the most suitable for probiotic selection. If an isolate harboured any antimicrobial resistance genes, there needed to be proof that these were not transposable to other bacteria. However, the *tetM* and *ermB* genes have been previously shown to be present on plasmids and other mobile genetic elements which can not only be transferred between *Lactobacillus* isolates, but also to other lactic acid bacteria such as *Enterococcus faecalis* and *Lactococcus lactis* (Gevers *et al*., 2003). Therefore, this would render any isolates harbouring these genes unsuitable as commercial probiotics, however this did not limit their usefulness when exploring the mechanisms of probiotic action against *Brachyspira*.

Resistance to tetracycline and erythromycin is most widely detected in *Lactobacillus* isolates, potentially resulting from the wide use of these antibiotics in animals, both as therapeutic

agents and growth promoters (Comunian *et al.*, 2010). The genes responsible for these resistances are most frequently identified as *tetM, tetW* and *ermB*; these genes have been associated with conjugative transposons on both chromosomes and plasmids (Roberts, 2008). The tetracycline resistance genes, *tetM* and *tetW* encode ribosomal protection proteins that interfere with the tetracycline binding to the ribosome to prevent the inhibitory effect on translation (Xi *et al.*, 2009). The macrolide resistance gene *ermB*, results in the methylation of the ribosomal binding site of the antibiotic, rendering it infective (Desjardins *et al.*, 2004). Therefore, *Lactobacillus* isolates harbouring these genes pose the risk of transferring these resistance genes to other bacteria in the gastrointestinal tract.

Importantly, many lactobacilli, including *L. crispatus, L. reuteri* and *L. salivarius* are intrinsically resistant to vancomycin and transfer of this resistance has not been observed (Tynkkynen *et al*., 1998). Vancomycin resistance is well characterised in lactic acid bacteria; vancomycin acts on the peptidoglycan precursors on the cell wall side of the cytoplasmic membrane and binds to D-alanine/D-alanine terminus of the pentapeptide, preventing polymerisation of peptidoglycan precursors. In many *Lactobacillus* species the terminal D-alanine is replaced with D-lactate or D-serine, preventing vancomycin binding and conferring resistance innately (Gueimonde *et al.*, 2013).

As previously mentioned, following the identification of putative AMR genes, the MICs for nine different antibiotics were tested in accordance to the EFSA guidelines. *L. reuteri* isolates showed the most suitability as probiotic candidates due to the absence of genotypic and phenotypic antimicrobial resistance. However, *L. salivarius* isolates were resistant to four of the antibiotics tested: kanamycin, streptomycin, tetracycline and erythromycin, resulting in these isolates being less suitable as probiotic candidates. It has long been recognised that the use of antibiotic growth promoters in the livestock industry has resulted in the increased

incidence of antimicrobial resistance in bacteria isolated from poultry (Gast and Stephens., 1988). Therefore, the high incidence of antimicrobial resistance was unsurprising and highlighted the importance of genotypically and phenotypically screening potential probiotics for resistance to antibiotics of both human and veterinary importance.

Screening of genome sequences was not solely used to screen for negative gene traits but was also important in identifying positive traits such as the presence of putative bacteriocin genes. Bacteriocins are antibacterial peptides produced by bacteria such as *Lactobacillus* which are inhibitory against other closely related bacteria. These peptides are ribosomally synthesised and primarily inhibit bacteria through pore formation or inhibition of cell wall synthesis in target cells. However, they have been shown to have activity against Gram negative bacteria, especially those that have had their outer membrane permeabilised (Cotter *et al.,* 2005; Messaoudi *et al.*, 2013). Studies have indicated that chickens fed on a diet containing a bacteriocin derived from *L. salivarius* had lower counts of *C. jejuni* following challenge, compared to chickens that were fed diets that did not contain the bacteriocin (Stern *et al.*, 2006). Therefore, bacteriocins may contribute to the inhibition of *Brachyspira* in the studies presented here (this was further explored in Chapter 6).

Sixty-six different bacteriocins have been identified in the *Lactobacillus* genus and studies have shown that *Lactobacillus* isolates of human and animal origin encode for twice as many bacteriocin genes compared to those from other sources (Collins *et al.*, 2017). The pipelines used in these studies were therefore able to screen for and predict a range of bacteriocin peptides. *Lactobacillus* isolates that encode several bacteriocin genes may have a competitive advantage against other bacteria in the gastrointestinal tract as a result of having the ability to kill isolates competing for the same niche. However, it is important to note that these predicted genes may not translate to functional bacteriocins due to gene mutations.

Therefore, phenotypic assays were required to confirm their presence (discussed in Chapter 6).

Salivaricin, a class I lantibiotic, and class II bacteriocins are small, heat stable peptides thought to inhibit Gram positive cell wall biosynthesis, thus killing target cells (Barbour *et al.*, 2016). Enterolysin A and helveticin J are bacteriolysins (formally known as class III bacteriocins). Bacteriolysins are large molecular weight, heat-labile proteins which are sub-divided into two groups: group A which are bacteriolytic enzymes that will kill the target cell through bacterial cell wall lysis (Enterolysin A) (Khan *et al.*, 2013) and group B which are non-lytic, bacteriostatic proteins (Helveticin J) (Yang *et al.*, 2014). These bacteriolysins appear to be abundantly present in the different isolates presented here and across other species of *Lactobacillus* (Chung *et al.*, 2018). Many of the known functions of bacteriocins relate to inhibition of closely related Gram positive species, however inhibition of *C. jejuni* and *Salmonella* Typhimurium have been observed in *in vivo* and *in vivo* models (Natrajan and Sheldon, 2000; Stern *et al.*, 2006) and therefore may play a role in *Brachyspira* inhibition, this will be further explored in Chapter 6.

In order for *Lactobacillus* isolates to be considered as potential probiotics, there must be evidence health benefits to the host. Therefore, the ten isolates presented in these studies were screened for their inhibitory capabilities against *Brachyspira in vitro.* It is well documented that *Lactobacillus* inhibition is strain specific and therefore it was important to determine which isolates of *Lactobacillus* would be effective against which isolates of *Brachyspira* which were characterised in Chapter 3 (Ouwehand *et al.,* 2002; Mappley *et al.*, 2011; Campana, *et al*., 2017). These studies showed a wide range of activity against *Brachyspira* at different pH, as shown in Table 5.6. Using these data, it was possible to select

four *Lactobacillus* isolates that were the most inhibitory against *Brachyspira*, these were *L. reuteri* SAP 2114 and 2115 and *L. salivarius* 2116 and 2117.

The gizzard and duodenum of the avian gastrointestinal tract are harsh environments that probiotic bacteria must survive in order to reach the large intestine and caeca. As previously mentioned the pH of the gizzard is between 1.9 and 4.5 and in the duodenum, bacteria are exposed to bile acids, therefore it was advantageous to test probiotic tolerance to low pH and presence of bile to infer *in vivo* survival (Svihus, 2014). A 3-hour incubation was used in these experiments, although it is unlikely that *Lactobacillus* isolates would be exposed to these adverse conditions for this period of time. Feed is estimated to remain in the chicken's gizzard for 30-60 minutes, after which it is mixed with the pancreatic and bile secretions for less than five minutes before the pH begins to rise rapidly to approximately pH 6 to allow digestion in the small intestine to occur (Svihus, 2014). Although this was designed to be a rapid screen for acid and bile salt tolerance, it may have been more appropriate to complete a time course experiment to investigate the *Lactobacillus* viable counts at one and two hours post exposure to these adverse conditions. Furthermore, it would have been advantageous to test *Lactobacillus* survival in the presence of chicken gizzard contents, although logistically this was not possible in the studies presented here, it is an important future consideration for subsequent studies as this will reflect more closely, the real environment of the poultry gastrointestinal tract.

Of the four isolates tested, the *L. reuteri* isolates were more tolerant to a pH of 1.9 and to the presence of bile salts, compared to the *L. salivarius* isolates. The data here were consistent with the study by Heravi *et al.* (2011) whereby it was suggested that *L. reuteri* isolates were more tolerant to low pH than *L. salivarius* isolates. This may be as a result of several mechanisms designed to ensure *Lactobacillus* can survive unfavourable environments. Such

mechanisms include the accumulation of protective compounds such as compatible solutes for example glycine betaine, which act as osmoprotectants to protect against extreme osmotic stress (Sheehan *et al.*, 2006). Additionally, the presence of glutamate decarboxylases which convert glutamate to GABA and are also known the contribute to the acid tolerance of *E. coli*, *Listeria monocytogenes, Lactococcus lactis* and *L. reuteri* as this process consumes the H^+ protons that contribute to acidity (Su *et al*., 2011). Therefore, the varying acid tolerance and bile salt tolerance may be as a result of genes involved in one or more of these mechanisms, these would need further exploration in future studies.

To summarise, a panel of *Lactobacillus* isolates were isolated and characterised to determine their suitability as probiotic candidates. The characterisation methods were informed by the EFSA guidelines which clearly outline the EU requirements for probiotics intended as animal feed supplements. Furthermore, candidates had to demonstrate inhibition against *Brachyspira* species. Four *Lactobacillus* probiotic candidates: *L. reuteri* SAP 2114 and SAP 2115 and *L. salivarius* SAP 2116 and 2117 were selected to explore the mechanisms of *Brachyspira* inhibition. Overall, the *L. reuteri* isolates were more suitable as probiotic candidates due to their tolerance to bile salts and acid, furthermore, they did not harbour any negative gene traits, such as antimicrobial resistance genes. *L. reuteri* SAP 2115 and *L. salivarius* SAP 2117 were the most inhibitory against *Brachyspira* in the studies presented here. Therefore, all four isolates will be further explored in subsequent chapters to begin to elucidate a mechanism of *Brachyspira* inhibition by *Lactobacillus* probiotics.

Chapter 6: Investigating the ability of *Lactobacillus* to inhibit *Brachyspira* species implicated in avian intestinal spirochaetosis

6.1 Introduction

The prevalence of *Brachyspira* in laying hens is estimated to be as high as 90% in free range hens and 76% in caged hens (Burch, 2010). Despite attempts to control disease, as mentioned in Chapter 1, the incidence of disease continues to rise. Furthermore, in the UK tiamulin is the only licenced antibiotic for the treatment of avian intestinal spirochaetosis. Therefore, antimicrobial resistance is an emerging concern as increasing numbers of *Brachyspira* isolates are becoming less sensitive to tiamulin and other antibiotics used worldwide (Herbst *et al.*, 2017). As a result, alternative control strategies are urgently required to promote animal health and to reduce the need for antibiotics. Probiotics are one such intervention that have shown inhibitory effects against *Brachyspira in vitro* (Bernardeau *et al.*, 2009; Mappley *et al.*, 2011) and the ability to reduce symptoms and *Brachyspira* colonisation in *vivo* (Mappley *et al.*, 2013).

Probiotics are widely used in the livestock industry (Collins *et al.*, 2009) and the mechanisms underlying their activity appear to be multifactorial. These mechanisms include production of acids, bacteriocins and other inhibitory metabolites, in addition to modulation of the immune system and causing competition for nutrients and binding sites to prevent colonisation of a pathogen (Collins *et al.*, 2009; Campana *et al*., 2017). However, exact mechanisms of action have not been fully elucidated for *Brachyspira* and may differ depending on the probiotic strain, animal host and the pathogen.

The aim of this chapter was to determine the potential inhibitory mechanisms of action of the *Lactobacillus* isolates characterised in Chapter 5 against the *Brachyspira* isolates characterised in Chapter 3. Preliminary data from Chapter 5 resulted in the characterisation

and selection of four probiotic candidates that demonstrated inhibition against *Brachyspira*: *L. reuteri* SAP 2114 and 2115 and *L. salivarius* SAP 2116 and 2117. However, the mechanisms of inhibition were unknown and required further exploration. Additionally, with the commercial potential of these potential probiotics in mind, the inhibition of other common pathogens, namely *E. coli* and *Salmonella* Typhimurium were tested to explore the ability of these *Lactobacillus* isolates to inhibit a wide range of avian pathogens.

The aims and objectives of this chapter were:

- To investigate the *Lactobacillus* pH dependent and independent mechanisms of *Brachyspira* inhibition.
- To use NMR analysis to identify metabolites present in the cell free supernatant (CFS) of the *Lactobacillus* isolates which may be responsible for inhibition of *Brachyspira* growth.
- To investigate how physical interactions between *Lactobacillus* and *Brachyspira* may impact *Brachyspira* viability *in vitro*.

6.2 Results

6.2.1 Identifying pH dependent inhibition of *Brachyspira*

The cell free supernatants (CFS) from *L. reuteri* SAP 2114 and 2115 and *L. salivarius* 2116 and 2117 were approximately pH 3.8 after 18 hours of anaerobic culture in MRS broth at 37°C. To differentiate between pH effects and other mechanisms of inhibition, the inhibition assays were performed with CFS at pH 3.8 and CFS neutralised to pH 7.2

To initially determine the pH dependent mechanism of inhibition, *Brachyspira* were cultured in brain heart infusion (BHI) broth supplemented with 10% (v/v) MRS at pH 3.8 and MRS at pH 7.2. A pH dependent mechanism of inhibition was observed for all of the *Brachyspira* isolates tested, except for *B. pilosicoli* SAP 865, as shown in Figure 6.1. The growth of *B. pilosicoli* B2904 and SAP 859, *B. intermedia* SAP 919 and *B. innocens* SAP 924 was significantly reduced when BHI broth was supplemented with MRS at pH 3.8 compared to MRS at pH 7.2 (p value ≤ 0.0001). *B. innocens* SAP 927 and SAP 943 and *B. alvinipulli* ATCC 51933 were also susceptible to a pH 3.8, but to a lesser extent than other *Brachyspira* isolates (p value ≤ 0.05). Interestingly, *B. pilosicoli* SAP 865 was tolerant pH 3.8 as observed in Figure 6.1C, whereby there was no significant difference in bacterial growth when the media was supplemented with MRS at pH 3.8 compared to pH 7.2.

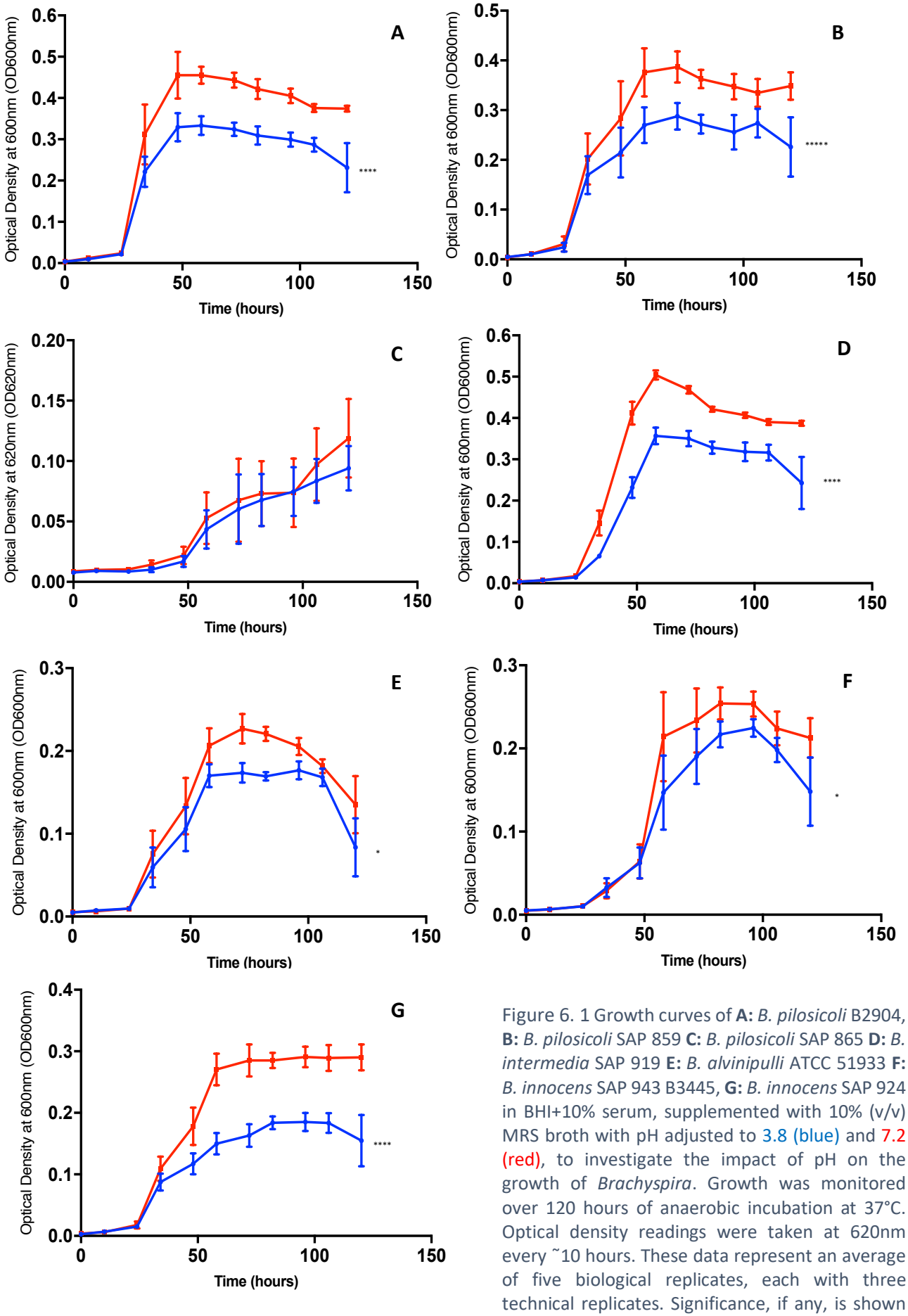


Figure 6. 1 Growth curves of **A:** *B. pilosicoli* B2904, **B:** *B. pilosicoli* SAP 859 **C:** *B. pilosicoli* SAP 865 **D:** *B. intermedia* SAP 919 **E:** *B. alvinipulli* ATCC 51933 **F:** *B. innocens* SAP 943 B3445, **G:** *B. innocens* SAP 924 in BHI+10% serum, supplemented with 10% (v/v) MRS broth with pH adjusted to 3.8 (blue) and 7.2 (red), to investigate the impact of pH on the growth of *Brachyspira*. Growth was monitored over 120 hours of anaerobic incubation at 37°C. Optical density readings were taken at 620nm every ˜10 hours. These data represent an average of five biological replicates, each with three technical replicates. Significance, if any, is shown for pH dependent growth between control groups * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

6.2.2 Identifying pH independent inhibition of *Brachyspira*

To determine the presence of a pH independent mechanism of inhibition (in addition to the pH dependent mechanism), *Brachyspira* were cultured in BHI broth supplemented with 10% (v/v) cell free supernatant at either pH 3.8 or pH 7.2 and growth was compared with the pH matched control (MRS at pH 3.8 or pH 7.2). The extent of pH independent inhibition was dependent on both the isolate of *Lactobacillus* and the isolate of *Brachyspira* it was tested against, as shown in Figure 6.2 and Table 6.1.

B. pilosicoli isolates displayed a range of susceptibilities to *Lactobacillus* cell free supernatant independent of pH as shown in Figure 6.2A-C and Table 6.1. *B. pilosicoli* B2904 was inhibited by all CFSs from each isolate of *Lactobacillus* (p value $\leq$ 0.0001), however the growth of *B. pilosicoli* SAP 859 and 865 was not significantly inhibited by a pH independent mechanism with the exception of SAP 859 which was inhibited by *L. salivarius* SAP 2116 (p value $\leq$ 0.0001).

B. intermedia SAP 919 was also inhibited by all *Lactobacillus* isolates independent of pH (p value $\leq$0.0001) (Figure 6.2D and Table 6.1). Additionally, *L. reuteri* SAP 2114 and 2115 were significantly more inhibitory at pH 3.8 than the *L. salivarius* isolates (p value $\leq$ 0.0001). *L. reuteri* SAP 2115 was also significantly more inhibitory than *L. salivarius* SAP 2116 (p value 0.0004) and SAP 2117 (p value 0.015) at pH 7.2, demonstrating clear phenotypic differences between the *L. reuteri* isolates.

The growth of *B. alvinipulli* ATCC 51933 was significantly reduced by the CFS from *L. reuteri* SAP 2115 and *L. salivarius* SAP 2116 at pH 3.8, compared to the pH matched controls (p value $\leq$0.05) (Figure 6.2E and Table 6.1). However, CFS at pH 7.2 did not significantly affect growth.

The growth of *B. innocens* SAP 943 and SAP 924 was significantly inhibited by all *Lactobacillus* CFSs at pH 3.8 compared to the pH matched control (p value $\leq$ 0.0001). *B. innocens* SAP 943

and SAP 924 were also significantly inhibited by *L. reuteri* SAP 2115 at pH 7.2, compared to control (p value 0.0004, $\leq$ 0.0001, respectively). Additionally, *B. innocens* SAP 924 was significantly inhibited by *L. reuteri* SAP 2114 and SAP 2115 and *L. salivarius* SAP 2117 at both pH 3.8 and pH 7.2 (p value $\leq$ 0.005). *B. innocens* SAP 927 was not included in these experiments because this isolate did not grow sufficiently in broth.

Overall, *L. reuteri* SAP 2115 was the most effective *Lactobacillus* isolate of the panel tested because it had the greatest inhibitory effects against multiple *Brachyspira* isolates (Table 6.1). There was a highly variable tolerance to the CFS amongst *Brachyspira* isolates and no specific *Lactobacillus* isolate was effective against one species of *Brachyspira*. *B. pilosicoli* B2904, *B. intermedia* SAP 919 and *B. innocens* SAP 924 and SAP 943 were the most susceptible to *Lactobacillus* CFS, whereas *B. pilosicoli* SAP 859 and 865 and *B. alvinipulli* ATCC 51933 were mostly tolerant to the CFS. Thus, demonstrating the need to test a range of *Lactobacillus* against a range of *Brachyspira.*

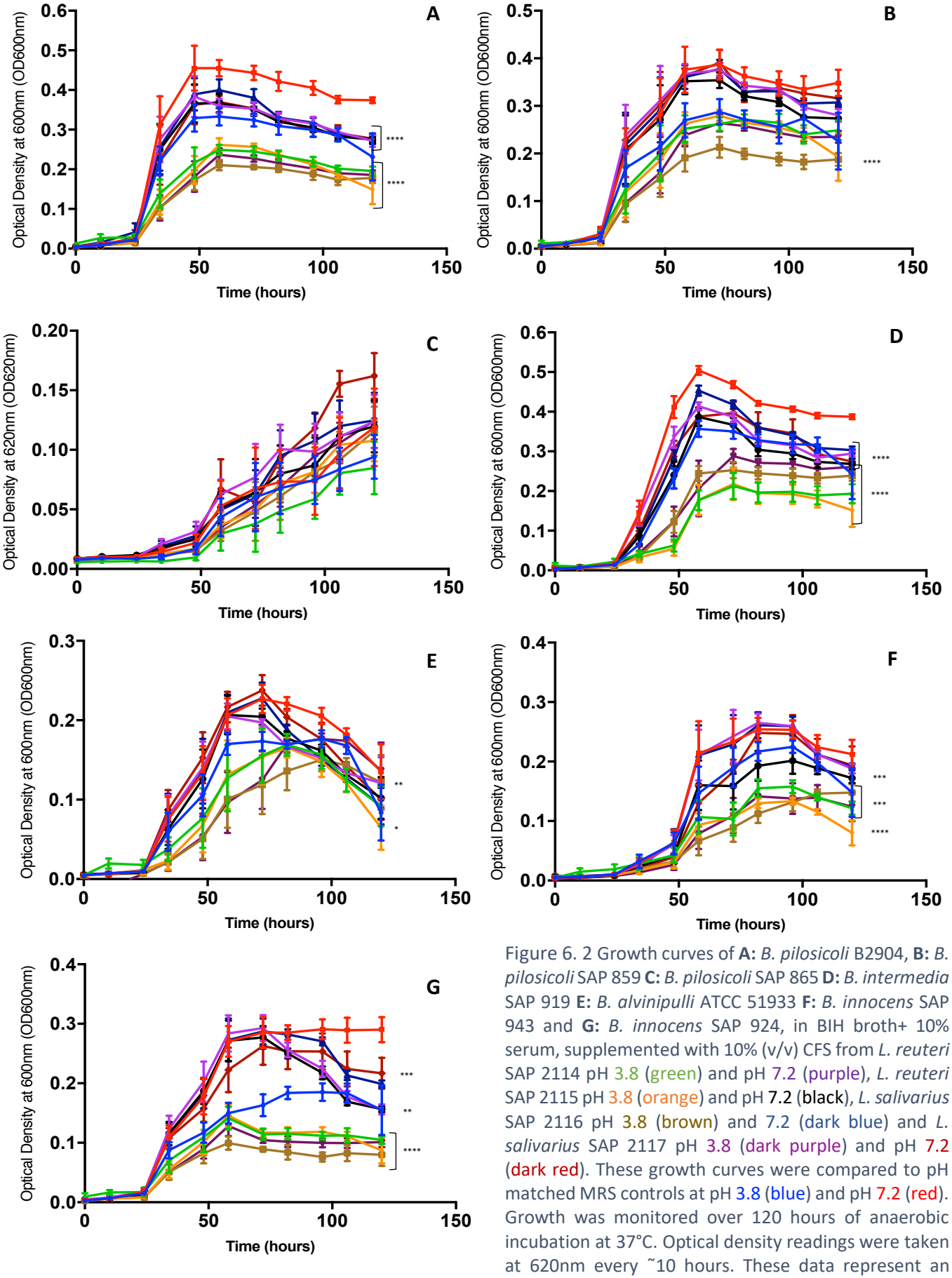


Figure 6. 2 Growth curves of **A:** *B. pilosicoli* B2904, **B:** *B. pilosicoli* SAP 859 **C:** *B. pilosicoli* SAP 865 **D:** *B. intermedia* SAP 919 **E:** *B. alvinipulli* ATCC 51933 **F:** *B. innocens* SAP 943 and **G:** *B. innocens* SAP 924, in BIH broth+ 10% serum, supplemented with 10% (v/v) CFS from *L. reuteri* SAP 2114 pH 3.8 (green) and pH 7.2 (purple), *L. reuteri* SAP 2115 pH 3.8 (orange) and pH 7.2 (black), *L. salivarius* SAP 2116 pH 3.8 (brown) and 7.2 (dark blue) and *L. salivarius* SAP 2117 pH 3.8 (dark purple) and pH 7.2 (dark red). These growth curves were compared to pH matched MRS controls at pH 3.8 (blue) and pH 7.2 (red). Growth was monitored over 120 hours of anaerobic incubation at 37°C. Optical density readings were taken at 620nm every ~10 hours. These data represent an average of five biological replicates, each with three technical replicates. Significance, if any, is shown for pH dependent growth between control groups * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

Table 6. 1 P values for the *Brachyspira* CFS inhibition assays presented in Figure 5.2. The area under the curve was calculated for each of the *Brachyspira* growth curves supplemented with the CFS from each of the four probiotic candidates at either pH 3.8 or pH 7.2. Statistical significance was calculated using a one-way ANOVA and data were highlighted in green if the p value was ≤ 0.05 and highlighted in red if the p value was > 0.05.

		P- Value ≤ 0.05						
		B. pilosicoli B2904	*B. pilosicoli* SAP 859	*B. pilosicoli* SAP 865	*B. intermedia* SAP 919	*B. alvinipulli* ATCC 51933	*B. innocens* SAP 943	*B. innocens* SAP 924
pH 3.8	MRS vs. SAP 2114 CFS	0.0001	0.9865	0.7107	0.0001	0.4054	0.0002	0.0001
	MRS vs. SAP 2115 CFS	0.0001	0.7829	0.999	0.0001	0.0419	0.0001	0.0001
	MRS vs. SAP 2116 CFS	0.0001	0.0001	0.999	0.0001	0.0035	0.0001	0.0001
	MRS vs. SAP 2117 CFS	0.0001	0.2341	0.999	0.0001	0.1687	0.0001	0.0001
pH 7.2	MRS vs. SAP 2114 CFS	0.0001	0.9995	0.4356	0.0001	0.1731	>0.9999	0.0053
	MRS vs. SAP 2115 CFS	0.0001	0.2607	0.4356	0.0001	0.0951	0.0004	0.0001
	MRS vs. SAP 2116 CFS	0.0001	0.9824	0.456	0.0001	0.3186	>0.9999	0.222
	MRS vs. SAP 2117 CFS	0.0001	>0.9999	0.4356	0.0001	0.9983	0.0654	0.0004

6.2.3 Investigating the mechanism of pH independent inhibition of *Brachyspira*

To explore the pH independent mechanisms of inhibition presented in these studies in more detail, *Lactobacillus* cell free supernatants were treated with trypsin or heat inactivated to denature any proteins that may have been responsible for *Brachyspira* inhibition. The hypothesis was that proteins, such as those identified in Chapter 5 may contribute to the inhibition of *Brachyspira*. Both trypsin and heat treatment were used to ensure the removal of heat stable proteins such as class I and class II bacteriocins and heat labile proteins such as bacteriolysins as identified in Tables 5.4 and 5.5 in Chapter 5. The data from these experiments are summarised in Figure 6.3, Appendix IV and Tables 6.2 and 6.3.

Trypsin treatment of the *L. reuteri* SAP 2114 only had an impact on *Brachyspira* inhibition at pH 3.8. The inhibition of *B. pilosicoli* B2904, *B. intermedia* SAP 919 and *B. innocens* SAP 924 was significantly reduced with this treatment (p value ≤ 0.0001, ≤ 0.0001 and 0.0445, respectively). Heat treatment of this CFS increased to inhibition of all *Brachyspira* isolates

except for *B. intermedia* SAP 919, where the inhibition was significantly reduced (p value 0.0065).

The trypsin treatment of *L. reuteri* SAP 2115 had an impact on *Brachyspira* inhibition at both pH 3.8 and 7.2; the inhibition of *B. intermedia* SAP 919 was significantly reduced at both pH 3.8 (p value $\leq$ 0.0001) and 7.2 (p value $\leq$ 0.0001), in addition to the inhibition of *B. innocens* SAP 943 being reduced at pH 7.2 (p value 0.0282). Interestingly, heat treatment of *L. reuteri* SAP 2115 CFS increased the inhibition of all *Brachyspira* isolates whereby the growth of each isolate was below the limit of detection.

Treatment of *L. salivarius* SAP 2116 with trypsin resulted in decreased inhibition of *B. pilosicoli* SAP 859 and *B. innocens* SAP 924 at pH 3.8 (p value 0.0039 and 0.0002, respectively), however this treatment had no effect on CFS at pH 7.2. Heat treatment of this CFS did not have any impact on the inhibition of any *Brachyspira* isolates tested.

Neither trypsin or heat treatment of *L. salivarius* SAP 2117 had a significant impact on the inhibition of *Brachyspira* isolates at pH 3.8. However, at pH 7.2 trypsin treatment significantly reduced the inhibition of *B. intermedia* SAP 919 and *B. innocens* SAP 943 and 924 (p value 0.0214, $\leq$ 0.00001 and 0.0052, respectively). Heat treatment of the CFS significantly reduced the inhibition of *B. pilosicoli* B2904, *B. intermedia* SAP 919 and *B. innocens* SAP 943 and 924 (0.003, $\leq$ 0.0001, 0.0027 and $\leq$ 0.0001, respectively).

It is important to note that *B. pilosicoli* SAP 865 was not tested in these assays due to there being no evidence of pH independent inhibition.

To summarise, these studies were used to determine whether the mechanism of *Brachyspira* inhibition by *Lactobacillus* CFS was in part, attributed to proteinaceous compounds such as bacteriocins. Putative bacteriocin genes were identified following whole genome sequence analysis, however the presence of these genes was not necessarily indicative of translated

protein. Therefore, denaturation of these potential proteins using trypsin and heat treatment was used to further investigate the inhibition of *Brachyspira.* It was evident from these data that the effect of these treatments on the *Lactobacillus* CFS was very much dependent on the isolate of *Lactobacillus* and the target *Brachyspira* isolate. Overall, trypsin treatment of *L. reuteri* CFSs resulted in reduced *Brachyspira* inhibition, suggesting the inhibitory role of bacteriocins. However, heat treatment increased *Brachyspira* inhibition. Trypsin treatment of *L. salivarius* SAP 2116 decreased *Brachyspira* inhibition, but heat treatment had no effect, suggesting that heat stable bacteriocins may contribute to inhibition. Treatment of *L. salivarius* SAP 2117 CFS had no effect on *Brachyspira* inhibition, therefore proteins did not play a role in inhibition by this isolate. Therefore, the hypothesis was that this inhibition could be attributed to non-protein metabolites produced by the *Lactobacillus* isolates. Thus, NMR analysis was employed to identify metabolites in the cell free supernatant of the four *Lactobacillus* isolates to determine which, if any, may be responsible for *Brachyspira* inhibition.

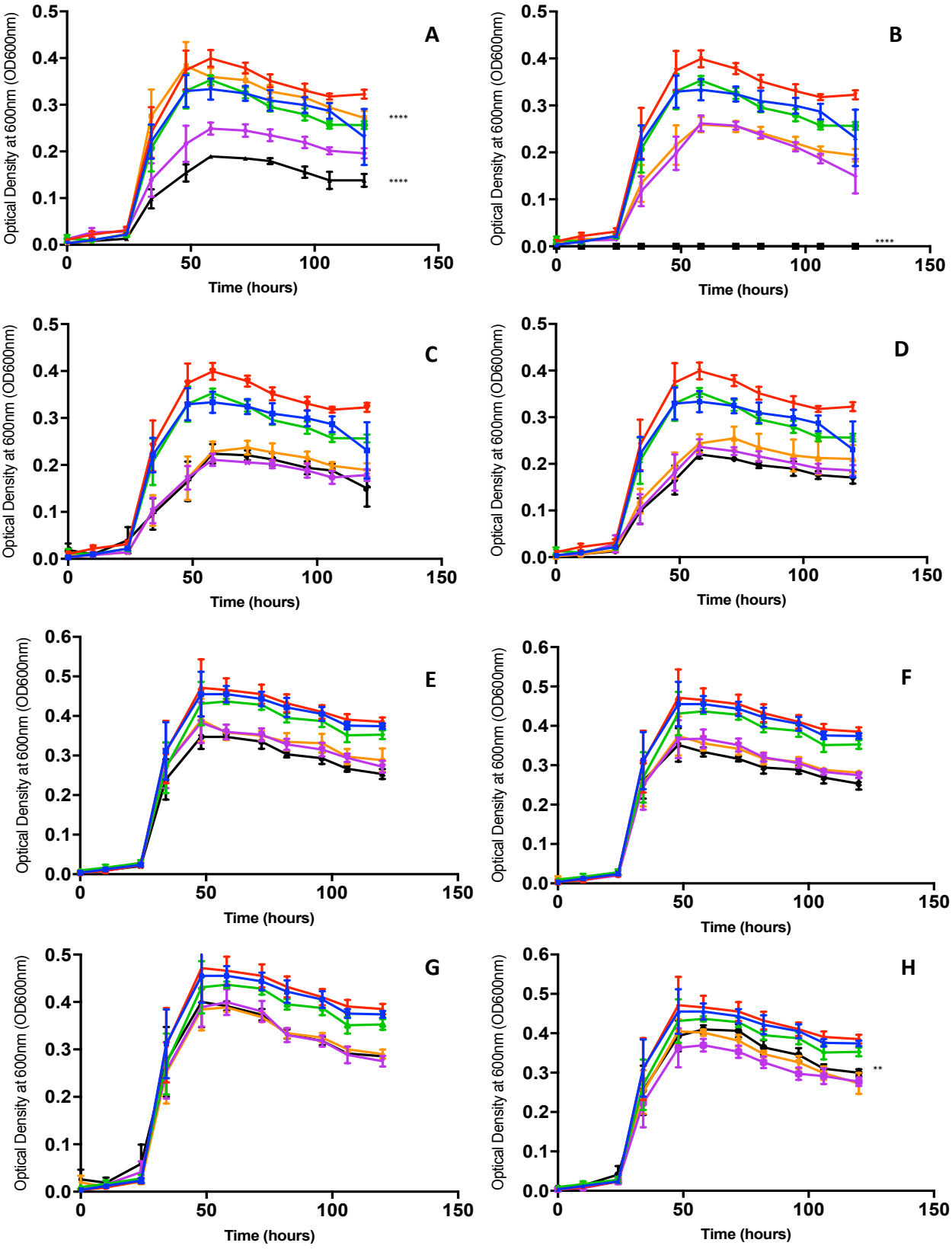


Figure 6. 3 Growth curves of *B. pilosicoli* B2904 supplemented with **A:** *L. reuteri* SAP 2114 CFS at pH 3.8 **B:** *L. reuteri* SAP 2115 CFS at pH 3.8 **C:** *L. salivarius* SAP 2116 CFS at pH 3.8 **D:** *L. salivarius* SAP 2117 CFS at pH 3.8 **E:** *L. reuteri* SAP 2114 CFS at pH 7.2 **F:** *L. reuteri* SAP 2115 CFS at pH 7.2 **G:** *L. salivarius* SAP 2116 CFS at pH 7.2 **H:** *L. salivarius* SAP 2117 CFS at pH 7.2 in BIH broth+ 10% serum. Each CFS was either treated with trypsin or heat treated to denature potential antimicrobial proteins produced by each *Lactobacillus* isolate. MRS (blue), MRS trypsin treated (red), MRS heat treated (green), CFS (purple), CFS trypsin treated (orange) and CFS heat treated (black). Growth was monitored over 120 hours of anaerobic incubation at 37°C. Optical density readings were taken at 620nm every ˜10 hours. These data represent an average of five biological replicates, each with three technical replicates. Significance, if any, is shown for changes in growth when comparing CFS without treatment to CFS with either trypsin or heat treatment * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

Table 6. 2 P values for the *Brachyspira* inhibition assays where *Lactobacillus* CFS was treated with trypsin prior to utilisation in these assays. The area under the curve was calculated for each of the growth curves shown in Figure 6.3 and Appendix IV and growth of *Brachyspira* was compared between the untreated CFS and the CFS treated with trypsin to determine if denaturation of proteins impacted *Brachyspira* growth. Statistical significance was calculated using a one-way ANOVA and data were highlighted in green if the p value was ≤ 0.05 and highlighted in red if it was > 0.05.

