EXPLORING THE ECOLOGIES OF *CAMPYLOBACTER* AND *EIMERIA* INFECTIONS IN UK SHEEP

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SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY, JULY 2017

Table of contents

List of Tables

List of Figures

Acknowledgments

First of all, I would like to thank God for everything in my life.

This work is dedicated to my country (IRAQ) who has all my love and loyalty, my beloved wife and children, all my family and friends, thanks for your love and support.

My dearest appreciation to my supervisor Professor Richard Birtles for his generous help, encouragement and guidance throughout the whole PhD period and through the process of writing this thesis.

Special thanks to Dr. Ian Goodhead, and Dr. Kevin Bown for their help and support.

Many thanks to farmers Carole, Johns and Arthurs for their help in the field.

Declaration

No portion of this work referred to in this report has been submitted in support of an application for another degree or qualification of this or any other university or another institute of learning.

Abbreviations

× g	Units of times gravity
µl	Microliter
ºC	Celsius
aspA	Aspartase A-encoding gene
ATR	Adaptive tolerance response
BE	Barrett's eosophagus
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
bp	Base pairs
CadF	Fibronectin-binding outer membrane protein
CCD	Charge coupled device
CCDA	Charcoal cefoperazone deoxycholate agar
CD	Crohn's disease
CDT	Cytolethal distending toxin
CdtA	Cytolethal distending toxins A
CdtB	Cytolethal distending toxins B
CdtC	Cytolethal distending toxins C
Cia	*Campylobacter* invasive antigens
CPS	Capsular polysaccharide
Cq	Quantification cycle
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA

EPS	Extracellular polymeric substances
FD	Functional dyspepsia
FlaA	Flagellin A protein
FlaB	Flagellin B protein
g	Gram
GBS	Guillian-Barre syndrome
GERD	Gastroesophageal reflux disease
GLM	Generalized linear model
glnA	Glutamine synthetase-encoding gene
gltA	Citrate synthase-encoding gene
glyA	Serine hydroxymethyltransferase-encoding gene
h	Hour
HGP	Human genome project
HK	Histidine kinase
HSPs	Heat shock proteins
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ITS-1	Internally transcribed spacer-1
ITS-2	Internally Transcribed spacer-2
KatA	Catalase
kb	Kilobase
LOS	Lipooligosaccharides
MFS	Miller Fisher syndrome

min	Minute
ml	Milliliters
MLST	Multi-locus sequencing typing
mtCOI	Cytochrome C oxidase subunit I-encoding gene
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NGS	Next generation sequencing
nM	Nanometre
OPG	Oocyst per gram
PCR	Polymerase chain reaction
PFGD	Post infectious functional gastrointestinal disorders
pgm	Phosphoglucomutase-encoding gene
pH	Potential of hydrogen
PMNs	Polymorphonuclear neutrophils
PNPase	Polynucleotide phosphorylase
qPCR	Quantitative polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
rDNA	Ribosomal RNA-encoding gene
RR	Response regulator
RSB	Resuspension buffer
SCAR	Sequence characterized amplified region
Sec	Second
SOL	Sialylation of lipooligosaccharides

ST	Sequence type
TBE	Tris/Borate/EDTA
T-cell	T lymphocyte
TCS	Two component regulator system
Th1	T helper cell (1)
Th2	T helper cell (2)
tkt	Transketolase-encoding gene
UC	Ulcerative colitis
UK	United Kingdom
uncA	ATP synthase A subunit-encoding gene
USA	United State of America
UV	Ultraviolet
VBNC	Viable but non-culturable
w/v	Weight/volume

Abstract

Several gastrointestinal parasites of sheep have veterinary and zoonotic importance, including coccidia belonging to the genus *Eimeria* and proteobacteria belonging to the genus *Campylobacter*. In the UK, both *Eimeria* and *Campylobacter* are both frequently isolated from sheep faeces, and studies have shown that infections or co-infections by 10 or more *Eimeria* species may occur.

Campylobacter jejuni and *Campylobacter coli* are the most frequently encountered sheep-associated *Campylobacter* species. Despite their potential veterinary and public health importance, little work has been reported to date exploring the ecologies of these microorganisms.

This project addressed this shortfall by completing cross-sectional and longitudinal surveys of *Eimeria* and *Campylobacter* infections in sheep flocks in southern Cumbria. In total almost 1000 ovine faecal samples were collected on 27 visits to three farms in the region. Infections were diagnosed, and infecting species of both genera delineated, using well-established methods. These results were collated with information about the timing of sample collection and the age of sheep, and climate data. Significant seasonal trends in the epidemiology of *Eimeria* and *Campylobacter* infections were observed. Furthermore, the intensity of *Eimeria* infections was also found to be significantly correlated with season, but, in addition with sheep age, rainfall prior to sample collection and, interestingly, to *Campylobacter* co-infection.

Another strand of the study was to assess the role of wildlife as reservoirs for sheep-associated campylobacters. A survey of red and roe deer living in the vicinity of the three farms studied failed to implicate either species in this role, suggesting they do not contribute to the natural persistence of these bacteria in Cumbrian sheep populations.

Finally, in an attempt to develop new molecular methods for the delineation of sheep-associated *Eimeria* species, next-generation sequencing (NGS) was used to try and attribute 18S rDNA sequences to *Eimeria* species present in multi-*Eimeria* species co-infections. In initial studies, 18S rDNA libraries derived from mock communities of four chicken-associated *Eimeria* species were analysed to assess how accurately NGS data matched the relative abundance of each *Eimeria* species, determined using traditional oocyst counting methods. Unfortunately, no suggestion of a correlation was apparent.

Overall the project clarified the epidemiology of two genera of significant sheep-associated pathogens and was able to identify some important ecological determinants of this epidemiology.

CHAPTER 1: Introduction

Livestock production constitutes a very important component of the world's agricultural economy. The contribution of livestock is primarily direct food production but extends to include the production of skins, fibre, fertilizer and fuel, as well as capital accumulation. Furthermore, livestock are closely linked to the social and cultural lives of farmers for whom animal ownership is a basis of sustainable farming and economic stability (Herrero *et al.*, 2013). The global demand for livestock continues to rise, with demand in developing countries for animal foods projected to double over the next 20 years (Herrero *et al.*, 2013). This rising demand has important economic implications including the potential to offer many of the world's poor a route out of poverty. However, disease outbreaks pose significant threats to livestock sectors throughout the world, both in terms of the economic impacts of the disease itself and also the preventative measures taken to reduce the risk of disease (Rich & Perry, 2011). Infections underlie a significant proportion of livestock disease and numerous approaches have been taken to quantifying their burden (Perry & Grace, 2009). For example, Bennett and Ijpelaar (2005) used a system of models for the economic analysis of endemic diseases of livestock in the UK to show that mastitis had the greatest burden in cattle farming (almost £200 million per annum), whereas for sheep farmers enzootic abortion was the most costly infection (approximately £24 million per annum) and, for poultry farmers, salmonellosis was the most burdensome, with a national cost of just over £100 million per annum (Bennett & Ijpelaar, 2005).

Sheep are key livestock species, with an estimated 1 billion animals living around the world, of which about 33 million are farmed in the UK (NSA, 2017). The UK sheep farming industry employs about 34,000 people on sheep farms and another 111,405 in allied industries, contributing £291.4 million to employment in the country (NSA, 2017). Infection has a not insignificant impact on UK sheep farming. As detailed above, enzootic abortion carries the greatest burden; this burden is also reflected in a study on Welsh farms (HCC, 2017) that attributed 26% of perinatal lamb losses to abortions or stillbirths caused by *Chlamydia abortus*, *Toxoplasma gondii* or *Campylobacter* species. Sheep are also susceptible to numerous other bacterial, viral and parasitic diseases (Taylor *et al.*, 2007). Although some of these are obligate pathogens, most are opportunistic and reside within the microbiome/virome/parasitome of healthy animals. Co-infection of an individual host by multiple species of microorganisms represents the natural state (Viney & Graham, 2013), and these co-infection "guilds" are likely to involve many different species (Pedersen & Fenton, 2007). As well as carrying disease threats in themselves, interactions within these

guilds of microorganisms have been shown to have the potential to affect an individual's susceptibility to infection or the consequences of infection (Cox, 2001; Pedersen & Fenton, 2007).

1.1 *Eimeria* species and coccidiosis

Apicomplexan protozoan parasites belonging to the genus *Eimeria* cause different diseases in humans, farm animals and pets, thus are parasites of considerable medical and economic importance (Müller & Hemphill, 2013). Coccidiosis is the generic name given to disease caused by *Eimeria* species. However, the economic importance of *Eimeria* species is not just due to the impact of coccidiosis but also because they commonly persist as chronic, sub-clinical infections that compromise livestock growth rates (Chartier & Paraud, 2012; Souza *et al.*, 2015). The high mortality and morbidity associated with coccidiosis present a serious threat challenge to animal production. *Eimeria* infections cause a major problem to the poultry industry (Bera *et al.*, 2010; Dalloul & Lillehoj, 2006), with coccidiosis being one of the most frequently reported diseases worldwide and being present wherever poultry are raised (Shirley *et al.*, 2007). The annual cost of coccidiosis globally is estimated to be approximately $2.4 billion, of which 76% is caused by clinical or sub-clinical coccidiosis and 24% by drug-related costs (Shirley *et al.*, 2007). Seven *Eimeria* species are commonly encountered in poultry, of which three are considered the most pathogenic, *Eimeria brunetti*, *Eimeria necatrix* and *Eimeria tenella* (Chengat *et al.,* 2017). In cattle (including buffaloes), the economic cost of *Eimeria* infections has been calculated to be approximately $400 million worldwide (Matjila & Penzhorn, 2002), again split between the loss to productivity and the cost of treatment/prevention (Fitzgerald, 1980). It is not uncommon for the prevalence of infection to reach 100% in calves, with the associated severe diarrhoea threatening death (Cornelissen *et al.*, 1995). Twelve *Eimeria* species have been detected in cattle in Europe, of which three are considered particularly pathogenic, *Eimeria alabamensis, Eimeria bovis* and *Eimeria zuernii* (Daugschies & Najdrowski, 2005; Taylor & Catchpole, 1994). In goats, *Eimeria* infections are also common and lead to economic losses due to a reduction in animal weight gain, diarrhoea, dysentery and anaemia (Chhabra & Pandey, 1992). As many as 17 *Eimeria* species have been reported in goats, of which nine have been associated with clinical disease and one, *Eimeria arloingi*, is considered the most pathogenic (Taylor *et al.*, 2007).

1.1.1 The epidemiology of *Eimeria* infections in sheep

Eimeria infections are highly prevalent in sheep worldwide (Dittmar *et al.*, 2010; Pfister & Flury, 1985). In South Australia, 80% of sheep surveyed were infected with *Eimeria* (O'Callaghan *et al.*, 1987), whereas in New Guinea, 89% of examined sheep were infected (O'Callaghan *et al.*, 1987; Varghese & Yayabu, 1985). Similarly, a survey in Kars province in Turkey reported an infection prevalence of 90% in adult sheep (Arslan *et al.*, 1999), and in China, a study conducted in 8 localities encountered *Eimeria* oocysts in between 85%-100% of sheep (Wang *et al.*, 2010).

In young animals, with little previous exposure to *Eimeria*, infections are thought to be particularly intense, as demonstrated by higher concentrations of oocysts in faeces compared to adult sheep (Catchpole *et al.*, 1993; Gregory *et al.*, 1980a; O'Callaghan *et al.*, 1987). Studies have shown that lambs can shed as many as 10^5 *Eimeria* oocysts per gram of faeces even in the absence of clinical symptoms (Gregory & Catchpole, 1987a; Kaya, 2004).

There are 15 *Eimeria* species associated with sheep worldwide (Kaufmann, 2013; Khan *et al.*, 2011; Reginsson & Richter, 1997; Saratsis *et al.*, 2011); *Eimeria ahsata, Eimeria granulosa, Eimeria gilruthi, Eimeria intricate, Eimeria gonzalezi, Eimeria marsica, Eimeria punctate, Eimeria dali, Eimeria pallida, Eimeria weybridgensis, Eimeria parva, Eimeria bakuensis, Eimeria faurei, Eimeria ovinoidalis* and *Eimeria crandallis*. All these species are thought to be host specific (Fayer, 1980). The species most frequently associated with coccidiosis are *E. ovinoidalis, E. bakuensis, E. parva, E. ahsata* and *E. crandallis* (Skirnisson, 2007), while *E. intricate* and *E. faurei* are thought to be moderately pathogenic in sheep (Levine, 1985; O'Callaghan *et al.*, 1987; Vercruysse, 1982).

The relative frequencies of which these species are encountered varies. In general, *E. crandallis* and *E. parva* appear to be the most dominant species (Gul & Deger, 2002; Kaya, 2004), although in Europe *E. weybridgensis, E. ovinoidalis* and *E. bakuensi* are also common (Berriatua *et al.*, 1994; Gauly *et al.*, 2001; Reeg *et al.*, 2005b; Taylor & Catchpole, 1994), in Australia and Brazil *E. ovinoidalis* appears to be equally common (Amarante & Barbosa, 1992; O'Callaghan *et al.*, 1987), and in South Africa *E. bakuensis* is also common (Bakunzi *et al.*, 2010). Importantly infections are not mutually exclusive; co-infections involving two or more *Eimeria* species are the normal (Arslan *et al.*, 1999; Gregory *et al.*, 1980b).

Various factors have been implicated as being determinants of *Eimeria* infection epidemiology. Housed lambs tend to be at higher risk of infection, particularly those reared

on straw (Berriatua *et al.*, 1994; Platzer *et al.*, 2005), probably because of the higher temperature and humidity within lambing sheds that favour oocyst sporulation and survival in the environment (Berriatua *et al.*, 1994; Platzer *et al.*, 2005). Furthermore, the intensity of rearing employed also appears to be important; several studies have shown sheep raised under extensive conditions have a significantly higher prevalence of *Eimeria* infections than sheep raised under less intense conditions (de Souza *et al.*, 2015), with one study reporting prevalences of 93%, 78% and up to 59% in intensive, semi-intensive and free range systems respectively (de Souza *et al.*, 2015). In agreement with these data, the prevalence of infection appears to be generally lower in sheep maintained in the UK on upland hill farms compared to lowland farms, on which sheep are more confined and thus at a much higher stocking density (Taylor, 1995). Moreover, sheep breed is thought to have an influence on the susceptibility of the animal to infection, with a higher prevalence of *Eimeria* infections reported in exotic breeds compared to local breeds (Li *et al.*, 2001; Reshi & Tak, 2014). Climatic parameters like temperature, humidity and rainfall may also have a marked influence on the prevalence and intensity of coccidiosis in different locations (Ibrahim & Afsa, 2013). An increased prevalence of infection is associated with wet, humid and hot weather (Khan *et al.*, 2011).

The shedding of oocysts varies depending on the age of a sheep and its physiological status. Adult females shed increased numbers of oocysts during the periparturient period (Rommel, 2000). The prevalence of disease and the intensity of oocyst excretion increases during and reaches a peak when females are weaning their lambs, then the intensity of excretion decreases but does not altogether cease (Reeg *et al.*, 2005b). However, in general, adults shed fewer oocysts than immature animals, an observation attributed to the suppression of *Eimeria* infection due to acquired immunity over a period of time (Maingi & Munyua, 1994). Nonetheless, this acquired immunity does not lead to a cessation in oocyst shedding, which persists throughout an animal's life (Catchpole *et al.*, 1993).

The high intensity of *Eimeria* infections in lambs is thought to reflect low immunity and low resistance (Kanyari, 1988; Maingi & Munyua, 1994). In lambs, the intensity of *Eimeria* oocysts peaks around the period of weaning, and then decreases (Reeg *et al.*, 2005b). Lambs acquire infection immediately after birth by contact either with oocysts being actively excreted by co-housed lambs or their mothers or with oocysts surviving in an environment previously occupied by shedding animals (Platzer *et al.*, 2005). Young animals (4 to 8 weeks of age) are also considered to be more likely to develop coccidiosis (Gregory *et al.*, 1980a),

particularly when infected with *E. crandallis* or *E. ovinoidalis* (Gregory & Catchpole, 1989). Work exploring the implications of *Eimeria* to the emergence of twin births as common practice in UK sheep farming has been published (Taylor & Catchpole, 1994).

The shedding of high numbers of oocysts by sheep is not considered as a symptom of coccidiosis. Sheep may not show any clinical symptoms even if they are heavily infected with non-pathogenic or low pathogenic species (Nurzaty *et al.*, 2014). The occurrence of disease is dependent on the pathogenic potential of the infecting *Eimeria* species (Berriatua *et al.*, 1994), the dose of oocysts acquired and the innate susceptibility of the individual (Skirnisson, 2007). Thus, both healthy and diseased animals produce *Eimeria* oocysts in their faeces (Craig, 1986). The continuous shedding of *Eimeria* oocysts by animals with chronic subclinical infections represents a continuous source of infection to the other sheep (Kaya *et al.*, 2007; Platzer *et al.*, 2005).

The severity of coccidiosis is affected by the presence of other co-infecting parasites like bacteria, viruses, and helminths (Taylor, 1995). Infection by *Eimeria* has been shown to provoke changes in microflora (Mohammed *et al.*, 2000) as well as predisposing secondary bacterial and parasitic infections (Taylor *et al.*, 1973; Yang *et al.*, 2014b). In addition, coccidiosis results in staining of the area around the perineum and hind legs with faecal material, increasing the chances of fly strike (Andrews, 2013).

1.1.2 The life cycle of *Eimeria*

The life cycle of *Eimeria* parasites consists of two phases, the endogenous phase and the exogenous phase (Figure 1). This life cycle requires only one host. In the endogenous phase, which consists of asexual and sexual multiplication, the sporulated oocysts are ingested either through contaminated water or food and enter the digestive tract. Here, the oocyst wall is weakened by the digestive enzymes leading to release of active sporozoites (Fitzgerald, 1980). *Eimeria* species have the ability to invade and develop within epithelial cells of the intestine (Andrews, 2013). The sporozoites penetrate the epithelial cell and become schizonts. Many nuclei will form inside each schizont by asexual multiplication leading to the development of merozoites from each nucleus. This multiplication will repeat more than one time leading to the asexual generation (Figure 1). At that stage, the animal may show clinical signs even before shedding of the oocyst in the faeces (Bowman, 2014). Merozoites will penetrate a new host cell to form gametogony. Most of the gametogony either become macrogametes which represent the female in sexual phase or microgametes that represent

the male. The oocysts will form after fertilization of macrogametes and after the formation of the oocyst wall, are released into the gut lumen (Fayer, 1980).

In the exogenous phase, the oocysts are shed in the faeces of an infected animal. The non-sporulated oocysts that are passed in faces need around 2-7 days to sporulate. Each contains four sporocysts, inside each of which there are two sporozoites (Fayer, 1980) (Figure 1). Oocyst sporulation time depends on *Eimeria* species and environmental conditions such as temperature, moisture and oxygen. For example, 10% of oxygen is needed in order to sporulate the oocyst in normal rate (Marquardt *et al.*, 1960). While in the complete absence of oxygen, no development takes place. If the weather is cold the unsporulated oocysts may need several weeks to sporulate (Wright & Coop, 2007). The passed oocysts are able to survive for months or even for a year in the environment (Foreyt, 1990). Theoretically, it was estimated that ingestion of single oocyst may lead to producing 30 million oocysts in animal faecal excretion (Gregory *et al.*, 1987).

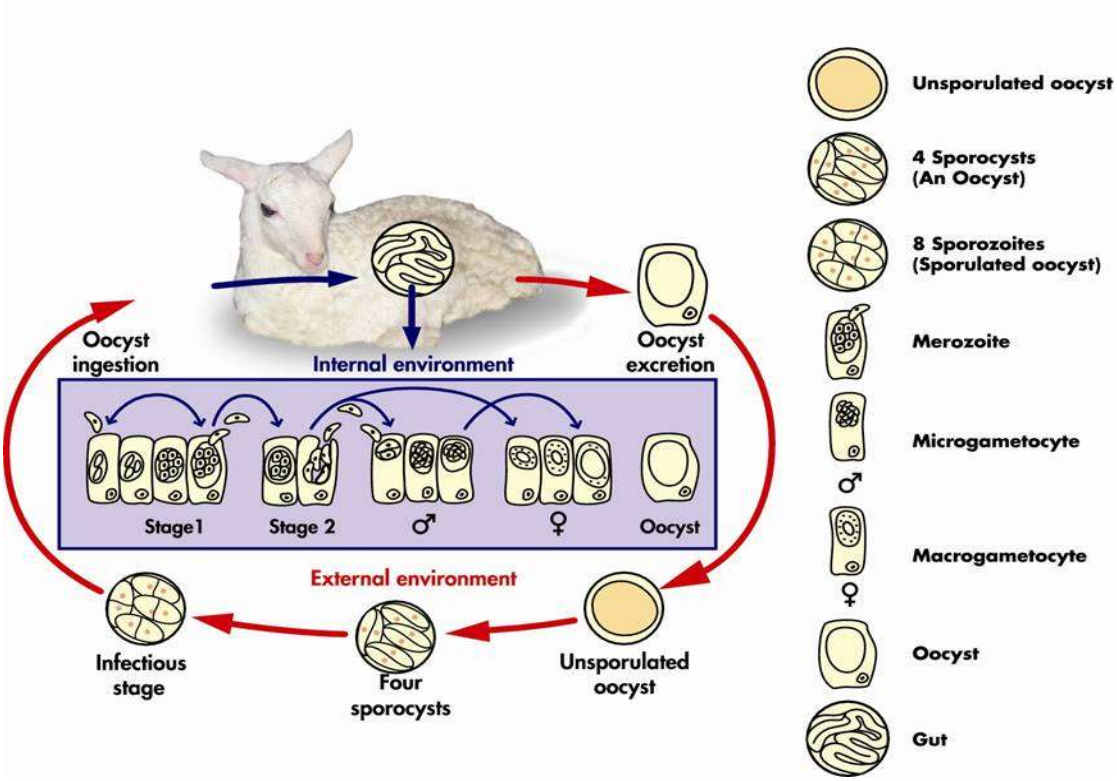


Figure 1: Life cycle of *Eimeria* parasite (BAHC, 2008).

1.1.3 Clinical signs and diagnosis of coccidiosis

The first clinical signs that appear during the early stage of acute infection are listlessness and loss of appetite. Diarrhoea is considered the most common clinical sign of infection,

together with weakness, weight loss, anemia, fever and a roughed coat (More *et al.*, 2015; Taylor *et al.*, 2007; Wang *et al.*, 1990). The animal may still show diarrheal signs even if there is a reduction in the oocyst output to a low level and that is because of the long time needed for intestinal recovery (Gregory & Catchpole, 1987b). The area around the perineum and hind legs become stained with fecal material. Proliferative enteritis is considered the most common pathology in infected animals (Tafti & Mansourian, 2008). Profuse watery diarrhea containing streaks of blood will occur in animals suffering from severe coccidiosis (Taylor & Catchpole, 1994). After recovery, the growth rate of an animal may be impaired and this may lead to low milk and meat production (Wang *et al.*, 2010).

Different factors determine the pathogenesis of the disease such as the immune status of the animal, age, the dose of ingested oocysts, *Eimeria* species and location of affected tissue (Kusiluka & Kambarage, 1996). Post-mortem examination of large intestine and cecum shows thickening of their mucosal surfaces and hemorrhage. White dots are frequently observed in the small intestine of the infected animal (Andrews, 2013). Histological examination reveals mucosal scrapes with a number of gamonts and oocysts, decrease in the size of intestinal villous and reduction in the size of the intestinal mucosa (Andrews, 2013).

The clinical manifestations of coccidiosis result from the ability of *Eimeria* to destroy the intestinal epithelial cells of the host leading to poor absorption of the nutrition, electrolyte loss and anemia (Engidaw *et al.*, 2015).

The diagnosis of coccidiosis is based on different parameters including the occurrence of clinical manifestations, the history of the flock/herd and identification of the oocyst number and species of *Eimeria*. The occurrence of sudden mortality in young animals should also suggest coccidiosis especially around the weaning period (Chartier & Paraud, 2012). There is an association between excretion of a high number of oocysts and occurrence of clinical coccidiosis. In small ruminants, 50000- 100000 oocysts per gram of faeces has been cited as being the threshold above which clinical coccidiosis occurs (Chartier & Paraud, 2012). However, the relative abundance of pathogenic *Eimeria* species among the shed oocysts needs to be considered (Chartier & Paraud, 2012).

1.1.4 Immunity against *Eimeria*

Immunity against coccidiosis develops after repeated exposure to *Eimeria* oocysts and protects the animal against clinical disease (Taylor *et al.*, 2011). However immune animals continue to be infected, remain infectious and shed oocysts (Catchpole *et al.*, 1993). During

the first few weeks of a lamb's life, it is offered passive immunity via its mother's colostrum, but subsequently it becomes highly susceptible to the disease before it is able to form an immunity of its own. It is during this non-immune window that coccidiosis is most threatening, with death rates of 20% reported in some instances (Kommuru *et al.*, 2014). The mechanism by which the immune response prevents coccidiosis developing in infected individuals is not well understood but is primarily mediated by lymphocytes (T-cells). Innate immune responses represented by natural killer cells, macrophages and neutrophils may control the disease in the early stage (Ovington *et al.*, 1995). Cells that play a role in combatting *Eimeria* infections are mainly polymorphonuclear neutrophils (PMNs) and macrophages (Hermosilla *et al.*, 2006). In one study, the interaction between *Eimeria* and PMNs was shown to be mediated by the expression of adhesion molecules on the surface of infected endothelial cells (Hermosilla *et al.*, 2006). Another study reported that in a model comprising of endothelial cells infected with *Eimeria falciformis*, sporozoites were effectively killed by introduced PMNs (Bekhti *et al.*, 1992). Similarly, the accumulation of PMNs in the early stages of *Eimeria* schizont formation has been noted in rodent infection models (Blagburn & Todd, 1984; Schito & Barta, 1997).

1.1.5 Treatment and control of *Eimeria* infections

Good husbandry and the use of anticoccidial drugs are the most widely used practices for the prevention of coccidiosis. Anticoccidial drugs may interfere with different stages of coccidia life cycle (sexual and asexual stages). One of the most common types of drug used against first and second stage schizonts is sulphonamide. These have a coccidiostatic effect at a low dose and are coccidiocidal at high dose (Yolande, 2005).

Three drugs are licensed for use in sheep in the United Kingdom. Firstly, decoquinate (6-ethyl-(decycloxy)-7-ethoxy-4-hydroxy-3-quinolinecarboxylate) is used as a premix powder, but cannot be used on sheep producing milk for human consumption (Andrews, 2013). Decoquinate is coccidiostatic, affecting *Eimeria* sporozoites (Taylor, 2012) by arresting the release of *Eimeria* sporozoites from sporulated oocyst at day one of parasite life cycle. It also prevents parasites forming merozoites by stalling meront development (Fitzgerald & Mansfield, 1986). Moreover, the treatment can act on gametocyte stage and reduce their damage to intestinal epithelial cells (Taylor & Bartram, 2012). Studies have demonstrated that the administration of decoquinate to sheep improves weight-gain and milk production (Morand-Fehr, 2005) and increases growth rate in lambs (Mage *et al.*, 1995). Secondly, diclazuril (benzeneacetonitrile derivative) is used as an oral suspension. the component can

be used as anticoccidial to control the disease, clinical outbreaks and reduce production losses (Le Sueur *et al.*, 2009; Taylor *et al.*, 2011). The treatment is used as metaphylactic medication that reduces oocyst shedding and improves the growth rate of targeted animals (Platzer *et al.*, 2005; Taylor *et al.*, 2003). The oral suspension use in sheep is available commercially in concentration of 0.25%. while in poultry, 0.5% premix with in the feed at a concentration of 1 mg/kg (Croubels *et al.*, 2002). On calve the diclazuril used as anticoccidaial compound that reduces the oocysts shedding significantly and improve the animals growth rate (Daugschies *et al.*, 2007). Finally, toltrazuril (1-[3-methyl-4- (4'-trifluoromethylthiophenoxy)-phenyl]-3 methyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione), which is used as oral suspension with a single dose. Toltrazuril is either used before the occurrence of *Eimeria* species as metaphylactic treatment or after oocyst shedding commences as therapeutic treatment (Mundt *et al.*, 2009). Toltazuril is coccidiocidal and is directed toward the sexual and asexual stages of the parasite's life cycle. Like decoquinate, toltrazuril should not be administered to sheep used to produce milk for human consumption (Andrews, 2013).

The specific livestock management system adopted by a farmer is recognized as being influential in the outcome of *Eimeria* infection (Gregory, 1990). Studies have shown that coccidiosis increases in the young lambs as a result of increased stocking densities and reduction in pasture availability, and that coccidiosis can be effectively controlled by keeping lambs indoors, in clean and dry pens (Andrews, 2013). However, to prevent the environmental accumulation of sporulated oocysts, lamb bedding should be changed on a regular basis and faecal contamination should be regularly removed from drinking troughs and feeding bowls (Engidaw *et al.*, 2015). Another key husbandry control measure is keeping lambs away from heavily contaminated pasture (Taylor & Catchpole, 1994).

1.1.6 Identification of *Eimeria* species

In general, the parasite is identified on the basis of oocyst morphology (Eckert *et al.*, 1995; Saratsis *et al.*, 2012) (Figure 2). Different parameters can be used to delineate *Eimeria* species, such as (i) the dimensions of oocysts and sporozoites, (ii) presence or absence of a micropyle cap on the oocysts, (iii) the shape of the oocysts (ovoid, urn-shaped, ellipsoidal or broadly ellipsoidal and spherical or subspherical), (iv) sporozoites lying head to head or head to tail, (v) oocyst wall colour (colourless, pale yellow, yellowish brown or brown), (vi) oocyst surface (smooth or granular), and (vii) presence or absence of polar granules (Eckert *et al.*, 1995; Reginsson & Richter, 1997). Determination of the morphology and size of

sporulated oocysts has been the most widely used approach to delineating *Eimeria* species (Souza *et al.*, 2015).

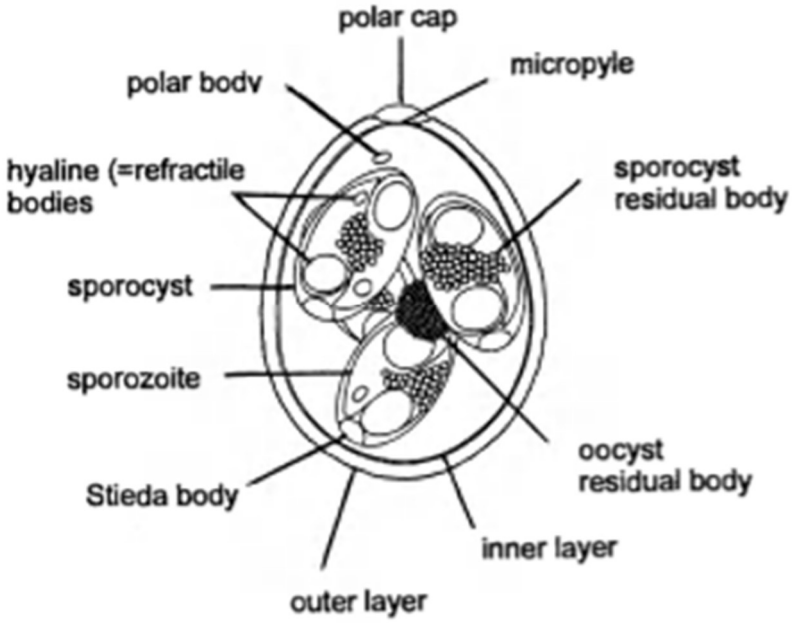


Figure 2: Schematic of the basic structure of an *Eimeria* oocyst. In this example, the oocyst contains four sporocysts, each containing two sporozoites (Eckert *et al.*, 1995).

Molecular methods have also been applied to the identification of *Eimeria* species. However, to date, only very few studies have described their use with sheep-associated *Eimeria* (Kaupke *et al.*, 2017; Yang *et al.*, 2014b). Most studies have focused on delineating *Eimeria* species in chickens (Godwin & Morgan, 2015; Moraes *et al.*, 2015; Prakashbabu *et al.*, 2017). Numerous genetic loci have been exploited in these molecular methods. Among the most common targets are the nuclear 18S rRNA-encoding gene (18S rDNA) (Miska *et al.*, 2010; Ruttkowski *et al.*, 2001) or the ribosomal internal transcribed spacer-1 (ITS-1) region (Khaier *et al.*, 2016; Kumar *et al.*, 2014; Lew *et al.*, 2003) or targeting the 5S rRNA repeat region (Blake *et al.*, 2006). However, other loci including mitochondrial genes such as cytochrome c oxidase subunit I-encoding gene (*mtCOI*) and a second internal transcript spacer region (ITS-2) have also been exploited (Woods *et al.*, 2000). More recently, approaches involving multiple loci have been developed (Ogedengbe *et al.*, 2015). Although comparative sequence analysis of 18S rDNA and ITS-1 PCR products are widely used, the sensitivity of these approaches has been questioned, especially for delineating closely-related *Eimeria* species, and alternatives have been proposed (El-Sherry *et al.*, 2013). One

of the most useful molecular assays currently available for detecting and differentiating chicken-associated *Eimeria* species is a realtime multiplex PCR assay (Fernandez *et al.*, 2003) and quantitative real-time PCR assays (Vrba *et al*., 2010) using sequence characterized amplified region (SCAR) markers. The SCAR markers are derived from randomly amplified polymorphic DNA (RAPD) fragments (Welsh & McClelland, 1990; Williams *et al.*, 1990). These markers are amplified using specific pairs of primers in order to differentiate between seven chicken-associated *Eimeria* species (Fernandez *et al.*, 2003).

1.2 *Campylobacter* species and campylobacteriosis

1.2.1 Microbiology of *Campylobacter* species

Campylobacters are curved or spiral shaped Gram-negative rods, ranging from 0.5 to 8 µm in length and 0.2 to 0.5 µm in wide (Penner, 1988). The bacterial cells may appear as S-shaped rods (Figure 3A) or "gull-wings" when short chains are formed by two or more cells (da Silva *et al.*, 2016). Moreover, the other forms of *Campylobacter jejuni* such as spherical or coccoid may occur in response to stressful conditions (Ikeda & Karlyshev, 2012) (Figure 3B). *Campylobacter* species are motile by a polar flagellum present at one or both ends of the bacterium (Alm & Guerry, 1993). *Campylobacter* species are fastidious bacteria that can only be grown in the laboratory using complex/rich media (Park, 2002) at incubation temperatures of between 35-42 °C (Inglis & Kalischuk, 2003; Khan *et al.*, 2013). Most species require microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) (Garénaux *et al.*, 2008) to grow in the laboratory, but some are aerobic (Epps *et al.*, 2013). *Campylobacter* species are non-fermenters of carbohydrates, producing energy from amino acid oxidation (Stanley & Jones, 2003).

Historically, it is believed that Theodore Escherich was the first to recognize *Campylobacter* species in 1886; he described vibrio-like spiral shaped bacteria in the stools of children with diarrhoea (Kist, 1986). Although organisms we know recognize as *Campylobacter* were first isolated as early as 1913 by McFadyean and Stockman from samples collected from aborted cattle and sheep (Kist, 1986; Skirrow, 2006), the genus itself did not come into existence until 1963 when Sebald and Veron proposed its creation (On, 2001). There are currently 34 species within the genus (LPSN, 2017). The genus of *Campylobacter* lies within the Class Epsilonproteobacteria and is closely related to the genera *Arcobacter, Sulfurospirillum, Helicobacter* and *Wolinella*.

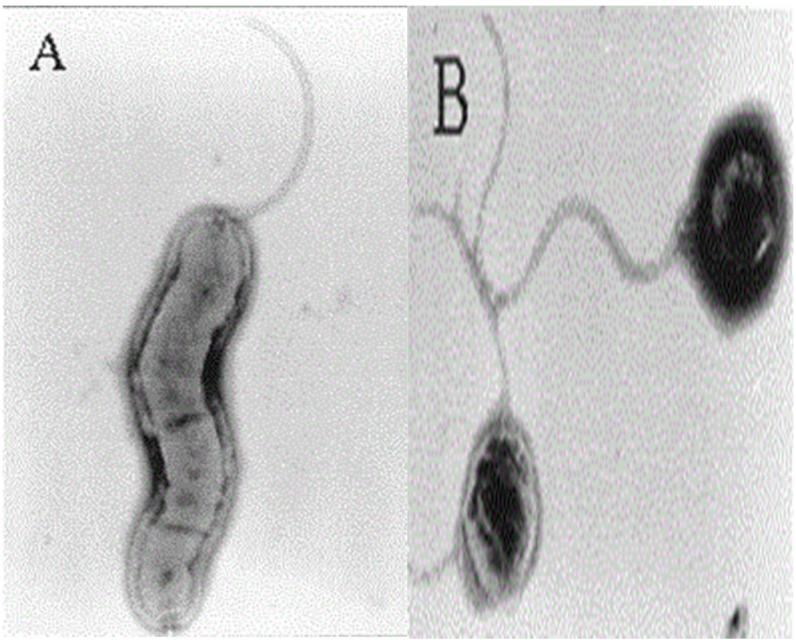


Figure 3: Transmission electron microscopic pictures of *C. jejuni*, [A], spiral form, and [B] coccoid form adapted from (Lázaro *et al.*, 1999).

1.2.2 Natural transmission of *Campylobacter* species

The substantial impact of the infectious pathogens from wildlife to the livestock and human health has become important in recent years (Billinis, 2013). That impact affects the health and productivity of livestock. Changes occurring in human populations, wildlife, livestock, and interference between wildlife and livestock leading to the emergence of diseases in livestock and human (Jones *et al.*, 2008). It is recorded that *C. jejuni* can be transmitted between wildlife, livestock and human (Weis *et al.*, 2016). Sharing of resources and habitat between domestic livestock and wild ungulates can play a particular role in disease transmission.

