Anthelmintic treatment in horses: efficacy and effects on intestinal health

Simon Paul Daniels

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

School of Veterinary Medicine
Faculty of Health and Medical Sciences
University of Surrey
Date of Submission: December 2017
Declaration of originality

By signing the below I confirm that:

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their original originator in the text, list of references or in footnotes. This thesis has not been submitted in whole or in any part for an academic degree or professional qualification. I agree that the University has the right to submit my work to the plagiarism detection service Turnitin UK for originality checks. Whether or not the drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above.

Simon Daniels
Abstract

Cyathostomins are ubiquitous in horses and high strongyle burdens are associated with colic. Parasite control, often using anthelmintic drugs, is an important element of equine healthcare. Anthelmintic dosing is also a risk factor for colic, which may be due to disruption of the mutualistic microbiota within the hindgut. This thesis describes studies to evaluate the current efficacy of equine anthelmintics and interactions with intestinal bacterial populations which may underlie the reported association with colic.

Faecal egg count data from horses throughout Great Britain was used to calculate strongyle egg reappearance periods (ERP) for ivermectin and moxidectin. For both drugs reduced ERP was detected compared to earlier studies. The ovicidal efficacy of fenbendazole was evaluated in resistant cyathostomins. Using an in vitro egg hatch viability assay, an 80% reduction of egg hatch rate was detected but this only persisted for three days following treatment. These studies further characterise the growing problem of anthelmintic resistance in equine parasites.

Microbiota-anthelmintic interaction was evaluated over three studies: i) In vitro feed fermentation was used to detect a decrease in fermentation rate and gas production following moxidectin administration; ii) 16S rRNA sequencing of faecal microbiota before and after moxidectin treatment revealed no change in bacterial community profile; and iii) 1H NMR metabolomics identified no significant differences in urinary and faecal metabolite profiles pre- and post-moxidectin treatment but in-vitro fermentations did show differences in alanine, ethanol, 5-HT, formate and maltose following moxidectin treatment.

These studies further characterise reduced efficacy of anthelmintics against equine cyathostomins and suggest that although moxidectin treatment has no measurable effect on bacterial community profile, it may be associated with functional changes in the gut microbiome.
Acknowledgements

Firstly I would like to acknowledge my supervisor Professor Chris Proudman for all of his guidance during this project. I would like to acknowledge my employer the Royal Agricultural University, specifically Professor Meriel Moore-Colyer and Dr Andrew Hemmings for providing time in my workload to dedicate to this research.

I would like to thank MSD Animal Health, Zoetis and Technologies Strategies Board (KTP) for the funding and anthelmintics provided that made this work possible. I would also like to thank ELPA Ltd for providing faecal egg count data and treatment records.

I would like to thank Professor Meriel Moore-Colyer for her advice and laboratory assistance for the in vitro gas production work. I would like to thank Dr Joy Leng for her help in the laboratory and advice with the bioinformatics and analysis of my sequencing and NMR data. I would like to thank Dr Jon Swann at Imperial College for processing the NMR samples and Dr Richard Ellis at APHA for doing the 16S sequencing. For the pure parasitology aspect I would like to acknowledge the team in the Diagnosteq laboratory at the University of Liverpool and Dr Laura Peachey for her help with the egg hatch assay protocol. I would like to acknowledge the laboratory technicians at the RAU and Hartpury College for their assistance with these studies.

Finally I would like to acknowledge my wife, and latterly daughter, who have had to put with my workaholic tendencies throughout this research project.
List of Contents

Section                                      Page

Abstract                                      iii
Acknowledgements                             iv
List of Figures                               vii
List of Tables                                xi
List of abbreviations                        xii

1.0 Introduction

1.1. The equine gastrointestinal tract        1
1.2. Intestinal disease in horses             2
1.3. Parasitism and colic                     5
1.4. Cross species comparison                 24
1.5. Combination anthelmintics               26
1.6. Summary                                 28
1.7. Research aims                           29

2. Review of methods                          30

2.1. Parasitology                             30
2.2. Measuring fermentation parameters in vitro 34
2.3. Microbiome community profiling          39
2.4. Metabolomics                             44

3. Nationwide surveillance for evidence of reduced macrocyclic lactone efficacy in horses in the UK  51

3.1. Introduction                             51
3.2. Methods and Materials                   53
3.3. Results                                 58
3.4. Discussion                              63
3.5. Conclusion                              67

4. Ovicidal efficacy of fenbendazole after treatment of horses naturally infected with cyathostomins  68

4.1. Introduction                             68
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2. Materials and Methods</td>
<td>69</td>
</tr>
<tr>
<td>4.3. Results</td>
<td>75</td>
</tr>
<tr>
<td>4.4. Discussion</td>
<td>80</td>
</tr>
<tr>
<td>4.5. Conclusions</td>
<td>84</td>
</tr>
<tr>
<td><strong>5. The effect of moxidectin administration on in vitro equine hindgut</strong></td>
<td>85</td>
</tr>
<tr>
<td>5.1. Introduction</td>
<td>85</td>
</tr>
<tr>
<td>5.2. Materials and Methods</td>
<td>86</td>
</tr>
<tr>
<td>5.3. Results</td>
<td>96</td>
</tr>
<tr>
<td>5.4. Discussion</td>
<td>106</td>
</tr>
<tr>
<td>5.5. Conclusions</td>
<td>112</td>
</tr>
<tr>
<td><strong>6. The effect of moxidectin administration on equine faecal microbiota</strong></td>
<td>113</td>
</tr>
<tr>
<td>6.1. Introduction</td>
<td>113</td>
</tr>
<tr>
<td>6.2. Materials and Methods</td>
<td>114</td>
</tr>
<tr>
<td>6.3. Results</td>
<td>118</td>
</tr>
<tr>
<td>6.4. Discussion</td>
<td>131</td>
</tr>
<tr>
<td>6.5. Conclusions</td>
<td>135</td>
</tr>
<tr>
<td><strong>7. The effect of moxidectin administration on the equine metabolome</strong></td>
<td>136</td>
</tr>
<tr>
<td>7.1. Introduction</td>
<td>136</td>
</tr>
<tr>
<td>7.2. Materials and Methods</td>
<td>137</td>
</tr>
<tr>
<td>7.3. Results</td>
<td>141</td>
</tr>
<tr>
<td>7.4. Discussion</td>
<td>152</td>
</tr>
<tr>
<td>7.5. Conclusions</td>
<td>157</td>
</tr>
<tr>
<td><strong>8. General discussion</strong></td>
<td>158</td>
</tr>
<tr>
<td>8.1. Parasites and microbiota</td>
<td>161</td>
</tr>
<tr>
<td>8.2. The future of equine parasite control?</td>
<td>163</td>
</tr>
<tr>
<td>8.3. Parasitism, anthelmintic treatment and intestinal disease</td>
<td>165</td>
</tr>
<tr>
<td>8.4. Future study</td>
<td>165</td>
</tr>
<tr>
<td><strong>9. Conclusions</strong></td>
<td>166</td>
</tr>
<tr>
<td><strong>10. References</strong></td>
<td>167</td>
</tr>
<tr>
<td><strong>11. Appendix 1 – Publications from this thesis</strong></td>
<td>204</td>
</tr>
</tbody>
</table>
**List of Figures**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 The anatomy of the equine gastrointestinal tract.</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Fermentation pathways of dietary carbohydrates in the horses’ hindgut.</td>
<td>4</td>
</tr>
<tr>
<td>1.3 Cyathostomin lifecycle.</td>
<td>8</td>
</tr>
<tr>
<td>1.4 Schematic diagram of the over dispersion of cyathostomins within a horse population.</td>
<td>16</td>
</tr>
<tr>
<td>2.1 Example of cumulative gas production and the fitted France <em>et al.</em> (1993) model.</td>
<td>39</td>
</tr>
<tr>
<td>2.2 Schematic of the 16S gene identifying variable regions (V1-V9), primers, amplicons and sequencing of those amplicons using the illumina platform.</td>
<td>40</td>
</tr>
<tr>
<td>2.3 Schematic representation of sequencing by synthesis, part of the illumina sequencing technology.</td>
<td>41</td>
</tr>
<tr>
<td>2.4 Graphical output from QIIME, alpha rarefraction curve and beta diversity PCoA plot.</td>
<td>42</td>
</tr>
<tr>
<td>2.5 Schematic representation of LEfSe.</td>
<td>43</td>
</tr>
<tr>
<td>2.6 Cladogram obtained from LEfSe, this plot identifies the relationship between the biomarkers identified within the model.</td>
<td>44</td>
</tr>
<tr>
<td>2.7 Schematic representation of the use of NMR spectroscopy within the field of systems biology.</td>
<td>47</td>
</tr>
<tr>
<td>2.8 Schematic representation of a PCA.</td>
<td>49</td>
</tr>
</tbody>
</table>
2.9. Schematic representation of an OPLS-DA model. 50

3.1 Locations of all premises tested for ivermectin and moxidectin ERP. 56

3.2 Faecal egg count reduction (%) yearly trend for moxidectin from 2008-2011. 62

4.1 Box and whisker plot illustrating the distribution of egg hatch rates following fenbendazole treatment. 78

4.2 Box and whisker plot illustrating distribution of eggs and larvae pre and post fenbendazole treatment. 79

5.1 Schematic representation of the *in vitro* gas production experiment. 89

5.2 *In vitro* gas production set up for gas readings. 92

5.3 Modelled gas production prior to moxidectin treatment. 96

5.4 Mean gas produced per hour (ml) prior to moxidectin treatment for both hay and oat substrates. 98

5.5 Panel plot for mean modelled gas production curves for hay and oats post treatment. 99

5.6 Panel plot of gas produced per hour for both feed substrates after moxidectin administration. 100

5.7 Dry matter loss (%) of hay substrate after moxidectin administration. 101

5.8 pH of faecal inoculum after hay fermentation post moxidectin administration. 102

5.9 pH of faecal inoculum after oat fermentation post moxidectin administration. 103
5.10 Fractional rate of gas production halfway through fermentation of hay.

5.11 Degradation rate of oats following moxidectin administration.

6.1 Panel plot of alpha diversity measures.

6.2 Beta diversity PCoA of treatment groups.

6.3 PCoA plot of beta diversity between the treatment and control groups over the four sampling points.

6.4 PCoA plot of jack-knifed beta diversity between treatment groups over the four time points.

6.5 Beta diversity at horse level identifying clustering by horse.

6.6 Panel plot of OTU abundances (%) for the two treatment groups over the four sampling points.

6.7 LEfSe discriminate analysis of the treatment and control groups 16 hours after moxidectin administration.

6.8 Panel plot of alpha diversity indices for samples used in the in vitro gas production experiment.

6.9 Beta diversity indices for samples used in the in vitro gas production experiment.

6.10 Panel plot of phyla, order and family level OTU abundances from the treatment groups used in the in vitro gas production experiment.

7.1 Scores plot of urine samples from both treated and control horses over the four sampling points.

7.2 Horse variability of urinary metabolites over the four sampling points.
7.3 Scores plot of faecal water NMR for the horses used in the *in vitro* fermentation experiment over the four time points. 144

7.4 Scores plot of faecal metabolite output of moxidectin treated horses used in the *in vitro* fermentation experiment following moxidectin administration. 145

7.5 Scores plot of faecal samples and hay fermentation inoculums split into treatment groups and sampling time points. 146

7.6 OPLS-DA loadings plot of metabolite output for faeces versus fermentations. 147

7.7 Scores plot of hay fermentations 16 hours post moxidectin and OPLS-DA loadings plot. 148

7.8 Scores and loadings plots for hay fermentations from the moxidectin treatment group at 16 hours post administration vs 160 hours post administration. 150
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3.1</strong> Egg reappearance tests at premises level, classified by premises, drug and then by time point post dosing.</td>
<td>59</td>
</tr>
<tr>
<td><strong>3.2</strong> Egg reappearance data from single animal premises analysed collectively by drug and time point.</td>
<td>60</td>
</tr>
<tr>
<td><strong>3.3</strong> Egg reappearance data for all horses tested collectively within the data set, classified by drug and then by time point post dosing.</td>
<td>61</td>
</tr>
<tr>
<td><strong>3.4</strong> Egg reappearance data for ivermectin at 8 weeks and moxidectin at 13 weeks over the four year period.</td>
<td>62</td>
</tr>
<tr>
<td><strong>4.1</strong> Population data including parasite control strategies employed and premises FECR.</td>
<td>76</td>
</tr>
<tr>
<td><strong>5.1</strong> Chemical composition of the solutions used in the culture medium.</td>
<td>90</td>
</tr>
<tr>
<td><strong>5.2.</strong> France <em>et al.</em> (1993) model fit $R^2$ values.</td>
<td>104</td>
</tr>
<tr>
<td><strong>6.1</strong> Mean faecal pH values for the moxidectin treated and control groups over the four sampling time points.</td>
<td>118</td>
</tr>
<tr>
<td><strong>7.1</strong> Ingredients used in the phosphate buffer solution.</td>
<td>138</td>
</tr>
<tr>
<td><strong>7.2</strong> Summary of OPLS-DA models, metabolites, resonances, concentrations and functions.</td>
<td>151</td>
</tr>
</tbody>
</table>
List of abbreviations

5-HT: 5-hydroxyindole-3-acetate

(1H) NMR: (proton) nuclear magnetic resonance

° C: degrees centigrade

µ: fractional rate of gas production

µl: microliters

µM: micromolar

A: asymptote of gas production

AAEP: American Association of Equine Practitioners

ANOVA: analysis of variance

b: gas production rate constant

B: gas production rate parameter

BW: body weight

BZ: benzimidazole

c: gas production rate constant

CH₄: methane

CHO: carbohydrate(s)

Cl⁻: chloride

CO₂: carbon dioxide

D-Lactate: dietary derived lactate

DIY: do it yourself

DM: dry matter

DNA: deoxyribonucleic acid

EL3: early third stage larvae

EL4: early fourth stage larvae
ELISA: enzyme-linked immunosorbent assay
EHA: egg hatch assay
EHT: egg hatch test
EPG: eggs per gram
ERP: egg reappearance period
Ext D: extent of degradation
FEC: faecal egg count
FECR: faecal egg count reduction
FECRT: faecal egg count reduction test
FRGP: fractional rate of gas production
g: gram(s)
g: force
h: hour (s)
H: hydrogen
HMDB: human metabolome database
IL-1β: interleukin 1 beta
IL-4: interleukin 4
IL-6: interleukin 6
IL-10: interleukin 10
IVM: ivermectin
Kg: kilograms
L3: third stage larvae
L4: fourth stage larvae
L5: fifth stage larvae
LBS: *Bacillus-Lactobacillus-Streptococcus* group
LDA: linear discriminate analysis

LED: light emitting diode

LEfSe: linear discriminate analysis coupled with effect size measurement

LL3: late third stage larvae

LSD: least significant difference

$L_T$: lag time

MDR: multidrug resistance

min: minute

mg: milligram(s)

ml: millilitre(s)

ML: macrocyclic lactones

mm: millimetres

MOX: moxidectin

MS: mass spectrometry

NMDA: N-Methyl-D-aspartic acid

$O_2$: oxygen

OH: hydroxide

OPLS-DA: orthogonal projections to latent structures discriminate analysis

OTU: operational taxonomic unit

P: pressure

psi: pounds per square inch

PCA: principal component analysis

PCoA: principal coordinate analysis

PCR: polymerase chain reaction

ppm: parts per million
Q²Y: OPLS-DA model prediction ability parameter
QIIME: quantitative insights into microbial ecology
R²: correlation coefficient
RDP: ribosomal database project
RPM: revolutions per minute
RTA: replication and transcription applicator
rRNA: ribosomal ribonucleic acid
SCFA: short chain fatty acid
s.e.d: standard error of difference
SD: standard deviation
t: time
\( t_{50} \): time taken to produce 50% of the total gas production
\( t_{95} \): time taken to produce 95% of the total gas production
Th-type: type of inflammatory response
THP: tetrahydropyrimidines
TNFα: tumour necrosis factor alpha
TSP: 3-trimethylsilyl-1-[2,2,3,3,\(^{2}\)H₄]-propionate
UPGMA: unweighted paired group method arithmetic mean
V: volume
VFA: volatile fatty acid
WAAVP: World Association for the Advancement of Veterinary Parasitology
Y: gas produced at time t
Chapter 1
1.0 Introduction

1.1 The equine gastrointestinal tract

The horse is a mono-gastric hindgut fermenter (McDonald et al., 2011), its large intestine comprising an enlarged anaerobic fermentation chamber that contains a highly complex community of microbiota, Figure 1.1. (Daly et al., 2001). The hindgut microbiota consists of bacteria, fungi, archaea and protozoa, all organisms that ferment digesta, predominantly structural carbohydrates, producing short chain fatty acids (SCFAs, also known as volatile fatty acids, VFAs). The predominant SCFAs in the horse are acetate, propionate and butyrate (Frape, 2010) which can provide 60-70% of the horses’ daily energy requirements (Bergman, 1990).

Figure 1.1. The anatomy of the equine gastro-intestinal tract. Image supplied by Hartpury College (Pers com).
The hindgut ecosystem is complex and until recently was relatively uncharacterised (Shepherd *et al*., 2012). In humans the gut microbiome has received greater attention and there is a growing body of evidence that the human microbiome plays a major role in many aspects of human health (Jones *et al*., 2014). Extrapolating to the horse, it is likely that the gut microbiome plays a much more important role than just fermenting digesta.

It is now known that the metabolic impact of human gut microbiota on the host involves the gut, liver, muscle and brain (Nicholson *et al*., 2012). Furthermore in humans a number of drugs that are metabolised or co-metabolised by the gut microbiota have recently been identified (Li and Jai, 2013). In horses the gut microbiota have been characterised in conditions such as laminitis, colic and equine grass sickness (Steelman *et al*., 2012; Biddle *et al*., 2013; Leng *et al*., 2015). Unsurprisingly systemic antimicrobial use has also been shown to alter the composition of faecal microbiota (Costa *et al*., 2015; Arnold *et al*., 2017). Recent work has also started to look at the effect of drugs other than antimicrobials on equine hindgut microbiota (Tyma *et al*., 2017).

Parasitic nematodes (macrobiota) have evolved to co-reside within the hindgut microbiome (Bancroft *et al*., 2012; Nielsen 2012; Peachey *et al*., 2017a). Commensal and symbiotic microbiota aim to condition their environment with minimal effect on the host, whereas parasites, living off their host, frequently negatively impact upon their host’s health (Reynolds *et al*., 2015).

1.2. Intestinal disease in horses

Acute intestinal disease, commonly referred to as colic, is the single biggest cause of equine mortality (Tinker *et al*., 1997; Goncalves *et al*., 2002; Hillyer *et al*., 2002; Archer and Proudman, 2006). “Colic” is an umbrella term for a number of different conditions, the aetiology of which is often multi-factorial in nature (Archer and Proudman, 2006). This
condition is recognised by both veterinarians and horse owners as potentially life threatening (Gonclaves et al., 2002; Al Jassim and Andrews, 2009). Previous colic research has focussed on risk factors, prevention and medical and surgical management. The focus of this thesis is on factors associated with impaired parasite control which may affect the risk of colic.

1.2.1. Risk factors for colic

One of the biggest risk factors for colic within routine daily horse management is change to the diet (Tinker et al., 1997; Cohen et al., 1999; Hudson et al., 2001; Hillyer et al., 2002). Feeding practices have long been associated with colic, Gamgee (1857) stated “too much hay and an excessive quantity of corn may induce violent indigestion and gripes… new hay and new oats combined are proverbially known to be injurious”. When the diet is changed rapidly, especially when altering starch rich concentrate feeds, this alters the hindgut microbiota. High levels of hydrolysable carbohydrates (CHOs) and fermentable CHO found predominantly in cereals can reach the hindgut and be rapidly fermented (Potter et al., 1992; Longland et al., 1999; Hoffman et al., 2001; Julliand et al., 2001; de Fombelle et al., 2003). Rapid fermentation of these CHO leads to an excess of lactic acid as a fermentation product which leads to hindgut acidification (Julliand et al., 2001; de Fombelle et al., 2003).

The environment in the normal equine hindgut is usually around pH 7.0 (Julliand et al., 2001: Medina et al., 2002) and anaerobic. Fibrobacter spp., which are cellulolytic bacteria, and members of the Ruminococcaceae are acid-intolerant and as pH decreases, these bacteria are suppressed and completely inhibited at pH <5.9 (Shirazi-Beechey, 2008; Daly et al., 2012). Similarly as rapidly fermentable CHO increases, the saccharolytic Bacillus-Lactobacillus-Streptococcus (LBS) group proliferates (Shirazi-Beechey 2008). These saccharolytic species are responsible for the production of lactate and favour hydrolysable CHO as substrates. They do not ferment structural carbohydrates, instead they use oligosaccharides released by
fibrolytic organisms (Daly et al., 2012). Members of the LBS group, including *Streptococcus bovis* are associated with acidosis of the hindgut (Daly et al., 2012; Biddle et al., 2013). An overview of the fermentation pathways of dietary carbohydrates in the horses’ hindgut can be seen in Figure 1.2. below.

**Figure 1.2.** Fermentation pathways of dietary carbohydrates in the horses’ hindgut (Shirazi-Beechey, 2008).

Figure 1.2 demonstrates the conversion by *Veillonella* of lactate to the SCFA propionate, a source of useful energy to horses. In humans and ruminants as lactic acid in the hindgut increases the abundance of *Veillonellaceae* spp. also increases, forming up to 10% of the hindgut microbiota (Duncan et al., 2007; Daly et al., 2012). However in the horse the population of *Veillonellaceae* spp. appears to remain around 1% of the hindgut microbiota even when the lactate content increases (Daly et al., 2012; Proudman et al., 2015).
Clinical observations and the studies cited above suggest that the equine hindgut microbiota may not adapt well to abrupt dietary change.

1.3. Parasitism and colic

Parasitism is a well-documented cause of colic in the horse (Archer and Proudman, 2006), having been associated with significant burdens of strongyles, *Anoplocephala perfoliata* and *Parascaris equorum* (Reinemeyer and Nielsen, 2009). Historically the large strongyle, *Strongylus vulgaris*, was believed to be responsible for up to 90% of all colic episodes in domesticated horses (White, 1997). In the 1960s *Strongylus vulgaris* was deemed the most prevalent and the most pathogenic parasite of the horse. The larvae of this parasite migrate from the large intestine through the mesenteric arteries causing thrombosis and arteritis which can lead to thrombo-embolism resulting in severe colic and death due to infarction and necrosis of the intestinal wall (Proudman and Matthews, 2000; Nielsen et al., 2008). Prophylactic anthelmintic use was employed to target *Strongylus vulgaris*. This approach had the desired effect as by the early 1980s the small strongyles, cyathostomins, frequently made up almost 100% of a horses strongyle egg output (Nielsen et al., 2008; Kaplan and Nielsen, 2010). However the reduction in prevalence of *Strongylus vulgaris* did not lead to a reduction in the number of colic cases presented to veterinary surgeons. Nevertheless epidemiological studies have identified that well-structured parasite control programmes do reduce the risk of colic occurrences (Uhlinger 1990; Cohen et al., 1999; Hillyer et al., 2002).

Cyathostomins have also been implicated in the onset of colic (Murphy et al., 1997). The first report of cyathostomins associated colic was in France in 1913 (Herd, 1990). In a multiyear study Uhlinger (1990) reported that the use of effective anthelmintic schedules to suppress faecal egg counts in horses resulted in a reduced incidence of colic. Furthermore as the
anthelmintic schedule improved and the faecal egg counts reduced, the incidence of colic
decreased proportionally (Uhlinger, 1990).

Cyathostomins have previously been associated with caecocaecal intussusceptions and non-
strangulating infarctions (Lyons et al., 1994; Mair and Pearson, 1995). While it has been
suggested that colic associated with cyathostomins is rare (Reinemeyer and Nielsen, 2009),
further epidemiological studies have identified a reduced risk of colic when targeting
cyathostomins with prophylactic anthelmintic treatment (Reeves et al., 1996). Murphy and
Love (1997) identified a pathological role of L3 larvae invading the hindgut, supporting earlier
studies linking cyathostomins to changes in intestinal motility (Bueno et al., 1979).

1.3.1. Cyathostomins

Cyathostomins are ubiquitous in grazing horses, and currently the most prevalent and
pathogenic parasites of the horse (Love et al., 1999; Kaplan and Nielsen, 2010; Matthews,
2011; Relf et al., 2011). With over 50 different species identified, it is only recently that
research has started to provide a greater understanding of the contribution that each
cyathostomin species plays in the parasites epidemiology, clinical disease and in anthelmintic
resistance (Matthews, 2008; 2011). The most pathogenic stage of the cyathostomes non-
migratory lifestyle is the larval emergence from the intestinal mucosa, Figure 1.3. Infective L3
larvae are ingested by the horse from pasture. The L3 larvae travel to the large intestine where
they penetrate the submucosa and mucosa into the intestinal wall. Once encysted in the mucosa
the L3 develop into Early L3 (EL3) stages, known as hypobiosis, and can remain in this state
for two years or greater (Love and McKeand, 1997). However EL3 can move straight to late
L3 phase (LL3), then on to Early L4 (EL4) where they then re-emerge into the lumen and
undergo one final moult before becoming sexually reproducing adult cyathostomins, Figure
1.3. (Matthews, 2008; 2011). The adult parasites have a much lower pathogenicity than the
larval stages but are easier to target with anthelmintics which prevent further egg shedding and re-infection (Kaplan and Nielsen, 2010).

While horses are susceptible to cyathostomin infections throughout their lives, young animals have higher burdens compared to mature animals. This suggests that some immunity is gained from previous exposure (Lyons et al., 1999; Klei and Chapman, 1999). An immunological mechanism to prevent infection has been proposed; upregulation of cytokines by Th-2-type T-cells. Cytokines IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 have been identified in both murine models and in horses when looking at strongyle infections (Klei and Chapman, 1999). However this immunity mechanism is still not well understood.
Figure 1.3. (A) The non-migratory cyathostomin lifecycle, the red boxes depict the most pathogenic aspect of the life cycle. (B) The gut lumen showing ingested L3 larvae becoming encysted and either continuing to develop or going into hypobiosis before re-emerging as L4 larvae into the gut lumen. A mass emergence of L4 can lead to clinical disease cyathostominosis. Figure adapted from Fort Dodge (2008).
While cyathostomins are classed as the most pathogenic parasite of equids, clinical disease is uncommon (Reinemeyer and Nielsen, 2009). Larval cyathostominosis is the clinical disease that occurs when encysted cyathostomin larvae synchronously re-emerge from the mucosa into the gut lumen (Love et al., 1999). Horses present with diarrhoea and weight loss (Murphy et al., 1997; Proudman, 1999) and this condition is most commonly seen in younger animals in the early spring (Murphy et al., 1997; Matthews, 2011). In some animals this syndrome will present as colic (Matthews, 2011). The mechanisms behind larval cyathostominosis are not fully understood. Changes in ingested L3 cyathostomins linked to the external environment e.g. cold conditioning, the numbers of adult nematodes in the gut lumen providing negative feedback to encysted larvae, and recent anthelmintic treatment have all been implicated in the onset of disease (Murphy et al., 1997; Love et al., 1999; Matthews, 2008; 2011). Up to 90% of a horse’s cyathostomin burden can be encysted (Duncan et al., 1998; Dowdall et al., 2002; Matthews, 2008). Horses that appear clinically healthy can harbour cyathostomin burdens numbering hundreds of thousands of larvae or worms without showing signs of clinical disease (Fog et al., 2011; Stratford et al., 2011). However it is well-established that the higher the cyathostomin burden the greater the risk of clinical disease (Matthews, 2008; 2011).