Trypsin Treated		P value < 0.05			
		SAP 2114	SAP 2115	SAP 2116	SAP 2117
pH 3.8	*B. pilosicoli* B2904	0.0001	0.9965	0.4261	0.4575
	B. pilosicoli SAP 859	0.6728	0.832	0.0039	0.9875
	B intermedia SAP 919	0.0001	0.0001	0.8124	0.597
	B. alvinipulli ATCC 51933	0.9946	0.986	0.0938	0.887
	B. innocens SAP 943	0.0524	0.3638	0.8582	0.1473
	B. innocens SAP 924	0.0455	0.672	0.0002	0.4661
pH 7.2	*B. pilosicoli* B2904	0.968	0.9999	0.9476	0.0854
	B. pilosicoli SAP 859	0.2887	0.8276	0.9906	0.9999
	B intermedia SAP 919	0.999	0.0001	0.2824	0.0214
	B. alvinipulli ATCC 51933	0.053	0.999	0.997	0.9909
	B. innocens SAP 943	0.4222	0.0282	0.9939	0.0001
	B. innocens SAP 924	0.999	0.9538	0.999	0.0052

Table 6. 3 P values for the *Brachyspira* inhibition assays where *Lactobacillus* CFS was heat treated prior to utilisation in these assays. The area under the curve was calculated for each of the growth curves shown in Figure 6.3 and Appendix IV and growth of *Brachyspira* was compared between the untreated CFS and the heat treated CFS to determine if denaturation of proteins impacted *Brachyspira* growth. Statistical significance was calculated using a one-way ANOVA and data were highlighted in green if the p value was ≤ 0.05 and highlighted in red if it was > 0.05.

Heat Treated		P value < 0.05			
		SAP 2114	SAP 2115	SAP 2116	SAP 2117
pH 3.8	*B. pilosicoli* B2904	0.0001	0.0001	0.9329	0.4641
	B. pilosicoli SAP 859	0.0021	0.0001	0.3006	0.9999
	B intermedia SAP 919	0.0065	0.0001	0.0776	0.996
	B. alvinipulli ATCC 51933	0.0092	0.0001	0.0978	0.99
	B. innocens SAP 943	0.7062	0.0001	0.2479	0.0999
	B. innocens SAP 924	0.0001	0.0001	0.5591	0.9938
pH 7.2	*B. pilosicoli* B2904	0.1095	0.3733	0.998	0.003
	B. pilosicoli SAP 859	0.7972	0.9853	0.999	0.9974
	B intermedia SAP 919	0.999	0.9856	0.298	0.0001
	B. alvinipulli ATCC 51933	0.9718	0.857	0.992	0.9579
	B. innocens SAP 943	0.9486	0.0009	0.9529	0.0027
	B. innocens SAP 924	0.5656	0.999	0.3113	0.0001

6.2.4 NMR

The data presented in section 6.2.3 suggested that a pH independent mechanism, other than bacteriocin production in the CFS may have also been responsible for *Brachyspira* inhibition. Therefore, this mechanism was further explored using a metabolomic approach to investigate the metabolites present in *Lactobacillus* cell free supernatant which may have contributed to *Brachyspira* inhibition.

A nuclear magnetic resonance (^{1}H- NMR) based approach was employed to determine the small metabolites present in the cell free supernatants of the ten *Lactobacillus* isolates presented in Chapter 5, following 18 hours of anaerobic culture at 37°C. This was coupled with multivariant analysis to determine if taxonomic differences and antimicrobial activity against *Brachyspira* could be predicted from the *Lactobacillus* metabolic profiles.

From this analysis, six metabolites were clearly identified in adequate quantities as illustrated in the NMR spectra presented in Figure 6.6. The most abundant metabolites were acetate, which appeared as a singlet peak at 1.91, lactate, which appeared as a quartet peak at 4.11 and a doublet peak at 1.33 and ethanol, which appeared as two quartet peaks at 3.6 and 1.2. The abundance of these metabolites was expected due to the fermentative metabolic pathways utilised by these bacteria (Caplice and Fitzgerald, 1999). *Lactobacillus* species can be homofermentative or heterofermentative and this could be easily identified from the spectra shown in Figure 6.6. Homofermentative *Lactobacillus* species such as *L. salivarius* and *L. crispatus* fermented sugars to produce only lactic acid and heterofermentative *Lactobacillus* species such as *L. reuteri* fermented sugars to produce lactic acid and ethanol, these metabolic pathways are illustrated in Figure 6.4. Other metabolites, identified in smaller quantities were succinate, which appeared as a singlet peak at 2.41 and acetoin, which appeared as a singlet peak at 2.2. These metabolic pathways are illustrated in Figure 6.5,

whereby pyruvate is metabolised in several different pathways to produce succinate and acetoin, in addition to acetate, lactic acid and ethanol.

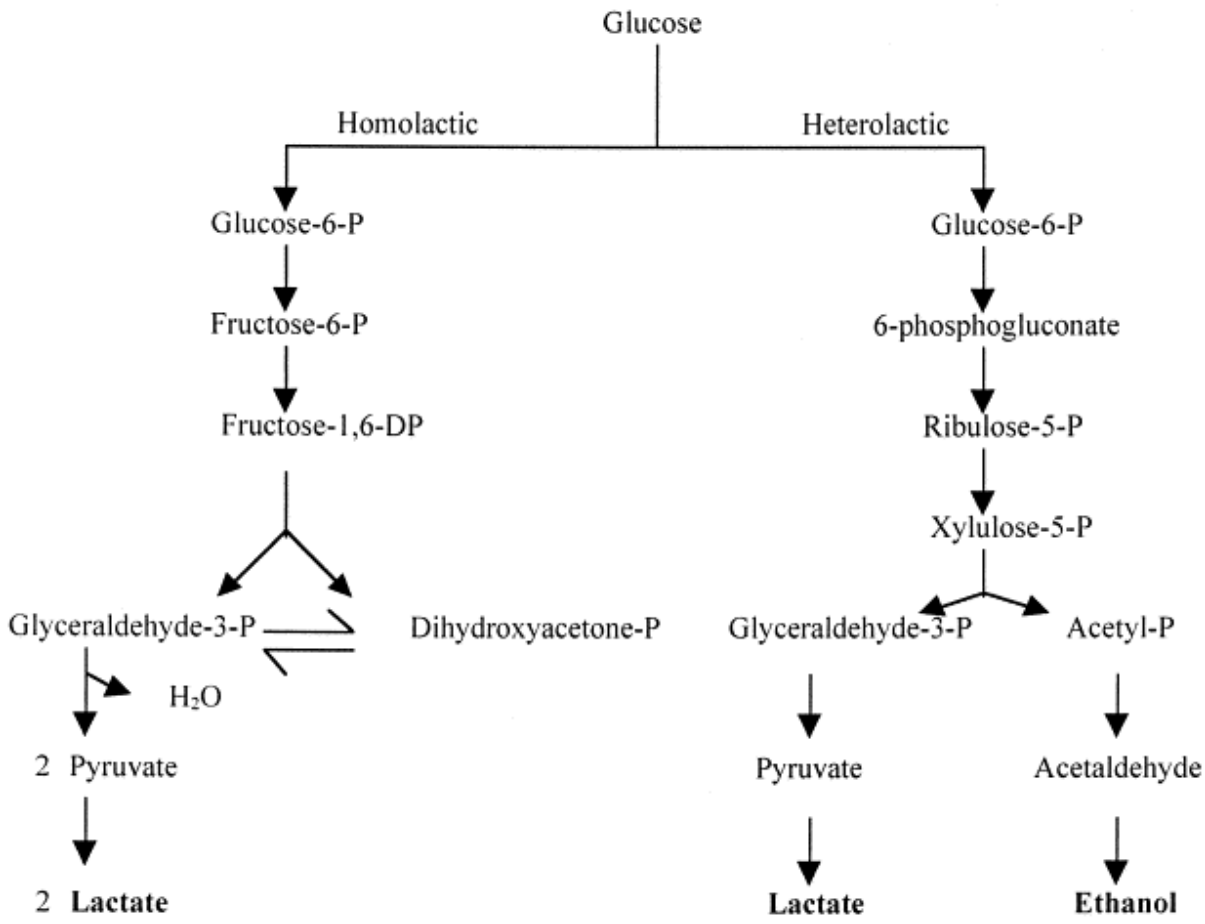


Figure 6. 4 The pathways by which glucose is metabolised by *Lactobacillus*. Homofermenters such *L. salivarius* and *L. crispatus* produce lactic acid as a major product of glucose fermentation. These species use the glycolysis pathway to generate two moles of lactate per mole of glucose. Heterofermenters such as *L. reuteri* produce one mole of lactate, ethanol and CO2 per mole of glucose via the pentose phosphate pathway. These two groups of *Lactobacillus* can be easily distinguished using NMR as *L. reuteri* produced high concentrations of ethanol (Caplice and Fitzgerald, 1999).

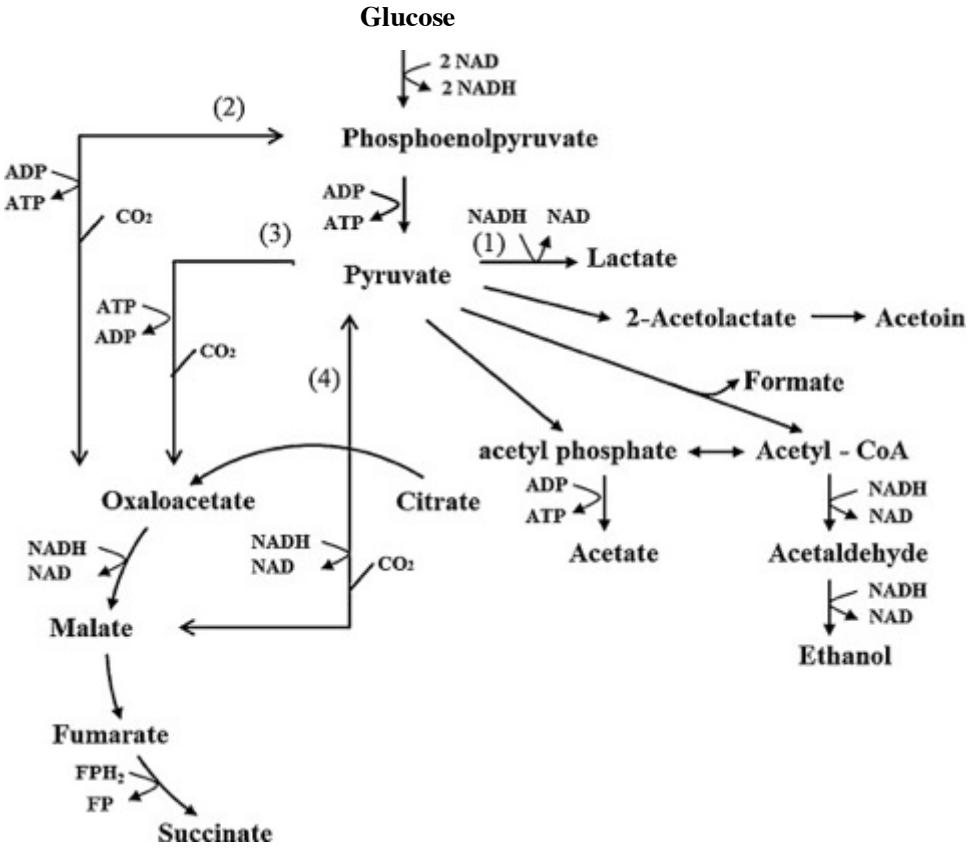


Figure 6. 5 The metabolic pathways of *Lactobacillus*, highlighting the pathways involved in the production of lactate, acetate, ethanol, acetoin and succinate (Tsuji *et al.*, 2013).

The observable differences between the metabolic profiles of the *Lactobacillus* isolates were not necessarily in the types of metabolites produced, as these are fairly consistent within the isolates tested, but in the differing concentrations in which these metabolites were produced. This was demonstrated in Figure 6.6, where six spectra are overlaid to illustrate that inhibition of *Brachyspira* may be correlated to the concentration of metabolites produced by different *Lactobacillus* isolates. These six spectra were from the CFS of *L. reuteri* SAP 2114 and 2115, *L. salivarius* SAP 2116 and 2116, *L. crispatus* SAP 2107 and the MRS media. As previously discussed, these *L. reuteri* and *L. salivarius* isolates were shown to be the most inhibitory against *Brachyspira* out of the panel of *Lactobacillus* isolates tested. Conversely the *L.*

crispatus SAP 2107 isolate was shown to be the poorest inhibitor of *Brachyspira*, as indicated in Table 5.6. On comparing these two phenotypes, it could be noted that these differences in inhibition may be, in part, attributed to the concentrations of the metabolites produced. For example, *L. crispatus* SAP 2107 produced a lactate concentration of 10mM, the lowest concentration of all of the isolates tested and no acetoin or succinate. *L. reuteri* SAP 2114 produced one of the highest concentrations of acetate, 38.50mM and the highest succinate concentration, 1.23mM. The lactate production by this isolate was the lowest of the isolates selected as potential probiotics at a concentration of 24mM, however this was still nearly 2.5x higher than the concentration produced by *L. crispatus* SAP 2107, the poor inhibitor of *Brachyspira*.

L. reuteri SAP 2115 was previously shown to be the best inhibitor of *Brachyspira* and produced the highest concentration of acetate of all isolates tested, 39.33mM. This isolate also produced one of the highest concentrations of lactate of all of the isolates tested, 27.67mM and the highest concentration of ethanol of the *L. reuteri* isolates tested, 70.67mM. This isolate did not produce any detectable succinate or acetoin.

L. salivarius SAP 2116 produced the lowest concentration of acetate, 25mM but produced one of the highest concentrations of lactate, 26mM, of all isolates selected at potential probiotic candidates. Low concentrations of succinate, 0.59mM and acetoin, 1.5mM were also detected. *L. salivarius* SAP 2117 was the most inhibitory of the *L. salivarius* isolates tested and produced a high acetate concentration, 29.67mM and the highest lactate concentration of all isolates tested, 28mM. Additionally, this isolate produced the highest concentration of acetoin, 2.07mM and a small amount of succinate, 0.63mM.

Table 6. 4 The concentrations of the key metabolites determined using NMR analysis. The key metabolites identified were acetate, lactate, ethanol, acetoin, succinate and glucose. These concentrations were calculated using Chenomx and are presented as averages of three biological replicates for the four potential probiotics selected in Chapter 4; *L. reuteri* SAP 2114 and 2115 and *L. salivarius* SAP 2116 and 2117. These values were compared to a *Lactobacillus* isolate unable to inhibit *Brachyspira*; *L. crispatus* SAP 2017.

		Metabolite concentration (mM)					
		Acetate	Lactate	Ethanol	Glucose	Acetoin	Succinate
***Lactobacillus* species**	**Isolate number**						
L. crispatus	SAP 2107	26.00	10.00	0.00	46.67	0.00	0.00
L. reuteri	SAP 2114	38.50	24.00	48.5	26.50	0.00	1.23
L. reuteri	SAP 2115	39.33	27.67	70.67	21.00	0.00	0.00
L. salivarius	SAP 2116	25.00	26.00	0.00	31.00	1.50	0.59
L. salivarius	SAP 2117	29.67	28.00	0.00	40.33	2.07	0.63

Glucose was the most abundant, detectible carbon source present in the MRS media, shown in yellow in Figure 6.6. On the NMR spectra, glucose appeared as fourteen separate peaks between 3.2 and 5.2 and each *Lactobacillus* isolate was shown to utilise glucose, indicated by a depletion in concentration after 18 hours of culture. Glucose utilisation varied depending on the isolate of *Lactobacillus* and low levels of glucose utilisation often resulted in lower concentrations of metabolite production. Interestingly, *L. reuteri* SAP 2115 CFS had the most inhibitory effects against *Brachyspira* and was also one of the highest utilisers of glucose in the panel of isolates tested. *L. crispatus* SAP 2107 CFS (shown in Table 6.4 and illustrated in Figure 6.6) was one of the weakest inhibitors of *Brachyspira* at a low pH and was also one of the poorest utilisers of glucose compared to all of the *Lactobacillus* isolates tested.

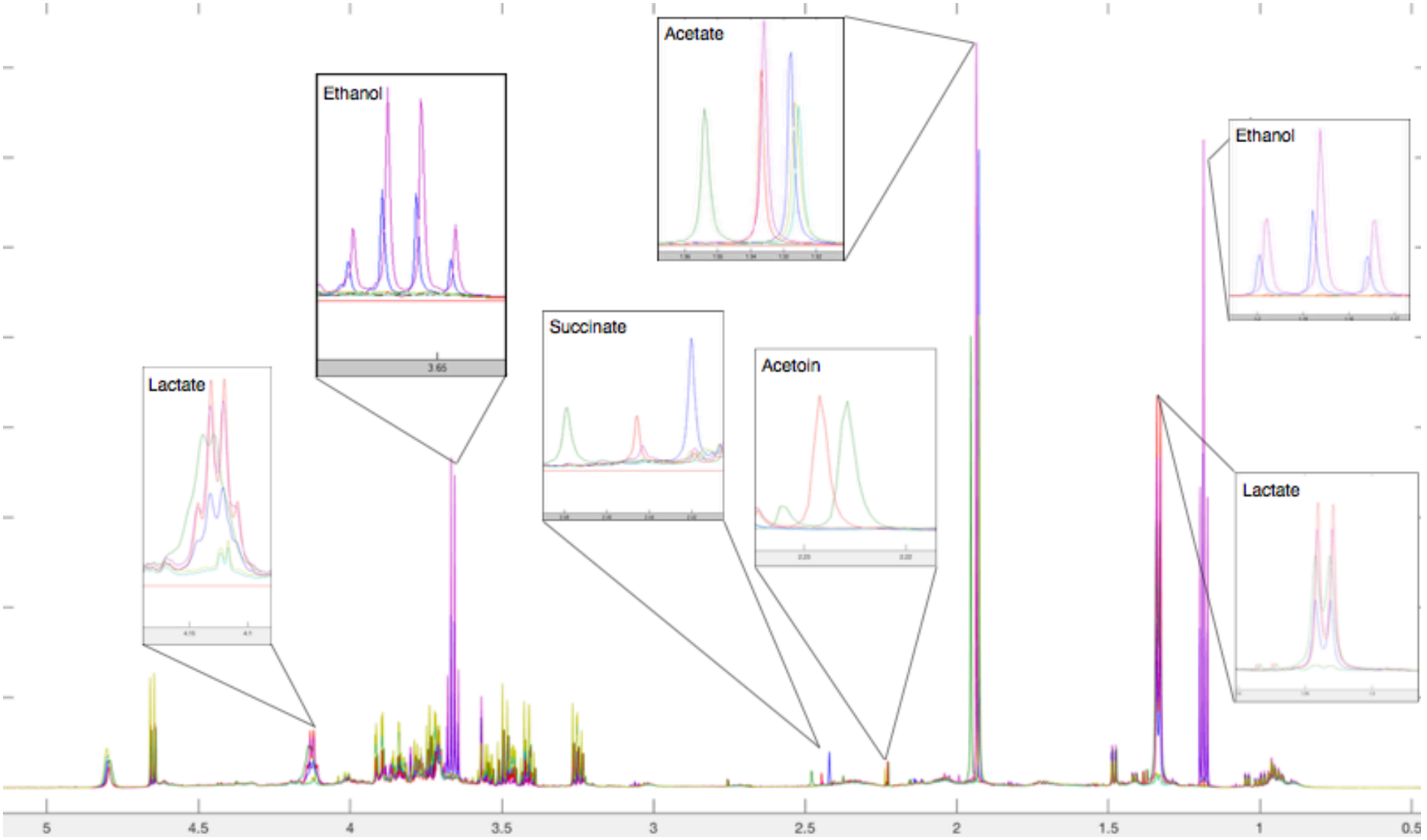


Figure 6. 6 H^{1} Nuclear magnetic resonance (NMR) spectra of *Lactobacillus* metabolites. The cell free supernatants from all *Lactobacillus* isolates underwent NMR analysis to determine metabolite production following 18 hours of anaerobic culture at 37°C. As an example, six spectra are presented (overlaid) above: the four *Lactobacillus* isolates selected as potential probiotic candidates: *L. reuteri* SAP 2114 (blue), *L. reuteri* SAP 2115 (purple), *L. salivarius* SAP 2116 (green) and *L. salivarius* SAP 2117 (red). In addition to one lactobacilli that was unable to inhibit *Brachyspira: L. crispatus* SAP 2017 (teal) and the MRS media control (yellow). The metabolites lactate, acetate, ethanol, succinate and acetoin are highlighted as potential metabolites that contributed to *Brachyspira* inhibition.

Having identified the metabolites present in the *Lactobacillus* cell free supernatant using NMR, principle component analysis was conducted with samples from each of the ten *Lactobacillus* isolates fully characterised in Chapter 5. Recent studies have used NMR to measure the taxonomic differences between *Lactobacillus* isolates and to predict their antimicrobial activity against pathogens (Parolin *et al.*, 2015; Nardini *et al.*, 2016). This approach was applied in the studies presented here to determine if the metabolic profiles of *Lactobacillus* isolates could be used to predict inhibition of *Brachyspira.*

The first principle component (PC1) and the second principle component (PC2) accounted for 58% of the total variance of the investigated samples, as shown in Figure 6.7. This multivariant analysis showed an interesting correlation which has been previously reported in *Lactobacillus* species (Parolin *et al.*, 2015; Nardini *et al*., 2016). The metabolome correlated with the taxonomy; this was illustrated by the separation of the different *Lactobacillus* species in PC1, which explained 38% of the variance. The clearest separation could be observed with the *L. crispatus* isolates, which were high in PC1, with the exception of SAP 2105. This showed variance from *L. reuteri* and *L. salivarius* which were low in PC1. Some separation between *L. reuteri* and *L. salivarius* was demonstrated, but not sufficiently to distinguish between the species. Parolin *et al*. (2015) also reported that the variance in PC2 correlated with the inhibition of the pathogens, however in the studies presented here, there was insufficient data to support this hypothesis, although there was some separation in PC2 according to the *Brachyspira* inhibition. For example, L. *salivarius* SAP 2116 and 2117 were low in PC2 and *L. crispatus* SAP 2107 was high in PC2, thus suggesting that those isolates high in PC2 may be less inhibitory. However, *L. reuteri* SAP 2115, the most inhibitory isolate, was in the middle of PC2, suggesting that the principle component analysis was able to separate good and bad

inhibitors of *Brachyspira* but not able to separate the extent of inhibition in the good inhibitors.

Following the principle component analysis, a colour plot was generated to identify the metabolites responsible for the variance observed in PC1, as seen in Figure 6.8. It was evident that there were two key differences between the cluster of *L. reuteri* and *L. salivarius* (both low in PC1) and *L. crispatus* (high in PC1). The first difference was in the production of lactate, whereby *L. reuteri* and *L. salivarius* isolates produced significantly more lactate than *L. crispatus*. The exception to this was *L. crispatus* 2105; two of the three biological replicates clustered with the majority of the *L. salivarius* isolates, however one replicate clustered with the remaining *L. crispatus* isolates. This highlights the need for more biological replicates to strengthen these data; it is proposed that at least six replicates it optimal. The second difference was in the glucose utilisation, whereby *L. reuteri* and *L. salivarius* isolates utilised more glucose than *L. crispatus* isolates. Therefore suggesting, as previously mentioned, that isolates capable of utilising more glucose were more inhibitory towards *Brachyspira*. Furthermore, the utilisation of glucose is directly linked to the production of lactate, thus the more glucose consumed, the higher the lactate concentration. However, when analysing the raw data, it was clear that other factors, such as acetate production may play a role in *Brachyspira* inhibition. Although the production of ethanol was associated with isolates low in PC1, this metabolite was not significant in highlighting the difference in PC1. This is most likely because isolates low in PC1 were both *L. reuteri* and *L. salivarius* and only *L. reuteri* isolates produce ethanol as discussed earlier in the chapter.

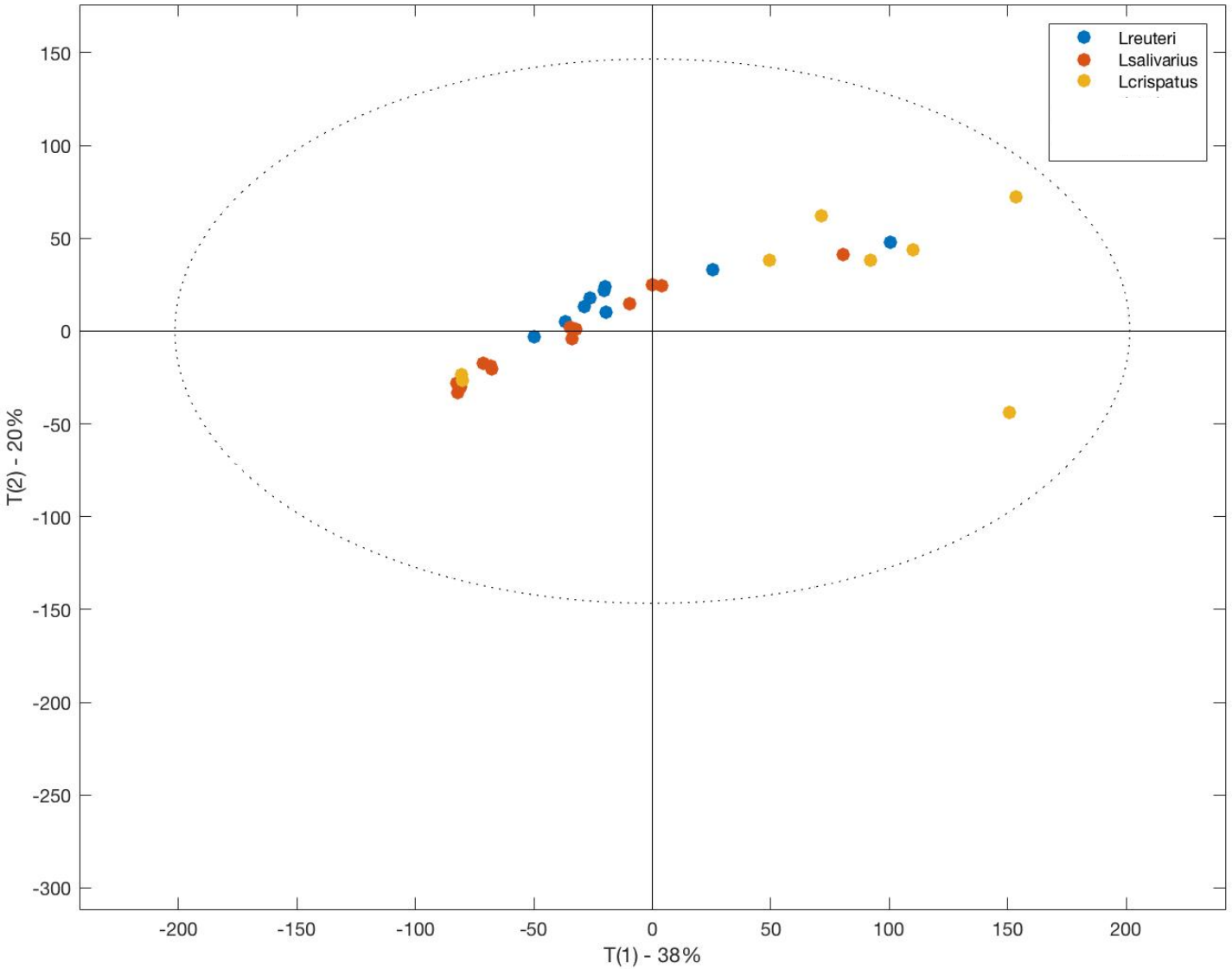


Figure 6. 7 PCA score plot for *Lactobacillus* cell free supernatant samples. The plot shows the distribution of eleven isolates from three species of *Lactobacillus*, five isolates of *L. salivarius* (orange), three isolates of *L. crispatus* (yellow) and three isolates of *L. reuteri* (blue) in principle component 1 (PC1) and principle component 2 (PC2). These two principle components account for 58% of the total variance between the metabolite spectra, with PC1 totally 38% of the variance and PC2 totalling 20% of the variance. These data show three biological replicates of all of the *Lactobacillus* CFSs tested.

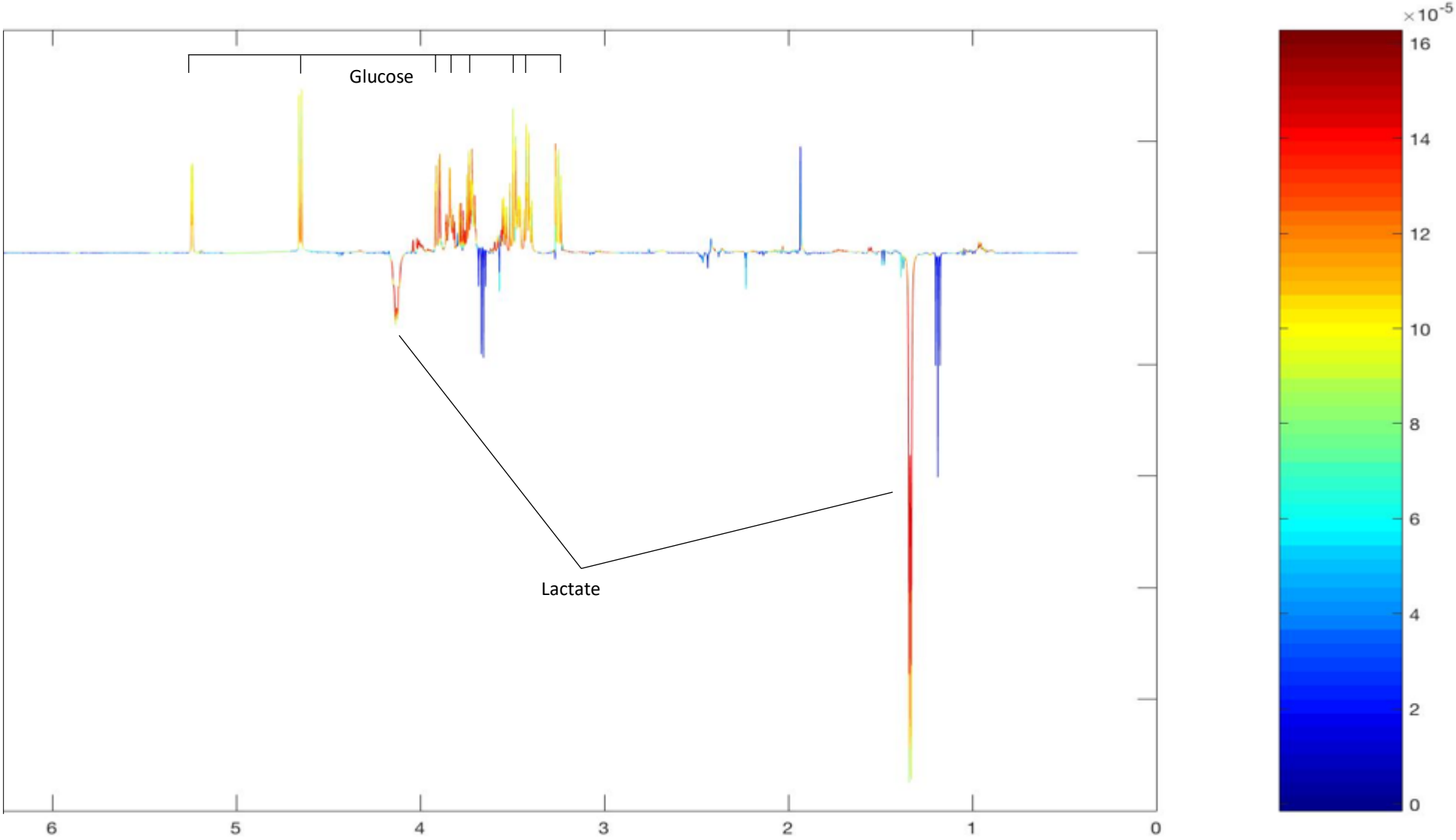


Figure 6. 8 Associated metabolites of the principle component 1 (PC1). PC1 accounted for the most variance between the metabolic profiles of the *Lactobacillus* isolates, there were two clear clusters of isolates in PC1, the *L. reuteri*/*L. salivarius* cluster, which was low in PC1 and the *L. crispatus* cluster, which was high in PC1. The metabolites associated with this variance were identified as lactate, associated with the *L. reuteri*/*L. salivarius* cluster (indicated by the downwards peaks) and glucose, associated with the *L. crispatus* cluster (indicated by the upwards peaks). Other peaks, identified in blue were indicative of ethanol and acetate, however these differences were not significant.

6.2.5 *Lactobacillus* antagonise the viability and growth of *Brachyspira*

In addition to investigating the effects of cell free supernatant and the secreted metabolites by *Lactobacillus* isolates, the effects of *Lactobacillus* cells on the viability and growth of *Brachyspira* were also determined using the 'spot test' method described by Bernardeau *et al.* (2009). Unlike the data presented by Bernadeau *et al.* (2009), there were no differences between the viability of *Brachyspira* when co-cultured with *Lactobacillus* for 4 hours and 24 hours, therefore the results for 4 hours were presented in Figure 6.9.