Campylobacter species are commonly found in the gastrointestinal tract of many mammals, birds, reptiles and other vertebrates without causing visible clinical symptoms of the disease. Thus, these animals are considered as natural reservoirs for the bacteria (Gilbert *et al.*, 2014; Tu *et al.*, 2004). Transmission between reservoir hosts is thought to be direct, via the faecal-oral route. However, some *Campylobacter* species, including *C. jejuni* and *Campylobacter coli* have also been encountered in natural and man-made aquatic environments such as lakes, streams, water troughs and water pipes, indicating that at least some strains of these species are able to persist outside vertebrate reservoir hosts system (Figure 4) (Costerton *et al.*, 1987; Costerton *et al.*, 1995; Gilbert *et al.*, 1993).

Numerous vertebrates have been implicated as reservoirs for *C. jejuni* and *C. coli* including poultry which is considered one of the major reservoirs colonized by *Campylobacter*, particularly *C. jejuni* and *C. coli* (Hald et al., 2000; Ridley et al., 2011). In the UK, the high prevalence of *Campylobacter* was detected in broiler chicken (Lawes *et al.*, 2012; Powell *et al.*, 2012). Other types of birds were also infected by *Campylobacter*, such as turkey, ducks, geese and wild birds (Sahin *et al.*, 2015). The prevalence of *Campylobacter* in chicken flock ranged from 2% to 100% depending on different parameters such as season of the year, regions, the age of flock and the type of production system (Fonseca *et al.*, 2016). It was reported that *C. jejuni* was the most dominant species in broiler at slaughter across the EU followed by *C. coli* (Hald, 2010). In North Europe countries, a clear seasonal pattern is recognized, in which the high rate was in summer (Barrios *et al.*, 2006; Patrick *et al.*, 2004). The epidemiological studies identified different sources that may play a role in the infection and maintain the *Campylobacter* in broiler chicken such as on farm puddles and farm surround (Bull *et al.*, 2006; Johnsen *et al.*, 2006), flies (Hald *et al.*, 2004) and the water system in the broiler house (Ogden *et al.*, 2007). The environmental contamination with *Campylobacter* regarding broiler house is un clear, which is either from or into the chicken house (Ridley *et al.*, 2011). An epidemiology study regarding broiler house found possible contamination from livestock such as cattle (Ellis-Iversen *et al.*, 2009) as well as the presence of pigs in the farm (Gregory *et al.*, 1997). In a study conducted in the UK suggested the presence of cattle near the broiler house increase the risk of infection in the flock (Ellis-Iversen *et al.*, 2009).

C. jejuni and *C. coli* infections in healthy sheep are common; (Garcia *et al.*, 2010; Jones *et al.*, 1999; Oporto *et al.*, 2007; Rosef *et al.*, 1983; Rotariu *et al.*, 2009; Schilling *et al.*, 2012; Sproston *et al.*, 2011; Stanley & Jones, 2003; Stanley *et al.*, 1998; Turkson *et al.*, 1988; Zweifel *et al.*, 2004). Survey of lambs at slaughter revealed a significant seasonal periodicity in intestinal colonisation rates by *Campylobacter* species (Stanley *et al.*, 1998), and this periodicity was also detected in a survey of *Campylobacter* shedding by sheep on pasture in the same region (North Lancashire, UK) (Jones *et al.*, 1999). In this survey, the highest shedding rates occurred in spring, coinciding with lambing, whereas the lowest shedding rates were detected when sheep were fed on hay or silage. Furthermore, there was a variation in shedding rate on different farms, located in different habitats (Jones *et al.*, 1999). This survey also specifically monitored shedding in ewe/lamb pairs, finding shedding of

Campylobacter began in new-born lambs by day three, and that, in birthing ewes, the amount of *Campylobacter* shed increased markedly after lambs were delivered (Jones *et al.*, 1999).

Other surveys of sheep in the UK and elsewhere have made similar seasonal observations. Sproston *et al.* (2011) surveyed sheep in Aberdeenshire, Scotland, between May and September, observing a significant decline in the proportion of sheep shedding *Campylobacter* and in concentration of shed *Campylobacter* during the period.

Campylobacter infections in cattle are also extremely common. Various researchers have isolated *Campylobacter* species from the faeces of healthy cattle e.g. (Atabay & Corry, 1998; Besser *et al.*, 2005; Garcia *et al.*, 1985; Giacoboni *et al.*, 1993; Grove-White *et al.*, 2010; Humphrey & Beckett, 1987; Kwan *et al.*, 2008; Nielsen, 2002; Rotariu *et al.*, 2009; Sproston *et al.*, 2011; Stanley *et al.*, 1998) and *Campylobacter* species have been recovered from cattle offal, beef and veal (Bolton *et al.*, 1985; Fricker & Park, 1989; Lammerding *et al.*, 1988; Stern *et al.*, 1985).

Campylobacter species have also been isolated from other livestock including pigs, which is recognized as a reservoir for the bacteria (Quintana-Hayashi & Thakur, 2012). The most frequently encountered species in pigs is *C. coli* (Gebreyes *et al.*, 2005; Thakur & Gebreyes, 2005). In a survey of pigs in two production systems (conventional and antimicrobial-free systems), it was confirmed that *C. coli* could persist in the environment as well as in pigs (Quintana-Hayashi & Thakur, 2012). The study confirmed the potential role of the environment as a source of livestock *Campylobacter*.

In addition to farmed animals, *Campylobacter* infections have been reported in various wildlife species. Particularly *C. jejuni* which is identified in a wide range of birds and wild animals (Dingle *et al.*, 2002b; Griekspoor *et al.*, 2013; Sheppard *et al.*, 2009; Waldenstrom *et al.*, 2002). *Campylobacter* infections have also been detected in red and roe deer in Poland (Koronkiewicz, 2004). In the UK, there are six species of deer. Two of them are considered as native (red deer and roe deer) whereas the others have been introduced (chinese water deer, sika deer, fallow deer and reeves muntjac deer) (Ratcliffe, 1987; Ward, 2005). *C. jejuni* has also been isolated from other wildlife species including badgers, squirrels, hares, foxes and woodland rodents (French *et al.*, 2009; Petersen *et al.*, 2001).

Invertebrates may also have a role in the natural transmission of *C. jejuni* and *C. coli*. (Rosef & Kapperud, 1983; Sproston *et al.*, 2010). A study conducted in the UK showed the role of flies and slugs in spreading *Campylobacter,* in which flies may contaminate surfaces and slug can contaminate fruits or crops (Sproston *et al.*, 2010).

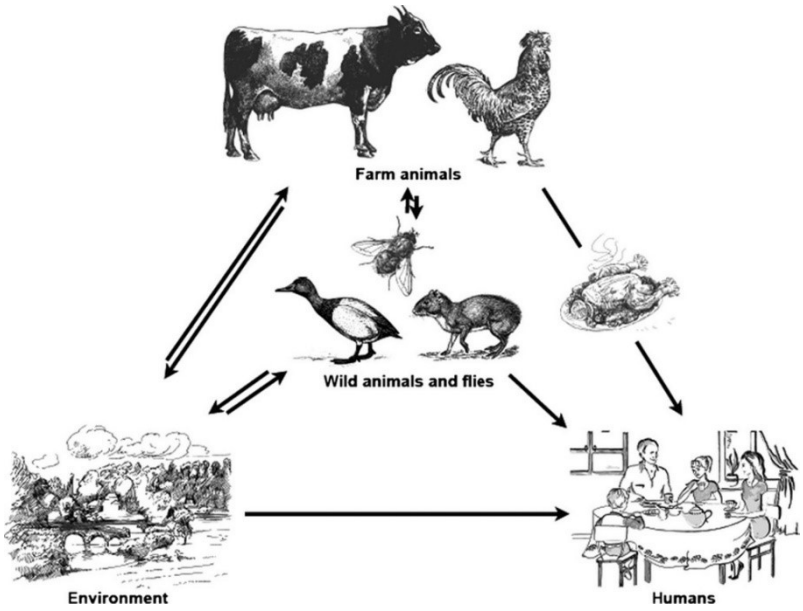


Figure 4: Routes of transmission for *Campylobacter jejuni* complex transmission route between environment, farm animals and wild animals through to humans (Bronowski *et al.*, 2014).

Studies have demonstrated that *Campylobacter* can survive in faeces of livestock and poultry for as long as 14 days in a range of temperatures between 10°C and 20°C and in the presence of heavy rain (Ahmed *et al.*, 2013; Moriarty *et al.*, 2011) and in farm slurry (Stanley *et al.*, 1998). However, it is known that some campylobacters can also persist in the farm environment and beyond, including in natural water sources (Kemp *et al.*, 2005) and soil (Jensen *et al.*, 2006).

The application of molecular epidemiological tools such as pulsed-field gel electrophoresis and, more importantly multi-locus sequence typing (MLST), and very recently, whole genome data (Thépault *et al.*, 2017); Yahara *et al.* (2017) has revolutionised our understanding of the transmission cycle of *C. jejuni* and *C. coli* as well as the accurate attribution of the relative importance of different putative sources of human infections (see 2.1.3). MLST is based on the construction and comparison of fingerprints (sequence types, STs) generated on the basis of sequence variation in PCR products derived from

approximately 500 base-pair fragments of multiple (typically seven) housekeeping genes lying in distinct loci across the bacterial genome (Maiden *et al.*, 1998). MLST was first applied to *C. jejuni* and *C. coli* in 2001 (Dingle *et al.*, 2001) and targeted fragments of *aspA* (encoding aspartase A), *glnA* (encoding glutamine synthetase), *gltA* (encoding citrate synthase), *glyA* (encoding serine hydroxymethyltransferase), *pgm* (encoding phosphoglucomutase), *tkt* (encoding transketolase) and *uncA* (encoding ATP synthase a subunit) (Dingle *et al.*, 2001). To date, almost 8,000 *C. jejuni* STs have been recognized (Harvala *et al.*, 2016).

Miller *et al.* (2005) developed an expanded MLST scheme to identify other species of *Campylobacter*, such as *C. coli* (Miller *et al.*, 2005). According to the MLST analysis of *Campylobacter* species population, some identified STs can be encountered in many different reservoirs and/or environmental niches, for example ST-21. However, other STs appear to have a more restricted distribution, for example ST- 179, which belongs to specific environmental isolate such as sand on bathing beaches in the UK (Bolton *et al.*, 1999). Other examples are ST-42 and ST-61 complexes which belong to isolate from human disease, cattle, and sheep; the ST-45 and ST-257 complexes which include isolates from human disease and isolates from poultry (Dingle *et al.*, 2002b). The ST-61 complex was recorded to be predominant among sheep and cattle isolates (Colles *et al.*, 2003).

1.2.3 Epidemiology of human campylobacteriosis

Among the zoonotic *Campylobacter* species, *C. jejuni* and, to a lesser extent, *Campylobacter coli* present by far the greatest public health importance (CDC, 2005; Mohan, 2015). These two species are the most common cause of foodborne gastroenteritis in many industrialised and developing countries (Guerrant *et al.*, 1990; Lazou *et al.*, 2014; Moore *et al.*, 2005). Campylobacteriosis is the most common enteric infection in the UK (Ketley, 1997). Estimates of the public health burden of campylobacteriosis suggest that the illness costs the economy £500 million pounds a year in the UK and $8 billion dollars a year in the USA (Sheppard *et al.*, 2009). Recent estimation showed *Campylobacter* infection lead to 100 cases of death annually, lose about £900 million per annum of the UK economy and cause more than 280,000 cases of food poisoning disease (Romero *et al.*, 2016).

Patients of all age groups can be infected with *C. jejuni* and *C. coli*. However, infections in toddlers and young adults are more prevalent than other age groups (Nielsen *et al.*, 2013). Humans acquire infections from a variety of sources including consumption of undercooked

contaminated meat or untreated contaminated milk, direct contact with infected animals, or through environmental exposure (Friedman, 2000) (Figure 5). People who live in rural areas have a greater risk of *Campylobacter* infection, possibly due to more frequent and direct contact with livestock (Devane *et al.*, 2005; Ethelberg *et al.*, 2005). The incidence of *Campylobacter* infection in young children is seen to be source related. The majority of recorded cases in urban areas have chicken-associated genotypes compared to those living in rural areas which have ruminant and wild-bird attributed genotypes (Strachan *et al.*, 2009). The major risk factors for campylobacteriosis in humans according to the case control studies and metaanalysis is, inproper handling and consumption of chicken meat (Domingues *et al.*, 2012; Gras *et al.*, 2012). Many studies have found that 50-70% of campylobacteriosis occurs as a result of poultry meat consumption (Adak *et al.*, 1995; Deming *et al.*, 1987; Harris *et al.*, 1986). Other studies show that in Europe about 30% of human campylobacteriosis cases results from consumption or preparation of poultry meat (Wagenaar *et al.*, 2013). Moreover, consumption of unpasteurized dairy product and daily contact with chicken or hens (Studahl & Andersson, 2000), and consumption of raw vegetables and fruit may lead to campylobacteriosis (Fullerton *et al.*, 2007; Verhoeff-Bakkenes *et al.*, 2011), in addition to untreated drinking water (Friedman *et al.*, 2004; Jacobs-Reitsma *et al.*, 2008; Pebody *et al.*, 1997). Waterborne campylobacteriosis may occur due to recent fecal contamination of water source by waterfowl and runoff of animal farm due to fecal material present (Hellein *et al.*, 2011).

A seasonal pattern of campylobacteriosis was reported in the age group 1-4 years, in which the incidence of the disease is temperature related. Moreover, spring peaks of incidence may occur in the children age less than one year (Louis *et al.*, 2005). The peak incidence of campylobacteriosis was observed to be in spring in England and Wales, which is in line with the increase of temperature which, in turn, is associated with agriculture activities (Louis *et al.*, 2005).

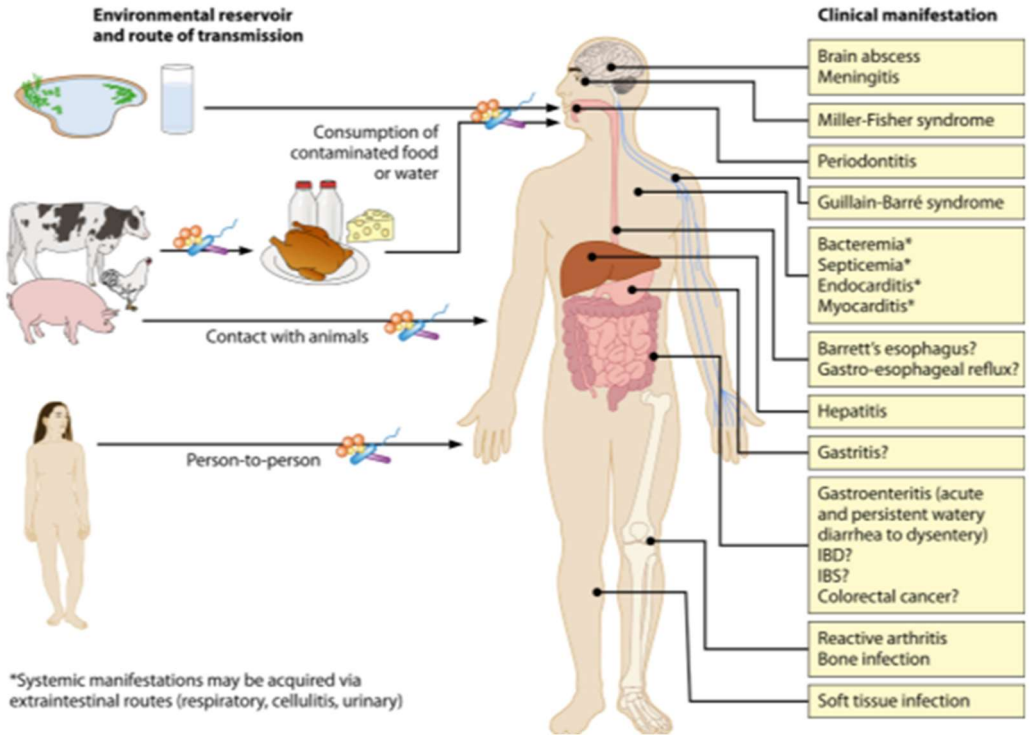


Figure 5: The epidemiology of *Campylobacter* infections and source of contamination, adapted from (Kaakoush *et al.*, 2015).

Molecular epidemiological studies have been used to explore the relative importance of these sources, and have concluded that the most common route of human infection is via contaminated poultry meat (e.g. Sheppard *et al*., 2009) but other sources, particularly meat from other farmed animals, are also important and should not be ignored (Thépault *et al.*, 2017).

The application of MLST helps in identifying *Campylobacter* infection source by exploiting the occurrence of specific *Campylobacter* strains in different reservoir (McCarthy *et al.*, 2007). The application of technique on samples from different sources revealed a significant correlation between *C. jejuni* isolated from human and different other sources in the term of (STs). For example, *C. jejuni* ST-21 and ST-45 have been isolated from human and different sources including chicken, wild birds, livestock, milk and environmental sources (Colles *et al.*, 2003; Dingle *et al.*, 2001).

In humans, the burden of campylobacteriosis caused by *C. jejuni* is much higher compared to other *Campylobacter* species, in which 90% of cases are associated with *C. jejuni* as showed in a study conducted in 2012 in the United Kingdom (Cody *et al.*, 2015). Source correlation analyses in the UK has shown that the majority of campylobacteriosis is caused

by the consumption of contaminated poultry meat (Gras *et al.*, 2012; Kittl *et al.*, 2013; Levesque *et al.*, 2013). In a study conducted by Wilson *et al.* (2008) in Lancashire, England revealed that most cases of human *Campylobacter* infections can be attributed to *C. jejuni* in chicken and cattle.

Although there has been a large amount work exploring the epidemiology of *C. jejuni* infections, far less progress has been made clarifying the determinants of *C. coli* infection, despite its contributing up to 10% of human *Campylobacter* infections. Nonetheless, the findings of a handful of *C. coli*-focused epidemiological studies have been published. In Scotland, a case-control study found an increased risk of *C. coli* infection in people older than 19 years, and during the summer months, while residing in an urban area decreased the risk (Roux *et al.*, 2013). A case-case epidemiological study that compared *C. coli* and *C. jejuni* cases showed the same trends (Roux *et al.*, 2013).

In terms of attributing sources of human infection, MLST has indicated that sheep and chicken *C. coli* sequence types (STs) are most frequently found in humans whilst those from cattle and pigs were rarer (Roux *et al.*, 2013). Combination of these data with those generated in the epidemiological surveys discussed above suggested 40% of cases of *C. coli* campylobacteriosis was acquired from chicken, and 54% of cases being acquired from ruminants (Roux *et al.*, 2013). A subsequent *C. coli* source attribution study carried out in New Zealand also identified ruminants and poultry as the main infection sources (Nohra *et al.*, 2016). The results of these two studies suggest that, unlike the situation with *C. jejuni*, ruminant sources might have a greater relative contribution to human *C. coli* infection burden than poultry, thereby underlining differences between the aetiology of human *C. coli* and *C. jejuni* infections.

Other *Campylobacter* species, including *Campylobacter upsaliensis, Campylobacter concisus, Campylobacter helveticus, Campylobacter lari,* and *Campylobacter fetus* have also been implicated in zoonoses (Fouts *et al.*, 2005) but their medical relevance is vastly overshadowed by that of *C. jejuni* and *C. coli*.

1.2.4 Epidemiology of campylobacteriosis in sheep and other livestock

Although, as detailed above, sheep serve as an asymptomatic reservoir for *Campylobacter* species, occasionally colonisation can progress to systemic infection and overt disease. *Campylobacter* species represent the third most common cause of infectious abortion in the United Kingdom and has increased in relative importance in recent years (AHVLA, 2017), and are equally important elsewhere (Kirkbride, 1993; Van den Brom *et al.*, 2012). The two species most frequently associated with abortion are *Campylobacter fetus* subspecies *fetus* and (increasingly recognized) *C. jejuni* (Sahin *et al.*, 2008). Abortion "storms" can occur, with, typically 20% of ewes being affected, although, as the pathogen is highly contagious, as many as 90% of the herd can succumb (Skirrow, 1994). Once an abortion storm starts, healthy ewes can be exposed to high levels of *Campylobacter* organisms through contact with the aborted foetus, placenta, and uterine discharges (Sahin *et al.*, 2008). Different STs of *C. fetus* was recognized worldwide from different hosts such as (ST-2, ST-3, ST-6, ST13, ST-14 ST-4 and ST-9) (Van Bergen *et al.*, 2005).

Livestock may be infected through many sources such as pasture contaminated with faeces of infected animals (including livestock and wildlife) or sharing the source of drinking water with infected animals. The high density of *Campylobacter* in stream water and pasture represents important source of infection to the grazing sheep, as confirmed by the regular presence (30%) of bacteria in sheep rumen samples at abattoir, indicating continuous ingestion of *Campylobacter* (Stanley *et al*., 1998). Stress factors such as lambing, waning in addition to moving of flock to new pastures increase the rate of *Campylobacter* shedding (Jones *et al.*, 1999). Lambs are born free of *Campylobacter*, indicating they acquire infection horizontally not vertically (Jones *et al.*, 1999). The husbandry practices by the farmer may affect the risk of *Campylobacter* infection to the herd (Garcia *et al.*, 1985; Jones, 1999), for example the spreading of untreated slurry during the winter on the farm land that may lead to infection of animals subsequently feeding on the contaminated pasture.

Although poultry and farm animals are the main reservoirs of *Campylobacter*, the bacteria has been also detected in different wildlife species (Wilson *et al.*, 2008). A few studies found that wild birds may play a minor role in infection of the cattle with pathogenic *C. jejuni* (French *et al.,* 2005; Kwan *et al.,* 2008; Sippy *et al.,* 2012). Grazing of animals on pasture contaminated by faecal material from a wild birds that visit the farm may introduce a new genotype to the herds or flocks (Stanley & Jones, 2003). The presence of wildlife may also contribute to the transmission of livestock-associated campylobacters; the same STs (such

as ST-61 and ST- 618) have been isolated from rabbits and cattle (French *et al*., 2005), and its been suggested that brown rats and house mice may also contribute to on-farm transmission of *Campylobacter* infections (Meerburg *et al*., 2006). A study conducted by Humphrey and Beckett (1987) showed that water sources may play a role in the transmission of *Campylobacter* infection. The study showed that herds drinking from river water shed *Campylobacter,* whereas those drinking from tap water were infection free.

1.2.5 Medical consequences of *Campylobacter* infection

The major symptoms of campylobacteriosis in humans are fever, abdominal pain, vomiting, diarrhoea, headache, muscle and joint pain (Black *et al.*, 1988; Bless *et al.*, 2014; Graves, 2013). The clinical signs appear after an incubation period between 1-10 days, with most of patients starting to show clinical symptoms by day four. The symptoms disappear within a week of occurring and in most cases the disease is self-limiting (Moore *et al.*, 2005). Moreover, the symptoms due to infection with *C. jejuni* differ depending on many factors such as infectious dose, the age of the patient, immunity, virulence of isolates and a history of infection. The infectious dose of the pathogen needed to induce the disease is recorded to be as few as 800 bacterial cells (Gharst *et al.*, 2013; Humphrey *et al.*, 2007; Salim *et al.*, 2014). Occasionally, more profound medical complications may occur due to infection with *C. jejuni* or other species of *Campylobacter*, those complications are divided into intra-intestinal sequelae and extraintestinal sequelae (Figure 5). Irritable bowel syndrome (IBS) is a common gastroenterological dysfunction that occurs as a post-infection complication of several bacterial pathogen infections including *Campylobacter*. The main clinical sign is abdominal pain which is related to both changes in the intestinal motility of patient and consistency of the stool (Smith & Bayles, 2007). *Campylobacter* infection may also lead to various forms of inflammatory bowel disease (IBD), which is a chronic condition in the digestive tract (Kaakoush *et al.*, 2015; Mukhopadhya *et al.*, 2011). Extraintestinal sequelae include reactive arthritis (Mortensen *et al.*, 2009), Guillian-Barre syndrome (Nyati & Nyati, 2013) and Miller-Fisher syndrome (Lo, 2007; Tatsumoto *et al.*, 2015) (Figure 5).

1.2.6 Veterinary consequences of *Campylobacter* infection

Although, as described above, *Campylobacter* species are commonly found in the gastrointestinal tract of many livestock without provoking overt disease, opportunistic infections with marked pathology have been associated with campylobacters. For example,

C. fetus is associated with bovine venereal campylobacteriosis, which is venereal disease causing temporary infertility of animal, the death of embryo and female abortion (Campero *et al.*, 2005; Eaglesome *et al.*, 1992).

In goats, *Campylobacter* can produce abortion. The abortion may happen in the last stage of gestation. If the animal not aborted, weak kids delivered and may die after a while. However, the infection of the male may occur without any clinical signs (Matthews, 2016).

In pregnant sheep, *Campylobacter* infection can progress to bacteremia with subsequent placentitis, fetal infection, and abortion. *Campylobacter*-induced abortion usually occurs during late gestation, although some lambs are carried to full-term and are born weak and succumb soon after birth (Kimberling, 1988). In severe cases, pregnant ewes may die due to septicaemia as a consequence of retention of a dead foetus (Hedstrom *et al.*, 1987; Skirrow, 1994). About 70 % of pregnant ewes may abort when a flock is subjected to bacteria for the first time (Dennis, 1990). *Campylobacter* species have also been implicated as agents of enterocolitis and mastitis in sheep (Dennis, 1975; Gressler *et al.*, 2012; Hedstrom *et al.*, 1987; Raji *et al.*, 2000).

Presence of *Campylobacter* in the gut of chicken may have an effect on the bird's health (Colles *et al.*, 2016; Wearne, 2013). In a study conducted by Humphrey *et al.* (2014) it was confirmed that *C. jejuni* can induce infection in modern broiler breeds. Different factors such as immunity of the host, presence of co-infection, the diet of chicken and pathogenicity of bacteria can determine the pathogenisity of *C. jejuni* (Wigley, 2015). Infection of poultry with *Campylobacter* may lead to a reduction in the bird growth rate (Colles *et al.*, 2008) and diarrhoea as a result of an inflammatory response which leads to damage of the feet and legs of the bird, due to the accumulation of poor wet litter (Humphrey *et al.*, 2014; Wearne, 2013).

1.2.7 *Campylobacter* virulence in humans and host

Humans are not a natural reservoir for campylobacters, thus human campylobacteriosis must be considered the result of an accidental infection that is of no value to the natural persistence of *Campylobacter*. Thus, human campylobacteriosis reflects the lack of adaptation of campylobacters to the human gut. *Campylobacter* species are unlikely to possess any human-specific virulence factors but rather human disease is provoked by the errant

interactions between factors used by campylobacters to exploit natural reservoirs and the human intestinal mucosa and immune system. Although the molecular basis of *Campylobacter* pathogenicity is not as well understood as it is for other pathogens, numerous studies have explored the mechanisms by which bacteria interact with hosts and the diversity of potential virulence factors used for this interaction. Invasion of the epithelial cells provokes an inflammatory response by the host (Harvey *et al.*, 1999; Kopecko *et al.*, 2001) in which pro-inflammatory cytokines are produced to activate the recruitment of macrophages, neutrophils and other immune cells to the site of infection (MacCallum *et al.*, 2006). The inflammatory response is activated by an IL-8 response which is thought to be triggered by cytolethal distending toxin (CDT) of *C. jejuni* (Zheng *et al.*, 2008). Capsular polysaccharide and flagella of bacteria play a vital role in epithelial cell attachment and invasion (Carrillo *et al.*, 2004). The polysaccharide capsule increases the ability of *C. jejuni* to survive, attach and invade host cells (Karlyshev *et al.*, 2000). Outer membrane proteins, toxins (such as cytolethal distending toxin and enterotoxin) and pili are considered other important virulence factors (Ketley, 1995; Wallis, 1994). Moreover, *Campylobacter* flagella play an important role in entry and colonization of bacteria to the mucosal layer of the intestine (Szymanski *et al.*, 1995). Flagella help the organism move efficiently in the viscous conditions inside gut thereby assisting in the establishment of infections in the caecal crypt or intestine (Lee *et al.*, 1986). *Campylobacter* species also produce a CDT that is able to bind with cholesterol-rich micro-domains on the cytoplasmic membrane of host cells and has DNase activity (Lai *et al.*, 2016). The toxin has a direct effect on the host epithelial cells by inducing arrest of cell cycle, distension and cell death (Hickey *et al.*, 2000). In addition to biofilm formation and adhesion (Mahdavi *et al.*, 2014).

1.2.8 Mechanisms of persistence in reservoir hosts and the environment

The mechanism of persistence of *Campylobacter* in the reservoir is not well understood, however, recent study of *Campylobacter* infection in chicken clarify that point. When *Campylobacter* infection occurs in chicken it provokes immune responses, inflammatory responses and, in turn, diarrhoea (Lacharme-Lora *et al.*, 2017). Shedding of the bacteria will help in the persistence of the pathogen from one host to another. Hermans *et al.* (2012) found that the immune response of chicken to tolerate the *Campylobacter* infection help in colonization and persistence of the bacteria in the chicken gut. Moreover, wild birds may also help in persistence of infection (Cody *et al.*, 2015).

Environmental persistence of *Campylobacter* species appears to be prolonged, particularly in presence of unfavourable conditions of bacterial survive. This persistence in the environment may relate to different strategies for survival of bacteria in harsh environments. Enter of bacteria to a viable but nonculturable (VBNC) state occur when experience unfavorable conditions such as entry into stationary phase and lake in the nutrient molecules. (Griffiths, 1993; Rollins & Colwell, 1986). The VBNC state of *Campylobacter* species has been recognized in water supply were induce infection to the chicken after drinking from that water (Pearson *et al.*, 1993). The cells cannot be recovered by various cultivation but they are metabolically active and show signs of respiratory activity (Cox *et al.*, 2015).

Biofilms are an aggregation of microorganisms such as bacteria, protozoa and fungi embedded in an extracellular matrix that occurs widely in natural and man-made aquatic environments (Costerton *et al.*, 1987; Costerton *et al.*, 1995; Gilbert *et al.*, 1993). Studies have indicated that *C. jejuni* has the ability to form biofilms and survive for several weeks within biofilms, even at low temperatures (Buswell *et al.*, 1998; Lehtola *et al.*, 2006; Maal-Bared *et al.*, 2012).

Campylobacters are considered thermotolerant and have the ability to tolerate thermal stress under 4 °C and above 50 °C regarding intracellular mechanisms that increase the persistence of these organisms in the environment (Murphy *et al.*, 2006). *Campylobacter* responds to heat stress via heat shock proteins which are the most highly conserved protein-coding genes that mediate bacterial response to thermal stress and increase as a respond to the rise of the temperature and environmental stresses (Murphy *et al*., 2006). Under cold stress pathogens increase the production of genes that are involved in energy metabolism, suppress and control the virulence genes transcription (Chaisowwong *et al*., 2012). Studies show the ability of *C. jejuni* to survive up to four months at low temperature in water (Buswell *et al.*, 1998; Hazeleger *et al.*, 1998).

C. jejuni overcomes a wide range of environmental conditions even if it leaks the classical mechanisms to coop stress which present in other bacteria (Kassem & Rajashekara, 2011). *Campylobacter* needs to tolerate the oxygen stress in order to survive and colonize the host.

1.2.9 Treatment and control of *Campylobacter* infections

Usually, the disease in humans is self-limiting so that maintaining hydration and electrolyte balance is a key element of *Campylobacter* enteritis treatment (Allos, 2001). In severe cases, the antimicrobial agents of choice for *Campylobacter* enteritis are macrolides (e.g., erythromycin) and fluoroquinolones (e.g., ciprofloxacin) (Allos, 2001; Engberg *et al.*, 2001; Florez-Cuadrado *et al.*, 2016). *C. jejuni* shows antimicrobial resistant to a wide range of antibiotics such as sulfamethoxazole, vancomycin, bacitracin, polymyxin/colistin, penicillins, trimethoprim and most cephalosporins. This either due to modification in antimicrobial targets, the fail of antibiotic to reach the target, efflux of antibiotic, inactivation and changing in antibiotics (Iovine, 2013). The develop of anti-*Campylobacter* vaccine prototypes occur as a result of emerging of antibiotic-resistant strains (Jagusztyn-Krynicka *et al.*, 2009). Recently, there is no global regulatory authority approved vaccine against *Campylobacter* association illness (Riddle & Guerry, 2016).

The *Campylobacter* associatied infection in ewes is treated and prevented by administrating chlortetracycline or tetracycline throughout the last stage of pregnancy (Washburn *et al.*, 2014). Moreover, to prevent the distribution of infection in the flock, uterine discharged, aborted fetus and placental membrane should be removed as soon as possible. Although there are promising results to develop a vaccine against *Campylobacter* infection, there is no accurate vaccine developed against the infection in chicken (Meunier *et al.*, 2016). There is no vaccine available in the UK, however, killed adjuvanted vaccines are used in New Zealand and North America (Mearns, 2007).

1.3 Co-infections

Eimeria species and *Campylobacter* species are just two examples of the diverse community of micro-organisms that are associated with sheep in a naturally healthy condition. Recent work has demonstrated that interactions between parasite community represent a strong influence on its composition and transmission (Graham *et al.*, 2007). Thus, the likelihood that an individual is infected by a particular micro-organism, is determined not just by the individual's innate susceptibility to that micro-organism, but also by what other micro-organisms co-infect that individual (Telfer *et al.*, 2010). The mechanisms that underlie these interactions are not yet well understood, but are likely to be both direct and indirect (via the immune system) (Ezenwa, 2016).

An example of the indirect interaction is what happens in mice when co-infection occurs with helminth parasites and viruses. In this case, the presence of the parasitic worm shifts the immune response such that immunity against viruses becomes impaired (Mueller, 2014). Virus-helminth co-infection was also tested in order to identifiy whether the immunomodulatory effects of helminth infection activates the immunity of the host or the activation occures as a result of change in the microbiota. The study showed that helminths reduce immunity against viruses in the germ-free mice in addition, and induce changes in the gut microbiota as well as stimulating alternative activation of machrophages (Osborne *et al.*, 2014).

Another example of an indirect effect of co-infection is the enhancement of immunity against pathogens. In a study conducted using mouse model infected with *Fonsecaea pedrosoi*, it was found that injection of intraperitoneal or intravenous bacterial lipopolysaccharide (LPS) enhanced the immune response against the fungal infection leading to eliminate the infection (da Glória Sousa *et al.*, 2011).

Identifying the co-infection consequences between microorganisim in the host, may help in designing a proper control program as well as increasing understanding of the ecology and evaluation of both parasite and host (Graham, 2002; Pedersen & Fenton, 2007; Read & Taylor, 2001).

The presence of different species of coccidia in the same host may increase the potential of the disease. Moreover, the severity of the disease may increase due to infection with another pathogen such as bacteria, helminths or viruses (Taylor, 1995).

Moreover, infection of an animal by *Eimeria* may lead to increase a Gram negative microflora (Mohammed *et al.*, 2000), as well as secondary bacterial infection (Taylor *et al.*, 1973; Yang *et al.*, 2014b). An experiment conducted to examine the interaction between microflora and *E. ovinoidalis*, revealed that the pathogenic expression of *E. ovinoidalis* increases in the presence of digestive microflora (Gouet *et al.*, 1984).

1.4 Aims of the research

1. To use cross-sectional and longitudinal surveys of sheep flocks in North-West England to determine the diversity of infecting *Eimeria* and *Campylobacter* and the epidemiology of the infections they cause.
2. To identify ecological determinants of observed diversity and epidemiology.
3. To explore the extent to which sheep-associated *C. jejuni* strains infect wildlife living in/adjacent to farmland/pasture.
4. To develop molecular tools to delineate *Eimeria* species.

CHAPTER 2: Materials and Methods

2.1 Farms

Surveys were carried out on three sheep farms located in Lake District (Southern Cumbria) in North West England. The first of these (Threlkeld Farm) was located in village of Satterthwaite in the Rusland valley, whereas the second (Stock Farm) was located about 6 km south west on the southern edge of Bethecar Moor above the village of Nibthwaite in the Coniston valley. Abbott Park Farm, the third farm, was located in Bandrake Head, Ulverston (Figure 6).

Threlkeld Farm maintains a flock of 500 Swaledale sheep. The ewes spend most of the year on higher ground (Figure 6, blue star) but are moved to lower-lying land (Figure 6, red star) at the end of October for three weeks for "tupping" (mating with rams) (Figure 6). Subsequently the ewes return to the higher ground for the winter, but are moved again to more sheltered pasture for lambing. Lambs are reared primarily on lowland pasture. The sheep in Threlkeld farm have no access to the Bethecar Moor.

Stock Farm maintains a flock of about 600 Swaledale sheep that graze across Bethecar Moor, a large expanse of unenclosed "common" upland grassland. The ewes spend almost all their time on the Moor, being rounded up only five times a year for brief periods:

In early November, the sheep spend three weeks on by-land adjacent to the farm during which time ewes over 12 months in age are tupped. During this period, all sheep are vaccinated against *Clostridium* and *Pasteurella* species (Heptavac P PLUS) and are treated for *Fasciola hepatica* and *Oestrus ovis* larvae (Flukiver).

The sheep remain on the Moor between late November and mid-January, then are briefly gathered in the by-land again to be pregnancy checked and to receive ectoparasite control (Crovect Cypemethrin).

In March, the sheep return to the by-land in preparation for lambing, and receive endoparasite and ectoparasite treatment (Ivermectin and Dysect). Young (non-pregnant) ewes are vaccinated against louping ill virus then are returned to the Moor. If delivery is uncomplicated, mothers and their lambs return to the Moor within two days of birth. Animals involved in more complicated deliveries remain in the by-land or are moved indoors. However, all mothers and lambs are returned to the Moor as soon as possible. All lambs are treated for ectoparasites (Dysect).

In July, the sheep are once again brought into the by-land for sheering, which takes about 10 days to complete.