The pathogenesis of cyathostomin infections is as an inflammatory enteropathy due to larval penetration or larval emergence from the large intestinal mucosa (Love and Murphy, 1997; Love et al., 1999). Differences in the levels of inflammatory cytokines, specifically IL-4, IL-10 and TNFα have been identified in horses harbouring cyathostomins compared to untreated controls (Matthews et al., 2004). Mast cells have also been identified as playing a role in cyathostomin infected horses (du Toit et al., 2007).
1.3.2. Parasite control mechanisms

Since the 1960s anthelmintic dosing has been the predominant form of equine endoparasite control. In an attempt to reduce the prevalence of *Strongylus vulgaris* and reduce incidence of colic, the interval dose programme was proposed. Drudge and Lyons (1966) suggested anthelmintic treatment every 8 weeks to prevent *Strongylus vulgaris* egg shedding and prevent the parasite reaching L5 maturity. This regimen shaped anthelmintic use and is still in use by many horse owners today.

However there are other forms of parasite control to consider alongside anthelmintics. Strongyle infections are picked up on the pasture, only one study has reported that L3 larvae could survive in deep littered stables (McGirr et al., 2015). Pasture management consisting of dung removal has previously been identified as the most effective form of parasite control (Herd, 1986; Coles, 2002; Lloyd et al., 2000; Nielsen et al., 2010a). Removal of faeces is preferred to chain harrowing which spreads infective larvae and increases pasture fouling (Herd 1990). Cross grazing with ruminants is also an effective method of parasite control (Coles, 2002).

There has been some work on the use of the nematophagous fungus *Duddingtonia flagrans* which was identified by Braga et al. (2009) to have efficacy against L3 larvae *in vitro*. Further reports by Cazapal-Monteiro et al. (2012) and Arias et al. (2012) indicated that spreading *D. flagrans* on pasture increased the egg reappearance time of horses treated with moxidectin. Maderia de Carvalho et al. (2012) supplemented horses daily with *D. flagrans* and also showed increased anthelmintic egg reappearance times. A daily dose of *D. flagrans* of a sufficient quantity, $<3 \times 10^5$ per kg of body weight, has been reported to significantly reduce cyathostomin L3 larvae (Buzatti et al., 2015). However Nielsen (2012) reported that while this biological technique was patented in the early 2000s no commercial product has yet reached
the market. The reason behind this is unknown, however survival through the gastro intestinal tract would require investigation and calculation of a sufficient dose that survives digestion also requires consideration.

There has been some focus on the use of tannin rich forages in reducing strongyle egg viability (Collas et al., 2015). There has also been some work into the use of plant extracts, in vitro, on cyathostomins with significant effects on egg hatch viability and larval inhibition (Peachey et al., 2015:2016).

While many different methods of parasite control have been explored over the past 50 years, there is still a heavy reliance on anthelmintics as the primary strategy for intestinal nematode control.

1.3.3. Anthelmintics

The first broad spectrum group of anthelmintics, the benzimidazoles (BZs), were introduced to the market in the 1960s (Herd, 1990). Benzimidazoles act on β-tubulin proteins and interfere with glucose metabolism starving the parasite to death (Martin, 1997; Brady and Nichols, 2009). Fenbendazole is licensed for treatment against large and small strongyles in horses. It is also licensed to treat the larval stages of cyathostomins whilst encysted in the gut mucosa. For this larvacidal efficacy the anthelmintic is administered orally over five consecutive days.

Early studies into the efficacy of BZs identified 97-100% efficacy on adult cyathostomins and 46-78% efficacy on L4 larvae using both faecal egg counts and worm counts at necropsy (Colgazier et al., 1977). The lower efficacy on the L4 larvae was proposed to be down to L4 emergence from the mucosa between treatment and necropsy (Colgazier et al., 1977). Larvacidal efficacy of fenbendazole has been reported as >91.5% on early 3rd stage larvae and >99.4% on late 3rd stage and developmental 4th stage encysted larvae (Duncan et al., 1998).
BZs also claim ovicidal efficacy unlike other anthelmintic classes licensed for horses (Lacey et al., 1987).

The tetrahydropyrimidines (THPs) were the second group of broad spectrum anthelmintics licensed for equine nematode treatment in the 1970s. When introduced to the market the active drug pyrantel was shown to eliminate 89-96% of adult cyathostomins in a single dose (Lyons et al., 1974). Pyrantel is only efficacious against adult nematodes, it acts as an agonist on acetylcholine receptors of nematode muscle cells leading to paralysis (Martin, 1997).

In the 1980s the macrocyclic lactone (ML) group of broad spectrum anthelmintics were introduced. Ivermectin was licensed for equine nematode treatment in the early 1980s and moxidectin was licensed in the UK in the late 1990s (Kaplan, 2004). The two compounds are chemically related and share similar structural features. Ivermectin is a modified avermectin that possesses a disaccharide linked to the carbon ring structure. Moxidectin lacks this sugar and is classified as a milbemycin (Cobb and Boeckh, 2009). Avermectins and milbemycins collectively make up the ML group (Sangster, 1999).

Both of these anthelmintics also share a common mode of action targeting the nematodes pharynx and binding with glutamate-gated Cl⁻ channels increasing Cl⁻ currents leading to paralysis (Sangster, 1999; Schumacher and Taintor, 2008; Cobb and Boeckh, 2009). Ivermectin also causes paralysis to the somatic musculature but this site is suggested to be less sensitive to the compound than the pharynx. In both in vitro and in vivo studies of ruminant species, resistance to ivermectin also is associated with side-resistance to moxidectin, thus suggesting the common mode of action. This side-resistance has not yet been reported in horse cyathostomins (Sangster, 1999).

Early studies indicated that both ivermectin and moxidectin had 99% efficacy against adult cyathostomins and 98% efficacy against luminal L4 cyathostomins. For the encysted stages
neither ivermectin nor moxidectin were effective against EL3 larvae and only moxidectin was efficacious against encysted LL3 (62.6-79.1%) (Xiao et al., 1994). Further work by Bairden et al. (2006) identified 99% moxidectin efficacy on adults and L4 in the lumen and 92.2% for encysted EL3 larvae. This work identified that moxidectin was useful as a larvicidal treatment for cyathostomins. Its lipophilic nature means that efficacy against cyathostomin larvae may persist for 2-3 weeks post administration (Schumacher and Taintor, 2008). Different studies have evaluated larvicidal efficacy on necropsied horses at different time periods post dosing which has shown differences in the efficacy of the EL3 and LL3, DL4 stages of encysted development (Matthews, 2008).

The egg reappearance period of ivermectin was 8-10 weeks when it was first marketed (Borgsteede et al., 1993). The datasheets for Ivermectin products in the UK no longer contain ERP times (NOAH compendium, 2017a). Moxidectin egg reappearance was reported in excess of 13 weeks, and up to 24 weeks, post treatment when it was first introduced (Jacobs et al., 1995; DiPietro et al., 1997; Demeulenaere et al., 1997; Boersema et al., 1998). The current marketing authorisation for moxidectin states 90 days egg suppression for cyathostomins (NOAH Compendium, 2017b).

The larvacidal efficacy of both a five consecutive day dose of fenbendazole and a single dose of moxidectin has previously been compared (Steinbach et al., 2006). This study highlighted that both of these treatments were efficacious against mucosal stages of cyathostomin development, fenbendazole for 4-6 days post treatment and moxidectin for 6-14 days post treatment.

1.3.4. Anthelmintic control strategies

Between the 1960s and the 1990s the continual addition of new anthelmintics being licensed for equine nematode treatment meant that prophylactic use of anthelmintics was the main focus
for parasite control. Over time horse owners and veterinarians have developed an irrational fear of parasites (Kaplan and Nielsen, 2010), leading to regular anthelmintic treatment to prevent colic. Veterinarians would often answer questions on parasite control with ‘there is a drug for that’ (Sangster, 2003). This compulsion to treat horses for parasites, without the knowledge of the horse’s parasite status, aiming treatment at a parasite that is no longer prevalent, is a practice that is 40 years out of date (Kaplan and Nielsen, 2010).

In 1999 Biggin et al. surveyed pony club members to identify parasite control practices and knowledge. Ivermectin was the predominant anthelmintic used within this group, moxidectin was not available when this data was collected. Respondents reported they changed the brand of anthelmintic with each treatment, however brand does not necessarily identify with anthelmintic class. The majority of respondents were confused as to the efficacy of the different anthelmintics for specific parasites. There was some understanding of pasture management but faecal removal from pasture was less frequent than recommended to reduce pasture contamination (Biggin et al., 1999). Similarly Lloyd et al. (2000) surveyed horse owners on parasite control practices and found that ivermectin was the predominant anthelmintic used. Again, at the time of data collection moxidectin was not yet available. This study reported a greater focus on twice weekly dung removal from pasture compared to the findings of Biggin et al. (1999). Most of the respondents of Lloyd’s study dosed horses with anthelmintic at a frequency of eight weeks or less. In a similar study by Allison et al. (2011) owners predominantly selected moxidectin followed by ivermectin for anthelmintic dosing. Of 574 respondents less than 60% were comfortable with their current anthelmintic schedule but 39% never used faecal egg counts and 20% only used FECs sporadically. These findings suggest that while some horse owners have started to realise that reliance on anthelmintics is unsustainable for nematode control, many horse managers are still using outdated control strategies.
1.3.5. Sustainable parasite control strategies

With anthelmintic resistance becoming a greater concern there has been a shift in focus away from prophylactic anthelmintic treatment. Faecal egg counts were reported as a useful economical tool to identify horses that shed high numbers of nematode eggs and therefore selective chemotherapy with anthelmintics could be targeted at these animals (Gomez and Georgi, 1991). Further work by Duncan and Love (1991) identified that this selective treatment approach may also be more economically viable than an interval dose regimen. Part of the rationale for a targeted anthelmintic approach is that the parasite distribution within horses tends to be over-dispersed, thus concentrated to few animals, Figure 1.4. (Duncan and Love, 1991; Matthews, 2008; Stratford et al., 2011). Typically on a horse premises 80% of the parasite population resides within 20% of the horses present on that premises (Stratford et al., 2011). Young animals in particular are more susceptible to infection, (Klei and Chapman, 1999). These points collectively suggest that diagnostic testing should be employed to identify horses within a population that are the high egg shedders, in order to target treatments at these animals.
Figure 1.4. Schematic diagram of the over dispersion of cyathostomins within a horse population, shaded horses harbouring cyathostomin burdens of concern, unshaded harbouring very low cyathostomins burdens. Treating high shedders and leaving low shedders untreated creates a refugium (Matthews, 2008).

Faecal egg counts are considered by many to be the cornerstone of equine parasitological diagnostic techniques. There are numerous different egg counting techniques including McMaster, Stoll and Wisconsin (Nielsen et al., 2010a; Nielsen 2014). Modifications of these techniques, commercial kits (Presland et al., 2005) and refined methods with increased sensitivity have all been employed. Fundamentally all of these techniques are designed to float nematode eggs to allow quantification via microscopy (Nielsen et al., 2010a; Stratford et al., 2011; Nielsen 2014).

Faecal egg counting is a low cost diagnostic option that is quick and simple to perform. Sample collection is straightforward and non-invasive making it possible for horse managers to collect samples and submit these for analysis. For greatest accuracy samples should be tested within 12 hours of faecal excretion, however samples have been successfully refrigerated in air tight containers for up to 120 hours without significant alteration in egg numbers (Nielsen et al., 2010b). This diagnostic technique holds limitations but forms part of a holistic approach to
parasite control. When using faecal egg counts and interpreting the data several limitations need to be considered: the lower limit for egg detection, repeatability of results, consideration of the horse’s age, previous anthelmintic dosing history and the time of year the sample was taken.

Lloyd (2009) reported that some horses continually shed low levels of faecal eggs, but their egg shedding predictability could not be defined without 12 months of monitoring. Similarly their previous anthelmintic history also influenced high or low egg shedding when moving to a monitoring-based programme (Lloyd, 2009). Concerns over fluctuations in daily nematode egg output have previously been reported (Uhlinger, 1993). Recent studies have evaluated variability in faecal egg output over time; Carstensen et al. (2013) found that from a group of six horses FECs undertaken on five consecutive days were not significantly different. Using a longitudinal approach over a two year period Lester et al. (2017) screened the faeces of a group of 573 horses four times over two years. They reported that 73% of horses remained in the same egg shedding category throughout the study and 94% remained in the same treatment category. For those horses that received no anthelmintic treatment over the study, 90.4% remained within the same egg shedding category. Horses that changed egg-shedding category were predominantly young animals. The overall results of this study suggest that horses with low strongyle burdens are likely to consistently remain low over time.

Historically concerns over using a FEC monitoring approach have centred on the limitation of high proportions of cyathostomins being encysted within the mucosa and therefore undetectable. Recent work has identified the potential to detect encysted cyathostomin larvae using an ELISA assay, however this test is not yet commercially available (Mitchell et al., 2016). Until such a time that this test is commercially available faecal egg counts remain the gold standard for estimating strongyle burdens.
Earlier studies reported that there was no correlation between FEC and total cyathostomin burden, luminal and mucosal stages, (Uhlinger, 1993). However Nielsen et al. (2010c) found that FECs of 500 epg and below were correlated with lower total worm burdens.

There is debate in the literature concerning the threshold FEC reading which should trigger anthelmintic intervention. The first point to consider here is each horse is an individual, therefore age, previous anthelmintic dosing history, pasture management, stocking densities time of year and faecal egg count history will all play a role in clinical decision-making. Early studies suggested treating horses with a FEC >100 epg (Gomez and Gerogi, 1991). This has subsequently been modified to >200 epg, now the most commonly cited cut-off in the literature (Matthews, 2008; Kaplan and Nielsen, 2010; Nielsen, 2014). However both Uhlinger (1993) and Nielsen (2012) recommend treatment cut-offs could be >500 epg in mature horses. It is important to note here that these cut-off points are arbitrary, “best guess” figures based upon the limited information available (Uhlinger, 2007). Taken together our limited understanding of cyathostomin biology and infection pathogenicity mean that ideal cut offs for anthelmintic treatment are currently unknown.

1.3.6. Refugia

A fundamental concept of sustainable parasite control is having a proportion of parasites within a population not exposed to anthelmintics, thus remaining “in refugium” (Sangster, 1999; Matthews, 2008; Nielsen et al., 2010a; Nielsen et al., 2014). These parasites that remain unexposed to anthelmintic reduce the selection pressure for anthelmintic resistance by keeping drug susceptible parasites within the population (Figure 1.4.). By only treating high egg shedding horses the nematodes in the rest of the horse population remain in refugia (Nielsen et al., 2010a). As refugia refers to any nematodes not exposed to anthelmintic, mucosal stage cyathostomins remain in refugia when the horse is dosed with ivermectin, pyrantel, or a single
19
dose of fenbendazole as these drugs are not efficacious against the encysted mucosal stages (Kaplan and Nielsen, 2010).

1.3.7. Application of sustainable parasite control

One of the greatest challenges in implementing sustainable parasite control measures is owner uptake. Over the past 20 years there have been studies published identifying the need to move to sustainable parasite control programmes. The findings of Biggin et al. (1999) identified that horse owners were frequently using anthelmintics but worm burdens were not a great concern. Allison et al. (2011) reported that horse owners were concerned about anthelmintic use and the risk of resistance development but were constrained by livery yard rules, or misinformed on how to use faecal egg counts which meant they remained reliant on anthelmintics (Allison et al., 2011). Although Duncan and Love (1991) identified the potential for financial savings taking this approach, horse owners still express hesitation and concerns over the costs implicated in moving to a monitoring-based approach (Nielsen et al., 2010a). It has been reported that owners who bought anthelmintics from their veterinarian were more likely to use faecal egg counts (Easton et al., 2016). The same study identified owners who bought anthelmintics via all of the possible routes e.g. veterinarian, suitably qualified person or pharmacist were all using faecal egg count monitoring to some extent, but there was limited uptake of efficacy testing (Easton et al., 2016).

A recent study by Vineer et al. (2017) concluded that focussing on emphasising the risk of anthelmintic resistance was not enough to change horse owner behaviour. More effort needs to be focussed on appropriate knowledge transfer and training mechanisms and more research focus on owner opinions and attitudes towards equine parasite control are needed to support behavioural change.
1.3.8. Shortened egg reappearance periods

Anthelmintic resistance is a process that occurs over time, it is a manifestation of adaptive evolution. The stage between drug-sensitive parasites and drug-resistant parasites is referred to as reduced efficacy (Lyons et al., 2011). Reduced efficacy of anthelmintics is seen as shortened egg reappearance periods and viewed as an early sign of anthelmintic resistance (Sangster 1999; Kaplan and Nielsen 2010; Lyons et al., 2011).

When anthelmintics are first licensed they all have an evidence-based efficacy period (egg reappearance period, ERP) stated on the marketing authorisation which is reproduced as a product data sheet. The ERP is the period post treatment during which egg shedding is below a given threshold. The ERP period varies with each anthelmintic, guide e.g. for BZs it is 6-8 weeks, for THPs 6 weeks, IVM 8-10 weeks and MOX >13 weeks (Stratford et al., 2011).

One problem with using ERP is the lack of definition of ERP. In a range of studies that have set out to investigate efficacy, ERP has been interpreted differently. Some view ERP as the first point where any eggs are seen post treatment, e.g. Little et al. (2003). Other studies use the point the group arithmetic mean FEC >10% of the Day 0 value (Borgsteede et al., 1993; Jacobs et al., 1995; Boersema et al., 1998; Mercier et al., 2001; von Samson-Himmelstjerna et al., 2007; Larsen et al., 2011). A third approach is to look for a percentage faecal egg count reduction (FECR) within the stated egg reappearance period for the product (Larson et al., 2011; Molento et al., 2012; Nielsen et al., 2013a; Relf et al., 2014). This lack of definition makes it difficult to compare studies.

Several studies have been conducted in the USA to determine the current ERP of ML anthelmintics. In 2011 Lyons et al. conducted a field study on 369 horses at 14 farms in Kentucky. Overall the ERP for ivermectin was similar on most farms returning positive counts at 4 weeks post treatment and moxidectin returning positive FECs at 5 weeks post treatment.
The farms had all used ML anthelmintics extensively in prophylactic programmes in the past. Lyons et al. (2011) reported that most farms had used ivermectin but some had not used moxidectin before yet still showed reduced moxidectin efficacy. Little et al. (2003), over a 3 year period, detected positive faecal egg counts before 8 weeks following ivermectin at a North Carolina stud farm. More recently Lyons et al. (2017) reported ERP for moxidectin at 4-6 weeks.

In Australia Edward and Hoffmann (2008) looked at FEC patterns in 50 horses at 10 farms post treatment over 3 years. The study highlighted that overall there was shortened egg reappearance following ivermectin treatment, with eggs reappearing from 26 days post treatment in horses that were regularly dosed at 6-8 week intervals (Edward and Hoffmann, 2008). In the UK Dudeney et al. (2008) were the first to highlight reduced efficacy to ivermectin. More recent studies have identified egg reappearance from 6 weeks post moxidectin treatment (Tzelos et al., 2017).

1.3.9. Anthelmintic resistance

The first reports of anthelmintic resistance in equine cyathostomins was to phenothiazine in 1958 by Poynter and Hughes in the UK and in 1961 by Drudge and Elam in the USA (Kaplan, 2004), therefore the concept of anthelmintic resistance is not new. Anthelmintic resistance is inherited from one generation to the next via the genome. Because cyathostomins have high levels of genetic diversity and are generally present in populations of hundreds of thousands in most healthy animals, resistant genes can develop and spread quickly within populations (Matthews 2008; 2011).

Benzimidazole resistance in cyathostomins was first reported in 1965 in Kentucky (Herd, 1990; Kaplan, 2004). Resistance to one member of the benzimidazole family confers resistance to other members of that class, with the exception of oxibendazole which appears to remain
efficacious against resistant cyathostomins for a limited time (Prichard, 1990; Tarigo-Martinie et al., 2001). Resistance to this group of anthelmintics has continued to spread and has now been documented as widespread and worldwide (Matthews, 2008). In some areas prevalence of benzimidazole resistant strains of cyathostomins has been found on 100% of premises investigated (Kaplan et al., 2004; Kaplan and Nielsen, 2010).

Cyathostomin resistance to pyrantel is documented in the USA (Kaplan, 2002; Kaplan et al., 2004; Relf, et al., 2011). However in the USA low dosages of pyrantel were historically given daily as a feed additive which increased selection pressure for resistance (Kaplan et al., 2004; Kaplan and Nielsen, 2010). It has been postulated that resistance to pyrantel and ivermectin has been slow to develop in cyathostomins due to the lack of larvacidal efficacy creating a larger refugium (Kaplan and Nielsen 2010; Stratford et al., 2011). There is growing concern about the development of multi drug resistance (MDR) in cyathostomins following the reports of widespread MDR in sheep (Matthews, 2008). This is especially critical due to the limited drug classes licensed for equine nematode treatment.

A significant concern for equine clinicians is efficacy of the larvicidal properties of fenbendazole, given at a five consecutive day dose at 7.5mg/kg, and moxidectin. The most pathogenic stage of the cyathostomin lifecycle is larval re-emergence from encysted development. With limited anthelmintic treatment options available to target these encysted larval stages, resistance to these larvacidal drugs is a serious concern. There have been no controlled efficacy or critical trials conducted on the larvacidal efficacy of fenbendazole in resistant populations. Studies have documented continuous egg shedding following five continuous days of treatment in known resistant populations (Matthews, 2008). A study by Hodgkinson et al. (2005) used stabled horses that were known to harbour BZ resistant cyathostomins. These horses were treated with pyrantel to remove any adult parasite population. Efficacy of pyrantel was tested using a faecal egg count reduction test which
resulted in an average 98% reduction. Seven days post pyrantel treatment these horses were
given a five day larvical dose of fenbendazole. At 28 days post FBZ treatment, strongyle
eggs were reappearing in faecal egg counts. As these horses were stabled, and therefore
unlikely to become infected within their environment, this egg appearance was suggested to be
due to a failure of the FBZ on the mucosal stages (Matthews, 2008). This contrasts to the
findings of Duncan et al. (1998) where in BZ susceptible cyathostomins, larvicidal efficacy on
encysted larvae was >91.5% on early 3rd stage larvae and >99.4% on late 3rd stage and
developmental 4th stages.

A study in Kentucky compared both five day fenbendazole and moxidectin larvicidal
treatments in a group of yearlings at the University of Kentucky. All of the yearlings had faecal
samples taken on day 0 and a proportion of the yearlings were given a larvicidal dose of
fenbendazole as part of a faecal egg count reduction test. There was no significant difference
in faecal egg count results between day 0 and day 14 in the FBZ treated horses (Rossano et al.,
2010). Further faecal samples were collected from each animal weekly, after 14 days
moxidectin was administered to all of the yearlings on the farm. During the moxidectin trial
the FEC results on days 0 and 42 did not significantly differ from those horses that had been
treated with fenbendazole. Overall moxidectin egg suppression persisted for up to 21 days, at
35 days all but two of the 15 yearlings had some egg reappearance (Rossano et al., 2010). The
findings of this study suggest fenbendazole resistance and reduced efficacy of moxidectin in
this horse population (Rossano et al., 2010).

In a more recent study Reinemeyer et al. (2015) evaluated the larvicidal efficacy of
fenbendazole and moxidectin in a BZ resistant population in the USA. The results of this study
indicated lower BZ larvicidal efficacy than historically reported, 71.2% against LL3 – L4
compared to 85.2% for moxidectin. EL3 efficacy was 38.6% for fenbendazole versus 63.6%
for moxidectin. The faecal egg count reduction tests were 44.6% and 99.9% for fenbendazole
and moxidectin respectively. While it may appear that there was still good moxidectin efficacy, the larvicidal effect was not significantly different between fenbendazole, moxidectin and the untreated controls (Reinemeyer et al., 2015). The authors conclude that the reason for the apparent reduction in larvicidal activity of both anthelmintics was most likely due to the time between treatment and necropsy. The findings of these studies taken collectively are a significant concern for the future treatment of encysted cyathostomin larvae.

1.3.9.1. ML resistance

The first reports of moxidectin and ivermectin resistant cyathostomins came from Brazil in 2008 in a group of naturally infected horses (Molento et al., 2008). Faecal egg count reduction was 16% following moxidectin treatment and 56% following ivermectin treatment.

In the UK there has been one case report of a failure of MLs to reduce strongyle egg output in donkeys (Trawford et al., 2005). However the authors of this study acknowledge that the moxidectin administered was “off-label” as it was an intra-muscular cattle formulation for injection. The pharmacokinetics of moxidectin in donkeys is unknown, horses and donkeys require different dosage rates. An extrapolation of horse data alone is not reliable when dosing donkeys (Molento et al., 2012). Therefore the findings of this study should be interpreted with extreme caution.

Macrocyclic lactone resistance in cyathostomins identified through the faecal egg count reduction test have been reported in Brazil, Finland and Italy (Peregrine et al., 2014). These findings suggest ML resistance is spreading within the parasite population.

1.4. Cross Species comparisons

Anthelmintic resistance in nematode species of small ruminants is much better understood than for horses. Ruminant species harbour strongylid nematodes closely related to cyathostomins
with a high prevalence of BZ, THP and ML resistance worldwide (Kaplan and Nielsen 2010; Molento et al., 2012). From the situation of ML resistance in trichostrongyloids in sheep and the similarities to equine cyathostomins, it is likely than anthelmintic resistance to MLs will occur in equine cyathostomins (Sangster, 1999). The differences between cyathostomins and trichostrongylids need to be considered in that i) cyathostomins have a longer pre-patent period that varies with each species, ii) cyathostomins spend more time in inhibited development which provides greater refugia, iii) horse management, e.g. dung collection can reduce re-infection that is not possible with sheep and iv) horse movement between properties allows greater risk of spreading resistant parasites (Sangster, 1999; Molento et al., 2012; Nielsen et al., 2014).