As predicted, the results from the 'spot test' assay showed that the inhibition of *Brachyspira* was dependent on the isolate of *Lactobacillus* tested. The *L. reuteri* isolates SAP 2114 and 2115 were the most inhibitory against all *Brachyspira* isolates, significantly inhibiting seven of the eight *Brachyspira* isolates when viable cells were used (*B. pilosicoli* B2904 p value 0.0003, *B. pilosicoli* SAP 865, p value $\leq$0.0001, *B. intermedia* SAP 919, p value 0.0006, *B. alvinipulli* ATCC 51933, p value $\leq$ 0.0001 and *B. innocens* SAP 924, SAP 927 and SAP 943, p value $\leq$ 0.0001). Co- culture with viable *L. reuteri* SAP 2114 and 2115 isolates prevented the growth of *B. pilosicoli* SAP 865, *B. alvinipulli* ATCC 51933 and *B. innocens* SAP 924, SAP 927 and SAP 943. Therefore, it was evident that live *L. reuteri* isolates were able to inhibit all *Brachyspira* species implicated in poultry infection, but not all isolates within a species, as *B. pilosicoli* SAP 859 was not inhibited by any *Lactobacillus* isolate. Furthermore, heat-inactivated *L. reuteri* SAP 2114 and SAP 2115 were able to significantly inhibit six of the eight *Brachyspira* isolates, namely *B. pilosicoli* B2904 (p value 0.039) and SAP 865 (p value $\leq$ 0.0001), *B. alvinipulli* ATCC 51933 (p value $\leq$ 0.0001) and *B. innocens* SAP 943, SAP 924 and SAP 927 (p value $\leq$ 0.0001). Of the *L. salivarius* isolates, only SAP 2117 significantly inhibited *B. pilosicoli* B2904, both when the cells were viable (p value 0.0072) and heat-inactivated (p value 0.0314). This demonstrated that physical interactions between *L. salivarius* and *Brachyspira* species were

not as inhibitory as with *L. reuteri* and that once again the *L. reuteri* isolates were more inhibitory against *Brachyspira* and therefore, have the potential to be the most suitable probiotic candidates for an intervention against avian intestinal spirochaetosis.

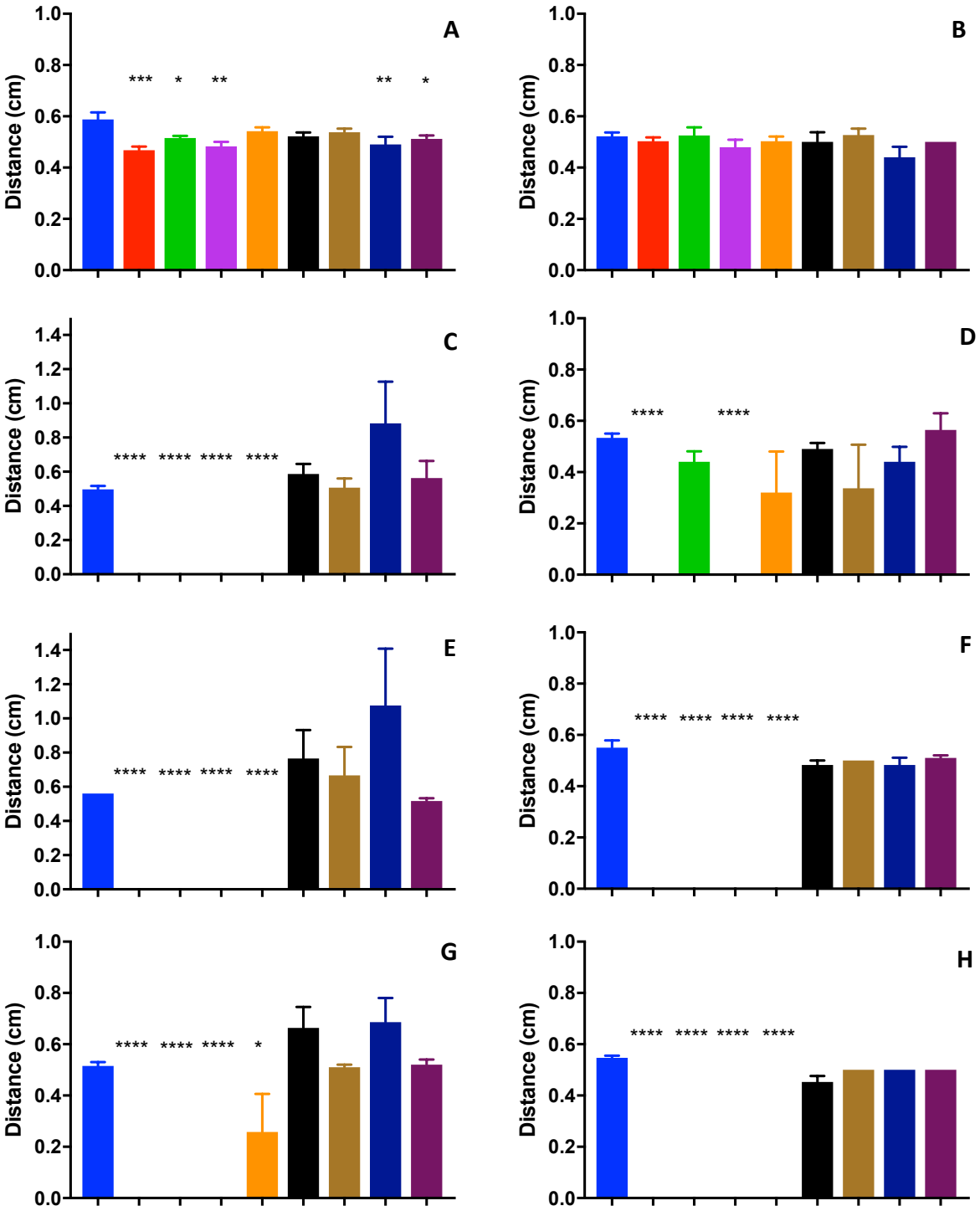


Figure 6. 9 The effect of live and heat inactivated *Lactobacillus* isolates on the viability of A: *B. pilosicoli* B2904, B: *B. pilosicoli* SAP 859, C: *B. pilosicoli* SAP 865, D: *B. intermedia* SAP 919, E: *B. alvinipulli* ATCC 51933, F: *B. innocens* SAP 943, G: *B. innocens* SAP 924 and H: *B. innocens* SAP 927 after four hours of co-incubation with *L. reuteri* SAP 2114 live cells (red) or heat inactivated cells (green), *L. reuteri* SAP 2115 live cells (purple) or heat inactivated cells (orange), *L. salivarius* SAP 2116 live cells (black) or heat inactivated cells (brown) and *L. salivarius* SAP 2117 live cells (dark blue) or heat inactivated cells (dark purple). *Brachyspira* culture with PBS was used as a control (blue). These data are an average of five biological replicates, each with three technical replicates. Significance, if any was determined by a one-way ANOVA and is shown for the reduction of *Brachyspira* viability compared to culture with PBS alone, * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

6.2.6 Efficacy of probiotic candidates against other avian pathogens

The four probiotic candidates presented in these studies were shown to be inhibitory against *Brachyspira*, however in addition their efficacy against *Salmonella* and *E. coli* was determined to assess these probiotic candidates as multi-purpose probiotics.

As observed with *Brachyspira*, there was a pH dependent mechanism of *Salmonella* and *E. coli* inhibition, whereby MRS or CFS at pH 3.8 was significantly more inhibitory that at pH 7.2 (p value $\leq$ 0.001), as shown in Figure 6.10. *Salmonella* Typhimurium SAP 16 was both highly susceptible to low pH and the CFSs of all four *Lactobacillus* isolates (p value $\leq$ 0.001). Unlike for *Brachyspira*, *L. reuteri* SAP 2115 was the least effective against *Salmonella* Typhimurium SAP 16 and *L. reuteri* SAP 2114 and *L. salivarius* SAP 2116 and SAP 2117 were significantly more effective than SAP 2115 at pH 3.8 (p value $\leq$ 0.001).

E. coli isolates had a range of susceptibilities to the different *Lactobacillus* CFSs, however were all significantly inhibited by low pH (p value $\leq$ 0.001). *E. coli* A2 was the most tolerant of the *E. coli* isolates tested, with only *L. reuteri* SAP 2114 and *L. salivarius* SAP 2117 significantly inhibiting this isolate at pH 3.8 (p value $\leq$ 0.001). *E. coli* B1 was significantly inhibited by the *L. salivarius* isolates SAP 2116 and 2117 at both pH 3.8 and pH 7.2 (p value $\leq$ 0.001). *E. coli* B3 was the most susceptible isolate tested and was significantly inhibited by the CFS of *L. reuteri* SAP 2115 and *L. salivarius* SAP 2116 and 2117 at both pH 3.8 and pH 7.2 (p value $\leq$ 0.001). The data presented here also demonstrate significant phenotypic differences between all of the *Lactobacillus* isolates tested both within and between species (p value $\leq$ 0.001).

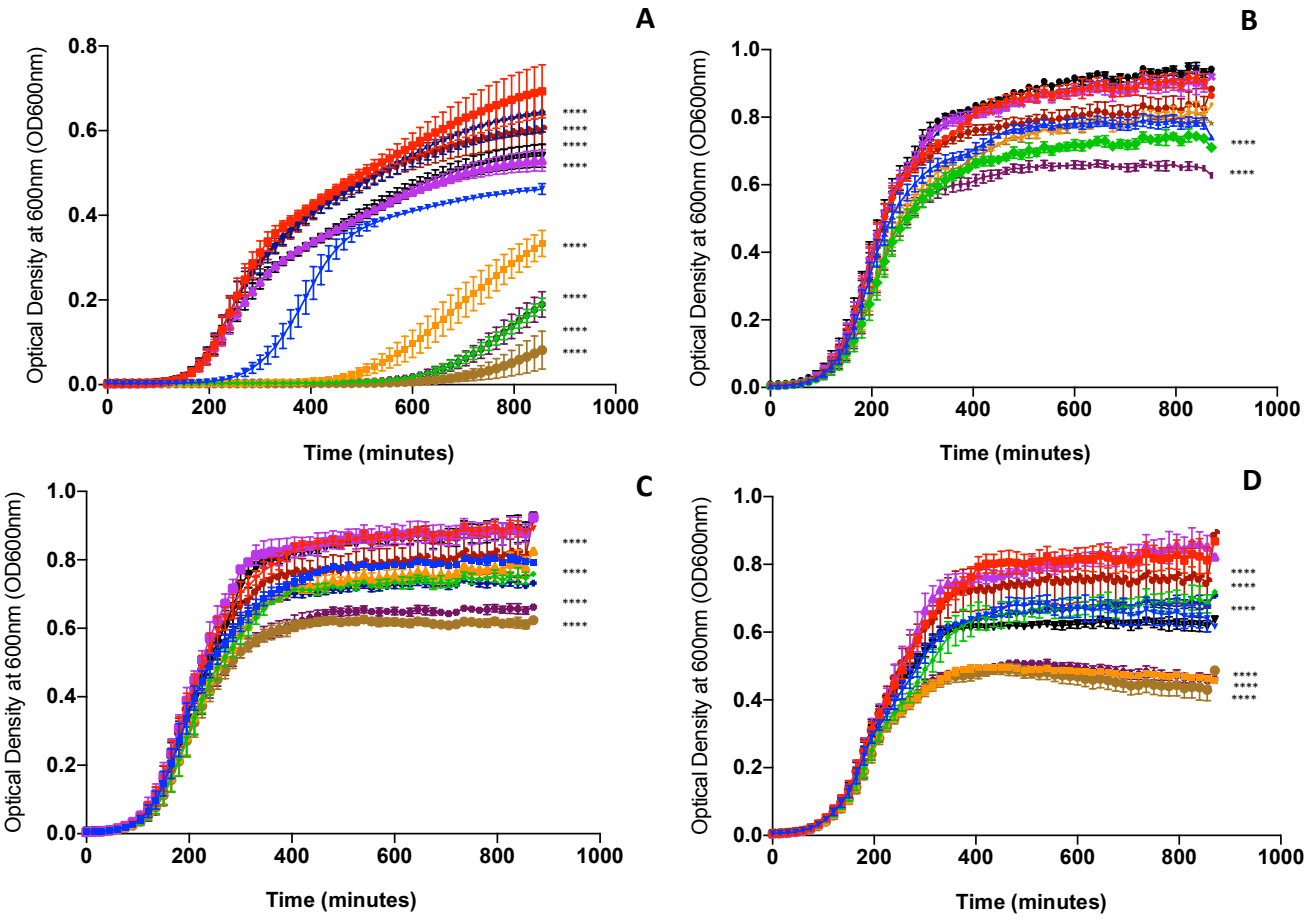


Figure 6. 10 Growth curves of **A:** *Salmonella* Typhimurium SAP 16, **B:** *E. coli* A2 (APEC; serogroup O1), **C**: *E. coli* B1 (APEC; serogroup O78) and **D:** *E. coli* B3 (APEC; serogroup O2) in nutrient broth, supplemented with 10% (v/v) CFS from *L. reuteri* SAP 2114 pH 3.8 (green) and pH 7.2 (purple), *L. reuteri* SAP 2115 pH 3.8 (orange) and pH 7.2 (black), *L. salivarius* SAP 2116 pH 3.8 (brown) and pH 7.2 (dark blue) and *L. salivarius* SAP 2117 pH 3.8 (dark purple) and pH 7.2 (dark red). Control wells were supplemented with MRS pH 3.8 (blue) and MRS pH 7.2 (red). Growth was monitored over 18 hours of aerobic culture at 37°C. Optical density readings were taken at 600nm every 15 minutes. These data represent an average of three biological replicates, each with three technical replicates. Significance, if any, is shown for the pH independent growth between control and test groups* $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

6.3 Discussion

The studies presented in this chapter focussed on beginning to identify the mechanisms by which potential *Lactobacillus* probiotics may exert inhibitory effects against *Brachyspira* species. The mechanisms underlying the activity of *Lactobacillus* probiotics against pathogens such as *Brachyspira* appeared to be multifactorial and included the production of antimicrobial metabolites and bacteriocins, in addition to physical interactions with pathogen, as explored in this chapter (Campana *et al.,* 2017).

A pH dependent mechanism of *Brachyspira* inhibition was first described by Mappley *et al* (2011) whereby the growth of *Brachyspira* was significantly reduced when growth media was supplemented with MRS broth and CFS at pH 3.8 compared with a pH of 7.2. The same mechanism has been described in the studies presented here, whereby the majority of *Brachyspira* isolates grew significantly better when media was supplemented with MRS broth and CFS adjusted to pH 7.2 compared to pH 3.8 (p value ≤ 0.05). *B. pilosicoli* B2904 and SAP 859, *B. intermedia* SAP 919 and *B. innocens* SAP 924 were the most highly susceptible to a low pH (p value ≤ 0.0001), suggesting that *Lactobacillus* probiotics may exert the greatest inhibitory effects on those *Brachyspira* isolates that are most susceptible to acidic conditions. Moreover, acidic conditions have been shown to induce a stress response in *Brachyspira* isolates, demonstrated by the formation of spherical bodies and damage to the cell membrane (Bernardeau *et al.*, 2009).

The *L. reuteri* isolates tested here demonstrated a greater inhibitory effect against *Brachyspira* species, compared to *L. salivarius* isolates. This was further evidence to support that probiotic activity of *Lactobacillus* isolates can be dependent on the properties of both the probiotic bacteria and the strain of the pathogen tested (Wine *et al.*, 2009; Mappley *et al.*, 2011; Campana *et al.,* 2017).

In addition to a pH dependent mechanism of inhibition of *Brachyspira*, it was evident that there was also a pH independent mechanism of inhibition. This was demonstrated by a significant decrease in *Brachyspira* growth (p value ≤ 0.05) compared to the pH matched control. One of the most important considerations for the selection of probiotic bacteria is the protection against bacterial pathogens in the gastrointestinal tract of the host (Šušković *et al.*, 2010). The role of antimicrobial compounds such a proteins and metabolites are crucial to the characterisation of probiotic bacteria. Thus, elucidating these pH independent mechanisms and improving the understanding of how probiotics exert their activity against pathogens is becoming of increasing importance. Therefore, one of the key objectives of this chapter was to explore the mechanisms involved in this inhibition and this was achieved using two different approaches. The first was to investigate the potential influence of proteins in the CFS of the *Lactobacillus* isolates. Whole genome sequence analysis of the panel of *Lactobacillus* isolates identified several putative bacteriocin gene clusters, as observed in Tables 5.4 and 5.5. Three classes of bacteriocins were identified from this analysis, one class I bacteriocin, one class II bacteriocin and two bacteriolysins (formally class III bacteriocins). As previously mentioned in Chapter 5, class I and II bacteriocins, are heat stable peptides and bacteriolysins such as enterolysin A and helveticin J, are heat labile proteins (Khan *et al.*, 2013; Yang *et al.*, 2014; Barbour *et al.*, 2016). Therefore, two approaches to denature these proteins were used, firstly to trypsin treat the CFSs (Gopal *et al.*, 2001) to denature any potential heat stable proteins and secondly to heat treat the CFS to denature any potential heat labile proteins. These treatments had varying effects on the inhibitory ability of the CFS against *Brachyspira*, suggesting that both secreted proteins and metabolites may be important in pathogen inhibition. Furthermore, demonstrating that there were multiple isolate specific activities contributing to *Brachyspira* inhibition. For example, the trypsin treatment of *L.*

reuteri SAP 2114 cell free supernatant at pH 3.8 reduced the inhibitory effect against three of the *Brachyspira* isolates tested, *B. pilosicoli* B2904, *B. intermedia* SAP 919 and *B. innocens* SAP 924. Thus, suggesting that proteinaceous antimicrobial metabolites may have been secreted from *L. reuteri* SAP 2114 and were contributing to the inhibition of these *Brachyspira* isolates. Interestingly, heat treatment of *L. reuteri* SAP 2114 increased the inhibition of *B. pilosicoli* B2904 and *B. innocens* SAP 924 and showed a small, non-significant reduction in inhibition of *B. intermedia* SAP 919. Therefore, it may be suggested that the inhibition of these three *Brachyspira* isolates may be, in part, attributed to the production of a heat stable bacteriocin (Collins *et al.*, 2017) that was denatured during trypsin treatment. However, whole genome sequence analysis did not identify any of these potential bacteriocins in *L. reuteri* SAP 2114. This highlights the importance of combining both phenotypic and genotypic testing as these *in silico* analysis databases may be incomplete or may not be able to identify all gene present from contiguous genome assemblies. A study by Gopal *et al.* (2001) also demonstrated a species-specific reduction in antimicrobial activity against *E. coli* following trypsin treatment which corresponds to the data in the studies presented here. Trypsin treatment of *L. reuteri* SAP 2115 at pH 3.8 only significantly reduced the inhibition of *B. intermedia* SAP 919, demonstrating that *L. reuteri* SAP 2114 and 2115 have slightly different mechanisms of action against different *Brachyspira* isolates. Interestingly, *L. reuteri* SAP 2115 was the most inhibitory of the four potential probiotics tested in these studies, this significant inhibition of multiple *Brachyspira* isolates is therefore unlikely to solely be as a result of bacteriocin production by this *Lactobacillus* isolate. The heat treatment of *L. reuteri* SAP 2115 CFS at pH 3.8 prevented the growth of all of the *Brachyspira* isolates tested. This is a surprising result that has not been reported in the literature, and furthermore many studies have stated that heat treating *Lactobacillus* CFS has no difference on its antimicrobial activity (Forestier *et al.*,

2001; Mariam *et al.*, 2014). Technical failure which, given the repeats done, is an unlikely explanation and so it must be surmised that heating may have changed the conformation of a metabolite present in the CFS, resulting in increased inhibitory activity. It is known there are many bacterial toxins that require processing to be effective. Cleavage of a precursor molecule can generate active toxins in the case of toxins such as *Pseudomonas* exotoxin A and Shiga-like toxins (Gordon and Leppla, 1994). However, the production of toxins from *Lactobacillus* species is not well documented and it seems counterintuitive that *Lactobacillus* isolates would produce a product that requires such dramatic processing to be effective. It is possible that a conformational change induced by heating 'accidentally' created a more toxic product. Heat treated MRS at pH 3.8 did not demonstrate enhanced activity against *Brachyspira* compared to the untreated, pH matched control, therefore that rules out heat treatment potentially changing the pH of the CFS and thus resulting in increased inhibition.

Trypsin and heat treatment of *L. salivarius* SAP 2116 CFS at pH 3.8 reduced the inhibition of two of the *Brachyspira* isolates tested, *B. pilosicoli* SAP 859 and *B. innocens* SAP 924, CFS treatment had no effect on the other *Brachyspira* isolates tested. This suggested that other secreted metabolites may play a more significant role in the inhibition attributed to this *Lactobacillus*. However, these results suggest the presence of both heat stable and heat labile proteins, as identified in Chapter 5. The *in-silico* analysis of the *L. salivarius* SAP 2116 genome using Bagel 4 and Anti-SMASH identified both putative class II bacteriocins and enterolysin A. As previously stated, class II bacteriocins are heat stable and thus trypsin treatment, but not heat treatment, could denature them. Bacteriolysins are heat labile and thus heat treatment and trypsin treatment may denature these proteins. Therefore, there was evidence that both class II bacteriocins and bacteriolysins may be contributing to *Brachyspira* inhibition with *L. salivarius* SAP 2116 CFS. Although many bacteriocins are known to have activity against other

Gram positive or closely related bacteria, there is evidence to suggest they can affect more divergently related Gram negative bacteria (Cotter *et al*., 2005; Messaoudi *et al.*, 2013). Bacteriocins have been shown most often to contribute to the inhibition of Gram negative bacteria when the outer membrane has been permeabilised (Cotter *et al.,* 2005), this may be an important mechanism of *Brachyspira* inhibition because the CFS of *Lactobacillus* has been shown to cause damage to *Brachyspira* cell membranes (Bernardeau *et al.*, 2009). Furthermore, lactic acid has been shown to be a potent membrane permeabiliser in *E. coli* (Alakomi *et al.*, 2000) and thus this mechanism may apply to *Brachyspira*, although further tests would be needed to confirm this. The inhibition of *Brachyspira* at pH 3.8 by *L. salivarius* SAP 2117 CFS was not affected by either trypsin or heat treatment, thus it was hypothesised that the inhibition may be solely attributed to production of other inhibitory metabolites.

The trypsin and heat treatment of the CFS at pH 3.8 in many incidences, significantly reduced the inhibition of *Brachyspira*, but the same was not observed at pH 7.2. This was further evidence for the activity of bacteriocins as a mechanism of *Brachyspira* inhibition as they are known to have a higher activity at a lower pH range (Houlihan *et al*.,2004). Thus, if the bacteriocins had been denatured the effect on *Brachyspira* inhibition may be more prominent at pH 3.8 compared to pH 7.2.

The inhibitory effects of the *L. reuteri* isolates tested in these studies were more affected by trypsin and heat treatment than the *L. salivarius* isolates, suggesting that the inhibition by these *L. reuteri* isolates may be multifactorial, whereas *L. salivarius* inhibition may be more primarily associated with the secretion of non-protein metabolites.

Therefore, the second approach to investigate the mechanisms by which *Lactobacillus* CFS inhibit *Brachyspira* was to identify the metabolites secreted by each isolate following 18 hours of anaerobic culture. The NMR analysis identified several key metabolites, such as acetate

and lactate, known to lower the pH of the CFS and other inhibitory compounds such as ethanol, succinate and acetoin, with proposed antibacterial activity (Šušković *et al.*, 2010). Organic acid production by *Lactobacillus* species is a direct result of glucose metabolism either by glycolysis for homofermenters or by the pentose phosphate pathway for heterofermenters (Caplice and Fitzgerald, 1999). Therefore, increased glucose utilisation results in increased acid production, thus increasing the inhibition of susceptible pathogens such as *Brachyspira* (Bernardeau *et al.*, 2009). The data presented here demonstrated this, whereby *L. reuteri* SAP 2115 utilised the most glucose, which subsequently generated the highest concentrations of acetic acid and lactic acid, in addition to high concentration of ethanol, resulting in the greatest inhibition of *Brachyspira.*

Acetoin has been reported as a potential antimicrobial compound produced by *Lactobacillus* and is documented to have activity against Gram negative bacteria (Collins *et al.*, 2009a; Šušković *et al.*, 2010). This metabolite is formed when acetaldehyde condenses with a molecule of pyruvate to form 2-acetolactate, subsequently acetoin is formed by the decarboxylation of the 2-acetolactate.

The final metabolite produced in sufficient quantities to be detected by NMR was succinate. Succinate is produced by some *Lactobacillus* isolates via an incomplete TCA cycle (Tsuji *et al.*, 2013) and while it has no known antimicrobial activity against pathogens, short chain fatty acids including succinate have been shown to produce up to 15% of the energy requirements in humans and up to 30% in pigs (Rinttila and Apajalahti, 2013). This could be of great importance to the productivity and feed conversion of livestock such as poultry and pigs. Furthermore, short chain fatty acids are weak acids and represent a potential acid stress on sensitive pathogens due to their high concentration and the low pH already established by lactic acid. Uncharged protonated weak acids are diffusible across cell membranes,

potentially lowering the internal pH of a bacterial cell, resulting in cell death (Ricke, 2003; Bearson *et al.,* 2006).

Principle component analysis was used to analyse and compare the metabolome of *Lactobacillus* isolates. Principle component 1 (PC1) and principle component 2 (PC2) accounted for 58% of the total variance in the investigated samples. The first component, which accounted for 38% of the total variance, was found to be mainly influenced by the taxonomy of the *Lactobacillus* isolates, these findings are in agreement with Parolin *et al.* (2015) and Nardini *et al.* (2016). Parolin *et al.* (2015) and Nardini *et al.* (2016) also reported the second component to be mainly influenced by the activity against *Candida* and *Chlamydia*, respectively. Although there was a suggestion of this in the data presented here, the distribution of the data do not show a strong link to anti-*Brachyspira* activity in the second component. However, the first component showed some clustering of isolates that were highly inhibitory against *Brachyspira* from those that poorly inhibited *Brachyspira*. *L. reuteri* and *L. salivarius* isolates clustered low in PC1 and were associated with good *Brachyspira* inhibition whereas *L. crispatus* isolates clustered high in PC1 and were associated with poor *Brachyspira* inhibition. In order to visualise the metabolites responsible for these differences, a colour plot was generated and illustrated that these key differences were primarily as a result of lactate and un-utilised glucose in the samples.

The utilisation of glucose and production of organic acids are metabolically interrelated and represent defensive strategies against pathogens such as *Brachyspira* (Caplice and Fitzgerald, 1999). Therefore, isolates that were unable to utilise high concentrations of glucose adequately, such as *L. crispatus* SAP 2107, were unsuitable as probiotics for several reasons. Firstly, they may not be commercially viable as they may not grow in sufficient quantities. Secondly, a low utilisation of glucose could lead to poor production of organic acids. Finally,

poor glucose utilisation could be linked to increased glucose concentrations in the gastrointestinal tract, thus potentially promoting the growth of undesirable bacteria. This could be important in *Brachyspira* infection as these bacteria utilise glucose for metabolic processes as indicated in Chapter 3.

High levels of glucose utilisation have been linked to *Lactobacillus* isolates with the greatest inhibition of pathogens. Nardini *et al.* (2016) supplemented the CFS of high glucose utilising *Lactobacillus* with glucose to 'reset the sugar consumption', which resulted in a significant increase in *C. trachomatis* infectivity. Suggesting that glucose depletion in the CFS may result in pathogen inhibition *in vivo.* Of the isolates selected for characterisation as potential probiotics, *L. reuteri* SAP 2114 and 2115 and *L. salivarius* SAP 2116 were high utilisers of glucose and subsequently produced high concentrations of organic acids. *L. salivarius* SAP 2117 was a poor utiliser of glucose, yet produced sufficient concentrations of organic acids, this was the proposed primary mechanism of inhibition, as previous studies deemed protein denaturation to be ineffective at reducing inhibition.

Another proposed mechanism by which probiotics exert their inhibitory effects is by physical interactions with the pathogen. This mechanism could prove to be of great importance against *Brachyspira in vivo* because motility and chemotaxis are important virulence factors for this organism (Naresh and Hampson, 2010). In order to colonise and attach to colonic enterocytes, *Brachyspira*, in particular *B. pilosicoli*, need to penetrate and move through the mucus layer. The cork-screw like motility of *Brachyspira* allows them to move through highly viscous substances such as mucus (Chunhao Li *et al.*, 2000) and chemotaxis towards mucin allows the bacteria to target desired colonisation sites. *Lactobacillus* have been shown to physically interact with *B. pilosicoli, B. innocens* and *B. hyodysenteriae* and cause coaggregation of bacteria. This interaction has been demonstrated to directly reduce the

motility of *Brachyspira* species, both when *Lactobacillus* cells are viable and heat inactivated (Bernardeau *et al.*, 2009; Mappley *et al.*, 2011). The results presented in this study show similar results whereby many *Brachyspira* isolates were inhibited by both viable and heat inactivated *L. reuteri* isolates. However, *L. salivarius* isolates had very little effect on the viability of *Brachyspira*. In most incidences, where *Brachyspira* viability was reduced by live *Lactobacillus* cell, heat inactivated cells also caused inhibition, suggesting that this effect was not as a result of competition for nutrients or production of antimicrobial compounds and was likely to be as a result of the passive coaggregation of *Brachyspira* and *Lactobacillus* as previously demonstrated (Bernardeau *et al.*, 2009). Despite this, it was evident that some *B. pilosicoli* isolates may be tolerant of this mechanism of inhibition. For example, *B. pilosicoli* SAP 859 was the only isolate tested that was unaffected by co-culture with *Lactobacillus* isolates, suggesting that this mechanism is not universally inhibitory.

Finally, it is well documented that probiotics are very much strain specific in their activity and the pathogens that they inhibit. With this in mind, it was interesting to determine if the potential probiotics characterised in Chapter 5 could be inhibitory against pathogens other than *Brachyspira.* It is widely reported that probiotics have activity against other avian pathogens such as *E. coli* and *Salmonella* (Rantala and Nurmi, 1973; Resta-Lenert and Barrett, 2003; La Ragione and Woodward, 2003), therefore *L. reuteri* SAP 2114 and 2115 and *L. salivarius* SAP 2116 and 2117 were tested against these species to determine if they had a broad spectrum of inhibition. Once again, all isolates tested had both pH dependent and pH independent mechanisms of inhibition with *Salmonella* Typhimurium SAP 16 being the most susceptible to all *Lactobacillus* isolates at a low pH compare to *E. coli*. This may be as a result of *E. coli* having greater acid tolerance as it can inhabit the gastrointestinal tract as a commensal. Overall, it was evident that all *Lactobacillus* isolates tested significantly inhibited

both *E. coli* and *Salmonella* isolates and thus they have the potential against a range of poultry pathogens. It is important to note that inhibition was once again dependent on the isolate of *Lactobacillus*, and therefore an appropriate isolate, or combination of isolates would need to be selected for prophylactic use based on the desired target pathogens.

To summarise, the mechanisms underlying the activity of *Lactobacillus* isolates against *Brachyspira* and other avian pathogens appeared to be multifactorial and included the production of antimicrobial compounds such as lactic acid and potential bacteriocin proteins. Furthermore, *Lactobacillus* isolates can coaggregate with *Brachyspira*, potentially interfering with two of its key virulence factors (Li *et al.*, 2000; Bernardeau *et al.*, 2009; Naresh and Hampson, 2010). In these studies, *L. reuteri* isolates appear to be the most effective, with evidence to suggest all three of these mechanisms were involved in *Brachyspira* inhibition *in vitro*, moreover, they were effective against other known avian pathogens, making them the most suitable probiotic candidates.

Chapter 7: Investigating the immune response to *Brachyspira* in tissue culture and the protective effective of *Lactobacillus* in a *Brachyspira* infection model using *Galleria mellonella*.

7.1 Introduction

There is a significant lack of understanding regarding the stimulation of the innate avian immune response to *Brachyspira* infection (Hampson, 2018). However, severe inflammatory symptoms have been reported following infection including: typhlitis and necrotic inflammation of the large intestine (Buckles *et al*., 1997; Nemes *et al.*, 2006). The inflammatory immune responses that result in these severe clinical manifestations are poorly understood. However, it is reported that the innate immune response is the first line of defence against *Brachyspira* once they reach the lower gastrointestinal tract. Histological analysis of human *Brachyspira* infections demonstrate infiltration of macrophages into the lamina propria in response to infection, and detection of *Brachyspira* within macrophages demonstrate these cells play a significant role in response to infection (Antonakopoulos *et al*, 1982; Padmanabhan *et al.*, 1996). Furthermore, evidence from porcine infections suggests that neutrophils have a role in the inflammatory immune response to *Brachyspira,* a possibly significant role given that these cells are the predominant cell found in *Brachyspira* infected tissue (Costa *et al.*, 2014). To date there is no evidence that suggests *Brachyspira* can survive and replicate within immune cells.

There is a growing body of evidence documenting the immunomodulatory properties of probiotics in the innate immune system, with a number of studies demonstrating their ability to modulate the immune system by both upregulating immune responses, proposed to help fight infection, and downregulating immune responses, proposed to help prevent inflammation *in vitro* (Haller *et al.*, 2000; Zhang *et al.*, 2005; Ma *et al.,* 2011). This is an

important balancing act to ensure that the pathogen is cleared by the immune system with a sufficient inflammatory response, but that this response does not result in extensive pathology to the host (Vanderpool *et al.,* 2008). Moreover, recent probiotic studies using *Galleria mellonella* have suggested immunomodulatory properties of *Lactobacillus* and subsequent protection from pathogens (Rossoni *et al.*, 2017; Scalfaro *et al.*, 2017).

This chapter aimed to characterise the host innate immune responses to *Brachyspira* using an avian *in vitro* model of infection to investigate the macrophage response to infection, additionally the ability of *Lactobacillus* to alter these immune responses was investigated. An *in vivo* model of infection (*Galleria mellonella)* was used as a rapid screen for *Brachyspira* virulence and to investigate how a probiotic intervention may be able to protect from the morbidity and mortality associated with *Brachyspira* infection in the context of a functional immune system.

The aims and objectives of this chapter were:

- To establish an *in vitro* HD11 avian macrophage model for studying *Brachyspira* infection and to characterise the macrophage cytokine responses to all species implicated in avian intestinal spirochaetosis.
- To investigate how the cytokine gene expression is affected by exposing the HD11 cells to *Lactobacillus* prior to *Brachyspira* infection.
- To establish an *in vivo G. mellonella* infection model for *Brachyspira* infection and to characterise the morbidity and mortality in response to all species implicated in avian intestinal spirochaetosis.
- To investigate how the morbidity and mortality associated with *Brachyspira* infection are affected when *G. mellonella* are infected with *Lactobacillus* prior to infection with *Brachyspira*.

7.2 Results

7.2.1 HD11 avian macrophage cytokine responses to *Brachyspira*

The cytokine response to *Brachyspira* was characterised for one isolate of *B. alvinipulli*, *B. intermedia, B. innocens* and *B. pilosicoli*, as shown in Figure 7.1. When compared to the control, infection with *B. pilosicoli* SAP 859 resulted in the greatest expression of all of the cytokines tested, in particular IFNγ (Figure 7.1E). *B. intermedia* SAP 919 elicited the lowest expression of IL1β, IL10 and IFNγ, showing no significant difference from the control and *B. alvinipulli* elicited the lowest expression of IL6 and IL8. *B. innocens,* which is currently thought to be non-pathogenic, elicited similar cytokine expression as those species considered to be pathogenic in poultry, including the second largest IFNγ response. The responses to the different species of *Brachyspira* were largely similar, except for in a few incidences. For example, the IFNγ expression in response to *B. innocens* SAP 924 was significantly higher compared to *B. intermedia* SAP 919 (p value 0.0215). Additionally, the IFNγ expression in response to *B. pilosicoli* SAP 859 was significantly greater than that of *B. intermedia* SAP 919 (p value $\leq$ 0.0001), *B. alvinipulli* ATCC 51933 (p value $\leq$ 0.0001) and *B. innocens* SAP 924 (p value 0.0009).

Brachyspira that had not entered the macrophages survived for the duration of the experiment, as determined by culture of the tissue culture media following the three-hour infection. By contrast, they did not survive within the macrophages, as determined using a gentamicin protection assay. This suggested that in this model, macrophages were capable of killing internalised *Brachyspira*, although electron microscopy should be used to confirm this.