Finally, in early September, sheep are herded so that 6 months old male lambs can be removed for meat production. Ewes and female lambs treated with Heptavac, Flukiver and Dysect and returned to the Moor.

Thus, typically, sheep on Stock Farm will spend about 300 days untended on Bethecar Moor.

Abbot Park Farm maintains a flock of about 200 sheep, mostly Texel but with a small number of Swaledales. These animals graze on pasture on the edge of Bethecar Moor and on the south-eastern corner of the Moor itself, but are not managed in the traditional husbandary practices used on Stock Farm. Sheep are far more regularly herded and spend far longer on by-land/pasture. Tupping takes place in November and sheep remain on pasture until December before returning to the Moor after treatment with Flukiver. Pregnancy checks follow in January and the sheep spend March and April on pasture during lambing. Following lambing, all sheep are treated with Flukiver, some form of ectoparasitic control and Heptivac P PLUS vaccination then are either returned to the Moor or left on pasture. Typically, sheep will receive further endoparasite control (Ricobendazole) monthly between April and September. Six month old male lambs are removed in later August and during September, after which remaining females are treated for *Fasciola hepatica* using Closantel sodium dehydrate. A final Flukiver treatment is carried out in October.

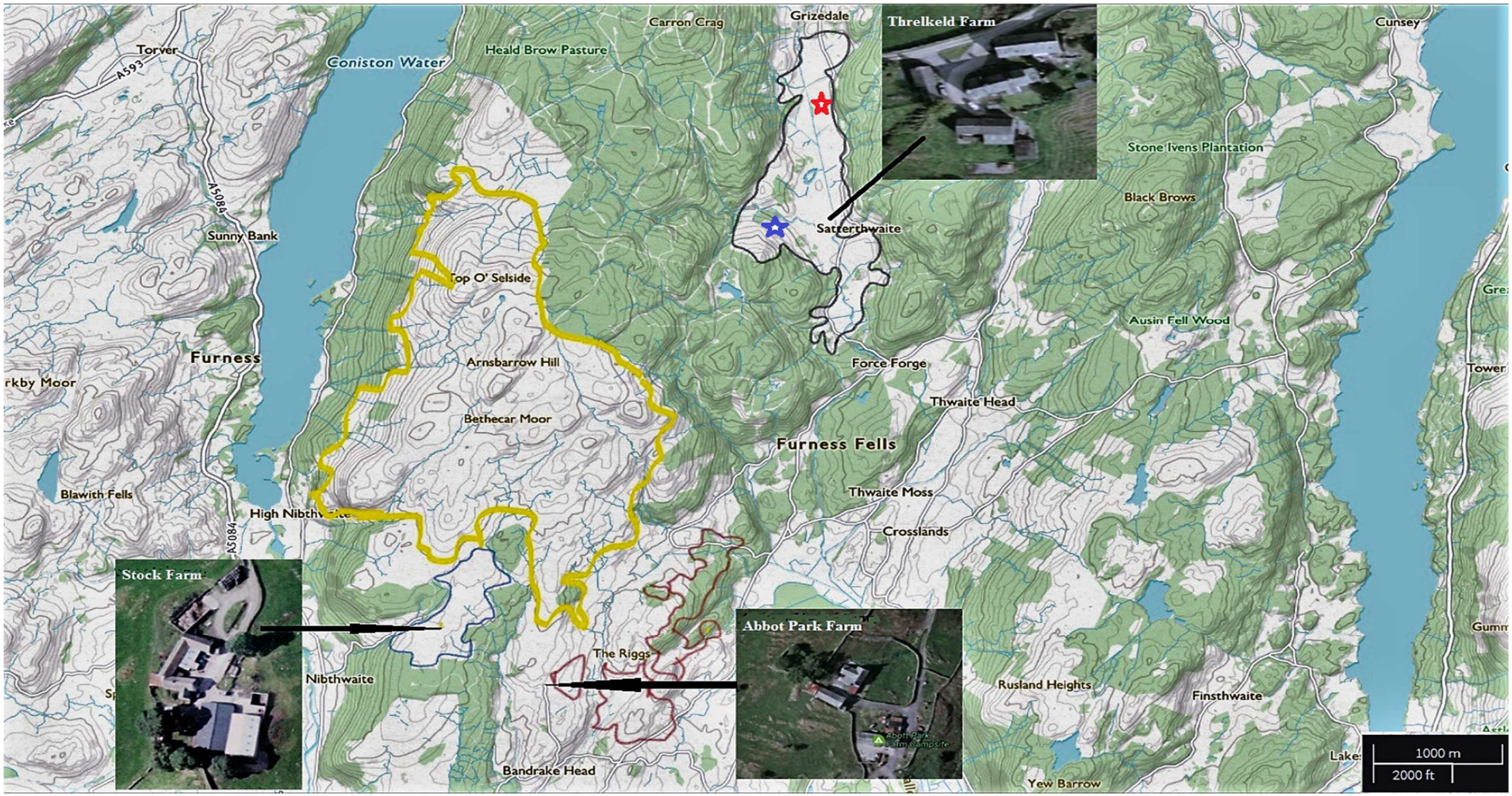


Figure 6: Map of study area including satellite images of Threlkeld Farm, Stock Farm and Abbot Park Farm. The extent of grazing used by each farm is marked on the map.

2.2 Survey of sheep

Each farm was used to address different objectives within the study. Threlkeld Farm was primarily used as a pilot project as it was available at the start of the study and, because sheep were enclosed on pasture, collection of faecal samples could be carried out without help from the farmer. Initially, samples collected from this farm were used to optimise the diagnostic assays throughout the study. Threlkeld Farm was visiting on four occasions between June 2014 and January 2015.

Stock Farm was chosen as the husbandry practices employed on this farm were traditional and widespread across Cumbria (and beyond), thus it best represented the majority of upland sheep farms in the UK. The aim of this study was to collect samples from sheep every time they were herded onto by-land over a two years period between September 2014 and September 2016. This aim was largely fulfilled, however, due to logistical problems, a survey was not carried out during lambing in 2015.

Abbot Park Farm was added to the study as the farmer was willing to allow sampling of sheep on a monthly basis, thereby allowing more intensive quantification of seasonal epidemiological trends. The aim of the study was to collect faecal samples every month between July 2015 and September 2016, however, it was not possible to collect samples in December 2015 and January 2016 due to excessive rainfall and widespread flooding across Cumbria at this time. (Table 1).

Table 1: Timing of surveys carried out during the study.

Farm name	Survey number	Date
Threlkeld Farm	1	June 2014
	2	July 2014
	3	August 2014
	4	January 2015
Stock Farm	1	September 2014
	2	November 2014
	3	January 2015
	4	February 2015
	5	July 2015
	6	September 2015
	7	November 2015
	8	February 2016
	9	May 2016
	10	July 2016
	11	September 2016
Abbot Park Farm	1	July 2015
	2	August 2015
	3	September 2015
	4	October 2015
	5	November 2015
	6	February 2016
	7	March 2016
	8	April 2016
	9	May 2016
	10	July 2016
	11	August 2016
	12	September 2016

On each visit to each farm, between 20 and 60 fresh faecal samples were opportunistically collected from the ground immediately after being voided. Approximately 5 g of faeces was placed in a 60 ml collection tube then immediately chilled on ice and held on ice during transport back to the laboratory. When possible, the ear tag number of sheep that produced the faeces was recorded.

2.3 Survey of Deer

Much of the land across the study area is managed by the Forestry Commission for wood production. The study area supports sizeable populations of red and roe deer (*Cervus elaphus* and *Capreolus capreolus*), and deer move freely across the entire study area such that they frequent, probably on a daily basis, the grazing land used by all three study farms. Both deer species are managed by the Forestry Commission to limit deer damage to young trees by regular culling carried out by rangers. Between November 2014 and August 2016, these rangers agreed to collect faecal samples from just-shot deer for the study. Approximately 5 g of faeces were collected soon after death during "gralloching" (removal of the alimentary tract in the field to avoid faecal contamination of the carcass) from the rectum into a 60 ml collection tube. These samples were dated, packed and were sent to the laboratory via first class post. Samples were collected only on a Monday, Tuesday or Wednesday to ensure they were received by the laboratory within 48 of collection.

2.4 Isolation and identification of *Eimeria*

2.4.1 Parasitological examination

About 4 g of faecal material were added to 56 ml of distilled water and mixed well to create a suspension. The suspension was left for 30 min at room temperature, mixed well again then transferred to clean container through a tea strainer. Ten ml of this filtrate were centrifuged for 5 min at 172 x g. The supernatant was discarded and the pellet was resuspended in 4ml of flotation fluid (saturated NaCl solution containing 500g per litre of glucose). Fifty µl of the parasite egg suspension were transferred to two chambers of a McMaster slide then left for 5 min to allow flotation of eggs. Eggs were then counted by microscopic observation under 100 x magnification. The number of eggs counted was multiplied by 20 to obtain the number of eggs per gram of faeces (Vadlejch et al., 2011).

2.4.2 Identification of *Eimeria* species

Eimeria species were identified on the basis of morphology and size of sporulated oocysts. Faecal samples in which *Eimeria* eggs were observed were subjected to an induced sporulation process. Ratio of one volume of faecal material to two volumes of 2.5% (w/v) potassium dichromate solution was mixed well, sieved to remove course material then poured as a thin layer into a petri dish. Egg suspensions were held at room temperature for 10 days to allow sporulation to occur (Andrade *et al.*, 2012; Toulah, 2007).

The solution of sporulated oocysts was centrifuged for 5 min at 1077 x g and the supernatant was discarded. The sediment was placed into a centrifuge tube and flotation fluid was added until a meniscus formed then a cover slide was placed on top of the tube and left for 5 min. The cover slide was then carefully removed and observed under 40 x or 100 x magnification to determine the identity of the oocysts present. At least 30 oocysts were observed for each sample, and identified at species level on the basis of shape, size and appearance by reference to a taxonomic key (Eckert *et al.*, 1995).

2.4.3 DNA Extraction from *Eimeria* oocysts

Sporulated oocysts were washed three times by suspension in 50 ml of sterile distilled water then centrifuging at 1077 x g for 5 min. DNA extracts were prepared from washed oocysts using a commercial spin column kit protocol (Isolate Faecal DNA Kit, Bioline, UK) with minor modifications. Briefly, 300 µl of each oocyst suspension was added to a bashing bead lysis tube containing 750 µl of lysis buffer and bead beating was carried out using a Tissuelyser II (QIAGEN) at maximum speed for 5 minutes. The tube was centrifuged for 1 min at 10000 x g then 400 µl of supernatant was transferred into a spin filter orange top (the preparation of spin filter orange top was done by snapping off its base, placed in collection tube and centrifuged for 1 min at 7000 x g). Next, 1200 µl of faecal DNA binding buffer was added to the collection tube, then 800 µl of this mixture were transferred to a spin column fitted with a collection tube. The tube was centrifuged at 10000 x g for 1 min and the flow-through was discarded from collection tube and the remaining mixture was added into the spin column. The tube was centrifuged at 10000 x g for 1 min again then the flow-through was discarded from collection tube. 200 µl of DNA pre-wash buffer were added to the spin column fitted with new collection tube and centrifuged at 10000 x g for 1 min. Then, 500µl of faecal DNA wash buffer was added and the column was centrifuged at 10000 x g for 1 min. Finally, 100µl of DNA elution buffer was added directly onto the column matrix of spin

column tube that fitted with 1.7 ml Eppendorf tube. The DNA was eluted by centrifugation of the tube for 30 sec at 10000 x g. The eluted DNA was transferred to green top spin filter (the preparation of spin filter green top was conducted by snapping off its base then centrifuged at 8000 x g for 3min), placed in 2ml collection tube and centrifuged for 1 min at 8000 x g. DNA was eluted in a final volume of 100µl and stored at -20 ^{0}C.

2.4.4 Electrophoresis

PCR products were identified following their electrophoretic resolution on 1% (w/v) agarose gels. One gram of agarose powder was mixed with 100ml of 1 x TBE buffer (prepared from 10 x TBE stock solution) (Bioline, UK) buffer then heated in a microwave oven until the agarose power had completely dissolved. Once the molten agar had cooled to 50ºC, 100µl of Gel Red (Cambridge Bioscience Ltd.) were added to the mixture, which was then poured into the gel casting tray using casting dams and a comb, and allowed to cool and set for 30 min at room temperature. The solid gel was placed in an electrophoresis tank filled with 1 x TBE buffer and PCR products loaded onto the wells of the gel. A 1 kilobase hyper ladder DNA marker (Bioline, UK) was also included on each gel. Electrophoresis was allowed to proceed at 110 volts for up to 75 min. DNA present in the gel was visualized by exposure to a UV transillumnator (SynGene).

2.4.5 PCR product purification

PCR products were purified using an Isolate II PCR and gel kit according to manufacturer's instructions (Invitrogen). Four volumes of binding buffer were mixed with one volume of PCR product (typically 20µl) then the mixture was added into the spin column. The spin column was centrifuged at 10000 x g for 1 min and the flow-through was discarded. The DNA that bound to the column, was washed by the addition of 650 µl of wash buffer followed by centrifugation at 10000 x g for 1 min. Again, the flow through was discarded and any residual wash buffer was removed by centrifugation at 10000 x g for 2 min. After that, the DNA was eluted from the column into a 1.7 ml Eppendorf tube by the addition of 15 µl of sterile distilled water into the centre of the column, incubated at room temperature for 1 min, then centrifuged at 10000 x g for 2 min. Purified PCR products were stored at -20ºC.

2.4.6 Sequencing of PCR products

The purified PCR products were sequenced commercially using Sanger sequencing (Source Bioscience) using the same primers as used for amplification.

2.4.7 Analysis of DNA sequence data

Sequence data were returned from the commercial sequencing service in the form of .ab1 files. These file, which contained chromatograms, were visualised using ChromasPro software (Technelysium DNA sequencing software). ChromasPro was used to align chromatograms obtained for each strand of a PCR product using F and R primers. This alignment was used to (i) assess the accuracy and reliability of base calling on each strand by the software, and (ii) to identify then remove primer sequences. If both stands yielded an unambiguous consensus sequence, the data were stored as a ChromasPro files and were analysed using the NCBI BLAST sequence searching tool through the ChromasPro programme.

2.5 Isolation and identification of *Campylobacter* species

2.5.1 Isolation by selective enrichment

Approximately 0.5g of faecal material was placed in 5ml of modified Preston broth, consisting of Brain Heart Infusion (BHI) (Oxoid, UK) broth supplemented with *Campylobacter* growth supplement (X115, Oxoid), modified Preston *Campylobacter* supplement (X114, Oxoid) and 5% lysed horse blood (TCS Biosciences Ltd., UK). This suspension was vortexed until the faecal material formed a homogenous suspension, then broths were incubated for 24h at 42 ºC under microaerophilic conditions in microaerophilic chamber (Molecular Atmosphere Controlled System, DW Scientific) (Stanley *et al.*, 1998). Putative isolates were identified on the basis of colonial morphology and microscopical features of vegetative bacteria cells using Gram staining.

Next, 100µl of the incubated suspension were plated onto *Campylobacter* blood-free selective agar containing charcoal cefoperazone deoxycholate agar (CCDA) selective supplement (SR 155E, Oxoid, UK) (Bolton *et al.*, 1984), and the plates were incubated for 48h at 42 ºC under microaerophilic conditions.

Putative *Campylobacter* colonies appearing on these plates were subcultured onto Colombia blood agar (Oxioid, UK) plates containing 5% lysed horse blood in order to obtain a single colony growth (Uaboi-Egbenni *et al.*, 2010). These plates were incubated for 48h at 42 ºC under microaerophilic conditions. Again, putative campylobacters were identified on the basis of their colonial morphology and microscopical features of vegetative bacteria cells using Gram staining. Isolates obtained in this manner were stored at -80ºC in BHI broth supplemented with 20% glycerol (Uaboi-Egbenni *et al.*, 2010).

2.5.2 Gram staining

Presumptive *Campylobacter* isolates were subjected to Gram staining (Fisher Scientific, UK) in order to visualize the morphological characteristics of the bacteria. A suspension of a colony of the presumptive isolate was prepared on a glass microscope slide and heat fixed. The slide was swamped with crystal violet for 1 min, briefly washed with tap water then swamped with Gram's iodine for another 1 min. The slide was then washed with 96% ethanol followed by water and flooded with a safranin counterstain for 10 min. Finally, the slide was washed with water, dried and observed under x1000 magnification (100x objective under oil immersion, 10x eye-piece lenses) by light microscopy (Brucker, 1986).

2.5.3 Amplification and characterization of *Campylobacter* 16S rDNA fragments

Boiled suspensions (1µl) of colonies of presumptive *Campylobacter* isolates were incorporated as template into *Campylobacter* genus-specific PCRs targeting an 857 bp 16S rDNA fragment (Linton *et al.*, 1997). Each reaction mixture comprised of 0.5µl of a 10pmol μl^{-1} solution of each primer (md16S1F, 5'- ATC TAA TGG CTT AAC CAT TAA AC -3' and md16S2 R, 5'- GGA CGG TAA CTA GTT TAG TAT T-3'), 12.5µl My Taq Red Mix and 10.5µl sterile distilled water. Reaction mixtures were subjected to a thermal programme consisting of an initial denaturation step at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 1 min, with incorporation of a final extension cycle at 72°C for 1 min. A positive control (*C. coli* strain 11068) and a reagent negative control (sterile distilled water) were incorporated into every PCR. Gel electrophoresis, purification, PCR product sequencing and collation and analysis of sequence data were performed using the same protocols described above.

For deer samples, generic eubacterial 16S rDNA PCR was used to confirm the identity of the isolates that incorporated the 27F Forward (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R Reverse (5'- GGTTACCTTGTTACGACTT revers primers) pair. PCR reagent concentrations and the thermal programme used were as described above.

2.5.4 Multi-locus sequence typing for *Campylobacter* isolates

PCR products generated from fragments of seven housekeeping genes were used for MLST, namely *aspA* (aspartase A), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase) and *uncA* (ATP synthase A subunit) using the protocols described by (Miller *et al.*, 2005). For each locus, a 25µl reaction mixture was prepared as follows: 1µl of a boiled bacterial suspension, 1µl of a 10pmol μl^{-1} solution of each of 2 locus-specific primers (Table 2), 12.5µl My Taq Red Mix and 9.5µl sterile distilled water.

Table 2: Oligonucleotide primers used in MLST. The nucleotide cods were ordered as follow B= C or G or T; D= A or G or T; H=A or C or T; K= G or T; M= A or C; R= A or G; S= G or C; V= A or C or G; W= A or T; Y= C or T

Primer	Primer sequence (5' -> 3')	Amplicon size (bp)
aspAF1	GAGAGAAAAGCWGAAGAATTTAAAGAT	676
aspAR1	TTTTTTCATTWGCRSTAATACCATC	
glnAF	TGATAGGMACTTGGCAYCATATYAC	700
glnAR	ARRCTCATATGMACATGCATACCA	
gltAF	GARTGGCTTGCKGAAAAYAARCTTT	706
gltAR	TATAAACCCTATGYCCAAAGCCCAT	
glyAF	ATTCAGGTTCTCAAGCTAATCAAGG	716
glyAR	GCTAAATCYGCATCTTTKCCRCTAAA	
pgmF1	CATTGCGTGTDGTTTTAGATGTVGC	720
pgmR1	AATTTTCHGTBCCAGAATAGCGAAA	
tktF1	GCAAAYTCAGGMCAYCCAGGTGC	730
tktR	TTTTAATHAVHTCTTCRCCCAAAGGT	
atpAF	GWCAAGGDGTTATYTGTATWTATGTTGC	700
atpAR	TTTAADAVYTCAACCATTCTTTGTCC	

Each reaction mix was subjected to thermal programme consisting of an initial denaturation cycle for 3 min at 95 ºC followed by 30 denaturation cycles for 20 sec at 94 ºC, annealing for 20 sec at 50°C, extension for 1 min at 72°C and a final extension cycle for 5 min at 50°C (Miller *et al.*, 2005).

Sanger sequencing of PCR products was performed commercially (Source Bioscience) using the same primers as used for amplification. Sequence data for each strand were visualised using Chromas Pro as described section 2.4.8 above, and these data were inputted into the *Campylobacter* MLST database (http://campylobacter.mlst.net/) to be ascribed allele number, sequence type and clonal complex.

2.5.5 Differentiation of *C. jejuni* and *C. coli*

The 16S rDNA-based assay described above for the identification of *Campylobacter* species is unable to reliably distinguish between the sister species *C. jejuni* and *C. coli*. Thus, for isolates on which complete MLST, as described above, was not attempted, comparison of sequence data derived from a single locus, *aspA*, was used to delineate these two species (methods as described above). Furthermore, *C. jejuni* and *C. coli*-specific PCRs, as previously described (Vondrakova *et al.*, 2014; Yang *et al.*, 2004) were also used on occasion.

In the *C. coli*-specific assay (Vondrakova et al., 2014), a 25 µl of reaction mixture comprising of 10.5 µl of sterile distilled water, 12.5 µl of My Taq Red Mix, 0.5 µl of a 10pmol μl^{-1} solution of Forward primer (5'- CATATTGTAAAACCAAAGCTTATCGTG-3'), 0.5 µl of a 10pmol μl^{-1} solution of Reverse primer (5'-AGTCCAGCAATGTGTGCAATG- 3') and 2µl of boiled DNA was prepared. Reaction mixtures were subjected to a thermal programme that comprised of an initial denaturation step for 5 min at 95ºC followed by 30 denaturation cycles for 15 sec at 95 ºC, annealing for 15 sec at 60°C, extension for 20 sec at 72°C and a final extension cycle for 10 min at 72°C. PCR products were resolved by electrophoresis and visualised on a transilluminator as described above. The expected product size of the assay was 133 bp.

In the *C. jejuni* specific assay (Yang et al., 2004), a 25 µl reaction mixture comprising of 9.5 µl of sterile distilled water, 12.5 µl of My Taq Red Mix, 0.5 µl of a 10pmol μl^{-1} solution of primer VS15 (5'-GAATGAAATTTTAGAATGGGG-3'), 0.5 µl of a of 10pmol μl^{-1} solution of primer VS16 (5'-GATATGTATGATTTTATCCTGC-3') (Yang *et al.*, 2004) and 2µl of boiled DNA was prepared. Reaction mixtures were subjected to a thermal programme that comprised of an initial denaturation step for 5 min at 95ºC followed by 30 denaturation cycles for 15 sec at 95 ºC, annealing for 15 sec at 56°C, extension for 20 sec at 72°C and a final extension cycle for 10 min at 72°C. PCR products were resolved by electrophoresis and visualised on a transilluminator as described above. The expected product size of the assay was 358 bp.

2.6 Epidemiological data analysis

2.6.1 Assembly of data

The data generated from lab work, such as presence or absence of *Eimeria* and *Campylobacter* were used for diagnostic purpose. These data were used to investigate prevalence and intensity of *Eimeria*, prevalence of *Campylobacter* and identification of *C. jejuni* and *C. coli*. Data like age of animals obtained from the farmers and the information of climate was retrieved from Met Office (MetOffice, 2017).

2.6.2 Univariant analyses

Minitab 16.2.4 was used in order to analyse the data. In which, chi square test was used in order to compare the positive and negative data. Kruskal-Wallis/ Mann-Whitney test were used to compare the quantitative data. Finally, scatter plot was used for correlation analysis and then liner regression was used.

2.6.3 Multivariant analysis

To investigate those factors that influence an individual's probability of testing positive for infection (prevalence models) with *Eimeria* spp. and *Campylobacter* spp., generalized linear models (GLMs) were used that assumed a binomial error term and a logit link. Factors considered included co-infection, age (categorised as ≤1-12 months, 13-24 months and > 24 months), season (summer = June, July and August; autumn= September, October and November; winter= December, January and February; spring= March, April and May), farm, Rainfall and species.

To consider intensity of infection (Intensity Models) with *Eimeria* spp., GLMs with a negative binomial error term and a log link were employed. The same predictors were considered as for the prevalence models above.

All analyses were carried out using R 3.4 (R. Development Core Team, 2016) using either the glm.nb function from the MASS library for the negative binomial GLMs (intensity models) or the glm function for the models investigating infection. Model selection was based on backward stepwise model selection with variables dropped according to P-value, with only those variables significant at the $p<0.05$ level being retained in the final model.

To investigate whether the potential non-independence of samples from the same site was important, additional models using GLMMs (Generalised Linear Mixed Models) with a binomial

error term and logit link were employed. However, as these had no effect on identifying the factors that were significantly predicting infection, they are not included in the results section.

CHAPTER 3: Sheep Survey Results

3.1 Threlkeld Farm

3.1.1 *Eimeria* infections

A total of 90 faecal samples, collected during two visits to Threlkeld Farm, were tested for the presence of *Eimeria* species (Table 3).

Table 3: Prevalence of *Eimeria* infections in ovine faecal samples on Threlkeld Farm.

Survey	Date	Nº samples collected	Nº samples infected (%)	Exact binomial 95% confidence interval (%)
1	12/6/2014	50	42 (84)	70- 92
2	22/1/2015	40	29 (73)	56- 85

A Chi-squared test was conducted and did not reveal a significant difference infection prevalence between the two surveys (χ^2= 1.76, DF= 1, P= 0.184). The intensity of *Eimeria* infections was also quantified (Table 4). A Mann-Whitney test was applied to these data and revealed a significant difference between the two surveys (P< 0.000, W= 2999.0).

Table 4: The mean intensity of *Eimeria* oocyst shedding at Threlkeld Farm.

Survey	Mean intensity (oocysts gram^{-1})	Standard error	Range
1	7890	1012	30000
2	138	21	480

A total of 10 *Eimeria* species were identified in these samples on the basis of microscopic observation of non-sporulated (i) and sporulated (ii) oocysts (Figure 7 and Table 5). Of these, *Eimeria ovinoidalis* was the most frequently encountered species in both surveys (Table 6), infecting 37% of the samples tested. Infections caused by all species were less prevalent in Survey 2 than in Survey 1, but this difference was only statistically significant for *Eimeria faurei* (Table 6). The majority of the samples contained two or more *Eimeria* species (Table 7). Although the prevalence of single, dual and multiple infections varied between Survey 1 and Survey 2, these variations were not statistically significant (Table 7).

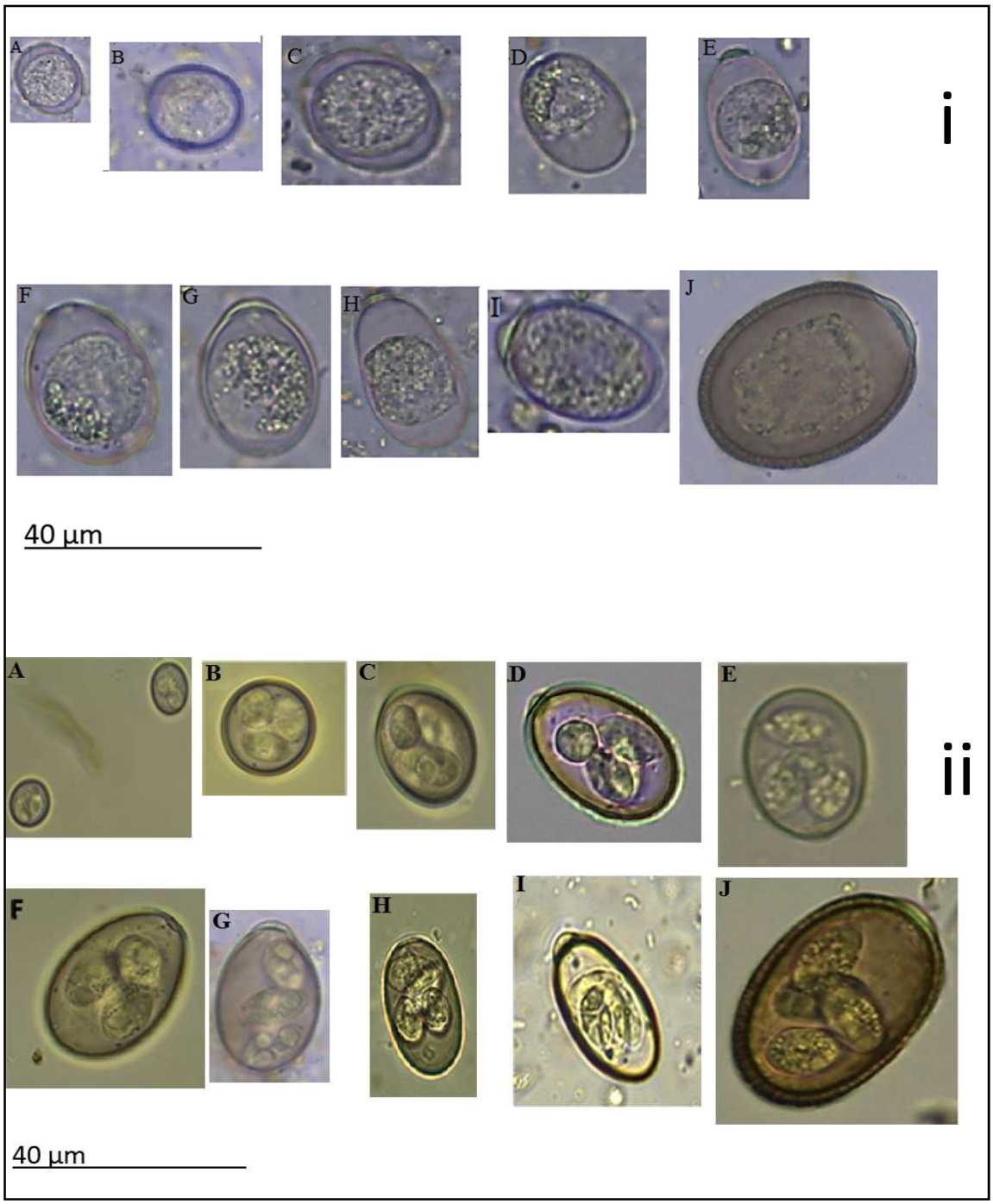


Figure 7: Microscopic appearance of (i) non-sporulated and (ii) sporulated oocytes of *Eimeria* species, (A) *Eimeria pallida*, (B) *Eimeria parva*, (C) *Eimeria ovinoidalis*, (D) *Eimeria weybridgensis*, (E) *Eimeria crandallis*, (F) *Eimeria fauri*, (G) *Eimeria granulosa*, (H) *Eimeria bakuensis*, (I) *Eimeria ahsata*, (J) *Eimeria intricata*. Magnification x1000.

Table 5: The identification key of different *Eimeria* species (Eckert *et al.*, 1995).

***Eimeria* species**	**Morphological characteristics**	**Oocyst dimensions**
Eimeria pallida	Ellipsoidal oocyst, no polar cap, colourless to pale yellow	12-20 x 8-15 µm
Eimeria parva	Spherical or sub spherical oocyst, no polar cap, colourless	13-22 x 11-13 µm
Eimeria ovinoidalis	Ellipsoidal oocyst, no polar cap, Presence of micropyle, colourless to pale yellow	17-25 x 13-20 µm
Eimeria weybridgensis	Ellipsoidal or subspherical broadly oocyst, presence of polar cap, presence of micropyle	17-30 x 14-19 µm
Eimeria crandallis	Ellipsoidal or subspherical broadly oocyst, with or without presence of polar cap, presence of micropyle	17-23 x 17-22 µm
Eimeria faurei	Ovoidal shape oocyst, no polar cap, presence of micropyle, yellowish brown colour	28-37 x 21-27 µm
Eimeria granulosa	urn-shaped oocyst, presence of the large polar cap on the broad end, presence of micropyle, yellowish brown colour	22-35 x 17-25 µm
Eimeria bakuensis	Ellipsoidal oocyst, presence of polar cap, presence of micropyle, yellowish brown colour	23-36 x 15-24 µm
Eimeria ahsata	Ovoidal shape oocyst, presence of distinct polar cap, presence of micropyle, yellowish brown colour	29-37 x 17-28 µm
Eimeria intricata	Ellipsoidal oocyst, presence of polar cap, presence of micropyle, thick and striated wall, brown colour	40-56 x 30-41 µm

Table 6: Frequency of *Eimeria* species in ovine faecal samples on Threlkeld Farm.

***Eimeria* species**	**Frequency (%)**			**Statistical results**
	Survey 1	**Survey 2**	**Overall**	
E. pallida	10 (20)	4 (10)	14 (15)	χ^2= 1.692, DF= 1, P= 0.193
E. parva	13 (26)	10 (25)	23 (26)	χ^2= 0.012, DF= 1, P= 0.914
E. ovinoidalis	19 (38)	17 (43)	36 (40)	χ^2= 0.188, DF= 1, P= 0.665
E. faurei	11 (22)	1 (3)	12 (13)	χ^2= 7.312, DF= 1, P= 0.007
E. ahsata	12 (24)	4 (10)	16 (18)	χ^2= 2.980, DF= 1, P= 0.084
E. crandallis	14 (28)	8 (20)	22 (24)	χ^2= 0.770, DF= 1, P= 0.380
E. granulosa	8 (16)	2 (5)	10 (11)	χ^2= 2.722, DF= 1, P= 0.099
E. bakuensis	14 (28)	10 (25)	24 (27)	χ^2= 0.102, DF= 1, P= 0.749
E. intricata	2 (4)	0 (0)	2 (2)	Counts too low
E. webridgensis	4 (8)	0 (0)	4 (4)	Counts too low

Table 7: Frequency of single, dual and triple *Eimeria* infections in ovine faecal samples on Threlkeld Farm.

Types of infection	**Frequency (%)**		**Statistical results**
	Survey 1	**Survey 2**	
Non-infected	8(16)	11(28)	χ^2= 1.765, DF= 1, P= 0.184
Single	11 (22)	8 (20)	χ^2= 0.053, DF= 1, P= 0.817
Dual	16 (32)	15 (38)	χ^2= 0.298, DF= 1, P= 0.585
Multiple	15 (30)	6(15)	χ^2= 2.795, DF= 1, P= 0.095
TOTAL	50	40	

Shannon's Weiner diversity index of *Eimeria* species present in ovine faecal samples on Threlkeld Farm was calculated for Survey 1 and Survey 2. The index values were 2.19 and 1.85 respectively indicating a richer diversity of species was encountered in Survey 1.

3.1.2 *Campylobacter* infections

A total of 208 faecal samples, collected during four visits to Threlkeld Farm, were tested for the presence of *Campylobacter* species (Table 8). The overall prevalence of infection in these samples (as confirmed by culture then genus-specific PCR) was 69/208 (33%). The prevalence of infection varied significantly between surveys (χ^2= 9.52, DF= 3, P= 0.023), peaking at 50% in Survey 2 and dropping to 9% in Survey 3 (Table 8). Pairwise comparisons revealed that the infection prevalence observed in Survey 3 was significantly lower than those observed in Surveys 2 and 4 (χ^2= 8.26, DF= 1, P= 0.004 and χ^2= 5.393, DF= 1, P= 0.020 respectively).

Table 8: Prevalence of *Campylobacter* infections in ovine faecal samples on Threlkeld Farm.

Survey	Date	Nº samples collected	Nº samples infected (%)	Exact binomial 95% confidence interval (%)
1	22/06/14	42	16 (38)	23-54
2	24/07/14	42	21 (50)	34-65
3	08/8/14	44	9 (20)	9-35
4	22/01/15	80	23 (29)	19-39

3.2 Stock Farm

3.2.1 *Eimeria* infections

A total of 350 faecal samples, collected from Stock Farm on 11 occasions, were tested for the presence of *Eimeria* species (Table 9).

Table 9: Prevalence of *Eimeria* infections in ovine faecal samples on Stock Farm.

Survey	Date	Nº of samples collected	Nº of infected samples (%)	Exact binomial 95% confidence interval (%)
1	16/09/14	35	24 (69)	50-83
2	10/11/14	19	13 (68)	43-87
3	22/01/15	28	22 (79)	59- 91
4	18/02/15	41	40 (98)	87- 99
5	23/07/15	62	54 (87)	76- 94
6	11/09/15	33	28 (85)	68- 94
7	09/11/15	18	14 (78)	52- 93
8	24/02/16	29	20 (69)	49- 84
9	09/05/16	23	21 (91)	71- 98
10	25/07/16	32	25 (78)	60- 90
11	12/09/16	30	20 (67)	47- 82

The prevalence of *Eimeria* infections ranged between 68% and 98% and varied significantly between surveys (χ^2= 22.55, DF= 10, P= 0.013). The intensity of *Eimeria* infections was also quantified (Table 10). A Kruskal-Wallis was applied to these data and revealed a significant difference between surveys (H= 55.43, DF= 10, P<0.001).

Table 10: The mean intensity of *Eimeria* oocyst shedding at Stock Farm.

Survey	Mean intensity (oocysts gram^{-1})	Standard error	Range
1	927	325	7216
2	325	91	1080
3	212	31	480
4	135	19	480
5	1203	274	7960
6	285	71	1460
7	106	25	380
8	253	102	2060
9	294	60	1100
10	2676	502	5940
11	61	9	140

The same 10 *Eimeria* species reported on Threlkeld Farm were encountered on Stock Farm (Table 5). Of these, *E. ovinoidalis* was the most frequently encountered species in all bar one survey (Table 11). The prevalence of infection for each species varied markedly between surveys, and for four species, *E. pallida, E. granulosa, E. bakuensis* and *E. weybridgensis* this variation was statistically significant (Table 11).

Samples containing infection by two or more *Eimeria* species were also encountered (Table 12). Although the prevalence of single, dual and multiple infections varied between surveys, these variations were not statistically significant (Table 12).

Table 11: *Eimeria* species observed in ovine faecal samples on Stock Farm.