The first reports of multi-drug resistance (MDR) in sheep were in the 1980s (Kaplan, 2004); since then the phenomenon has become widespread among sheep and goats and has been reported worldwide. In the strongylid nematodes of sheep, benzimidazoles were licensed for use in 1961; resistance was first reported in 1964. Ivermectin was licensed for use in sheep in 1982, the first report of resistance to IVM in these nematodes was in 1985. Moxidectin was licensed for use in sheep in 1991 and resistance was reported in 1995 (Kaplan, 2004). In 2001 Sargison et al. reported a case of MDR parasites at a sheep farm in Scotland with resistance to BZs, THPs and ivermectin. In 2005 Sargison et al. conducted a second study on the same farm to investigate the efficacy of moxidectin; resistance was confirmed and the decision was made to close the farm, highlighting the first case of MDR in sheep in the UK. The lessons learnt from sheep nematode control suggest that if sustainable parasite control mechanisms are not implemented for horses, then MDR parasites of horses will develop.
1.5. Combination Anthelmintics

One of the current approaches to preserving the efficacy on new groups of anthelmintics licensed for sheep is to combine two active ingredients that act on different sites of the parasite (Leathwick, 2012). The concept is to combine anthelmintics from different classes with different modes of action, to reduce the speed of anthelmintic resistance (Leathwick, 2012).

This theory arose from the concept that if there is reduced efficacy to one anthelmintic class that is part of the combination the second anthelmintic class will remain lethal to the parasite (Leathwick, 2012). This approach has been successfully used with anti-malaria drugs, (Hastings, 2011).

The use of combination anthelmintics has been modelled using a computer simulation and has been shown to preserve the duration of efficacy of new anthelmintic classes licensed for sheep in New Zealand (Leathwick, 2012). In a situation where there is adequate refugia and high efficacy to MLs, the addition of a new active ingredient showed almost no increase in the frequency for the resistant gene (Leathwick, 2012). However where the efficacy of the combination falls below 70% combined, then the effect of the combination is lost (Leathwick et al., 2012). One problem with this approach is the potential for cross resistance to develop between classes (Leathwick, 2012).

An interesting consequence of combination anthelmintics is the development of side resistance between the MLs and BZs (Mottier and Prichard, 2008). It appears that the use of and resistance to MLs in Haemonchus contortus increases the frequency of the β tubulin alleles containing the dominant codon for BZ resistance (Mottier and Prichard, 2008). This has raised concern over the use of combination therapy as these unpredicted mutations could increase the speed of MDR through cross resistance (Mottier and Prichard, 2008; Leathwick, 2012).
Lyons et al. (2016) reported that combinations of benzimidazoles, piperazine and pyrantel were not effective against strongyles of Thoroughbred foals in Kentucky. This is the first report of an attempt to use a combination anthelmintic in horses.

1.6. Effect of anthelmintic treatment on the gastrointestinal tract

Epidemiological studies into colic have identified anthelmintic treatment as a risk factor for colic (Proudman, 1991; Cohen et al., 1999; Hillyer et al., 2002). For each of these studies colic episodes followed within one week of anthelmintic administration. However the reason behind colic following nematode treatment is unknown.

Studies have also investigated the effects of larvicidal activity on the intestine. Steinbach et al. (2006) found that a five day larvicidal dose of fenbendazole led to mucosal inflammation and ulceration to the mucosa, this inflammation was not reported after treatment with moxidectin. It was suggested that the severe tissue damage caused by fenbendazole treatment could mimic larval cyathostomosis (Steinbach et al., 2006). A more recent report by Nielsen et al. (2013b) evaluated the inflammatory response following anthelmintic treatment in an experimental herd of ponies in Kentucky. Ponies were dosed with moxidectin and blood samples taken to evaluate cytokine expression. The findings of this study highlighted no systemic inflammatory effect of anthelmintic treatment. In another study, also in Kentucky, inflammatory cytokines were characterised following moxidectin or fenbendazole treatment from blood samples. Moxidectin had no systemic effect on inflammatory cytokines with the exception of an increase on TNFα 14 days following treatment. However there was a significant increase in TNFα, IL-1β and IL-6 following a five day larvicidal dose of fenbendazole (Betancourt et al., 2015). These findings support the previous findings of Steinbach et al. (2006). Betancourt et al. (2015) propose that it is likely the moxidectin had an anti-inflammatory effect on the intestine. Anti-inflammatory
effects have previously been identified for macrocyclic lactones in a murine model (Yan et al., 2011).

1.7. Summary

It is apparent that cyathostomins have a complicated biology that currently is not fully understood. There is an association between cyathostomins and colic however the mechanisms behind this are not fully understood either. It is apparent that some level of control over cyathostomins is required to maintain horse health. However the association between parasites and colic has left horse owners with an irrational fear of parasites, compelling them to treat horses regularly with anthelmintics. The lack of understanding of the cyathostomin lifecycle and fear of colic by horse managers has inevitably played a role in the progression of anthelmintic resistance. To ensure it is possible to treat mucosal encysted cyathostomin larvae into the future sustainable parasite control is essential to reduce the risk of intestinal disease occurring. It is also important to understand the current efficacy of the most recent anthelmintics licensed for equine nematode treatment within the UK, to identify if resistance is developing within the macrocyclic lactones. Furthermore anthelmintic classes such as the benzimidazoles that have novel modes of action may still be useful, potentially in combination with other classes, against resistant populations. However the ovicidal efficacy of benzimidazoles on BZ resistant cyathostomins has not been characterised.

Curiously, as well as cyathostomins being implicated in colic, anthelmintic treatment has also been implicated in the onset of colic. It is possible that anthelmintic treatment may upset the complex relationship between nematodes and microbiota in the hindgut of the horse. To date the effect of anthelmintic treatment on the equine microbiome is not well reported. As post dosing colic is likely to be multifactorial in nature it is important to understand if anthelmintics play a role in disease onset. This information would allow veterinarians to inform horse
managers on sustainable and effective parasite control mechanisms without compromising gut health.

1.8. Research Aims

The aim of this research was split into two distinct parts, firstly: to identify current efficacy of the macrocyclic lactones against cyathostomins in UK pleasure horses, and to characterise the ovicidal efficacy of the benzimidazole group in the face of anthelmintic resistance.

Secondly, to use a multi ‘omics approach to characterise the microbiological and biochemical response within the hindgut when horses are treated with anthelmintic. This includes identification of any functional changes in fermentation patterns following anthelmintic treatment.

Objectives:

- Evaluate the egg reappearance times of strongyles following treatment with macrocyclic lactones in a field situation.
- Test the ovicidal activity of fenbendazole in horses harbouring BZ resistant cyathostomins.
- Compare fermentation kinetics of feeds fermented in faeces from horses treated with anthelmintic compared to untreated controls.
- Define the composition of microbiota in horses following anthelmintic administration
- Identify potential metabolite biomarkers associated with anthelmintic administration.
Chapter 2

2.0 Review of Methods

In order to meet the objectives of this thesis, a range of different methods and techniques were used. These span from more fundamental parasitology to \textit{in vitro} techniques for identifying feed substrate fermentation patterns and “next-generation” ‘omics technologies to evaluate microbial community profiling and metabolite abundances. Each of the applied methods is briefly reviewed within this following section.

2.1. Parasitology

One of the difficulties with evaluating parasite burdens and anthelmintic efficacy is that there are no universally accepted guidelines on methods. In 1992 Coles \textit{et al.} published the first version of the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines. These guidelines were updated in 2002 specifically for evaluating efficacy of equine anthelmintics using the critical test (Duncan \textit{et al.}, 2002). However critical tests are not commonly used due to the requirements of slaughter and necropsy. Thus the use of faecal egg tests remains prominent. In 2006 Coles \textit{et al.} reviewed the 1992 WAAVP guidelines adding greater detail to molecular methods to detect BZ resistance but with limited practical application in the field. Outside of these broad-reaching guidelines, reviews and recommendations specifically for equine parasitology have been published by Kaplan and Nielsen (2010), Nielsen \textit{et al.} (2010a), Stratford \textit{et al.} (2011), Matthews \textit{et al.} (2012) and Nielsen \textit{et al.} (2013a). Thus within the literature there are varied methods and cut off values reported but a lack of standardisation which makes comparing studies difficult.
2.1.1. Faecal egg counts

In chapter 1 it was identified that there are a range of variations employed for diagnostic tests within equine parasitology. The most commonly reported faecal egg counting technique is the McMaster method (Stratford et al., 2011), this is a faecal flotation method allowing quantification of nematode eggs using microscopy. There are a range of modifications to this method including centrifugation, increasing the number of counting grids on a slide and increasing the weight of the faecal sample to be tested. These modifications aid the clarity and therefore speed of the test and have allowed for lower egg detection limits (Nielsen, 2014). Modifications to the standard McMaster protocol also improve the reliability and repeatability of the test (Denwood et al., 2012).

2.1.2. Faecal Egg Count Reduction Test (FECRT)

Currently the only method widely used in the field to detect anthelmintic resistance is the Faecal Egg Count Reduction Test (FECRT) (Coles et al., 2006; Matthews, 2011; Stratford et al., 2011). This is considered to be the “gold standard” for evaluating anthelmintic efficacy (Nielsen et al., 2010a).

The WAAVP set out guidelines for the FECRT applied to horses in 1992 (Coles et al., 1992). The original WAAVP guidelines were set out to standardise the testing of new anthelmintics, however it was recognised that a standardised protocol for equine FECRT was required for efficacy evaluation.
The FECRT is a simple *in vivo* test that can be used in all grazing animals and all anthelmintics that treat nematodes. The WAAVP approved protocol:

1. Identify animals with positive FEC >150 epg
2. Treat with correct dosage of anthelmintic
3. Take a second FEC 10-14 days post dosing
4. Use the following calculation:

\[
\text{FECRT (\%) = } \frac{100 \times (\text{Baseline FEC - Post Treatment FEC})}{\text{Baseline FEC}}
\]

(Kaplan and Nielsen, 2010; Stratford *et al.*, 2011)

The WAAVP (1992) equine guidelines are based on detecting BZ resistance as this was the only concern when these guidelines were written. The WAAVP guidelines were originally designed for farm livestock kept and tested in large groups. The WAAVP 2006 review (Coles *et al.*, 2006) on the detection of anthelmintic resistance in nematodes of veterinary importance updated the equine FECRT and recommends using a sample size of at least 6 horses where possible (Coles *et al.*, 2006; Nielsen *et al.*, 2013a). Coles *et al.* (2006) also suggest if new animals are introduced to a group it is acceptable to test just the one horse using a FECRT to gauge efficacy before introducing the new animal into a herd. The 2006 review suggested different FECRT cut offs for resistance are required for the different chemical groups, however these were not stated. Post-treatment FEC were recommended to be conducted 3-7 days after THPs, 8-10 days after BZs and 14-17 days after MLs (Coles *et al.*, 2006).
In 2010 Kaplan and Nielsen suggested cut off values for the different anthelmintic group when using the FECRT on groups of 5-10 horses on each farm, where possible. Where the group mean FECRT is below the cut off value for the anthelmintic being tested then this is indicative of resistance. The suggested percentage reduction cut offs are:

<table>
<thead>
<tr>
<th>Anthelmintic</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzimidazoles:</td>
<td>90%</td>
</tr>
<tr>
<td>Pyrantel:</td>
<td>90%</td>
</tr>
<tr>
<td>Ivermectin:</td>
<td>95%</td>
</tr>
<tr>
<td>Moxidectin:</td>
<td>95%</td>
</tr>
</tbody>
</table>

The American Association of Equine Practitioners (AAEP) guidelines (Nielsen et al., 2013a) outline recommendations for faecal egg counting, the FECRT and egg reappearance times. This document is the only current set of practical guidelines on egg reappearance times. However the cut off values for reduced efficacy and resistance in these guidelines differ to other figures in the literature. The AAEP guidelines also clearly state that there are no specific equine FECRT guidelines and these are awaited from WAAVP.

While the FECRT is used as the gold standard for detecting resistance, it does have limitations when used in the field. Time and cost need to be factored in, as well as poor data quality with low precision and reproducibility (Craven et al., 1999; Nielsen et al., 2010a). Furthermore it does not allow for inter-animal variation and pharmacodynamics within the host (Craven et al., 1999). The FECRT will not detect resistance in a population if its prevalence is less than 25% of that population (Martin et al., 1989).

2.1.3. Egg Hatch Assay (EHA)

The WAAVP guidelines (1992) identified an in vitro assay, the Egg Hatch Assay (EHA) to measure anthelmintic efficacy. Eggs are collected from faecal samples and subjected to different concentrations of BZ anthelmintic in vitro. The discriminating dose will prevent
hatching of 99% of the eggs exposed to the anthelmintic and therefore those that do hatch are resistant (Coles et al., 1992; 2006). Originally validated for sheep, it has been successfully used for nematodes infecting horses (Coles et al., 1992; Matthews et al., 2012).

The EHA is a more technically demanding, but more reliable than the FECRT (Craven et al., 1999). The EHA provides no more early warning of anthelmintic resistance than the FECRT as studies have found 25% of the population need to be resistant before this assay will detect it (Taylor et al., 2002). The EHA is only suitable for use with BZs, other classes do not have ovicidal efficacy, however the EHA has been modified to work with tetrahydropyrimidines, specifically levamisole for ruminants (Taylor et al., 2002). As widespread BZ resistance is documented, the value of developing the egg hatch assay may be limited (Matthews et al., 2012). Therefore most suitable diagnostic tool for monitoring ML efficacy remains the FECRT (Coles et al., 2006).

2.2. Measuring fermentation parameters in vitro

In vitro techniques are useful research tools to non-invasively identify changes within the hindgut microbiome associated with diet and orally delivered medications. Currently Leng et al. (2017) are working on a multi-chamber model of the horse’s hindgut. Prior to this, hind gut models have been much simpler and focussed upon fermentation kinetics to measure digestibility of substrates in vitro. The original in vitro fermentation technique was described by Tilly and Terry (1963). This two stage process was designed to model microbial digestion in rumen inoculum followed by enzymatic digestion. As enzymatic digestion precedes fermentation in the horse the Tilly and Terry method is of limited use in equids.

A second in vitro model for fermentation is the gas production technique. The manual transducer gas production technique developed by Theodorou et al. (1994) provides a simple
way of determining *in vitro* fermentation of feedstuffs providing detailed information of fermentation kinetics.

### 2.2.1. *In vitro* gas production technique

The *in vitro* gas production technique is based upon the measurement of gas, CH$_4$ and CO$_2$, produced by the fermentation of a feed substrate that is buffered in bicarbonate medium, inoculated with rumen/caecal fluid or faeces. The original model was developed for ruminants however the technique has successfully been adapted for use with equids. Early equid studies relied upon horses cannulated into the caecum to provide caecal fluid. Lowman (1996) compared the fermentation kinetics of equine caecal fluid compared to faecal inoculum and concluded that faeces were a suitable source of inoculum for equine gas production studies.

A potential concern for the use of faeces to create a faecal inoculum is the exposure of anaerobic bacteria to air directly following defecation. The formation of equine faeces in faecal balls means the excreted bacteria remain anaerobic for some time and remain viable for several hours after voiding (Holter, 1991; Theodorou *et al*., 1993). If the faecal sample used to create a faecal inoculum is from the centre of a freshly voided dung pile the bacteria should remain viable.

The *in vitro* gas production technique uses 125ml Wheaton bottles containing feed substrate, faecal inoculum, buffer and a reducing agent which are sealed with crimp top rubber seals. These bottles are then incubated at 39°C and as fermentation occurs gas is produced. Gas starts to accumulate within the headspace in the bottles. The volume of gas and pressure inside the bottle is periodically measured and released from the bottle during incubation using a hypodermic needle and pressure transducer. The speed and the amount of gas produced are determined by the substrate being fermented. Thus there is a close relationship between the pattern of gas production and substrate degradation (Jessop and Herrero, 1997). The process of
gas production within culture bottles is not thoroughly understood. Direct gas production from fermenting feed substrate produces CH₄ and CO₂, other indirect gasses are also produced from the buffering of SCFAs within the closed environment i.e. not directly from the feed substrate but from fermenting VFAs. It is thought that the SCFAs produced by fermentation of the substrate react with carbonates in the buffer producing more CO₂ than would be found in vivo (Rymer et al., 1997). This is only problematic when comparing the in vitro gas production technique to degradation in vivo. When comparing differing feed substrates or animal treatment groups both in vitro, findings will be comparable as both groups will be subject to the same conditions (Moore-Colyer, Pers. Comm.). At the end of incubation the fluid medium can be sampled for pH and SCFA analysis and remaining substrate dried and weighted to determine end-point dry matter loss.

During incubation bottles are maintained at 39°C, this figure was originally derived from ruminant studies as the rumen temperature is normally maintained between 39°C – 40.5°C (McDonald et al., 2007). Where the technique has been used with equids the incubation temperature has remained at 39°C even though the horses’ caecum is approximately 38.5°C (Lowman et al., 1999; Biddle et al., 2013). Furthermore Lowman et al. (1999) found lower gas production at 25, 30 and 45°C using equine faeces as inoculum. Therefore the optimum incubation temperature used for in vitro gas production appears to be 39°C.

When considering the source of inoculum it is also important to consider the diet of the donor animals and the substrate to be fermented in vitro. As diet directly impacts upon the composition of the hindgut microbiota the diet to be fermented in vitro should be similar to that of the donor animal (Rymer et al., 2005).
2.2.2. Mathematical models to describe gas production data

Cumulative gas production is the most common way to present gas production profiles. There is a direct linear relationship between pressure and gas produced. However when the pressure in the headspace of the bottles increases above seven psi the relationship with gas production is less predictable (Theodorou et al., 1994). To describe the relationship between gas volume and pressure the following equation $V=XP+I$, where $V$ is volume (ml), $P$ is pressure (psi), $X$ is slope and $I$ is the intercept (bias correction factor), could be used to describe the relationship between volume and pressure for all bottles (Theodorou et al., 1994). The bias correction factor allows for slight manufacturing differences in the headspace of bottles or operator error when dispensing media during inoculation. As there is a linear relationship between gas produced and pressure in the bottle headspace the bias correction regression equation removes erroneous values from the data (Theodorou et al., 1994). Following this bias correction, cumulative gas profiles from each bottle should then be summed and a cumulative gas production curve formed (Theodorou et al., 1994).

Cumulative gas production curves only identify gas per gram of substrate and are only comparable to other substrates or conditions that were fermented within the same experiment. To allow comparison of fermentation parameters between different experiments and more detailed information on fermentation kinetics, mathematical modelling can be applied to cumulative gas profiles to produce rate parameters.

There are differing gas models but all work on the principle that a substrate (S) is fermented to yield a volume of gas (Y) at a rate $\mu$ ($\mu$ - fractional rate of degradation per hour) at time (t) (Thornley and France, 2007). The model of France et al. (1993) was designed specifically for the manual pressure transducer technique of Theodorou et al. (1994). The France et al. (1993)
model models the sigmoidal response frequently encountered when fermenting feed substrates \textit{in vitro}. The France \textit{et al.} (1993) model assumes that when bottles are inoculated there is a lag time before fermentation begins. The fractional rate of substrate degradation is postulated to vary with time. Rate parameters are calculated within a maximum likelihood programme (Ross, 1987) using the data from the inoculation period, gas produced and substrate loss over the experiment. The constants refer to the extent of substrate degradation linked to the lag time.

The equation of the France \textit{et al.} (1993) model is detailed below:

\begin{equation}
Y = A \{1 - \exp \left[ -b \left( t - L \right) - (\sqrt{t} - \sqrt{L}) \right]\}
\end{equation}

\begin{itemize}
  \item Y = gas produced (ml) at time t
  \item A = asymptotic value for gas pool size
  \item b = rate constant (h\(^{-1}\)) which is independent of time
  \item c = rate constant (h\(^{-0.5}\)) whose influence decreases with time
  \item t = time
  \item L\(_T\) = lag time (hours)
\end{itemize}

The quadratic equation can also be presented as: \(Y = A - B Q^t Z^{\sqrt{t}}\)

From this model, using the rate constants, the fractional rate of gas production (FRGP or \(\mu\)) half way through incubation can be calculated:

\begin{equation}
\text{FRGP} = b + \left(\frac{c}{2\sqrt{t_{50}}}\right)
\end{equation}

The rate constants in the model b and c both influence the FRGP. As c changes with time it changes the shape of the curve. If the value for c becomes negative then the curve is sigmoidal and quadratic equation is used. When the value for c is positive then an exponential rate of gas
production has occurred (Figure 2.1.) and the following equation is applied to obtain the relevant parameters: \[ Y = A - B Q \]

**Figure 2.1.** Example of sigmoidal and quadratic curves and an example of a quadratic observed gas curve and the fitted France et al. (1993) model shown in red triangles.

This modelling technique also allows the calculation of the extent of substrate degradation, which is not possible in other gas models. The extent of degradation considers the flow rate of digesta within its equation. This is specifically useful when considering feedstuffs for horses where the rate of passage through the gastrointestinal tract is much quicker than that of ruminants. Overall the France et al. (1993) model allows a greater insight into early stage fermentation as well as end point fermentation which is more comparable to the horses mean retention time compared to that of ruminants.

### 2.3. Microbiome community profiling

Over the past decade there has been a significant increase in the use of ’omics technologies within scientific research. The use of DNA sequencing allows the profiling of whole bacterial communities without the need to culture them. High-throughput sequencing has significantly
enhanced our understanding of microbial ecology (Langille et al., 2013). There are two differing approaches to DNA microbiome sequencing, full metagenomics or metataxonomics. Metagenomics is the study of all of the genomes within a given sample. This therefore provides a rich information set on the structure of the population and the functions of this population through gene expression (Kuczynski et al., 2012). Targeted amplicon studies, metataxonomics, analyse of all taxa within a sample by sequencing a specific gene (Kuczynski et al., 2012). Most commonly, for microbiota profiling, the bacterial 16S rRNA gene is targeted for metataxonomics (Langille et al., 2013).

For targeted DNA sequencing the initial step is to amplify a desired region on the 16S gene. Within this thesis the V4 region was the specific target, this can be visualised in Figure 2.2 below. Post amplification the amplicons are deep sequenced using next generation sequencing.

![Figure 2.2. Schematic of the 16S gene identifying variable regions (V1-V9), primers, amplicons and sequencing of those amplicons using the illumina platform. Adapted from Kuczynski et al. (2012).](image-url)
Illumina MiSeq uses sequencing by synthesis to produce a large number of sequencing reads from a relatively small amount of DNA (Buermans and den Dunnen, 2014). This sequencing makes use of complementary fluorescent-labelled nucleotides which are added to the anchored parallel strands. Each type of nucleotide base has different fluorescent terminator dyes and these are imaged and cleaved when the nucleotide is added. All four types of terminator-bound nucleotides are present during this processing, allowing for natural competition and reducing bias. This occurs simultaneously to millions of DNA strands in different cells allowing the generation of a large number of sequencing reads to be acquired (Buermans and den Dunnen, 2014). Figure 2.3 depicts sequencing by synthesis.

**Figure 2.3.** Schematic representation of sequencing by synthesis, part of the illumina sequencing technology. Adapted from www.cegat.de/en/services/next-generation-sequencing/ [accessed 14/10/2017].
2.3.1. Bioinformatics

Following sequencing, raw reads require bioinformatics to provide meaningful data. One of the most common bioinformatics pipelines for analysing MiSeq derived sequences is Quantitative Insights into Microbial Ecology (QIIME). QIIME is an open source pipeline that allows quality filtering. QIIME also facilitates the picking of operational taxonomic units (OTUs) which are clusters of sequence reads with 96% similarity or greater. This similarity is expected to correspond with a particular species. OTUs are picked from the sequence reads against large databases such as the Ribsomal Database Project (RDP). Following OTU picking taxonomic assignment is applied and phylogenetic reconstruction before analysis through graphic representation (Caprosa et al., 2010). Figure 2.4. demonstrates visual analysis of alpha and beta diversity outputs. The final stages of QIIME bioinformatics provides an OTU table that can be used for further analysis.

**Figure 2.4.** Graphical output from QIIME (A) alpha rarefaction curve (B) beta diversity PCoA plot of two groups from a dataset plotted between principal coordinate one (PC1), PC2 and PC3. Spatial grouping on this scores plot suggest differences in diversity of OTUs between these two groups. Adapted from www.github.com [accessed 14/10/17].
Once an OTU table has been generated, further statistical analysis can be carried out on the data to identify differences in microbial profiles within groups. However due to the distribution of OTU abundances within these types of data sets a non-parametric model that is also robust in the face of multiple comparisons is required. Linear Discriminant Analysis (LDA) effect Size (LEfSe) uses a Kruskall Wallis rank-sum test followed by a pairwise Willcoxon rank-sum test and finally linear discriminate analysis to estimate the effect size of each differently abundant feature, Figure 2.5. (Segata et al., 2011). This platform allows the identification of statistical significance, biological consistency and bacterial biomarkers of disease. The final output from LEfSe generates three plots, two different histograms identifying OTUs of significantly differing abundances and differing treatments/conditions and a cladogram of how differences are phylogenetically related. The primary histogram of differences in OTU abundances and cladogram can be seen in Figure 2.6. (Segata et al., 2011).

**Figure 2.5.** Schematic representation of LEfSe (a) Kruskall Wallis on data to identify OTUs of differing distributions, (b) Wilcoxon pairwise testing of those distributions identified in a. (c) The resulting vectors are used to build a LDA model from which the relative difference among classes is used to rank the features (Segata et al., 2011).
2.4. Metabolomics

Metabolomics uses a systems biology approach by viewing ‘top down’ complex biological biochemistry integrated with computational systems and mathematical modelling, with the aim of characterising and quantifying small molecules within living systems (Nicholson and Lindon, 2008). Traditional study approaches were ‘bottom up’ selecting a specific target to form a hypothesis and then setting about testing that hypothesis, without consideration for the other interactions within that biological system. Systems biology takes a holistic approach with the idea that the complex networks within a biological system are greater than the individual parts that make up that system.

Metabolomics allows the characterisation of metabolites within a biological sample e.g. a biofluid, to identify metabolites within that system. This may be applied to the study of disease, medical or dietary interventions or to study the effects of lifestyle, exercise or ageing (Markley et al., 2017). In the horse metabolomics is in its infancy, but to date the ‘normal’ metabolome
of the Thoroughbred race horse has been characterised using blood plasma, faeces and urine, providing a reference library for the healthy horse (Escalona et al., 2015). Both faecal and urine samples can be collected non-invasively and are useful for metabolomics. Urine is the primary biofluid and provides information on the metabolic state of the whole organism and includes metabolites derived from the diet and from the gut microbiome. Similarly faeces offer the same advantages but also provide further information on excretory metabolites and those closely linked with the gut microbiome (Wu et al., 2010). The gut bacteria have a huge contribution to the metabolites excreted in urine, Escalona et al. (2015) reported that from a metabolomics perspective, urine was the most informative biofluid in the horse.