7.2.2 HD11 avian macrophage cytokine responses to *Lactobacillus*

Probiotic bacteria are generally recognised as safe (GRAS) and are used very widely (Mattia and Merker, 2008; EFSA *et al.*, 2017). However, these bacteria are also recognised by the immune system and as one aim of this study was to exploit certain *Lactobacillus* isolates as possible control agents against *Brachyspira,* therefore, it was important to characterise the immune response to *Lactobacillus* in this model. *L. reuteri* SAP 2115 was selected for study in this model because it was significantly more inhibitory against a range of *Brachyspira in vitro* than other isolates tested and *L. salivarius* SAP 2117 was selected because it was the most inhibitory of the *L. salivarius* isolates against *Brachyspira*.

By comparison to the responses elicited by *Brachyspira* isolates, *L. reuteri* SAP 2115 elicited similar expression of IL1β, IL8 and IL10 whereas the expression of IL6 was lower, but not significantly. The expression of IFNγ was significantly lower compared to that of *B. pilosicoli* SAP 859 (p value $\leq$ 0.0001), *B. alvinipulli* ATCC 51933 (p value $\leq$ 0.0014) and *B. innocens* SAP 924 (p value $\leq$ 0.0001). *L. salivarius* SAP 2117 elicited a similar expression of IL8 to *Brachyspira*, however the expression of IL1β, IL6 IL10 and IFNγ were significantly lower. For example, the IL1β response to *L. salivarius* SAP 2117 was significantly lower compared to *B. pilosicoli* SAP 859 (p value 0.0115), *B. alvinipulli* ATCC 51933 (p value 0.0194) and *B. innocens* SAP 924 (p value 0.0487). Furthermore, the expression of IL10 was significantly lower than *B. pilosicoli* SAP 859 (p value 0.0168) and finally the IFNγ response was significantly lower than *B. pilosicoli* SAP 859 (p value $\leq$ 0.0001), *B. alvinipulli* ATCC 51933 (p value 0.0103) and *B. innocens* SAP 924 (p value 0.0004) (Table 7.1). These data show that there were key differences in the cytokine gene expression between pathogenic bacteria such as *Brachyspira* and probiotic bacteria such as *Lactobacillus*. In addition, there were differences in gene

expression between *Lactobacillus*, which may demonstrate the need to carefully select probiotic strains dependent on their immunomodulatory abilities.

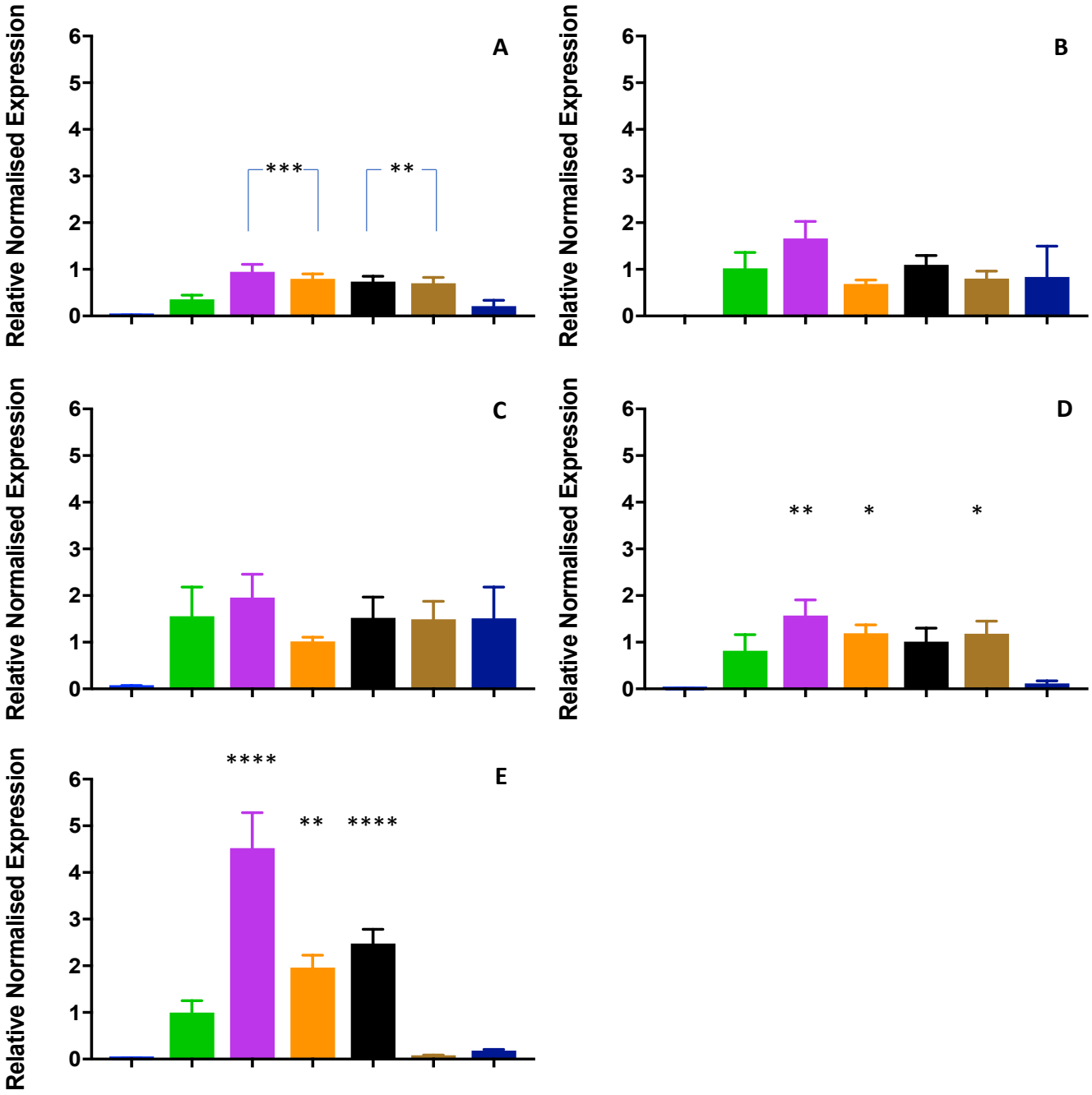


Figure 7. 1 Expression of cytokine transcripts from HD11 avian macrophages 4-hours post infection with *Brachyspira* species. **A:** Expression of IL1β **B:** Expression of IL6 **C:** Expression of IL8 **D:** Expression of IL10 and **E:** Expression of IFNγ. The bars represent the following infections: blue- mock infection with PBS, green- infection with *B. intermedia* SAP 919, purple- infection with *B. pilosicoli* SAP 859, orange- infection with *B. alvinipulli* ATCC 51933, black- infection with *B. innocens* SAP 924, brown- infection with *L. reuteri* SAP 2115 and dark blue- infection with *L. salivarius* SAP 2117. Data are representative of three biological replicates each with three technical replicates. Mean values and SEM are displayed. P values are presented in Table 7.1. Significance, if any, is shown between infection with PBS, compared to *Brachyspira* infection * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

Table 7. 1 P values for the comparison of cytokine expression between all *Brachyspira* isolates and the control infection with PBS. Expression of IL1β, IL6, IL8, IL10 and IFNγ were compared between *Brachyspira* isolates using a one-way ANOVA to investigate the HD11 cell responses to different *Brachyspira* species. P values are presented whereby values highlighted in green were ≤ 0.05 and those highlight in red were > 0.05.

	IL1β	IL6	IL8	IL10	IFNγ
***B. intermedia* SAP 919**	0.3902	0.691	0.4844	0.4007	0.3536
***B. pilosicoli* SAP 859**	0.0004	0.1768	0.2486	0.0042	0.0001
***B. alvinipulli* ATCC 51933**	0.0004	0.9445	0.8986	0.0413	0.0018
***B. innocens* SAP 924**	0.0011	0.6156	0.5127	0.128	0.0001
***L. reuteri* SAP 2115**	0.006	0.8812	0.5395	0.0451	>0.9999
***L. salivarius* SAP 2117**	0.9351	0.8834	0.5755	>0.9999	>0.9999

7.2.3 HD11 avian macrophage cytokine responses to *Brachyspira* following pre-exposure to *Lactobacillus*

In the experiments presented here, pre-exposure of HD11 cells to *Lactobacillus* resulted in a marked effect on subsequent cytokine responses to *Brachyspira* infection. The most significant reductions in cytokine expression were observed in the expression of IFNγ (Figure 7.2E). Infecting the HD11 cells with both *L. reuteri* SAP 2115 and *L. salivarius* SAP 2117 significantly reduced the IFNγ expression in response to *B. pilosicoli* SAP 859, *B. alvinipulli* ATCC 51933 and *B. innocens* SAP 924 (p value ≤ 0.0001). The IFNγ response to *B. intermedia* SAP 919 was also reduced, however not significantly (p value 0.177 and > 0.99, respectively). *L. reuteri* SAP 2115 was able to reduce the expression of IFNγ to a greater extent than *L. salivarius* SAP 2117. The IL6 response to *B. pilosicoli* SAP 859 was also significantly reduced by the pre-exposure to both *L. reuteri* SAP 2115 and *L. salivarius* SAP 2117 (p value ≤ 0.0005) (Figure 7.2B). Furthermore, this response was significantly reduced to *B. innocens* SAP 924 after prior infection with *L. salivarius* SAP 2117 (p value ≤ 0.05). The overall gene expression of IL6 was reduced when cells were infected with both *Lactobacillus* isolates prior to infection with *Brachyspira*, despite no significance being observed. The expression of IL8 was

predominantly unaffected by *Lactobacillus* pre-exposure, although gene expression in response to *B. pilosicoli* SAP 859, the strongest inducer of IL8 expression was significantly reduced by *L. reuteri* SAP 2115 (p value $\leq$ 0.05) (Figure 7.2C). The response to IL10 gene expression was similar to that of IL6, there was an overall decrease in expression as a result of pre-exposure to both *Lactobacillus* isolates (Figure 7.2D). However, significance was only observed when cells infected with *B. pilosicoli* were pre-exposed to *L. salivarius* SAP 2117. *L. salivarius* SAP 2117 was not able to induce a high level of IL10 expression, compared to *L. reuteri* SAP 2115, but was still able to modulate the cytokine response in response to *Brachyspira* infection. As previously mentioned, immunomodulation can be the up or downregulation of an immune response. In the studies presented here, IL6, IL10 and IFNγ are significantly downregulated in response to *Brachyspira*, following exposure to *Lactobacillus*. However, IL1β was significantly upregulated in response to infection with *B. intermedia* SAP 919 (p value $\leq$ 0.005) (Figure 7.2A). An increase in IL1β expression in response to *B. pilosicoli* SAP 859 was also observed after exposure to *L. salivarius* SAP 2117, however this was not significant (p value 0.330). The expression of IL1β in response to *B. alvinipulli* ATCC 51933 and *B. innocens* SAP 924 remained largely unchanged.

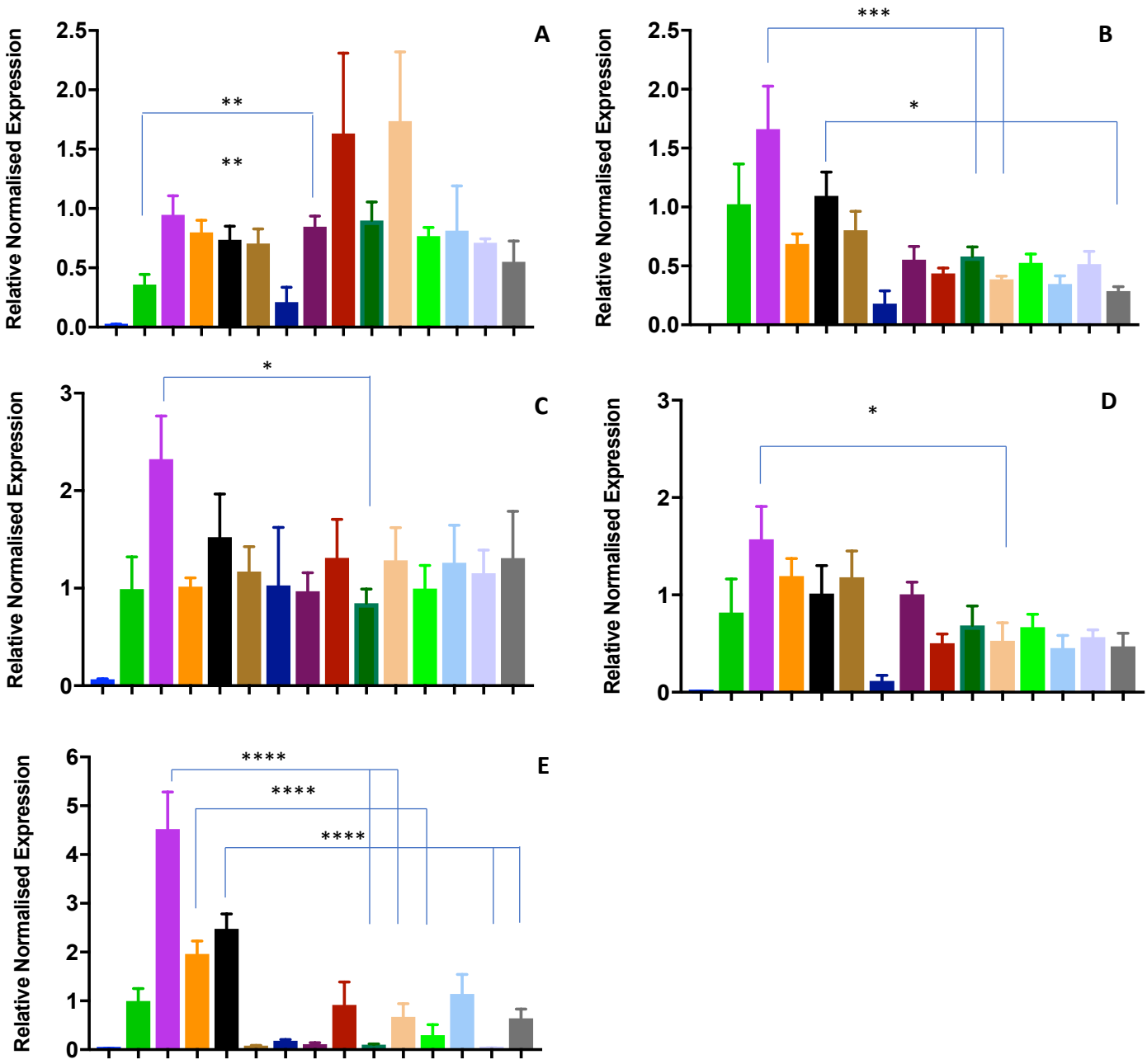


Figure 7. 2 Expression of cytokine transcripts from HD11 avian macrophage cells after pre-treatment with either *L. reuteri* SAP 2115 or *L. salivarius* SAP 2117 for 30 minutes prior to infection with *Brachyspira* species for 4 hours. **A:** Expression of IL1β **B:** Expression of IL6 **C:** Expression of IL8 **D:** Expression of IL10 and **E:** Expression of IFNγ. The bars represent the following infections: blue- mock infection with PBS, green- infection with *B. intermedia* SAP 919, purple- infection with *B. pilosicoli* SAP 859, orange- infection with *B. alvinipulli* ATCC 51933, black- infection with *B. innocens* SAP 924, brown- infection with *L. reuteri* SAP 2115, dark blue- infection with *L. salivarius* SAP 2117, dark purple- infection with *L. reuteri* SAP 2115 followed by *B. intermedia* SAP 919, dark red- infection with *L. salivarius* SAP 2117 followed by *B. intermedia* SAP 919, dark green- infection with *L. reuteri* SAP 2115 followed by *B. pilosicoli* SAP 859, peach- infection with *L. salivarius* SAP 2117 followed by *B. pilosicoli* SAP 859, light green- infections with *L. reuteri* SAP 2115 followed by *B. alvinipulli* ATCC 51933, light blue- infection with *L. salivarius* SAP 2117 followed by *B. alvinipulli* ATCC 51933, lilac- infection with *L. reuteri* SAP 2115 followed by *B. innocens* SAP 924 and grey- infection with *L. salivarius* SAP 2117 followed by *B. innocens* SAP 2117. Data are representative of three biological replicates each with three technical replicates. Mean values and SEM are displayed. Significance, if any, is shown between infection with *Brachyspira* alone, compared to *Lactobacillus* pre-treatment * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

Table 7. 2 A summary of the P values to compare the cytokine responses from HD11 cells infected with *Brachyspira* alone, compared to cells pre-treated with different *Lactobacillus* isolates. Significance was determined using a one-way ANOVA with a p value of ≤ 0.05 indicating a significant difference (highlighted in green) and a p value of > 0.05 indicating no significant difference (highlighted in red).

	IL1β	IL6	IL8	IL10	IFNγ
SAP 919 vs. SAP 919+ SAP 2115	0.7595	0.3624	>0.9999	0.9986	0.1775
SAP 919 vs. SAP 919+ SAP 2117	0.0043	0.1838	0.996	0.9581	>0.9999
SAP 859 vs. SAP 859 + SAP 2115	>0.9999	0.0002	0.0134	0.058	0.0001
SAP 859 vs. SAP 859 + SAP 2117	0.3306	0.0001	0.2753	0.0313	0.0001
ATCC 51933 vs. ATTC 51933+ SAP 2115	>0.9999	0.9952	>0.9999	0.5417	0.0004
ATCC 51933 vs. ATCC 51933 + SAP 2117	>0.9999	0.8751	0.9992	0.1432	0.2524
SAP 924 vs. SAP 924 + SAP 2115	>0.9999	0.1038	0.972	0.7293	0.0001
SAP 924 vs. SAP 924 + SAP 2117	0.999	0.019	0.9997	0.4152	0.0001

7.2.4 Developing a *Galleria mellonella* model to study *Brachyspira* infection

The *Galleria mellonella* (greater wax moth) larvae insect model has been previously used to assess the infection of a number of pathogenic bacteria including, *Campylobacter jejuni* and *E. coli* (Senior *et al.*, 2011; Alghoribi *et al.*, 2014; Mehat *et al.*, 2018). This model has a number of advantages in that *G. mellonella* possess haemocytes that have been likened to mammalian neutrophils and neutrophils are considered analogous to heterophils (Montali, 1988; Brooks *et al.,* 1996). Therefore, *G. mellonella* haemocytes may respond to *Brachyspira* in a similar way to avian heterophils.

The first stage in developing a model to study *Brachyspira* infection was to determine the dose of *Brachyspira* that would show a gradual increase in *G. mellonella* mortality over time. Three different doses of *Brachyspira* were selected; 1 x 10^5, 1 x 10^6 and 1 x 10^7 CFU/ larvae and the mortality in response to these bacterial doses are shown in Figure 7.3. It was evident from these experiments that the different *Brachyspira* doses had a distinct effect on the mortality of the larvae. Doses of 1 x 10^5 and 1 x 10^6 CFU/larvae did not result in gradual increases in mortality over 168 hours for any isolate except for *B. innocens* SAP 924 (Figure

7.3E), which was shown to have the highest mortality when infected with low doses of *Brachyspira*. Therefore, a dose of 1 x 10^7 CFU/ larvae was selected because in all isolates tested there was a gradual increase in mortality over time. Furthermore, a time point of 120 hours as selected as the time course for this experiment because the majority of mortality occurred in this time frame. From this preliminary data, it was clear that each *Brachyspira* isolate resulted in distinct mortality profiles in the larvae and that despite *B. innocens* being proposed as non-pathogenic in the avian host, it was indeed pathogenic in this host.

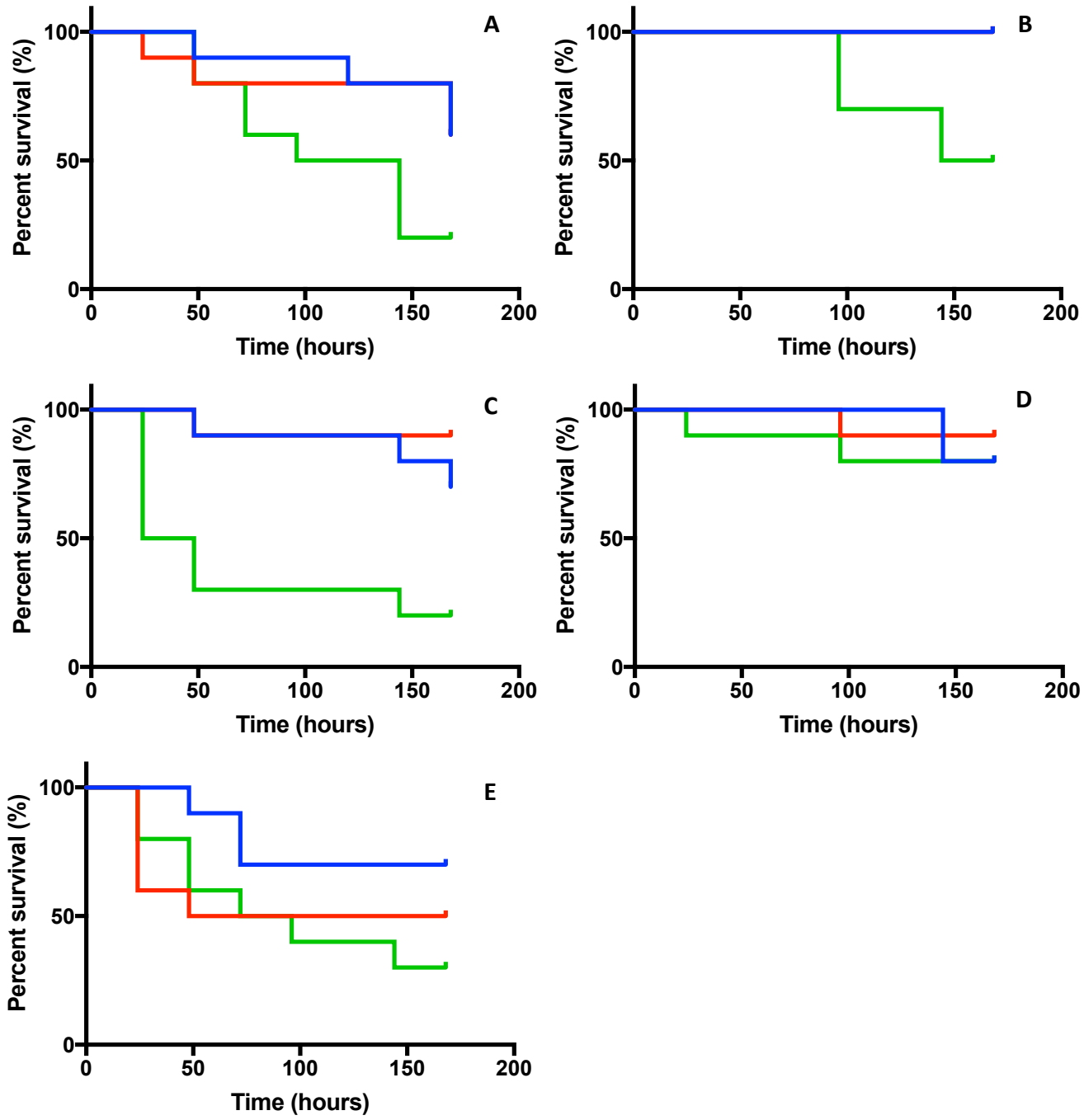


Figure 7. 3 *Galleria mellonella* percent survival after infection with multiple doses of *Brachyspira* species. **A:** Infection with *B. pilosicoli* B2904, **B:** Infection with *B. pilosicoli* SAP 859, **C:** Infection with *B. intermedia* SAP 919, **D:** Infection with *B. innocens* SAP 943 and **E:** Infection with *B. innocens* SAP 924. Blue lines represent infection with $1x10^5$ CFU/ larvae of *Brachyspira*, red lines represent infection with $1x10^6$ CFU/ larvae of *Brachyspira* and green lines represent infection with $1x10^7$ CFU/ larvae of *Brachyspira*. Data are representative of three biological replicates each with 10 larvae per infection group. Mortality scores were recorded every 24 hours for 168 hours of incubation at 37°C.

7.2.5 *Galleria mellonella* infection model

Following on from the preliminary studies, all eight *Brachyspira* isolates were screened using the *G. mellonella* model at a dose of 1 x 10^7 CFU/ larvae, as shown in Figure 7.4. *G. mellonella* that were not injected and those that were injected with PBS showed 100% survival and no associated morbidity, as seen in Figure 7.4A and Figure 7.4B. Each *Brachyspira* isolate significantly reduced the survival of *G. mellonella* larvae, but to varying degrees (p value $<$ 0.0001). Additionally, each *Brachyspira* isolate significantly increased the melanisation score of the larvae, also to varying degrees (p value $<$ 0.0001).

Of the three *B. pilosicoli* isolates, infection with SAP 859 and SAP 865 resulted in the highest survival rates, 78% and 83% respectively and the lowest melanisation scores, 23 and 19, respectively. Whereas, *B. pilosicoli* B2904 had one of the lowest survival rates of all of the isolates tested, 60% and one of the highest melanisation scores, 31. Additionally, of the three *B. innocens* isolates tested, SAP 943 resulted in the lowest survival rate, 45% and highest morbidity score of all isolates tested, 34. *B. innocens* SAP 924 and SAP 927 were less virulent to the *G. mellonella* larvae and resulted in a survival rate of 55% and 62% and melanisation scores or 29 and 27, respectively. *B. intermedia* SAP 919 was the second most virulent isolate in this model with a survival rate of 48% and a melanisation score of 32. *B. alvinipulli* ATCC 51933 elicited similar responses to *B. pilosicoli* SAP 859, with a survival rate of 77% and a melanisation score of 23. Overall, it was not possible to correlate the survival rate and melanisation scores to a particular species of *Brachyspira*. As observed in previous assays, each isolate exhibited unique characteristics that could not be correlated to a particular species of *Brachyspira*.

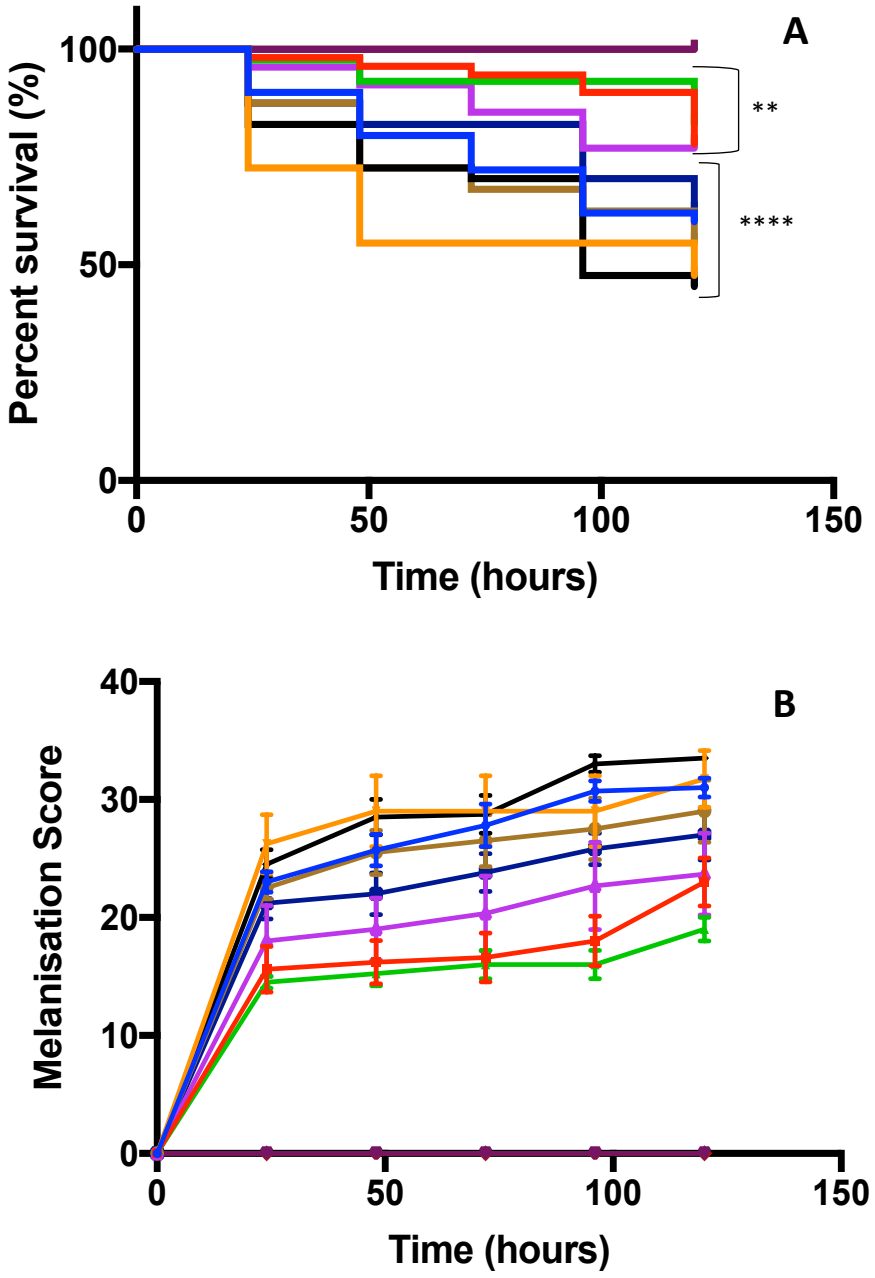


Figure 7. 4 *Galleria mellonella* **A:** percent survival and **B:** morbidity scores following infection with 10^7 CFU/ larvae of *B. pilosicoli* B2904 (blue), *B. pilosicoli* SAP 859 (red), *B. pilosicoli* SAP 865 (green), *B. alvinipulli* ATCC 51933 (purple), *B. intermedia* SAP 919 (orange), *B. innocens* SAP 943 (black), *B. innocens* SAP 924 (brown) and *B. innocens* SAP 927 (dark blue). Control larvae were injected with sterile PBS (dark purple) to control for injection technique and non-injected controls (dark red) were used to control for the rate of larval pupation for the 120-hour duration of incubation at 37°C. The percent survival and morbidity scores were calculated from five biological replicates, each with 10 larvae per experimental condition. Scores were recorded every 24 hours. Significance, if any, is shown between infection with *Brachyspira*, compared to the PBS control infection * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

In addition to observing the morbidity and mortality associated with *Brachyspira* infection in *G. mellonella* larvae, attempts to culture *Brachyspira* from the haemolymph of the larvae were conducted to determine the duration of survival of *Brachyspira* within the larvae. One isolate from each species of *Brachyspira* was selected for these studies; *B. pilosicoli* SAP 859, *B. intermedia* SAP 919, *B. alvinipulli* ATCC 51933 and *B. innocens* SAP 924. These isolates were selected due to range of morbidity and mortality observed in Figure 7.4, in order to determine if probiotic interventions would be suitable for *Brachyspira* infections of differing virulence. These data were summarised as qualitative data in Table 7.3 due to the difficulty and inaccuracy of counting individual colonies of *Brachyspira*. It was evident that *Brachyspira* remained viable in the *G. mellonella* haemolymph for up to 48 hours post infection for *B. pilosicoli* SAP 859 and *B. alvinipulli* ATCC 51933 and up to 24 hours post infection for *B. intermedia* SAP 919 and *B. innocens* SAP 924. Interestingly, the associated morbidity and mortality following *Brachyspira* infection continued to increase after the bacteria were no longer viable in the haemolymph. Following four hours of infection, the numbers of all *Brachyspira* isolates began to decrease until approximately 24-48 hours post infection, after which viable *Brachyspira* were below the limits of detection.

Table 7. 3 Qualitative summary of *Brachyspira* species cultured from the haemolymph of the *Galleria mellonella* larvae. Haemolymph from three larvae was pooled at each of the time points, cultured on to FABA and incubated anaerobically at 37°C for 3-5 days.

Time (hours)	*B. pilosicoli* SAP 859	*B. intermedia* SAP 919	*B. alvinipulli* ATCC 51933	*B. innocens* SAP 924
0	+++	+++	+++	++
1	+++	+++	+++	++
2	++	+++	+++	++
4	+	++	+	+
6	+	++	+	+
24	+	+	+	+
48	+	-	+	-
72	-	-	-	-
96	-	-	-	-
120	-	-	-	-

+++ strong *Brachyspira* growth, ++ intermediate *Brachyspira* growth, + weak *Brachyspira* growth and – no *Brachyspira* growth.

Histopathology was also conducted to investigate the immune responses and pathology associated with *Brachyspira* infection in this model and to determine where *Brachyspira* localised in the larvae. Histopathology scores assessed the bacteria in the gut, the inflammatory bodies and the clusters of haemocytes circulating within the larvae, as shown in Table 7.4. These scores can be seen in detail in Section 2.4, however in brief, a score of 0 corresponds to no bacteria in the gut, inflammatory bodies or haemocyte clusters, a score of 1 corresponds to a very small number of bacteria, inflammatory bodies and haemocytes clusters, a score of 2A corresponds to a small number bacteria, inflammatory bodies and haemocytes clusters, a score of 2B corresponds to a moderate number bacteria, inflammatory bodies and haemocytes clusters and a score of 3 corresponds to an abundance of bacteria, inflammatory bodies and haemocytes clusters.

The inflammatory/ melanin bodies developed rapidly post infection and were observed in very low numbers immediately after infection with each *Brachyspira* species. Within two hours of infection, there were small numbers of inflammatory/ melanin bodies in response

to all *Brachyspira* isolates. This concurred with the observable colour change (melanisation) of the larvae within two hours post infection. Between 48 and 96 hours the numbers of inflammatory/melanin bodies increased to a moderate number, depending on the species of *Brachyspira*. Overall, these scores were similar following infection with each species of *Brachyspira* tested.

The numbers of bacteria in the gut of the *G. mellonella* increased over the duration of these experiments following infection with *B. pilosicoli* SAP 859, *B. intermedia* SAP 919 and *B. alvinipulli* ATCC 51933. However, no bacteria were observed in the gut following infection with *B. innocens* SAP 924. Importantly, these bacteria were not identified as *Brachyspira,* but as Gram positive cocci, likely to be Enterococci as these are known to colonise the gastrointestinal tracts of *G. mellonella*. Infection with *B. pilosicoli* SAP 859 resulted in the highest numbers of bacteria in the gut. The clustering of haemocytes occurred later on in the infection process compared to the development of inflammatory bodies. Small clusters of haemocytes appeared 24 hours after infection with *B. pilosicoli* SAP 859, *B. intermedia* SAP 919 and *B. innocens* SAP 924, whereas only very small numbers of haemocytes were observed after 48 hours of *B. alvinipulli* ATCC 51933 infection. Overall, the histological analysis showed the development of a cellular immune response over time, however there were no histological indicators that correlated with the morbidity or mortality scores associated with infection by different *Brachyspira* species. For example, *B. pilosicoli* SAP 859 was the least virulent of the isolates selected in this model yet showed some of the highest scores for bacteria in the gut, inflammatory/ melanin bodies and haemocyte clusters.

Table 7. 4 Histology scores from infection with different *Brachyspira* species. This scoring system scored the presence of bacteria in the gut, the inflammatory/ melanin bodies and the clusters of haemocytes observed in the larvae after infection with *B. pilosicoli* SAP 859, *B. intermedia* SAP 919, *B. alvinipulli* ATCC 51933 and *B. innocens* SAP 924. A score of 0 denotes no sign of infection, a score of 1 denotes minimal signs of infection with very small numbers of the descriptors below, a score of 2A denotes a mild infection with small numbers of the descriptors below, a score of 2B denotes moderate infection, with moderate numbers of the descriptors below and a score of 3 denotes severe infection with high numbers of the descriptors below.