Species name	Frequency (%)											
	Survey 1	**Survey 2**	**Survey 3**	**Survey 4**	**Survey 5**	**Survey 6**	**Survey 7**	**Survey 8**	**Survey 9**	**Survey 10**	**Survey 11**	**Statistical results**
E. pallida	3 (9)	3 (16)	3(11)	3 (7)	6 (10)	3 (9)	3 (17)	8 (28)	3 (13)	1 (3)	0 (0)	χ^2= 1.608, DF= 10, P< 0.001
E. parva	3 (9)	1 (5)	5(18)	4 (10)	12 (19)	4 (12)	2 (11)	8 (28)	9 (39)	6 (19)	4 (13)	χ^2= 17.345, DF= 10, P= 0.067
E. ovinoidalis	13 (37)	9 (47)	14(50)	20 (49)	38 (61)	12 (36)	10 (56)	16 (55)	16 (70)	19 (60)	12 (40)	χ^2= 4.211, DF= 10, P= 0.164
E. faurei	5 (14)	3 (16)	5(18)	8 (20)	8 (13)	5 (15)	3 (17)	5 (17)	3 (13)	3 (9)	0 (0)	χ^2= 7.465, DF= 10, P= 0.681
E. ahsata	5 (14)	1 (5)	7(25)	11 (27)	6 (10)	6 (18)	2 (11)	7 (24)	5 (22)	3 (9)	9 (30)	χ^2= 4.660, DF= 10, P= 0.145
E. crandallis	8 (23)	3 (16)	6(21)	13 (32)	15 (24)	12 (36)	4 (22)	12 (41)	5 (22)	5 (16)	10 (33)	χ^2= 0.492, DF= 10, P= 0.398
E. granulosa	2 (6)	1 (5)	5(18)	7 (17)	6 (10)	3 (9)	2 (11)	12 (41)	5 (22)	7 (22)	4 (13)	χ^2= 23.882, DF= 10, P= 0.008
E. bakuensis	10 (29)	8 (42)	7(25)	18 (44)	27 (44)	21 (64)	9 (50)	11 (38)	5 (22)	14 (44)	6 (20)	χ^2= 2.048, DF= 10, P= 0.015
E. intricata	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	Counts too low
E. webridgensis	0 (0)	0 (0)	5 (18)	2(5)	6 (10)	3 (9)	1 (6)	8 (28)	17 (74)	1 (3)	10 (33)	χ^2= 0.528, DF= 10, P< 0.001

Table 12: Frequency of single, dual and multiple *Eimeria* infections in ovine faecal samples on Stock Farm.

Types of infection	Frequency (%)											Statistical results
	Survey 1	Survey 2	Survey 3	Survey 4	Survey 5	Survey 6	Survey 7	Survey 8	Survey 9	Survey 10	Survey 11	
Non-infected	11 (31)	6 (32)	6 (21)	1 (2)	8 (13)	5 (15)	4 (22)	9 (31)	2 (9)	7 (22)	10 (33)	χ^2= 22.552, DF= 10, P= 0.013
Single	6 (17)	2 (11)	2 (7)	7 (17)	8 (13)	3 (9)	3 (17)	0 (0)	3 (13)	3 (9)	4 (13)	χ^2= 7.591, DF= 10, P= 0.669
Dual	11 (31)	6 (32)	9 (32)	20 (49)	25 (40)	12 (36)	4 (22)	3 (10)	5 (22)	9 (28)	8 (27)	χ^2= 16.438, DF= 10, P= 0.088
Multiple	7 (20)	5 (26)	11 (39)	13 (32)	21 (34)	13 (39)	7 (39)	17 (59)	13 (57)	13 (41)	8 (27)	χ^2= 17.382, DF= 10, P= 0.066
TOTAL	35	19	28	41	62	33	18	29	23	32	30	

Shannon's Weiner diversity index of *Eimeria* species present in ovine faecal samples at Stock Farm was calculated. The index varies between surveys (Figure 8), peaking at 2.17 in Survey 8 whilst being lowest (1.74) in Survey 2.

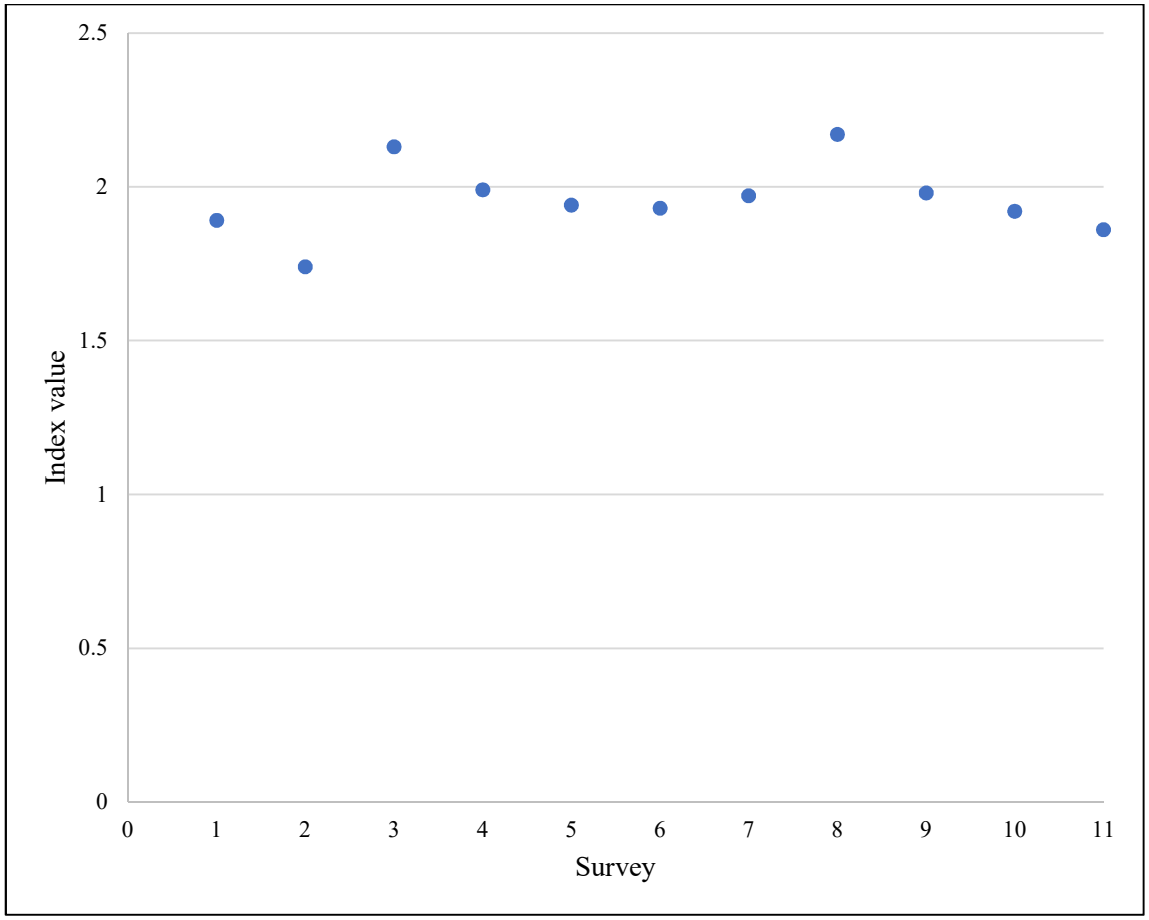


Figure 8: Shannon Weiner diversity index distribution for *Eimeria* species diversity obtained in each survey on Stock Farm.

3.2.2 *Campylobacter* infections

A total of 353 faecal samples, collected during 11 visits to Stock Farm, were tested for the presence of *Campylobacter* species (Table 13). The overall prevalence of infection in these samples (as confirmed by culture then genus-specific PCR) was 115/353 (33%). The prevalence of infection varied significantly between surveys (χ^2= 24.88, DF= 10, P= 0 .006), peaking at 63% in Survey 4 and dropping to 16% in Survey 7 (Table 13).

Table 13: Prevalence of *Campylobacter* infections in ovine faecal samples on Stock Farm.

Survey	Date	Nº samples collected	Nº samples infected (%)	Exact binomial 95% confidence interval (%)
1	16/09/14	41	10 (24)	12-40
2	10/11/14	20	7 (35)	15-59
3	22/01/15	30	10 (50)	17-52
4	18/02/15	38	24 (63)	45-78
5	23/07/15	60	17 (28)	17-41
6	11/09/15	32	7 (22)	9-39
7	09/11/15	19	3 (16)	3-39
8	24/02/16	29	12 (41)	23-61
9	09/05/16	23	7 (30)	13-52
10	25/07/16	31	7 (23)	9-41
11	12/09/16	30	11 (37)	19-56
Total		353	115 (33)	27-37

Eighteen of the isolates obtained in survey 4 (1-18), 11 of the isolates obtained in survey 5 (19-29), and five (30-34) of the isolates obtained in survey 6 were randomly selected for further characterisation using, in the first instance, *aspA* sequence analysis (Table 14). In which, 6 *aspA* allele were obtained from which four of them associated with *C. jejuni* and two of them associated with *C. coli*, all of these alleles were previously reported. MLST was then completed on nine of these isolates, four of which were *C. jejuni* and five of which were *C. coli* (Table 15). Two STs were obtained from the four *C. jejuni* isolates, ST42 and ST61. Four STs were obtained from the five *C. coli* isolates, ST827, ST828, ST1837 and a new ST, designated ST5351. All STs bar the new one lay within the ST828 clonal complex. ST5351could not be ascribed to any currently recognised clonal complex.

Table 14: Species identity and *aspA* allele number of 34 *Campylobacter* isolates obtained from sheep on Stock Farm.

Isolate number	*aspA* allele	Species identity	Isolate number	*aspA* allele	Species identity
1	9	*C. jejuni*	18	33	*C. coli*
2	9	*C. jejuni*	19	1	*C. jejuni*
3	1	*C. jejuni*	20	53	*C. coli*
4	4	*C. jejuni*	21	1	*C. jejuni*
5	33	*C. coli*	22	33	*C. coli*
6	1	*C. jejuni*	23	9	*C. jejuni*
7	1	*C. jejuni*	24	1	*C. jejuni*
8	33	*C. coli*	25	1	*C. jejuni*
9	7	*C. jejuni*	26	4	*C. jejuni*
10	7	*C. jejuni*	27	33	*C. coli*
11	33	*C. coli*	28	1	*C. jejuni*
12	53	*C. coli*	29	33	*C. coli*
13	33	*C. coli*	30	1	*C. jejuni*
14	1	*C. jejuni*	31	7	*C. jejuni*
15	1	*C. jejuni*	32	33	*C. coli*
16	4	*C. jejuni*	33	1	*C. jejuni*
17	1	*C. jejuni*	34	33	*C. coli*

Table 15: MLST data for nine selected *Campylobacter* isolates obtained from sheep on Stock Farm.

Isolate number	ST	MLST locus							Clonal complex	Species
		aspA	*glnA*	*gltA*	*glyA*	*pgm*	*tkt*	*uncA*		
15	42	1	2	3	4	5	9	3	ST- 42	*C. jejuni*
12	5351	53	38	30	81	566	71	36	New	*C. coli*
17	61	1	4	2	2	6	3	17	ST- 61	*C. jejuni*
27	1837	33	39	30	82	113	171	17	ST- 828	*C. coli*
28	42	1	2	3	4	5	9	3	ST- 42	*C. jejuni*
18	827	33	39	30	82	104	56	17	ST- 828	*C. coli*
8	828	33	39	30	82	113	171	17	ST- 828	*C. coli*
11	828	33	39	30	82	113	171	17	ST- 828	*C. coli*
25	61	1	4	2	2	6	3	17	ST- 61	*C. jejuni*

3.3 Abbot Park Farm

3.3.1 *Eimeria* infections

A total of 423 faecal samples, collected from Abbot Park Farm on 12 occasions, were tested for the presence of *Eimeria* species (Table 16). The prevalence of *Eimeria* infections ranged between 53% and 100%. Moreover, there is a significant difference between surveys (χ^2= 62.547, DF= 1, P< 0.001).

Table 16: Faecal samples collected in this study.

Survey	Date	Nº of samples collected	Nº of infected samples (%)	Exact binomial 95% confidence interval (%)
1	28/07/2015	41	41 (100)	92-100
2	20/08/2015	47	47 (100)	93-100
3	02/09/2015	55	38 (69)	55-80
4	30/10/2015	30	16 (53)	34-71
5	20/11/2015	23	14 (61)	38-80
6	04/02/2016	35	26 (74)	56-87
7	23/03/2016	33	24 (73)	54-86
8	27/04/2016	35	23 (66)	47-80
9	27/05/2016	42	36 (86)	71-94
10	08/07/2016	25	25 (100)	88-88
11	18/08/2016	30	17 (57)	37-74
12	22/09/2016	37	20 (54)	36-70

The intensity of *Eimeria* infections at Abbot Park Farm was also quantified (Table 17). A Kruskal-Wallis test was applied to these data and revealed significant differences between surveys ($H= 87.88$, $DF= 11$, $P< 0.001$).

Table 17: The mean intensity of *Eimeria* oocyst shedding at Abbot Park Farm

Survey	Mean intensity (oocysts gram^{-1})	Standard error	Range
1	460	124	4960
2	159	39	1280
3	180	25	600
4	209	75	1200
5	86	20	230
6	240	70	1720
7	329	94	2180
8	361	84	1200
9	451	92	2660
10	353	70	1420
11	240	45	580
12	62	8	100

A total of 10 *Eimeria* species were identified in these samples on the basis of microscopic observation (Table 5). These were the same *Eimeria* species as encountered on Threlkeld and Stock Farms. Of these, *E. ovinoidalis* and *E. bakuensis* where the most frequently encountered except on survey 12 where *E. parva* was the most frequent species (Table 18). The prevalence of infection for each species varied markedly between surveys, and for six species this variation was statistically significant (Table 18). Samples containing infection by single, two or more of *Eimeria* species were also encountered (Table 19). The prevalence of single, dual and multiple infections varied between surveys, and all were statistically significant (Table 19).

Table 18: *Eimeria* species observed in ovine faecal samples on Abbot Park Farm.

Species name	Frequency (%)												Statistical results
	Survey 1	Survey 2	Survey 3	Survey 4	Survey 5	Survey 6	Survey 7	Survey 8	Survey 9	Survey 10	Survey 11	Survey 12	
E. pallida	4 (10)	8 (17)	11 (20)	3 (10)	3 (13)	3 (9)	6 (18)	6 (17)	12 (29)	6 (24)	4 (13)	3 (8)	χ^2= 11.353, DF= 11, P= 0.414
E. parva	7 (17)	17 (36)	7 (13)	2 (6.7)	3 (13)	10 (29)	10 (30)	12 (34)	22 (52)	9 (36)	13 (43)	14 (38)	χ^2= 33.424, DF= 11, P< 0.001
E. ovinoidalis	19 (46)	20 (43)	14 (26)	14 (47)	10 (44)	21 (60)	16 (49)	19 (54)	16 (38)	20 (80)	14 (47)	9 (24)	χ^2= 37.792, DF= 11, P< 0.001
E. faurei	5 (12)	5 (11)	5 (9)	3 (10)	1 (4)	1 (2)	8 (24)	6 (17)	3 (7)	1 (4)	0 (0)	4 (11)	χ^2= 17.921, DF= 11, P= 0.083
E. ahsata	6 (15)	7 (15)	4 (7)	3 (10)	1 (4)	4 (11)	14 (42)	5 (14)	11 (26)	16 (64)	2 (7)	5 (14)	χ^2= 62.255, DF= 11, P< 0.001
E. crandallis	3 (7)	5 (11)	4 (7)	0 (0)	0 (0)	6 (17)	6 (18)	7 (20)	3 (7)	3 (12)	3 (10)	5 (14)	χ^2=13.860, DF= 11, P= 0.241
E. granulosa	12 (29)	9 (19)	3 (6)	6 (20)	8 (35)	10 (29)	6 (18)	5 (14)	11 (26)	2 (8)	3 (10)	2 (5)	χ^2=27.379, DF= 11, P= 0.004
E. bakuensis	25 (61)	29 (62)	21 (38)	13 (43)	10 (44)	14 (40)	12 (36)	17 (49)	26 (62)	14 (56)	8 (27)	9 (24)	χ^2= 31.216, DF= 11, P< 0.001
E. intricata	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	2 (5)	1 (4)	0 (0)	0 (0)	Counts too low
E. webridgensis	1 (2)	1 (2)	1 (2)	2 (7)	0 (0)	7 (20)	14 (42)	9 (26)	22 (52)	9 (36)	9 (30)	3 (8)	χ^2= 88.046, DF= 11, P< 0.001

Table 19: Frequency of single, dual and multiple infections in sheep at Abbot Park Farm.

Types of infection	Frequency (%)												Statistical results
	Survey 1	**Survey 2**	**Survey 3**	**Survey 4**	**Survey 5**	**Survey 6**	**Survey 7**	**Survey 8**	**Survey 9**	**Survey 10**	**Survey 11**	**Survey 12**	
Non-infected	0 (0)	0 (0)	17 (31)	14 (47)	9 (39)	9 (26)	9 (27)	12 (34)	6 (14)	0 (0)	13 (43)	17 (46)	χ^2= 67.856, DF= 11, P< 0.001
Single	10 (24)	13 (28)	12 (22)	2 (7)	1 (4)	5 (14)	2 (6)	0 (0)	1 (2)	2 (8)	0 (0)	2 (5)	χ^2= 40.959, DF= 11, P< 0.001
Dual	20 (49)	18 (38)	21 (38)	5 (17)	6 (26)	5 (14)	3 (9)	8 (23)	8 (19)	4 (16)	3 (10)	7 (19)	χ^2= 35.962, DF= 11, P< 0.001
Multiple	11 (27)	16 (34)	5 (9)	9 (30)	7 (30)	16 (46)	19 (58)	15 (43)	27 (64)	19 (76)	14 (47)	11 (30)	χ^2= 58.854, DF= 11, P< 0.001
TOTAL	41	47	55	30	23	35	33	35	42	25	30	37	

Shannon's Weiner diversity index of *Eimeria* species present in ovine faecal samples at Abbot Park Farm was calculated for twelve occasions. The index shows the high diversity of *Eimeria* species encountered on survey 7 in which the values was 2.17 compared to the lowest *Eimeria* species diversity on survey 1 in which the index value was 1.29 (Figure 9).

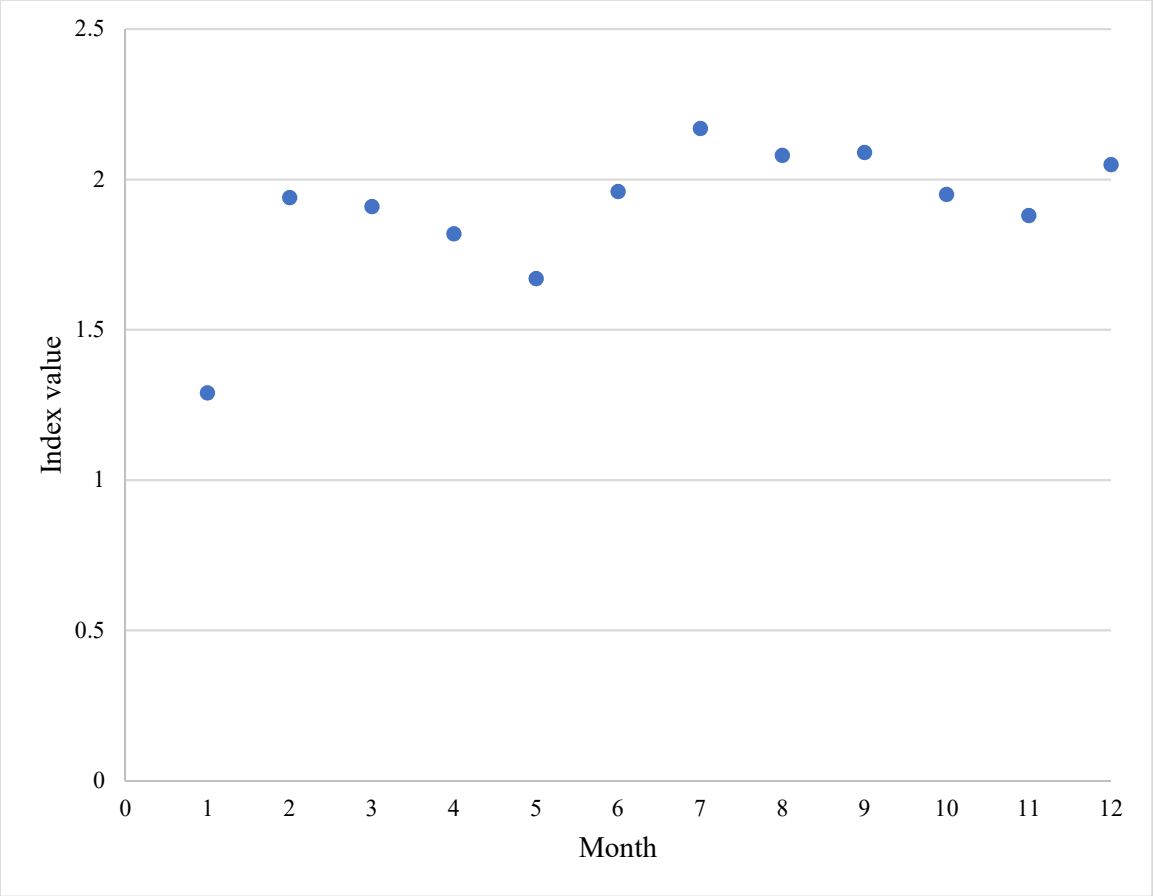


Figure 9: The Shannon's Weiner diversity index distribution according to the months at Abbot Park Farm.

3.3.2 *Campylobacter* infections

A total of 430 faecal samples, collected from Abbot Park Farm on 12 occasions, were tested for the presence of *Campylobacter* species. *Campylobacter* infections were detected in all surveys at prevalence ranging from 10.8 to 41.9%. No significant inter-survey variation in prevalence was detected (χ^2= 15.468, DF= 11, P= 0.162) (Table 20).

Table 20: Prevalence of *Campylobacter* infections in sheep at Abbot Park Farm.

Survey	Date	Nº of samples collected	Nº of infected samples (%)	Exact binomial 95% confidence interval (%)
1	28/07/2015	40	10 (25)	12-41
2	20/08/2015	52	20 (39)	25-52
3	02/09/2015	55	15 (27)	16-40
4	30/10/2015	30	7 (23)	9-42
5	20/11/2015	23	5 (22)	7-43
6	04/02/2016	36	10 (28)	14-45
7	23/03/2016	34	13 (38)	22-56
8	27/04/2016	31	13 (42)	24-60
9	27/05/2016	37	14 (38)	22-55
10	08/07/2016	25	7 (28)	12-49
11	18/08/2016	30	7 (23)	9-42
12	22/09/2016	37	4 (11)	3-25
TOTAL		430	125 (29)	24-33

PCR-based delineation of *C. coli* and *C. jejuni* from one another and from other *Campylobacter* species was performed on all 125 *Campylobacter* isolates obtained from Abbott Park Farm using species-specific PCRs. *C. coli* and/or *C. jejuni* were detected in most (105) of the samples (Table 21). Although *C. jejuni* infections were more common than *C. coli* infections, dual infections involving both species, were significantly more common than single species infections (or infections by other *Campylobacter* species) (χ^2= 32.0, DF= 3, P< 0.001) (Table 21).

Table 21: Relative abundance of *C. coli* and *C. jejuni* with coinfection in sheep surveyed on Abbott Park Farm.

Survey	Nº cultures tested	*C. coli* only	*C. jejuni* only	*C. coli* + *C. jejuni*	Other species
1	10	3	1	6	0
2	20	0	3	17	0
3	15	3	3	8	1
4	7	1	3	2	1
5	5	2	1	2	0
6	10	1	3	6	0
7	13	1	7	5	0
8	13	1	3	3	6
9	14	0	4	4	6
10	7	2	2	1	2
11	7	7	0	0	0
12	4	0	0	0	4
Total	125	21	30	54	20

Further characterisation of a subset of isolates, six from survey 1(1-6), 16 from survey 2 (7-22) and seven from survey 4 (23-29) was carried out using *aspA* sequence analysis (Table 22). The same isolates were identified by using Species-specific PCR. In which nine of isolates identified as *C. jejuni*, four as *C. coli*, 15 as *C. jejuni*/ *C. coli.* However, sample 29 identified as neither of *C. jejuni* nor *C. coli* by the use of Species identity (species-specific PCR), in contrast with the data of *aspA* in which the sample was identified as mixed infection. This may be related to ambiguous of *aspA* data.

Table 22: Species identity and *aspA* allele number of 29 *Campylobacter* isolates obtained from sheep on Abbott Park Farm.

Isolate number	***aspA* allele**	**Species identity (*aspA*)**	**Species identity (species-specific PCR)**	**Isolate number**	***aspA* allele**	**Species identity (*aspA*)**	**Species identity (species-specific PCR)**
1	7	*C. jejuni*	*C. jejuni*	16	53	*C. coli*	mixed
2	33	*C. coli*	*C. coli*	17	33	*C. jejuni*	mixed
3	33	*C. coli*	*C. coli*	18	53	*C. jejuni*	mixed
4	53	*C. coli*	mixed	19	33	*C. jejuni*	mixed
5	53	*C. coli*	*C. coli*	20	33	*C. coli*	mixed
6	33	*C. coli*	*C. jejuni*	21	53	*C. coli*	mixed
7	33	*C. coli*	mixed	22	2	mixed	*C. jejuni*
8	1	*C. jejuni*	*C. jejuni*	23	2	*C. jejuni*	*C. jejuni*
9	53	*C. coli*	mixed	24	1	*C. jejuni*	*C. jejuni*
10	2	*C. jejuni*	*C. jejuni*	25	1	*C. jejuni*	*C. jejuni*
11	1	*C. jejuni*	*C. jejuni*	26	33	*C. coli*	*C. coli*
12	4	*C. jejuni*	mixed	27	53	*C. coli*	mixed
13	1	*C. jejuni*	mixed	28	1	*C. jejuni*	mixed
14	1	*C. jejuni*	mixed	29	2	mixed	other
15	33	*C. coli*	mixed				

3.4 Combined data

3.4.1 Seasonal trends

3.4.1.1 *Eimeria* prevalence

Eimeria infection prevalence data for the three farms were stratified by season (March to May = spring, June to August = summer, September to November = autumn and December to February = winter) (Figure 10). Infection prevalence varied significantly by season (χ^2= 35.309, DF= 3, P< 0.001).

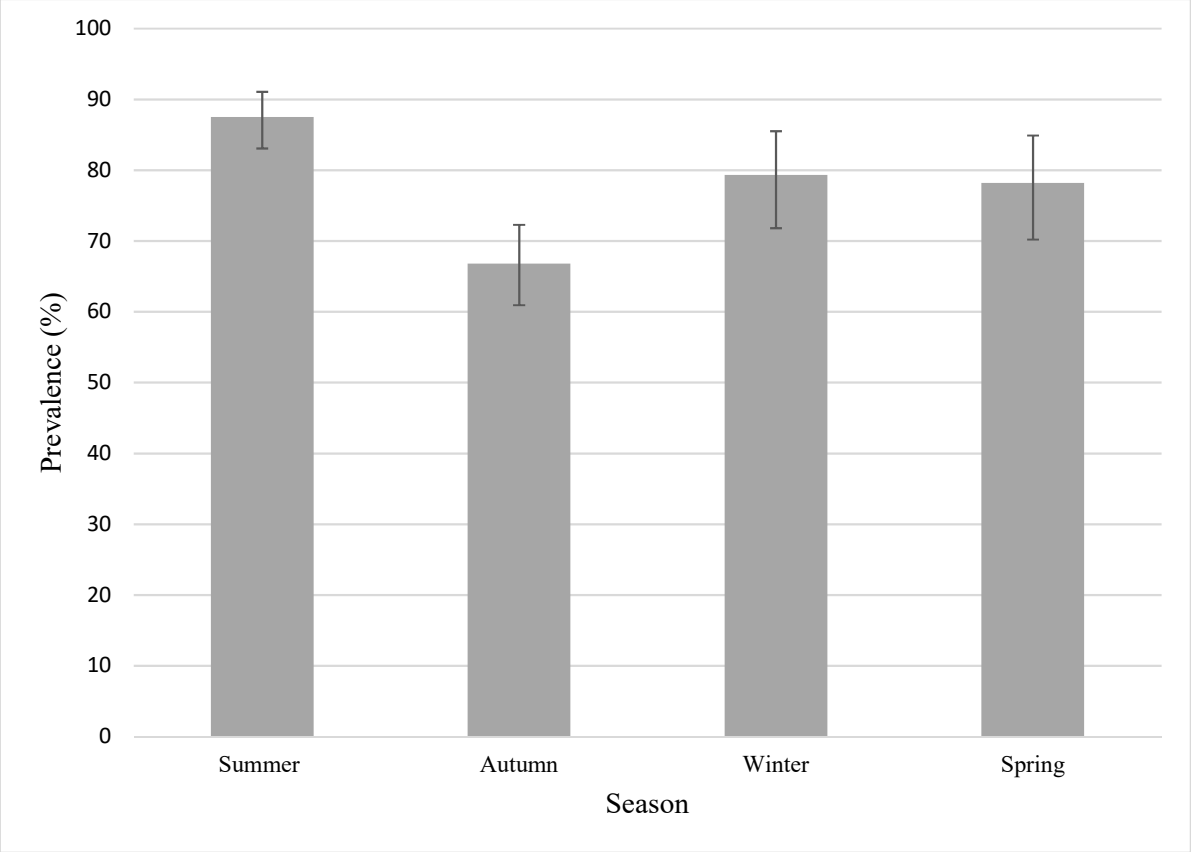


Figure 10: The overall seasonal prevalence of *Eimeria* infection at three farms.

The pairwise comparison indicated *Eimeria* prevalence was significantly greater in summer than autumn, winter or spring. Moreover, the prevalence of *Eimeria* infection was also significantly lower in autumn than in either spring or winter (Table 23).

Table 23: Pairwise comparison of *Eimeria* prevalence in different seasons.

Seasons		Statistical results
summer	winter	χ^2= 4.939, DF= 1, P= 0.026
summer	autumn	χ^2= 34.456, DF= 1, P< 0.001
summer	spring	χ^2= 5.959, DF= 1, P= 0.0146
winter	autumn	χ^2= 7.287, DF= 1, P= 0.007
winter	spring	χ^2= 0.052, DF= 1, P= 0.819
autumn	spring	χ^2= 5.640, DF= 1, P= 0.017

3.4.1.2 *Campylobacter* prevalence

Campylobacter infection prevalence data for the three farms were also stratified by season. As observed for *Eimeria* infections, the overall prevalence of *Campylobacter* infections varied significantly by season (χ^2= 12.682, DF= 3, P= 0.005) (Figure 11).

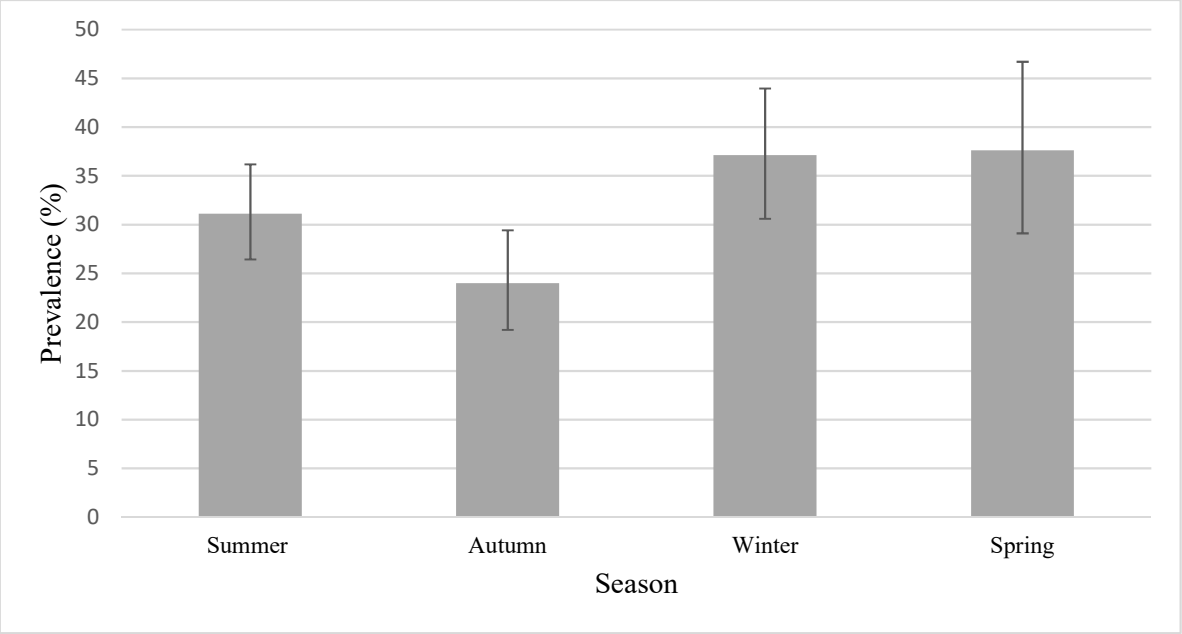


Figure 11: The seasonal prevalence of *Campylobacter* infections on the three study farms.

Pairwise comparisons revealed that although *Campylobacter* infection prevalence was highest in spring, this prevalence did not vary significantly from the prevalence in either summer or winter. However, the *Campylobacter* infection prevalence in autumn was significantly lower than those in the other three seasons (Table 24).

Table 24: Pairwise comparison of *Campylobacter* prevalence in different seasons.

Seasons		Statistical results
summer	winter	χ^2= 2.139, DF= 1, P= 0.143
summer	autumn	χ^2= 4.027, DF= 1, P= 0.044
summer	spring	χ^2= 1.760, DF= 1, P= 0.184
winter	autumn	χ^2= 9.988, DF= 1, P= 0.001
winter	spring	χ^2= 0.009, DF= 1, P= 0.924
autumn	spring	χ^2= 7.913, DF= 1, P= 0.004

3.4.1.3 *Eimeria* infection intensity

The mean intensity of *Eimeria* infection for the three farms was stratified by season (Figure 12). The Kruskal-Wallis test was used to explore the differences in the overall intensity of *Eimeria* infections at three farms according to the season, and demonstrated a significant difference (H= 77.16, DF= 3 P<0.001). Pairwise comparison indicated *Eimeria* intensity was significantly greater in summer than in either autumn, spring or winter (Mann Whitney test, W= 31303, P< 0.001, W= 15977, P= 0.004, W= 19983, P< 0.001 respectively). Similarly, mean *Eimeria* infection intensity was significantly greater in spring than either autumn or winter (W= 24630, P> 0.000, W= 14722, P< 0.001 respectively). There was no significant difference between the overall *Eimeria* infection intensities in autumn and winter. (W= 29587, P= 0.206).

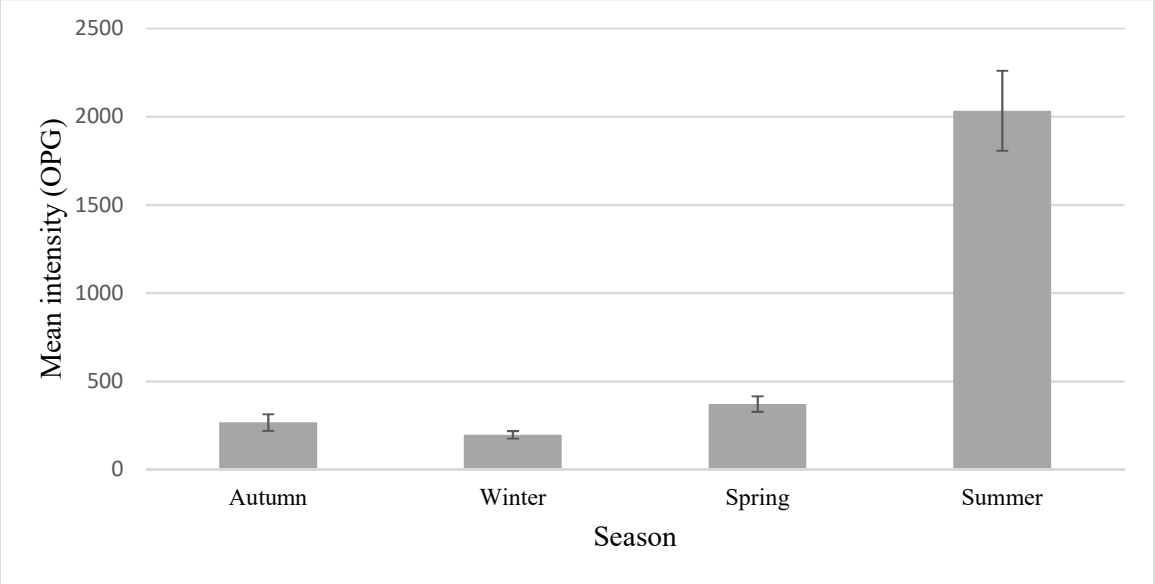


Figure 12: The overall seasonal intensity (mean ± SE) of *Eimeria* infections on the three study farms.

3.4.1.4 Prevalence of *Eimeria* species

The seasonally-stratified prevalence data for each of the ten *Eimeria* species encountered in this study were compared (Table 25). *E. ovinoidalis* was the most frequently encountered species in all seasons except for autumn, in which *E. bakuensis* was the most frequently encountered (Table 25). For seven of the 10 species, significant inter-seasonal variation in prevalence was observed (Table 25).

Table 25: The seasonal prevalence of the 10 *Eimeria* species detected in ovine faecal samples collected from the three farms included in this study.