There are two analytical techniques commonly used for metabolomics, mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Markley et al., 2017). Both of these techniques allow unbiased profiling of the majority of metabolites present within a biological system. Within this thesis NMR was the analytical technique applied. Proton nuclear magnetic resonance (\(^1\text{H}\) NMR) uses the spin property possessed by all protons contained in the metabolites within a given sample (Levitt, 2005). These spinning protons can be oriented into a higher or lower energy state. NMR applies an electromagnetic field to these protons and they are separated into two energy levels (Levitt, 2005). There are always an excess of nuclei in the lower energy state, this excess of low energy state spins provides the signal measured by \(^1\text{H}\) NMR spectroscopy. Using electromagnetic radiation low energy nuclei can be excited to resonate into a higher energy level. By removing (pulsing) the electromagnetic energy field the nuclei relax back to their normal energy state, emitting a small magnetic field. This signal is collected as waves which undergo Fourier transformation to convert the data from time to frequency, thus giving rise to peaks within a spectrum (Levitt, 2005). The chemical shift (position of the peak) in the spectrum is dependent on the electrons surrounding the nucleus. The chemical shift of spectral peaks are calibrated to an internal standard, commonly 3-
trimethylsil-1-[2,2,3,3\textsuperscript{2}H4] propionate (TSP). NMR standards give a single strong resonance as all of their nuclei have identical protons that are strongly shielded (Levitt, 2005).

The multiplicity of a peak is dependent on the spin–spin coupling affecting a proton (Levitt, 2005). Coupling is caused by the interaction of neighbouring nuclei resulting in the NMR frequency being split into different multiplicities. The multiplicity of a peak can be in the form of a singlet, doublet, triplet, quartet, quintet or multiplet (Levitt, 2005). Figure 2.7 provides a schematic representation of the use of \textsuperscript{1}H NMR in metabolomics.
Figure 2.7. Schematic representation of the use of NMR spectroscopy within the field of systems biology. Simple schematic representation of how the NMR applies magnetic fields to nuclei which, under pulse, causes waves that are detected by the spectrometer. Waves are converted by Fourier transformation into peaks within the NMR spectra. From these peaks metabolite abundances can be identified. Multivariate statistics and modelling of metabolite abundances allows detection of differences these can then be related back to the system. Figures adapted from Nicholson and Lindon (2008); www.systemsbiology.org [accessed 15/10/2017]; Institute of Chemistry (2017); www.ceitec.eu/ [accessed 15/10/17].
2.4.2. Multivariate statistics for metabolomics analysis

The datasets produced by $^1$H NMR provide information on thousands of possible metabolite variables. In order to identify differences in metabolic abundances multivariate statistics are applied (Trygg et al., 2007). Multivariate analysis allows simultaneous analysis of multiple measurements/variables within a sample of interest. When applied to NMR spectroscopy data, multivariate statistics allows the detection of peaks within an NMR spectra that vary between groups of metabolic phenotypes. Peaks within spectra represent metabolites, thus multivariate models allow the identification of metabolites with significantly different abundances.

Unsupervised multivariate models are used for primary data analysis, e.g. principal component analysis (PCA). This provides an overview summary of multivariate data e.g. NMR spectra, without detailed class membership of the samples within it (Holmes and Antti, 2002). Differences are identified between principal components rather than on a two dimensional X, Y axis. A principal component represents the plane of greatest variance between observations within the spread of the data. The plane that shows the greatest variation is referred to as the first principal component, PC1. The relation of each variable in PC1 to the plane with the second most variance (PC2) is also plotted, for ease of interpretation this can be plotted two dimensionally. If the PCA identifies differences in metabolic phenotype between groups then a loadings plot can be created to identify observations between these two principal components, Figure 2.8. The loadings plot identifies the differences in metabolite abundances between the principal components and the multiplicity and resonance can then be used to identify that specific metabolite.
Figure 2.8. Schematic representation of a PCA (A) first two components in a data set, (B) two-dimensional scores plot showing the relationship between the observations and the first two principal components and (C) the loadings plot identifying the variables contributing to the position of the observation in the first principal component. Adapted from Trygg et al. (2007).

Following such preliminary data exploration, a supervised model is used to include information of the class membership of the samples/groups. Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) uses a prediction and regression method between the descriptor ($X$ data) and the response ($Y$ data). In context $X$ data may represent a control and $Y$ data a treatment group. OPLS-DA models use an orthogonal filter to remove any systematic variation present in $X$ that is not present in $Y$. The orthogonal filter rearranges the original 3D data in to 2D while using information from the control group to filter out systematic variation from the treatment group. This yields a multivariate model with improved interpretation and less complexity (Trygg and Wold, 2002). OPLS-DA models allow the visualisation of differences between metabolite abundances between two groups on a spectra plotted as covariance with class (Cloarec et al., 2005). The loadings plot has an $R^2$ heat map, the stronger
the correlation coefficient between a metabolite and a treatment group is reflected by colour in the loadings plot. Figure 2.9. depicts an OPLS-DA model.

**Figure 2.9.** Schematic representation of OPLS-DA (A) multidimensional dataset, (B) orthogonal filter applied to the dataset to remove variation not orthogonal to Y, (C) two-dimensional plot showing separation between groups and (D) the OPLS-DA loadings plot showing differences between groups identified within the model. Adapted from Trygg et al. (2007).
Chapter 3.0

Nationwide surveillance for evidence of reduced macrocyclic lactone efficacy in horses in the UK

3.1. Introduction

All anthelmintic drugs have a specific egg reappearance period post treatment which were established when the drug was first licensed. Shortened egg reappearance period (ERP), from that stated on the marketing authorisation, or compared to reports published when the drug was introduced, is an early indicator of anthelmintic resistance (Sangster, 1999). Monitoring egg reappearance period post treatment provides early indication of reduced drug efficacy prior to the onset of resistance (Sangster, 1999).

In the UK the macrocyclic lactones ivermectin and moxidectin are the most recent anthelmintics to be licensed for nematode treatment in horses. These anthelmintics are also the drugs of choice used by horse managers in the UK for worm control (Allison et al., 2011). Ivermectin and moxidectin were licensed for use in the 1980s and 1990s respectively (Kaplan, 2004). Ivermectin was reported to have an egg reappearance period of 8-10 weeks when it was first marketed (Borgsteede et al., 1993). Moxidectin egg reappearance was reported in excess of 13 weeks, and up to 24 weeks, post treatment when it was first introduced (Jacobs et al., 1995; DiPietro et al., 1997). An ERP of 13 weeks is used on the moxidectin datasheet.

Currently there are no universally adopted guidelines specifically for the evaluation of resistance or egg reappearance in horse strongyles (Relf et al., 2014). Published methods have been adapted from ruminant work from the WAAVP guidelines (Coles et al., 1992). The American Association of Equine Practitioners (AAEP) (Nielsen et al., 2013a) has set out guidelines for resistance and efficacy testing in horses. The suggestion for egg reappearance testing uses the faecal egg count reduction (FECR) test at intervals beyond 14 days using a 90% efficacy cut-off for macrocyclic lactones.
To date, there have been no published reports of macrocyclic lactone resistance within cyathostomins in the UK. Only one UK study to date has suggested moxidectin resistance within cyathostomins from FECR data. This was in a population of donkeys being treated “off-label” with a formulation designed for cattle, for which the pharmacokinetics in equids are not well understood (Trawford et al., 2005). However, there have been reports in North and South America, central Europe and more recently the UK of shortened egg reappearance periods for both ivermectin and moxidectin within cyathostomins (Molento et al., 2008; Lyons et al., 2009, 2011; Rossano et al., 2010; Relf et al., 2014).

Resistance to the benzimidazole group of anthelmintics is reported as widespread within cyathostomins (Osterman Lind et al., 2007; Relf et al., 2014; Matthews, 2014). There are three new anthelmintic classes that have been licensed for animal use since the macrocyclic lactones but there appear to be no plans for these classes to be licensed for equine nematode treatment in the near future (Epe and Kaminsky, 2013). The reliance of prophylactic anthelmintic use as a colic prevention mechanism was originally introduced by Drudge and Lyons (1966). Interval dosing regimens are frequently used by many horse managers today (Nielsen, 2012). It is therefore important to understand the current efficacy of the macrocyclic lactones in equine strongyles in the UK.

3.1.1 Objectives

This retrospective study was performed with two objectives:

(1) To determine egg reappearance periods after dosing horses with ivermectin or moxidectin under field conditions.

(2) To identify trends in egg reappearance over a four year period.
The target population for both objectives was UK pleasure horses with persistently positive faecal egg counts in the face of anthelmintic treatment.

3.2. Materials and Methods

3.2.1. Ethical approval

Ethical approval was granted by the University of Liverpool Ethics Committee, RETH000363.

3.2.2. Parasite control programmes

Parasite control records of horses tested for ivermectin and/or moxidectin efficacy by a commercial provider between 01/01/2008 – 29/08/2011 were reviewed (n=200). Parasite control programmes were designed around FEC data, animal age, breed, weight and pasture management. For each animal FECs were scheduled throughout the year to monitor nematode status and specific anthelmintic treatments for pre-patent cyathostomin spp. specifically those in hypobiosis and for Anoplocephala spp. were also scheduled. Where animals demonstrated persistently positive FEC results from monitoring FECs and when FECs were directly after the stated egg reappearance period of an anthelmintic, the commercial provider placed these animals under additional FEC monitoring. This involved additional FECs to check the length of the egg reappearance periods for anthelmintics to check the efficacy of these drugs. This process worked backwards from the end of the anthelmintics ERP, in weeks, to identify the time point of egg reappearance. All FEC results, drug dosing data and dates were recorded in the horses’ record. Sample collection and anthelmintic treatment were undertaken by the client, instructed by the commercial provider. The instructions given to clients for sample collection and dosing, FEC kits and anthelmintic were supplied by the commercial provider. When FECs were required clients were asked to randomly sub-sample from a freshly voided faecal pile and place into an airtight container which was then posted back to the laboratory. Prior to
anthelmintic dosing, clients were asked to estimate weight with a girth tape. From the weight measurement anthelmintic dosage was increased by 50 kg for small breeds and 100 kg for large breeds to prevent under dosing through inaccurate weight estimation and allow for some loss during administration. Clients were given specific dates for FEC sampling and anthelmintic administration.

3.2.3. Faecal egg counts

All FECs during this period were processed at the University of Liverpool following the WAAVP guidelines for the modified McMaster technique (Coles et al., 1992) repeated in duplicate and taking the mean result, thus each egg represented 25 eggs per gram (epg). No larval cultures were performed therefore all strongyle eggs were reported collectively.

3.2.4. Database formation

FEC results and treatment data were extracted from paper records of parasite control programmes and entered into a database for analysis. Faeces for FECs were taken between 4 and 10 weeks post treatment for ivermectin and between 3 and 13 weeks for moxidectin. These time points had been selected by the commercial provider as a pragmatic efficacy testing tool (Nielsen et al., 2010a). FECs had been scheduled considering the animal’s age, the grazing conditions and management, presence of companion animals and the animals’ treatment history.

FEC results were categorised by treatment i.e. ivermectin or moxidectin. Following recommendations for efficacy testing, horses with initial pre-treatment faecal egg counts less than 150 epg were excluded from the data set (Coles et al., 2006). An additional exclusion criterion was any horse that had received anthelmintic within 90 days of initial faecal egg count.
3.2.5. Study population demographics

Of 200 records, 153 met the inclusion criteria, categorised as moxidectin \((n=95)\), ivermectin \((n=58)\). Records comprised of Thoroughbreds, including cross breeds \((n=33)\), warmbloods, including cross breeds \((n=40)\), draft horses \((n=10)\), native ponies \((n=22)\) and cob types \((n=35)\), with some records no breed was recorded \((n=13)\). There were 72 females and 81 males ranging from 1-31 years \((\text{mean } 10 \pm 7.2 \text{ years})\). Premises were distributed throughout Great Britain (Figure 3.1). Premises were predominantly DIY livery yards. Horses were deemed as high risk for parasitic infection due to persistently positive faecal egg counts. Some premises had records of the presence of benzimidazole resistant nematodes. DIY livery yards carry an increased risk of spreading resistant nematodes due to movement of horses between different DIY establishments. Collectively these factors were considered to leave these horses at high risk of parasitic infection.
Figure. 3.1. Locations of all premises tested, squares represent moxidectin and circles represent ivermectin. Adapted from www.sketchmap.co.uk.

3.2.6. Calculation of egg reappearance period (ERP)

Egg reappearance period was described using three previously reported parameters: (1) the first time point a positive faecal egg count was recorded post treatment (Lyons et al., 2008; Molento et al., 2008), (2) the group arithmetic mean FEC >10% of Day 0 value (Borgsteede et al., 1993; Jacobs et al., 1995; Boersema et al., 1998; Mercier et al., 2001; von Samson-Himmelstjerna et al., 2007; Larsen et al., 2011), and (3) <90% FECR within the stated egg reappearance period for the product (Larson et al., 2011; Nielsen et al., 2013a; Relf et al., 2014).
FECR was calculated as:

\[
\text{Pre-treatment FEC} - \text{Post treatment FEC} \times 90 \\
\text{Pre-treatment FEC}
\]

ERP metrics were evaluated at premises level (n=16 moxidectin, n=10 ivermectin). When only one test record per premises existed, data were reported at animal level (n= 61 moxidectin; n= 31 ivermectin). All single animal premises results were analysed collectively.

3.2.7. Longitudinal data analysis

The FEC reduction data and percentage difference between pre-treatment and post treatment FECs for both ivermectin and moxidectin were analysed over the four year time period (2008-2011). Mean FEC reduction and percentage difference data at 8 weeks post ivermectin treatment and 13 weeks post moxidectin treatment were analysed using the Mann-Kendall trend test in R (version 3.2.2).
3.3. Results

3.3.1. Egg reappearance times

Shortest observed ERP (time to first positive faecal egg count observed) for both ivermectin and moxidectin was 5 weeks. Five of the 16 moxidectin premises displayed ERP (measured as >10% of day 0 FEC and FECR <90%) at 12-13 weeks (Table 3.1.). For moxidectin individual animal data collectively (Table 3.2.), the earliest ERP was 5 weeks, with a 67% FECR (n=1). At six weeks post dosing the mean FECR was 87% and there was 16% difference between pre-treatment and post-treatment FEC (n=3). Most tests (n=87) were conducted between 10 and 13 weeks post moxidectin, giving a mean FECR 80-85% and pre and post dosing FEC difference of 11-22%. Overall for moxidectin (Table 3.3.) 5% of animals had an ERP of 5-7 weeks, 95% of horses had an ERP of ≤13 weeks.

3.3.2. Egg reappearance trends over the four year period

At 8 weeks post ivermectin treatment the mean FEC reduction for each year over the four year period were 74.4%, 100%, 87% & 68.2% and the FEC % differences were 22%, 0%, 8% and 33% respectively (Table 3.4.). This was not statistically significant, \( P= 0.54 \) \( (r=0.0581) \). For moxidectin the mean FEC reduction at 13 weeks post treatment were 93.3%, 89.9% , 86.5% and 79.5% respectively (Figure 3.2.) this demonstrated a significant negative trend \( (r=-0.172) \) \( (P=0.022) \). For FEC % difference at 13 weeks results were 2%, 9%, 13% & 31 % respectively (Table 3.4.).
Table 3.1. Egg reappearance tests at premises level, classified by premises, drug and then by time point post dosing. Superscripts denote shortened egg reappearance \(^a\) both FECR <90\% and pre-treatment and post treatment FEC difference >10\%. \(^b\) indicates egg reappearance by one metric only.

<table>
<thead>
<tr>
<th>Premises</th>
<th>Number of animals tested</th>
<th>Drug</th>
<th>Pre FEC (mean)</th>
<th>Post FEC (mean)</th>
<th>Time point (Weeks) post dosing</th>
<th>Percentage difference between pre and post dose FECs (%)</th>
<th>Mean FECR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Mox</td>
<td>375</td>
<td>50</td>
<td>13</td>
<td>13(^a)</td>
<td>87(^a)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Mox</td>
<td>350</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Mox</td>
<td>408</td>
<td>267</td>
<td>13</td>
<td>65(^a)</td>
<td>35(^a)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Mox</td>
<td>344</td>
<td>13</td>
<td>10</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>Mox</td>
<td>238</td>
<td>0</td>
<td>13</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>Mox</td>
<td>180</td>
<td>55</td>
<td>12</td>
<td>31(^a)</td>
<td>69(^a)</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>Mox</td>
<td>400</td>
<td>42</td>
<td>13</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>Mox</td>
<td>625</td>
<td>25</td>
<td>12</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>Mox</td>
<td>375</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>Mox</td>
<td>400</td>
<td>100</td>
<td>10</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>Mox</td>
<td>525</td>
<td>88</td>
<td>13</td>
<td>17(^a)</td>
<td>83(^a)</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>Mox</td>
<td>1563</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>Mox</td>
<td>313</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>Mox</td>
<td>850</td>
<td>263</td>
<td>13</td>
<td>31(^a)</td>
<td>69(^a)</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>Mox</td>
<td>325</td>
<td>17</td>
<td>13</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>16</td>
<td>7</td>
<td>Mox</td>
<td>364</td>
<td>14</td>
<td>13</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>Ivm</td>
<td>713</td>
<td>125</td>
<td>5</td>
<td>18(^a)</td>
<td>82(^a)</td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>Ivm</td>
<td>388</td>
<td>38</td>
<td>9</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>19</td>
<td>2</td>
<td>Ivm</td>
<td>200</td>
<td>13</td>
<td>7</td>
<td>6</td>
<td>94</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>Ivm</td>
<td>413</td>
<td>38</td>
<td>8</td>
<td>9</td>
<td>91</td>
</tr>
<tr>
<td>21</td>
<td>5</td>
<td>Ivm</td>
<td>300</td>
<td>250</td>
<td>10</td>
<td>83(^a)</td>
<td>17(^a)</td>
</tr>
<tr>
<td>22</td>
<td>2</td>
<td>Ivm</td>
<td>188</td>
<td>463</td>
<td>8</td>
<td>247(^a)</td>
<td>-147(^a)</td>
</tr>
<tr>
<td>23</td>
<td>2</td>
<td>Ivm</td>
<td>313</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>2</td>
<td>Ivm</td>
<td>263</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>Ivm</td>
<td>475</td>
<td>175</td>
<td>10</td>
<td>37(^a)</td>
<td>63(^a)</td>
</tr>
<tr>
<td>26</td>
<td>4</td>
<td>Ivm</td>
<td>606</td>
<td>131</td>
<td>8</td>
<td>22(^a)</td>
<td>78(^a)</td>
</tr>
</tbody>
</table>
Table 3.2. Egg reappearance data from single animal premises analysed collectively by drug and time point. Grouped by drug and week for analysis. Superscripts suggest early egg reappearance.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Time point (Weeks) post dosing</th>
<th>Number of animals tested</th>
<th>Pre dose FEC (mean)</th>
<th>Post dose FEC (mean)</th>
<th>Difference between pre and post dose FECs (%)</th>
<th>Mean FECR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ivm</td>
<td>4</td>
<td>2</td>
<td>550</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Ivm</td>
<td>6</td>
<td>2</td>
<td>763</td>
<td>100</td>
<td>13\textsuperscript{a}</td>
<td>87\textsuperscript{a}</td>
</tr>
<tr>
<td>Ivm</td>
<td>7</td>
<td>3</td>
<td>775</td>
<td>75</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>Ivm</td>
<td>8</td>
<td>13</td>
<td>521</td>
<td>121</td>
<td>23\textsuperscript{a}</td>
<td>77\textsuperscript{a}</td>
</tr>
<tr>
<td>Ivm</td>
<td>9</td>
<td>3</td>
<td>533</td>
<td>342</td>
<td>64\textsuperscript{a}</td>
<td>36\textsuperscript{a}</td>
</tr>
<tr>
<td>Ivm</td>
<td>10</td>
<td>8</td>
<td>516</td>
<td>159</td>
<td>31\textsuperscript{a}</td>
<td>69\textsuperscript{a}</td>
</tr>
<tr>
<td>Mox</td>
<td>3</td>
<td>1</td>
<td>275</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mox</td>
<td>5</td>
<td>1</td>
<td>150</td>
<td>50</td>
<td>33\textsuperscript{a}</td>
<td>67\textsuperscript{a}</td>
</tr>
<tr>
<td>Mox</td>
<td>6</td>
<td>3</td>
<td>1613</td>
<td>250</td>
<td>16\textsuperscript{a}</td>
<td>85\textsuperscript{a}</td>
</tr>
<tr>
<td>Mox</td>
<td>7</td>
<td>1</td>
<td>325</td>
<td>50</td>
<td>15\textsuperscript{a}</td>
<td>85\textsuperscript{a}</td>
</tr>
<tr>
<td>Mox</td>
<td>8</td>
<td>1</td>
<td>1850</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mox</td>
<td>9</td>
<td>1</td>
<td>150</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mox</td>
<td>10</td>
<td>15</td>
<td>433</td>
<td>58</td>
<td>13\textsuperscript{a}</td>
<td>87\textsuperscript{a}</td>
</tr>
<tr>
<td>Mox</td>
<td>11</td>
<td>4</td>
<td>444</td>
<td>50</td>
<td>11\textsuperscript{a}</td>
<td>89\textsuperscript{a}</td>
</tr>
<tr>
<td>Mox</td>
<td>12</td>
<td>11</td>
<td>250</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mox</td>
<td>13</td>
<td>23</td>
<td>490</td>
<td>107</td>
<td>22\textsuperscript{a}</td>
<td>78\textsuperscript{a}</td>
</tr>
</tbody>
</table>
Table 3.3. Egg reappearance data for all horses tested collectively within the data set, classified by drug and then by time point post dosing. Superscript letters denote shortened egg reappearance. \(^a\) highlights shortened egg reappearance calculated by FECR <90% and >10% difference between pre-treatment and post treatment FEC from a small sample. \(^b\) indicates shortened egg reappearance agreed by both metrics from a larger sample. \(^c\) indicates early egg reappearance by one metric only.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Time point (Weeks) post dosing</th>
<th>Number of animals tested</th>
<th>Pre dose FEC (mean)</th>
<th>Post dose FEC (mean)</th>
<th>Difference between pre and post dose FECs (%)</th>
<th>Mean FECR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ivm</td>
<td>4</td>
<td>2</td>
<td>550</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Ivm</td>
<td>5</td>
<td>2</td>
<td>713</td>
<td>125</td>
<td>18(^a)</td>
<td>82(^a)</td>
</tr>
<tr>
<td>Ivm</td>
<td>6</td>
<td>7</td>
<td>490</td>
<td>100</td>
<td>8</td>
<td>80(^c)</td>
</tr>
<tr>
<td>Ivm</td>
<td>7</td>
<td>6</td>
<td>441</td>
<td>100</td>
<td>23(^c)</td>
<td>93</td>
</tr>
<tr>
<td>Ivm</td>
<td>8</td>
<td>19</td>
<td>488</td>
<td>138</td>
<td>28(^b)</td>
<td>72(^b)</td>
</tr>
<tr>
<td>Ivm</td>
<td>9</td>
<td>4</td>
<td>544</td>
<td>275</td>
<td>51(^a)</td>
<td>49(^a)</td>
</tr>
<tr>
<td>Ivm</td>
<td>10</td>
<td>18</td>
<td>433</td>
<td>172</td>
<td>40(^b)</td>
<td>60(^b)</td>
</tr>
<tr>
<td>Mox</td>
<td>3</td>
<td>1</td>
<td>275</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mox</td>
<td>5</td>
<td>1</td>
<td>150</td>
<td>50</td>
<td>33(^a)</td>
<td>67(^a)</td>
</tr>
<tr>
<td>Mox</td>
<td>6</td>
<td>3</td>
<td>1613</td>
<td>250</td>
<td>16(^a)</td>
<td>85(^a)</td>
</tr>
<tr>
<td>Mox</td>
<td>7</td>
<td>1</td>
<td>325</td>
<td>50</td>
<td>15(^a)</td>
<td>85(^a)</td>
</tr>
<tr>
<td>Mox</td>
<td>8</td>
<td>1</td>
<td>1850</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mox</td>
<td>9</td>
<td>1</td>
<td>150</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mox</td>
<td>10</td>
<td>24</td>
<td>447</td>
<td>41</td>
<td>9</td>
<td>91</td>
</tr>
<tr>
<td>Mox</td>
<td>11</td>
<td>4</td>
<td>444</td>
<td>50</td>
<td>11(^a)</td>
<td>89(^a)</td>
</tr>
<tr>
<td>Mox</td>
<td>12</td>
<td>11</td>
<td>250</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Mox</td>
<td>13</td>
<td>48</td>
<td>479</td>
<td>88</td>
<td>18(^b)</td>
<td>82(^b)</td>
</tr>
</tbody>
</table>
Figure 3.2. Faecal egg count reduction (%) yearly trend for moxidectin from 2008-2011, trend significantly decreasing ($P=0.022$) r-0.172 over time. The green line represents the moxidectin trend over time, the black line is the 90% FECR threshold.

Table 3.4. Egg reappearance data for ivermectin at 8 weeks and moxidectin at 13 weeks over the four year period. Superscript letters denote shortened egg reappearance. \(^a\) highlights shortened egg reappearance calculated by FECR <90% and >10% difference between pre-treatment and post treatment FEC. \(^b\) indicates shortened egg reappearance from one metric only.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Year</th>
<th>Time point (weeks) post dosing</th>
<th>Number of animals tested</th>
<th>Difference between pre and post dose FECs (%)</th>
<th>Mean FECR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ivm</td>
<td>2008</td>
<td>8</td>
<td>6</td>
<td>22(^a)</td>
<td>74(^a)</td>
</tr>
<tr>
<td>Ivm</td>
<td>2009</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Ivm</td>
<td>2010</td>
<td>8</td>
<td>9</td>
<td>8</td>
<td>87(^b)</td>
</tr>
<tr>
<td>Ivm</td>
<td>2011</td>
<td>8</td>
<td>36</td>
<td>33(^a)</td>
<td>68(^a)</td>
</tr>
<tr>
<td>Mox</td>
<td>2008</td>
<td>13</td>
<td>5</td>
<td>2</td>
<td>93</td>
</tr>
<tr>
<td>Mox</td>
<td>2009</td>
<td>13</td>
<td>13</td>
<td>9</td>
<td>88(^b)</td>
</tr>
<tr>
<td>Mox</td>
<td>2010</td>
<td>13</td>
<td>50</td>
<td>13(^a)</td>
<td>87(^a)</td>
</tr>
<tr>
<td>Mox</td>
<td>2011</td>
<td>13</td>
<td>27</td>
<td>21(^a)</td>
<td>80(^a)</td>
</tr>
</tbody>
</table>
3.4. Discussion

This study suggests shortened ERP following treatment with ivermectin and moxidectin compared to original published values. Sangster (1999) suggested that a reduction in the ERP of an anthelmintic is an early indicator of reduced efficacy, which longer term will lead to resistance. Shortened egg reappearance in Kentucky was reported by Lyons et al. (2011) in strongyle populations in Thoroughbred yearlings that were displaying an ERP half the length of that published in the marketing authorisation for both ivermectin and moxidectin. Traversa et al. (2009a) reported shortened egg reappearance times for macrocyclic lactones in Italy. Results of this present study, the work of Relf et al. (2014) and Tzelos et al. (2017) all suggest that there is a reduction in the egg reappearance time after macrocyclic lactone treatment in strongyle populations of some horses in the UK. All three studies report egg reappearance for both drugs starting at 35 days post dosing. This present study’s findings for ivermectin are consistent with the work of Von Samson-Himmelstjerna et al. (2007) and Relf et al. (2014), both reporting early egg reappearance from ivermectin treatment at 5 and 6 weeks respectively. Recent UK studies suggest that resistance is not present in strongyles to macrocyclic lactones when tested with the FECR test at 14 days post dosing (Lester et al., 2013; Stratford et al., 2013; Tzelos et al., 2017) however findings from the present study and those of Relf et al. (2014) and Tzelos et al. (2017) suggest reduced efficacy.