	Time (hours)	Bacteria in gut (cocci)	Inflammatory/ melanin bodies	Clusters of haemocytes
B. pilosicoli	0	1	1	0
SAP 859	1	1	1	0
	2	1	2a	1
	4	2a	2a	1
	6	2a	2a	1
	24	1	2a	2a
	48	1	2a	2a
	72	1	2b	2a
	96	2a	2b	2b
	120	2a	3	2b
B. intermedia	0	0	1	0
SAP 919	1	0	1	0
	2	0	2a	1
	4	1	2a	1
	6	2a	2a	1
	24	1	2a	2a
	48	1	2b	2a
	72	1	2b	2a
	96	1	2b	2a
	120	1	2b	2a
	0	1	1	0
B. alvinipulli	1	0	1	0
ATCC 51933	2	0	2a	0
	4	1	2a	0
	6	0	2a	0
	24	1	2a	0
	48	1	2b	1
	72	1	2a	1
	96	2a	2b	1
	120	0	2b	1
B. innocens	0	0	1	0
SAP 924	1	0	2a	0
	2	0	1	1
	4	0	2a	1
	6	0	2a	1
	24	0	2a	2a
	48	0	2a	2a
	72	0	2a	2a
	96	0	2b	2b
	120	0	2b	2b

Haematoxylin and Eosin (H&E) staining of histology sections at 24-hour intervals for 120 hours showed the development of an immune response in the *G. mellonella* larvae. This was characterised by the infiltration of haemocytes in the haemolymph and the early development of inflammatory nodules at 24 hours post infection (Figure 7.5C), numerous pigmented bodies and clotted haemolymph at 48 hours (Figure 7.5D), increased pigmented bodies and increasing haemocyte circulation at 72 hours (Figure 7.5E), development of pigmented bodies throughout the larvae at 96 hours and 120 hours (Figures 7.5F and G). Control sections that were not infected with *Brachyspira* showed no signs of infection, although there were some circulating haemocytes in the haemolymph (Figures 7.5A and B). There was clear evidence that *Brachyspira* induced an immune response in this model over the course of 120 hours. Therefore, the *G. mellonella* model was deemed suitable for the study of *Brachyspira* infection.

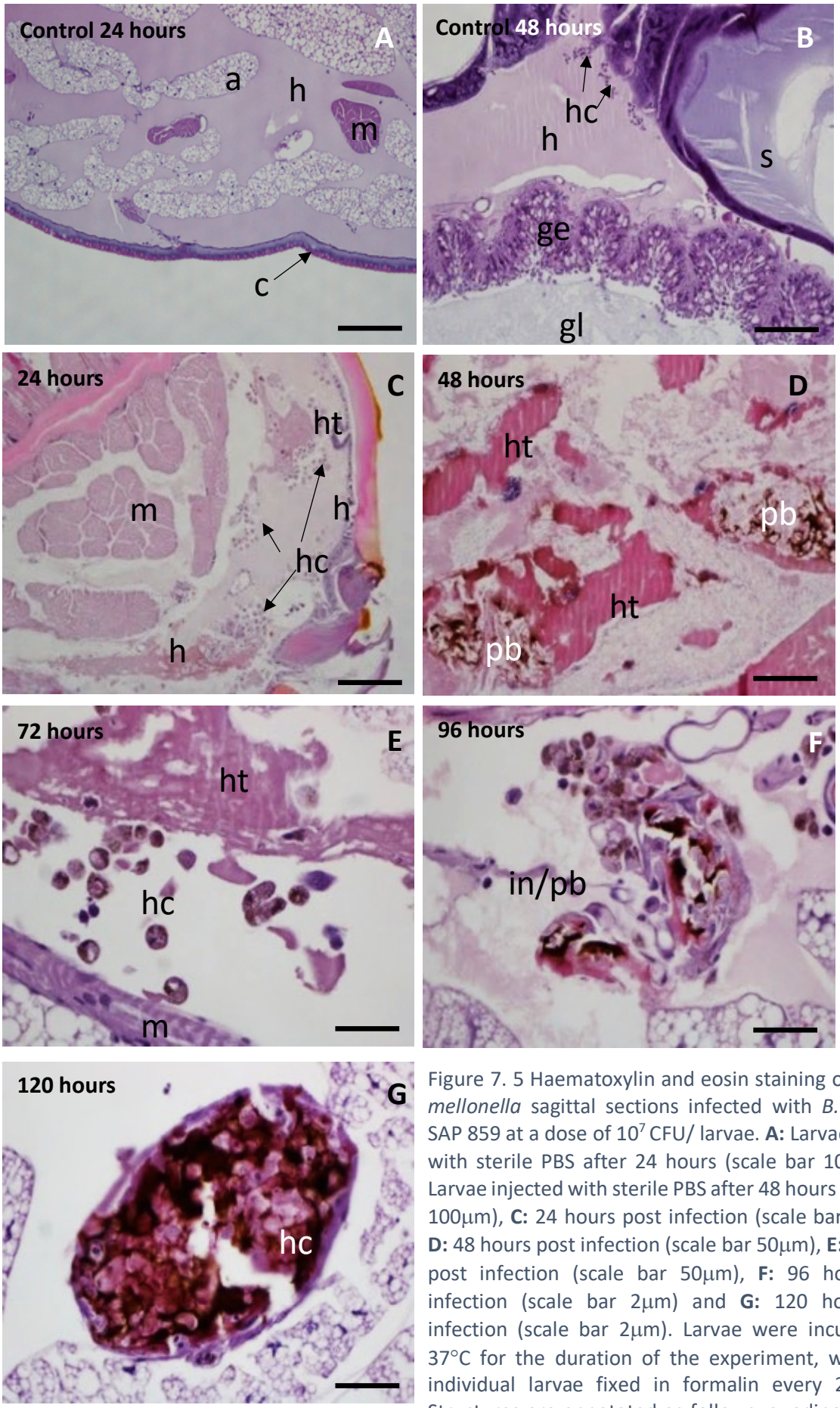


Figure 7. 5 Haematoxylin and eosin staining of *Galleria mellonella* sagittal sections infected with *B. pilosicoli* SAP 859 at a dose of 10^7 CFU/ larvae. **A:** Larvae injected with sterile PBS after 24 hours (scale bar 100μm), **B:** Larvae injected with sterile PBS after 48 hours (scale bar 100μm), **C:** 24 hours post infection (scale bar 100μm), **D:** 48 hours post infection (scale bar 50μm), **E:** 72 hours post infection (scale bar 50μm), **F:** 96 hours post infection (scale bar 2μm) and **G:** 120 hours post infection (scale bar 2μm). Larvae were incubated at 37°C for the duration of the experiment, with three individual larvae fixed in formalin every 24 hours. Structures are annotated as follows: a: adipose tissue, c: cuticle, ge: gut epithelium, gl: gut lumen, h: haemolymph, hc: haemocytes, ht: haemolymph clotted,

The H&E staining was unable to detect *Brachyspira* in the larvae, this was most likely due to poor uptake of the stain by the bacteria, this was also notable for Gram stains. Therefore, silver stains were conducted on a small number of slides due to its specificity for spirochaetes, as mentioned in Chapter 3. The sliver stains, shown in Figure 7.6, show that in addition to circulating in the haemolymph, *Brachyspira* are located in the muscle of the larvae. Moreover, these bacteria showed classic spirochaete morphology, exemplified by their long spiral shape devoid of spherical bodies.

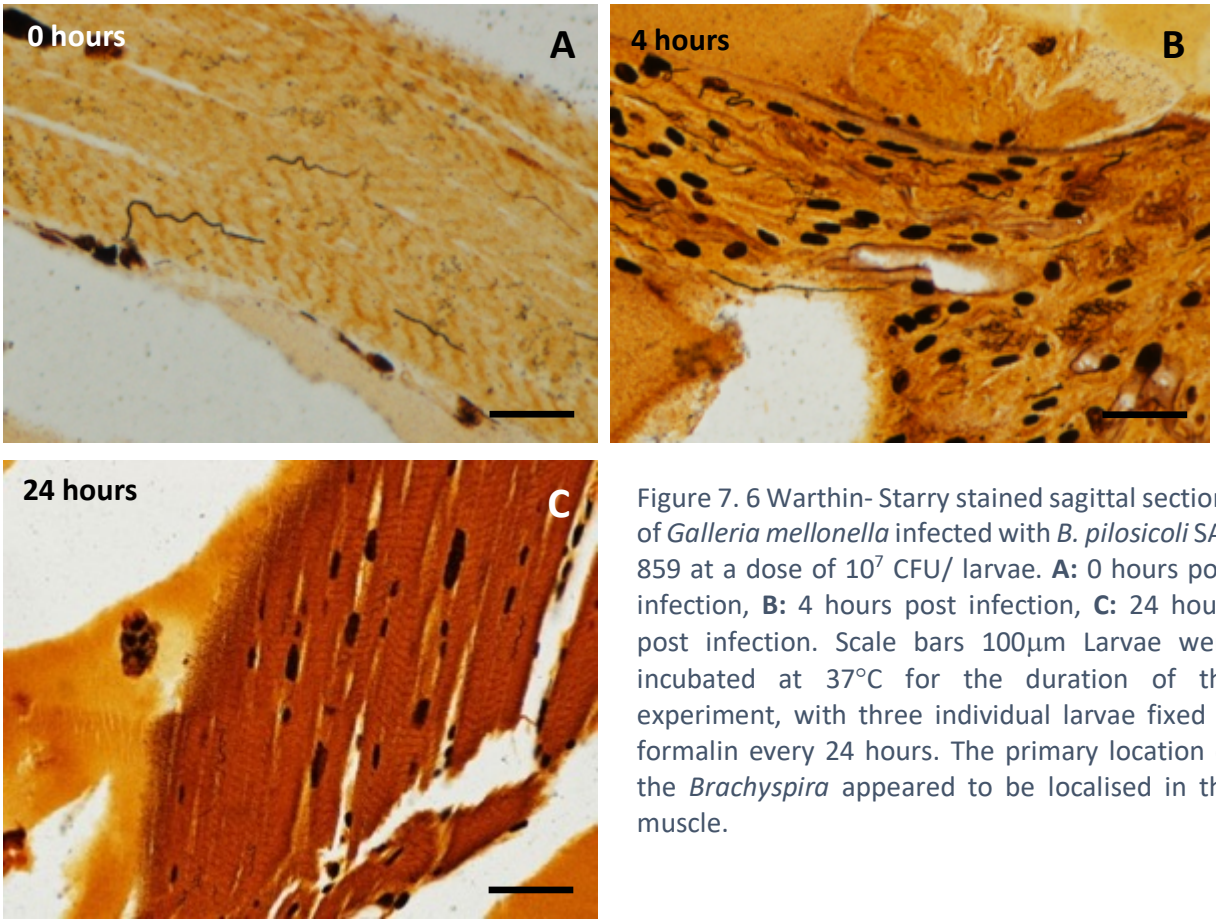


Figure 7. 6 Warthin- Starry stained sagittal sections of *Galleria mellonella* infected with *B. pilosicoli* SAP 859 at a dose of 10^7 CFU/ larvae. **A:** 0 hours post infection, **B:** 4 hours post infection, **C:** 24 hours post infection. Scale bars 100µm Larvae were incubated at 37°C for the duration of the experiment, with three individual larvae fixed in formalin every 24 hours. The primary location of the *Brachyspira* appeared to be localised in the muscle.

7.2.6 Developing a *G. mellonella* model to study probiotics as an intervention against *Brachyspira* infection

Having determined the *G. mellonella* model as a model to study the *Brachyspira* infection, it was important to determine whether a probiotic intervention may be able to protect the larvae from this pathogen.

Initially, the effects of each of the four *Lactobacillus* isolates was tested to determine the dose of *Lactobacillus* that would not induce mortality or any associated morbidity in the larvae. A range of doses from 1 x 10^5 to 1 x 10^2 CFU/ larvae were tested, as shown in Figure 7.7. These data showed that infections with *Lactobacillus* at doses of 1 x 10^2, 1 x 10^3 and 1 x 10^4 CFU/ larvae resulted in 90 to 100% survival in the larvae, whereas infection with 1 x 10^5 induced significant mortality in the larvae (p value < 0.0001), most likely due to bacterial load and rapid replication of *Lactobacillus*. Morbidity scores were zero throughout these experiments. As a result of these preliminary experiments, a dose of 1 x 10^4 CFU/ larvae was selected as this was the highest dose of injectable *Lactobacillus* that did not induce significant mortality in this model. Previous probiotic experiments in *G. mellonella* have utilised a range of *Lactobacillus* species and doses against a range of pathogens including *Candida albicans*, *Salmonella* Typhimurium, *E. coli*, *Staphylococcus aureus* and *Listeria monocytogenes* (Grounta *et al.*, 2016; Rossoni *et al.*, 2017; Scalfaro *et al.*, 2017). Thus, emphasising the requirement to optimise *Lactobacillus* doses and species for the designated pathogen.

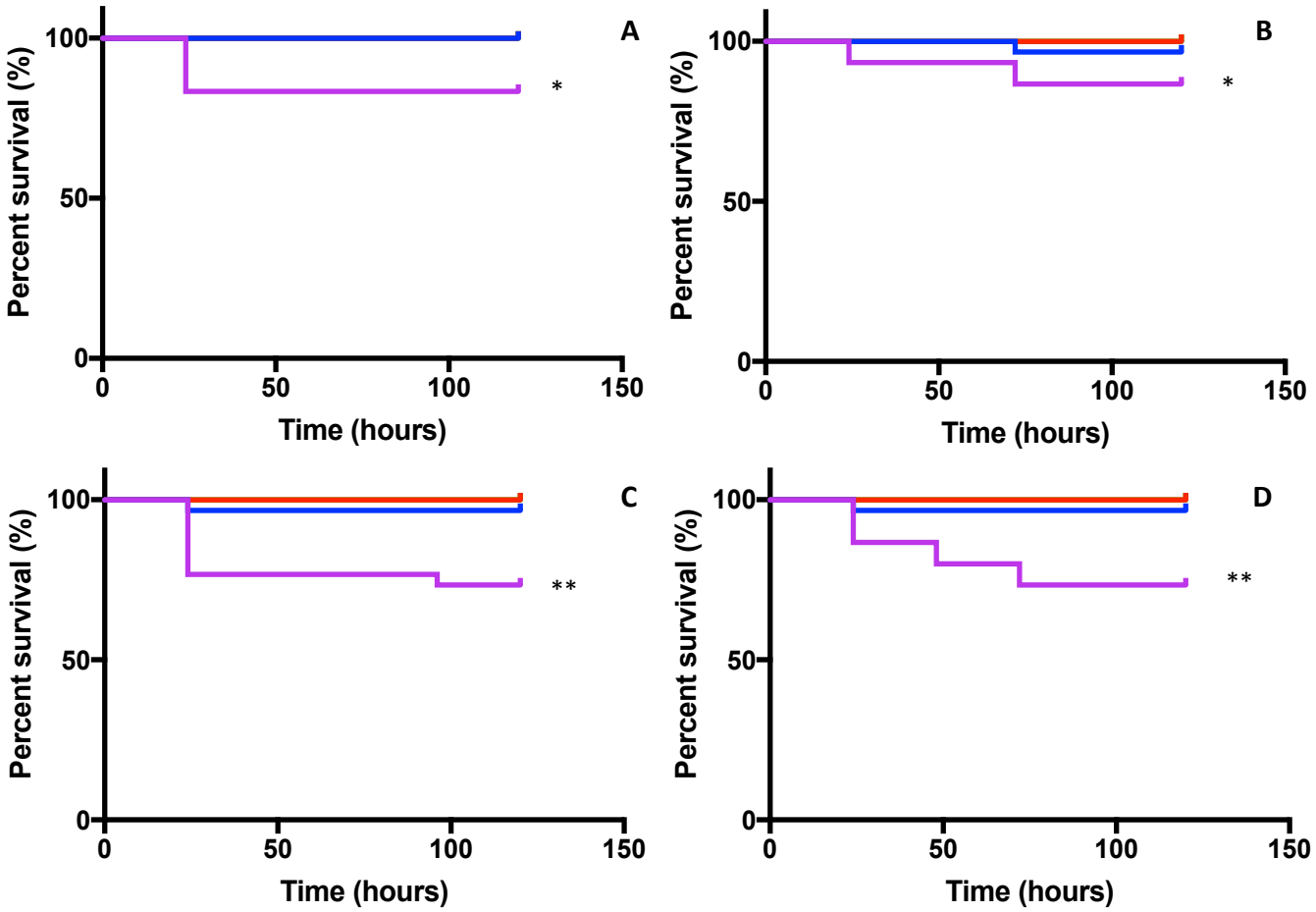


Figure 7. 7 *Galleria mellonella* survival after infection with between 10^2 and 10^5 CFU/ larvae of **A:** *L. reuteri* SAP 2114, **B:** *L. reuteri* SAP 2115, **C:** *L. salivarius* SAP 2116 and **D:** *L. salivarius* SAP 2217. The green lines represent infection with 10^2 CFU/ larvae, the red lines represent infection with 10^3 CFU/ larvae, the blue lines represent infection with 10^4 CFU/larvae and the purple lines represent infection with 10^5 CFU/ larvae. Data are representative of three biological replicates each with 10 larvae per infection group. Mortality scores were recorded every 24 hours for 168 hours of incubation at 37°C. Significance, if any, is shown between infection with different *Lactobacillus* doses, compared to the PBS control infection * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

7.2.7 *Lactobacillus* probiotics as an intervention against *Brachyspira* in the *G. mellonella* model

To assess the ability of the four probiotic candidates selected in Chapter 5 to protect against *Brachyspira* infection in this model, *G. mellonella* were initially infected with *Lactobacillus*, followed by infection with *Brachyspira*.

It was evident that a one-hour pre-treatment with *Lactobacillus* afforded some protection for larvae prior to *Brachyspira* infection, compared to the controls injected with PBS, as shown in

Figure 7.8. As demonstrated in assays described in Chapters 5 and 6, the protective effects of the chosen probiotics were dependent on the isolate of *Lactobacillus* and the isolate of *Brachyspira*. *Lactobacillus* infection prior to infection with *B. pilosicoli* SAP 859 (Figure 7.8A) did not have any significant protective effects on the *G. mellonella* larvae, however *L. reuteri* SAP 2114 demonstrated a small protective effect. Larvae infected with *L. reuteri* SAP 2114 and *L. salivarius* SAP 2117 were significantly protected from mortality associated with infection with *B. intermedia* SAP 919 (p value $\leq$ 0.0001) (Figure 7.8B). Whereas, *L. reuteri* SAP 2115 and *L. salivarius* SAP 2116 did not elicit any protection against *B. intermedia* SAP 919, in fact *L. salivarius* SAP 2116 slightly decreased the survival rate of the larvae. Despite this, *L. salivarius* SAP 2116 was the only *Lactobacillus* isolate to significantly protect the larvae from infection with *B. alvinipulli* ATCC 51933 (p value $\leq$ 0.00332). *L. reuteri* SAP 2115 and *L. salivarius* SAP 2117 demonstrated the ability to protect larvae after 72 hours and increase their overall survival. *L. reuteri* SAP 2114 decreased the survival rate compared to the PBS control (Figure 7.8C). Infections with *L. salivarius* SAP 2116 and SAP 2117 were able to significantly improve the survival of larvae infected with *B. innocens* SAP 924 (p value $\leq$ 0.0332, $\leq$ 0.0021, respectively) compared to the control. *L. reuteri* SAP 2115 also conferred protection against *B. innocens* SAP 924, although this was not significant (Figure 7.8D). The larvae infected with *Lactobacillus* showed no significant mortality compared to the PBS control. Furthermore, there were no observable differences in the morbidity scores when larvae were infected with any of the *Lactobacillus* isolates prior to *Brachyspira* infection.

It is worth noting that *G. mellonella* were also injected with *Lactobacillus* CFS from all four probiotic candidates prior to *Brachyspira* infection. Despite the CFS alone not causing any associated morbidity or mortality, co-infection with *Brachyspira* resulted in 100% mortality within 48 hours post infection, thus the CFS could not protect the larvae.

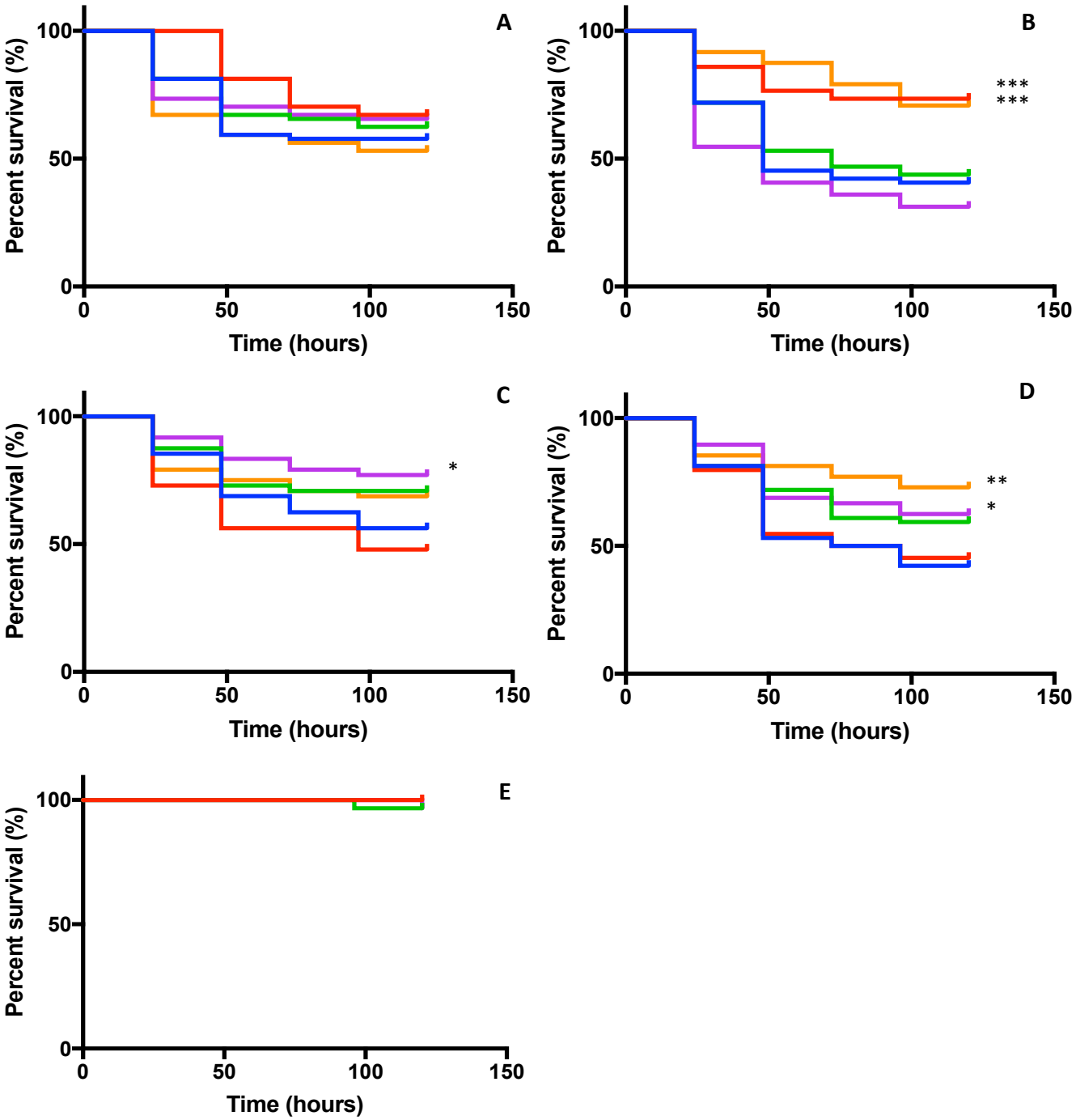


Figure 7. 8 *Galleria mellonella* percent survival following a one-hour pre-infection with 10^4 CFU/ larvae of *Lactobacillus*, before infection with *Brachyspira* species at a dose of 10^7 CFU/ larvae. **A:** Infection with *B. pilosicoli* SAP 859, **B:** infection with *B. intermedia* SAP 919, **C:** infection with *B. alvinipulli* ATCC 51933, **D:** infection with *B. innocens* SAP 924 and **E:** infection with *Lactobacillus* isolates. The blue lines represent the control infection of PBS followed by *Brachyspira* infection, the red lines represent infection with *L. reuteri* SAP 2114 followed by *Brachyspira* infection, the green lines represent infection with *L. reuteri* SAP 2115 followed by *Brachyspira* infection, the purple lines represent infection with *L. salivarius* SAP 2116 followed by *Brachyspira* infection and the orange lines represent infection with *L. salivarius* SAP 2117 followed by *Brachyspira* infection. Percent survival was calculated from five biological replicates, each with 16 larvae per experimental condition. Scores were recorded every 24 hours. Significance, if any, is shown between infection with *Lactobacillus* followed by *Brachyspira* infection and injection with sterile PBS followed by *Brachyspira* infection * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

7.3 Discussion

The studies presented in this chapter focussed on improving the understanding of the innate immune responses to *Brachyspira* infection using both *in vitro* and *in vivo* models. The data generated here from the *in vitro* HD11 avian macrophage studies indicated that *Brachyspira* elicited immune responses in cells of the innate immune system and that pre-exposure to *Lactobacillus* probiotics were able to alter these responses.

To further study the pathobiology of *Brachyspira* infections and probiotics an *in vivo G. mellonella* infection model was developed in order to ascertain the virulence of each *Brachyspira* isolate in the context of a functional immune system and investigate whether pre-exposure to *Lactobacillus* could protect the larvae from the morbidity and mortality associated with infection, given the immunomodulatory properties already observed in the HD11 cells.

Host immune responses against *Brachyspira* species are poorly understood (Hampson, 2018). There has been no significant research conducted to determine the innate immune responses to *Brachyspira*, although the adaptive immune response is known to generate antibodies against *Brachyspira* in low levels (Hampson, 2018). Therefore, the studies presented here characterised the macrophage response to *Brachyspira in vitro* using HD11 avian macrophages in culture. The HD11 cell line is a macrophage-like cell line created by transforming chicken haematopoietic cells with the avian myelocytomatosis type MC29 virus (Beug *et al.*, 1979).

Infections with *Brachyspira* in both humans and animals have been associated with the infiltration of immune cells such as macrophages in the lamina propria, which lies just under the mucosal epithelium (Antonakopoulos *et al*, 1982; Padmanabhan *et al.*, 1996; Duhamel, 2001; Hampson, 2017), demonstrating the importance of macrophages in defence against

Brachyspira. *Brachyspira* have shown the ability to cause chronic caecal and colonic inflammation as a result of invading the lamina propria (Duhamel, 2001). Therefore, understanding the macrophage response to infection and investigating mitigation strategies may improve treatment and control of avian intestinal spirochaetosis.

In order to determine the immune responses to *Brachyspira* species, the production of a selection of pro- and anti-inflammatory cytokines were measured. After four hours of infection all *Brachyspira* isolates were capable of inducing expression of the pro-inflammatory cytokines IL1β, IL6, IL8 and IFNγ and the anti-inflammatory cytokine IL10. Both pathogenic and non-pathogenic *Brachyspira* elicited similar cytokine responses from the HD11 cells. *B. innocens* is often regarded as a commensal bacteria as no clinical disease has been associated with infection, similarly *C. jejuni* is considered as a commensal bacteria in the avian hosts, however studies have shown that *Campylobacter* were also capable of eliciting immune responses in HD11 cells (Smith *et al.*, 2005). *B. pilosicoli* SAP 859 elicited the greatest cytokine responses, in particular the IFNγ response, which was significantly higher than other isolates. The differences in immune response may be as a result of the response to lipooligosaccharide (LOS) on the outer membrane of *Brachyspira*. LOS have been shown to be antigenically distinct both between species and within species of *Brachyspira* (Lee and Hampson, 1999), thus may have an impact on the subsequent immune response. LOS activates toll-like receptor 4 (TLR4) which is expressed on a number of immune cells, including macrophages. This activation produces a pro-inflammatory response and may be, in part attributed to the differences in the pro-inflammatory responses induced by the different species of *Brachyspira* (both pathogenic and non-pathogenic) (John *et al.*, 2017).

A gentamicin protection assay was utilised to determine whether *Brachyspira* could survive intracellularly. As previously mentioned, *Brachyspira* have been located within macrophages

using microscopy techniques but no reports of intracellular replication or survival have been made (Antonakopoulos *et al.,* 1982). The assay utilised in these studies demonstrated that *Brachyspira* do not survive within the HD11 cells, however remained viable when cultured from the tissue culture media following three hours of infection. Following this, a fluorescent oxidative burst assay was performed in an attempt to measure phagocytic activity of the macrophages in response to *Brachyspira* in order to further characterise the immune response to this pathogen. However, control experiments containing *Brachyspira* alone resulted in higher background fluorescence than a co-culture with the pathogen and HD11, and thus this assay did not give accurate results. This may have been as a result of the *Brachyspira nox* gene activity in the presence of oxygen. This gene aids in the conversion of oxygen to water via a superoxide intermediate which would oxidise the fluorescent dye in this experiment, resulting in this assay being an unsuitable method to measure oxidative burst (Hajjar *et al.*, 2017).

Immunomodulation is one of many proposed mechanisms of probiotic action. Probiotic bacteria are known to stimulate the immune responses in the gastrointestinal tract without causing disease and are hypothesised to prime the immune system, but also to modulate responses to control the production of cytokines and chemokines (Haller *et al.*, 2000). The cytokine responses to *L. reuteri* SAP 2115 and *L. salivarius* SAP 2117 were investigated as these two probiotic candidates were the most successful of their species throughout all experiments conducted in Chapters 5 and 6. It was initially important to establish the cytokine response elicited by these selected *Lactobacillus* isolates. Both isolates were able to induce the expression of both pro- and anti-inflammatory cytokines, except for IFNγ which is a potent pro-inflammatory cytokine (Tau and Rothman, 1999). *L. salivarius* SAP 2117 was unable to induce the expression of IL10, an anti-inflammatory cytokine capable of preventing

inflammation and restoring homeostasis during infection (Iyer and Cheng, 2012). Whereas, *L. reuteri* SAP 2115 was able to induce significantly higher levels of IL10 expression, thus suggesting that this probiotic candidate may be more suitable because IL10 expression may play a significant role in controlling immune responses as a result of *Brachyspira* infection. However, this would need to be shown in an appropriate animal model. Increased IL10 production has been associated with the decreased expression of proinflammatory cytokines such as IFNγ (de Moreno de Leblanc *et al.*, 2011) and these studies have suggested that IFNγ is the most expressed pro-inflammatory cytokine (of those tested here) in *Brachyspira* infection. Therefore, *Lactobacillus* isolates that induce the expression of IL10 may be more favourable as probiotic candidates to mitigate avian intestinal spirochaetosis because they have the potential to reduce the inflammatory response, thus may contribute to reducing the severity of the disease.

The experiments conducted in these studies suggested evidence of alterations in cytokine expression following probiotic pre-treatment as many of the cytokine responses were significantly altered when the HD11 cells were pre-exposed to either *L. reuteri* SAP 2115 or *L. salivarius* SAP 2117. As previously highlighted, the IFNγ response appears to be of significant importance in *Brachyspira* inflammation as this was the most highly expressed cytokine, especially in response to *B. pilosicoli* SAP 859. The infection of HD11 cells with *Lactobacillus* prior to *Brachyspira* infection reduced the expression of IFNγ in response to all *Brachyspira* species. IFNγ signalling is mediated through the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway which is known to be inactivated by some isolates of *Lactobacillus* in response to infection (Lee *et al.*, 2010; Llewellyn and Foey, 2017). *L. acidophilus* and *L. plantarum* isolates have been shown to activate STAT1 and STAT3 and inactivate JAK2, which is essential for IFNγ signalling (Llewellyn and Foey, 2017). The

expression of IL6 following pre-exposure to *Lactobacillus* was also reduced in response to *Brachyspira*. In addition to the inhibition of the JAK/STAT pathways, *Lactobacillus* probiotics have also been shown to attenuate the NFκB and ERK pathways which are responsible for the production of pro-inflammatory IL6, therefore, reducing its expression. (Lee *et al.*, 2010; Llewellyn and Foey, 2017). The expression of IL1β and IL8 remained largely unchanged after pre-exposure to *Lactobacillus*. The expression of these cytokines has been linked to NFκB and p38 MAPK pathways (Takanashi *et al.*, 2013) and therefore the *Lactobacillus* isolates used in these studies may have been ineffective against the branches of these pathways involved in IL1β and IL8 production. As with all experiments conducted thus far, the impact on cytokine gene expression was dependent on both the *Brachyspira* and *Lactobacillus* isolates, as previously observed by Llewellyn and Foey (2017). Probiotic bacteria have been known to induce the expression of suppressor of cytokine signalling (SOCS) genes. These proteins are known to be inhibitory regulators of the JAK/STAT pathway and TLR4 mediated cytokine responses (Dimitriou *et al.*, 2008), thus downregulating the expression of pro-inflammatory cytokines. SOCS genes, in particular SOCS3 is known to be inducible by IL10. The anti-inflammatory response is initiated by the phosphorylation of STAT3 which inhibits the production of pro-inflammatory cytokines using the JAK/STAT pathway, this in turn upregulates the gene expression of SOCS genes. In human macrophages, *L. rhamnosus* GG was shown to increase the phosphorylation of STAT3 and increase the expression of SOCS3, which was proposed to be a mechanism of immune modulation in human health (Latvala *et al.*, 2011). It is important to note that much of this research has been conducted in human or murine models both *in vitro* and *in vivo* and thus it can only be speculated that similar responses occur in the avian host.

These studies have demonstrated that there is careful regulation of the innate immune system in response to infection. *Lactobacillus* isolates downregulated the expression of pro-inflammatory cytokines which *in vivo* may prevent a severe pro-inflammatory response. They also resulted in an overall downregulation of IL10 which may be fundamental in controlling the immune response to ensure there is enough of a response to clear infection, but that it is not dampened so significantly that the infection can proliferate. It is imperative to repeat the studies described in whole animal *in vivo* studies to test the hypotheses derived by studies in *in vitro* tissue culture albeit HD11 cells are chicken derived.

The *Galleria mellonella* infection model has been previously shown to be a powerful and effective infection model for a number of pathogens such as *Campylobacter jejuni*, *E. coil* and *Candida albicans* (Brennan *et al.*, 2002; Senior *et al.*, 2011; Alghoribi *et al.*, 2014). Furthermore, this model has proven useful in investigating antibiotic and probiotic interventions to treat and prevent disease (Benthall *et al.*, 2015; Scalfaro *et al.*, 2017). This infection model is becoming a more popular alternative to mammalian and avian models of infection due to the simplicity and reliability, in addition to being an inexpensive tool that is more ethically accepted. The *G. mellonella* model is useful for studying infection in the context of a functional immune system, although no studies of *Brachyspira* infection have been conducted in this model. *G. mellonella* are capable of innate immune responses, these responses consist of two parts (Tsai *et al*., 2016). The first is the cellular immune response, mediated by immune cells know as haemocytes, which are present in the haemolymph of the insects (Ramarao *et al*., 2012). The second is the humoral immune response, which is mediated by molecules such as melanin. Haemocytes are capable of phagocytosis and can produce small antimicrobial peptides. These cells are functionally analogous to mammalian neutrophils, which are analogous to avian heterophils (Brooks *et al*., 1996); therefore, this

model may be able to give an insight to *Brachyspira* infection in both mammalian and avian hosts. The humoral immune response can be measured as the production of melanin results in a visible colour change in the larvae. Melanin encapsulates pathogens at the site of infection, which is followed by the coagulation of haemolymph (Tsai *et al.,* 2016).

In order to begin to develop a *Brachyspira* infection model using *G. mellonella* larvae, all eight *Brachyspira* isolates used throughout the studies presented here were injected into the larvae to assess their virulence in this model. Pathogens such as *C. jejuni* and *C. albicans* have been well researched in this model and has been used to identify virulence trends in isolates (Brennan *et al.*, 2002; Champion *et al.*, 2010; Mehat *et al.*, 2018). Therefore, similar approaches for *Brachyspira* infection were undertaken. Interestingly, the virulence observed in the *G. mellonella* had some key differences to that of avian infection, namely that *B. innocens* resulted in the highest mortality in this model, however this species is proposed to be non-pathogenic in the avian host (Mappley *et al.,* 2014). Although *B. innocens* is deemed non-pathogenic in the avian host, there has been some evidence that this species of *Brachyspira* is linked to poor egg production in flocks, especially in free-range hens (Burch *et al.*, 2009) and thus may be causing disease in chickens. *B. innocens* was capable of eliciting pro-inflammatory responses in HD11 cells also, showing that it does cause an immune response comparable to that of pathogenic species. In chickens, disease caused *B. alvinipulli*, *B. intermedia* and *B. pilosicoli* manifests as similar clinical symptoms, but can range in severity, therefore, the range of morbidity and mortality associated with these isolates in the *G. mellonella* model may show some similarities to avian infection. However, many more isolates of *Brachyspira* would need to be screened using this model in order to reach a conclusion, furthermore, the virulence of these *Brachyspira* isolates would need to be

determined in a chicken host to be able to confirm the similarity of the avian and the insect host.