***Eimeria* species**	**Autumn**	**Winter**	**Spring**	**Summer**	**Statistical results**
E. pallida	32(11)	21 (12)	27 (21)	39 (14)	χ^2= 6.888, DF= 3, P= 0.076
E. parva	40(14)	37 (21)	53 (41)	77 (27)	χ^2= 36.063, DF= 3, P< 0.001
E. ovinoidalis	103(37)	88 (51)	67 (51)	149 (52)	χ^2= 16.440, DF= 3, P< 0.001
E. faurei	29(10)	20 (12)	20 (15)	33 (12)	χ^2= 2.115, DF= 3, P= 0.549
E. ahsata	36(13)	33 (19)	35 (27)	52 (18)	χ^2= 11.942, DF= 3, P <0.001
E. crandallis	46(16)	45 (26)	21 (16)	48 (17)	χ^2= 8.437, DF= 3, P= 0.038
E. granulosa	31(11)	36 (21)	27 (21)	47 (16)	χ^2= 10.022, DF= 3, P= 0.018
E. bakuensis	107(38)	60 (35)	60 (46)	131 (46)	χ^2= 7.551, DF= 3, P= 0.056
E. intricata	0 (0)	0 (0)	3 (2)	5 (2)	χ^2= 9.047, DF= 3, P= 0.029
E. webridgensis	20(7)	22 (13)	62 (47)	31 (11)	χ^2= 122.118, DF= 3, P >0.001

3.4.1.5 *Eimeria* co-infection prevalence

The overall prevalence of single, dual and multiple infections varied between seasons, and these variations were significantly different (Table 26). The single, dual and multiple infections by *Eimeria* species were significantly more frequent in the summer compared to other seasons on the three farms (Table 26).

Table 26: Frequency of single, dual and multiple infections in sheep at three farms according to the season.

Types of infection	**Frequency (%)**				**Statistical results**
	Autumn	**Winter**	**Spring**	**Summer**	
Single	35 (13)	22 (13)	6 (5)	47 (16)	χ^2= 11.623, DF= 3, P= 0.009
Dual	80 (29)	52 (30)	24 (18)	109 (38)	χ^2= 17.853, DF= 3, P< 0.001
Multiple	72 (26)	63 (36)	74 (56)	104 (36)	χ^2= 37.772, DF= 3, P< 0.001

Pairwise analyses between seasons according to the type of infection were conducted. These revealed significant differences between spring and each of autumn, winter and summer in single species infections. However, there was no significant difference between autumn and each of winter and summer or between winter and summer (Table 27). For dual-species *Eimeria* infections, there was a significant difference between autumn and each of spring and summer in addition to spring and each of winter and summer. However, there was no significant difference between winter and each of autumn and summer (Table 27). A significant difference was recognised in the prevalence of multiple *Eimeria* species infections between autumn and each of winter and spring in addition to spring and each of winter and summer. However, there was no significant difference between summer and each of autumn and winter (Table 27).

Table 27: The statistical results of pairwise comparison between different single, dual and multiple *Eimeria* infections in different seasons.

Season		Type of infection/ Statistical results		
		Single	**Dual**	**Multiple**
autumn	winter	χ^2= 0.005, DF= 1, P= 0.946	χ^2= 0.114, DF= 1, P= 0.735	χ^2= 5.854, DF= 1, P= 0.016
autumn	spring	χ^2= 6.436, DF= 1, P= 0.011	χ^2= 5.303, DF= 1, P= 0.021	χ^2= 35.331, DF= 1, P< 0.001
autumn	summer	χ^2= 1.721, DF= 1, P= 0.190	χ^2= 5.645, DF= 1, P= 0.018	χ^2= 2.334, DF= 1, P= 0.127
winter	spring	χ^2= 6.900, DF= 1, P= 0.014	χ^2= 5.812, DF= 1, P= 0.016	χ^2= 11.237, DF= 1, P<0.001
winter	summer	χ^2= 1.134, DF= 1, P= 0.287	χ^2= 2.977, DF= 1, P= 0.084	χ^2= 1.332, DF= 1, P= 0.248
spring	summer	χ^2= 11.603, DF= 1, P< 0.001	χ^2= 16.689, DF= 1, P< 0.001	χ^2= 23.760, DF= 1, P< 0.001

3.4.2 Age effects

3.4.2.1 *Eimeria* prevalence

Eimeria infection prevalence data were collated from sheep surveyed on Stock Farm and Abbot Park Farm and were stratified by age group (1-12 months, 13-24 months and more than 24 months) (Figure 13). Overall infection prevalence of *Eimeria* varied significantly by age group (χ^2= 10.971, DF= 2, P= 0.004). Pairwise comparison indicated *Eimeria* prevalence was significantly greater in animals aged of 1-12 months than either animals aged 13-24 months or animals of greater than 24 months age. However, there was no significant difference in *Eimeria* prevalence between animals aged from 13-24 months and older animals (Figure 13).

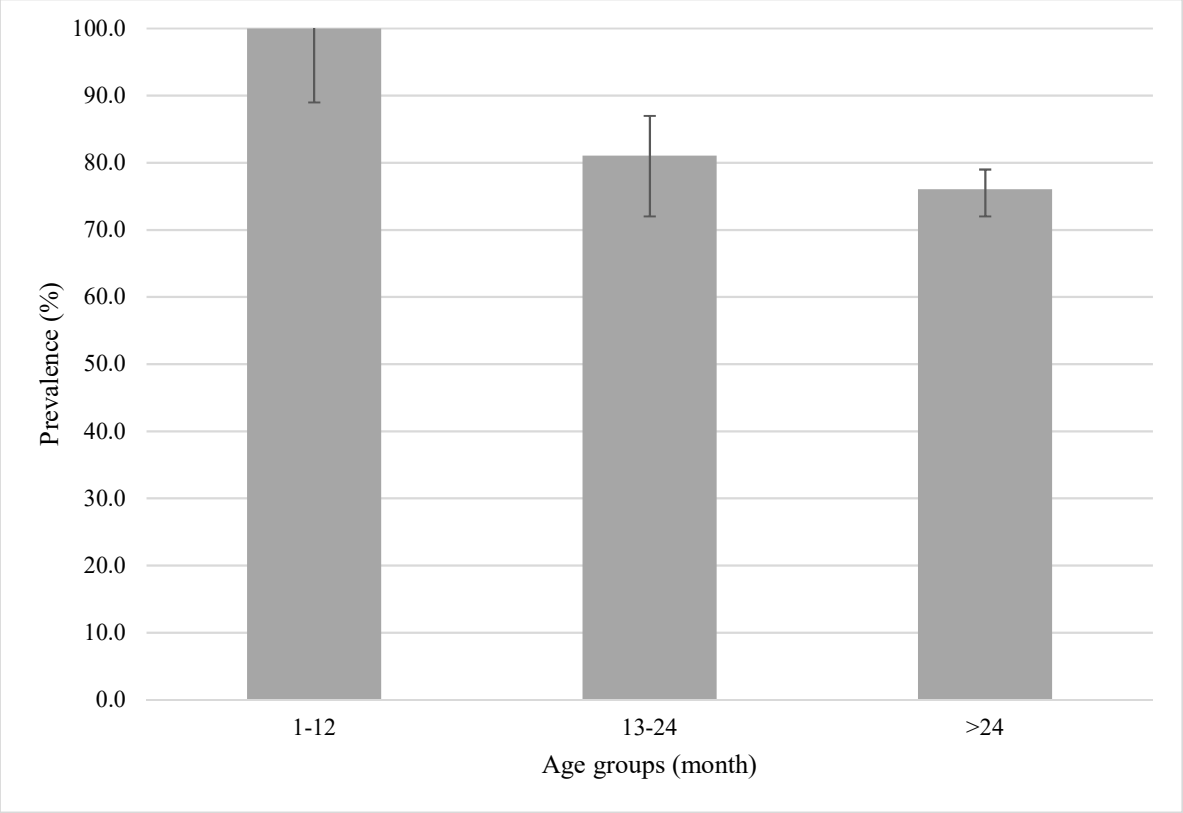


Figure 13: The overall age-related prevalence (± SE) of *Eimeria* infections in sheep on Stock Farm and Abbot Park Farm.

3.4.2.2 *Campylobacter* prevalence

Campylobacter infection prevalence data were collated from sheep surveyed on Stock Farm and Abbot Park Farm and were stratified by age group, as described above. Although the prevalence of *Campylobacter* infections varied between age groups, with the mean prevalence being lowest for animals under 12 months old and highest for animals over 24 months old, these variations were not significantly different (χ^2= 0.996, DF= 2, P= 0.608) (Figure 14).

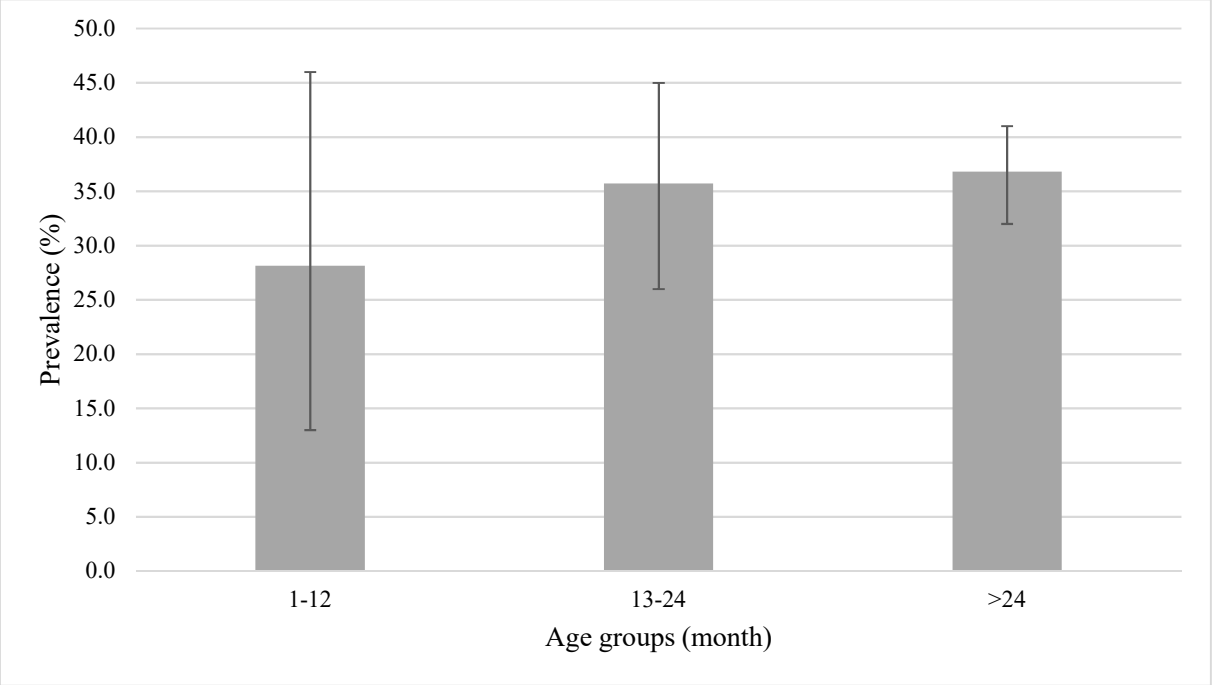


Figure 14: The overall age-related prevalence (± SE) of *Campylobacter* infections in sheep on Stock Farm and Abbot Park Farm.

3.4.2.3 *Eimeria* infection intensity

The intensities of *Eimeria* infections in sheep on Abbot Park Farm and Stock Farm were also stratified by age group (Table 28), and statistical analysis of these data (Kruskal-Wallis test) indicated significant variation between the age groups (H= 83.33 DF= 2, P< 0.001) (Table 28). Pairwise comparison (using the Mann-Whitney test) indicated *Eimeria* shedding intensity was significantly greater in animals aged from 1-12 months than either animals aged between 13-24 months or animal aged > 24 months (W= 3241, P< 0.001 and W= 15093, P< 0.001 respectively). Moreover, animals aged between 12 and 24 months shed a significantly higher number of *Eimeria* oocysts than animals aged over 24 months (W= 32814, P< 0.000).

Table 28: *Eimeria* infection intensites in the three age groups of sheep on Abbot Park Farm and Stock Farm.

Age (month)	Mean intensity	Standard error	Range
1-2	14684	9379	299980
13-24	548	97	7260
>24	327	104	49980

3.4.2.4 Prevalence of *Eimeria* species

The prevalence of each of the ten *Eimeria* species encountered in sheep on Abbot Park Farm and Stock Farm was compared in animals belonging to different age groups (Table 29). *E. ovinoidalis* was the most frequently encountered species in all age groups. Statistical analyses (using Chi-squared test) indicated that infection prevaelnce varied significantly between age groups for four *Eimeria* species, namely *E. ovinoidalis, E. faurei, E. bakuensis* and *E. webridgensis* (Table 29). Pairwise comparison between different *Eimeria* species according to the age group was conducted (Table 30).

Table 29: The prevalence (%) of individual *Eimeria* species observed in ovine faecal samples on both Abbot Park Farm and Stock Farm stratified by sheep age.

***Eimeria* species**	**Age groups (month)**			**Statistical results**
	1-12	**13-24**	**>24**	
E. pallida	2 (6)	22 (19)	81 (13)	χ^2= 2.748, DF= 2, P= 0.253
E. parva	11 (34)	30 (26)	143 (23)	χ^2= 2.777, DF= 2, P= 0.249
E. ovinoidalis	25 (78)	54 (47)	291 (46)	χ^2= 12.723, DF= 2, P= 0.002
E. faurei	4 (13)	25 (22)	61 (10)	χ^2= 3.736, DF= 2, P< 0.001
E. ahsata	6 (19)	24 (21)	110 (17)	χ^2= 0.760, DF= 2, P= 0.684
E. crandallis	7 (22)	25 (22)	106 (17)	χ^2= 1.986, DF= 2, P= 0.370
E. granulosa	8 (25)	14 (12)	108 (17)	χ^2= 3.433, DF= 2, P= 0.180
E. bakuensis	21 (66)	51 (44)	261 (41)	χ^2= 7.559, DF= 2, P= 0.023
E. intricata	1 (3)	3 (3)	4 (1)	Count too low
E. webridgensis	0 (0)	21 (18)	103 (16)	χ^2= 6.541, DF= 2, P= 0.038

Table 30: The statistical results of pairwise comparison between different *Eimeria* species according to the age group of animals.

Age groups (month)	*Eimeria ovinoidalis*	*Eimeria faurei*	*Eimeria bakuensis*	*Eimeria webridgensis*
(1-12)- (13-24)	χ^2= 10.047, DF= 1, P= 0.002	χ^2= 1.304, DF= 1, P= 0.253	χ^2= 4.710, DF= 1, P= 0.030	χ^2= 6.75, DF= 1, P= 0.009
(1-12)- (>24)	χ^2= 12.687, DF= 1, P< 0.000	χ^2= 0.287, DF= 1, P= 0.592	χ^2= 7.464, DF= 1, P= 0.006	χ^2= 6.150, DF= 1, P= 0.013
(13-24)- (>24)	χ^2= 0.017, DF= 1, P= 0.897	χ^2= 13.748, DF= 1, P< 0.000	χ^2= 0.316, DF= 1, P= 0.574	χ^2= 0.245, DF= 1, P= 0.621

3.4.2.5 *Eimeria* co-infection prevalence

Samples containing infection by single, two and multiple *Eimeria* species were also stratified according to the age of animals (Table 31). There is no significant difference in the prevalence of single, dual and multiple infections according to the age groups (Table 31).

Table 31: Frequency of single, dual and multiple *Eimeria* infections in sheep on Abbot Park Farm and Stock Farm stratified by age.

Type of infection	Frequency (%)			Statistical results
	1-12months	13-24months	>24months	
Single	0 (0)	16 (14)	75 (12)	χ^2= 4.899, DF= 2, P= 0.086
Dual	14 (42)	33 (28)	172 (27)	χ^2= 3.658, DF= 2, P= 0.161
Multiple	19 (58)	44 (38)	234 (37)	χ^2= 5.691, DF= 2, P= 0.058

3.4.3 Co-infection effects

The frequencies with which sheep were infected with either *Eimeria* or *Campylobacter* or both, or neither, were calculated (Table 32). No significant correlation between infections by the two microorganisms was detected (Table 32).

Table 32: Frequency of co-infections between *Campylobacter* and *Eimeria* species in sheep surveyed in this study.

Farm	***Eimeria* infection status**	***Campylobacter* infection status**		**Statistical results**
		Infected	Uninfected	
Abbot Park Farm	Infected	96	222	χ^2= 0.250, DF= 1, P= 0.617
	Uninfected	29	76	
Stock Farm	Infected	94	180	χ^2= 0.093, DF= 1, P= 0.761
	Uninfected	22	46	
Threlkeld Farm	Infected	22	49	χ^2= 0.002, DF= 1, P= 0.960
	Uninfected	6	13	
Total	Infected	212	451	χ^2= 0.362, DF= 1, P= 0.548
	Uninfected	57	135	

3.4.4 Climate effects

3.4.4.1 Correlation between *Eimeria* prevalence and mean monthly temperature

For each of the 25 surveys of *Eimeria* infections carried out on the three study farms, the mean temperature of the month of the survey. Temperature data were combined with *Eimeria* infection prevalence data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 15). The Pearson correlation for the association between *Eimeria* infection prevalence and mean monthly temperature was 0.072, hence no correlation was apparent (P= 0.734).

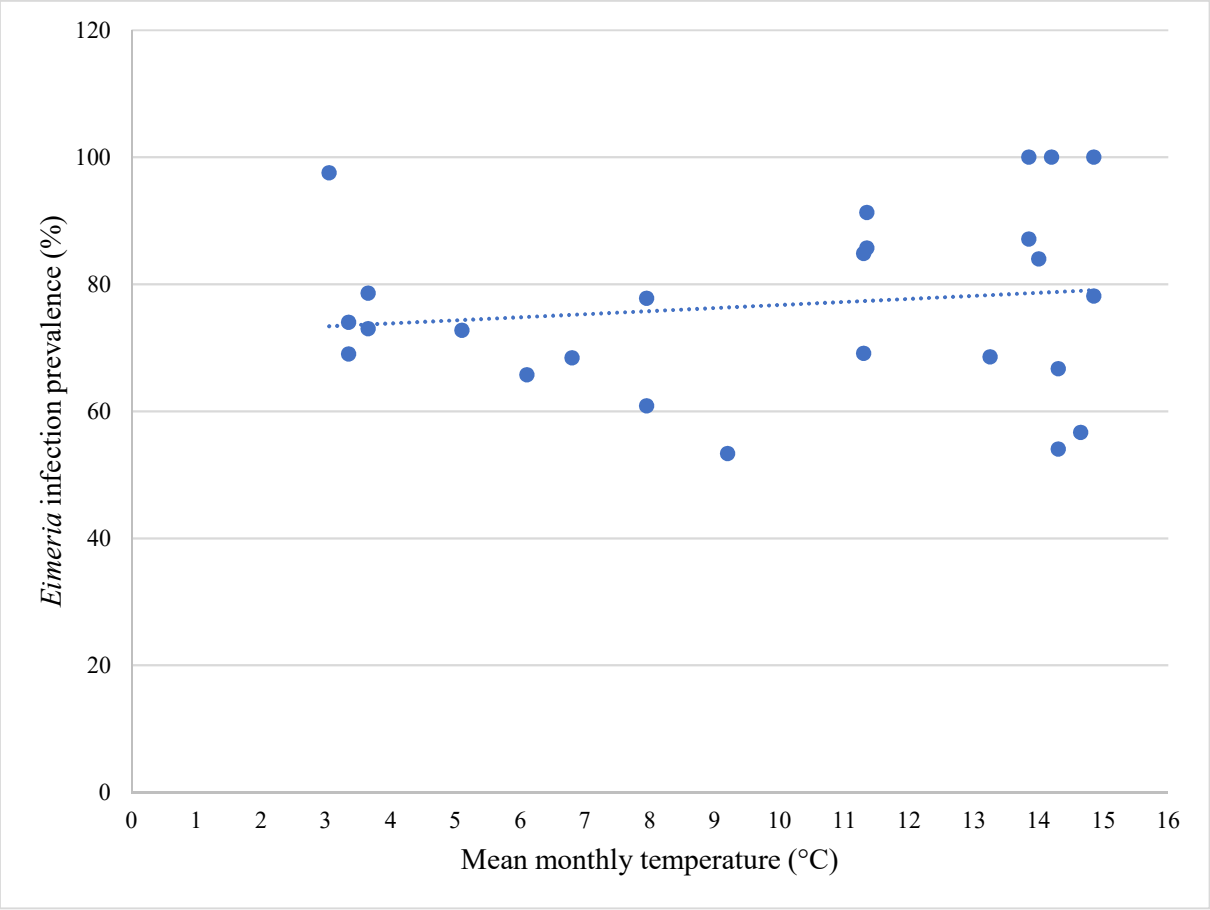


Figure 15: Scatterplot of *Eimeria* infection prevalence in each survey and mean monthly temperature for the month the survey took place.

3.4.4.2 Correlation between *Eimeria* infection prevalence and mean temperature in month prior to survey

For each of the 25 surveys of *Eimeria* infections carried out on the three study farms, the mean temperatures of the months prior to those in which the survey took place were combined with *Eimeria* infection prevalence data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 16). The Pearson correlation for the association between *Eimeria* infection prevalence and mean temperature in the month prior to the survey was -0.118, hence no correlation was apparent (P= 0.574).

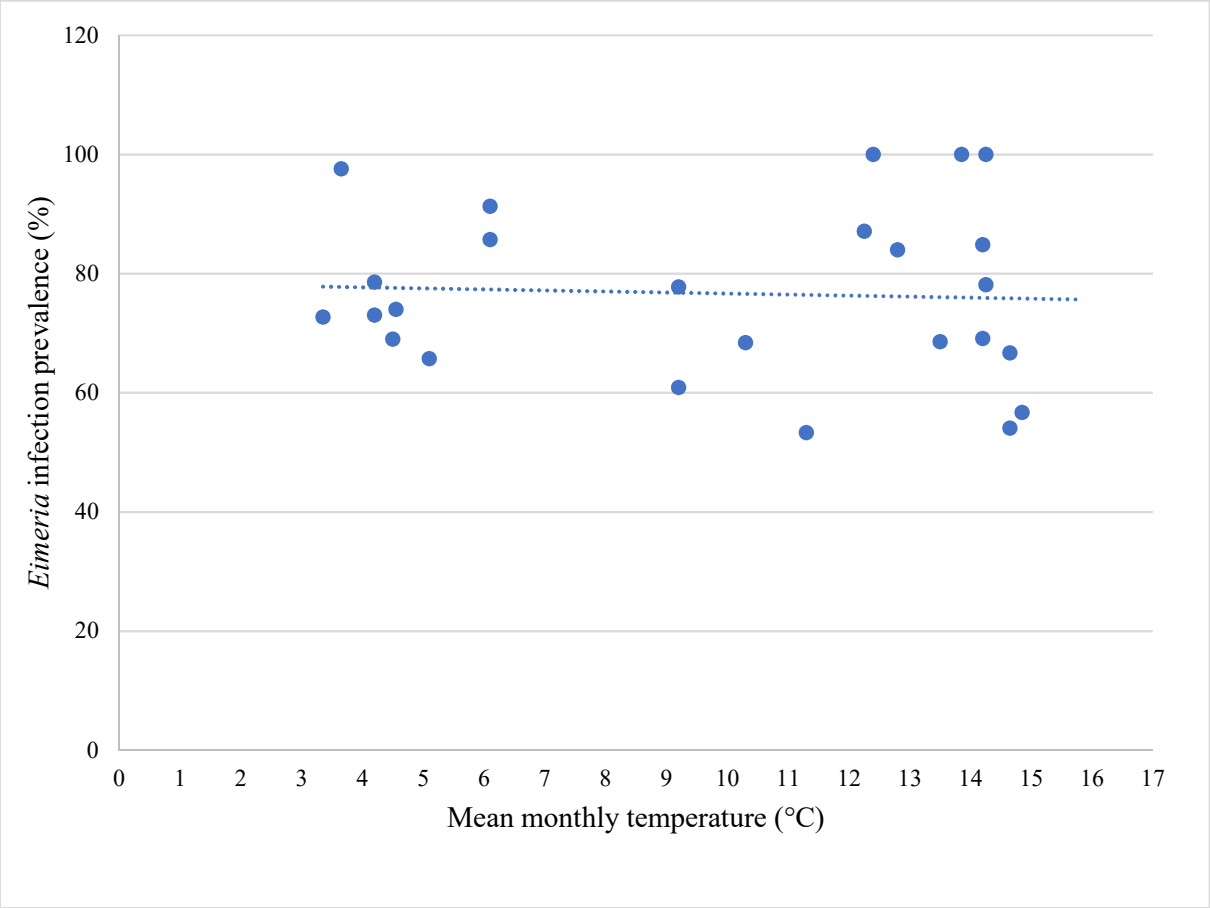


Figure 16: Scatterplot of *Eimeria* infection prevalence in each survey and mean temperature for the month prior to that in which the survey took place.

3.4.4.3 Correlation between *Campylobacter* prevalence and mean monthly temperature

For each of the 27 surveys of *Campylobacter* infections carried out on the three study farms, the mean temperature of the month of the survey was obtained and these data were combined with *Campylobacter* infection prevalence data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 17). The Pearson correlation for the association between *Campylobacter* infection prevalence and mean monthly temperature was -0.380, hence a weak non-significant negative linear correlation was apparent (P= 0.051).

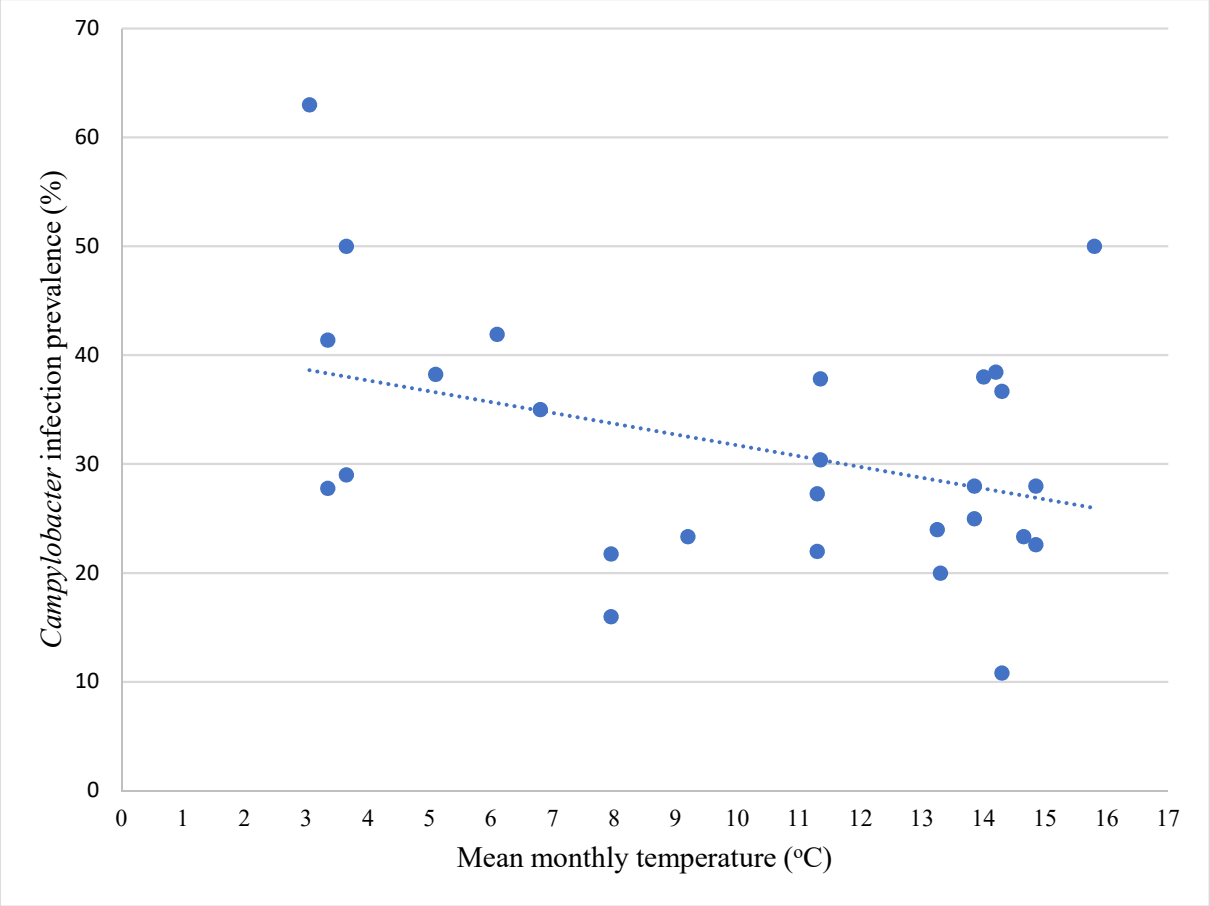


Figure 17: Scatterplot of *Campylobacter* infection prevalence in each survey and mean monthly temperature for the month the survey took place.

3.4.4.4 Correlation between *Campylobacter* infection prevalence and mean temperature in month prior to survey

For each of the 27 surveys of *Campylobacter* infections carried out on the three study farms, the mean temperature of the month prior to that in which the survey took place was obtained, and these data were combined with *Campylobacter* infection prevalence data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 18). The Pearson correlation for the association between *Campylobacter* infection prevalence and mean temperature in the month prior to the survey was -0.568, hence a moderate but significant negative correlation was apparent (P= 0.003).

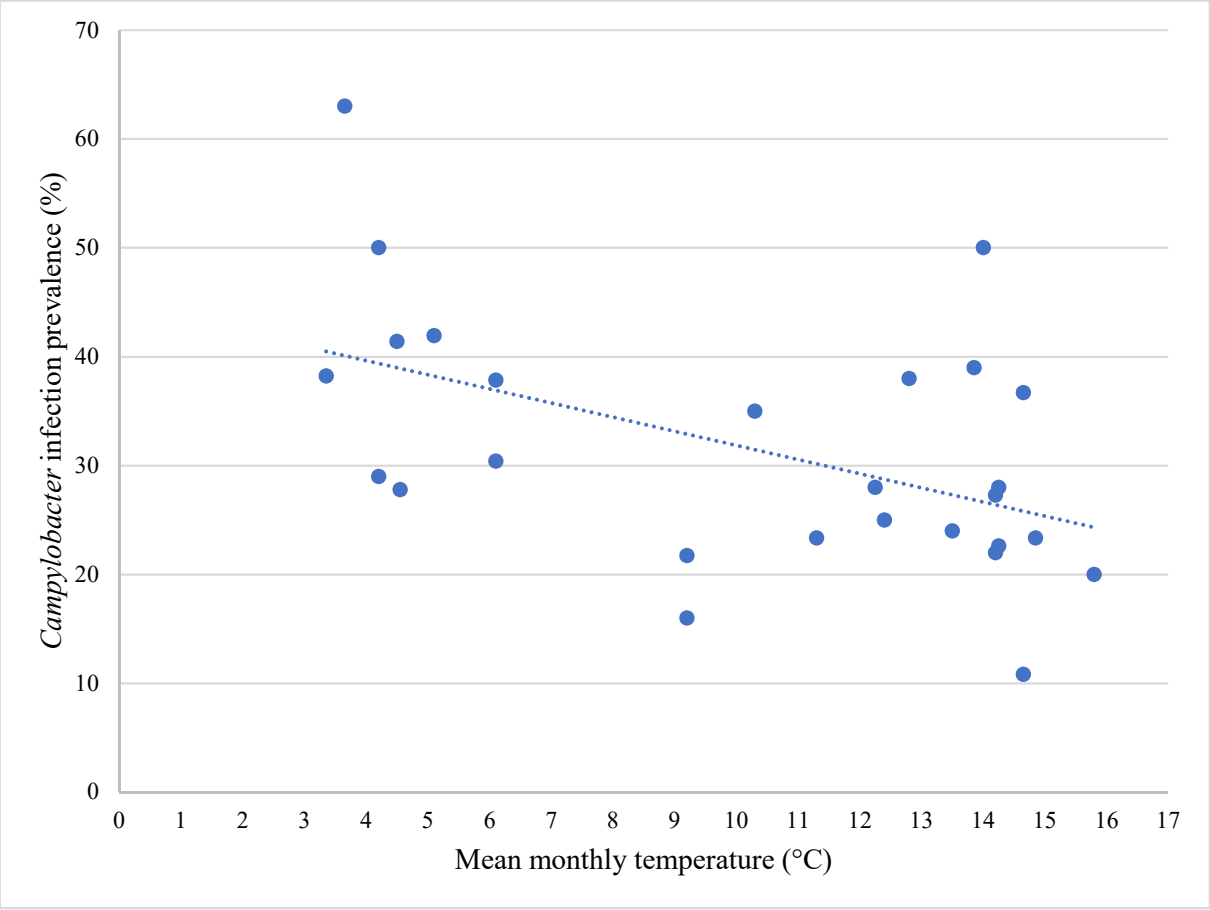


Figure 18: Scatterplot of *Campylobacter* infection prevalence in each survey and mean temperature for the month prior to that in which the survey took place.

3.4.4.5 Correlation between *Eimeria* prevalence and monthly rainfall

For each of the 25 surveys of *Eimeria* infections carried out on the three study farms, the rainfall for the month of the survey was obtained and these data were combined with *Eimeria* infection prevalence data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 19). The Pearson correlation for the association between *Eimeria* infection prevalence and monthly rainfall was -0.110, hence no correlation was apparent (P= 0.600).

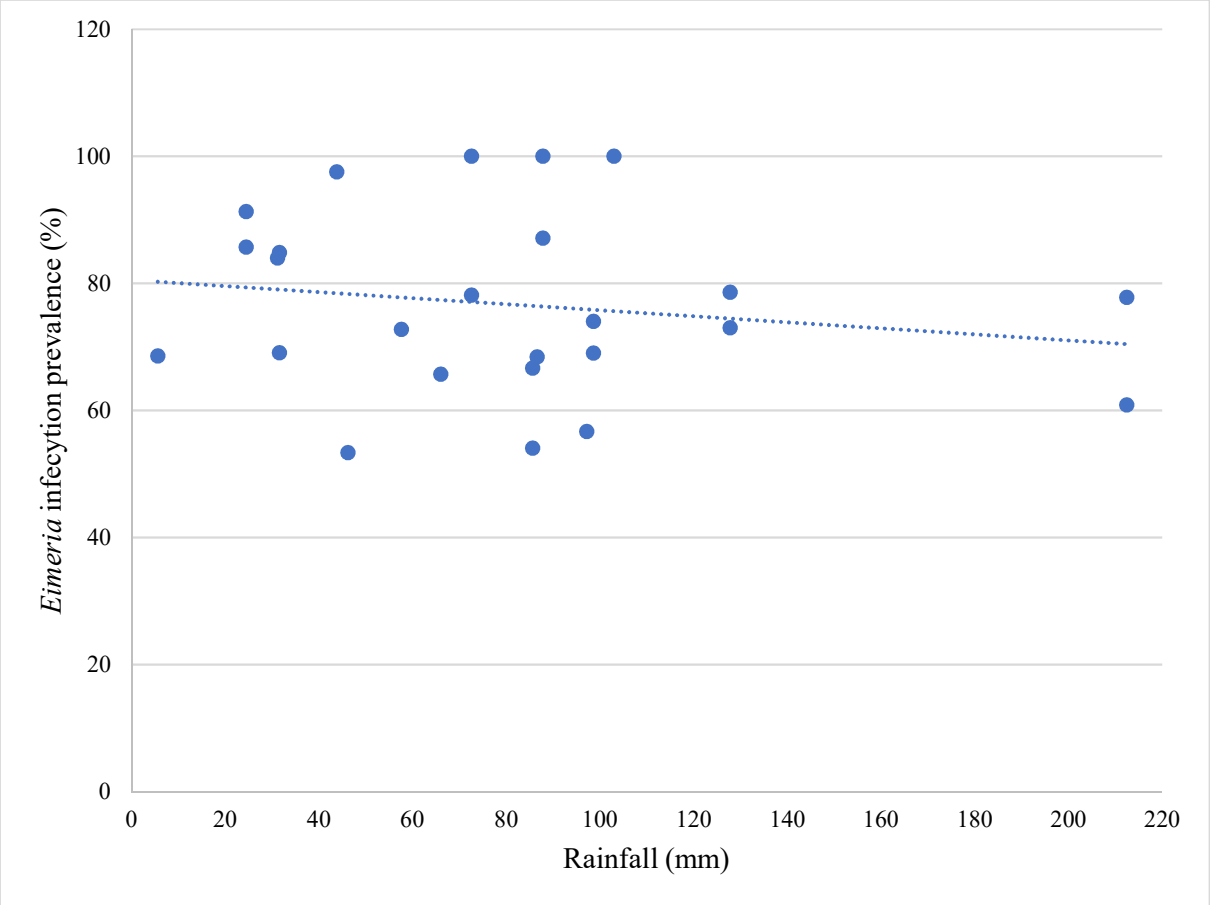


Figure 19: Scatterplot of *Eimeria* infection prevalence in each survey and rainfall for the month the survey took place.

3.4.4.6 Correlation between *Eimeria* infection prevalence and rainfall in month prior to survey

For each of the 25 surveys of *Eimeria* infections carried out on the three study farms, the rainfall in the month prior to that in which the survey took place was obtained, and these data were combined with *Eimeria* infection prevalence data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 20). The Pearson correlation for the association between *Eimeria* infection prevalence and rainfall in the month prior to the survey was -0.022, hence no correlation was apparent (P= 0.919).