When moxidectin was introduced for equine nematode treatment the ERP was reported as 13 weeks which is used on the product datasheet. ERP was reported up to 24 weeks when moxidectin was first introduced (Jacobs et al., 1995; DiPietro et al., 1997; NOAH Compendium, 2013). Ivermectin ERP has been reported up to 10 weeks (Borgsteede et al., 1993) however DiPietro et al. (1997) reported ERP at only 6 weeks, suggesting some variability in the ERP of this drug.
The results of the present study suggest that the ERP for ivermectin was from 7 weeks post-treatment, less than the originally reported 8-10 weeks (Borgsteede et al., 1993) with some cases of egg reappearance at 5 weeks. Moxidectin ERP within high risk UK pleasure horses in the present study was 11-13 weeks compared to in excess of 13 weeks (DiPietro et al., 1997) and up to 24 weeks (Jacobs et al., 1995) when first marketed. Boersema et al. (1998) reported egg reappearance at 9 weeks after moxidectin administration in some individual animals, our present study highlighted egg reappearance for moxidectin was as low as 5 weeks in individual animals.

When looking at the Mann Kendall trend for reduced egg reappearance over the four year period, there was a significant reduction in the moxidectin ERP over time. Some caution should be taken in the interpretation of these findings as the number of tests per year varied. However in the case of moxidectin, as the number of tests increased the ERP decreased. This would suggest that the ERP was reducing over the time period, or was already reduced and was being detected as more efficacy tests were carried out. The ivermectin data over time also had few tests in 2008 compared to 2011, however as the number of horses tested increased the identification of shortened ERP increased again suggesting a reduction over time.

Ivermectin has been used extensively for treating equine nematodes for the past 20 years, yet resistance has been slow to develop when compared to the benzimidazoles. Kaplan (2004) suggested that the reason for ivermectin retaining high efficacy against cyathostomins is its lack of efficacy against the encysted mucosal stages of larvae, which are in refugia. Lyons et al. (2009) reported from critical efficacy tests that ivermectin was efficacious against L5 cyathostomins but failed to remove L4 larvae at six days post-treatment. One yearling that was necropsied 25 days post treatment harboured 19,150 adult parasites suggesting that the lack of L4 efficacy allowed these larvae to mature (Lyons et al., 2009).
The results of our present study indicate that ivermectin treatment was associated with earlier egg reappearance for strongyles than moxidectin; the reasons for this are likely to be multifactorial. One reason could be low efficacy against L4 larvae as reported by Lyons et al. (2009). Early egg reappearance could also be linked to overuse of ivermectin over the past three decades, increasing selection pressure from repeated use. Epidemiological studies indicate that ivermectin and moxidectin are the drugs most frequently used by horse owners in the UK (Biggin et al., 1999; Allison et al., 2011; Relf et al., 2011; Ireland et al., 2013)

The parasite control programmes of our study population had an emphasis on the use of macrocyclic lactone anthelmintics especially on premises where BZ resistance had previously been reported. These treatment trends are consistent with reported UK pleasure horse parasite control practices (Allison et al., 2011).

The animals in this study comprised a range of ages, 1-31 years mean 10±7.2 years. The pharmacokinetics of anthelmintics can differ in younger animals compared to older animals (Gonzalez Canga et al., 2009). As the age range in this study was so great the effect of age on pharmacokinetics may have influenced our findings in this study.

The AAEP guidelines (Nielsen et al., 2013a) for FECR test results state these can only be evaluated at premises level. However Coles et al. (2006) previously reported that the test could be used at animal level in horses, where necessary, to indicate anthelmintic efficacy. In this present study the animal level results were very similar to those at premises level and more reflective of a field situation where horses are often kept alone or in pairs. In these situations efficacy testing could not normally be conducted using the AAEP guidelines. There is a lack of standardisation for calculating egg reappearance, this limits the ability to compare many of the published reports. In the present study three different, previously validated methods were adopted (Borgsteede et al., 1993; Jacobs et al., 1995; Boersema et al., 1998; Mercier et al., 2001; von Samson-Himmelstjerna et al., 2007; Lyons et al., 2008; Molento et al., 2008; Larsen
et al., 2011; Relf et al., 2014). Interestingly in these results 99% of the FECR calculations and the pre-treatment and post treatment FEC percentage differences, both at animal and premises level, were in agreement with each other for detecting the ERP. It should be remembered that all the faecal egg count efficacy tests make the assumption that if few eggs are excreted, that this is due to high anthelmintic efficacy; it could be due to many other factors influencing faecal egg output for example pre-patent nematodes and faecal egg distribution within faeces (Vidyashankar et al., 2007).

The author recognises the limitations to this study. Data were analysed retrospectively, original sampling and dosing was carried out by horse owners, thus sampling and dosing may not have been accurate which could influence these findings. Data represent field cases throughout Great Britain, FECs were not planned experimentally and ERP may have occurred earlier than we report due to the timing of the scheduled FECs. As this study was not planned as an experiment this limits the ability to draw robust conclusions on some of these findings. There could be alternative reasons for early egg reappearance outside of reduced anthelmintic efficacy, e.g. incorrect weight estimation and dosing which would also influence these findings. There was also a possibility that the animals in this study had persistently positive FECs due to poor anthelmintic efficacy, rather than true early egg reappearance, however, true 14 day FECR data was not available.

At premises level sample numbers were small; where only one record per premises was available, results were analysed collectively, and therefore conclusions should be drawn with some caution. However it is common for horses to be kept in small groups and for practical identification of anthelmintic efficacy this dataset suggests that it is possible to gain an indication of egg reappearance times on premises with very few animals.
When looking at the changes in ERP over time the number of tests in 2008 and 2009 were few for both ivermectin and moxidectin and this is a limitation. However the data from a greater numbers of tests in 2010 and 2011 suggest a reduction in the ERP over time.

Data from this present study suggests that both the adapted % FECR method and the >10% difference between pre- and post-treatment FEC method are acceptable tools for evaluating the egg reappearance period. Furthermore, these two metrics used together may provide a more robust metric for defining reduced efficacy.

This sample population may not be representative of the whole UK horse population; horses were selected that had signs of early egg reappearance thus biasing the sample towards horses harbouring strongyles demonstrating possible reduced efficacy. However all of these horses were following a targeted worm control strategy based upon faecal egg counting and pasture management before anthelmintic intervention. Notwithstanding the limitations described above, these data do provide an insight into ivermectin and moxidectin efficacy in a field situation.

3.5. Conclusion

These results provide evidence for reduced strongyle ERPs following macrocyclic lactone treatment in pleasure horses in a field situation over a four year period. This work further supports the recent development of reduced efficacy of macrocyclic lactones in the UK.
Chapter 4.0

Ovicidal efficacy of fenbendazole after treatment of horses naturally infected with cyathostomins

4.1. Introduction

Benzimidazoles (BZ) are the only class of anthelmintics with ovicidal efficacy (Lacey, 1990). Recent reports of BZ resistance in cyathostomins in the UK have highlighted its increasing prevalence (Lester et al., 2013; Stratford et al., 2013). However reports by Garcia et al., (2013) in Texas USA and Milillo et al, (2009) in Italy highlighted that BZ susceptibility was present at horse premises within their respective studies. The marketing authorisation for fenbendazole states that this product is only suitable for use in animals with BZ-sensitive cyathostomins (NOAH Compendium, 2013), however efficacy testing is not common practice by horse managers in the field (Biggin et al., 1999; Allison et al., 2011; Relf et al., 2011).

Previous equine nematode work has focused upon adulticidal and larvicidal efficacy (Traversa et al., 2009b; Matthews, 2011, Stratford et al., 2011). Although rarely reported, ovicidal activity of BZs as measured by the egg hatch assay (EHA) (Le Jambre, 1976), was highlighted as a useful marker of anthelmintic resistance for research purposes. To date, most of the work investigating BZ ovicidal activity has been conducted on ruminant nematodes, particularly *Haemonchus contortus*. To the best of the author’s knowledge there are no published studies on BZ ovicidal efficacy on equine cyathostomins in the face of resistance.

In ruminant nematodes Leathwick et al. (2012) and Bartram et al. (2012) proposed a mechanism to slow down anthelmintic resistance by the use of combination anthelmintics. The rationale behind this being one product containing two different drugs with differing modes of action to attack nematodes. The combined drug would have good efficacy against nematodes and resistance to this combination approach would be slower to develop. For this to be successful both anthelmintics in the combination require good efficacy against nematodes from
the outset (Leathwick et al., 2012; Bartram et al., 2012). With mounting evidence for reduced efficacy of macrocyclic lactones, (Lester et al., 2013; Stratford et al., 2013; Relf et al., 2014; Daniels and Proudman, 2016; Tzelos et al., 2017), it is important to understand whether ovicidal efficacy, a unique element to the BZ class of anthelmintics, still performs in the face of resistance.

4.1.1. Objective

The objective of this study was to investigate the ovicidal efficacy of fenbendazole in horses under field conditions when given at 7.5 mg/kg BW as a single dose, or on five consecutive days.

4.2. Materials and Methods

4.2.1. Pilot Study

A pilot study (n=6) was carried out to inform sample size estimates, time points for sampling and method for strongyle egg collection. Horses with faecal egg counts ≥ 150 eggs per gram (epg) were dosed with either a single dose (n=3) or five day dose of fenbendazole (n=3) according to the manufacturer’s instructions. Results indicated that after dosing with a single dose of fenbendazole at 7.5mg/kg per os, hatch rates returned to baseline level within five days. After dosing with fenbendazole at 7.5mg/kg for five consecutive days within feed, hatch rates returned to baseline levels within 10 days of the last dose. The WAAVP Egg Hatch Assay protocol (Coles et al., 1992) was adapted and successfully used to collect and incubate eggs for hatch analysis. Pilot data gave a hatch rate standard deviation of 0.1414, a value that was used in subsequent sample size calculations.
4.2.2. **Sample size estimates**

Estimates were conducted in SiZ (Cytel v 1.0), using a Wilcoxon-Mann-Whitney model. Assuming a standard deviation of 5 % in egg hatch rate, a 5-fold difference in mean % egg hatch with 95% confidence and 80% power, could be detected with a sample size of 40 horses (20 in each group). A 2-fold difference could be detected under the same assumptions with a sample size of 100 horses (50 in each group). The model was very sensitive to standard deviation indicating the need for a pilot study to establish parameter values for modelling sample size. This led to the adoption of a sample size of 40 horses (20 in each group) which gave 80% power and 95% confidence to detect a 7-fold difference in egg hatch rate for this study.

4.2.3. **Ethical consent**

This study received ethical approval from the University of Liverpool Ethics Committee, RETH000363.

4.2.4 **Sample Population**

From 109 animals at four premises in South-West England and one in South Wales, 40 horses met the inclusion criteria of: faecal egg count (FEC) ≥150 eggs per gram faeces (epg) (Coles et al., 2006), no anthelmintics administered within the previous 13 weeks and in good health (Table 4.1). All farms harboured BZ resistant cyathostomins identified post hoc by faecal egg count reduction test. Horses were sampled between August and October 2012. The study population consisted of 20 females and 20 males, aged 0.5-20 years, mean 6 ± 4.7 years. Breeds comprised: 20 warmbloods, four Thoroughbreds, two warmblood cross Thoroughbreds, seven native ponies, five cobs and one Irish draft horse. Pre-treatment FEC for the population ranged
from 150 epg to 1300 epg with an arithmetic mean of 550 epg ± 328 epg and a median of 475 epg. A further 70 horses were screened but did not meet the inclusion criteria.

At all five premises horses were out at pasture during the day, three premises horses were maintained only at pasture and the other two premises the horses were stabled at night. Grazing was mixed with other horses on the premises, which were screened but did not meet our inclusion criteria. All horses were in fields where dung was not removed from the pasture.

Premises one, two and three had not used BZ anthelmintics for 10 years, premises four and five used BZ infrequently (Table 4.1). Horse exposure to BZ prior to arrival on these premises was unknown. None of the premises quarantined new animals on arrival. Time on the current premises varied, <1 month to >5 years. Premises were deemed high risk for intestinal parasite transmission due to a high proportion of young animals and frequent movement of horses on or off the premises (Nielsen et al., 2010a; Relf et al., 2011).

Body weight was estimated by weigh tape and rounded up to the nearest 50 kg for light/small breeds and 100 kg for heavy/large breeds to counter inaccuracy (Ellis and Hollands, 1998).

4.2.5 Treatment group allocation

At the time of treatment BZ susceptibility was unknown. Treatment was not randomized; approximately 50% of treatments at each premises were single doses and the remaining 50% received a five day dose (Table 4.1). Allocation of delivery route depended upon management, e.g. feasibility of offering medicated feed once daily.
4.2.6. FEC sample collection and analysis

Prior to treatment a single, fresh, spontaneously voided, faecal sample was collected from each animal for FEC analysis. Samples were refrigerated at 4°C prior to analysis. A modified McMaster method (Coles et al., 1992) was used, where each egg represented 25 epg.

4.2.7. Method

For each sample 3g of faeces were weighed out and added to 42 ml of tap water. The samples were agitated with a spatula and then sieved though a 0.250 mm aperture to remove debris. The liquid collected was then divided between two 17 ml test tubes and centrifuged for 10 minutes at 1800 x g. After centrifugation the supernatant was discarded, the pellet was vortexed and then re-suspended in saturated sodium chloride solution. Samples were homogenised and 1ml pipetted from the meniscus into a two-chamber McMaster slide. Nematode eggs were then counted under a microscope using the x10 objective. Strongyle eggs were recorded for each replicate of the sample, multiplied by 50 and then the mean epg per animal calculated, thus each egg observed represented 25 epg.

4.2.8. Faecal egg count reduction test

A second FEC was conducted 14 days post-treatment, as described above, to calculate FEC reduction (FECR). The mean pre-treatment and post treatment results, per premises, were used to calculate FECR. FECR was calculated as:

\[
\text{Pre-treatment FEC} - \text{Post treatment FEC} \times 90 \\
\text{Pre-treatment FEC}
\]
A cut-off value of 90% faecal egg count reduction was used to determine anthelmintic resistance as recommended by Coles et al. (1992) and Stratford et al. (2011) for BZ. From FECR results, strongyle populations per premises were categorized post hoc as “BZ susceptible” or “BZ resistant”. FECR results indicated that on no premises could the strongyle population (in all tested horses) be classified as BZ susceptible. Two groups were considered in the analysis of the results from this study: a single dose group (n=21) and a five day dose group (n=18).

4.2.9. Anthelmintic treatment and sample collection for egg hatch tests

Before treatment a fresh, spontaneously voided, faecal sample was collected from all animals. Samples were placed in air tight, screw top plastic beakers and filled to the top with water to keep the contents under anaerobic conditions. In the laboratory samples were stored at room temperature (Coles et al., 1992). All 21 horses in the single dose group were administered a dose of 18.75% w/w fenbendazole at 7.5 mg/kg on day 0 orally using the oral dosing device supplied, ensuring that the entire dose was swallowed. Further faecal samples were collected, and stored, for five consecutive days from the day after dosing with fenbendazole. All 18 of the horses receiving five consecutive days’ treatment were administered fenbendazole (10% w/v) at 7.5 mg/kg BW daily for five consecutive days (day -4 to 0), orally in feed following the manufacturer’s recommendations. Feed intake was monitored to ensure the full dose was ingested. Further faecal samples were collected, as previously described, and stored from one day after the last dose of fenbendazole and then on days 3, 5, 7 and 9 post-treatment for the five consecutive day dosing group. All samples were stored as described by Coles et al. (1992) and were used for egg hatch testing.
4.2.10. Egg hatch test

The egg hatch assay described by Matthews et al. (2012), was modified to an egg hatch test (EHT) that did not expose eggs to anthelmintics, simply tested the ability of excreted strongyle eggs to hatch *in vitro*.

Strongyle eggs were extracted by sieving each faecal sample mixed with tap water through a stack of sieves from 2 mm, 0.75 mm, 0.35 mm, 0.075 mm and 0.038 mm apertures which separated faecal matter from nematode eggs. The lining of the 38 micron sieve was then backwashed with tap water into a collecting jug and the contents divided equally between two soft polyalyme 15 ml centrifuge tubes.

Following centrifugation at 1800 x g for 2 minutes, forceps were used to pinch the polyalyme tubes above the pellet thus separating the pellet from the supernatant. The supernatant was discarded and the pellet re-suspended in 15 ml saturated sodium chloride solution. Re-suspended pellets were then centrifuged again for a further 2 minutes at 1800 x g.

After centrifugation, using forceps to pinch the tubes below the meniscus, content above this point was poured onto a 0.038 mm aperture sieve and eggs were washed with tap water to remove the salt solution. The eggs and a small amount of water were then backwashed from the sieve and transferred into beakers.

Twenty-four well cell culture plates, with lines scored on the underside to aid counting, were used for the incubation of the eggs. For each sample, duplicates of 3 ml of water containing approximately 100 eggs per ml were transferred by pipette into the plate wells and the plate was incubated for 48 hours at 26°C. This volume of egg suspension was used to ensure that a minimum of 100 eggs per well were incubated at each time point.
After incubation the plates were removed from the incubator and examined using an inverted microscope. For each well the number of hatched larvae and eggs were recorded. While it was possible to distinguish between viable and non-viable eggs all eggs were recorded as unhatched for the purposes of this study. Data were entered into a Microsoft® Excel spreadsheet (Microsoft Excel 2007) and the percentage egg hatch was automatically calculated. The mean percentage hatch was calculated from the two replicates for each animal at each time point. The mean number of eggs and larvae per animal per time point were also calculated. From faecal cultures no large strongyles were identified, therefore all strongyles were classified as cyathostomins.

4.2.11. Statistical analysis

Differences pre and post treatment within each group were analysed using the Friedman non-parametric ANOVA with post hoc testing and Bonferroni correction applied. All tests were carried out in SPSS (IBM v 20).

4.3. Results

One mare on premises two was sold just as the trial started therefore the results represent 39 animals instead of 40. All premises demonstrated BZ-resistant parasites (Table 4.1.). On three premises 11 animals had individual FECR values of 91.6-100% after a five consecutive day dose of fenbendazole. At day 14 post-treatment five of the horses from the 39 (8%) had a FEC greater than before treatment and a further two had the same FEC as before treatment indicating a 0% FEC reduction. Mean FEC at 14 days post-treatment for all 39 horses was 250 ± 270 epg with 34 of the 39 horses recording a positive result (≥25 epg).
<table>
<thead>
<tr>
<th>Premises</th>
<th>Type</th>
<th>Total Animals on Premises</th>
<th>Number Sampled (%)</th>
<th>Number of animals given a single dose of FBZ</th>
<th>Median Age years (range)</th>
<th>Treatment History</th>
<th>FECs in use</th>
<th>FECR % Mean of treated animals</th>
<th>FECR % Median of treated animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stud</td>
<td>10</td>
<td>6 (60)</td>
<td>3</td>
<td>6.5 (0.5-15)</td>
<td>Blanket chemical – No BZ</td>
<td>No</td>
<td>-27</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>Dealer</td>
<td>21</td>
<td>8 (38)</td>
<td>4</td>
<td>7 (5-20)</td>
<td>Blanket chemical – No BZ</td>
<td>Random</td>
<td>61</td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>Stud</td>
<td>4</td>
<td>4 (100)</td>
<td>2</td>
<td>8 (4-15)</td>
<td>Blanket chemical – No BZ</td>
<td>No</td>
<td>54</td>
<td>64</td>
</tr>
<tr>
<td>4</td>
<td>Stud</td>
<td>26</td>
<td>4 (15)</td>
<td>2</td>
<td>1 (0.5-7)</td>
<td>Blanket chemical - BZ</td>
<td>No</td>
<td>35</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>Stud</td>
<td>48</td>
<td>17 (35)</td>
<td>10</td>
<td>5 (1-14)</td>
<td>Targeted from FEC - BZ</td>
<td>Yes</td>
<td>38</td>
<td>83</td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>109</td>
<td>39*(36)</td>
<td>10</td>
<td>5 (1-14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Only 39 out of the 109 horses had FEC ≥150 epg
Median egg hatch rate after treatment is illustrated for both treatment groups in Figure 4.1. In the single dose group there was a significant difference between the pre- and post-treatment egg hatch rates ($P=0.0001$). There was a significant reduction in the number of eggs and larvae post treatment ($P=0.001$), at day five total eggs and larvae remained significantly lower than at day zero ($P=0.003$) (Fig. 4.2A).

Five consecutive daily doses of fenbendazole led to significant differences between pre- and post-treatment egg hatch rate ($P=0.001$). Significant differences between sampling points are indicated in Fig. 4.1B. There was a significant reduction in eggs and larvae immediately post dosing ($P=0.0001$) and levels of eggs and larvae remained significantly lower than pre-treatment at day nine ($P=0.004$) (Fig. 4.2B).
Figure 4.1. X Box and whisker plot illustrating the distribution of egg hatch rates, following (A) single dose fenbendazole in animals harbouring resistant cyathostomins; and following (B) treatment for 5 consecutive days in horses with BZ resistant cyathostomins. Population values for egg hatch rate that are significantly different from the previous sampling point are labelled “#”. For both the single dose and five day dose groups’ egg hatch was significantly reduced for three days after treatment, before returning to baseline levels.
Figure 4.2. Box and whisker plot illustrating distribution of eggs and larvae pre and post treatment with a (A) single dose of fenbendazole and (B) five consecutive daily doses of fenbendazole. Significant differences compared to the previous sampling point are labelled “#”. Numbers of eggs and larvae at day five and nine days post-treatment remain significantly lower than at day zero following single dose and five consecutive days dosing respectively.
4.4. Discussion

Data from this present study show a significant reduction in egg hatch rate for three days following a single dose or five consecutive day dose of fenbendazole in horses with resistant cyathostomins. While all premises were classified as resistant based on FECR tests, there was nonetheless a significant reduction in the number of eggs and larvae present in samples post treatment.

There is limited published data on the ovicidal effect of BZ on susceptible cyathostomins, making it difficult to compare findings from this present study to previous work. Miller and Morrison (1992) reported fenbendazole treatment resulted in little or no development of strongyle eggs in cattle faeces from 12 to 72 hours after treatment. In comparison, in the present study at 72 hours post treatment egg hatch significantly increased, returning to baseline hatch rate. This could be due to species variation or due to the influence of the resistant proportion of cyathostomins within this population. It should be assumed that most BZ-susceptible cyathostomins were removed by the fenbendazole treatment, thus skewing the population to the resistant proportion of cyathostomins which continued to hatch post treatment. It is important to consider that horses commonly harbour multiple species of cyathostomins. Previous work has identified that the efficacy of benzimidazoles and susceptibility to anthelmintic resistance can differ between cyathostomin species (Traversa et al., 2009b).

Hotson et al. (1970) reported a 70% FECR in small ruminants demonstrating BZ resistance, however the BZ treatment showed little effect on the adult parasite burden in a post slaughter worm count. Compared to untreated controls the female worms had fewer eggs present. The authors suggested that fenbendazole treatment temporarily suppressed egg laying but allowed the parasites to survive (Hotson et al., 1970). This suggests anthelmintics have the ability to temporarily damage adult parasites and parasite eggs in resistant nematodes. To the author’s
knowledge there have been no such reports from equine cyathostomins of the viability of the eggs shed post-treatment with BZ (Chandler et al., 2000; Chandler and Love, 2002; Rossano et al., 2010). When considering the numbers of eggs and larvae post treatment from this present study it is important to remember that there is no direct linear correlation between the numbers of nematode eggs excreted and the number of intestinal worms present within horses (Nielsen et al., 2010). However results from this present study do indicate the presence of egg-laying cyathostomins within these horses post treatment.

In the present study the population of horses comprised animals with a range of ages. The pharmacokinetics of anthelmintics can differ in younger animals in comparison to older animals (Gonzalez Canga et al., 2009). Older animals often have lower FEC results due to immunological defence from previous cyathostomin exposure (Klei and Chapman, 1999), yet some animals never develop a sufficient immune response. It has previously been suggested that resistance is more prevalent in immunologically immature animals (Klei and Chapman, 1999; Chapman et al., 2003; Blanek et al., 2006) of which there were many within this study population. Premises where Garcia et al., (2013) reported 100% FECR to BZ was made up of a population of 11 horses with a mean age of 18 years that therefore would have developed some immunity to cyathostomin infections. The age range and mean age of the animals that did not meet the inclusion criteria for the present study were similar to that of rest of the study population. As these animals also resided on the same five premises, this would suggest that age alone did not influence FEC. Therefore the grazing environment, also known to influence parasite burden (Osterman Lind et al., 1999), may have played a significant role.

Both the single dose and five day doses of fenbendazole were administered according to the manufacturer’s instructions. There is evidence in other species, dogs and small ruminants, that fenbendazole administration with food significantly increases bioavailability compared to an oral bolus (McKellar et al., 2008). McKellar et al. (2002) suggested that in horses
administering fenbendazole by oral bolus directly followed by food appeared to reduce bioavailability, however these conclusions were drawn from a very small sample. Differences in the administration routes should be considered when directly comparing these groups.

Use of any anthelmintic product should be based on local, regional and farm-level, epidemiological information about susceptibility of nematodes (NOAH Compendium, 2013). In the present study the mean FEC at 14 days post-treatment was $250 \pm 270$ epg with 34 of the 39 horses recording a positive result ($\geq 25$ epg). Lester et al. (2013) reported FECR for BZ at day 14 where the FEC was higher than at day 0 (pre-treatment). This was also a finding here: at day 14 post-treatment, of 39 horses with BZ-resistant strongyles five (8%) had a FEC greater than before treatment and a further two had the same FEC as before treatment indicating a 0% reduction.