Brachyspira remain viable in the *G. mellonella* haemolymph for between 24 and 48 hours post infection, suggesting that the cellular and humoral immune responses in these larvae are capable of killing this pathogen. Notably, for many of the isolates tested, the highest mortality rates were observed within the first 48 hours of infection. This could be associated with the infiltration of increasing numbers haemocytes and development of inflammatory/ melanin bodies to clear the infection. This can be observed in the histology scoring and also in the H&E stains which showed the increasing numbers of haemocytes at 24 hours and the development of pigmented bodies at 48 hours post infection. It is also important, however to consider that *Brachyspira* are fastidious anaerobes and thus bacterial numbers may also be declining due to lack of nutrients and presence of oxygen.

In the avian host, *Brachyspira* are primarily found in the caeca and large intestine, however they have been known to enter the bloodstream and cause systemic infection and as a result have been found in the spleen and liver of infected birds (Mappley *et al.*, 2013). The *G. mellonella* infection model is a systemic infection model as the bacteria are injected directly into the haemolymph. Sliver staining of *G. mellonella* tissue sections showed that the *Brachyspira* were primarily located in the muscle tissue and no spirochaetes were observed in the gut. Silver stains were conducted at 0, 4 and 24 hours post infection to observe where the bacteria were localised in this model, ideally more time points would have been stained in order to qualitatively monitor *Brachyspira*, however financial constraints limited this. The silver stains of *B. pilosicoli* SAP 859 suggested that the bacterial cells were healthy as they displayed a long spirochaete morphology; *Brachyspira* are known to form spherical bodies and become more cocci in shape in response to stress (Wood *et al.*, 2006; Bernardeau *et al.*,

2009). It is also worth noting that the muscle of birds following *Brachyspira* infection has not been extensively investigated for the presence of *Brachyspira,* although one study found that chicken carcasses were contaminated with *Brachyspira* (Verlinden *et al.*, 2012). Therefore, the localisation of these bacteria in the *Galleria mellonella* model may inform avian infection studies in future *in vivo* testing as this may be a possible route of zoonotic infection. *Brachyspira* were not found in the gastrointestinal tracts of the larvae with H&E, Gram or Silver staining. However, there were increased numbers of Gram positive cocci in the gut, as noted during the histology scoring. *Enterococcus faecalis* is known to be a commensal of the gut of *G. mellonella* larvae (Jarosz, 1979) and therefore, it could be suggested that an infection with *Brachyspira* could have compromised the immune system, thus allowing the proliferation of *E. faecalis*.

This model of *G. mellonella* infection was systemic in nature, and although it proved to be a useful model to assess the virulence of different isolates, avian intestinal spirochaetosis is primarily a gastrointestinal disease, despite there being some evidence of systemic infection. Interestingly, analogies have been found between the epithelial cells of the larvae's midgut and the intestinal cells of the mammalian digestive system (Ramarao *et al.,* 2012) and therefore may prove to be a useful gut model in future studies. However previous studies have demonstrated that the ingestion of foodborne pathogens such as *L. monocytogenes* and *P. aeruginosa* does not induce mortality in this model (Fedhila *et al.*, 2010). Other drawbacks would include the technical difficulties of feeding the larvae and that exact infection doses would be difficult to obtain (Tsai *et al.,* 2016).

G. mellonella were determined as a suitable model for *Brachyspira* infection, therefore *Lactobacillus* probiotics were investigated as a suitable intervention for the mortality and morbidity associated with pathogen infection. Previous studies using *Lactobacillus* in *G.*

mellonella have tested several doses of these bacteria and their ability to protect against the chosen pathogen, ranging from 10^2 to 10^9 CFU/ larvae, with mortality associated with higher doses of *Lactobacillus* (Grounta *et al.*, 2016; Rossoni *et al.*, 2017; Scalfaro *et al.*, 2017). In the studies presented here, a range of 10^2 to 10^5 CFU/ larvae were tested with only 10^5 CFU/ml causing any associated mortality, therefore a dose of 10^4 CFU/ml was selected as this was the highest dose of *Lactobacillus* that did not result in any associated morbidity and mortality.

As with the assays presented in Chapter 6, the effects of the different *Lactobacillus* isolates were very much specific on both the isolate of *Lactobacillus* and the isolate of *Brachyspira*. *B. intermedia* SAP 919 was the most sensitive to probiotic intervention whereby *L. reuteri* SAP 2114 and *L. salivarius* SAP 2117 were able to protect the larvae against *Brachyspira* infection. *L. salivarius* SAP 2116 was able to protect against infection with both *B. alvinipulli* ATCC 51933 and *B. innocens* SAP 924, in addition to *L. salivarius* SAP 2117 being able to protect against *B. innocens* SAP 924 infection. Previous studies have also demonstrated that pre-treatment with *Lactobacillus* could protect against *C. albicans, L. monocytogenes* and *S. aureus* (Grounta *et al.*, 2016; Rossoni *et al.*, 2017; Scalfaro *et al.*, 2017). It has been proposed by Rossoni *et al.* (2017) and Scalfaro *et al*. (2017) that pre-treatment with *Lactobacillus* may be able to prime the immune system by increasing the numbers of haemocytes in the haemolymph, thus when the larvae are challenged with *C. albicans* the infection can be rapidly addressed, however this was not investigated in these studies. Additionally, physical interactions between *Brachyspira* and *Lactobacillus* could have resulted in the protective effect observed in the larvae, as observed the co-culture experiments in Chapter 6 and in tissue culture experiments conducted by Mappley *et al.* (2011). Finally, studies presented earlier in Chapter 6 demonstrated that the *Lactobacillus* isolates used in these studies were able to produce

antimicrobial compounds that were shown to inhibit *Brachyspira* species, therefore these may be contributing to the protective effect following *Brachyspira* infection.

Interestingly, *L. reuteri* SAP 2115, which was the most successful inhibitor of *Brachyspira* in the *in vitro* experiments presented in Chapter 6 did not demonstrate any significant protection of *G. mellonella* larvae in this model. Suggesting that multiple mechanisms interplay in the protective effects of probiotics and that not all probiotics elicit effects by the same mechanisms. Throughout the studies presented in Chapter 6, *B. pilosicoli* SAP 859 was one of the most tolerant isolates to probiotic intervention and the same trends in data were seen in these studies whereby probiotic intervention did not protect the larvae from infection with *B. pilosicoli* SAP 859.

Notably, this model was utilised to characterise the *G. mellonella* response to *Brachyspira* with the intention of optimising this alternative *in vivo* model which could be used to potentially explore isolate virulence and probiotic protection in the context of a functional immune system. It would be important to validate these results in a more realistic *in vivo* chicken model, however the *G. mellonella* model provided useful preliminary data.

Further studies with a larger number of probiotics and a larger number of *Brachyspira* isolates are needed to validate the *G. mellonella* model to conclude if *Lactobacillus* probiotics would be a suitable intervention in this model, however these experiments have made progress towards this aim. It is important to note that the reproducibility of results between batches of *G. mellonella* purchased from live food stores can be poor, which may have accounted for the variability in results between biological replicates. To overcome this, future studies would be conducted with TruLarv™ as these *G. mellonella* larvae are standardised by age and weight and come from a breeding colony devoid of hormone or antimicrobials, thus improving the reproducibility of experiments.

In summary, this chapter described the investigation of innate immune response to *Brachyspira* and *Lactobacillus* using two models of infection and demonstrated that pre-infection with *Lactobacillus* was able to significantly alter the immune responses to *Brachyspira* species. Probiotic bacteria have been well documented to be involved in many immunomodulatory processes, involving many pathways to control pro-inflammatory immune responses. Further studies are required to determine the exact mechanisms of protection elicited in these experiments. However, significant progress has been made in understanding the innate immune response to *Brachyspira* infection and how *Lactobacillus* probiotics may be able to mitigate disease.

Chapter 8: Final discussion and future perspectives

8.1 Summary of results, limitations and discussion

Brachyspira species pose a significant economic burden in the UK and globally and yet remain poorly understood. Therefore, this study aimed to gain greater understanding of those *Brachyspira* species associated with economic impacts in the poultry industry and to investigate mitigation strategies to ease this burden and improve poultry health and welfare. The studies presented here aimed to characterise and thereby improve the understanding of *Brachyspira* species implicated in avian intestinal spirochaetosis, with the genome studies presenting the largest number of *Brachyspira* whole genome sequences to date, to highlight the genetic variance between species in the genus. They also aimed to investigate probiotic interventions and their mechanisms of action. It was shown that probiotics have the potential to mitigate *Brachyspira* infection by several mechanisms including acid production, the activity of bacteriocins and other inhibitory metabolites independent of pH and co-aggregation of *Brachyspira*. Furthermore, the data generated in this study were the first reports on the immune interactions of *Brachyspira* with HD11 cells in a tissue culture model and the development of a *Galleria mellonella* model to investigate *Brachyspira* infection and potential probiotic interventions.

8.1.1 Phenotypic characterisation of *Brachyspira*

Traditional phenotypic methods of identification were employed (Fellström and Gunnarsson, 1995) but these were unable to identify many of the isolates tested possibly because the tests used were developed for *Brachyspira* isolates of porcine origin which have been shown and confirmed here to be phenotypically distinct from poultry isolates (Atyeo *et al.*, 1999; Feberwee *et al.*, 2008).

Current phenotypic methods proved ineffective when identifying *Brachyspira* species isolated from poultry in the studies presented here. Therefore, as a continuation of the work published by Mappley *et al.* (2012), Biolog technology was utilised with the aim of identifying metabolic capabilities unique to each species of *Brachyspira* that could be targeted for the differential diagnosis of species responsible for poultry disease. Thirty-nine carbon sources were utilised by all *Brachyspira* isolates, many of these associated with glycolysis and the pentose phosphate pathway; metabolic pathways previously mapped in genome studies (Bellgard *et al.*, 2009; Wanchanthuek *et al.*, 2010). Unfortunately, Biolog data analysis did not reveal any unique metabolic targets that could distinguish *Brachyspira* species which is likely to be a result of the small number of isolates tested and the metabolic diversity observed within different species. A greater understanding of the metabolic pathways and this diversity could be achieved by investigating more isolates of each species, in addition to combining these data with the genotypic data available for each isolate. *B. pilosicoli* isolates were capable of respiring on sixty-six carbon sources on the PM1 and PM2 Biolog plates, whereas the other species were capable respiring on between fifty-seven and fifty-nine carbon sources. This may be related to the larger host range of *B. pilosicoli,* whereby utilising more carbon sources may provide adaptability for different ecological niches.

Interestingly, the pangenome analysis of *B. pilosicoli* demonstrated the genotypic diversity within this species, with 53% of the pangenome consisting of accessory genes and only 47% consisting of core genes. This is not characteristic of *Brachyspira* species because the *B. hyodysenteriae* core consisted of 70% of the genes. These findings highlight the plasticity of the *B. pilosicoli* genome, which reflects the metabolic diversity observed in phenotypic studies. Further work could identify the metabolic genes within the accessory genome to potentially explain the phenotypic differences observed between isolates of *B. pilosicoli* to

confirm this hypothesis. Also, there is a need to understand what metabolic genes are present and active or present but redundant through accumulation of mutations. Similarly, if present but inactive, would certain environments select revertants as this too contributes to dynamic plasticity of the phenotype and as yet nothing is known of this. Phenotypic analysis clearly has limitations and requires a more detailed genotypic approach to support them and answer some of the questions arising.

8.1.2 Genotypic characterisation of *Brachyspira*

Whole genome sequencing has proved to be a very useful tool, not only for the identification of *Brachyspira* (discussed in Chapter 3), but also for improving the understanding of the genus (Bellgard *et al.*, 2009; Pati *et al.*, 2010; Wanchanthuek *et al.*, 2010; Mappley *et al.*, 2012). The studies presented here have contributed fifteen novel *B. pilosicoli* genomes to the five available on Genbank, making the comparative genomics more robust than previous studies (Wanchanthuek *et al.*, 2010; Mappley *et al.*, 2012). In order to make more accurate comparisons additional genome sequences from *B. alvinipulli, B. innocens* and *B. intermedia* are required. Comparison of all available genome sequences (Chapter 4) revealed that *B. pilosicoli* isolates were distinct from other species of *Brachyspira* and also that this species had a larger accessory genome (53% of the genes) compared to other species such as *B. hyodysenteriae* (30% of the genes). This may account for *B. pilosicoli* being the only species that is capable of infecting human, porcine and avian species (Trott *et al.*, 1996), in addition to being able to form end-on attachments to the intestinal epithelium (Trampel *et al.,* 1994; Muniappa *et al.*, 1996). *B. aalborgi* is the only other species capable of this, however no genome sequences are available for this species and thus this interaction cannot be further investigated (Mappley *et al.,* 2014). Novel comparisons between *B. pilosicoli*, *B.*

hyodysenteriae and the "Other" *Brachyspira* species, including *B. alvinipulli*, *B. intermedia, B. innocens, B. hampsonii, B. murdochii* and *B. suanatina* suggested that *B. hyodysenteriae* and the "Other" species of *Brachyspira* shared more genes with one another (1329 genes) than with *B. pilosicoli* which shared 210 genes with the "Other" species and only thirty-five genes with *B. hyodysenteriae*. This was the first pangenome analysis highlighting the differences between the species, as previous studies have only used one isolate from each species, which is not representative of the intra-species diversity (Wanchanthuek *et al.*, 2010).

8.1.3 *Lactobacillus* as a probiotic intervention against *Brachyspira*

Four *Lactobacillus* isolates (Chapter 5) were selected for further investigation primarily due to their ability to inhibit multiple *Brachyspira* species, in addition to their suitability as commercial products as described in the EFSA guidelines (EFSA *et al.*, 2017). *L. reuteri* isolates are likely to be suitable probiotic candidates for commercialisation, as also suggested by Mappley *et al.* (2011; 2013), because of their greater inhibition of *Brachyspira*, the absence of antimicrobial resistance genes and tolerance to bile salts and acid, in addition to showing the greatest immunomodulatory potential in the HD11 avian macrophage assays. *L. salivarius* isolates were used in these studies as a comparison between hetero- and homofermentative *Lactobacillus* but were shown to be less inhibitory than *L. reuteri.* Recently, *in vitro* studies have investigated the effects of probiotics as intervention strategies for *Brachyspira* infection for both porcine and avian species of *Brachyspira*. Inhibition has been proposed to be as a result of several mechanisms, including acid production (primarily attributed to lactic acid), hydrogen peroxide production and production of other antimicrobial compounds such as bacteriocins (Bernardeau *et al.*, 2009; Klose *et al.*, 2009, 2010; Mappley *et al.*, 2011).

The inhibitory ability of organic acids produced by lactic acid bacteria such as *Lactobacillus* is well established (Collins *et al.*, 2009). The studies presented here suggested that acid production by the *Lactobacillus* isolates contributed significantly to the inhibition of *Brachyspira*. *Lactobacillus* cell free supernatant and MRS media were significantly more inhibitory at pH 3.8 compared to pH 7.2; this was observed for all *Brachyspira* isolates except for *B. pilosicoli* SAP 865, which appeared to be acid tolerant, therefore making it unsuitable for probiotic intervention. If time permitted an analysis of acid tolerance response genes in this isolate would have been of interest in investigating this unusual phenotype. Additional studies could utilise SEM to identify spherical bodies or evidence of cell membrane disruption to confirm the acid stress responses previously identified in *Brachyspira* isolates (Bernardeau *et al.*, 2009). Furthermore, NMR analysis showed that the most inhibitory *Lactobacillus* isolates produced the highest concentrations of organic acids, subsequently consuming the highest concentrations of glucose. *Lactobacillus* isolates that consume more glucose may be more inhibitory against *Brachyspira in vivo* as there would be greater competition for this essential nutrient.

The production of antimicrobial peptides was also investigated in these studies using techniques to denature proteins present in the *Lactobacillus* cell free supernatant. These studies suggested that *L. reuteri* isolates produced heat stable bacteriocins as trypsin treatment of the cell free supernatant significantly reduced the inhibition of a number of *Brachyspira* isolates. *L. salivarius* SAP 2117 produced heat labile bacteriocins as heat treatment of the cell free supernatant reduced the inhibition of *Brachyspira* isolates. Bacteriocin activity was generally greater at pH 3.8, supporting the hypothesis that acid may permeabilise the cell membrane and enhance the activity of bacteriocins (Cotter *et al.,* 2005). Conversely, the peptides produced by *L. salivarius* SAP 2117 were more effective at pH 7.2,

suggesting that bacteriocins can inhibit Gram negative bacteria independent of pH. Future work would aim to identify these proteins and characterise their efficacy against *Brachyspira* in more detail. Physical interactions between *Brachyspira* and potential *Lactobacillus* probiotics have shown that co-aggregation is an important mechanism of inhibition, both in simple *in vitro* assays (Bernardeau *et al.*, 2009) and in tissue culture models (Mappley *et al.*, 2011). This co-aggregation impairs the pathogen's motility, as previously discussed, motility is regarded as a key virulence factor of *Brachyspira* and is vital for its survival and colonisation in the large intestine. The *L. reuteri* isolates selected for these studies showed the ability to impair *Brachyspira* viability following co-culture with viable and heat inactivated cells suggesting that this effect was not as a result of competition for nutrients or production of antimicrobial compounds and was likely to be as a result of the passive coaggregation of *Brachyspira* and *Lactobacillus* as previously demonstrated (Bernardeau *et al.*, 2009). Some *B. pilosicoli* isolates were tolerant of this mechanism of inhibition, for example *B. pilosicoli* SAP 859 was unaffected by co-culture with *Lactobacillus* isolates, suggesting that this mechanism is not universally applicable to all *Brachyspira*. *L. salivarius* isolates were not effective in these studies, demonstrating that the ability to co-aggregate with *Brachyspira* is isolate specific. The results from this study are limited as the culture conditions do not represent those of the gastrointestinal tract, however these preliminary results could be confirmed using an *in vitro* intestinal cell line which may be a closer representation of the bacterial interactions at the epithelium. In addition to the mechanisms mentioned above, *Lactobacillus* species are robust and grow rapidly, compared to the slow growing, fastidious nature of *Brachyspira* species. This therefore makes *Lactobacillus* probiotics ideal candidates to mitigate *Brachyspira* as they have the potential to outcompete *Brachyspira* in the large intestine (Mappley *et al.*, 2013).

The HD11 avian macrophage cell line has been used to explore the innate immune response to a number of avian pathogens including, *Salmonella* species and *Campylobacter jejuni* in order to explore host-pathogen interactions (Kaiser *et al.*, 2000; Smith *et al.*, 2005). Therefore, the initial aim of the studies presented here was to develop a novel tissue culture model using HD11 cells to investigate the *in vitro* macrophage response to *Brachyspira* and *Lactobacillus* species. Subsequently, the immunomodulatory properties of the *Lactobacillus* isolates were investigated by pre-treating the cells with the probiotic candidates prior to infection with *Brachyspira*. *Brachyspira* species demonstrated the ability to induce both pro- and anti-inflammatory cytokine responses in the HD11 cells with the greatest level of expression observed in IFNγ. IL1β and IL10 expression were also significantly upregulated compared to the control, however no significant differences were observed in IL6 or IL8 expression. A similar study to investigate the macrophage response to *C. jejuni* demonstrated the induction of pro-inflammatory cytokines such as IL1β, IL6 and IL8 from these cells, however IFNγ and IL10 responses were not significantly different from the control infection (Smith *et al.*, 2005). *L. reuteri* SAP 2115 significantly upregulated IL1β and IL10, with the upregulation of IL10 potentially contributing to the control of the pro-inflammatory immune response. *L. salivarius* SAP 2117 did not induce significant changes in cytokine expression compared to the control, suggesting that this isolate may not be as effective at modulating the immune response as *L. reuteri* SAP 2115.

Studies where cells were pre-treated with *Lactobacillus* prior to infection with *Brachyspira* suggested that these potential probiotics could not only directly inhibit *Brachyspira*, as demonstrated in Chapters 5 and 6, but could also modulate the innate immune response following infection with this pathogen. As previously mentioned, *Brachyspira* species induced the expression of several pro-inflammatory cytokines from the HD11 macrophages, with the

most marked effect on the expression of IFNγ. This cytokine is important in both the innate and adaptive immune responses. It is an important activator of macrophages and also a potent activator of Class II major histocompatibility complex (MHC) expression on an antigen presenting cell which presents antigens to T cells (Holling *et al.,* 2004). When HD11 cells were pre-treated with *Lactobacillus* isolates before challenge with *Brachyspira*, the IFNγ response from the HD11 cells was significantly reduced. Thus, demonstrating the ability of *Lactobacillus* to modulate the immune response and reduce the pro-inflammatory cytokine expression. This modulation is hypothesised to be via the JAK/STAT pathway which mediates IFNγ signalling, which is known to be inhibited by some isolates of *Lactobacillus* (Lee *et al.*, 2010; Llewellyn and Foey, 2017). *Lactobacillus* isolates, including *L. acidophilus* and *L. plantarum* activate STAT1 and STAT3 and inactivate JAK2 inhibiting the production of pro-inflammatory cytokines via the JAK/STAT pathway. This has been proposed to be a key anti-inflammatory mechanism of probiotics which may prove useful in the mitigation of *H. pylori* infection, although *in vivo* experiments are required to test this hypothesis (Lee *et al.*, 2010). Further investigation of inflammatory markers in an experimental chicken model may help to quantify the protective response against *Brachyspira in vivo*. The study by Mappley *et al*. (2013) showed reduced inflammation in birds treated with the probiotic, although this was attributed to reduced *Brachyspira* numbers as immune responses were not considered in this study.

The *Galleria mellonella* model has been used increasingly to assess the virulence of pathogens including *Campylobacter* and *E. coli*, and has been used to identify a number of virulence determinants in pathogens such as *C. jejuni* (Champion *et al.*, 2010; Senior *et al.*, 2011; Alghoribi *et al.*, 2014; Mehat *et al.*, 2018). Therefore, the aim of this study was to determine if this model would be suitable for the study of *Brachyspira* and possible probiotic

interventions. Recent studies have suggested that the pre-treatment of *G. mellonella* larvae with probiotics can modulate the immune system by increasing the numbers of circulating haemocytes and protecting the larvae from mortality associated with pathogen infection (de Oliveira *et al.*, 2017; Ribeiro *et al.*, 2017; Rossoni *et al.*, 2017). The study presented here demonstrated that protection by *Lactobacillus* species was not only dependent on the isolate of *Lactobacillus*, but also by the isolate of *Brachyspira* the larvae were infected with. For example, *L. salivarius* SAP 2117 had a significant protective effect against *B. intermedia* SAP 919 and *B. innocens* SAP 924 but not against the other *Brachyspira* isolates. Interestingly, the *L. reuteri* isolates were the most inhibitory against *Brachyspira* isolates in the *in vitro* experiments however, in the *G. mellonella* model, they showed little or no protection. Conversely, *L. salivarius* isolates were the least inhibitory against *Brachyspira* isolates *in vitro* but were able to protect larvae. The associated immune responses require further investigation and these findings emphasise the need for a more representative model of infection. That said, this model provides a number of advantages over mammalian and avian models; there are few ethical considerations, the model is easily manipulated and inexpensive and the larvae are capable of a cellular immune response comparable to that of mammalian neutrophils and avian heterophils (Montali, 1988; Brooks *et al.,* 1996). However, there are a number of considerations and limitations with this model, primarily it is not representative of mammalian or avian infection as it does not resemble to route of infection or provide the optimal ecological niche for *Brachyspira*. This was not the intention for this model as the aim was to explore its suitability as a model for *Brachyspira* infection in the context of a functional immune system and to investigate the protective effects of potential probiotics. Results from this model did, however, raise some questions regarding *Brachyspira* infection in chickens which would require further investigation. For example, *Brachyspira* were located in the

muscle tissue of the *G. mellonella* larvae, therefore it would be important to determine if this occurs in chickens. Interestingly, there have been reports of chicken carcass contamination with *Brachyspira* (Verlinden *et al.*, 2012), however this could also be as a result of faecal contamination.

Additionally, this model requires further validation through testing an increased number of *Brachyspira* isolates from all known species. Unfortunately, this was not possible in the studies reported here due to the limited number of *Brachyspira* isolates available in the SAP collection at Surrey University. The data produced demonstrated that no correlation between *Brachyspira* species and morbidity and mortality scores were observed, this may be as a result of the small numbers of isolates initially used in these studies. Alternatively, as revealed by the pangenome analysis of *B. pilosicoli*, the accessory genome for these species constitutes approximately 53% of its genes which therefore may result in the varied virulence observed in both the *G. mellonella* model and experimental infection models with *B. pilosicoli*, as previously discussed.

Overall, this thesis has aimed to explore the potential for novel probiotics to mitigate avian intestinal spirochaetosis caused by *Brachyspira* species. This was achieved by characterising a panel of *Brachyspira* isolates and a panel of *Lactobacillus* isolates in order to explore the mechanisms by which probiotics may exert their effects. A number of mechanisms contributed to the inhibition of *Brachyspira* and it was evident that inhibition was multifaceted and depended on the isolate of *Lactobacillus* tested and the susceptibility of the *Brachyspira* isolate. On reflection, each of these assays were performed at 37°C, the optimal temperature for mammals, despite avian species having an internal temperature of 42°C. This was a limitation because antimicrobial metabolite production may differ at 42°C compared to 37°C. Therefore, future work would focus on optimising assays at the optimal avian

temperature. For the studies described here, 37°C was utilised as all previous protocols utilised this temperature for the growth of *Brachyspira*; this organism was difficult to grow during initial isolation and therefore, once this protocol was optimised at 37°C this was used throughout all assays presented here. Additionally, from a commercial perspective, antimicrobial metabolites such as bacteriocins could be produced and purified at 37°C *in vitro* and then added as feed supplements to poultry diets. Therefore, the use of 37°C for the growth of *Lactobacillus* in these studies may be advantageous.

An alternative approach would be to consider testing a multi-species probiotic product. The four probiotics used throughout these studies have demonstrated a range of different inhibitory abilities, via a range of mechanisms. Therefore, suggesting that a mixed species probiotic may be a more suitable mitigation strategy because several mechanisms of action would be involved in *Brachyspira* inhibition. These isolates also demonstrated inhibition against *E. coli* and *Salmonella* isolates and thus it would be important to consider these *Lactobacillus* isolates for several pathogens infecting poultry, however *in vivo* confirmation of these findings is essential.

8.2 Conclusion

In conclusion, the studies presented here have demonstrated that novel *Lactobacillus* candidates were suitable as potential probiotic intervention strategies against *Brachyspira* species implicated in poultry disease. *Lactobacillus* isolates used in the studies showed the ability to exert their inhibitory effects via several different mechanisms: (i) the production of organic acids such as lactic acid (ii) the production of inhibitory metabolites, independent of pH (iii) the secretion of bacteriocins and bacteriolysins (iv) the physical interactions with *Brachyspira* to impair motility and (v) modulation of the immune response to *Brachyspira*

infection. It is important to note that there was not one probiotic candidate that was effective against all *Brachyspira* isolates or that inhibited *Brachyspira* via all of the mechanisms described. *L. reuteri* SAP 2115 was overall the most successful probiotic candidate. However, investigating a multispecies probiotic may be a more suitable approach for further studies.

8.3 Future perspectives

The results presented in this thesis provide the foundation for further investigation in a number of key areas:

(1) Additional isolates from each of the species implicated in avian intestinal spirochaetosis require whole genome sequencing in order to conduct more in-depth comparative genomics. The studies presented here added fifteen novel *B. pilosicoli* genomes to the analysis which allowed direct comparisons with the twenty-five available *B. hyodysenteriae* genomes. Additionally, these studies contributed one *B. intermedia* genome and three *B. innocens* genomes for comparative analysis. However, this was not sufficient to compare these individual species, thus they were grouped which limited the comparisons that could be made. Furthermore, an increased number of genome sequences for non-pathogenic *Brachyspira* would allow for comparisons between pathogenic and non-pathogenic species which be an important advancement in the knowledge of this genus.

(2) *Lactobacillus* cell free supernatants require further interrogation in order to purify and identify inhibitory proteins. Screening whole genome sequences for putative bacteriocin genes identified several that may be inhibitory against *Brachyspira* species. Additionally, trypsin and heat treatment of cell free supernatants from certain *Lactobacillus* isolates decreased the inhibition of *Brachyspira*. Therefore, protein

precipitation and fractionation could be used to isolate the proteins present in the cell free supernatant and these could be tested against *Brachyspira* to determine inhibition.

(3) The *G. mellonella* infection model needs further validation with an increased number of *Brachyspira* isolates from all species. Additionally, the mechanism of probiotic protection in this model requires further characterisation. For example, haemocyte counts would be conducted to support the theory that immunomodulation by *Lactobacillus* contributes to the protection against *Brachyspira.* Histopathology of infection with *Lactobacillus* alone and in combination with *Brachyspira* needs to be analysed and compared with that of *Brachyspira* infection alone. It would also be important to determine the location of *Lactobacillus* and whether they co-aggregate with *Brachyspira* in this model.

(4) The findings of this thesis should be validated in an *in vivo* chicken model and in particular the results from the *in vitro* HD11 tissue culture and the *in vivo Galleria mellonella* model. An experimental chicken model such as that published by Mappley *et al.,* 2013 would validate if the probiotic candidates characterised in these studies would have a protective effect in the desired host.

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Appendix I (Chapter 3)

Table 9. 1 48-hour end point utilisation of carbon sources of PM1 and PM2 plates for 8 isolates of *Brachyspira* using the Biolog Phenotypic Microarray™ (PM) technology. The extent of respiration is colour-coded whereby dark purple indicates high levels of respiration on a carbon source, purple indicates moderate levels of respiration on a carbon source, pale purple indicates low levels of respiration on a carbon source and white indicates no respiration on a carbon source.

Carbon Source PM1	*B. alvinipulli* ATCC 51933	*B. pilosicoli* B2904	*B. pilosicoli* SAP 859	*B. pilosicoli* SAP 865	*B. intermedia* SAP 919	*B. innocens* SAP 924	*B. innocens* SAP 927	*B. innocens* SAP 943
Neg control	0	0	0	0	0	0	0	0
L- Arabinose	0.62193333	0.82995	0.7591	0.84463333	0.87433333	0.85176667	0.81513333	0.78701111
N-Acetyl-D-glucosamine	0.21293333	0.26695	0.2896	0.1017	0.16463333	0.03083333	0.04026667	0.01155556
D- Saccharic Acid	-0.0079333	-0.0015	-0.01025	0.00653333	-0.0074	0.00986667	0.00393333	0.00081111
Succinic Acid	-0.0082667	0.00165	-0.01055	0.00446667	-0.0017	0.007	0.00406667	0.00195556
D- Galactose	0.39813333	0.6524	0.7053	0.58403333	0.56306667	0.76503333	0.63486667	0.64202222
L- Aspartic Acid	-0.0069333	0.0014	-0.0007	0.0017	-0.0054667	0.0013	0.00323333	0.00084444
L- Proline	0.0028	0.00085	-0.0121	-0.0014667	-0.0067	0.00106667	0.0061	0.00293333
D- Alanine	0.01406667	-0.00345	-0.01495	0.00066667	-0.0053667	0.00063333	0.00323333	-0.0006889
D- Trehalose	-0.0007333	0.0062	-0.0018	0.1047	0.1542	0.00113333	0.0056	0.0013
D- Mannose	0.42693333	0.7299	0.71855	0.5275	0.55223333	0.714	0.70043333	0.65344444
Dulcitol	0.06306667	0.0018	-0.0144	0.03376667	-0.0067	-0.0101667	0.00276667	0.00252222
D-Serine	-0.0026333	-0.00485	0.03305	-0.0023667	-0.0069	0.0034	0.0059	-0.0007667
D- Sorbitol	-0.0065	-0.00225	-0.0106	-0.0059333	0.09283333	-0.0020667	0.0051	-0.0007667
Glycerol	0.15256667	-0.0016	-0.0045	-0.003	-0.0047667	0.00773333	0.00873333	0.00504444
L- Fuctose	0.30753333	0.31715	0.47415	0.3664	0.5218	0.6088	0.56013333	0.48344444
D- Glucuromic Acid	0.6736	0.4602	0.5852	0.5423	0.5992	0.70936667	0.65496667	0.61788889
D- Gluconic Acid	-0.0028	-0.00285	-0.01505	0.00393333	-0.0048333	0.00176667	0.00253333	0.00051111
D,L-a-Glycerol-Phosphate	0.03996667	0.05725	0.03115	0.22826667	0.16673333	0.17133333	0.44226667	0.14792222
D-Xylose	0.64953333	0.8524	0.7652	0.8409	0.87376667	0.84483333	0.85766667	0.80648889
L- Lactic Acid	0.24823333	-0.0016	0.0031	-0.0016	-0.0054667	0.00243333	0.00563333	0.00254444
Formic Acid	-0.0031	0.01665	0.00435	0.0036	-0.0056	0.00773333	0.0061	0.00023333
D-Mannitol	0.06586667	0.00635	-0.0137	0.00146667	0.0267	-0.0022667	0.00466667	-4.444E-05
D- Gluamic Acid	0.2025	-0.00645	-0.00385	-0.0005333	0.00056667	-0.0079	0.00716667	0.00682222
D- Glucose-6-Phosphate	0.28086667	0.5174	0.5017	0.38796667	0.4464	0.52036667	0.448	0.408
D- Galactonic Acid-y-Lactone	0.1142	0.0891	0.0955	0.1384	0.09633333	0.1454	0.15516667	0.15025556
D, L-Malic Acid	0.00086667	0.00115	-0.0002	0.00603333	-0.0008	0.00246667	0.00686667	0.00315556
D-Ribose	0.91806667	0.7422	0.5364	0.7208	0.8796	0.82143333	0.73836667	0.71392222
Tween 20	-0.0132333	-0.0319	-0.052	-0.0197667	-0.0323667	-0.0338667	-0.0191	-0.0187667
L- Rhamnose	0.54293333	0.7179	0.72445	0.7142	0.7495	0.82706667	0.77576667	0.75548889
D- Fructose	0.4549	0.5525	0.68415	0.52696667	0.62296667	0.75106667	0.7452	0.68256667
Acetic Acid	0.05576667	-0.004	-0.0066	0.00133333	-0.0033333	-0.0007	0.01076667	0.00272222
a-D-Glucose	0.27066667	0.55425	0.62435	0.2099	0.3766	0.60703333	0.38053333	0.32891111
Maltose	0.2271	0.00655	-0.00085	0.11563333	0.20893333	0.00906667	0.01176667	0.00388889
D- Melibiose	0.26146667	0.17535	0.44905	0.2289	0.34656667	0.46393333	0.28756667	0.22525556
Thymidine	0.24326667	0.001	-0.0087	-0.0036333	-0.0080333	-0.0138667	0.00513333	-0.0003889
L- Asparagine	-0.0044667	-0.00395	-0.0065	0.00233333	-0.0039	-0.0016	0.00646667	0.00098889
D-Aspartic Acid	-0.0040667	-0.0053	-0.00655	0.004	-0.0089667	0.01406667	0.0062	0.00113333
D- Glucosaminic Acid	-0.0026	0.003	0.0029	0.0021	0.02803333	0.00286667	0.01563333	0.00661111
1,2- Propanediol	0.23913333	0.0002	-0.00015	0.00526667	-0.0032667	0.01476667	0.0107	0.00453333
Tween 40	-0.012	-0.02945	-0.0399	-0.0157333	-0.0289	-0.0264333	-0.0103	-0.0120667
a-Keto-Glutaric Acid	0.31523333	0.00285	0.00055	0.05616667	0.01986667	0.0184	0.01993333	0.01117778
a-Keto- Butyric Acid	0.26896667	0.0628	0.1174	0.29276667	0.09846667	0.08296667	0.0636	0.10096667
a-Methyl-D0 Galactoside	0.00076667	0.0094	0.01225	0.0057	0.01166667	0.0123	0.01586667	0.00575556
a-D-Lactose	0.26036667	0.3641	0.2673	0.19866667	0.1753	0.298	0.18163333	0.06094444
Lactoulose	0.1729	0.15665	0.29005	0.21716667	0.22456667	0.1279	0.30453333	0.12117778
Sucrose	-0.0025333	0.00975	0.0129	0.01583333	0.01483333	-0.0015333	0.01353333	0.00334444
Uridine	-0.0066667	0.0705	0.0049	-0.0024333	-0.0011333	-0.0135333	0.0155	0.00536667
L- Glutamine	-0.0056	0.0026	0.0188	-0.0030667	-0.0058	0.00223333	0.00933333	0.00174444
m-Tartaric Acid	-0.0036667	-0.00655	-0.0018	0.00563333	-0.0060333	-0.0009333	0.01203333	0.00251111
D-Glucose-1-Phosphate	-0.0035667	0.0086	-0.0042	0.00803333	-0.0025667	0.01376667	0.01106667	0.00598889
D- Fructose-6-Phosphate	0.35986667	0.54165	0.644	0.59096667	0.5278	0.6575	0.65016667	0.58465556
Tween 80	0.26903333	-0.0269	-0.0387	-0.0191667	-0.0259667	-0.0247333	-0.0084667	-0.0111889
a-Hydroxy Glutatic Acid-y-Lactone	0.00323333	-0.00065	-0.0084	0.01456667	-0.0029333	0.02163333	0.0166	0.00733333
a-Hydroxy Butyric Acid	-0.0021333	-0.0042	-0.0105	0.00526667	-0.0061333	0.01466667	0.01486667	0.01105556
B-Methyl-D-Glucoside	0.00433333	-0.0002	-0.005	0.2196	0.0508	0.10096667	0.19953333	0.06631111
Adinitol	-0.0009333	-0.00675	-0.0206	0.02263333	-0.0057667	0.00333333	0.01016667	0.00235556
Maltotriose	0.25926667	0.0176	0.0936	0.2402	0.21546667	0.06403333	0.0516	0.01736667
2- Deoxy Adenosine	0.0155	-0.00255	-0.01545	0.00013333	-0.0084333	-0.0006	0.00866667	-0.0003778
Adenosine	0.28293333	0.4727	0.02245	-0.0012333	-0.0028667	-0.0089	0.00766667	0.00145556
Glycyl-:-Aspartic Acid	0.00073333	-0.00025	0.0019	0.00096667	-0.0001333	0.00753333	0.0047	0.0024
Citric Acid	-0.0095667	-0.006	0.00065	-0.0058333	-0.0036667	-0.0054	0.0039	-0.0012333
m-Inositol	-0.0005667	-0.0024	-0.00295	0.0025	-0.0117333	-0.0007667	0.0072	0.00053333
D- Threonine	-0.0019	-0.00935	-0.01165	0.00206667	-0.0088333	0.00806667	0.01053333	0.00231111