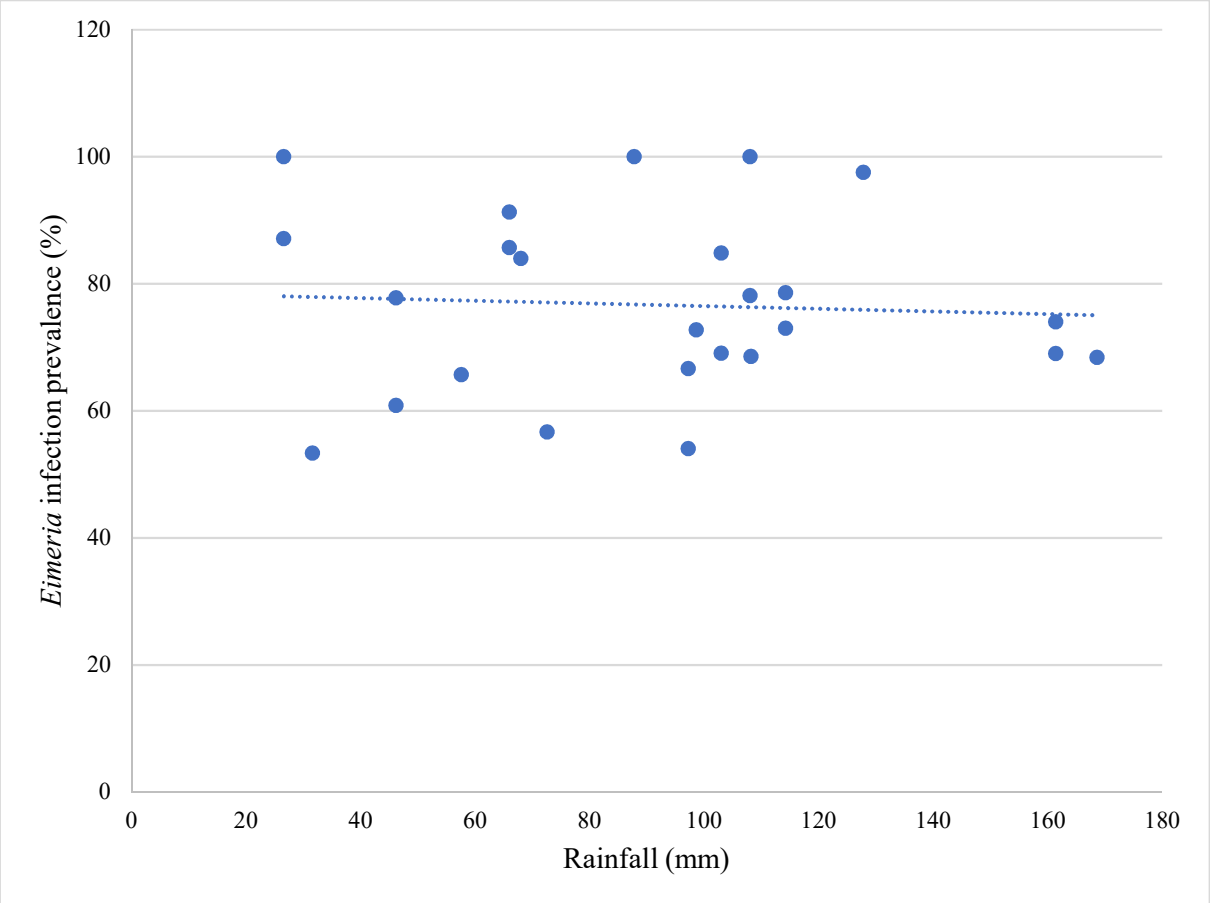


Figure 20: Scatterplot of *Eimeria* infection prevalence in each survey and rainfall in the month prior to that in which the survey took place.

3.4.4.7 Correlation between *Campylobacter* prevalence and monthly rainfall

For each of the 27 surveys of *Campylobacter* infections carried out on the three study farms, the rainfall in the month of the survey was obtained, and these data were combined with *Campylobacter* infection prevalence data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 21). The Pearson correlation for the association between *Campylobacter* infection prevalence and monthly rainfall was -0.281, hence no linear correlation was apparent (P= 0.155).

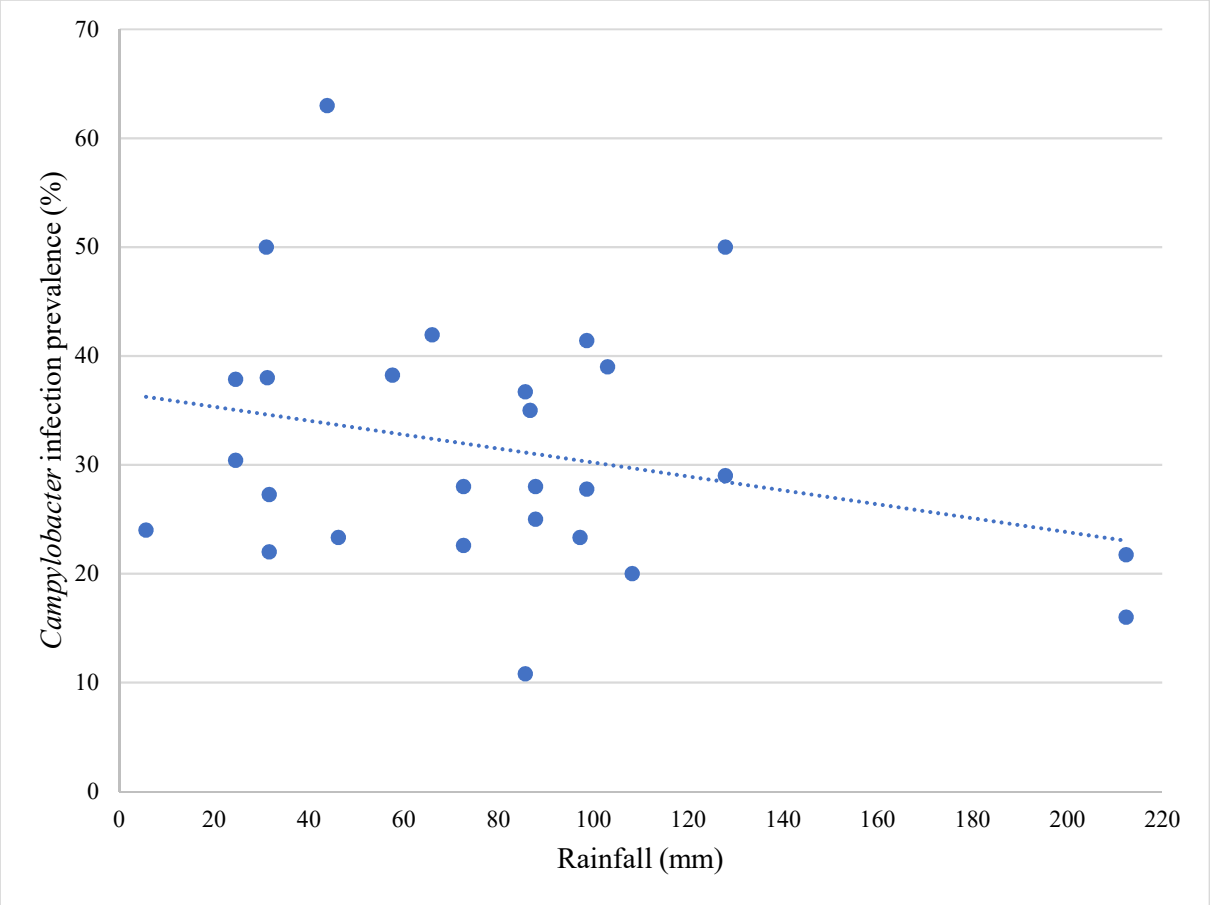


Figure 21: Scatterplot of *Campylobacter* infection prevalence in each survey and rainfall in the month the survey took place.

3.4.4.8 Correlation between *Campylobacter* infection prevalence and rainfall in month prior to survey

For each of the 27 surveys of *Campylobacter* infections carried out on the three study farms, the rainfall in the month prior to that in which the survey took place was obtained, and these data were combined with *Campylobacter* infection prevalence data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 22). The Pearson correlation for the association between *Campylobacter* infection prevalence and mean temperature in the month prior to the survey was 0.242, hence no correlation was apparent (P= 0.224).

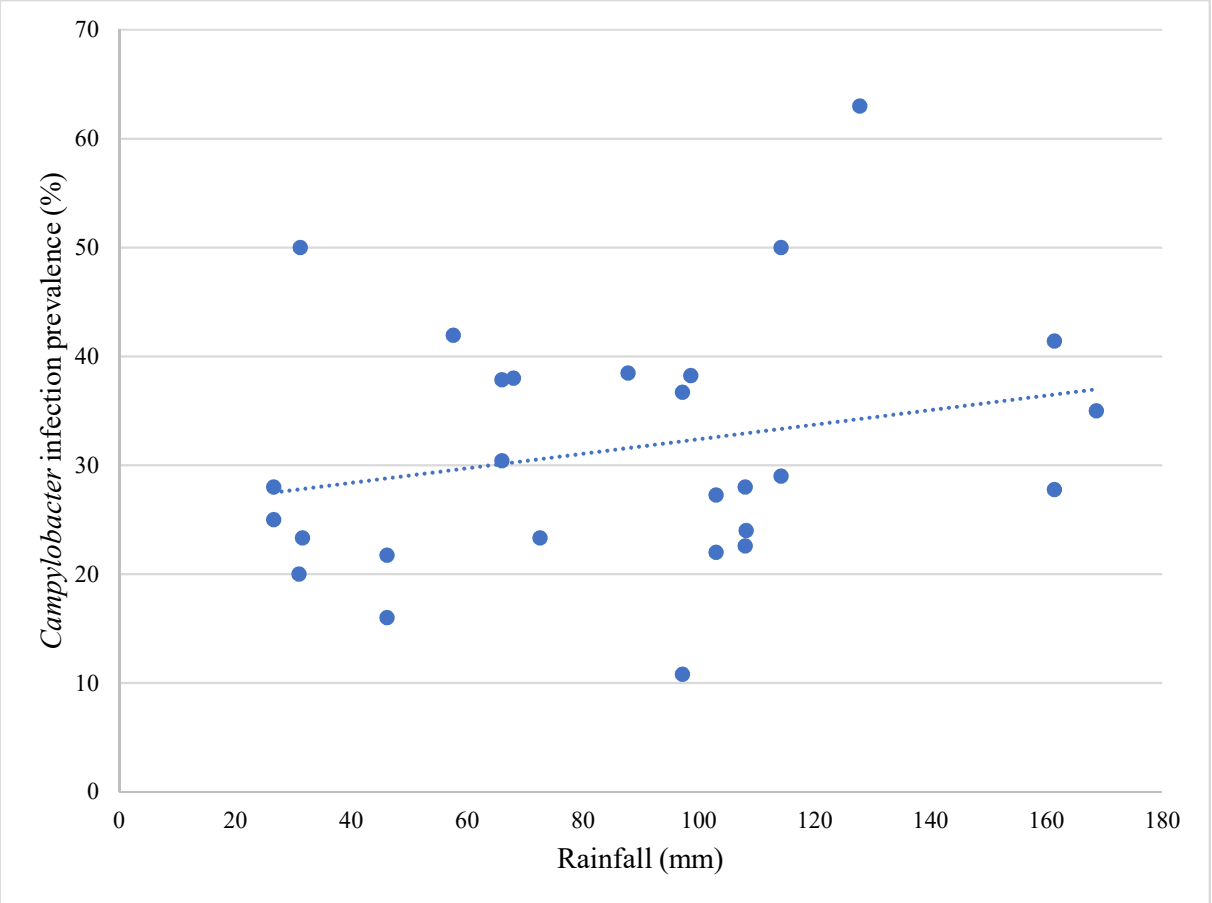


Figure 22: Scatterplot of *Campylobacter* infection prevalence in each survey and rainfall in the month prior to that in which the survey took place.

3.4.4.9 Correlation between *Eimeria* infection intensity and monthly rainfall

For each of the 27 surveys of *Eimeria* infections carried out on the three study farms, the rainfall in the month in which the survey took place was obtained, and these data were combined with mean *Eimeria* infection intensity data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 23). The Pearson correlation for the association between *Eimeria* infection intensity and rainfall was -0.256, indicating a weak but non-significant negative linear relationship (P= 0.216).

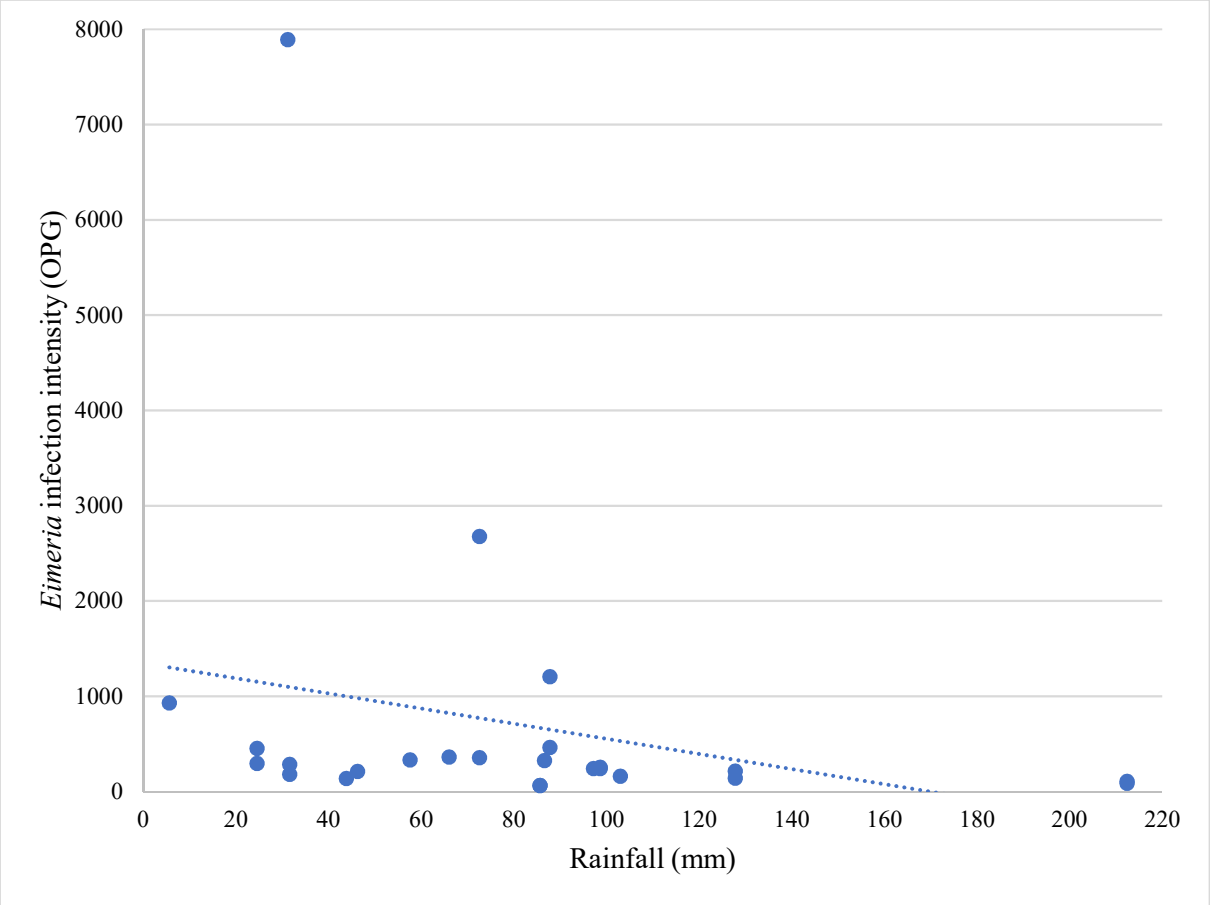


Figure 23: Scatterplot of *Eimeria* infection intensity in each survey and rainfall for the month the survey took place.

3.4.4.10 Correlation between *Eimeria* infection intensity and rainfall in month prior to survey

For each of the 25 surveys of *Eimeria* infections carried out on the three study farms, the rainfall in the month prior to that in which the survey took place was obtained, and these data were combined with *Eimeria* infection intensity data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 24). The Pearson correlation for the association between *Eimeria* infection prevalence and rainfall in the month prior to the survey was -0.124, hence no correlation was apparent (P= 0.556).

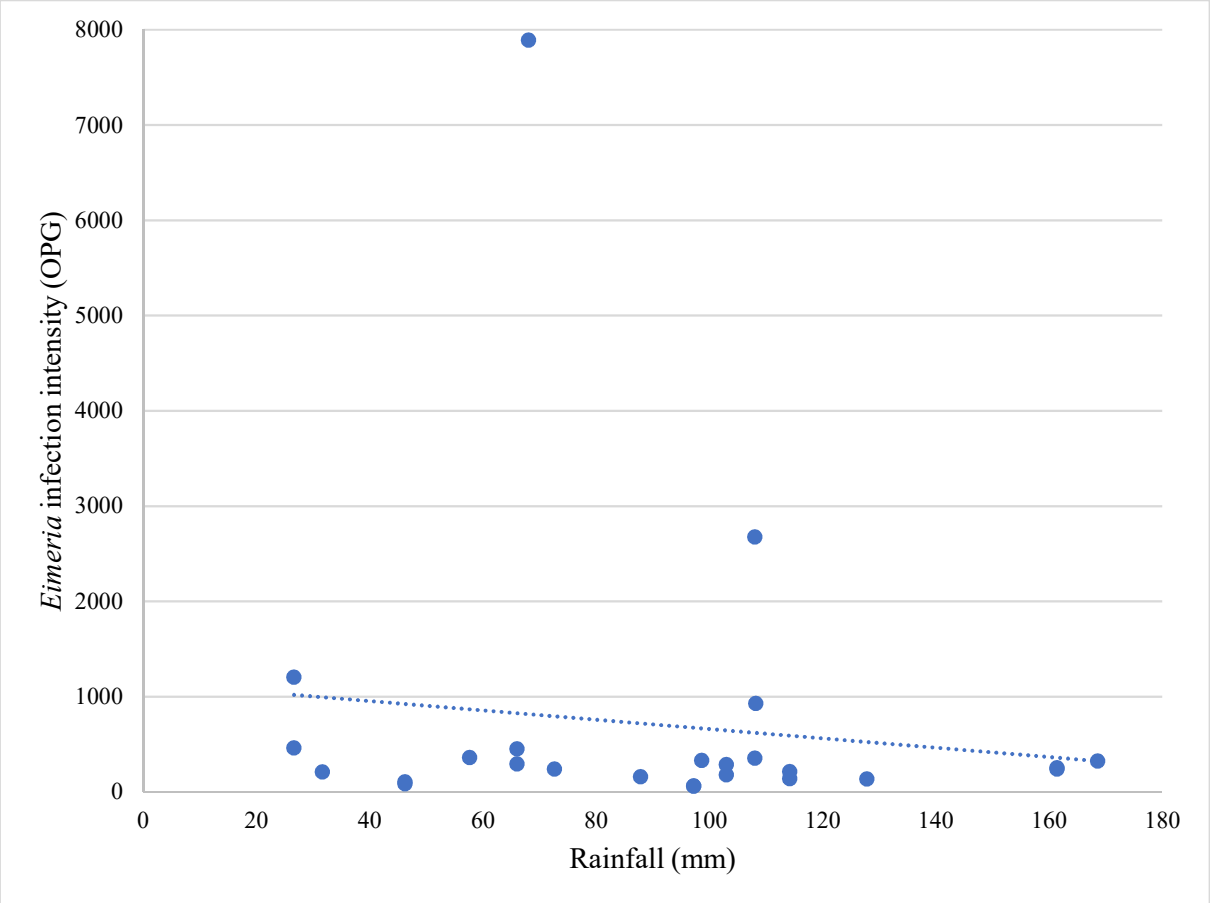


Figure 24: Scatterplot of *Eimeria* infection intesnity in each survey and rainfall in the month prior to that in which the survey took place.

3.4.4.11 Correlation between *Eimeria* infection intensity and mean monthly temperature

For each of the 25 surveys of *Eimeria* infections carried out on the three study farms, the mean temperature of the month of the survey. Temperature data were combined with *Eimeria* intensity data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 25). The Pearson correlation for the association between *Eimeria* intensity and mean monthly temperature was 0.300, hence no correlation was apparent (P= 0.145).

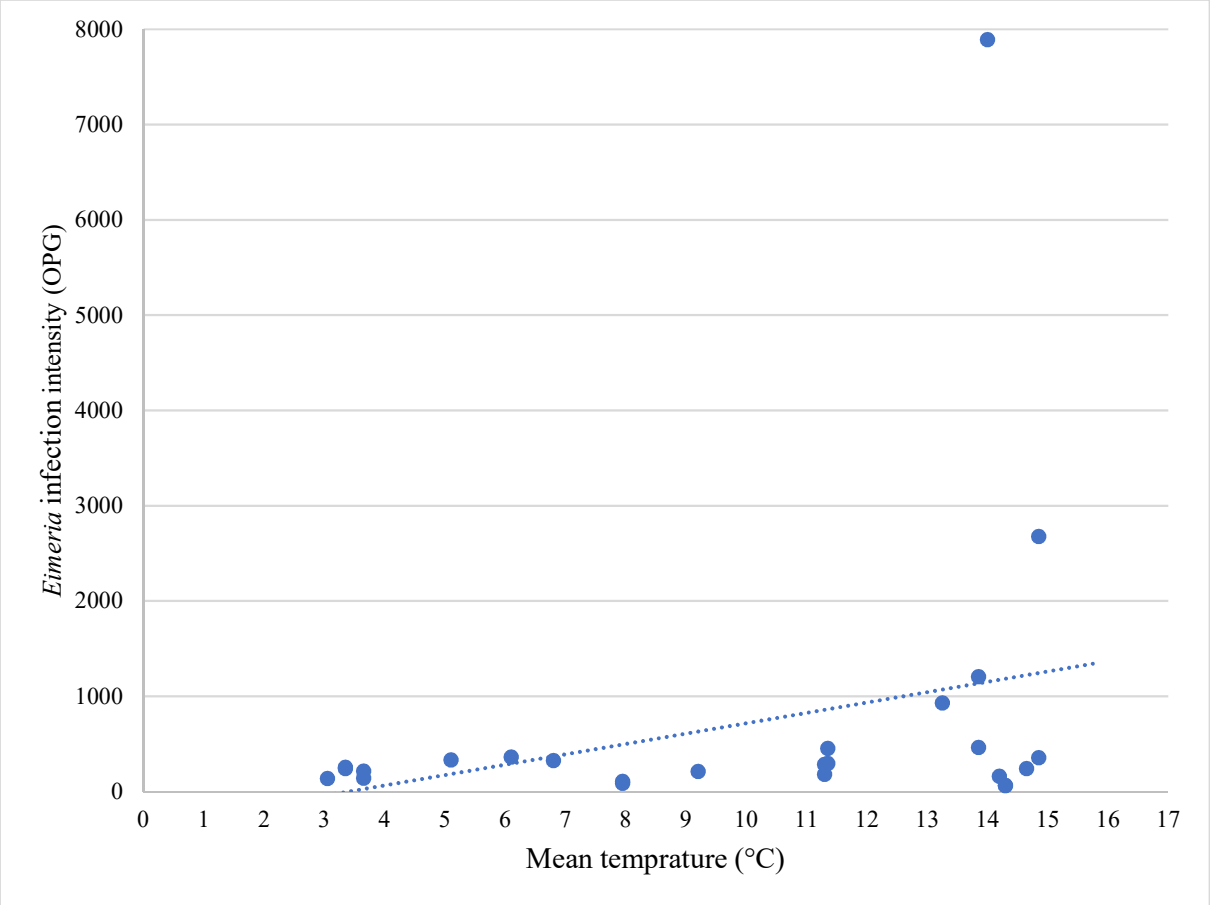


Figure 25: Scatterplot of *Eimeria* infection intesnity in each survey and mean monthly temperature for the month the survey took place.

3.4.4.12 Correlation between *Eimeria* infection intensity and mean temperature in month prior to survey

For each of the 25 surveys of *Eimeria* infections carried out on the three study farms, the mean temperature of the month of the survey. Temperature data were combined with *Eimeria* intensity data using a scatterplot and the nature of correlation between the two datasets was assessed using linear regression (Figure 26). The Pearson correlation for the association between *Eimeria* intensity and mean monthly temperature was 0.216, hence no correlation was apparent (P= 0.300).

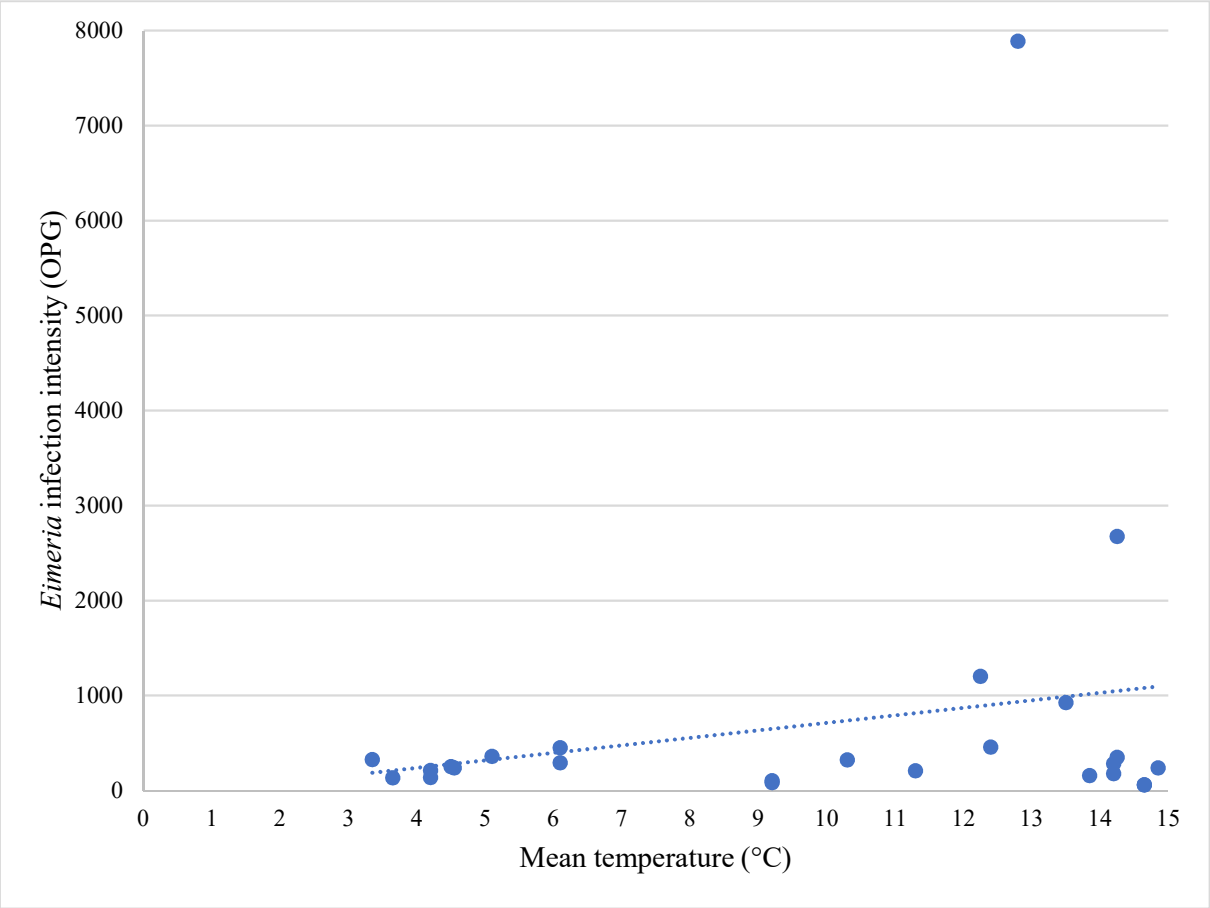


Figure 26: Scatterplot of *Eimeria* infection intesnity in each survey and mean temperature in the month prior to that in which the survey took place.

3.5 Generalized linear model (GLM)

GLM analyses were carried out to identify ecological determinants that were significantly correlated to *Eimeria* infection prevalence and intensity, *Campylobacter* infection prevalence, and the infection prevalence of the two most frequently enocuntered *Eimeria* species, *E. ovinoidalis* and *E. bakuensis*.

3.5.1 *Eimeria* infection prevalence model

An animal's age had a significant effect on the probability of infection with *Eimeria* with infection more common in young animals (odds ratio = 0.58) (Table 33). Season of the year also had an effect on the overall prevalence of *Eimeria* infection. Compared to autumn, a significantly higher prevalence of *Eimeria* infection was recorded in each of summer (odds ratio = 3.88), winter (odds ratio = 2.11) and spring (odds ration = 1.81) (Table 33).

Table 33: Parameter estimates and standard errors for GLM model of infection with *Eimeria* in sheep on Abbot Park Farm and Stock Farm.

Parameters	Estimate (SE)	Z value	P value	Odds ratio
Intercept	2.0795 (0.71)	2.915	0.003	
Age	-0.5416(0.23)	-2.259	0.023	0.58
Spring	0.5983 (0.25)	2.337	0.019	1.81
Summer	1.356 (0.24)	5.471	<0.001	3.88
Winter	0.7488 (0.26)	2.846	0.004	2.11

3.5.2 *Campylobacter* infection prevalence model

The season of the year had a significant effect on the probability of sheep being infected with *Campylobacter* (Table 34). Compared to autumn, the prevalence of *Campylobacter* infection was higher in each of winter (odds ratio = 2.71) and spring (odds ratio = 1.93) (Table 34). The model did not identify any other significant determinants of *Campylobacter* infection prevalence.

Table 34: Parameter estimates and standard errors for GLM model of infection with *Campylobacter* in sheep on Abbot Park Farm and Stock Farm.

Parameters	Estimate (SE)	Z value	P value	Odds ratio
Intercept	-1.1519(0.14)	-8.21	<0.001	
Spring	0.6582(0.23)	2.83	0.004	1.93
Winter	0.9977 (0.22)	4.43	<0.001	2.71

3.5.3 *Eimeria* infection intensity model

Interestingly, *Campylobacter* infections were negatively correlated to the intensity of *Eimeria* infections. Thus, the higher the intensity of *Eimeria* infections in sheep, the lower the probability of coinfection with the *Campylobacter* (Table 35). Moreover, the age of animals was significantly correlated to the intensity of *Eimeria* infection. As the age of sheep increased, the intensity of *Eimeria* infection decreased (Table 35). Season of the year was also significantly correlated to the intensity of *Eimeria* infection. The most intense infections were observed in summer followed by spring then winter. *Eimeria* infection intensities were lowest in autumn (Table 35). Finally, rainfall in the month prior to sampling also significantly correlated with the intensity of *Eimeria* infection. As rainfall increased, the intensity of *Eimeria* infection increased (Table 35).

Table 35: Parameter estimates and standard errors for GLM model of *Eimeria* infection intensity in sheep on Abbot Park Farm and Stock Farm.

Parameter	Estimate (SE)	Z value	P value
Intercept	8.12707 (0.46)	17.510	<0.001
Campylobacter infection	- 0.365685(0.14)	-2.462	0.013
Age	-1392(0.13)	-10.066	<0.001
Spring	0.93591(0.20)	4.467	<0.001
Summer	1.708051(0.18)	9.442	< 0.001
Winter	0.081368(0.23)	0.348	0.728
Rainfall previous month	0.008672(0.00)	3.823	<0.001

3.5.4 *E. ovinoidalis* infection prevalence model

Sheep infected with *E. ovinoidalis* were significantly more likely to be also infected with *E. bakuensis* than uninfected sheep (odds ratio = 2.06) (Table 36). An effect of farm was also observed, with sheep on Stock Farm being more likely to be infected with *E. ovinoidalis* than sheep on Abbot Park Farm (odds ratio = 1.63) (Table 36). The model also detected a negative correlation between *Campylobacter* infection and *E. ovinoidalis* infection, hence *E. ovinodalis* infection prevalence was significantly lower in sheep infected with *Campylobacter* (odds ratio = 0.70) (Table 36). Finally, season of the year was also found to have a significant effect on the prevalence of the *E. ovinoidalis*. Compared to autumn, the likelihood of infection was significantly greater in summer (odds ratio = 2.22) and winter (odds ratio = 1.81) (Table 36).

Table 36: Parameter estimates and standard errors for GLM model of infection *with E. ovinoidalis* in sheep on Abbot Park Farm and Stock Farm.

Parameters	Estimate (SE)	Z value	P value	Odds ratio
Intercept	-1.0019 (0.16)	-5.912	<0.001	
Eimeria bakuensis	0.7248 (0.15)	4.722	<0.001	2.06
Stock Farm	0.4945 (0.16)	3.064	0.002	1.63
Campylobacter	-0.3553 (0.16)	-2.142	0.032	0.70
Summer	0.7995 (0.18)	4.254	<0.001	2.22
Winter	0.5966 (0.22)	2.639	0.008	1.81

3.5.5 *Eimeria bakuensis* infection prevalence model

In agreement with the outputs from the *E. ovinoidalis* model described above, the *E. bakuensis* model indicated that sheep infected with *E. bakuensis* were significantly more likely to be infected with *E. ovinoidalis* than sheep not infected with *E. bakuensis* (odds ratio = 2.08) (Table 37). Furthermore, an effect of farm was also observed, with sheep on Stock Farm being less likely to be infected with *E. bakuensis* than sheep on Abbot Park Farm (odds ratio = 0.72) (Table 37).

Table 37: Parameter estimates and standard errors for GLM model of infection *with E. bakuensis* in sheep on Abbot Park Farm and Stock Farm.

Parameters	Estimate (SE)	Z value	P value	Odds ratio
Intercept	0.2164 (0.42)	0.513	0.608	
Eimeria ovinoidalis	0.7353 (0.14)	4.935	<0.001	2.08
Stock Farm	- 0.3251(0.15)	2.157	0.031	0.72

3.6 Survey of deer faeces for *Campylobacter* infections

A total of 38 deer faecal samples, collected on eight occasions, were tested for the presence of *Campylobacter* species (Table 38). Seven of these samples yielded putative *Campylobacter* isolates. Only one of these isolates yielded a positive result when tested in the *Campylobacter* genus-specific PCR. This amplicon was sequenced and analysis of the partial 16S rDNA sequence obtained indicated 100% sequence similarity with *Acinetobacter* species (GenBank Accession number gi|441425454|KC128833.1).

Table 38: The summary of tested deer faecal samples for the presence of *Campylobacter* species according to the animal species, sex and age.

Survey	Date	Number of samples	Deer species		Sex		Age (months)		
			Red	Roe	Male	Female	≤1- 12	13-24	> 24
1	6/2014	6	0	6	6	0	4	1	1
2	11/2014	5	1	4	1	4	4	0	1
3	12/2014	11	2	9	0	11	6	1	4
4	1/2015	2	0	2	0	2	1	0	0
5	2/2015	7	4	3	1	6	5	0	2
6	6/2015	4	0	4	4	0	3	0	1
7	7/2015	1	0	1	1	0	0	0	1
8	8/2015	2	0	2	2	0	1	0	1

3.7 Summary of results obtained

Threlkeld Farm: Results obtained from what was primarily pilot work on this farm indicated that most sheep were infected with either *Eimeria* or *Campylobacter* or both. Ten *Eimeria* species were encountered. Analysis of data from the four surveys completed demonstrated that the prevalence and intensity of *Eimeria* infection varied significantly between surveys, as did the prevalence of *Campylobacter* infections.

Stock Farm: Results generally reiterated those obtained on Threlkeld. In addition, analysis of data from the 11 surveys completed demonstrated the abundance of some *Eimeria* species varied between surveys as did the overall diversity of *Eimeria* in circulation. The importance of *C. jejuni* and *C. coli* as the most common *Campylobacter* species in sheep faeces was also demonstrated, and molecular typing of strains belonging to these two species revealed an expected level of diversity.

Abbot Park Farm: A total of 12 surveys were completed on this farm and the results obtained from these were generally in agreement with those obtained at Threlkeld and Stock Farm. A more comprehensive exploration of the contributions of *C. coli* and *C. jejuni* to the *Campylobacter* infections detected helped clarify their relative importance.

Data from all three farms were combined and used to explore the significance of a range of determinants on the prevalence, intensity and diversity of *Eimeria* infections, and the prevalence of *Campylobacter* infections. Initially the significance of variation within these data was explored using univariant statistical analysis, but subsequently more sophisticated multivariant analyses were preformed using generalised linear models.

The key findings derived from univariant analysis were:

Significant seasonal trends in the epidemiology of *Eimeria* and *Campylobacter* infections, the intensity of *Eimeria* infections and the epidemiology of particular *Eimeria* species.

Significant variation in the epidemiology of *Eimeria* infections, intensity of *Eimeria* infections, and epidemiology of specific *Eimeria* species in sheep of different ages.

A significant albeit moderate correlation between the prevalence of *Campylobacter* infections and the mean temperature in the month prior to survey.

The key findings derived from multivariant analysis were:

In keeping with univariant analysis, significant seasonal trends in the epidemiology of *Eimeria* and *Campylobacter* infections, and specifically, in the epidemiology of *E. ovinoidalis*.

The intensity of *Eimeria* infections is significantly correlated with season, decline significantly in older animals, increase significantly when the month prior to sampling is wetter (greater rainfall) and, interestingly, decrease in animals with a *Campylobacter* co-infection.

The epidemiologies of *E. ovinoidalis* and *E. bakuensis* appear to interact, with co-infection being significantly more common than would be expected by chance alone. Furthermore, both species are more abundant on Stock Farm than elsewhere.

The epidemiology of *E. ovinoidalis* infections is also significantly correlated to that of *Campylobacter* infections season. As seen with *Eimeria* infections in general, the prevalence of this species was lower in animals co-infected with *Campylobacter* than animals without a *Campylobacter* infection.

Finally, survey of red and roe deer living in the vicinity of the three farms studied failed to implicate either species in the epidemiology of *Campylobacter* infections, suggesting they do not contribute to the natural persistence of these bacteria in Cumbrian sheep populations.