Resistance in equine cyathostomins appears highly prevalent within the UK horse population (Relf et al., 2011), indicating the need to understand how anthelmintics perform in the face of resistance. Treatment histories for premises in this study (Table 4.1) highlighted reliance on anthelmintics without diagnostic testing. Similar parasite control strategies were reported within commercial, high risk premises by Relf et al. (2011). Previous work has highlighted that BZs are less commonly used by today’s horse managers and have been in decline over the past 10 years (Biggin et al., 1999; Allison et al., 2011). Yet even with reduced exposure to BZs, resistance in cyathostomins appears to be highly prevalent within the UK horse population (Biggin et al., 1999; Allison et al., 2011; Relf et al., 2011; Wood et al., 2013). Greater education is required in the sector encouraging diagnostic testing and targeting of appropriate anthelminthic treatments (Nielsen et al., 2010a).

The FECRT used in this study to determine anthelmintic efficacy, was originally developed for small ruminants. The FECRT it is seen as the gold standard field test for equine nematodes (Coles et al., 2006; Matthews, 2011). The sensitivity of this test in the horse has previously
been criticised (Stratford et al., 2011; Matthews et al., 2012; Geurden et al., 2013). It has been suggested in small ruminants that 25% of the parasite population requires the resistant mutation before resistance is detected using this method (Martin et al., 1989), Stratford et al. (2011) suggested this can be extrapolated to equine nematodes. Within the literature there is a lot of variation in the interpretation of the requirement of the pre-treatment FEC for evaluating anthelmintic efficacy using FECRT. This present study followed the recommendation of Coles et al. (1992) WAAVP guidelines and Coles et al. (2006) of ≥150 epg. Other work suggests the minimum inclusion for efficacy testing to be ≥50 epg (Milillo et al., 2009; Traversa et al., 2009a; Stratford et al., 2014) or ≥100 epg (Canever et al., 2013). This differs again somewhat to current cut off recommendations of 200-500 epg for anthelmintic treatment of animals within the field (Uhlinger, 1993; Matthews, 2008). In the present study the WAAVP (1992) guidelines were chosen as they are the current, globally recognised gold standard for efficacy testing. This does however make it difficult when comparing results to other published work. Within this study both the median hatch rate and the arithmetic mean were selected as metrics to evaluate the data. Faecal egg count data rarely follows a normal distribution therefore the median is the most appropriate measure of central tendency (Denwood et al., 2010; Nielsen et al., 2010a).

The author acknowledges a number of limitations to this study which limit the extrapolation of findings. The sample size estimates indicated the need for a study of 20 horses per treatment group in order to detect a 7-fold difference in egg hatch rate with 95% confidence. The study comprised only 39 horses, allocated to two treatment groups, and was therefore underpowered to detect differences of this magnitude or smaller.

The study population may not be representative of the UK horse population as a whole, but represents a category of high risk horses within this population where sustainable anthelmintic treatment should be targeted. A study design issue here was the low prevalence of premises with cyathostomin populations that were demonstrably BZ-susceptible making comparisons
difficult. Notwithstanding these limitations, this study adds valuable information to our understanding of the ovicidal effect of fenbendazole in UK horses under field conditions.

4.5. Conclusion

These results suggest that the ovicidal effect of fenbendazole appears to be present but short lived in benzimidazole-resistant cyathostomins when dosed as a single oral dose, or for five consecutive days administered in feed. Numbers of eggs and larvae present immediately post treatment were significantly lower than before treatment. Hatch viability in post treatment eggs appeared to return to pre-treatment values within three days of a single dose or five consecutive day’s treatment at 7.5 mg/kg BW.
Chapter 5.0

The effect of moxidectin administration on in vitro equine hindgut fermentation kinetics of hay and oats.

5.1. Introduction

Microbiota within the horse’s hindgut play a complex role in digestion and metabolism (Proudman et al., 2015). Within the caecum and large colon resides a microbial population uniquely adapted to ferment dietary plant fibre (Shirazi-Beechey, 2008). Under normal conditions the hindgut ecosystem remains in balance producing short chain fatty acids (SCFAs), carbon dioxide and hydrogen (Shirazi-Beechey, 2008; Santos et al., 2011). There is very little lactate present under normal conditions and the hindgut pH remains ≥7 (Shirazi-Beechey, 2008; Daly et al., 2012).

Rapid changes in the hindgut microbiome, often brought about by management changes, have been associated with the onset of colic (Al Jassim and Andrews, 2009). Epidemiological studies have identified dietary changes, involving both forage and concentrates, to be the greatest management-associated risk factors for colic (Cohen, 1999; Hillyer et al., 2002).

Another key risk factor for colic is intestinal parasite burden. Both Uhlinger (1990) and Cohen et al. (1999) report that structured parasite control programmes, which included anthelmintic dosing, had a protective effect against colic. However anthelmintic treatment has also been identified as a risk factor for the onset of colic (Cohen et al., 1999; Hillyer et al., 2002). Both Cohen et al. (1999) and Hillyer et al. (2002) identified an increased risk of colic within seven days of anthelmintic treatment. Specifically the administration of the macrocyclic lactone anthelmintics ivermectin and moxidectin were associated with colic onset (Hillyer et al., 2002).
Little is known about the effect of anthelmintic treatment on the horse’s hindgut microbiome. To date studies in humans, goats and sheep using 16S rRNA sequencing have provided conflicting findings on the effect of anthelmintic on the gut microbiota (Cooper et al., 2013; Bartley et al., 2015; Li et al., 2016). A reduction in cellulolytic bacteria and an increase in Lactobacilli and Streptococci have previously been reported by Jacotot et al. (2004). Using bacterial counts after anthelmintic treatment these authors observed greater changes following moxidectin when compared to ivermectin or fenbendazole.

Fermentation patterns can serve as an indicator of microbial activity and digestibility of substrates. SCFA output and pH are readily measurable in vitro using the method described by Theodorou et al., (1994). Analysis of fermentation kinetics using this method has not been used previously to study the effect of anthelmintics on the hindgut microbiome.

5.1.1. Objective

The objective of this study was to describe the in vitro fermentation kinetics of hay and oats using samples from horses treated with moxidectin compared to untreated controls.

5.2. Method and Materials

5.2.1. Study population

From a group of 17 polo ponies managed exclusively on pasture, six were randomly selected using the sample function in R (i386 3.1.2). The horses ranged from 6-16 years (mean 11 ±3 years), comprised four mares and two geldings. Breeds represented were Argentinian polo pony cross Thoroughbred (n=5) and Argentinian polo pony cross quarter horse (n=1). Horses were not exercised and being kept in fields, no concentrate feeds were given and their diet was supplemented with meadow haylage ad libitum.
The healthcare plan for these horses for the previous three years included an interval dose worm control programme comprised of moxidectin three times per year. Two of the horses were imported from Argentina in spring 2015 with unknown anthelmintic history. In September 2015, once on pasture turnout, screening FECs were conducted on all horses using a modification of the McMaster method (detection sensitivity to 25 eggs per gram) as described in chapter 4. All horses underwent further FECs at the end of January and the end of February 2016, all FEC tests reported no eggs seen. Based upon these FEC results, animal age, pasture management information, lack of access to pasture during the polo season and past anthelmintic history the parasite status of this group of horses was categorised as low (Nielsen et al., 2013a).

5.2.2. Ethical approval

This study was given favourable ethical opinion by the University of Surrey Animal Welfare and Ethical Review Board NASPA-2015-008-SVM.

5.2.3. Treatment group allocation

From the six horses previously selected, treatment and control animals were randomly assigned using the sample function in R (i386 3.1.2). Three animals were dosed with moxidectin and three remained untreated controls.

5.2.4. Anthelmintic treatment

Moxidectin is the most recent anthelmintic licensed for equine nematode treatment, and the most commonly used by horse managers (Allison et al., 2011), therefore it was selected for use in this study. Prior to dosing, each horse in the treatment group had its weight estimated using a commercial weigh tape. Due to known inaccuracies of this method (Ellis and Hollands, 1998), treatment weights were rounded up ≥50 kg. Moxidectin (18.92mg/g oral paste) was delivered
orally using the dosing device provided as per the manufacturer’s instructions at a dose of 0.4 mg/kg body weight.

5.2.5. Sampling method

Sample collection took place in March 2016. Using previously published pharmacokinetics and faecal excretion times for moxidectin (Gokbulet et al., 2001; Perez et al., 2001) sampling time points were selected as: prior to treatment (time zero), 16 hours after anthelmintic treatment, 40 hours after anthelmintic treatment and 160 hours after anthelmintic treatment. Samples were collected in the morning on each of the sampling days. On day 0 prior to anthelmintic treatment a fresh, spontaneously voided, faecal sample was collected from each animal. For each horse a thermos flask was pre warmed by filling with boiling water, sealed and transported to the farm in an insulated polystyrene container containing two hot water bottles. As each animal defecated the water in the thermos flask was discarded and the flask filled with faeces to the top, sealed and placed back into the insulated container. Samples were then transported back to the laboratory, within two hours of collection. This sampling process was repeated at each of the faecal sampling time points.

On the afternoon of day zero at approximately 4pm the treatment group were dosed with moxidectin per os, as described above.

5.2.6. Experimental methods

5.2.6.1. Sample storage

On returning to the laboratory the thermos flasks were placed into a pre-warmed incubator at 39°C.
5.2.6.2. In vitro model feed preparation

Prior to sample collection two horse feeds, meadow hay and rolled oats were oven dried separately at 60°C for 24 hours and left to cool. Samples were then milled to 2 mm using a Fritsch Pulversette 19 universal cutting mill. One gram of feed was weighed out and placed into pre-labelled 125ml serum bottles. Each diet treatment, hay and oats, was run in triplicate for each horse. There were also two blank bottles, containing no substrate, per diet per horse, thus for each horse at each time point there were 10 bottles (Figure 5.1.). In total 240 bottles were used in this experiment.

![Figure 5.1. Schematic representation of donor horses and the number of bottles with differing substrates that was set up at each of the four sampling time points.](image)

5.2.6.3. Preparation of culture medium

Modified Van Soest culture medium as described by Theodorou et al. (1994) was prepared in a 6 litre flask at room temperature, by dissolving 0.2 g of trypticase peptone, 0.2 ml micro-
mineral solution, 400 ml buffer, 400 ml macro-mineral solution and 2 ml resazurin solution in 1 litre of distilled water. The flask contained a magnetic stirrer on a magnetic plate were used to ensure the each ingredient was fully dissolved, prior to the addition of the following ingredients (in the order presented above). CO₂ was continually passed through the solution until the medium turned pink, indicating the medium was anaerobic. The composition of the buffer, micro-mineral and macro-mineral solutions are detailed in Table 5.1. These solutions were stored in the refrigerator at 4⁰ C until required.

**Table 5.1.** Chemical compositions of the solutions used in the culture medium.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Chemical Compound</th>
<th>Formula</th>
<th>g/1.4 litres distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>Ammonium hydrogen carbonate</td>
<td>NH₄HCO₃</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Sodium hydrogen carbonate</td>
<td>NaHCO₃</td>
<td>49</td>
</tr>
<tr>
<td>Macro-mineral</td>
<td>Di-sodium hydrogen</td>
<td>Na₂HPO₄·12H₂O</td>
<td>13.23</td>
</tr>
<tr>
<td></td>
<td>orthophosphate dodeca-hydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Potassium di-hydrogen orthophosphate</td>
<td>KH₂PO₄</td>
<td>8.68</td>
</tr>
<tr>
<td></td>
<td>Magnesium sulphate 7-hydrate</td>
<td>MgSO₄·7H₂O</td>
<td>0.84</td>
</tr>
<tr>
<td>Micro-mineral</td>
<td>Calcium chloride 2-hydrate</td>
<td>CaCl₂·2H₂O</td>
<td>13.2g/100 ml</td>
</tr>
<tr>
<td></td>
<td>Manganese chloride 6-hydrate</td>
<td>MnCl₂·6H₂O</td>
<td>10g/100 ml</td>
</tr>
<tr>
<td></td>
<td>Cobalt chloride 6-hydrate</td>
<td>CoCl₂·6H₂O</td>
<td>1g/100 ml</td>
</tr>
<tr>
<td></td>
<td>Ferric chloride 6-hydrate</td>
<td>FeCl₃·6H₂O</td>
<td>8g/100 ml</td>
</tr>
</tbody>
</table>
5.2.6.4. Preparation of faecal inoculum

From individual thermos flasks 75g of faeces were taken from the centre of the sample and placed into a food blender with 375 ml of Van Soest medium. The blender was turned on for 40 seconds during which time the mixture was flushed with CO₂. The mixture was passed through a muslin bag in a funnel into a beaker to collect the liquid. This procedure was repeated for each horse at each sampling time point.

5.2.6.5. Preparation of culture bottles

Each culture bottle was flushed with CO₂ for approximately 4 seconds before adding 70 ml of culture medium from an automatic dispenser, 4 ml of reducing agent (2.5g cysteine HCl, 16 ml 1M NaOH, 2.5g sodium sulphate, 380 ml distilled water) was added to each bottle via a bottle top dispenser and 10 ml of faecal inoculum was added to each bottle using a disposable 10 ml syringe. Rubber stoppers were pushed firmly into the neck of each bottle and sealed with aluminium crimp seals. Bottles were then placed into a pre-heated incubator set at 39⁰C (Lowman et al., 1999). The sample preparation was followed for the blank bottles, these contained no feed substrate.

Bottles were adjusted to ambient pressure (zero reading on the pressure transducer display) using the transducer and a hypodermic needle. The start time, at ambient pressure, was recorded to inform gas reading times.
5.2.6.6. Gas accumulation measurements

Readings were taken using the manual pressure transducer technique of Theodorou et al. (1994). A three-way valve was attached to a pressure transducer containing an LED digital read-out meter. A syringe and hypodermic needle was set to one arm of the valve with the other set to allow gas from the bottle to flow through to the transducer. The needle was inserted through the rubber seal and the pressure reading recorded, Figure 5.2.

![Image of gas accumulation measurement setup](image)

**Figure 5.2.** *In vitro* gas production set up for gas readings.

The syringe plunger was then withdrawn until the LED read zero, whereupon the needle was removed from the bottle and the volume of gas withdrawn was recorded. The gas was discarded and the process repeated for each bottle. Culture bottles were only removed from the incubator for a short period of time for gas readings, during this time they were placed in a water bath to
ensure they remained at 39°C. The transducer pressure was recorded in pounds per square inch (psi). Readings were taken at approximately 4 hourly intervals up to 113 hours post inoculation. Once the bottles stopped producing gas the experiment was stopped.

5.2.6.7. After fermentation ceased

After completion of the last gas reading the rubber stoppers and crimps were removed from the bottles. The contents of each bottle was filtered using pre-weighed Whatman No.1 filter paper in a funnel, bottles were back washed with 20 ml of distilled water to collect remaining substrate. The pH of the faecal inoculum was recorded using a calibrated pH probe (Orion Star A211). Microfuge tubes, in duplicate, of the inoculum from the culture bottles were collected and frozen at -80°C for metabolite analysis at a later date.

5.2.6.8. Dry matter loss

Once the inoculum had passed through the pre weighed filter paper the papers were oven dried at 60⁰C. The filter papers were weighed and the weight of the residue, subtracted from the filter paper weight. This was recorded and used to calculate substrate dry matter loss using the equation below.

\[
DM \text{ loss (}) = \frac{DM \text{ into bottle} - DM \text{ residue in bottle}}{DM \text{ into bottle}} \times 100
\]
5.2.7. Data analysis

5.2.7.1. Cumulative gas production

Data were inputted into a Microsoft® Excel spreadsheet (Microsoft Excel 2010) to process the pressure and volume readings for each bottle. For each bottle gas volume readings were corrected against pressure readings, bias correction, using linear regression (Theodorou et al., 1994). Corrected volumes were then adjusted according to the readings from the blank bottles (those containing no food substrate) to remove background fermentation from the faecal inoculum itself. Values were then summed to provide a cumulative gas volume of each bottle.

The maximum likelihood program (MLP; Ross, 1987) was used to fit curves to the cumulative gas profiles using the France et al. (1993) model detailed below.

Where:

\( Y \) = cumulative gas pool in ml

\( t \) = time in hours

\( A \) = asymptote value for gas pool size (ml)

\( B = A \ e^{bT + c\sqrt{t}} \)

\( Z = e^{-c} \)

\( Q = e^{-b} \)

\( L_T = \text{lag time in hours} \)

\( b = \text{rate constant (h}^{-1}) \)

\( c = \text{rate constant (h}^{-0.5}) \)
Equation 1

\[ Y = A - BQ^{Z/L}T \]

Equation 2

\[ Y = A - BQ^{t} \]

When the initial rate of gas production was quadratic, i.e. the rate constant \( c \) was negative, equation 1 was used. If an exponential rate of gas production was noted i.e. when \( c=0 \), equation 2 was used.

The fitted France et al. (1993) parameters of \( L_T \), \( A \), \( B \), \( Q \) and \( Z \), the time to reach 50\% of gas produced \( (t_{50}) \), time to 95\% gas produced \( (t_{95}) \), fractional rate of gas production (FRGP) half way through fermentation were analysed for hay and oats separately, comparing the treatment and control groups.

For each inoculation time point \( (0, 16, 40 \text{ and } 160) \) comparable gas reading times within a 2 hour window were compiled in the database for repeated measures analysis. These time points were: 0, 10, 20, 30, 44, 60 and 70 hours post inoculation of the bottles.

5.2.7.2. Statistical analysis

Data demonstrated a normal distribution. For analysis of cumulative gas production for both treatment and control groups over time a repeated measures ANOVA was used. The feed treatments hay and oats were considered separately. The same approach was taken for the analysis of dry matter loss of substrate (%) and faecal inoculum pH and parameters derived from the France et al. (1993) model. Post hoc testing was conducted using Fishers least significant difference (LSD) test. All statistical analysis was conducted in GenStat 18\textsuperscript{th} ed.
5.3. Results

5.3.1. Pre-treatment gas production

At sampling point zero, for both diets the treatment and control groups demonstrated no significant differences in cumulative gas production pre-treatment with moxidectin (Figure 5.3.).

Figure 5.3. Modelled gas production prior to moxidectin treatment fermenting (a) hay and (b) oats in vitro, error bars represent 95% confidence limits.
At pre-treatment there were no significant differences in any of the fermentation kinetic parameters calculated in the France et al. (1993) model. There were no differences in dry matter loss or inoculum pH for either treatment group for both substrates. The fermentation model produced very similar results for hourly gas production for each treatment group for both of the feeds (Figure 5.4.). The France et al. (1993) model was a good fit to the cumulative gas produced, Table 5.2.

However the pre-treatment bottles only produced gas for 48 hours post inoculation, this was a minimum of 22 hours less than the other time points suggesting less than optimum in vitro fermentation. As these fermentations ran for a much shorter time than bottles inoculated at 16 hour, 40 hours and 160 hours post moxidectin the data were analysed separately using a T-Test and not included in the repeated measures ANOVA for cumulative gas production, dry matter loss and inoculum pH.
Figure 5.4. Mean gas produced per hour (ml) prior to moxidectin treatment for both hay and oat substrates for both the treatment and control groups. Hay fermentation for both treatment groups was less variable, demonstrated by the 95% confidence interval error bars, than oat fermentation. For hay and oats substrates neither treatment group was different from the other for gas produced per hour prior to moxidectin administration.

5.3.2. Post Treatment

Cumulative fermentation kinetics identified a significant reduction in gas produced 16 hours after moxidectin administration ($P=0.001$) for both hay and oat feed treatments. There was no significant difference in cumulative gas production at any other time points for either of the feed substrates, Figure 5.5.
Figure 5.5. Panel plot for mean modelled gas production curves for hay (plots a, b, c) and oats (plots d, e, and f). Plots a and d are mean gas production from bottles inoculated with faeces collected 16 hours post moxidectin treatment, plots b and e bottles inoculated from faeces collected 40 hours after treatment and plots c and f bottles inoculated from faeces collected 160 hours post moxidectin treatment. Error bars demonstrate 95% confidence intervals. Only plots A and D demonstrate significant differences between treatment and control groups ($P=0.001$).

The hourly rate of gas production post moxidectin treatment is illustrated in Figure 5.6. These graphs demonstrate that in fermentations inoculated with faeces collected 16 hours after moxidectin administration gas produced per hour is reduced compared to untreated controls. Fermentations inoculated with faeces collected at 40 and 160 hours after moxidectin administration there was no difference in gas production (Figure 5.6. B & C).
Figure 5.6. Panel plot of gas produced per hour for both feed substrates and both treatment groups at 16 (A), 40 (B) and 160 (C) hours after moxidectin administration. Error bars demonstrate 95% confidence intervals. Plot A treatment and control group differ in hourly gas production rate for both feed substrates up to 60 hours. Plot C the treatment groups differ in gas produced for the hay substrate up to 40 hours.
5.3.3. Dry matter loss

The percentage dry matter loss of substrate was calculated for each fermentation experiment after moxidectin administration for both treatment groups. There was a significant difference in dry matter loss of hay and interaction with time between treatment groups ($P=0.014$). Post hoc testing indicated this was in fermentations inoculated from faeces collected 40 hours after moxidectin administration, Figure 5.7. There was no difference between the treatment groups and time points for the oats substrate ($P<0.05$).

![Figure 5.7](image)

**Figure 5.7.** Dry matter loss (%) of hay substrate over the three sampling time points after moxidectin administration, between the two treatment groups. There was a significant interaction between time and treatment ($P=0.014$). Error bars represent 95% confidence intervals, significance is marked with a *.

5.3.4. Inoculum pH

Post moxidectin administration the pH of the faecal inoculum varied in both treatment groups over the time points, both treatment groups followed the same pattern of variance. Due to inter-horse variability the time points for the moxidectin treatment group were compared to each other post treatment and the same approach taken for the control group. There was a significant
increase in pH of faecal inoculum that fermented hay one week after moxidectin administration in the treated group ($P=0.002$). There was no significant difference in the pH of the faecal inoculum that fermented hay in the control group, Figure 5.8. For the oats substrate the pH of the moxidectin treated group was significantly lower the day after moxidectin administration ($P=0.005$) (Figure 5.9. A). The pH of the faecal inoculum for the control group was not significantly different over the time points.

**Figure 5.8.** pH of faecal inoculum after hay fermentation post moxidectin administration (A) the treated group and (B) the untreated controls. The moxidectin group pH significantly differed between time points ($P=0.002$) however the control group pH did not differ between time points ($P>0.05$). Error bars represent 95% confidence intervals, significance points are indicated by *. 


Figure 5.9. pH of faecal inoculum after oat fermentation post moxidectin administration for (A) the treated group ($P=0.005$) and (B) the untreated controls. The control group was not significantly different between the sampling points. Error bars represent 95% confidence intervals, significance points are indicated by *.

5.3.5. Model fit

To ensure that the France et al. (1993) model was a good fit to the cumulative gas production data, $R^2$ values were calculated during the modelling calculations. These values indicated the model was a good fit to the cumulative data over the consecutive fermentation experiments. The $R^2$ values are presented in table 5.2.
Table 5.2. $R^2$ values for cumulative gas production and the France et al. (1993) modelled data, indicating the model was a good fit to the data.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre Treatment</th>
<th>16 Hours Post Treatment</th>
<th>40 Hours Post Treatment</th>
<th>160 Hours Post Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oats Mox</td>
<td>0.993</td>
<td>0.998</td>
<td>0.996</td>
<td>0.996</td>
</tr>
<tr>
<td>Oats</td>
<td>0.991</td>
<td>0.997</td>
<td>0.996</td>
<td>0.989</td>
</tr>
<tr>
<td>Hay Mox</td>
<td>0.993</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hay</td>
<td>0.998</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

5.3.6. Hay fermentation kinetics

From the France et al. (1993) model at 16 hours after moxidectin administration there was a significant interaction in the fractional rate of gas production between the two treatment groups for hay substrate half way through incubation, this was also observed at 160 hours following moxidectin administration ($P=0.001$) Figure 5.10.

![Figure 5.10](image.png)

**Figure 5.10.** Fractional rate of gas production (FRGP) halfway through fermentation of hay for faecal inoculum exposed to moxidectin and untreated controls at 16, 40 160 hours post moxidectin administration. Interactions are identified by * identified by LSD. The error bars represent 95% confidence intervals.
5.3.7. Oat fermentation parameters

The models using a diet of oats demonstrated a significant interaction between the treatment and control groups for the extent of substrate degradation \((P=0.008)\) 16 hours after moxidectin administration, Figure 5.11. There were no differences in any of the parameters at 40 and 160 hours after moxidectin administration for the oats substrate.

**Figure 5.11.** Degradation rate of oats following moxidectin administration, time interaction at 16 hours \((P=0.008)\), error bars represent 95% confidence interval.
5.4. Discussion

Prior to dosing with moxidectin there was no difference between the fermentation profiles, dry matter loss (%) of substrate or pH of the faecal inoculum for either of the treatment groups. However the morning following moxidectin administration fermentation of hay and oats in our \textit{in vitro} model were significantly reduced in fermentations inoculated with faeces from treated animals. When looking at the repeated measurements of cumulative gas production there was no significant difference between the treatment and control groups from two days after moxidectin administration. However over the week after treatment and there were some differences between the treatment groups when looking at the fractional rate of gas production, rate and amount of substrate degradation.

Dry matter loss of oat substrate did not differ between the groups and time points after moxidectin administration but did significantly alter for hay substrate. The two treatment groups followed the same pattern initially for dry matter loss of hay substrate but two days after moxidectin administration the control group used significantly more substrate than the treated group. By one week after moxidectin administration the treatment groups were not significantly different in hay substrate loss and the moxidectin treated group were using more substrate than they had in both of the previous post treatment time points.

The pH of the faecal inoculum was measured at the end of fermentation for both substrates. Due to inter-horse microbiome variability (Willing \textit{et al.}, 2009; Blackmore \textit{et al.}, 2013) pH changes within groups were analysed over the sampling time points. For both hay and oats substrates the control group pH did not significantly differ over the sampling points. The pH values for the moxidectin treated group followed the same pattern over time as the control group. However with hay fermentation pH significantly increased a week after treatment.
compared to the first two days after treatment. With the oats substrate the pH of the faecal inoculum was significantly lower the morning after moxidectin administration. Jacotot et al. (2004) found moxidectin treatment significantly lowered the pH and altered the bacterial composition of the caecum, increasing Streptococci spp. and Lactobacilli spp. and significantly reduced cellulolytic bacteria for five hours after anthelmintic administration. In the present study the pH of the inoculum from fermenting oats was lower than that of hay around the time of moxidectin administration. This would be expected as starch fermentation would lead to a lower pH than forage (Julliand et al., 2001; Medina et al., 2002), thus the substrate would influence the inoculum pH. It should be noted here that while the pH of the faecal inoculum was significantly lower around the time of moxidectin administration it remained within the normal range for the horse hindgut (Daly et al., 2012). Biddle et al. (2013) reported that the pH of faecal inoculum in culture bottles decreased to a pH ≤ 6 within 12 hours of fermentation when exposed to starch and lactate, but by 50 hours had increased to pH ≥7 in some animals. These findings are a further illustration of the variable nature of hindgut pH and inter-horse variability. In the present study pH readings were taken at the end of fermentation, the readings gained may not reflect the pH of the faecal inoculum part-way through fermentation where the fermentation profiles between groups differed.