Fumaric Acid	0.05036667	5E-05	-0.0085	0.00543333	0.004	0.0052	0.01216667	0.00422222
Bromo Succinic Acid	0.00313333	0.0018	0.00635	0.02113333	0.00983333	0.0109	0.00706667	0.00565556
Propionic Acid	0.00096667	-0.00125	-0.0095	0.00716667	0.0018	0.00583333	0.0057	0.0029
Mucic Acid	0.00106667	-0.0026	-0.00915	0.00883333	-0.0112667	0.0089	0.00843333	0.00217778
Glycolic Acid	0.14993333	-0.0064	-0.01485	0.007	-0.0047333	0.00546667	0.0151	0.00623333
Glyoxylic Acid	0.1644	0.15045	0.16565	0.27713333	0.13423333	0.2155	0.29016667	0.16792222
D- Cellobiose	0.25966667	0.0691	0.11065	0.246	0.2112	0.13523333	0.044	0.017
Inosine	-0.0039333	0.17085	-0.0067	-0.0005	-0.0084667	-0.0051667	0.0029	-0.0021
Glycyl- Glutamic Acid	-0.0040667	0.005	0.0176	0.00043333	-0.0021333	0.0057	0.00456667	0.00122222
Tricarballylic Acid	-0.0062333	-0.00425	-0.00915	-0.0031333	-0.0107667	0.00213333	0.00266667	0.00545556
L- Serine	-0.0076667	-0.0083	-0.01775	0.00356667	-0.0098667	-0.0026333	0.00646667	-0.0004444
L- Threonine	-0.0041333	-0.0063	-0.0172	-0.0041	-0.0078333	-0.0031	0.01116667	0.00292222
L- Alanine	0.01233333	-0.0037	-0.0137	0.21716667	-0.0087333	-0.0001333	0.00853333	0.00251111
L- Alanyl-Glycine	0.00046667	-0.0059	-0.0052	0.00633333	-0.0052333	0.01093333	0.00983333	0.01077778
Acetoacetic Acid	0.01206667	0.0814	0.0954	0.02123333	0.0402	0.0601	0.07353333	0.05667778
N- Acetyl-B-D-Mannosamine	0.22203333	0.0035	0.01575	0.0855	0.2155	0.01336667	0.00523333	0.00367778
Mono Methly Succinate	-0.0008333	0.0037	-0.00935	0.00426667	-0.0043667	0.00166667	0.01186667	0.00348889
Methyl Pyruvate	0.2178	0.01305	-0.01225	0.15456667	0.04513333	0.28286667	0.23843333	0.08361111
D- Malic Acid	-0.0055667	-0.00085	-0.0102	-0.0034333	-0.0076	0.0099	0.00613333	0.00221111
L- Malic Acid	-0.0092333	5E-05	-0.00695	-0.0039	-0.0090333	-0.0126333	0.00363333	-0.0001556
Glycy- L -Proline	-0.0076667	0.0369	0.05475	-0.0012667	0.01243333	-0.0021667	0.0014	-0.0024667
p-Hydroxy Phenyl Acetic Acid	-0.003	0.0024	-0.0027	0.00093333	-0.0048	0.00126667	0.0084	0.0044
m-HydroyPhenyl Acetic Acid	-0.0014	0.004	0.00445	0.00366667	-0.0030333	-0.0015667	0.00873333	0.00281111
Tyramine	-0.0013	0.0031	0.034	0.01573333	0.00023333	0.01193333	0.0156	0.0115
D- Psicose	0.48013333	0.7883	0.7716	0.63973333	0.73103333	0.736	0.67186667	0.74225556
L- Lyxose	0.5868	0.5363	0.41325	0.6683	0.71096667	0.70013333	0.70043333	0.59871111
Glucuronamide	0.5134	0.72975	0.71145	0.72486667	0.7136	0.80546667	0.74646667	0.74148889
Pyruvic Acid	0.23033333	0.31985	0.5735	0.3541	0.09853333	0.43433333	0.2908	0.16366667
L- Galactonic Acid-y- Lactone	0.50976667	0.70305	0.6555	0.42653333	0.50753333	0.6194	0.47196667	0.48778889
D- Galacturonic Acid	0.2938	0.36635	0.4825	0.37676667	0.4611	0.5745	0.43316667	0.43522222
Phenylethyl-amine	0.0141	0.007	-0.01255	0.01756667	-0.0008333	0.02046667	0.01163333	0.00787778
2- Aminoethanol	-0.0026333	0.0383	0.10655	0.00923333	-0.0068667	0.00363333	0.00486667	0.00382222

Carbon Source PM2	***B. alvinipulli* ATCC 51933**	***B. pilosicoli* B2904**	***B. pilosicoli* SAP 859**	***B. pilosicoli* SAP 865**	***intermedia* SAP 919**	***B. innocens* SAP 924**	***B. innocens* SAP 927**	***B. innocens* SAP 943**
Neg Control	-0.0041	0.0142	-0.00435	0.00853333	0.0096	0.00676667	0.0077	0.0032
Chondrionitin Sulfate C	0.00263333	0.02	0.01485	0.02196667	0.007	0.01133333	0.01206667	0.00918889
a-Cyclodextrin	-0.0072	0.0027	-0.02285	0.0031	0.18253333	0.25506667	0.0733	0.0259
B- Cyclodextrin	-0.0015333	0.0171	-0.0157	-0.0003	0.05306667	0.00273333	0.00606667	0.00288889
y- Cyclodextrin	0.00236667	0.00285	-0.01825	0.00636667	0.02143333	0.0189	0.04193333	0.01591111
Dextrin	0.30306667	0.43515	0.40645	0.3095	0.3942	0.46803333	0.42976667	0.26792222
Gelatin	0.00123333	0.00385	0.0004	0.00826667	0.01163333	0.00836667	0.0084	0.00306667
Glycogen	0.00876667	0.0132	0.0041	0.02253333	0.01313333	0.01813333	0.02143333	0.01267778
Inulin	0.04693333	0.0279	0.02755	0.1428	0.03916667	0.03053333	0.04346667	0.02535556
Laminarin	0.08736667	0.1047	0.32065	0.07976667	0.20406667	0.23466667	0.15326667	0.05315556
Mannan	0.01496667	0.01105	0.00365	0.12606667	0.02893333	0.01563333	0.0159	0.00863333
Pectin	0.35686667	0.4377	0.47225	0.3449	0.35703333	0.47603333	0.41423333	0.25634444
N- Acetyl-D-Galactosamine	-0.0046333	0.0053	-0.0107	0.00893333	0.0039	0.02116667	0.00213333	-8.889E-05
A-Acetly-Neuraminic Acid	0.00683333	0.19515	0.2532	0.01123333	0.01263333	0.00363333	0.00603333	0.00367778
B-D-Allose	0.63073333	0.48785	0.5351	0.63866667	0.65423333	0.69596667	0.66753333	0.59457778
Amygdalin	0.00106667	0.0045	0.0032	0.03453333	0.1926	0.03396667	0.0093	0.00603333
D-Arabinose	0.8803	0.84385	0.79765	0.86176667	0.88923333	1.02913333	0.85976667	0.82035556
D-Arabitol	0.0034	0.00355	-0.0091	0.0417	0.01206667	0.02086667	0.01546667	0.00872222
L- Arabitol	0.00036667	0.0003	-0.01435	0.02173333	-0.0028667	0.0105	0.00953333	0.00107778
Arbutin	0.08363333	0.0376	0.0472	0.0875	0.05786667	0.105	0.10263333	0.08107778
2- Deoxy-D- Ribose	0.80123333	0.7587	0.7222	0.79606667	0.8427	0.84046667	0.8148	0.7802
i- Erythritol	0.00293333	0.00105	-.013	0.0297	0.00226667	0.08276667	0.01416667	0.00375556
D-Fucose	0.37696667	0.5291	0.5007	0.40983333	0.4026	0.56026667	0.48213333	0.33204444
3-0-B-D-Galacto-pyranosyl-D-Arabinose	0.6311	0.796	0.72295	0.6082	0.62386667	0.70133333	0.66043333	0.60747778
Gentiobiose	0.36343333	0.4865	0.46205	0.30106667	0.31816667	0.4064	0.3678	0.22893333
L- Glucose	0.3257	0.186	0.23885	0.3114	0.37233333	0.4491	0.39766667	0.25565556
Lactitol	0.0025	0.06965	0.00815	0.01483333	0.14133333	0.1846	0.26833333	0.09067778
D- Melezitose	0.00886667	0.0012	-0.00735	0.01466667	0.01313333	0.0124	0.0137	0.00626667
Maltitol	0.00566667	0.00295	-0.0086	0.01113333	0.0088	0.0136	0.01396667	0.00658889
a-Methyl-D-Glucoside	0.0087	0.00765	0.0047	0.0173	0.00026667	0.03016667	0.01666667	0.00752222
B-Methyl-D-Galactoside	0.00396667	0.23815	0.11045	0.0195	0.19443333	0.27883333	0.1773	0.059
3- Methyl Glucose	0.4208	0.4201	0.4254	0.4329	0.42073333	0.55706667	0.4697	0.29973333
B-Methyl-D- Glucuronic Acid	0.00226667	-0.00125	-0.00535	0.0302	0.01323333	0.01313333	0.01653333	0.00611111
a-Methyl-D-Mannoside	0.00266667	0.00365	-0.0076	0.01143333	0.00213333	0.16126667	0.0173	0.00716667
B-Methyl-D-Xyloside	-0.0009667	0.0042	-0.0034	0.02013333	0.00566667	0.19223333	0.055	0.01786667

Palatinose	0.75626667	0.77155	0.71565	0.73426667	0.65846667	0.81496667	0.7703	0.74213333
D-Raffinose	-0.0050333	0.117	0.09545	0.03126667	0.00743333	0.01203333	0.00783333	0.00267778
Salicin	0.25246667	0.00705	-0.0055	0.1145	0.15983333	0.17026667	0.16533333	0.05741111
Sedoheptulosan	0.00246667	0.00095	-0.0101	0.01166667	0.0043	0.01056667	0.01166667	0.00712222
L-Sorbose	0.76043333	0.68695	0.7037	0.7581	0.70943333	0.80286667	0.7735	0.7392
Stachyose	0.00496667	0.00445	-1E-04	0.0128	0.01156667	0.0224	0.0411	0.0165
D- Tagatose	0.86543333	0.84605	0.8064	0.8676	0.74216667	0.9248	0.8821	0.8452
Turanose	0.44073333	0.3008	0.2935	0.34566667	0.3603	0.5037	0.4771	0.29326667
Xylitol	0.00273333	-0.00225	-0.00775	0.0145	-0.0016667	0.01673333	0.01913333	0.00567778
N-Acetyl-D-Glucosaminitol	-0.0021333	0.00125	-0.0101	0.0099	-0.0001333	0.0101	0.0091	0.00376667
y-Amino Butyric Acid	0.002	-1E-04	-0.0074	0.01153333	-0.0038	0.0106	0.01463333	0.00447778
B- Amini Valeric Acid	-0.0026	0.0021	-0.01755	0.00613333	-0.0031667	0.01036667	0.01346667	0.00588889
Butyric Acid	0.0259	0.6031	0.70845	-0.0001667	0.19776667	0.29856667	0.2465	0.08163333
Capric Acid	-0.0043667	-0.01005	-0.03865	-0.0031	-0.0223	-0.0196	-0.0071333	-0.0065444
Caproic Acid	-0.0057333	0.0023	-0.00305	0.00756667	-0.0052	0.0068	0.01006667	0.00548889
Citraconic Acid	0.00103333	-0.00255	-0.01165	0.01256667	0.00803333	0.008	0.011	0.00466667
Citramalic Acid	0.00096667	-0.0006	-0.0101	0.01256667	-0.0070333	0.01053333	0.0109	0.00366667
D- Glucosamine	0.8518	0.7957	0.74845	0.84303333	0.71573333	0.9299	0.87813333	0.82651111
2-Hydroxy Benzoic Acid	0.0002	0.00265	-0.0094	0.0157	0.01206667	0.0105	0.01543333	0.00584444
4- Hydroxy Benzoic Acid	0.00373333	0.0016	-0.00565	0.03906667	0.00816667	0.0193	0.01556667	0.00685556
B- Hydroxy Butyric Acid	-0.0005	0.00045	-0.0076	0.0097	0.2322	0.0175	0.01466667	0.01235556
y-Hydroxy Butyric Acid	0.00126667	-0.00255	-0.0162	0.0125	0.00573333	0.00476667	0.00923333	0.00281111
a-Keto-Valeric Acid	0.0937	0.1323	0.1565	0.23876667	0.11613333	0.4229	0.1811	0.16003333
Itaconic Acid	0.03703333	0.0174	-0.0098	0.0451	0.01636667	0.01656667	0.0384	0.0334
5-Keto-D-Gluconic Acid	0.81756667	0.7784	0.6755	0.84176667	0.77786667	0.85766667	0.82013333	0.79877778
D-Lactic Acid Methly Ester	0.0097	0.0072	-0.0062	0.02083333	0.00776667	0.018	0.01453333	0.01297778
Malonic Acid	-0.0088667	-0.0045	0.002	-0.0033333	-0.0088333	0.00256667	0.0042	-1E-04
Melibionic Acid	0.00806667	0.0071	0.0061	0.02063333	0.0274	0.0239	0.0232	0.02383333
Oxaclic Acid	0.003	-0.002	-0.0026	0.01063333	0.0057	0.01126667	0.0113	0.00566667
Oxalomalic Acid	0.24603333	0.1977	0.1991	0.38716667	0.19903333	0.4281	0.2243	0.19576667
Quinic Acid	0.00056667	0.00125	-0.0087	0.01583333	-0.0033667	0.0156	0.01176667	0.00342222
D-Ribono-1,4-Lactone	0.01203333	0.00175	-0.0147	0.02116667	-0.0023	0.00873333	0.01466667	0.00868889
Sebacic Acid	0.00216667	0.0054	-0.00595	0.02333333	0.0066	0.01566667	0.0115	0.0059
Sorbic Acid	0.59163333	0.4928	0.48155	0.6022	0.5044	0.6841	0.6091	0.55886667
Succinamic Acid	0.00086667	0.00595	-0.0129	0.0175	0.00146667	0.00266667	0.012	0.00523333
D-Tartaric Acid	-0.0017	0.005	-0.02165	0.01516667	-0.0073	-0.0013	0.0051	0.00156667
L- Tartaric Acid	-0.0031	0.00205	-0.00695	0.01073333	-0.0059333	0.00163333	0.00853333	-0.0008222
Acetamide	-0.0004667	0.0039	-0.01455	0.00963333	0.00786667	0.0118	0.00756667	0.00188889
L-Alaninamide	-0.0041667	0.00025	-0.0007	0.0046	-0.0042	0.00433333	0.00133333	0.00074444
N-Acetyl-L-Glutamic Acid	-0.0043	0.0064	-0.00525	0.00416667	0.00323333	0.00803333	0.00633333	0.00414444
L- Arginine	-0.0003667	0.0016	0.00335	0.00696667	-0.0133	0.00213333	0.01023333	0.00357778
Glycine	-0.0013333	0.003	-0.0212	0.0164	0.0022	0.00366667	0.00616667	0.00248889
L- Histidine	0.0022	0.00475	-0.01575	0.08326667	0.00423333	0.00546667	0.01506667	0.00965556
L. Homoserine	-0.0065667	0.0021	-0.01595	0.00953333	-0.0098667	0.00506667	0.01073333	0.00427778
Hydroxy-L-Proline	-0.0047667	0.00515	-0.00845	0.01076667	-0.0076	0.00556667	0.0098	0.0049
L- Isoleucine	0.00193333	0.00785	-0.0138	0.0102	-0.0010667	0.00486667	0.0085	0.00343333
L- Leucine	-0.0034667	0.0044	0.01595	0.00763333	-0.0015	0.0018	0.0079	0.0047
L- Lysine	0.02163333	0.01155	0.021	0.01876667	0.00293333	-0.0019333	0.0078	0.00166667
L- Methionine	-0.0037667	0.00545	0.03705	0.01253333	-0.0090667	0.0018	0.0056	0.00366667
L- Ornithine	0.03326667	0.163	0.1895	0.07893333	0.02076667	0.00443333	0.00946667	0.00468889
L- Phenylalanine	-0.0024333	0.0384	0.004	0.0269	0.0008	0.00863333	0.00506667	0.00135556
L- Pyroglutamic Acid	-0.0022	0.0093	0	0.02183333	0.00413333	0.0052	0.00373333	0.00107778
L- Valine	-0.0037	0.02355	0.0219	0.00803333	-0.0087333	0.00853333	0.00563333	0.00057778
D,L- Carnitine	-0.0071333	0.00785	-0.0076	0.0012	-3.333E-05	0.00116667	0.00443333	0.00111111
Sec-Butylamine	-0.0091333	-0.0157	-0.02295	-0.0062333	-0.0088667	-0.0068333	0.00046667	-0.0039778
D,L- Octopamine	0.04246667	0.1334	0.1954	0.13536667	0.0244	0.02706667	0.01746667	0.00678889
Putrescine	0.00536667	0.135	0.0985	0.10456667	0.03383333	0.1224	0.03383333	0.01534444
Dihydroxy Acetone	0.65043333	0.46055	0.418	0.63586667	0.6846	0.73386667	0.6569	0.53883333
2,3- Butanediol	-0.0015333	0.0767	0.01685	0.0037	-0.0024	0.0017	0.0033	0.00186667
2,3- Butone	0.06543333	0.03505	0.02325	0.06123333	0.05346667	0.0431	0.04796667	0.03828889
3-Hydroxy-2- Butanone	0.00066667	0.03095	-0.01165	0.0101	0.0006	0.00563333	0.00303333	0.00101111
Total carbon sources utilised	59	66			57	57		
Percentage of carbon sources utilised	31.0526316	34.73684211			30	30		

Appendix II (Chapter 4)

Table 9. 2 Genome assembly statistics for all *Brachyspira* whole genome sequences used in the comparative genomic studies and exclusion criteria for the five genomes removed from these studies.

Assembly	# contigs	Largest contig	Total length	N50	L50	# N's per 100 kbp	reason for exclusion	excluded
Balvp001_ATCC51933	22	582949	3420763	342358	4	256.84		
Bhamp002_30446	4	1924647	3037350	1924647	1	595.88	very large number of N inserted	x
Bhamp003_30599	612	34622	2943158	7864	111	0.07	very low N50, poor quality genome sequencing	x
Bhamp004_NSH-16	77	224477	3161271	88495	13	0		
Bhamp005_NSH-24	178	103774	2969002	29547	29	0	low N50, poor quality genome sequencing	x
Bhamp006_P280-1	16	953127	3186631	690165	2	0.25		
Bhyod007_865	105	320136	3009507	111249	9	16.61		
Bhyod008_ATCC27164	2	3041447	3074045	3041447	1	0		
Bhyod009_B204	113	245629	3045413	86825	11	118.57	large number of N inserted	
Bhyod010_B6933	90	215281	3056944	124108	9	124.05	large number of N inserted	
Bhyod011_B78	94	419366	3056178	154115	7	14.4		
Bhyod012_B8044	165	191659	3018724	71132	14	1.33		
Bhyod013_BH718	2	2998332	3034648	2998332	1	0		
Bhyod014_FM88-90	118	303456	2993625	170197	6	0.77		
Bhyod015_FMV89-3323	111	367772	3167038	142819	6	11.05		
Bhyod016_G21	85	325507	3113349	122169	8	17.34		
Bhyod017_G44	135	237607	3002616	98426	11	18.98		
Bhyod018_JR1	427	417478	3173932	99212	9	11.03		
Bhyod019_JR2	474	266428	3193448	115365	10	38.64		
Bhyod020_JR3	446	179915	3158623	60136	19	18.24		
Bhyod021_NSW15	158	153661	3020963	71065	16	11.92		
Bhyod022_NSW5	155	380148	3023934	75956	11	7.61		
Bhyod023_NX	143	196673	3013691	78384	11	72.54	large number of N inserted	
Bhyod024_Q17	129	249263	3019999	80615	13	15.23		
Bhyod025_ST190	71	321703	3059185	155275	7	11.44		
Bhyod026_ST195	94	390917	3082158	148037	7	15.57		
Bhyod027_ST210	90	470364	3055385	151603	7	17.67		
Bhyod028_ST265	209	342883	3108081	57007	14	1.29		
Bhyod029_VIC2	79	239727	3023965	124009	9	17.53		
Bhyod030_WA100	187	316037	3130163	77247	13	11.5		
Bhyod031_WA1	2	3000694	3036634	3000694	1	0		
Binno032_B3453	139	171035	3203920	51776	20	0		
Binno033_B3650	95	379134	3150198	66364	13	0		
Binno034_B3652	72	361958	3200075	102553	11	0		
Binno035_ATCC29796	130	168341	3281611	52799	20	0		
Bintm036_B3445	144	217871	3493658	80677	15	0		
Bintm037_PWS-A	2	3304788	3308048	3304788	1	0		
Bmurd038_B-11	32	889148	3144515	720165	2	0		
Bmurd039_DSM12563	1	3241804	3241804	3241804	1	0		
Bpilo040_B-04	6	2342853	2598894	2342853	1	0		
Bpilo041_B-06	71	435730	2764670	185117	6	0		
Bpilo042_B-14	30	680182	2435627	170846	4	0		
Bpilo043_B2903	13	1226659	2756759	732242	2	0		
Bpilo044_B2904	74	198373	2739777	84986	11	0	duplicate	x
Bpilo045_B2905	10	1778376	2546415	1778376	1	0		
Bpilo046_B2953	69	319509	2729767	120589	7	0		
Bpilo047_B3078	13	637327	2542553	409203	3	0		
Bpilo048_B3084	22	698261	2545332	637106	2	0		
Bpilo049_B-31	8	1144602	2588278	522457	2	0		
Bpilo050_B3420	54	290230	2696933	150269	6	0		
Bpilo051_B3424	84	316261	2767941	101345	8	0		
Bpilo052_B3451	16	933847	2639689	809962	2	0		
Bpilo053_B3545	116	148079	2631575	50006	17	0		
Bpilo054_B-37	5	1651971	2590466	1651971	1	0		
Bpilo055_B-67	10	1112538	2549350	1037582	2	0		
Bpilo056_95-1000	1	2586443	2586443	2586443	1	0		
Bpilo057_B2904	1	2765477	2765477	2765477	1	0.54		
Bpilo058_P43-6-78	1	2555556	2555556	2555556	1	0		
Bpilo059_SP16	59	198665	2703361	106143	10	0.44		
Bpilo060_WesB	1	2889522	2889522	2889522	1	1026.71	very large number of N inserted	x
Bsuan061_BRSU_AN4859-03	34	2243936	3258009	2243936	1	0.55		

Appendix III (Chapter 5)

Table 9. 3 Protein sequences and BLAST sequence homology for the putative bacteriocin genes identified by the Anti-SMASH bacteriocin mining pipeline.

Species	Isolate Name	Predicted secondary metabolite gene clusters	Protein Sequence	BLAST sequence homology
L. salivarius	SAP 2103	Salivaricin	MMKEFTVLTECELAKVDGGYTPKNCAIAVGGGMLSGAIRG GMAGTVFGVGTGNLAGAFAGAHIGLVAGGLACIGGYLGSH	Multispecies bacteriocin (*Lactobacillus*) (100%)
L. crispatus	SAP 2105	None		
L. crispatus	SAP 2106	None		
L. crispatus	SAP 2107	None		
L. reuteri	SAP 2108	None		
L. salivarius	SAP 2113	Salivaricin	MMKEFTVLTECELAKVDGGYTPKNCAIAVGGGMLSGAIRG GMAGTVFGVGTGNLAGAFAGAHIGLVAGGLACIGGYLGSH	Multispecies bacteriocin (*Lactobacillus*) (100%)
L. reuteri	SAP 2114	None		
L. reuteri	SAP 2115	None		
L. salivarius	SAP 2116	Salivaricin	MMKEFTVLTECELAKVDGGYTPKNCAIAVGGGMLSGAIRG GMAGTVFGVGTGNLAGAFAGAHIGLVAGGLACIGGYLGSH	Multispecies bacteriocin (*Lactobacillus*) (100%)
L. salivarius	SAP 2117	Salivaricin	MMKEFTVLTECELAKVDGGYTPKNCAIAVGGGMLSGAIRGG	Multispecies bacteriocin (*Lactobacillus*) (100%)

MAGTVFGVGTGNLAGAFAGAHIGLVAGGLACIGGYLGSH

Table 9. 4 Protein sequences and BLAST sequence homology for the putative bacteriocin genes identified by the Bagel 4 bacteriocin mining pipeline.

Species	Isolate Name	Predicted bacteriocin gene clusters	Protein Sequence	BLAST sequence homology
L. salivarius	SAP 2103	Enterolysin A	VYLVKIRNGDETTVIHGTKNNTLGDAKVSMTVSAASSFTFIIYPNNTGYF KLREWTTYIDVTQSGKYIFRGRVIAVNPKHNEDGTFYKEITCESAMAYLN DSILSWEKVDKKPADFFVELINEHNKQMGDASKQFKIAENSVTNNTNNL YCYVEDGISILEEMKTDLLQNEDLGGEISIDYRNDGNYISWTRDKKVKGQ QVIKLAKNLKTLSAKPDISKICSVLYPFGATKEVPVDQKNDEKTNEVSTPRI NISSVNNGKNYIEDPELIKAIGRVSNTKTWENIKEPKNLLAKAQEYLKTM RNYRIAYELDAVDLQPLGLAVDSFECGNYYHVINPVINVDEWLRLVGVTI DLNKPLESTLTVGDQVKRLVDYSMDNIQTERNLRRLAAEQQVLKHQNN MLIDENNQLKQTIIKLWNDILSKQESNDSSGSAWNWPFKPPATIRFDGA QLFGVNPGGEFRPNGFHDGLDFGSVNWPGSEVKAIHDGTVTLKGAMD GLGNYFVTNGDGFNIVYQEAFGSASDIRVNIGDHVKVGDVVGIRTTDHL HVGVTKHDFNAALGSAFSNNGTWLDPKKLIEDGLKNNMGTTQPVNGD WGPVIRNAATKMKVNISDNDVNRIKALIANESGGNQTVTQQVWDQN MAAGTPAQGLLQYVPSTFNAYAVDGHRNIKSGFDQLLAFFNNSTWSSD ISLHGWGPNGSKRFDKIPA	M23 peptidase (*Lactobacillus salivarius*) (65%)
		Bacteriocin Class IIc	MIIMMKEFTVLTECELAKVDGGYTPKNCAIAVGGGMLSGAIRGGMAG TVFGVGTGNLAGAFAGAHIGLVAGGLACIGGYLGSH	Multispecies bacteriocin (*Lactobacillus*) (100%)
L. crispatus	SAP 2105	Enterolysin A Class III	VYLVKIRNGDETTVIHGTKNNTLGDAKVSMTVSAASSFTFIIYPNNTGYF KLREWTTYIDVTQSGKYIFRGRVIAVNPKHNEDGTFYKEITCESAMAYLN DSILSWEKVDKKPADFFVELINEHNKQMGDASKQFKIAENSVTNNTNNL YCYVEDGISILEEMKTDLLQNEDLGGEISIDYRNDGNYISWTRDKKVKGQ QVIKLAKNLKTLSAKPDISKICSVLYPFGATKEVPVDQKNDEKTNEVSTPRI NISSVNNGKNYIEDPELIKAIGRVSNTKTWENIKEPKNLLAKAQEYLKTM RNYRIAYELDAVDLQPLGLAVDSFECGNYYHVINPVINVDEWLRLVGVTI DLNKPLESTLTVGDQVKRLVDYSMDNIQTERNLRRLAAEQQVLKHQNN MLIDENNQLKQTIIKLWNDILSKQESNDSSGSAWNWPFKPPATIRFDGA QLFGVNPGGEFRPNGFHDGLDFGSVNWPGSEVKAIHDGTVTLKGAMD GLGNYFVTNGDGFNIVYQEAFGSASDIRVNIGDHVKVGDVVGIRTTDHL HVGVTKHDFNAALGSAFSNNGTWLDPKKLIEDGLKNNMGTTQPVNGD WGPVIRNAATKMKVNISDNDVNRIKALIANESGGNQTVTQQVWDQN	M23 peptidase (*Lactobacillus salivarius*) (65%)

		Bacteriocin Class IIc	MAAGTPAQGLLQYVPSTFNAYAVDGHRNIKSGFDQLLAFFNNSTWSSD ISLHGWGPNGSKRFDKIPA MIIMMKEFTVLTECELAKVDGGYTPKNCAIAVGGGMLSGAIRGGMAG TVFGVGTGNLAGAFAGAHIGLVAGGLACIGGYLGSH	Multispecies bacteriocin (*Lactobacillus*) (100%)
L. crispatus	SAP 2106	Enterolysin A Class III	MKFRKLIISLLGTALLTSSVGLSTTTASADTLDDSQNTTEVQPKNLKWAYP FKANKKNGVRPMYNAQTFGITNYMRSTTPPSYFHDGWDFGFSEVGHS NVYAIHQGTVKKVAYGNGLGWFIWVISPDNYVEVYQEGFNKKKDIYVK TGQKIKLGQKIGKLTGSHLHLGVTQTNKDYINKYGFPCKNWNVNNGTW LNPIEVIKSNLKK	M23 peptidase (*Lactobacillus crispatus*) (99%)
		Helveticin J	MVKNITPELVYRLNGMHHVVAQVGAVIDNHIFALQLLHSAHDVLVYRK HEGLTKNVDYSEPHLVMTGFGHTQTWVPANDKDEYFVGAKPNSGNW TTQIARVKYPRLLPERYTSNTQLPRLSHLNRATDVPYDGHNHLHRVEASV SPNGKYFMIAAIWDDDSGHFALYDLNEVNQKLDENGTTNTPITDLHCLS AFHIDNFDHPSVAPSEEAPQMIDSVQGYAIDDDKNIYISNQLSPKIDHAT GEVTTWSRKIVKFPWGETNPENWQVAMIDGIDLPDRYSEVESIHVQAP DDIYLTVAYHQKYVKDGEFKLRTLENQIFHISDLG	Bacteriocin (*Lactobacillus crispatus*) (96%)
L. crispatus	SAP 2107	Enterolysin A Class III	MKFRKLIISLLGTALLTSSVGLSTTTASADTLDDSQNTTEVQPKNLKWAYP FKANKKNGVRPMYNAQTFGITNYMRSTTPPSYFHDGWDFGFSEVGHS NVYAIHQGTVKKVAYGNGLGWFIWVISPDNYVEVYQEGFNKKKDIYVK TGQKIKLGQKIGKLTGSHLHLGVTQTNKDYINKYGFPCKNWNVNNGTW LNPIEVIKSNLKK	M23 peptidase (*Lactobacillus crispatus*) (99%)
		Helveticin J	MVKNITPELVYRLNGMHHVVAQVGAVIDNHIFALQLLHSAHDVLVYRK HEGLTKNVDYSEPHLVMTGFGHTQTWVPANDKDEYFVGAKPNSGNW TTQIARVKYPRLLPERYTSNTQLPRLSHLNRATDVPYDGHNHLHRVEASV SPNGKYFMIAAIWDDDSGHFALYDLNEVNQKLDENGTTNTPITDLHCLS AFHIDNFDHPSVAPSEEAPQMIDSVQGYAIDDDKNIYISNQLSPKIDHAT GEVTTWSRKIVKFPWGETNPENWQVAMIDGIDLPDRYSEVESIHVQAP DDIYLTVAYHQKYVKDGEFKLRTLENQIFHISDLG	Bacteriocin (*Lactobacillus crispatus*) (96%)
L. reuteri	SAP 2108	Enterolysin A Class III	MEGKLIFKPSHLKSKVLSTLTVCGGALFLLSGNAAADDQTTDVQQPVTP QQTTNEQPNASTVDVQTPNTYNGVAVSPQASEATQNTENYNVVPTQ GNNQEVQHKEATQPQNPNNYGYLDSVSLSNNELQVSGWQATNQAED KPYHYVIAYDNTAKAELGRQQVKNVSRPDVAQAYPDATNADNSGFNST IKINTTNEDYTNHSISVISRYSDATNGEGNHVDYWYPAFTFDQGNYAWL DDMHTENDQLHVTGWNATNQASNKDYHYVILYNKTKGHELSRQLVDE KNSQRPDVQKVYQNVNNATKSGFNVTFDLSKLSFDASDQLQVISRYSD	M23 peptidase (*Lactobacillus reuteri*) (99%)