CHAPTER 4: Exploration of multi-species *Eimeria* populations using next generation sequencing

4.1 Introduction

Next generation sequencing (NGS) has revolutionised life science, and is now universally applied across the discipline. NGS has made a dramatic impact on the fields of microbiology and infectious disease research in particular by providing a practical means of obtaining whole genome sequences and of assessing microbial diversity (Gullapalli *et al.*, 2012). However, the focus of NGS microbial diversity assessments have been communities of bacteria (e.g. the human microbiome) (Finotello *et al.*, 2016) and, to a lesser extent, viruses (Rascovan *et al.*, 2016). Far less progress has been made exploring the diversity of eurkayotic microorganisms, even parasites associated with human or livestock (Bass *et al.*, 2015; Tanaka *et al.*, 2014). Most of the eukaryotic microorganism NGS-based diversity studies to date have focused on fungi (Bálint *et al.*, 2014) or on environmental niches (Bråte *et al.*, 2010; Lecroq *et al.*, 2011). NGS has been exploited to help research interests in *Eimeria* species, but primarily in the generation of whole genome sequences (genomics) and for transcriptomics (Blake *et al.*, 2015). Only very recently has the application of NGS to explore the diversity of *Eimeria* species infecting natural hosts been described (Vermeulen *et al.*, 2016). Such a development is particularly important given the huge diversity of *Eimeria* species, the existence of several, or even many, species in the same host, and the current absence of any molecular data for the vast majority of species.

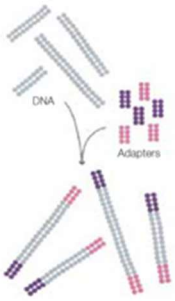
4.1.1 Sequencing technologies

The first DNA sequencing technology was developed in the early 1970s by Walter Gilbert (Maxam & Gilbert, 1977) and Frederick Sanger (Sanger & Coulson, 1975), and these were widely adopted in the 1980s following the advent of PCR, to sequence short fragments of cloned DNA. Sanger sequencing was used to generate the first microbial whole genome sequence (Fleischmann *et al.*, 1995), however, this achievement required a huge amount of effort and resource (Land *et al.*, 2015). Similarly, although Sanger sequencing could be utilise to assess microbial diversity (Ward *et al.*, 1990), it represented an extremely costly approach to doing so. The advent of NGS technologies, based on massive parallel sequencing, overcame these shortfalls and opened the door to cheap and quick DNA sequencing on a vast scale. Bioinformatics has undergone an equivalent revolution, facilitating the generation and analysis of whole genomes and the development of metagenomics to assess diversity in an extraordinarily sensitive manner. Several different NGS technologies have been developed. Polonator (http://www. polonator.org/), SOLiD (http://www. appliedbiosystems.com), 454 sequencing (http://www.my454.com/) and

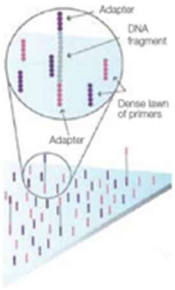
Solexa/ Illumina (http://www.illumina.com) were early innovators in the field (Gullapalli *et al.*, 2012), but now Illumina dominate. Such has been the increase in the throughput capability, that a recent estimate suggested that an Illumina Hiseq X Ten has the capacity to sequence 18,000 human genomes a year at cost of less than $1000 per genome (Glover & Adams, 2016). However, other technologies are now emerging as serious competitors to Illumina including Oxford Nanopore Technologies (https://nanoporetech.com/).

4.1.2 Illumina (Solexa) sequencing

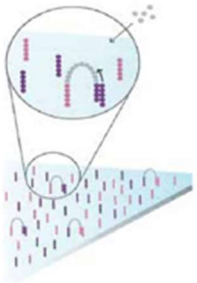
"Sequencing by synthesis technology" emerged in the late 1990s and was commercialised by Solexa in 2006. Solexa was acquired by Illumina in 2007 and for the last decade Illumina have been refining and optimizing sequencing by synthesis and single molecule amplification such that the latest generation of Illumina instruments can generate multiple terabases (Tb) of data per run. In the sequencing by synthesis approach (Figure 27), a flow cell receives the four nucleotides simultaneously in addition to the DNA polymerase, to integrate into the oligo-primed cluster fragments. The approach allows to sequencing the tens of millions of clusters in parallel. Each process of incorporation is a unique appearance because the nucleotides are labelled with the base unique fluorescent label and the 3-OH group which is blocked chemically. The process of adding each base is imaged and instrument optics is imaged each flow cell lane in three 100-tile segments at a cluster density of 30,000 per tile. The next incorporate of each strand by DNA polymerase is induce after chemically remove the 3-blocking group. According to the instrument settings, this process continues into the numbers of cycles, producing 25–35 bases length of reading (Mardis, 2008).



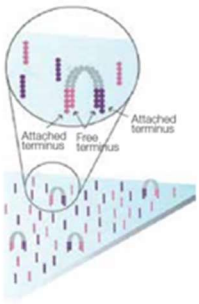
(A) Prepare genomic DNA sample



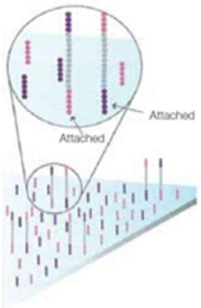
(B) Attach DNA to surface



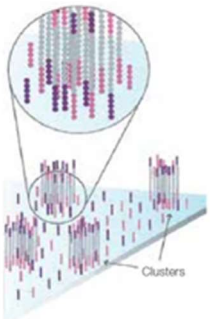
(C) Bridge amplification



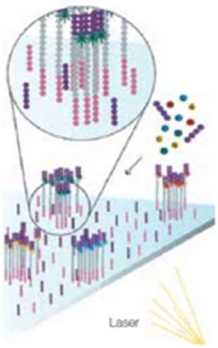
(D) Fragments become double strands



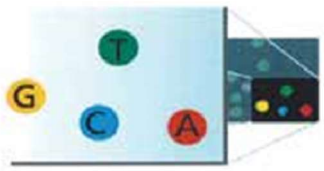
(E) Denature the double strands molecules



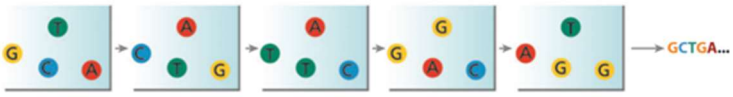
(F) Complement amplification



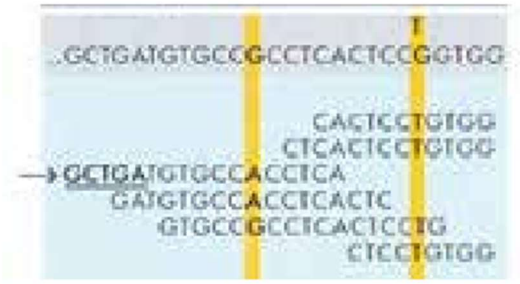
(G) Determine first base



(H) Image first base



(I) Sequencing over multiple chemistry cycles



(J) Align data

Figure 27: Bridge amplification of the cluster strands in the Illumina sequencing-by-synthesis approach. A: ligation adapters added to the sample, B: The single stranded DNA attached randomly to the surface of the flow cell, C: Bridge amplification is formed by add unlabelled nucleotides and enzyme, D: Build the double strand, E: Single strand tempates anchor to the surface after denaturation, F: Millions of double strand DNA clusters in each flow cell channel, G: Add the four labelled reversible terminators, primers and DNA polymerase to start the first sequencing cycle, H: Identify the first base from fluorescent emitted from each cluster I: The sequencing cycles are continued until identifying each base in a fragment, J: Differences in sequencing identified after alignment and comparison of data with references. Adapted from Illumina sequencing (2010).

This study attempts to use NGS to obtain 18S rDNA sequence data for the majority of sheep *Eimeria* species for which such data are currently unavailable. Sheep are naturally co-infected with multiple *Eimeria* species, thus a PCR/Sanger sequencing-based approach is unlikely to generate unambiguous data, and even if it did, ascribing a particular 18S rDNA sequence to a particular *Eimeria* species would be impossible.

4.2 Materials and Methods

4.2.1 Molecular delineation of species within mixed *Eimeria* communities infecting Cumbrian sheep.

Samples for NGS were retrieved from the routine samples that used during the surveys described in Chapters 2 and 3. Samples were selected on the basis of the relative abundance of different *Eimeria* species, as previously determined microscopically (see 3.1.1). Oocysts were harvested from selected samples and DNA extraction was carried out immediately (see 2.4.3), prior to sporulation in order to reduce the effect of inter-species variation in sporulation rates.

4.2.2 Source of chicken-associated *Eimeria* oocysts and quantification of oocyst suspensions.

Suspensions of oocysts from four chicken-associated *Eimeria* species, namely *Eimeria acervulina* (Houghton strain, harvested 2nd March 2016), *Eimeria mitis (*Houghton strain, harvested February 2010), *Eimeria necatrix (*Houghton strain, harvested March 2010) and *Eimeria tenella* (Wisconsin strain, harvested 28th April 2016), were kindly provided by Prof. Damer Blake of the Royal Veterinary College. These suspensions had been quantified by Dr Blake and their density was confirmed by counting oocysts, in triplicate. Numerous mock communities, containing known numbers of oocysts belonging to one or more of these *Eimeria* species (Table 39), were constructed and DNA was extracted from each as described in 2.4.3. Two "mock communities" containing no *Eimeria* were also included as background controls.

Table 39: The relative abundance of different chicken *Eimeria* species numbers in each sample used in the next generation sequence assay. Samples 1-16 were used in conjunction with the *Eimeria*-specific RA_EimeriaF/RA_EimeriaR primer pair, and samples 17-32 were used in conjunction with the 18Sv9_Euk 1391F/18sV9_EUKBR primer pair. *No oocysts, background controls

Sample number	***E.acervulina***	***E. mitis***	***E.necatrix***	***E. tenella***
1	0	2000	0	0
2	0	0	0	2000
3	15	60	30	90
4	2000	0	0	0
5	30	0	0	0
6	312	312	2500	2500
7	625	2500	312	312
8	5000	625	625	625
9	2500	5000	5000	5000
10	1000	1000	1000	1000
11	0	0	2000	0
12	15	30	15	60
13	30	90	150	300
14	0	0	0	30
15*	0	0	0	0
16	0	0	0	2000
17	0	2000	0	0
18	3012	3012	2500	2500
19	5000	625	625	625
20	2500	5000	5000	5000
21	1000	1000	1000	1000
22	0	0	2000	0
23	2000	0	0	0
24	15	60	30	90
25	15	30	15	60
26	30	90	150	300
27	60	120	80	30
28	60	30	30	30
29	0	0	0	30
30	0	0	30	0
31	0	30	0	0
32*	0	0	0	0

4.2.3 Isolation and quantification of mixed *Eimeria* populations from sheep faeces

About 4g of faecal material were processed as described in 2.4.1. Next, the filtrate was centrifuged for 5 min at 4000 x g. The supernatant was discarded and the pellet was resuspended to 50ml of distilled water and centrifuged for 5 min at 4000 x g, this process was repeated 4 times until a clear suspension was obtained. Then the supernatant was discarded and 10 ml of flotation fluid were added, the pellet was resuspended and the tube was centrifuged at 2000 x g for 2 min. Moreover, 5 ml of supernatant were transferred into 50 ml Falcon tube and the tube was completed with distilled water. Then, the tube was centrifuged for 5 min at 4000 x g in order to wash the oocyst. Finally, the supernatant was removed and the pellet was resuspended with 5ml of distilled water.

4.2.3.1 Sporulated *Eimeria* oocyst isolation

After sporulation of oocysts as described in 2.4.2, the suspension was processed as mentioned above and the pallet of sporulated oocysts was resuspended with 5ml of distilled water.

4.2.3.2 *Eimeria* oocyst count and species identification

Sporulated and non-sporulated *Eimeria* oocysts in each faecal sample were examined in triplicate for oocyst count and species identification. For each sample, 5ml of faecal suspension were mixed thoroughly and 60µl of that suspension were examined on the microscopical slide. *Eimeria* species and the number of oocysts was recorded.

4.2.4 DNA extraction from *Eimeria* oocysts

For each 5ml sample suspension, the tube was centrifuged for 5 min at 4000 x g. The supernatant was discarded and the pellet was resuspended in 300 µl of distilled water. Triplicate slides were made for each sample and the oocysts were again counted and examined for species identification. DNA was extracted from oocyst suspensions as described in 2.4.3.

4.2.5 Amplification of 18S rDNA fragments from *Eimeria* oocyst DNA extracts

Two PCRs were employed in an attempt to amplify *Eimeria* 18S rDNA fragments in an unbiased manner. The first of these incorporated primers previously described as having broad-range eukaryotic specificity (EarthMicrobiomeProject, 2017) and targeting a 260bp fragment of the V9 region of the molecule (Figure 29). Reaction mixes (20 µl) comprised

of 0.4 µl of a 10pmol μl^{-1} solution of primer 18Sv9_Euk 1391F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTACACACCGCC-3′) and 0.4 µl of a 10pmol μl^{-1} solution of primer 18sV9_EUKBR (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGATCCTTCTG-3′), 10µl of My Taq Red Mix and 7.2 µl double distilled H_2O. Reaction mixes were subjected to a thermal programme that consisted of an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec then a final cycle of extension at 72°C for 5 min

The second PCR incorporated primers previously described as being *Eimeria* genus-specific and targeted a 600bp 18SrDNA fragment (Figure 30) (Dr Damer Blake, RVC, personal communication).

The reaction mixes (20 µl) comprised of 0.4 µl of a 10pmol μl^{-1} solution of primer RA_EimeriaF (5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGCGCAAATTACCCAATGAA-3′), 0.4 µl of a 10pmol µl-1 solution of primer RA_EimeriaR(5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATGCCCCCAACTGTCCCTAT-3′), 10µl of My Taq Red Mix and 7.2µl double distilled H_2O. Reaction mixes were subjected to a thermal programme that consisted of an initial denaturation step at 95°C for 5 min followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 20 sec and extension at 72°C for 20 sec then a final extension step at 72°C for 10 min (Thabet *et al.*, 2015).

PCR products were resolved by electrophoresis and visualized by exposure to a UV transilluminator (SynGene) as described in 2.4.5.

4.2.6 Optimization of assay for NGS

In order to reduce the risk of PCR-induced errors in NGS sequencing templates, qPCR was used to determine, for each of the PCRs used, the thermal cycle number at which the exponential production of PCR products stopped.

The two 20µl PCR mixtures containing the two sets of primer pairs (18Sv9_Euk 1391F and 18sV9_EUKBR) and (RA_EimeriaF and RA_EimeriaR) were prepared by adding 10 µl of (sybr green containing) SensiFAST master mix (Bioline, UK), 7.2 µl nuclease-free water, 0.4 µl of a10pmol μl^{-1} solution of the forward primer, 0.4 µl of a 10pmol μl^{-1} solution of the

reverse primer and 2µl of template DNA. The assay was run in a Rota-Gene Q (QIAGEN) using a thermal programme comprising of one cycle of denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 62°C for 20 sec. and extension at 72°C for 20 sec. Emitted fluorescence was quantified at the end of each extension step. A positive control (*Eimeria* DNA) and a reagent negative control (sterile distilled water) were incorporated into qPCR (Figure 31).

4.2.7 Purification of PCR products

Unpurified PCR products were transferred to a 96-well PCR plate, which was then centrifuged for 1 min at 1000 x g at 20ºC. Next, a suspension of AMPure XP beads (Beckman Coulter, High Wycombe, UK) was brought to room temperature and mixed by vortexing for 30 sec then 20µl aliquots of this suspension were added to each well of the PCR plate using multichannel pipet and mixed thoroughly by pipetting up and down 10 times. The 96-well plate was incubated for 5 min at room temperature then transferred to a magnetic stand. Supernatants were removed from every well whilst the plate was on this stand and discarded. The plate was then removed from the magnetic stand and 200 µl of 80% (v/v) ethanol was used to resuspend the beads in each well. The plate was returned to the magnetic stand and after 30 sec the ethanol was removed from each well and discarded. This ethanol washing was repeated two more times after which the plate was left on a magnetic stand for 10 min to allow the beads to dry. The plate was then removed from the magnetic stand and 52.5 µl of 10mM Tris pH8.5 were added to each well and gently pipetted up and down 10 times, then the plate was incubated for 2 min at room temperature to allow purified PCR products to be recovered from beads into solution. Finally, the plate was again placed on its magnetic stand and, after 2 min, the supernatant (50 µl) in each well was transferred to a new 96-well PCR plate and stored at -20 ºC.

4.2.8 Labelling of purified PCR products

Dual indexes (Table 40) and Illumina sequencing adapters were attached to purified PCR products using the Nextera XT index kit (Illumina). This kit comes in a 96-well plate format (Figure 28, C). Tubes containing index 1 primers with a yellow solution and orange caps (Figure 28, A) were arranged horizontally with column 1 to 12 of 96 PCR plate. The index 2 primers tubes with clear solution and white caps (Figure 28, B) were arranged vertically with row A to H of 96 PCR plate.

Table 40: The dual indexing primers used for each sample in the 96-well plate in which each sample take one primer from index 1 and another primer from index 2 according to the indexes arrangement in Figure 28.

Index 1	Sequence	Index 2	Sequence
N701	N701 TAAGGCGA	S501	S501 TAGATCGC
N702	N702 CGTACTAG	S502	S502 CTCTCTAT
N703	N703 AGGCAGAA	S503	S503 TATCCTCT
N704	N704 TCCTGAGC	S504	S504 AGAGTAGA
N705	N705 GGACTCCT	S505	S505 GTAAGGAG
N706	N706 TAGGCATG	S506	S506 ACTGCATA
N707	N707 CTCTCTAC	S507	S507 AAGGAGTA
N708	N708 CAGAGAGG	S508	S508 CTAAGCCT
N709	N709 GCTACGCT		
N710	N710 CGAGGCTG		
N711	N711 AAGAGGCA		
N712	N712 GTAGAGGA		

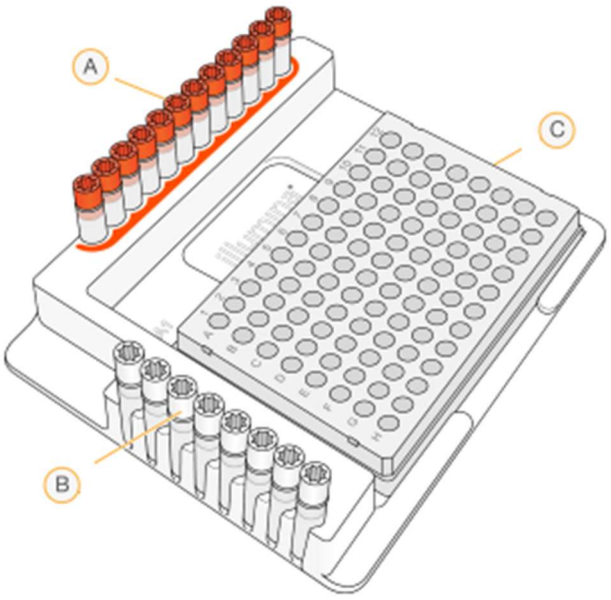


Figure 28: Nextera XT index kit consists of index 1 primers (A), index 2 primers (B) and 96-well plate (C). This figure was taken from the Nextera® XT DNA Library Prep Reference Guide (Illumina).

Into each well of the plate the following products were added; 5 µl of Nextera XT index 1primer, 5 µl of Nextera XT index 2primer, 25 µl of 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems) and 10 µl of PCR Grade water. The total volume in each well after the addition of reagents was 50 µl. The contents of each well were mixed by gently pipetted up and down 10 times. The plate was covered with Microseal A (BIORAD, UK) and centrifuged at 1000 x g for 1 min at 20°C.

The plate was transferred to a thermal cycler for PCR. The thermal programme consisted of an initial denaturation step at 95°C for 3 min, followed by 8 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, then a final extension step at 72°C for 5 min. Subsequent to PCR, plates containing the created *Eimeria* 18S rDNA libraries were held at 4°C.

4.2.9 Purification of labelled *Eimeria* 18S rDNA libraries

The *Eimeria* 18S rDNA PCR product libraries were purified using AMPure XP beads, as described above, except that 56µl instead of 20 µl aliquots of AMPure XP beads were added to each well of the 96-well plate and, after washing, DNA was dissolved in 27.5 µl rather than 52.5 µl of 10mM Tris pH8.5, with a supernatant volume of 25 µl being transferred from each well to a new 96-well plate then stored at -20°C.

4.2.10 Quantification of *Eimeria* 18S rDNA libraries

Eimeria 18S rDNA libraries were quantified using the Quant-iT dsDNA high sensitivity (HS) assay kit (Thermo Fisher SCIENTIFIC, UK) Qubit Fluorometer (Invitrogen, UK). All reagents were brought to room temperature. Firstly, 200 µl of Quant- iT working solution was prepared by adding 1 µl of Quant- iT reagent to 199 µl of Quant- iT buffer. Next, 200 µl of standards (standard one and standard two) were prepared by the addition of 190 µl of working solution into 10 µl of standards from the kit. The libraries were diluted by adding 1 µl of each to 199 µl of working solution. All tubes were briefly vortexed then incubated for 2 min at room temperature and transferred to a Qubit Fluorometer. The Qubit fluorometer is a sensitive tool based on the detection of target fluorescent DNA in the sample. From home screen, the high sensitivity double strand DNA was selected. Then the standards calibration reading was generated by reading the first and the second standard following the instruction on the screen. the values of standards were represented the actual fluorescent. After that, each sample tube was placed on the Qubit fluorometer reading space to calculate the concentration of the DNA.

4.2.11 Further quantification of *Eimeria* 18S rDNA libraries, normalization and pooling of libraries

A fluorometric quantification method that uses dsDNA binding dyes was conducted in order to further quantify libraries. Depending on the size of the DNA amplicons the DNA concentration was determined followed Agilent Technologies 2100 Bioanalyzer trace;

(concentration in ng/µl)/ (660g/mol × average library size) × 10^6 = concentration in nM

10 mM Tris pH 8.5 to 4 nM or Resuspension Buffer (RSB) was used to dilute the DNA concentration of the final library.

Pooling library (96 libraries) was conducted by mixing Aliquot 5 µl of diluted DNA from each library.

4.2.12 Verification of DNA fragment size in *Eimeria* 18S rDNA libraries

4.2.12.1 Preparation of tape station

In order to prepare the tape station (Agilent Technologies), Agilent 2200 Tape Station software was launched. Then tips and high sensitivity D1000 Screen Tape were placed into the 2200 Tape Station. The reagents were left for 30 min at room temperature in order to thaw then were vortexed in order to be mixed. To prepare ladder, 2µl of High Sensitivity D1000 sample buffer were added into 2µl of High Sensitivity D1000 Ladder. To prepare the sample, 2µl of High Sensitivity D1000 sample buffer were added to 2µl of DNA sample, which was vortexed for 1 min then briefly centrifuged to position samples at the bottom of the tube.

4.2.12.2 Sample analysis

The samples were loaded into 2200 Tape Station; the required samples were selected in the controller software and start bottom was clicked after the filename was entered in order to save the results. The average sizes of DNA bands were measured (Figure 32).

4.2.13 Quantitation of pooled library by qPCR assay

In order to quantify the pooled library, NEBNext Library Quant Kit for Illumina (New England Biolab, UK) was used. The kit reagents were thawed at room temperature, mixed well and then briefly centrifuged in order to collect the reagents from the sides of the tube. Then, all reagents were left on the ice.

4.2.13.1 Master Mix and primer mix preparation

First, 100 µl of NEBNext Library Quant Primer Mix were added into 1.5 ml NEBNext Library Quant Master Mix tube, the tube was vortexed for 10 sec in order to be mixed. After that, the date and time were written on the master mix tube as an indication of the addition of primer mix.

4.2.13.2 NEBNext Library Quant Dilution Buffer (1X) preparation

The NEBNext Library Quant Dilution Buffer (1X) was prepared by making 1:10 dilution through adding 150 ml of Quant Dilution Buffer into 1350 ml of water. For library preparation, 1.2 ml were used.

4.2.13.3 Preparation of library dilutions

Dilution of 1:1000 for the NEBNext library was prepared by adding 1 µl of pooled library samples to 999 µl NEBNext Library Quant Dilution Buffer (1X). In order to make 1: 10,000 and 1: 100,000 dilutions, two extra of the diluted Library samples dilutions were made by the addition of 10 µl of the 1: 1,000 dilution to 90 µl NEBNext Library Quant Dilution Buffer (1X) and 10 µl of the 1: 10,000 dilution to 90 µl NEBNext Library Quant Dilution Buffer (1X) respectively, which were used for qPCR analysis.

4.2.13.4 Preparation of qPCR assay

Each library sample and DNA standard were run in triplicate in order to get accurate results for the qPCR assay (Figure 33). The total volume of 20 µl mixture was prepared by adding 16 µl of NEBNext Library Quant Master Mix with primers and 4 µl of library dilution or DNA standard. To prepare the negative control, no template control (20 µl mixture) was prepared by adding 16 µl of NEBNext Library Quant Master Mix with primers to 4 µl of library dilution buffer (1X). The reaction mixtures were mixed by pipetting buffer or sample at least 5 times. After that qPCR Assay was run in a Real-time Thermal Cycler (Rota-gene).

For the standard primary reaction, one cycle of denaturation at 95°C for 1 min was followed by 35 cycles of denaturation at 95°C for 15 sec and extension at 63°C for 45 sec.

4.2.13.5 Quantifying the qPCR library

A special website tool (NEBioCalculator) was used in order identify the number of amplifiable templates in the pooled library. This calculator was used in order to identify the average of the undiluted concentration of the pooled library. The accurate quantification of the pooled library is crucial to the success of next generation sequencing (https://nebiocalculator.neb.com/#!/qPCR) (Table 41).

4.2.14 Library denaturing and MiSeq sample loading

NaOH (Fisher Scientific, UK) was used to denatured the pooled libraries, hybridization buffer was used for dilution and heating of denatured were used as steps to prepare for the generation of cluster and data sequencing. MiSeqv3 reagent kit was used as suggested by Illumina to improve run metrics.

4.2.14.1 Denaturation and dilution of DNA

A standard normalization followed according to the guide of denaturation and diluted library. The MiSeq reagent kit v3 was used that support concentration of DNA of 6-20 pM. This kit required at least 4nM of pooled library before dilution and denaturation.

The pooled library was diluted into the 4nM by mixing 1 µl of pooled library (concentration 26.4nM) and 5.6 µl water to obtain 6.5 µl of the diluted library.

Fresh (1M) NaOH was prepared as follow; In Falcon tube, 2 grams of Sodium hydroxide were mixed with 50ml of distilled water. The fresh 1M NaOH was diluted into 0.2M by adding 8ml of distilled water into 2ml of 1M NaOH.

4.2.14.2 Denaturation of diluted library

In a microcentrifuge tube 5 µl of 4nM of pooled library and 5 µl of fresh diluted 0.2N NaOH were combined, vortexed and then centrifuged at 280 x g for 1 min at 20°C. Further, the sample was incubated at room temperature for 5 min in order to obtain single strand throughout denature of the DNA.

Pre-chilled HT1 (990 µl) on ice water bath (3 parts ice and 1part water) was added to the 10 µl denatured DNA sample. The mixture 20 Pm was kept on ice until final dilution prepared.

A final concentration (10 Pm) of denatured and diluted DNA was obtained by a combination of 20 Pm (500 µl) denatured library and Pre-chilled HT1 (500µl). The samples were mixed, centrifuged and kept on ice.

4.2.14.3 Denaturation and dilution of PhiX control

The 10 nM PhiX library was denatured and diluted into the same concentration of the amplicon library were both mixed and contained at least 5% of phiX.

The process of denaturation and dilution of the PhiX library was performed by adding 3 µl of 10mM Tris pH 8.5 into 2 µl of 10 nM PhiX library. In a microcentrifuge tube, 5 µl of 4 nM PhiX library were combined with 5 µl of 0.2 N NaOH. The tube was mixed using the vortex.

A single strand was obtained by denaturing the PhiX library after incubated at room temperature for 5 min. The 20 pM PhiX library were obtained by mixing 10 µl of denatured PhiX library with 990 µl of pre-chilled HT1.

The final concentration of denatured and diluted 20 pM PhiX library was obtained by the combination of 20 pM PhiX library denatured and pre-chilled HT1. Finally, the samples were mixed, centrifuged and kept on ice.

4.2.14.4 Amplicon Library and PhiX control combination

In a microcentrifuge tube, 180 µl of denatured and diluted PhiX control was mixed with 420 µl denatured and diluted amplicon library. The tube was incubated at 96°C for min in the hot block, then mixed by inverted 1-2 times and incubated directly in the ice-water bath for 5 min. The step of heat denaturation of library and PhiX control was performed immediately before library loading into MiSeq reagent cartridge. Finally, the mixture of denatured and diluted PhiX control and denatured and diluted amplicon library was loaded into the MiSeq reagent cartridge and the Illumina MiSeq System was started.

4.2.15 Bioinformatic Analysis

The following information is provided by Dr. I Goodhead, who led this section of the study. DNA sequences were initially extracted using CASAVA 1.8 (Illumina) and converted to .fastq.gz format for further processing and trimming. All raw Illumina sequence fastq files were trimmed for the presence of adapter sequences using Cutadapt version 1.2 using option "-O 3" and quality-trimmed using Sickle version 1.200 with a minimum window quality score of 20. Any reads shorter than 10 bp after trimming were removed. Quality scores for all sequences were assessed using FASTQC v0.9.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc).

Reads pairs were joined using PANDASEQ using default parameters, which joins together each member of a read pair. Un joined reads were not used, but stored for further analysis.

For the mock community profiling experiment, a database of 18S rRNA sequences was created from the four known *Eimeria* species, by downloading the gene sequences from the NCBI database, specifically: EF210324: *Eimeria acervulina*; U67118: *Eimeria mitis*; U67119: *Eimeria necatrix* and EF210325: *Eimeria tenella*. These sequences were formatted for Centrifuge-based metagenomic classification (https://ccb.jhu.edu/software/centrifuge/) which uses a mapping based algorithm to assign classifications to sequences. PANDASEQ-joined read pairs were individually mapped against each 'reference sequence' (i.e. against the custom *Eimeria* 18S database) and counted, thereby allowing for relative abundance metrics to be calculated.

4.3 Results

4.3.1 Amplification of 18S rDNA fragments from *Eimeria* oocyst DNA extracts

Gel electrophoresis confirmed the success of both PCRs used for the amplification of *Eimeria* 18S rDNA fragments (Figure 29 and Figure 30).

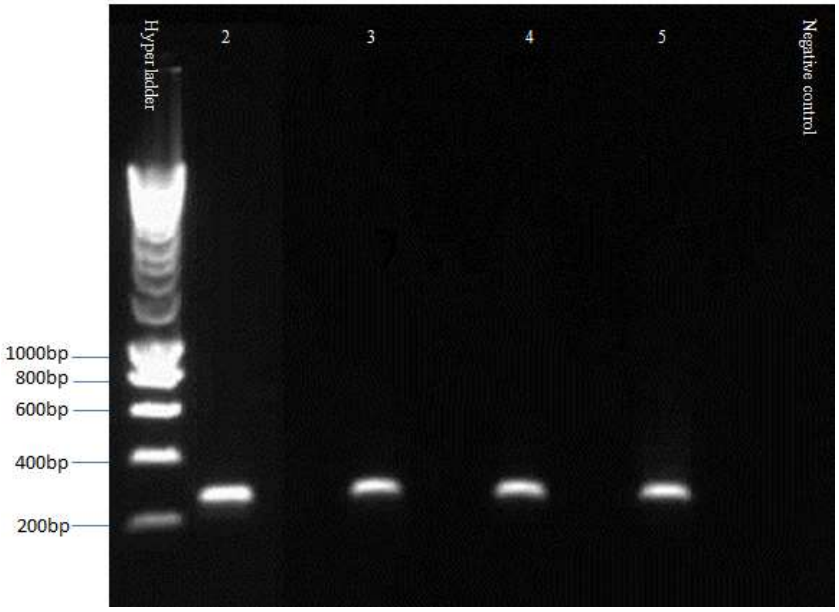


Figure 29: Gel electrophoresis of partial 18S rDNA fragments generated using the 18Sv9_Euk 1391F/18sV9_EUKBR primer pair. *Eimeria* samples appear in lanes 2, 3, and 4, and the positive control is in lane 5.

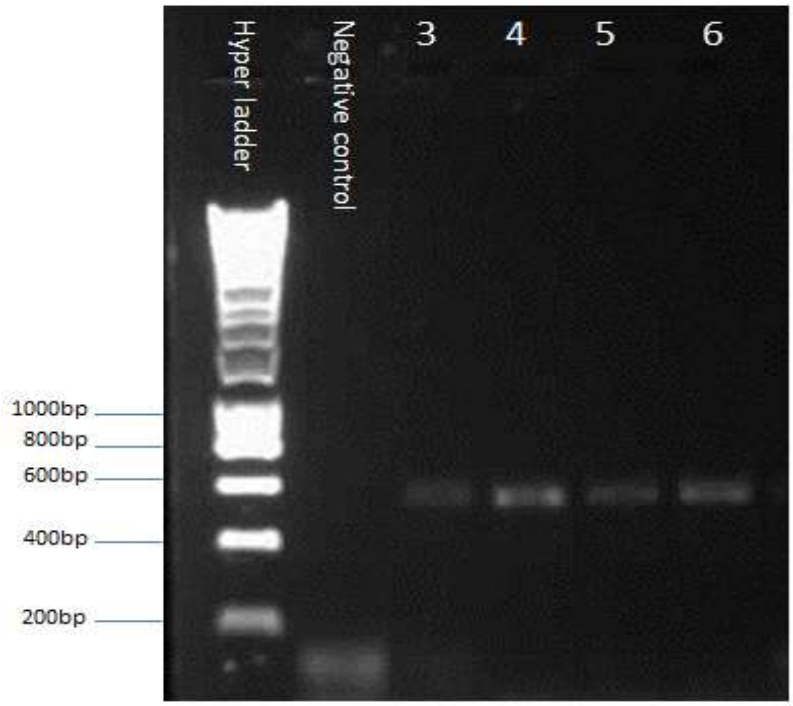


Figure 30: Gel electrophoresis of partial 18S rDNA fragments generated using the *Eimeria*-specific RA_EimeriaF/RA_EimeriaR primer pair. *Eimeria* samples appear in lanes 3, 4 and 5. Positive control is in lane 6.

4.3.2 Optimization of PCR assays for NGS

qPCRs indicated that for both PCRs, exponential production of products has ceased by cycle 25 (Figure 31). Thus, this number of cycles was incorporated in thermal cycles used to generate PCR products for NGS library preparation.

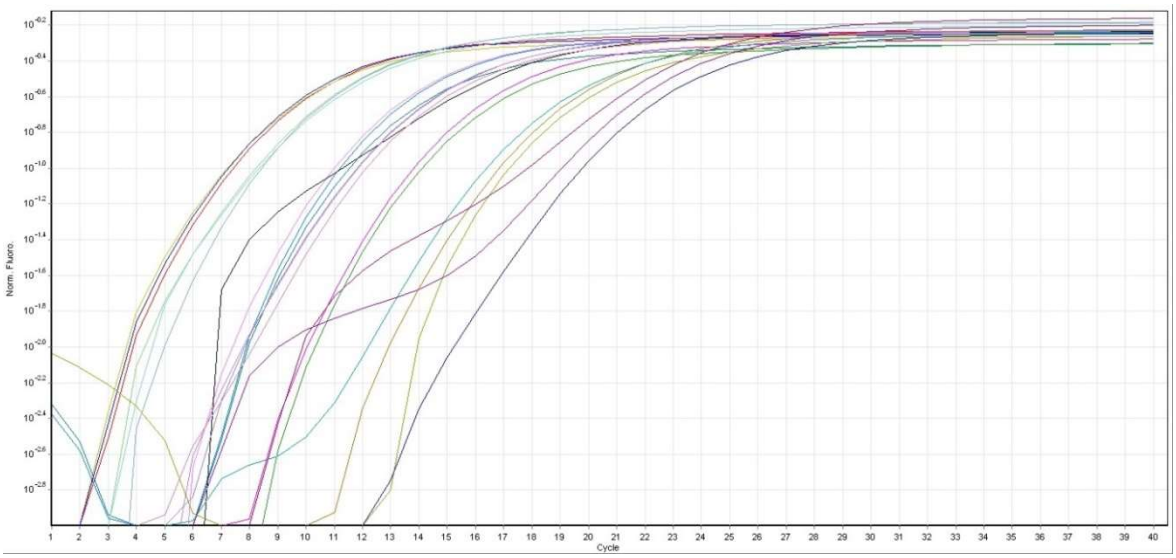


Figure 31: Amplification curve in semi-logarithmic view obtained from PCRs incorporating primer pairs 18Sv9_Euk 1391F/18sV9_EUKBR and RA_EimeriaF/RA_EimeriaR and various DNA extracts prepared from mock communities of *Eimeria* oocycts.

4.3.3 Verification of DNA fragment size in *Eimeria* 18S rDNA libraries

The average sizes of DNA bands of pooled *Eimeria* DNA were determined using the TapeStation system. As expected, bands of 297 bp and 600bp were obtained (Figure 32).