In this present study the bacterial composition of faecal inoculums was not analysed, however fermentation serves as a measure of hindgut microbial activity. Santos et al. (2011) stated that fermentation patterns can serve as an indicator of microbial activity and therefore a representation of the horse’s hindgut ecosystem. Therefore the reduction in gas production and alteration of other fermentation parameters in the present study suggests moxidectin administration was associated with alteration in the bacterial activity within the faecal inoculum. Interestingly while the main difference in the fermentation kinetics was detected at 16 hours after moxidectin administration, there were other changes in gas production over the
week following administration of moxidectin, predominantly within the hay substrate. Fermentation kinetics were reported by Biddle et al. (2013) as a measure of hindgut microbial activity alongside inoculum pH measurements over time and 16S rRNA sequencing to identify microbial composition change within the hindgut. In our present study pH was only measured at the endpoint of fermentation and microbial composition was not measured. Both pH and microbial composition warrant investigation in vivo from the findings of this in vitro study.

The combined results of this present study suggest that following moxidectin administration there was a fluctuation in the pH of the faecal inoculum within the in vitro system. This may be linked to the differences in fermentation observed in fermentations inoculated with faeces collected 16 hours after moxidectin treatment.

The findings for the hay substrate fit with the findings of Jacotot et al. (2004) who identified that cellulolytic bacterial counts decreased significantly 24 hours after moxidectin administration. One week after moxidectin administration the pH of the faecal inoculum for the hay substrate significantly increased. However there was still a difference in the fractional rate of gas production and a longer lag time for in the treated horses, suggesting the ecosystem had not fully returned to normal.

The results of the oat fermentations may also fit with the findings of Jacotot et al. (2004), initially fermentation was reduced around the time of moxidectin administration however pH fluctuations may have made the inoculum environment more suited to starch fermentation. Jacotot et al. (2004) reported as caecal pH dropped and the cellulolytic bacteria decreased there was an increase in Lactobacilli and Streptococci. In this situation it may be expected that starch fermentation would improve as the bacteria proliferating in the ecosystem are much more suited to starch as a substrate. The morning after moxidectin administration there was a significant reduction in gas production for the oats substrate, however beyond this time point
there were no differences in the fermentation kinetics for the treatment and control groups, thus suggesting the adaption of the hindgut microbiota to a more acidic environment within the treatment group. The oat substrate fermentation was much quicker to return to normal than the hay substrate, suggesting changes in fermentation activity were optimised to starch rather than structural carbohydrates.

Prior to and during the experiment the donor horses received no concentrate feed, therefore the bacterial ecosystem in the faecal inoculums would not have been used to starch as a substrate. This may explain some of the inter horse variability for starch fermentation. Changes in diet from grass to concentrates has shown increases in the relative abundance of the *Bacillus-Lactobacillus-Streptococcus* lactate producing bacteria and a 4-fold decrease of cellulolytic *Fibrobacter* spp., this change has been associated with lowered colonic pH (Shirazi-Beechey, 2008). Ideally the donor horses should be fed similar diets to that being fermented in the *in vitro* system (Rymer *et al*., 2005). The forage in the culture bottles differed slightly to that of the donor horses’ diet, however Muller *et al.* (2008) found no difference in fermentation kinetics between hay and haylage in horses so this should not have influenced the findings in this present study.

Changes in bacterial faecal inoculum composition were reported by Biddle *et al.* (2013) using a similar culture based technique when exposing inoculum from donor animals on a forage based diet to starch substrate *in vitro*. Using 16S rRNA sequencing Biddle *et al.* (2013) saw an increase in *Firmicutes* spp., specifically an increase in *Streptococcus* spp., during the first 24 hours after inoculation. This would also fit with the findings of the present study as these microbiota composition changes would be associated with reduced forage fermentation.

The *in vitro* gas production system was originally designed to analyse fermentation kinetics in ruminants (Theodorou *et al*., 1994) and has been successfully adapted to study equine
fermentation. Reports by Lowman *et al.* (1996) and Machboeuf *et al.* (1997) identified that equine faeces were a good substitute for caecal fluid for *in vitro* gas production models. Lowman *et al.* (1999) reported that the *in vitro* gas production technique was a good representation of *in vivo* fermentation in the horse. Since then this method has been adapted for a range of equine studies including pure nutrition, changes associated with the starch content of the diet and tracking microbial changes when simulating starch overload (Biddle *et al*., 2013; Murray *et al*., 2014).

In this sealed-system, *in vitro* model, the products of fermentation e.g. volatile fatty acids are retained within the faecal inoculum, *in vivo* these would be absorbed. Theodorou *et al.* (1994) identified that while volatiles remained in the *in vitro* environment this did not alter the pH or significantly impact fermentation kinetics. The media used in these experiments was also originally optimized for ruminants and this should also be considered when interpreting results as this might not allow the culturing of some bacteria normally present within the horse hindgut. One further limitation is that this system is totally anaerobic, Julliand *et al.* (2001) found that as cereal concentration in the diet was increased the number of aero-anaerobes significantly increased compared to horses fed only forage. It would be unlikely these organisms would thrive in the *in vitro* gas production environment due to the lack of oxygen.

A similar *in vitro* method to model bacterial changes associated with starch overload was successfully used by Biddle *et al.* (2013). Thus the model used in the present study gives a fair representation of the horse’s hind gut environment. In a more advanced *in vitro* model Leng *et al.* (2017) have developed a three chamber hind gut, in which each vessel demonstrated 80% similarity in bacterial composition and produced short chain fatty acids as a product of fermentation. This also suggests it is possible to simulate some of the complexity of hindgut microbial fermentation *in vitro*. In ruminants Danielsson *et al.* (2014) used the *in vitro* gas production method, sequencing bacterial DNA from rumen fluid prior to bottle inoculation and
harvested bottles during fermentation for further sequencing. The study found that bacterial composition in the *in vitro* gas production system was a good representation of the rumen. Taken together these previous studies suggest *in vitro* models give a fair representation of the horses’ hindgut microbiome.

Within our present study both hay and oats were chosen as a substrates for the *in vitro* system, hay representing a typical forage ration and oats because many horses are managed on concentrate diets containing starch from cereal grains. Hindgut microbiota are optimized for forage fermentation and the effect of high levels of starch reaching the hindgut is already known to be associated with microbial changes (Daly *et al.*, 2012). Cereal inclusion in the diet has previously been identified as a risk factor for colic. Furthermore anthelmintic administration has also been identified as a colic risk factor (Cohen *et al.*, 1999; Hillyer *et al.*, 2002). The findings of our present study show that anthelmintic administration briefly altered the activity of microbiota. Previous studies have identified that dietary changes alter both activity and composition of microbiota, increasing the risk of colic. As anthelmintic administration appears to alter the activity of microbiota further work is required to identify if this also leads to changes in microbiota composition.

During sampling at pre-treatment it took longer to collect samples than the following sampling time points. While the pre-warmed flasks were kept in a polystyrene container during the sampling process the pre-treatment samples may have dropped below optimum temperature therefore affecting the bacterial viability. The fermentation profile at this time point did show that there was no difference in the fermentation kinetics between the groups prior to moxidectin administration. By having an untreated control group throughout the experiment meant the limitations in the data prior to moxidectin administration did not significantly impact on the viability of this study. Furthermore the dry matter loss (%) was the same at 160 hours post treatment as it was at pre-treatment suggesting some reliability in this data. However
interpreting the gas production data from this pre-treatment time point and any other analysis from the fermentations from this time point should be taken with caution.

This present experiment did use a low number of donor animals, this is comparable to other studies using this culture based technique. One particular restriction to increasing the sample size in this experiment was the amount of time taken to collect samples, inoculate and collect readings from the culture bottles.

5.5. Conclusions

Notwithstanding limitations, the data from this present study suggest that moxidectin administration in vivo reduced cumulative gas production associated with the fermentation of both hay and oat substrates in an in vitro model 16 hours following moxidectin administration. While cumulative gas production did not differ between the treatment and control groups at 40 and 160 hours after moxidectin administration, there were some differences in fermentation kinetics and substrate losses. These findings suggest that moxidectin may alter hindgut microbial metabolism around the time of treatment. Further work is required to better characterise changes in microbial function and to determine whether they are associated with changes in microbial community profile.
Chapter 6.0

The effect of moxidectin administration on equine faecal microbiota composition using 16S rRNA sequencing

6.1. Introduction

There is limited understanding of the relationship and communication between nematodes and the hindgut microbiome within the horse (Peachey et al., 2017a). Currently there is limited knowledge on the effect of nematodes and anthelmintic treatment on the equine hindgut microbiome. Equine hindgut microbiota are extremely sensitive to change (Daly et al., 2012; Dougal et al., 2013), in chapter five moxidectin significantly altered fermentation patterns for both hay and oats around the time of administration. Alterations in microbiota and metabolome of pigs infected with *T. suis* have previously been reported when compared to uninfected controls (Li et al., 2012).

The interaction between parasites and gut microbiome is potentially perturbed by anthelmintic treatment. This phenomenon has been explored by Bartley et al. (2015) who reported changes within the microbiota of sheep infected with nematodes when compared to uninfected controls with some composition changes around anthelmintic administration. Cooper at al. (2013) identified in humans infected with *T. trichiura* that parasite burden and anthelmintic treatment did not alter the microbiota composition when compared to parasite free untreated controls. Goats infected with *Haemonchus contortus* were reported to have a significantly altered ruminal microbiota composition compared to uninfected controls (Li et al., 2016).

This interaction has been explored in horses by Jacotot et al. (2004) who reported a reduction in cellulolytic bacteria and reduction in caecal pH followed by an increase in *Lactobacilli* and *Streptococci* after treatment with moxidectin. These findings warrant further investigation into bacterial composition of equine gut microbiota following anthelmintic administration.
6.1.1. Objectives

There were two objectives of this study:

1) To characterise the composition and diversity of faecal microbiota prior to and post anthelmintic treatment in animals with very low intestinal parasites burdens.

2) To monitor faecal pH prior to and following moxidectin treatment.

6.2. Materials and methods

6.2.1. Study population

A group of 17 polo ponies managed exclusively on pasture at a farm in Gloucestershire were recruited on to this study. The population comprised both mares (n=12) and geldings (n=5) with a mean age of 12 ±3.5 years, and a range of breeds (Thoroughbreds n=4, Thoroughbred cross Argentinian polo pony n=8, Argentinian polo pony n=4, Argentinian polo pony cross Quarter horse n=1. Horses were not exercised and no concentrate feeds were given, their diets were supplemented with meadow haylage ad libitum. The healthcare plan, including parasite control and treatment history for these horses, was the same as described in chapter five.

6.2.2. Ethical approval

This study was given favourable ethical opinion by the University of Surrey Animal Welfare and Ethical Review Board NASPA-2015-008-SVM.

6.2.3. Treatment group allocation

Treatment and control animals were randomly assigned using the sample function in R (i386 3.1.2). Nine animals were dosed with moxidectin and eight remained untreated controls.
6.2.4. Anthelmintic treatment

Moxidectin was selected as the anthelmintic for this study as it is most recent anthelmintic licensed for equine nematode treatment, and the most commonly used by horse managers (Allison et al., 2011).

6.2.5. Sampling method

Sample collection took place in March 2016. On day 0 prior to anthelmintic treatment a fresh, spontaneously voided, faecal sample was collected from each animal. Faecal pH was recorded using a hand held pH monitor (Hanna pHep, HI98128) as described by Muller et al. (2008) in the fresh faecal pile. A small sample of faeces was collected into labelled 50 ml screw top vials and placed into an insulated container containing ice for transport to the laboratory. On arrival at the laboratory samples were frozen at -80°C. This process was repeated at each sampling time point, 16 hours, 40 hours and 160 hours post moxidectin treatment. On the afternoon of day 0 at approximately 4pm the treatment group were dosed with moxidectin, as previously described in chapter 5.

6.2.6. Next generation sequencing

6.2.6.1. Bacterial DNA Extraction

Bacterial DNA was extracted from faecal samples in preparation for 16S rRNA sequencing. Extraction was undertaken using PSP® Spin Stool DNA Kit (Stratec Molecular, Germany). All reagents and buffers were supplied within the kit and were prepared in line with the manufacturer’s instructions. For each sample 200 mg of faeces were weighed into a 2 ml microfuge tube and mixed with 1.2 ml of lysis buffer and then vortexed for one minute. Microfuge tubes were incubated on a pre-heated thermomixer at 95°C for 10 minutes whilst being shaken. Each microfuge tube had five Zirconia II beads added and samples were bead beaten for two minutes on a vortex. Samples were then centrifuged at 11,100 x g for one
minute. The supernatant was transferred to the inviAdsorb-tube and vortexed for 15 seconds. Samples were incubated at room temperature for one minute before centrifugation for three minutes at full speed (14,000 x g). The supernatant was transferred to a new microfuge tube and centrifuged again for three minutes at full speed. From the supernatant 400 µl was transferred into a new microfuge tube and 25 µl of proteinase k was added, the solution was mixed briefly by vortex. The sample was then incubated in a pre-heated thermomixer at 70°C for 10 minutes at 900 rpm.

After incubation 200 µl of binding buffer A was added and mixed briefly by vortex. The mixture was then transferred to RTA spin filter tubes and incubated at room temperature for one minute. Spin filters were then centrifuged at 11,000 x g for two minutes. The RTA filter was then transferred to a new receiver tube and the previous tube and filtrate discarded. Once fitted to a new receiver tube 500 µl of wash buffer one was added to the spin filter. Samples were then centrifuged at 11000 x g for one minute. Again the filter was transferred to a new receiver tube and the previous tube and filtrate were discarded. Then 700 µl of wash buffer II was added to the filter and centrifuged at 11,000 x g for one minute. The filtrate was again discarded and the receiver tube re-used, to ensure all ethanol from the wash buffers was removed the samples were centrifuged at 14,000 x g (full speed) for four minutes. After centrifugation the filtrate and receiver tubes were discarded and the filters placed on new microfuge tubes. Then 200 µl of pre warmed molecular biology grade water, at 70°C, was added to the filters to elute the DNA. Samples were incubated for one minute at room temperature and then centrifuged for one minute at 11,000 x g. The spin filter was removed and the eluted DNA contained in the microfuge tube. To ensure that DNA was present 1 µl of elution fluid was placed on a calibrated Nano-drop spectrometer to estimate DNA concentration in ng/ µl. Mean DNA concentration per sample was 89.6 ± 49.9 ng/ µl. Samples were then frozen at -20°C prior to sequencing.
6.2.6.2. *16S rRNA Sequencing*

Pre-sequencing PCR to amplify the V4 region of the 16S rRNA gene (using primers F515 (GTGYCAGCMGCCGCGGTA) and R927 (CAGCCCGYCAATTCTTTRAGT)), clean up and sequencing was performed at the Animal and Plant Health Agency (APHA) using the Illumina MiSeq platform.

6.2.7. *Data analysis*

6.2.7.1. *pH data*

Faecal pH data was recorded in a Microsoft® Excel spreadsheet (Microsoft Excel 2010). pH was analysed using a repeated measures ANOVA in GenStat 18th ed.

6.2.7.2. *Bioinformatics of sequencing data*

Filtering and processing of raw reads generated during sequencing was carried out using QIIME v. 1.6.0. Reads were initially aligned using Pandaseq and cut off at 380-420 reads, these were then combined into combined_fasta files. Using the USEARCH programme in QIIME v.1.8.0 operational taxonomic units (OTUs) were clustered at a level of 96% similarity using pick_otus.py. OTUs were then classified using the Ribosomal Database Project (RDP) classifier 2.0 (Wang et al., 2007). Once OTUs were assigned pick_rep_set.py was used to pick sets of representative sequences, one per OTU. This was then followed by assigning taxonomy using assign_taxonomy.py and then aligned using the PyNast programme align_seqs.py. The output of the aligned sequences was used to create an OTU table using make_otu_table.py. The ‘fasttree’ QIIME script was then used to build a phylogenetic tree using programme make_phylogeny.py.

Once the phylogenetic tree and OTU table were created alpha and beta rarefaction analysis was carried out using the QIIME scripts alpha_diversity.py, jacknifed_beta_diversity.py,
beta_diversity_through_plots.py and multiple_rarefaction. Two alpha rarefaction indices were calculated, Obs and Chao1. Chao1 rarefaction looks at species richness and evenness within the sample whereas Obs looks only at the richness within the sample. Following this taxa summary plots were created using summarize_taxa_through_plots.py for phyla, class, order and family levels of classification.

The Linear Discriminate Analysis (LDA) effect size (LEfSe) platform was then used to identify significantly different abundant OTUs between the treatment groups and time points. This non-parametric model is robust in the face of multiple comparisons (Segata et al., 2011).

6.3. Results

None of the horses showed any signs of colic after moxidectin administration. One of the horses in the treatment group excreted cyathostomins the day following treatment.

   6.3.1. pH data

From the faecal pH data there were no significant differences between the treatment and control groups identified (P>0.05). Mean pH values are displayed in table 6.1.

| Table 6.1. Mean faecal pH values for the Moxidectin treated and control groups over the four sampling time point. |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| **Group** | Pre-Treatment  | 16 Hours Post Treatment | 40 Hours Post Treatment | 160 Hours Post Treatment |
| Moxidectin | 6.8±0.13 | 6.9±0.29 | 6.7±0.33 | 6.7±0.10 |
| Control | 6.7±0.20 | 7.0±0.28 | 6.8±0.16 | 6.8±0.13 |
6.3.2. Sequencing data

Total sequences within the data set was 4,034,103, the minimum number of sequences per sample was 1,388, the maximum was 101,512. The mean number of sequences per sample was 62,063. After filtering and removal of chimeras 5746 OTUs were identified from the sequenced samples.

6.3.2.1. Alpha Diversity

Alpha diversity was calculated for each horse at each sampling point. Both Obs and Chao1 alpha rarefaction indices were plotted against the number of sequences for individual animals, and treatment groups. When looking at the rarefaction curves for both observed species richness and evenness moxidectin administration did not influence species richness or evenness within samples over the four sampling time points, Figure 6.1.
Figure 6.1. Panel plot of alpha diversity measures. (A) Species richness using Chao1 alpha diversity at subject level for both treatment and control groups over the four time points for individual animals. (B) Species richness and evenness using Chao1 alpha diversity for treatment groups over the four time points. (C) Observed species rarefraction curve for all horses, both treatment groups, at all four time points. (D) Rarefraction curve of observed species per treatment group at each sampling time point. In the legends 0, 16, 40 and 160 reflect the time points, M and C refer to treatment groups and the number defines the animal. Error bars represent 95% confidence interval.
6.3.2.2. Beta Diversity

Beta diversity defines diversity between groups, in comparison to alpha diversity which is within groups. Principle coordinate analysis of beta diversity, classified by treatment group, indicated no clustering between groups (Figure 6.2.). Beta-diversity between treatment groups across all time points was also explored (Figure 6.3.) and no clear clustering was identified. Jack-knifed beta diversity identifies robustness of Unweighted Paired Group Method with Arithmetic Mean (UPGMA) clusters. Jack-knifed beta-diversity PCoA plots again suggest no significant differences between treatment groups and time points, Figure 6.4. Inter-horse variability was also plotted using beta diversity, there was some clear animal level clustering over the time points for both treatment groups, Figure 6.5.

![Beta diversity PCoA plot](image)

**Figure 6.2.** Beta diversity PCoA plot separated by treatment group only, including all of the sampling time points.
Figure 6.3. PCoA plot of beta diversity between the treatment and control groups over the four sampling points. There are no distinct clustering between the treatment groups or time points. C0-3 identifies control group at time points 0, 16, 40 and 160, M0-3 identifies the moxidectin group at sampling points 0, 16, 40 and 160.

Figure 6.4. PCoA plot of jack-knifed beta diversity between treatment groups over the four time points. There appears to be no visually spaced clusters between treatment groups and time points. C0-3 identifies control group at time points 0, 16, 40 and 160, M0-3 identifies the moxidectin group at sampling points 0, 16, 40 and 160.
**Figure 6.5.** Beta diversity at horse level for each sample for the moxidectin treated horses (A) and the untreated controls (B). Within both treatment groups there is some clustering by horse. Within the key the number on the right refers to the animal, numbers in parenthesis reflect the number of samples from that animal.
6.3.3. *Taxa summary*

Taxa plots at phyla, order and family level can be seen in Figure 6.6. At phyla level there are no significant changes around the time of moxidectin administration. Moving down two taxonomic levels to order level there are no changes only some minor fluctuation within the *Firmicutes* phyla. The *Veillonellaceae* here represent approximately 1-2% of the microbiota and did not fluctuate around the time of treatment. These data suggest moxidectin administration did not alter the composition of the faecal microbiota.
Figure 6.6. OTU abundances (%) for the two treatment groups over the four sampling points, A is phyla level, B is order and C is family.
6.3.4. Discriminant analysis

There were no significantly differing OTU abundances identified between the two treatment groups over the four time points. As there was a significant change in fermentation identified in the *in vitro* fermentation experiment (Chapter 5), an OTU table specifically looking at 16 hours after treatment was analysed in LEfSe. At this time point the treatment group had three OTUs with differing abundances compared to the control group. Two of these OTUs were *archaea* one representing methane producing organisms and the second methane suppressing organisms. The third OTU a *Firmicutes, Coprobacillaceae* that ferment glucose to acetate and lactate. The control group also had a single OTU of increased abundance but this referred to an OTU only labelled at kingdom level, Figure 6.7.

![Figure 6.7. LEfSe discriminate analysis of the treatment and control group 16 hours after moxidectin administration.](image)

**Figure 6.7.** LEfSe discriminate analysis of the treatment and control group 16 hours after moxidectin administration.
6.3.5. In vitro fermentation samples

Sequence data from the samples used in the in vitro gas production experiment, chapter 5, were also analysed separately. Alpha diversity plots, Figure 6.7., showed no differences in diversity within the treatment groups. Beta diversity plots, Figure 6.8., also showed no differences between treatment groups, but there was some animal-level clustering for both treatment groups.

The OTU abundances of the two groups used in the in vitro fermentation experiment are presented at phyla, order and family level in Figure 6.9. At phyla and order level there is no evidence of marked differences in bacterial population structure compared to the samples described above in the complete study population. At family level there were some small fluctuations in Bacteroidetes Porphyromonadaceae and Bacteriodales BS11 which both decreased slightly at 16 hours after moxidectin administration. Ruminococcaceae and Veillonellaceae slightly increased 16 hours after moxidectin administration, the former in the treated group the latter in the controls. None of these fluctuations were significant. For linear effect size discriminate analysis (LEfSe) there were no significantly differing OTUs in the samples between treated and control animals from the in vitro gas production experiment.
Figure 6.7. Panel plot of alpha diversity indices for samples used in the *in vitro* gas production experiment. (A) Chao1 rarefaction index of alpha diversity for individual animals used in the *in vitro* gas production over the sampling time points. (B) Chao1 rarefaction index of alpha diversity for the treatment groups over the four sampling time points. (C) Alpha rarefaction index observed species individual animals used in the *in vitro* gas production experiment over the four time points. (D) Alpha rarefaction index observed species for the *in vitro* gas production treatment groups over the four time points. Error bars represent 95% confidence intervals.
Figure 6.8. Beta diversity indices for samples used in the *in vitro* gas production experiment. (A) Jack-knifed beta diversity of the treatment and control groups over the *in vitro* gas production experiment. (B) Jack-knifed beta diversity of the treatment and control groups over each of the four sampling points. (C) Beta diversity of the treatment and control groups over the four sampling time points. (D) Beta diversity of faecal samples used for *in vitro* gas production at horse level, mox treated (7, 9, 17) and controls (10, 11, 16).
Figure 6.9. Panel plot of (A) phyla (B) order and (C) family level OTU abundances from the treatment groups used in the *in vitro* gas production experiment over the four time points. No changes in OTU abundances occurred around moxidectin administration.
6.4. Discussion

In the present study faecal pH did not differ between the treatment groups or over the sampling time points. There were no changes in the bacterial composition of faecal microbiota following anthelmintic treatment between the treatment groups when compared over all four sampling points. The findings of our in vitro fermentation experiment suggest that some form of alteration in the horses’ hind gut ecosystem occurred around the time of moxidectin administration. The findings in this present study suggest that the changes were not in bacterial population composition. When focusing solely on the results from 16 hours after moxidectin administration there were four significantly differing OTUs identified using LEfSe. For one OTU within the control group labelled bacteria, details of the taxonomy below kingdom are unknown. Within the treated group three OTUs were identified, two archaea and Coprobacillaceae. Archaea are prokaryotes and within the gut are responsible for methane activity. In the present study 16 hours after moxidectin administration there was an increase in abundance of these methane producing bacteria within the Methanobacteriaceae family in the moxidectin treated horses. However there was also an increase in abundance of Archaea in the Thermoplasmata family which is responsible for methane suppression in the treated group. Coprobacillaceae are within the Firmicutes phyla, they ferment structural carbohydrates and produce acetate and lactate. While these OTU abundances were identified as significantly different between the treatment groups 16 hours after moxidectin administration, the overall effect was at such a small magnitude which was not detected when comparing groups over all of the sampling time points.

Both Chao1 and Obs rarefaction indices for alpha diversity were compared up to 50 000 sequences per sample, at which points reads were reaching a plateau. Beta diversity and jack-
knifed beta diversity highlighted no differences between the treatment groups over the sampling time points.

Previous studies have suggested that faecal pH is a reasonable indicator of the pH in the caecum and proximal colon (Berg et al., 2005). However Willing et al. (2009) found faecal pH did not alter around the time of a significant increase in starch within the diet. Willing et al. (2009) did find changes in bacterial composition the faecal microbiota using 16S rRNA sequencing. These past results suggest that faecal pH does not always replicate what is happening the hind gut ecosystem.

In the present study we detected minimal change in the faecal microbiota composition following moxidectin administration. The lack of change in the faecal microbiota in our study is consistent with no identified differences in faecal pH. The taxa summary for the horses used in the fermentation experiment, chapter 5, suggests minor fluctuations in fibre degrading bacteria. Whilst there were no differences in abundances of OTUs of fibre degrading bacteria these minor downward fluctuations may have played a role in the reduced fermentation seen in chapter 5. It is entirely possible that any changes within the gut microbiome around the time of anthelmintic administration are purely functional. This hypothesis would fit with the findings of the in vitro gas production experiment in chapter 5. By using the 16S rRNA approach this only allows profiling of bacterial composition, this method does not allow evaluation of functional changes. It is possible that moxidectin alters the metabolism of microbiota instead of altering composition. Full metagenomic sequencing would be required to evaluate gene expression to test this hypothesis.