			AQNGEGNRVDFWFAPTSRENQGNLDSANFSNGQLVVNGWHANDASI IAPNHFLILFDRTANKQAASLSASQVNRPDVAKAFGAIQTAGKAGFTGT FNANVIIPGHEYSLVSRYSTSANGNGDQGHYVDYWFNGLEFNQARYSV DSFTQNDKGFHVTGWMASDFAVNRPNAYVILLNNGKEVTRSKVTLTD RSDVAAVYPSLYNSRKSGFSTDLIVNPASLTGELSMILRFTGSNDGNSNY TDQNTNKYATNAGSFDTVNVSGNQIKVAGWHASTQTAGKDYQFIIVLDR NGHELTRQAVNTKDITRNDVQKVYPWLTNSVKS GFDTTVSINDQINHKVVRLIHRYSNQANGEGSYVDYYSNPISVYSEFQNE NGTTFYYDSRTGAKKTGWVNINNNNYYFDPSTGAMFTGTHTVDGKSY DFGNNGVAVENDKWGWPFPNVGEGHFGGAQLFGVNPGGQFRRNGF HDGLDFGSIDHPGSEVHAIHGGKVTQIGYTAGLDWYVLVDTGEYLTVY QEAFSNKNNIQVQVGQQINTGDVIGRRDTAHVHIGVTRQHNFNIALAN SFNNNGTWLNPLDLIRNGSK	
L. salivarius	SAP 2113	Enterolysin A Class III	MFQGKILVQGVNRAEKEPLNLFDPKSVQIQWKVNQTWSLQFTAYNDG SLAYQMLESEASIFLDNQEYIIKQVADDSSNGLDSIQVTATHVYFEVQKIR KYKDYVDPEDKDKQTDVKVLKDNTDSSKSDDSDNAKTDTSEKTEGNTTT KVTTKTTDETQQDNQNQVTYSIQDVLEHWLKDNKLGFTYEVIGSFEKKE LEELQDGTGADMLSKISDTWSNAIIYPDNRKIRVYSADKFNLNRGNRIDY LNNASEIKFSTDSTSLTNMAYCIGGKYSVETTTETTTTTTTTTTSGGWGW PFPSVGEGNFMQAQRFGNDGGYRQNGFHEGLDFGSVDHPGRDVHAI HGGKVTIKSYMGGLGNYVVISGGGYNVVYQEAFSSPNNIIVNVGDTVK VGDVIGYRDTSHLHVGVTKADFNVAVGKSFTNDGTWLDPLELIKNGPS DTDTETSSETNSNSNTQEYYYFAPFMYRDEESIKKYGEHPAEPIEDGRFK DKSAMIEYVKTKLQPEPSLSIDVTTTTDIKPIAGDVVHVMVKSQDISTNFT LTGFTWYPYSYPVDNPTSITLNSNVQNILDYQNSRQKQFNKAISELKSST NEAINNSNSFNEFGGNQQLKTWLNDFVGG	M23 peptidase (*Lactobacillus salivarius*) (97%)
		Bacteriocin Class IIc	MIIMMKEFTVLTECELAKVDGGYTPKNCAIAVGGGMLSGAIRGGMAG TVFGVGTGNLAGAFAGAHIGLVAGGLACIGGYLGSH	Multispecies bacteriocin (*Lactobacillus*) (100%)
L. reuteri	SAP 2114	Enterolysin A Class III	MEGKLIFKPSHLKSKVLSTLTVCGGALFLLSGNAAADDQTTDVQQPVTP QQTTNEQPNASTVDVQTPNTYNGVAVSPQASEATQNTENYNVVPTQ GNNQEVQHKEATQPQNPNNYGYLDSVSLSNNELQVSGWQATNQAED KPYHYVIAYDNTAKAELGRQQVKNVSRPDVAQAYPDATNADNSGFNST IKINTTNEDYTNHSISVISRYSDATNGEGNHVDYWYPAFTFDQGNYAWL DDMHTENDQLHVTGWNATNQASNKDYHYVILYNKTKGHELSRQLVDE KNSQRPDVQKVYQNVNNATKSGFNVTFDLSKLSFDASDQLQVISRYSD	M23 peptidase (*Lactobacillus reuteri*) (99%)

			AQNGEGNRVDFWFAPTSRENQGNLDSANFSNGQLVVNGWHANDASI IAPNHFLILFDRTANKQAASLSASQVNRPDVAKAFGAIQTAGKAGFTGT FNANVIIPGHEYSLVSRYSTSANGNGDQGHYVDYWFNGLEFNQARYSV DSFTQNDKGFHVTGWMASDFAVNRPNAYVILLNNGKEVTRSKVTLTD RSDVAAVYPSLYNSRKSGFSTDLIVNPASLTGELSMI LRFTGSNDGNSNYTDQNTNKYATNAGSFDTVNVSGNQIKVAGWHAST QTAGKDYQFIIVLDRNGHELTRQAVNTKDITRNDVQKVYPWLTNSVKS GFDTTVSINDQINHKVVRLIHRYSNQANGEGSYVDYYSNPISVYSEFQNE NGTTFYYDSRTGAKKTGWVNINNNNNYYFDPSTGAMFTGTHTVDGKSY DFGNNGVAVENDKWGWPFPNVGEGHFGGAQLFGVNPGGQFRRNGF HDGLDFGSIDHPGSEVHAIHGGKVTQIGYTAGLDWYVLVDTGEYLTVY QEAFSNKNNIQVQVGQQINTGDVIGRRDTAHVHIGVTRQHNFNIALAN SFNNNGTWLNPLDLIRNGSK	
L. reuteri	SAP 2115	Enterolysin A Class III	MEGKLIFKPSHLKSKVLSTLTVCGGALFLLSGNAAADDQTTDVQQPVTP QQTTNEQPNASTVDVQTPNTYNGVAVSPQASEATQNTENYNVVPTQ GNNQEVQHKEATQPQNPNNYGYLDSVSLSNNELQVSGWQATNQAED KPYHYVIAYDNTAKAELGRQQVKNVSRPDVAQAYPDATNADNSGFNST IKINTTNEDYTNHSISVISRYSDATNGEGNHVDYWYPAFTFDQGNYAWL DDMHTENDQLHVTGWNATNQASNKDYHYVILYNKTKGHELSRQLVDE KNSQRPDVQKVYQNVNNATKSGFNVTFDLSKLSFDASDQLQVISRYSD AQNGEGNRVDFWFAPTSRENQGNLDSANFSNGQLVVNGWHANDASI IAPNHFLILFDRTANKQAASLSASQVNRPDVAKAFGAIQTAGKAGFTGT FNANVIIPGHEYSLVSRYSTSANGNGDQGHYVDYWFNGLEFNQARYSV DSFTQNDKGFHVTGWMASDFAVNRPNAYVILLNNGKEVTRSKVTLTD RSDVAAVYPSLYNSRKSGFSTDLIVNPASLTGELSMILRFTGSNDGNSNY TDQNTNKYATNAGSFDTVNVSGNQIKVAGWHASTQTAGKDYQFIIVLD RNGHELTRQAVNTKDITRNDVQKVYPWLTNSVKSGFDTTVSINDQINH KVVRLIHRYSNQANGEGSYVDYYSNPISVYSEFQNENGTTFYYDSRTGAK KTGWVNINNNNNYYFDPSTGAMFTGTHTVDGKSYDFGNNGVAVENDK WGWPFPNVGEGHFGGAQLFGVNPGGQFRRNGFHDGLDFGSIDHPGS EVHAIHGGKVTQIGYTAGLDWYVLVDTGEYLTVYQEAFSNKNNIQVQV GQQINTGDVIGRRDTAHVHIGVTRQHNFNIALANSFNNNGTWLNPLDL IRNGSK	M23 peptidase (*Lactobacillus reuteri)* (99%)
L. salivarius	SAP 2116	Enterolysin A Class III	VYLVKIRNGDETTVIHGTKNNTLGDAKVSMTVSAASSFTFIIYPNNTGYF KLREWTTYIDVTQSGKYIFRGRVIAVNPKHNEDGTFYKEITCESAMAYLN DSILSWEKVDKKPADFFVELINEHNKQMGDASKQFKIAENSVTNNTNNL	M23 peptidase (*Lactobacillus salivarius*) (65%)

			YCYVEDGISILEEMKTDLLQNEDLGGEISIDYRNDGNYISWTRDKKVKGQ QVIKLAKNLKTLSAKPDISKICSVLYPFGATKEVPVDQKNDEKTNEVSTPRI NISSVNNGKNYIEDPELIKAIGRVSNTKTWENIKEPKNLLAKAQEYLKTM RNYRIAYELDAVDLQPLGLAVDSFECGNYYHVINPVINVDEWLRLVGVTI DLNKPLESTLTVGDQVKRLVDYSMDNIQTERNLRRLAAEQQVLKHQNN MLIDENNQLKQTIIKLWNDILSKQESNDSSGSAWNWPFKPPATIRFDGA QLFGVNPGGEFRPNGFHDGLDFGSVNWPGSEVKAIHDGTVTLKGAMD GLGNYFVTNGDGFNIVYQEAFGSASDIRVNIGDHVKVGDVVGIRTTDHL HVGVTKHDFNAALGSAFSNNGTWLDPKKLIEDGLKNNMGTTQPVNGD WGPVIRNAATKMKVNISDNDVNRIKALIANESGGNQTVTQQVWDQN MAAGTPAQGLLQYVPSTFNAYAVDGHRNIKSGFDQLLAFFNNSTWSSD ISLHGWGPNGSKRFDKIPA	
		Bacteriocin Class IIc	MIIMMKEFTVLTECELAKVDGGYTPKNCAIAVGGGMLSGAIRGGMAG TVFGVGTGNLAGAFAGAHIGLVAGGLACIGGYLGSH	Multispecies bacteriocin (*Lactobacillus*) (100%)
L. salivarius	SAP 2117	Enterolysin A Class III	MFQGKILVQGVNRAEKEPLNLFDPKSVQIQWKVNQTWSLQFTAYNDG SLAYQMLESEASIFLDNQEYIIKQVADDSSNGLDSIQVTATHVYFEVQKIR KYKDYVDPEDKDKQTDVKVLKDNTDSSKSDDSDNAKTDTSEKTEGNTTT KVTTKTTDETQQDNQNQVTYSIQDVLEHWLKDNKLGFTYEVIGSFEKKE LEELQDGTGADMLSKISDTWSNAIIYPDNRKIRVYSADKFNLNRGNRIDY LNNASEIKFSTDSTSLTNMAYCIGGKYSVETTTETTTTTTTTTTSGGWGW PFPSVGEGNFMQAQRFGNDGGYRQNGFHEGLDFGSVDHPGRDVHAI HGGKVTIKSYMGGLGNYVVISGGGYNVVYQEAFSSPNNIIVNVGDTVK VGDVIGYRDTSHLHVGVTKADFNVAVGKSFTNDGTWLDPLELIKNGPS DTDTETSSETNSNSNTQEYYYFAPFMYRDEESIKKYGEHPAEPIEDGRFK DKSAMIEYVKTKLQPEPSLSIDVTTTTDIKPIAGDVVHVMVKSQDISTNFT LTGFTWYPYSYPVDNPTSITLNSNVQNILDYQNSRQKQFNKAISELKSST NEAINNSNSFNEFGGNQQLKTWLNDFVGG	M23 peptidase (*Lactobacillus salivarius*) (97%)
		Bacteriocin Class IIc	MIIMMKEFTVLTECELAKVDGGYTPKNCAIAVGGGMLSGAIRGGMAG TVFGVGTGNLAGAFAGAHIGLVAGGLACIGGYLGSH	Multispecies bacteriocin (*Lactobacillus*) (100%)

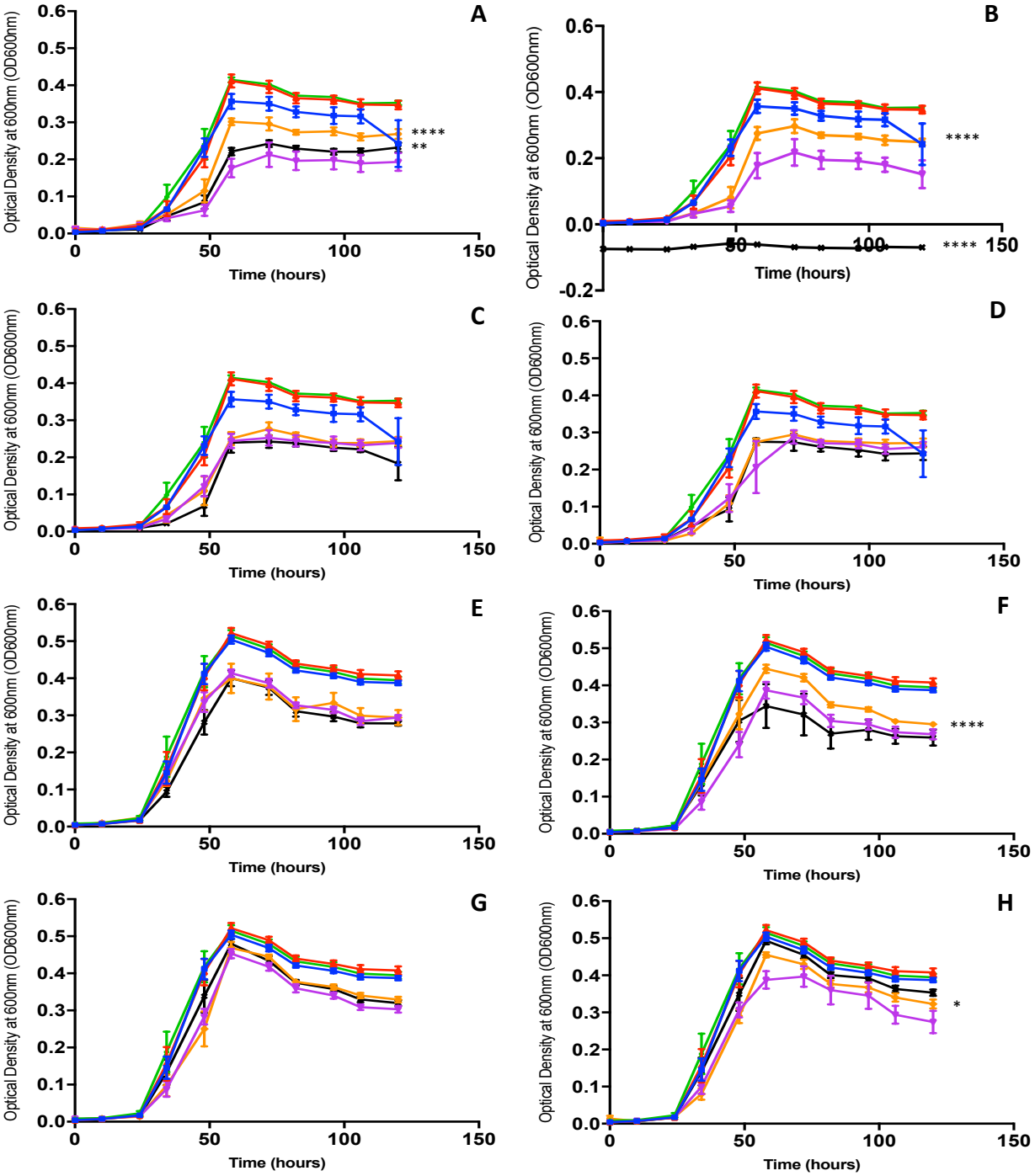

Figure 9. 1 Growth curves of *B. intermedia* SAP 919 supplemented with **A:** *L. reuteri* SAP 2114 CFS at pH 3.8 **B:** *L. reuteri* SAP 2115 CFS at pH 3.8 **C:** *L. salivarius* SAP 2116 CFS at pH 3.8 **D:** *L. salivarius* SAP 2117 CFS at pH 3.8 **E:** *L. reuteri* SAP 2114 CFS at pH 7.2 **F:** *L. reuteri* SAP 2115 CFS at pH 7.2 **G:** *L. salivarius* SAP 2116 CFS at pH 7.2 **H:** *L. salivarius* SAP 2117 CFS at pH 7.2 in BIH broth+ 10% serum. Each CFS was either treated with trypsin or heat treated to denature potential antimicrobial proteins produced by each *Lactobacillus* isolate. MRS (blue), MRS trypsin treated (red), MRS heat treated (green), CFS (purple), CFS trypsin treated (orange) and CFS heat treated (black). Growth was monitored over 120 hours of anaerobic incubation at 37°C. Optical density readings were taken at 620nm every ~10 hours. These data represent an average of five biological replicates, each with three technical replicates. Significance, if any, is shown for changes in growth when comparing CFS without treatment to CFS with either trypsin or heat treatment * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

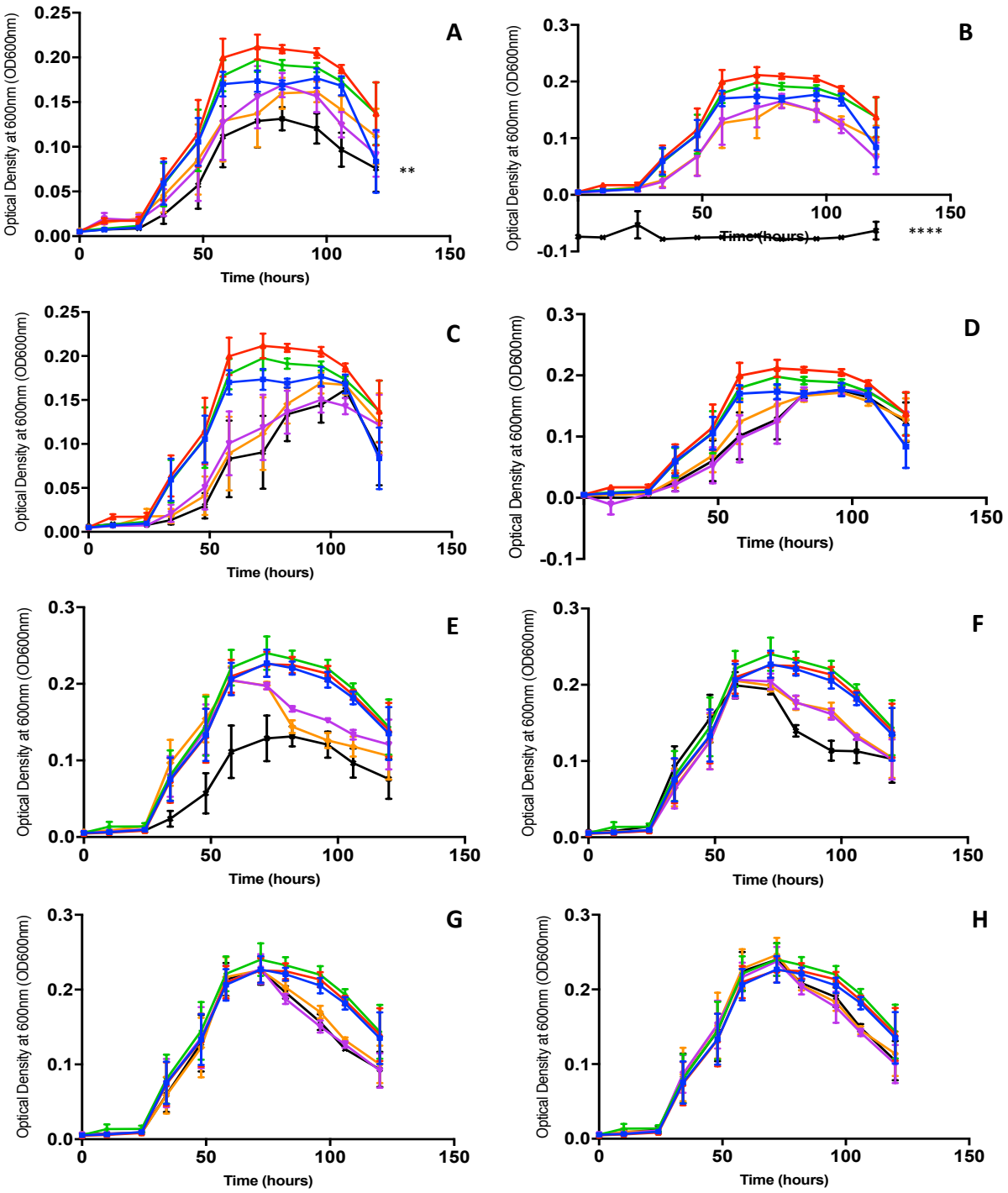

Figure 9. 2 Growth curves of *B. alvinipulli* ATCC 51933 supplemented with **A:** *L. reuteri* SAP 2114 CFS at pH 3.8 **B:** *L. reuteri* SAP 2115 CFS at pH 3.8 **C:** *L. salivarius* SAP 2116 CFS at pH 3.8 **D:** *L. salivarius* SAP 2117 CFS at pH 3.8 **E:** *L. reuteri* SAP 2114 CFS at pH 7.2 **F:** *L. reuteri* SAP 2115 CFS at pH 7.2 **G:** *L. salivarius* SAP 2116 CFS at pH 7.2 **H:** *L. salivarius* SAP 2117 CFS at pH 7.2 in BIH broth+ 10% serum. Each CFS was either treated with trypsin or heat treated to denature potential antimicrobial proteins produced by each *Lactobacillus* isolate. MRS (blue), MRS trypsin treated (red), MRS heat treated (green), CFS (purple), CFS trypsin treated (orange) and CFS heat treated (black). Growth was monitored over 120 hours of anaerobic incubation at 37°C. Optical density readings were taken at 620nm every ˜10 hours. These data represent an average of five biological replicates, each with three technical replicates. Significance, if any, is shown for changes in growth when comparing CFS without treatment to CFS with either trypsin or heat treatment * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

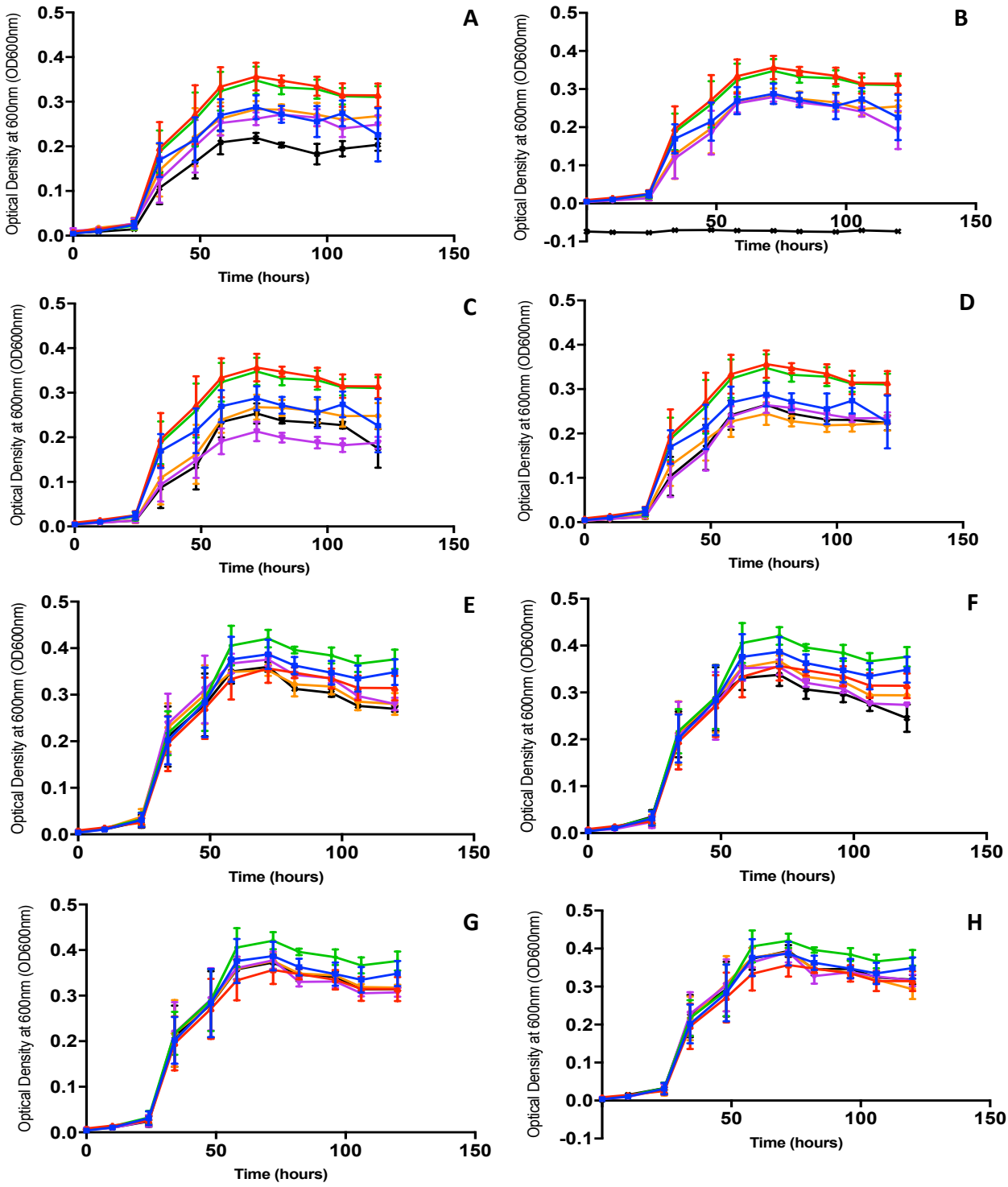

Figure 9. 3 Growth curves of *B. pilosicoli* SAP 859 supplemented with **A:** *L. reuteri* SAP 2114 CFS at pH 3.8 **B:** *L. reuteri* SAP 2115 CFS at pH 3.8 **C:** *L. salivarius* SAP 2116 CFS at pH 3.8 **D:** *L. salivarius* SAP 2117 CFS at pH 3.8 **E:** *L. reuteri* SAP 2114 CFS at pH 7.2 **F:** *L. reuteri* SAP 2115 CFS at pH 7.2 **G:** *L. salivarius* SAP 2116 CFS at pH 7.2 **H:** *L. salivarius* SAP 2117 CFS at pH 7.2 in BIH broth+ 10% serum. Each CFS was either treated with trypsin or heat treated to denature potential antimicrobial proteins produced by each *Lactobacillus* isolate. MRS (blue), MRS trypsin treated (red), MRS heat treated (green), CFS (purple), CFS trypsin treated (orange) and CFS heat treated (black). Growth was monitored over 120 hours of anaerobic incubation at 37°C. Optical density readings were taken at 620nm every ˜10 hours. These data represent an average of five biological replicates, each with three technical replicates. Significance, if any, is shown for changes in growth when comparing CFS without treatment to CFS with either trypsin or heat treatment * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

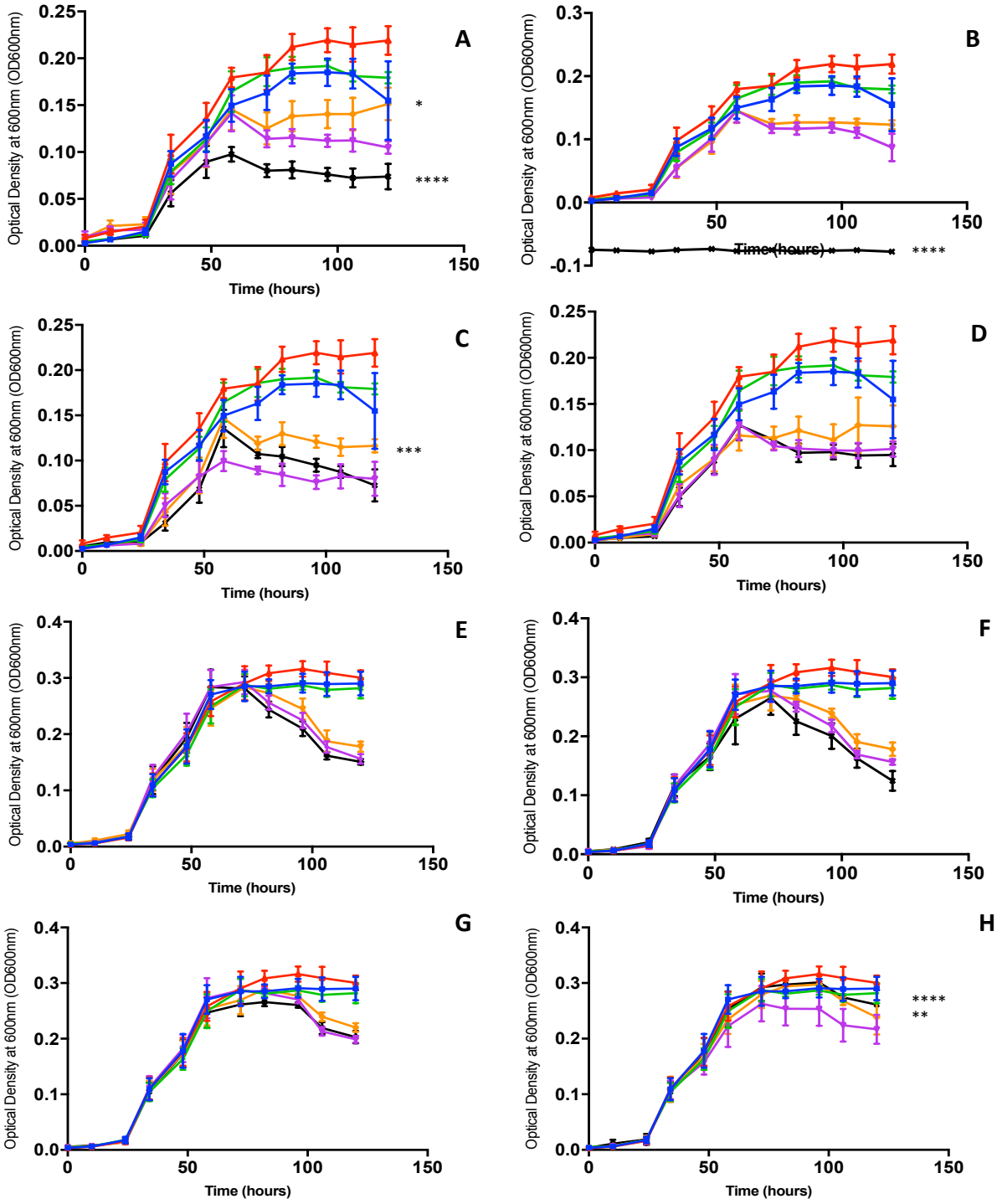

Figure 9. 4 Growth curves of *B. innocens* SAP 924 supplemented with **A:** *L. reuteri* SAP 2114 CFS at pH 3.8 **B:** *L. reuteri* SAP 2115 CFS at pH 3.8 **C:** *L. salivarius* SAP 2116 CFS at pH 3.8 **D:** *L. salivarius* SAP 2117 CFS at pH 3.8 **E:** *L. reuteri* SAP 2114 CFS at pH 7.2 **F:** *L. reuteri* SAP 2115 CFS at pH 7.2 **G:** *L. salivarius* SAP 2116 CFS at pH 7.2 **H:** *L. salivarius* SAP 2117 CFS at pH 7.2 in BIH broth+ 10% serum. Each CFS was either treated with trypsin or heat treated to denature potential antimicrobial proteins produced by each *Lactobacillus* isolate. MRS (blue), MRS trypsin treated (red), MRS heat treated (green), CFS (purple), CFS trypsin treated (orange) and CFS heat treated (black). Growth was monitored over 120 hours of anaerobic incubation at 37°C. Optical density readings were taken at 620nm every ˜10 hours. These data represent an average of five biological replicates, each with three technical replicates. Significance, if any, is shown for changes in growth when comparing CFS without treatment to CFS with either trypsin or heat treatment * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.

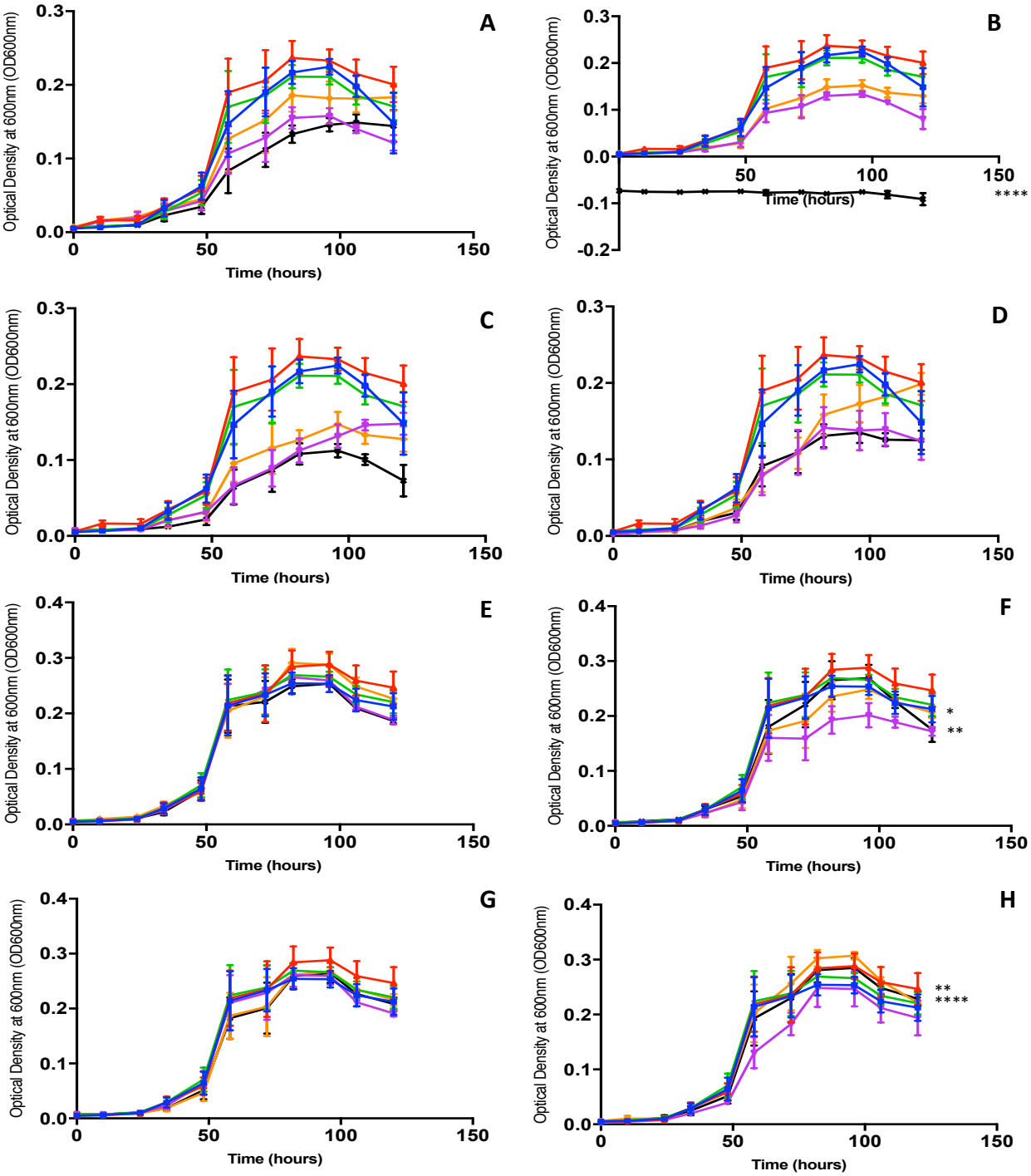

Figure 9. 5 Growth curves of *B. innocens* SAP 943 supplemented with **A:** *L. reuteri* SAP 2114 CFS at pH 3.8 **B:** *L. reuteri* SAP 2115 CFS at pH 3.8 **C:** *L. salivarius* SAP 2116 CFS at pH 3.8 **D:** *L. salivarius* SAP 2117 CFS at pH 3.8 **E:** *L. reuteri* SAP 2114 CFS at pH 7.2 **F:** *L. reuteri* SAP 2115 CFS at pH 7.2 **G:** *L. salivarius* SAP 2116 CFS at pH 7.2 **H:** *L. salivarius* SAP 2117 CFS at pH 7.2 in BIH broth+ 10% serum. Each CFS was either treated with trypsin or heat treated to denature potential antimicrobial proteins produced by each *Lactobacillus* isolate. MRS (blue), MRS trypsin treated (red), MRS heat treated (green), CFS (purple), CFS trypsin treated (orange) and CFS heat treated (black). Growth was monitored over 120 hours of anaerobic incubation at 37°C. Optical density readings were taken at 620nm every ˜10 hours. These data represent an average of five biological replicates, each with three technical replicates. Significance, if any, is shown for changes in growth when comparing CFS without treatment to CFS with either trypsin or heat treatment * $p \leq 0.05$; ** ≤ 0.01, *** ≤ 0.0005, **** ≤ 0.0001.