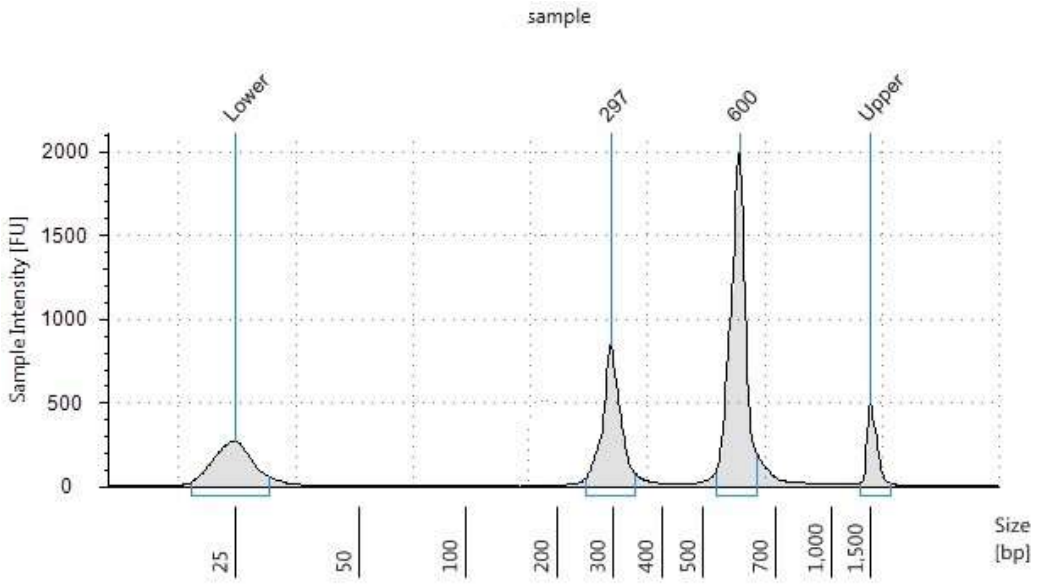


Figure 32: Electropherogram showing the analysis of pooled *Eimeria* DNA producing two concentrations (297 and 600 bp) with the Agilent High Sensitivity D1000 ScreenTape Assay and the Agilent 2200 TapeStation system. The system was used in order to calculate The average sizes of DNA bands range between the lower and the upper ladder values (25 to 1500 bp).

4.3.4 Quantification of *Eimeria* 18S rDNA libraries

To determine the average concentration of undiluted DNA of the pooled library, the Cq value (quantification cycle) was calculated using the qPCR (Table 41). The Cq values obtained from qPCR of each triplicate of the NEBNext library of different dilutions 1:1000; 1: 10,000 and 1:100,000 in addition to the Cq values for the 4 standards were interred into calculation tool in addition to the average fragment size of DNA obtained from Tapstation in order to calculate the average of undiluted DNA concentration of the pooled library.

Table 41: The calculations for estimating mean concentration of undiluted pooled library DNA.

Dilution (1:X)	Cq1	Cq2	Cq3	Average Cq	Undiluted concentration (nM)
1000	5.75	5.58	5.63	5.65	24.06
10000	8.35	9.32	8.65	8.77	30.53
100000	11.96	11.99	11.44	11.80	41.30
Average of undiluted concentration					26.4

In order to determine the efficiency of *Eimeria* extracted DNA, the standard curve of qPCR was created. In which, the *Eimeria* DNA (red boxes) was plotted against standard DNA (blue boxes) to calculate the efficiency. The efficiency (E) was 0.94 and the linear correlation (R2) was 0.97 (Figure 33).

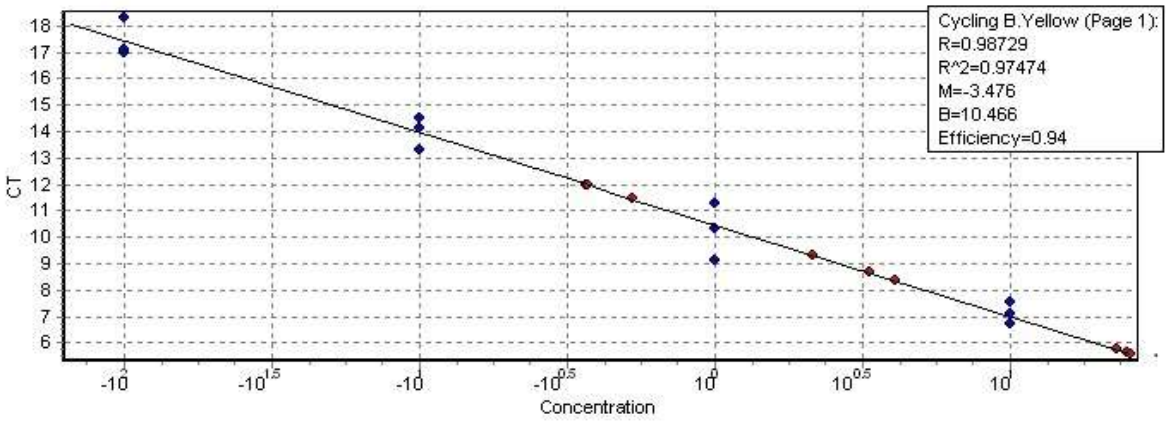


Figure 33: Quantitative polymerase chain reaction standard curve of *Eimeria* DNA.

4.3.5 Bioinformatic results for mock communities of chicken-associated *Eimeria* species

Oocycts from different species of chicken-associated *Eimeria* samples were mixed together in various ratios to generate a series of mock communites (Table 39). These samples were amplified using the *Eimeria*-specific RA_EimeriaF/RA_EimeriaR primer pair and the generic eukaryotic 18Sv9_Euk 1391F/18sV9_EUKBR primer pair. Bioinformatic analysis essentially analysed the resulting sequence data for the abundance of reads that could be specifically matched to the 18S rDNA sequence of one of the four *Eimeria* species within the mock communities. The number of reads assigned to each of these four species is presented in Tables 42 and 43. Far more reads could be assigned from the

RA_EimeriaF/RA_EimeriaR-generated dataset than the 18Sv9_Euk 1391F/ 18sV9_EUKBR-generated dataset. Indeed, typically only a few dozen reads from the 18Sv9_Euk 1391F/18sV9_EUKBR-generated dataset could be assigned to any of the four *Eimeria* species. For the RA_EimeriaF/RA_EimeriaR-generated dataset, occasionally >10,000 reads could be assigned to a specific *Eimeria* species, but more often this number was far lower. However, disappointingly, no correlation between the relative abundance of oocysts in mock communities and the relative number of NGS reads assigned to each of the four species could be discerned for either the RA_EimeriaF/RA_EimeriaR or the 18Sv9_Euk 1391F/ 18sV9_EUKBR-generated dataset.

Table 42: Species-specific reads obtained from NGS analysis of partial 18S rDNA amplification products generated using the Eimeria-specific RA_EimeriaF/RA_EimeriaR primer pair compared to relative contribution of species to mock communities, as calculated by counting oocyts. *no DNA, background control.

Sample No.	Results	*E. acervulina*	*E. mitis*	*E. necatrix*	*E. tenella*
1	NGS reads	25090	3112	245	448
	oocyst count	0	2000	0	0
2	NGS reads	3673	1692	21676	32106
	oocyst count	0	0	0	2000
3	NGS reads	4029	77	4294	1797
	oocyst count	15	60	30	90
4	NGS reads	66564	96	164	201
	oocyst count	2000	0	0	0
5	NGS reads	225	157	45	44
	oocyst count	30	0	0	0
6	NGS reads	3528	111	4111	1371
	oocyst count	312	312	2500	2500
7	NGS reads	32	2	12	5
	oocyst count	625	2500	312	312
8	NGS reads	50431	3465	8128	10821
	oocyst count	5000	625	625	625
9	NGS reads	9787	412	26242	9577
	oocyst count	2500	5000	5000	5000
10	NGS reads	23	4	4	4
	oocyst count	1000	1000	1000	1000
11	NGS reads	0	0	0	0
	oocyst count	0	0	2000	0
12	NGS reads	0	0	0	0
	oocyst count	15	30	15	60
13	NGS reads	220	1665	1656	3520
	oocyst count	30	90	150	300
14	NGS reads	3252	95	1000	152
	oocyst count	0	0	0	30
15*	NGS reads	0	0	0	0
	oocyst count	0	0	0	0

Table 43: Species-specific reads obtained from NGS analysis of partial 18S rDNA amplification products generated using the 18Sv9_Euk 1391F/18sV9_EUKBR primer pair compared to relative contribution of species to mock communities, as calculated by counting oocyts. *no DNA, background control.

Sample No.	**Results**	***E.acervulina***	***E. mitis***	***E.necatrix***	***E. tenella***
16	NGS reads	32	20	0	0
	oocyst count	0	0	0	2000
17	NGS reads	51	7	4	9
	oocyst count	0	2000	0	0
18	NGS reads	3	0	0	0
	oocyst count	3012	3012	2500	2500
19	NGS reads	8	6	0	1
	oocyst count	5000	625	625	625
20	NGS reads	30	16	1	5
	oocyst count	2500	5000	5000	5000
21	NGS reads	102	65	2	7
	oocyst count	1000	1000	1000	1000
22	NGS reads	14	0	2	4
	oocyst count	0	0	2000	0
23	NGS reads	16	8	1	0
	oocyst count	2000	0	0	0
24	NGS reads	0	0	0	0
	oocyst count	15	60	30	90
25	NGS reads	17	5	4	5
	oocyst count	15	30	15	60
26	NGS reads	15	2	1	1
	oocyst count	30	90	150	300
27	NGS reads	11	1	3	5
	oocyst count	60	120	80	30
28	NGS reads	68	6	11	9
	oocyst count	60	30	30	30
29	NGS reads	184	7	5	3
	oocyst count	0	0	0	30
30	NGS reads	22	3	9	6
	oocyst count	0	0	30	0
31	NGS reads	26	1	3	3
	oocyst count	0	30	0	0
32*	NGS reads	0	0	0	0
	oocyst count	0	0	0	0

As a result of this failure, NGS data generated from DNA extracts prepared from sheep samples were not analysed.

5. CHAPTER 5: Discussion

In this study, cross-sectional and longitudinal surveys of *Eimeria* and *Campylobacter* infections were used in sheep flocks in North-West England, in order to determine the diversity and the epidemiology of the infections they cause. In which, 10 *Eimeria* species were identified. The study showed, both *Campylobacter jejuni* and *Campylobacter coli* are the most frequently encountered *Campylobacter* species.

The study tried to explore the ecological determinants of the diversity and the epidemiology recognized. In which, a significant seasonal trend on the epidemiology of *Eimeria* infection and intensity was observed. A correlation between the intensity of *Eimeria* infection and each of animal age, rainfall prior to sample collection and the co-infection with *Campylobacter* was observed. Moreover, a clear seasonality of *Campylobacter* infection was recorded.

Although, this study attempt to quantify the epidemiology of *Campylobacter* infections in wildlife species (deer) living in the vicinity of sheep pasture to determine the importance of these species as sources of infection for livestock. However, neither of *Campylobacter* species was identified during the period of the study.

Finally, a molecular tool (NGS) was used in order to differentiate between *Eimeria* species. In which, no suggestion of a correlation between different *Eimeria* species was obvious.

5.1 The effect of seasonal trends

5.1.1 The association with prevalence

5.1.1.1 *Eimeria*

Season of the year has an effect on the overall prevalence of *Eimeria*. In which, the high prevalence of *Eimeria* infection was recorded in summer compared to autumn. Similar studies confirmed that the prevalence of *Eimeria* infections in addition to different other parasites was higher in summer, in which humid and hot weather are favourable for eggs to complete their cycle. (Khan *et al.*, 2011; Nahed-Toral *et al.*, 2003). The high prevalence of *Eimeria* infection that was recorded in summer in this study may be related to the higher shedding of oocyst in the feces of animal which increase the chance of infection with the infective oocyst due to the availability of favourable weather condition (warm and humid)

(Khan *et al.*, 2011; Nahed-Toral *et al.*, 2003). Moreover, the high infection rate of *Eimeria* in summer may relate to the presence of lambs in the flock that shedding a high number of *Eimeria* in the fecal material which increases the spread of *Eimeria* in the flock. A study conducted by Khan *et al.* (2011) confirmed that lamb shedding a high number of oocysts. Moreover, the presence of infected animals in the flock may increase the contamination of pasture with the oocyst leading to the spread of infection. Similar findings were observed previously, in which, grazing of ewe and lamb on contaminated pasture with *Eimeria* on spring and summer increase the coccidiosis (Nourollahi-Fard *et al.*, 2016).

5.1.1.2 *Campylobacter*

The present study showed that the infection with *Campylobacter* was detected throughout the year round, with an underlying pattern of seasonality in the prevalence of *Campylobacter.*

The season of the year has a significant effect on the probability of animals to be infected with *Campylobacter*. In this study, the highest prevalence of infection was recorded in summer and spring in both Threlkeld Farm and Abbot Park Farm as shown in the univariant results. This finding is consistent with data reported for grazing sheep in the UK and abroad in which the highest shedding of *Campylobacter* occurred in summer and warm weather (Jones *et al.*, 1999; Kudva *et al.*, 1997; Shahrokhabadi *et al.*, 2013). However, the result of Stock Farm in the univariant analysis showed an interesting observation of *Campylobacter* infection. In which the higher prevalence was observed in winter compared to summer. The same results were obtained from GLM analysis, which showed an increase of the *Campylobacter* prevalence in winter.

The increase in *Campylobacter* prevalence in both summer and winter is also in line with the suggestion that indicates that no seasonality variation in *Campylobacter* infection rate recognized in the grazing ewes (Stanley *et al.*, 1998). In a study conducted at Lancaster, UK, the rate of *Campylobacter* shedding varied at different times of the year, the highest rate (100%) was coinciding with lambing, weaning, and movement of the herd onto new pasture, while the lowest rate of shedding occurs when the herds feed on silage and hay (Jones *et al.*, 1999).

In Stock Farm, the sheep spend all time on fell during winter season compared to Abbot Park Farm where the sheep stay on by-land of the farm and sometimes indoor, that may

explain the difference in *Campylobacter* prevalence between the two farms. A study found temperature has an indirect effect on the prevalence of infection with a lower prevalence recorded when animals housed indoor during winter compared to animals living outdoor (Jones *et al.*, 1999). Grazing of animals in the fell may affect the *Campylobacter* shedding positively due to the challenge of harsh winter or type of food that depend mainly on pasture. Moreover, grazing of sheep of Stock Farm in fell most time of the year may increase the prevalence of *Campylobacter* infection compared to Abbot Park Farm in which the animals grazed most time of the year in the by-land which intern may increase the frequency of the tested species. This in line with the study conducted in Lancashire, where the prevalence of *Campylobacter* different depends on the type of pasture. In which the higher shedding was recognized on saltmarsh grazing compared to grazing on upland fell and lastly farm grazing (Jones *et al*., 1999). However, lower grass quality may increase *Campylobacter* shedding in sheep (Kaneene & Potter, 2003). Drinking of sheep grazed on fell, from *Campylobacter* contaminated stream and river increase the possibility of animal to be infected with the *Campylobacters* (Stanley & Jones, 2003), The high prevalence of *Campylobacter* on Stock Farm in winter may relate to the contact of sheep with wild animals or birds in the fell in which the wild birds considered as a source of infection to the farm animals. In a study conducted by Colles *et al.* (2008), it was confirmed that ST-42 was isolated from wild birds, in this study the same ST was isolated from sheep fecal samples at Stock Farm. A study conducted on cattle farm showed that several factors rather than the temperature may affect the *Campylobacter* shedding and increase the prevalence of a disease such as the presence of birds, insect and rodent (Stanley & Jones, 2003). In which, birds play an important role in contamination due to the presence of bacteria in sheep faeces that been picked by birds (Skirrow, 1994). The population density also has a positive impact on the prevalence of *Campylobacter* infection. A study conducted in cattle farm showed the high population density increase the *Campylobacter* prevalence. In which, the animals are highly exposed to *Campylobacter* from other animals (Grove-White *et al.*, 2010). By looking at the farm management practices, in Abbot Park Farm only adult ewes were sent to the fell during winter compared to the sheep in Stock Farm in which the adult and their lamb spent all winter in the fell. That may have an effect on the prevalence rate of infection with *Campylobacter* positively due to continuous exposure to the pathogen. Rainfall may have an effect on the prevalence of the disease which may increase the occurrence of *Campylobacter* in winter. In a study conducted by Taema *et al.* (2008), it was found some evidence of a correlation between temperature and rainfall that affect the prevalence of the

disease, in which the prevalence of the *Campylobacter* disease increase in the low temperature with the presence of rainfall.

5.1.2 The association with intensity

The data retrieved from the univariant and multivariant analysis showed that season of the year has a significant effect on the intensity of *Eimeria* in sheep. The study showed a significant difference in the overall intensity according to the season in which the highest *Eimeria* intensity was recorded in summer compared to autumn. The presence of lambs in the field that shed high numbers of oocyst may have an effect on the intensity of infection by *Eimeria*. It has been reported that, at the beginning of summer and in late autumn, oocysts intensity in adult sheep rises from low number to several hundred and this coincides with the apperance of lambs (Reginsson & Richter, 1997). In addition to the presence of infectious oocyst in the field due to a climatic condition which increases the chance of animal to have more *Eimeria* parasite present on contaminated pasture. In summer, the presence of a high number of infectious oocysts (due to wet and warm weather) shed by animals (Khan *et al.*, 2011) or from previously contaminated pasture lead to increase in the intensity of the disease.

The intensity and prevalence of *Eimeria* infection in sheep are significantly higher in wet season compared to dry seasons (Ibrahim & Afsa, 2013). As the oocysts excretion by animals is influenced by many factors such as the immunity status of the animals (Reeg *et al.*, 2005a) and infectious dose of *Eimeria* oocysts (Gregory & Catchpole, 1990); Immune stress factors such as crowding density may lead to a reduction in the immunity of animals, thereby provoking an increased intensity of *Eimeria* (Craig et al., 2008).

5.1.3 The association with *Eimeria* species

The information on prevalence and the species composition of *Eimeria* are important to implement effective control programs. Among 15 *Eimeria* species were described in sheep (Kaufmann, 2013; Khan *et al.*, 2011; Reginsson & Richter, 1997; Saratsis *et al.*, 2011) and 11 locally *Eimeria* species were recorded in the UK (Taylor, 2009), 10 *Eimeria* species were identified in this study. The association between *Eimeria* species and the season were determined. In which *E. ovinoidalis* was the most encountered species during all seasons except for autumn when *E. bakuensis* was the most prevalent. These findings are similar with (Catchpole *et al.*, 1976) in which *E. ovinoidalis* considered as predominant species due to the high ability to reproduce. However, *E. intricate* was the less prevalent species detected

in this study. Moreover, a similar result was found by Skirnisson (2007) in which *E. ovinoidalis* was predominant in all seasons and *E. bakuensis* was dominant in autumn and winter compared to spring and summer.

Although *E. ovinoidalis* and *E. bakuensis* were recorded to be the dominant species in many studies (Agyei, 2003; Reeg *et al.*, 2005a) and consider as a pathogenic *Eimeria* spp. that dominant in animals in this study, these animals did not show any clinical signs of coccidiosis. This might be due to the rate of development of immunity. Different parameters may affect the clinical coccidiosis such as weaning period, farm husbandry, hygiene and occurrence of another type of infection (Vercruysse, 1982).

5.1.4 The association with Co-infection

The co-infection between *Eimeria* species was stratified against season in which there was a significant difference between the type of infection on different seasons. Infection by triple or dual *Eimeria* species at three farms was more common in all seasons compared to single infection. This finding similar to other reports in which triple or dual infections were more common than single infection (Arslan *et al.*, 1999; Gregory *et al.*, 1980a; Toulah, 2007). It was confirmed that infection with more than one *Eimeria* species is considered as a normal state of infection by *Eimeria* (Vercruysse, 1982).

5.2 The effect of age trends

5.2.1 The association with prevalence

5.2.1.1 *Eimeria*

The combined data and the GLM analysis showed that age of animals had a significant effect on the probability of animal to be infected with *Eimeria*. The overall prevalence of *Eimeria* infection tends to be higher in young animals compared to older animals. However, the odds ratio of animals to be infected was 0.58. The Same result was obtained in other studies in which animals aged between 6 months and one year tend to have a higher prevalence of infection compared to older groups (Om *et al.*, 2010; Sisodia *et al.*, 1997; Wang *et al.*, 2010). The occurrence of high prevalence in young animals may relate to the weak immunity or

less resistance to *Eimeria* in young animals (Gregory *et al.*, 1980a; Maingi & Munyua, 1994).

5.2.1.2 *Campylobacter*

The overall prevalence of *Campylobacter* infection at both Abbot Park Farm and Stock Farm was investigated by combined data according to the age groups of animals. The study showed that all age groups were had an infection with *Campylobacter*. Although *Campylobacter* was more frequent in animals aged >24 months, there was no statistical variation in the prevalence between different age groups. However, the GLM analysis showed only the season of the year has an effect on *Campylobacter* infection compared to the age effect.

5.2.2 The association with *Eimeria* intensity

In this study, infection by *Eimeria* was detected in the all age groups which it was similar to the previously reported studies (Barutzki et al., 1990; Taylor & Catchpole, 1994). Although most of the examined sheep in this study shed oocysts, the oocyst output in the sheep is age related, in which the intensity of the oocyst shedding decrease with the increase of animal age. Similar findings had previously been reported in sheep in the United Kingdom and Papua New Guinea, in the oocyst output decrease as the age of animals increase (Pout, 1973; Varghese & Yayabu, 1985). The intensity of infection in lamb is tended to be higher compared with the adult animal (Hashemnia et al., 2014; Maingi & Munyua, 1994), this may due to the low immunity and low resistance of young animals (Kanyari, 1988; Maingi & Munyua, 1994).

5.2.3 The association with *Eimeria* species

Eimeria infection was observed in all age groups which is similar to reported data (Taylor & Catchpole, 1994). The infection of animal starts very early when lamb age between 2-3 weeks in which the prevalence and intensity of the *Eimeria* reach the peak around weaning period. The intensity of the disease is reduced clearly but not reach to zero in older sheep (Chartier & Paraud, 2012).

E. ovinoidalis is the most common species observed in all age groups followed by *E. bakuensis*. There is a significant difference in the prevalence between *E. ovinoidalis* in the animals age from 1-12 month and the other animal age groups. This result similar to other studies, in which the prevalence of *E. ovinoidalis* is higher in young animals (Gregory *et al.*,

1983; Reeg *et al.*, 2005a). Similarly, *E. bakuensis* in the animals of age group 1-12 month is significantly different from animals age 13-24 months and animals age more than 24 months. The same result found in Soay sheep (*Ovis aries L.*) on St Kilda in UK in which *E. bakuensis* and *E. weybridgensis* were dominant in young animals except for *E. granulosa* (Craig *et al.*, 2007). Distinct reduction in the number of *E. ovinoidalis* and other species may relate to the early onset of immunity toward this species in lamb (Reeg *et al.*, 2005a), which may indicate that immunity about certain *Eimeria* species is selective (species specific) similar to *Eimeria* in chicken (Lillehoj & Lillehoj, 2000). That may explain why this species is highly prevalent in the young animals.

5.2.4 The association with Co-infection

Infection by three or more of different *Eimeria* species was the highly prevalent in all age groups of examined animals, in which there is no significant difference between age groups according to the type of infection. However, the multiple infections were the most frequent type of infection in all age groups. This finding similar to other reports in which the dual or triple infections were more common than single infections (Arslan *et al.*, 1999; Gregory *et al.*, 1980a; Toulah, 2007; Wang *et al.*, 2010). The occurrence of mixed infection in the animal considered as a normal state of infection by *Eimeria* (Vercruysse, 1982).

5.3 The effect of climate trends

Person correlation analysis was performed in order to investigate the climate effect (temperature and rainfall) on each of prevalence and intensity of *Eimeria* infection. In this study, a significant correlation between rainfall and *Eimeria* intensity was observed. In which, *Eimeria* intensity increase significantly when the month prior to sampling is wetter (greater rainfall). This correlation may help to predict the occurrence of the disease next month. During the rainy and wet season, the high humidity and ambient temperature provide favourable conditions for sporulation of oocysts which in turn increase the burden of the disease (Ibrahim & Afsa, 2013; Nuvor *et al.*, 1998; Rodríguez-Vivas *et al.*, 1996). However, no correlation was identified with the prevalence of infection.

Moving to the effect of climate on *Campylobacter* infection, there is a moderate negative significant correlation between *Campylobacter* infection prevalence and the mean temperature in the month prior to the survey. A study conducted by Taema *et al.* (2008) on *Campylobacter* infection in a zoological collection in the UK, investigated the climate effect

(rain and temperature) on *Campylobacter* prevalence in the week prior to the onset of the disease (isolation) which showed an increase in the prevalence of disease at high temperature. However, the prevalence of the disease increases at low temperature coinciding with the occurrence of rain.

5.4 Correlation between *Campylobacter* and *Eimeria*

In this study, the GLM analysis showed that the intensities of *Eimeria* infections are lower in sheep co-infected with *Campylobacter* than in sheep without a concurrent *Campylobacter* infection. Similarly, as seen with *Eimeria* infections in general, the prevalence of *Eimeria ovinoidalis* was lower in animals co-infected with *Campylobacter* than animals without a *Campylobacter* infection.

The co-infection with different parasites at one time is considered as a normal state that may occur in human and different animals (Cox, 2001; Petney & Andrews, 1998; Telfer *et al.*, 2010). Microorganisms like *Campylobacter* and coccidia have represented an example of a wide community of pathogens that can habitat livestock digestive system with no clinical effect. The composition of this community depends on the interaction between them (Graham *et al.*, 2007). However, the interaction mechanism is not understood which may relate to direct and indirect interactions (Ezenwa, 2016). Many studies showed the negative correlations between co-infectors in which the presence of one parasite may reduce the presence of the another. In an experiment conducted on mice, a negative correlation was observed between *Schistosoma mansoni* and *Trichinella spiralis*, in which, infection of mice with *S. mansoni* increase the resistance of animal against *T. spiralis* (Christensen *et al.*, 1987). A study conducted on sheep found that the presence of naturally acquired infection with *Stileria hepatica* may induce resistance against experimental infection with *Fasciola. gigantica* (Hammond, 1973).

5.5 Correlation between *Eimeria* species

The epidemiologies of *E. ovinoidalis* and *E. bakuensis* was tested using the GLM analysis. The study showed the co-infection with the two species is being significantly more common than would be expected by chance alone. A study conducted by Skirnisson (2007) suggested that coccidiosis caused by *E. ovinoidalis* was commonly associated with infections by other *Eimeria* species including *E. bakuensis*.

Moreover, both species are more frequent on Stock Farm than Abbot Park Farm. The difference in the relative abundance of both species between farms may relate to different factors.

The large herd size (600 sheep) owned by farmer at Stock Farm compared to Abbot Park Farm (200 sheep) may affect the frequency of the two species. A number of studies found the prevalence of *Eimeria* is higher in the large herd size compared to smaller one, which in turn increases the environmental contamination (Kusiluka *et al.*, 1998; Rehman *et al.*, 2011). Moreover, the frequency of pathogenic *Eimeria* was higher in the large herd size which increases the prevalence of the disease (Klockiewicz *et al.*, 2007).

A previous study showed the difference in prevalence of infection between farms may relate to differences in the ecological conditions or different in management programs followed by sheep producers to control coccidiosis (Ibrahim & Afsa, 2013). In this study, farmer management may contribute to the prevalence rate of *Eimeria*. Grazing of sheep of Stock Farm in fell most time of the year may increase the prevalence rate of *Eimeria* infection compared to Abbot Park Farm, in which the animals grazed most time of the year in the by-land which intern may increase the frequency of the tested species. Moreover, contact with other animals from a different farm or with wild animals may increase the prevalence of the disease.

5.6 Differentiation between *Campylobacter jejuni* and *Campylobacter coli*

Campylobacter specific primers were used in order to differentiate between *C. jejuni* and *C. coli* isolates at Abbot Park Farm. The most common infection of tested samples was the mixed infection. Although the most frequent species was *C. jejuni*, however, there is no significant difference between the two species. This result similar to other studies in which *C. jejuni* was recorded to be the most common species responsible for campylobacteriosis in sheep that cause enterocolitis (Bailey *et al.*, 2003; Yang *et al.*, 2014a). In Lancaster, UK, *C. jejuni* was the main species isolated on pasture from sheep faeces followed by *C. coli* (Jones *et al.*, 1999). In a study conducted by Stanley *et al.* (1998), *C. jejuni* was estimated to comprise 87% of *Campylobacter* isolated from sheep fecal samples. Another study conducted in the North West of England revealed that most of the livestock sent to slaughter are infected with one or more species of *Campylobacter* (Stanley & Jones, 2003).

5.7 Survey of deer

Many studies indicate that *Campylobacter* can be isolated from deer in different part of the world (Jokinen *et al.*, 2011; Koronkiewicz, 2004; Petersen *et al.*, 2001). Despite this, few studies have reported the isolation of *Campylobacter* spp. from deer in the United Kingdom. A study by Taema *et al.* (2008) was conducted in order to investigate the presence of *Campylobacter* in wild animal populations from 1990-2003. In this study, *Campylobacter* isolates were obtained from three deer in a zoological collection over the period of the study. *Campylobacter* has not detected in deer fecal samples. That may suggest, deer do not contribute to the natural persistence of campylobacters in Cumbrian sheep populations. However, in the present study one bacterial isolate was detected in deer fecal sample using the md16S primer and confirmed again using universal 16S rDNA primer. This isolate belonged to the *Acinetobacter* species. This result is similar to another study, in which the *Acinetobacter* species was isolated from the internal organs of the fallow deer on Little St. Simons Island, Georgia, USA (Morse *et al.*, 2009).

5.8 Campylobacters STs

In this study, many *Campylobacter* STs were identified by applying MLST technique such as *C. coli* ST-828, *C. jejuni* ST-61 and ST-42. Similar STs were isolated from sheep faecal samples (Colles *et al.*, 2003; Sheppard *et al.*, 2009; Wilson *et al.*, 2008). These STs were identified to be associated with different reservoirs such as sheep, cattle, human stool, environmental water, chicken and other animals. *C. jejuni* ST-61 is responsible for 60% of human disease isolates (Dingle *et al.*, 2002a). The ST-61 complexes were recorded to be predominant among sheep and cattle (Colles *et al.*, 2003). That suggests ruminants may act as an important source of contaminated food and environment that lead to human infection (French *et al.*, 2005). In addition to the ST-61 complexes, ST-42 was mainly isolated from human disease, cattle, and sheep (Dingle *et al.*, 2002a). The importance of these *Campylobacter* strains (STs) is their ability to infect human (Rotariu *et al.*, 2009).

C. coli isolates belonging to ST-828 were isolated from different sources such as human, sheep, chicken, farm environments, pig, water, cattle, duck and turkey. Both humans and animals can be infected with ST-828 which is why this ST is important from the perspective of foodborne diseases (Sheppard *et al.*, 2009; Thakur *et al.*, 2006).

As the stream water is the only source of drinking water in these farms, this may play a role in the occurrence of these STs such as ST-61 through the contamination of water sources with faeces of infected animals and wild animals as well as seeping of agricultural water from farms. All these factors may have related to increasing infection with this ST (Carter *et al.*, 2009; Daczkowska-Kozon & Brzostek-Nowakowska, 2001; Pitkänen, 2013).

As the stream water is going through different farms in this area and it is the only source of drinking water for sheep that grazing and defecating around it, the chance of the presence of the STs mentioned above is increased. Finally, a new ST was identified (ST-5351) which has no clonal complex record reported previously when assign in the MLST website, which belongs to *C. coli*.

Campylobacter jejuni and C. coli are also delineated using *aspA* sequence comparison with using species- specific PCR. Delineation of the two-species allowed their relative abundance on Abbott Park Farm to be assessed. The study showed the sequence obtained from *aspA* allele identified the most of the isolates at Abbot Park Farm as a single type of *Campylobacter* species. Compared to the data obtained from using species-specific *Campylobacter* primers in which the mixed infection was the most frequent. That clarify there is a frequently mixed infection in the individual sheep. In which, the *aspA* identified the isolate as a single infection, that may due to differences in the relative abundance of *C. jejuni*/ *C. coli* in the sample in which the relative abundance of one species covered the occurrence of the other in sequence result.

5.9 Future work

5.9.1 Co-infections

Co-infection with different parasites at one time may normally occur in individual humans and animals (Cox, 2001; Petney & Andrews, 1998; Telfer *et al.*, 2010). Occurrence of multiple species in a single host is common for specific parasite and region compared to single host infection or non-infected host, in which the dynamic and structure of these multiple communities may be affected by host related factors and environmental factors (Petney & Andrews, 1998). The co-infection between different parasites interacts either positively or negatively as shown in different studies (Adams *et al.*, 1989; Christensen *et al.*, 1987; Frontera *et al.*, 2005). Understanding the interaction between those multiple communities may help to predict the occurrence of disease and chose the necessary control program (Pedersen & Fenton, 2007).

In order to evaluate the occurrence of the pathogenic parasites in nature and the interaction between them, experimental perturbation (such as evaluate the changes by adding or removing of other species) can apply in the laboratory to understand interspecific parasites interaction (Bender *et al.*, 1984).

Seting up an experiment in the laboratory may help in studying and understanding the effect of co-infection in sheep between *Campylobacter* and *Eimeria*. A longitudinal survey of individual sheep would help to investigate whether *Campylobacter* has an effect on *Eimeria* or if *Eimeria* has an effect on *Campylobacter*. The infection between *Campylobacter* and *Eimeria* observed in our study could be further explored experimentally to determine whether the increase in the concentration of *Eimeria* is a result of *Campylobacter* infection or whether increase in concentration of *Eimeria* predisposes animals to *Campylobacter* infection. However, such laboratory experiments are not accurate to give all picture of the type of interaction between the pathogens, due to different and uncontrol condition occurs in nature that cannot imitate in the experimental models such as burden of the disease and the time in which the co-infection occur (Fenton, 2013).

As many studies showed, that model system of laboratory experiment may help in understanding the relation of interaction between multiple parasites in the same host as well as study the infection period, clinical symptoms and the intensity of the diseases (Graham, 2008; Graham *et al.*, 2005; Rodriguez *et al.*, 1999). Predict the outcome of parasites co-infection will help in design a proper control program and increase the knowledge about the

ecology of both parasite and host as well as the evolution of both (Graham, 2002; Pedersen & Fenton, 2007; Read & Taylor, 2001). For example, a number of studies explain the interaction between helminth and microparasite. In which helminth may reduce the surface required by microparasite to attach and produce a disease (Roberts-Thomson *et al.*, 1976) or affect the microparasite replication by reducing the cell type required (Lwin *et al.*, 1982). The interaction between coccidia and different pathogens was observed. In which, coccidia infection severity may affect due to infection with a different type of pathogens such as bacteria, helminths and viruses (Taylor, 1995). Moreover, microflora such as Gram negative bacteria may increase due to infection by *Eimeria* (Mohammed *et al.*, 2000), as well as secondary bacterial infection (Taylor *et al.*, 1973; Yang *et al.*, 2014b). Iinteractions between microflora and *E. ovinoidalis* was recognized in experiments conducted on the lamb in which presence of digested microflora is necessary to develop the *E. ovinoidalis* pathogenic expression (Gouet *et al.*, 1984). However, difficulty in the prediction of how infection will affect the other may result from the complexity within the host (Graham, 2008).

5.10 NGS discussion

The Next Generation sequence technique was used in this study in order to identify and differentiate between different *Eimeria* species in sheep. Mock communities of chicken *Eimeria* species were created and used as control groups to imitate the community of *Eimeria* species in the sheep fecal samples. The mock community was designed to have identified an overall number of different *Eimeria* species, as well as varied in a number of oocyst between the low number and high number.

The comparison of the generated data (from using chicken *Eimeria* specific primer) against the constructed database from NCBI was not clear. Similarly, the matching of data of chicken *Eimeria* species (that generated from a broad-range eukaryotic specificity primer) with constructed database was also not clear.

By looking to the data generated from four *Eimeria* species using the 18Sv9_Euk 1391F/18sV9_EUKBR primer pair, is much less compared to the data generated by the use of Eimeria-specific primers. That may relate to the primer selectivity, in which the Eimeria-specific primers were more specific to generate much data. In study conducted by Junier *et al*. (2008) to evaluate the selectivity of PCR primer, in which more sequence read was observed when specific primers were used compared to combination of primers.

5.10.1 Why did it not work?

Although the four *Eimeria* species of chicken were sequenced, they had a huge amount of sequence variations. The read data that generated (to categorized by the software) are varied in length size, some of them are long read (example 400bp) and most of them are a short read. The software for some reasons was categorized those short reads to a specific group of *Eimeria* species while these short reads they can match any one of different other *Eimeria* species. Closing the gaps in the *Eimeria* genome (many thousands) still consider as a challenge for the sequence assembly process (Reid *et al.,* 2014).

One of the reasons that may lead to mismatching of the generated read with the specific sequences of different *Eimeria* species is the PCR conditions, that had been used to generate these reads. The hypothesis behind that, the PCR condition lead to generate a lot of short reads instead of long read that aimed to look for.

In a study conducted by Vermeulen *et al.* (2016) investigating the biodiversity of *Eimeria* communities. In which, from 158 samples, only 58 sample originate enough amount of DNA

in the PCR amplification which then undergo sequencing analysis, they emphasized that the reason of the failure of amplification for some samples may due to the occurrence of PCR inhibitors or the presence of overhang adapters that have been added to the primer, which may affect the primer efficiency during annealing in the PCR amplification.

5.10.2 What could be done in the future to get the results?

Need to design *Eimeria* primer specific for NGS application, forcing the MiSeq to read a great proportion or the complete sequence of data. So, if the MiSeq have not read the missing sequence (the short length), the ability to differentiate between species will fail. Moreover, targeting a part of 18S gene that believes to be better in term of differentiating between *Eimeria* species.

Targeting other gene rather than nuclear 18S rRNA-encoding genes were expected to see for more regular variation, such as ribosomal internal transcribed spacer-1 (ITS-1) region (Khaier *et al.*, 2016; Kumar *et al.*, 2014), a second internal transcript spacer region (ITS-2) , 5S ribosomal repeat or mitochondrial genes such as cytochrome c oxidase subunit I-encoding gene (mtCOI) in order to differentiate between different *Eimeria* species (Woods *et al.*, 2000).

5.10.3 The wider application of the experiment

Eimeria species are difficult to grow and animals almost always carry multiple *Eimeria* species. Also, it is known that identification of fastidious species may be biased as some may be better adapted to laboratory media than others, so the NGS may offer an alternative routine practice over *Eimeria* identification species and differentiation. If the primers are not biased then a non-bios alternative to cultural base in the assessment of the diversity of *Eimeria* species will be available.

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