The findings of our present study are similar to those of Cooper et al. (2013) who found that in humans ivermectin treatment did not significantly alter the composition of faecal microbiota when given to children infected with T. trichiura >2000 epg nor did it effect their control group who received the same treatment with no detectable parasite burden from FEC. In other animal
studies those that have reported changes around the time of an anthelmintic administration were treating animals with significant nematode burdens (Bartley et al., 2015). In this present study the horses had very low parasite burdens, therefore it is possible that at the time of anthelmintic treatment there was a limited potential for parasite death to affect the microbiota of the treated horses. In a recent pilot study in the USA Kunz et al. (2017) treated 6 Quarter horses with moxidectin and praziquantel combined and used 16S rRNA sequencing to characterize the effect on faecal microbiota. The study found no significant changes in bacterial diversity around the time of moxidectin administration. These researchers used different sampling points to the present study and lacked a control group. The findings of Kunz et al. (2017) are consistent with the present study i.e. moxidectin treatment does not cause a shift in the bacterial composition of the horses’ faecal microbiota. Another recent study reported changes in the faecal microbiota of Thoroughbreds infected with cyathostomins when compared to horses with low cyathostomins burdens (Peachey et al., 2017b).

The horse’s gut microbiome appears to be more sensitive to changes than that of humans and ruminants (Daly et al., 2012). When comparing these differing species the horse normally has little lactate present in the hindgut (Shirazi-Beechy, 2008). Previous studies have found that in the horse the lactate utilizing Veillonellaceae make up approximately 1% of the microbiome (Daly et al., 2012; Proudman et al., 2015), whereas in the human colon they can represent 10% of the microbiome (Duncan et al., 2007; Daly et al., 2012; Segata et al., 2012). Daly et al. (2012) proposed that where there is an increase in lactic acid in the hindgut, horses are unable to convert this rapidly to SCFAs which may play a role in the alterations in the hindgut ecosystem, which can lead to colic. Anthelmintic administration has previously been identified as a risk factor for colic (Kaenne et al., 1997; Cohen et al., 1999; Hillyer et al., 2002). However the reasons for this are not fully understood (Hillyer et al., 2002). In the present study anthelmintic administration did not significantly alter faecal microbiota composition or the
faecal pH. *Veillonellaceae* appeared in both treatment and control group at approximately 1-2% across the sampling time points.

Equine microbiota studies have previously reported inter-horse variability (Blackmore *et al.*, 2013; Dougal *et al.*, 2013), this was also a finding within the present study. The beta diversity PCoA panel plot, Figure 6.5, shows clustering by animal in both the treatment and control groups. Stability in faecal microbiota of ponies was reported by Blackmore *et al.* (2013) using two 72 hours sampling periods 11 weeks apart. Blackmore *et al.* (2013) suggested that using horses as replicates in treatment and control microbiome studies may be problematic due to the levels of inter-horse variability within treatment groups.

Faecal microbiota are commonly used in studies to represent the hindgut microbiota, reasons for this being the convenient non-invasive approach (Costa *et al.*, 2012; Shepherd *et al.*, 2012; Blackmore *et al.*, 2013; Proudman *et al.*, 2015). Faecal microbiota do not accurately represent the proximal part of the hindgut, however Julliand and Grimm (2017) report that the faecal microbiota was a sensitive indicator of dietary change. This is supported by Dougal *et al.* (2013) who reported that the bacterial composition in the caecum was different from faeces and the right dorsal colon. Whilst the use of faeces is convenient for studying hindgut microbiota, it should be remembered that the faecal microbiome might not accurately reflect the caecal microbiome. Therefore findings within the faecal microbiome that reflect expected caecal changes need to be interpreted with some caution.

Previous studies have identified a reduction in caecal pH and a change in bacterial composition identified in cannulated horses following moxidectin administration (Jacotot *et al.*, 2004). In the present study there were no significant changes in pH. This may reflect the use of faeces over caecal fluid when comparing to previous studies. It could be that changes associated with anthelmintic administration are of such a small magnitude they could not be detected using a
top down ‘omics approach, or that changes are functional and therefore unable to be detected by microbiota profiling.

6.5. Conclusions

The primary objective of this study was to determine whether moxidectin administration altered the hindgut bacterial composition. Our findings suggest that when administered to horses with a low cyathostomin burden, moxidectin did not significantly alter bacterial diversity or composition of the faecal microbiota, nor did it alter the faecal pH. None of the horses showed signs of colic post administration. Our study is consistent with recent findings in the USA where moxidectin and praziquantel combined did not alter the faecal microbiome following treatment.
Chapter 7.0.
The effect of moxidectin administration on the equine metabolome using $^{1}$H NMR spectroscopy

7.1. Introduction

Interactions between gut microbiota and mammalian metabolism can be analysed indirectly through the use of metabolic profiling of host biological samples for host-microbe co-metabolites (Wu et al., 2010). Metabolomics uses a top-down systems biology approach to simultaneously measure all low molecular weight metabolites present in a biofluid (Waldram et al., 2008; Escalona et al., 2015). This robust and well validated approach allows the untargeted characterisation of metabolic phenotypes and of multidimensional biochemical responses of complex biological systems to differing stimuli (Nicholson et al., 1999; Rezzi et al., 2007).

Our understanding of the role of gut microbiota is limited, metabolomics allows insight into the complex metabolic interactions that occur between the host and its microbiome (Escalona et al., 2015). As our understanding of the equine gut microbiome increases this raises further questions on the metabolic interactions within the gut microbiome and the effect of diet and drug treatments. In humans, metabolomics has allowed a much greater understanding of the host-microbiome metabolic axis which involves the gut, brain, liver and muscle (Nicholson et al., 2012). Within the past 10 years as the use of ‘omics technologies have taken off with development of differing areas of use. One of these areas is pharmacometabolomics which allows greater understanding of the impact of drug treatments and the pathways involved in the metabolome (Kaddurah-Daouk et al., 2008).

Within equine research previous studies have profiled metabolites from biofluids using $^{1}$H NMR spectroscopy, to provide normal values for healthy individuals (Escalona et al., 2015). Further studies have used this approach to explore metabolic conditions such as laminitis and metabolic syndrome (Hodervance et al., 2007; Keller et al., 2011).
Previous studies have indicated that gut microbiota play a role in drug metabolism (Rowland, 1986). Li and Jai (2013) identified drugs that are metabolised by the human microbiome and documented observed microbial transformations. One of the drugs featured in their study was the anthelmintic levamisole. To date no studies have reported the effect of anthelmintic treatment in the horse using metabolomics.

7.1.1. Objectives

There were two objectives of this study:

1) To profile bacterial metabolites in urine prior to and after anthelmintic treatment.
2) To profile faecal bacterial metabolites in faeces and hay fermentation inoculums following moxidectin administration.

7.2. Materials and methods

7.2.1. Ethical approval

This study was given favourable ethical opinion by the University of Surrey Animal Welfare and Ethical Review Board NASPA-2015-008-SVM.

7.2.2. Study population, treatment groups and sampling

The study population and treatment group allocation were the same as described in chapter 6. Briefly, 17 polo ponies which were predominantly Thoroughbreds with a mean age 12 ±3.5 years, managed exclusively at pasture, with very low parasite burdens that were randomly allocated into treatment and control groups. The sampling time points remained the same as described in chapters 5 and 6, sampling prior to treatment, then 16 hours, 40 hours and 160 hours following moxidectin treatment. At each sampling time point free catch urine samples were collected using a collection beaker on a stick and transferred into 50 ml screw top vials. These were transported to the laboratory on ice and aliquoted into 5 ml cryo-vials on arrival. Samples were then frozen at -80°C prior to analysis. Urine samples were
the primary biofluid chosen for this study as urine has previously been identified as the best biofluid for metabolomic studies in the horse, followed by faeces, (Escalona et al., 2015). Faecal samples from the horses used in the in vitro gas production experiment and the fermentation samples saved from the hay fermentations from chapter 5 were also used in this study. The hay fermentations were selected for metabolite analysis over the oat fermentations as hay was more representative of the horses’ forage based diets and therefore more comparable to the faecal samples.

7.2.3. Sample preparation

Phosphate buffer solution was made in a one litre flask by combining the ingredients listed in Table 7.1. A magnetic stirrer was used to ensure the solution was fully dissolved prior to sample preparation.

Table 7.1. Ingredients used in the phosphate buffer solution.

<table>
<thead>
<tr>
<th>Chemical Compound</th>
<th>Formula</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium phosphate</td>
<td>Na₂HPO₄</td>
<td>28.86</td>
</tr>
<tr>
<td>Monosodium phosphate</td>
<td>NaH₂PO₄</td>
<td>5.25</td>
</tr>
<tr>
<td>Sodium 3(Trimethylsilyl) propionate-d4) (TSP) (1 mM)</td>
<td>C₆H₁₄O₂Si</td>
<td>0.172</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>NaN₃</td>
<td>0.193</td>
</tr>
<tr>
<td>Heavy water</td>
<td>D₂O</td>
<td>500 ml</td>
</tr>
<tr>
<td>Water</td>
<td>H₂O</td>
<td>500 ml</td>
</tr>
</tbody>
</table>

7.2.3.1. Urine samples and fermentations

Samples were defrosted and then vortexed, 400 µl was then aliquoted into microfuge tubes and 200 µl of phosphate buffer was added. Samples were then vortexed again before centrifugation for 10 minutes at 10 000 x g. Following centrifugation 550 µl of supernatant was transferred by pipette into 5 mm internal diameter NMR tubes. The same method was used for preparing the fermentation samples.
7.2.3.2. **Faecal samples**

Faecal samples were defrosted, 0.3 g of faeces weighed out into a microfuge tube and 1.2 ml of phosphate buffer was added. To ensure lysis, two 1.7 mm Zirconia beads were added to the microfuge tubes which were then placed in a bead breaker for 10 minutes followed by centrifugation for 10 minutes at 16 000 x g. Supernatants were transferred into new microfuge tubes and the centrifugation process was repeated again two further times. After the final centrifugation 500 µl of supernatant was pipetted into 5 mm internal diameter NMR tubes.

7.2.4. **1H NMR Spectroscopy**

1H NMR spectroscopy was conducted at Imperial College London in the division of Computational and Systems Medicine within the Department of Surgery and Cancer. Spectroscopic analysis of all samples was carried out on a 700 MHz Bruker NMR spectrometer equipped with a cryo-probe. For each of the urine, faecal and fermentation samples standard one-dimensional 1H NMR spectra were acquired with water peak suppression using a standard pulse sequence. For each sample 8 dummy scans were followed by 256 scans and collected in 64K data point. A spectra width of 20 ppm was used for all sample types. Chemical shifts in spectra were referenced against the TSP singlet at δ 0.0.

7.2.5. **Data analysis**

Spectra were phased and baseline corrected in Topspin 3.0 before alignment and normalisation (total quotient method) was carried out in Mathworks Matlab R2014a. Samples were analysed using multivariate statistics to compare the profiles of the urine, faeces and fermentations between the treatment groups. Initially unsupervised principal component analysis (PCA) models were constructed. Where there was spatial clustering between groups, supervised models were constructed using orthogonal projections to latent-structures-discriminate analysis (OPLS-DA). OPLS-DA models were used to identify group-specific metabolic changes. Within OPLS-DA models goodness of fit is
measured by $R^2_Y$ and prediction ability is measured by $Q^2_Y$ (Trygg et al., 2007). Only OPLS-DA models with $Q^2_Y$ values >0.4 and metabolite abundances differences $R^2 >0.5$ are reported. It is possible with these complex models that over-fitting occurs, where too many latent variables are added reducing the predictive ability and creating bias within the model (Gowan et al., 2010). To ensure models were not over-fitting OPLS-DA models underwent permutation testing, to 1000 permutations, to ascertain the significance by comparing the permutated model to the original OPLS-DA model. Only models with good prediction ability and no over-fitting ($p <0.01$) were kept.

Reference metabolite resonances from Escalona et al. (2015) and the human metabolome database (HMDB) were used to identify metabolites from the groups that significantly differed in the OPLS-DA models.

Metabolite concentrations were then calculated between treatment groups and time points. Using Matlab a peak integral was obtained for the NMR peak of interest. This integral value could then be used along with integral of the internal standard TSP, and the number of protons giving rise to the measured resonance. These values were used to calculate the actual concentration of interest using the equation below. A Students t-test was used to identify significant differences between concentrations and metabolite values, using a Bonferroni correction for multiple comparisons ($p <0.02$).

$$\text{Concentration} = \frac{\text{peak area of metabolite \times protons represented by TSP peak}}{\text{peak area of TSP \times protons represented by metabolite peak}} \times \text{TSP}$$
7.3. Results

7.3.1. Urine samples

There were no significant shifts in bacterial metabolite abundance following treatment between treatment groups over any of the different samplings points, Figure 7.1.A. In the *in vitro* fermentation experiment chapter 5, at 16 hours following moxidectin administration there were significant changes in fermentation patterns, however in this experiment no differences in urinary metabolite abundances between treatment groups were identified at this time point in this experiment, Figure 7.1.B. Horse variability was plotted within groups, Figure 7.2. There was no effect of horse variability in urine samples over time.
Figure 7.1. Scores plot (A) of urine samples from both treated and control horses over the four sampling points. No significant clustering was observed between groups and time points, PC 1 21% of variation, PC 2 12% of variation. Plot (B) of treatment and control group urinary metabolites 16 hours after moxidectin administration. There was no significant clustering between groups, PC 1 37% of variation, PC 2 17% of variation.
Figure 7.2. Horse variability of urinary metabolites over the four sampling points (A) control group and (B) treatment group. Numbers refer to the donor horse. Similar clusters are present for both treatment groups over the time points with little inter-horse variability.
7.3.2. Faecal samples

7.3.2.1. Analysis between treatment groups

Faecal water samples from the horses used in the *in vitro* fermentation experiment were analysed over the four sampling time points, Figure 7.3. There was no clustering identifying metabolic differences between treatment groups or time points. There was some clustering by horse over the repeated time points, this is identified by horse number in Figure 7.3 below.

![Scores plot of faecal water NMR](image)

**Figure 7.3.** Scores plot of faecal water NMR for the horses used in the *in vitro* fermentation experiment over the four time points. Markers within the plot identify treatment group and sampling point, numbers next to markers represent the donor animal. Some clustering by animal can be seen over the sampling points, PC1 56% of variation, PC 2 12% of variation.

Within the *in vitro* fermentation experiment, chapter 5, 16 hours post moxidectin administration was the time point where significant changes in fermentation were identified between treatment groups. Faecal samples from this time point was then analysed for difference in metabolome; no significant difference between the treatment groups was identified.
7.3.2.2. Analysis within treatment groups

Within both treatment groups, separately, faecal metabolite output was also analysed over the time points. Within the control group there were no differences in metabolite abundances over any of the four sampling points. Within the moxidectin treated group some clusters identifying metabolic profiles can be seen between post administration time points, Figure 7.4. Clustering was identified between samples taken at 16 hours and 40 hours post moxidectin administration. This data was modelled using OPLS-DA but the model was rejected due to poor prediction ability. Due to insufficient sample numbers it was not possible to create an OPLS-DA model between 16 and 160 hours post moxidectin administration.

Figure 7.4. Scores plot of faecal metabolite output of moxidectin treated horses used in the in vitro fermentation experiment at time points following moxidectin administration. Clusters highlighted are between Mox 16 (X), and Mox 40 (⋆), PC 1 64% of variation, PC 2 11% of variation. The OPLS-DA model was rejected due to poor prediction ability.
7.3.3. Fermentation samples

7.3.3.1. Analysis of faecal samples compared to fermentations

Differences between the metabolic profile of faecal samples and the fermentations were observed, Figure 7.5. Inter-sample variation in metabolite profile was greater in the hay fermentation samples than in the faeces, Figure 7.5. The OPLS-DA model was of moderate fit ($Q^2_Y = 0.44$) and the following metabolites differed in abundance between the two sample types; alpha-methyl aspartate ($R^2 0.6$), butyrate and adipate ($R^2 0.55$) within the model (Figure 7.6). Calculated differences in metabolite concentrations can be found in table 7.2.

Figure 7.5. Scores plot of faecal samples and hay fermentation inoculums split into treatment groups and sampling time points. There is a significant cluster between sample type, faeces and hay fermentations, PC 1 63\% of variation, PC 2 11\% of variation.
7.3.3.2. Analysis of hay fermentations between treatment groups

Faecal fermentations from the in vitro fermentation of hay were analysed between treatment groups over the four sampling points. Differences in metabolic profiles were observed 16 hours following moxidectin administration between the treatment and control groups (OPLS-DA model $Q^2_Y = 0.48$). Metabolites responsible for this difference were ethanol, 5-hydroxyindole-3-acetate and alanine (Figure 7.7.). There were no significant differences between the treatment groups at any of the other time points.
Figure 7.7. (A) Scores plot of hay fermentations 16 hours post moxidectin for both treatment and control groups, PC 1 47% of variation, PC 2 23% of variation. (B) OPLS-DA loadings plot with a $Q^2_Y$ of 0.48 and $R^2$ of 0.94. Metabolites with differing abundances ($R^2 > 5$) were ethanol, alanine and 5-hydroxyindole-3-acetate (5-HT). Formate had an $R^2$ 0.45. Other common metabolites are labelled for reference only.
7.3.3.3. Analysis of hay fermentation within treatment groups

Within the untreated control group, no differences in metabolite profile were detected between any of the sampling time points. Within the moxidectin treated group there was a difference in metabolic profile between 16 hours and 160 hours after moxidectin administration within the treated horses, Figure 7.8. The OPLS-DA model had a $Q^2_Y$ of 0.77. Metabolites with differing abundances with an $R^2 > 0.8$ were formate, ethanol and maltose in the 16 hours post moxidectin administration fermentations. At 160 hours after moxidectin administration, metabolites present here were SCFAs butyrate, acetate and propionate. There were no differences in metabolic profiles between 0 and 16 hours, 16 and 40 hours or 40 and 160 hours after moxidectin administration in the hay fermentations.
Figure 7.8. (A) Scores plot of metabolites for hay fermentations inoculated with faeces from the moxidectin treatment group at 16 hours post administration vs 160 hours post administration. Loadings plot (B) identifying metabolites with differing abundances ($Q^2 Y = 0.77$, $R^2 = 1$).
Table 7.2. Summary of OPLS-DA models, metabolites, resonances, concentrations and functions. Resonance key s; singlet, d; doublet, t; triplet, q; quartet. Metabolites referenced to Human Metabolome Database (HMDB).

<table>
<thead>
<tr>
<th>OPLS-DA Model</th>
<th>$Q^2Y$ &amp; Permutation P values</th>
<th>Metabolite</th>
<th>$1^H$ Resonance (δ)</th>
<th>Correlation coefficient ($R^2$)</th>
<th>Concentration (mean &amp; SD)</th>
<th>Concentration Significance</th>
<th>Function &amp; reference to Human Metabolome Database</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faeces v Fermentations</td>
<td>0.44 P=0.008</td>
<td>Butyrate</td>
<td>0.9 (t)</td>
<td>-0.55</td>
<td>Faeces</td>
<td>0.08 ± 0.03</td>
<td>0.19 ± 0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adipate</td>
<td>1.56 (m)</td>
<td>-0.55</td>
<td></td>
<td>0.77 ± 0.35</td>
<td>0.92 ± 0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alphamethyl-aspartate</td>
<td>1.48 (s)</td>
<td>-0.6</td>
<td></td>
<td>1.63 ± 0.8</td>
<td>2.11 ± 0.73</td>
</tr>
<tr>
<td>Fermentations 16hrs mox v control</td>
<td>0.48 P=0.01</td>
<td>Alanine</td>
<td>1.46 (d)</td>
<td>-0.8</td>
<td>Mox</td>
<td>4.57 ± 0.82</td>
<td>5.14 ± 0.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td>3.65 (q)</td>
<td>-0.7</td>
<td></td>
<td>4.24 ± 0.86</td>
<td>5.19 ± 0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-HT</td>
<td>3.59 (s)</td>
<td>-0.7</td>
<td></td>
<td>3.01 ±0.76</td>
<td>3.49 ± 0.44</td>
</tr>
<tr>
<td>Fermentations mox 16hrs v 160hrs</td>
<td>0.77 P=0.01</td>
<td>Formate</td>
<td>8.46 (s)</td>
<td>-0.95</td>
<td>16h</td>
<td>16.38 ± 14.08</td>
<td>5.42 ± 8.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td>1.2 (t)</td>
<td>-0.95</td>
<td></td>
<td>3.05 ±0.59</td>
<td>1.87 ± 1.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maltose</td>
<td>3.41 (t)</td>
<td>-0.85</td>
<td></td>
<td>1.14 ± 0.22</td>
<td>0.7 ± 0.54</td>
</tr>
</tbody>
</table>
7.4. Discussion

No difference was detected in bacterial metabolite profiles within treatment groups or between treatment groups for urinary metabolites prior to or following moxidectin administration. There was no change in faecal bacterial metabolites within treatment groups or between treatment groups over the sampling time points. However we did observe a significant difference in metabolite profile in the fermentations initiated with faeces from moxidectin-treated horses. The observed difference was seen in abundance between alanine, ethanol and 5-HT from samples collected the morning after moxidectin treatment when compared to the control group. There was also a difference in metabolic profile within the moxidectin treatment group when comparing fermentations inoculated with faeces from 16 hours after moxidectin treatment to fermentation inoculated with faeces collected one week after moxidectin treatment. The metabolites responsible for this difference were formate, ethanol and maltose. There was also a difference in metabolic profile between faecal samples and fermentations. The metabolite responsible for this difference was butyrate but it is unlikely to be of biological significance as butyrate was also differentially produced between control faeces and control fermentations.

The differences between ruminants and mono-gastric mammals and differences in pharmacokinetics between species should be considered when reviewing previous veterinary studies. In a ruminant study by Lifshitz et al., (2005), neither ivermectin nor moxidectin were metabolized or degraded in ruminal or abomasal contents. However benzimidazoles, specifically albendazole, have been identified as being activated within the rumen by intestinal microbiota (Lanusse et al., 1992; Virkel et al., 1999). From these previous studies it would appear that while benzimidazoles and levamisole may be metabolised by the gut microbiota, this does not appear to be the case for macrocyclic lactone anthelmintics.
The faecal samples analysed in the present study were the same as those used to create the faecal inoculum for *in vitro* fermentation; we demonstrate a difference in metabolic profile between these two sample types. The fermentations are likely to have had increased levels of metabolites as *in vitro* gas production is a closed system, whereas the faeces would have lost many of the metabolites detected in the fermentation through absorption in the horses’ gastrointestinal tract prior to excretion. This is clearly seen in the difference in butyrate concentrations (Figure 7.6.). Theodorou *et al.* (1994) suggested that the accumulation of volatile fatty acids did not significantly alter pH or fermentation kinetics during *in vitro* gas production. However metabolite abundances would be likely to differ when sampling from this closed system at the end of fermentation.

Within the faecal samples the higher abundance of alpha-methyl aspartate between sampling points may be associated with moxidectin. The mode of action of moxidectin is on glutamate-gated chloride channels and alpha-methyl aspartate mimics the action of glutamate (Cobb and Boeckh 2009; Abongwa *et al.*, 2017). One possible reason for an increase in the abundance of alpha-methyl aspartate could be a product of the metabolized drug which acts upon glutamate-gated chloride channels that has been excreted. However there is limited detailed published data on excreted metabolites of moxidectin to support this hypothesis.

The greater abundance of adipate in the fermentations may be associated with the effect of treatment on fermentation kinetics. Clapperton (1977) observed that adipate altered methane production in rumen fermentation. The changes in gas production in chapter 5 could be due to the influence of treatment linked to an increase in adipate abundance during *in vitro* fermentation. This would support the findings in chapter 6 where changes in abundance of methanogenic organisms in the faecal microbiota were observed 16 hours after moxidectin administration.
Alanine is a non-essential amino acid and a product of lactate fermentation from anaerobic respiration (Brennan et al., 2002; Hansen and Owen, 2013). Normally lactate is transported to the liver and converted to glucose via the glucose alanine cycle (Hansen and Owen, 2013). Within the closed *in vitro* system any lactate produced may be fermented by microbiota and the products of this would remain within the system which may explain an increase in alanine within the fermentations. Within the fermentations inoculated with faeces from the horses that had been treated with moxidectin 16 hours previously there was an increase in abundance of 5-hydroxyindole-3-acetate (5-HT). 5-HT is the main metabolite of the neurotransmitter serotonin, which is a powerful vasoconstrictor and plays an important role in gastrointestinal motility. 5-HT is metabolized by gut microbiota and is specifically associated with *E.coli* and *clostridium* spp. playing a role in the brain-gut axis (Nicholson et al., 2012).

When comparing fermentation metabolic profiles of the moxidectin treated horses 16 hours post treatment to samples 160 hours post treatment there was a difference in formate, ethanol and maltose. Formate is a microbial metabolite that is involved in acetate and folate metabolism (Escalona et al., 2015). It also plays a role in metabolic acidosis (Escalona et al., 2015). Both formate and lactate are organic acids and in humans have been associated with parasite burdens (Holmes et al., 2011). In the present study the horses had low strongyle egg counts but this cannot account for pre-patent infection. There was evidence of cyathostomins excreted in faeces the day following moxidectin administration in one of the horses used in the *in vitro* fermentation experiment, demonstrating strongyle presence. However it is unknown if this individual animal influenced the difference in formate abundance between groups. This may explain the increase in formate at this time point. The presence of formate the day following moxidectin administration also suggests a drop in pH within the *in vitro* system. This finding supports the reduced pH identified in chapter 5 within these fermentations 16 hours after treatment. Formate and ethanol are both products of glucose fermentation, specifically products...
of the fermentation of D-lactate (Ward, 2015). Cross fermentation, where bacteria use more than one energy pathway to ferment glucose results in the production of formate and ethanol (Ward, 2015). This is a common route for facultative anaerobes and is the method by which *Clostridia* spp. produce butyrate. It is therefore possible that the increase in these metabolites was due to the anaerobic *in vitro* fermentation environment rather than the effect of moxidectin on the metabolome. However the timing of the observed difference in formate, the day following treatment, is suggestive of a causal relationship.

D–lactate, produced by species of *Lactobacilli* (Al Jassim et al., 2005), and is associated with carbohydrate-overload in laminitis. Both formate and ethanol are products of D-lactate fermentation, therefore it is possible that following moxidectin administration there was an increase in D-lactate in the fermentations which was then fermented to VFAs, formate and ethanol which were observed here. This hypothesis is supported by the findings of Jacotot *et al.* (2004) who saw an increase in *Lactobacilli* spp. in bacterial counts following moxidectin administration. However while there was a pH reduction in the fermentation inoculated with faeces collected 16 hours following moxidectin administration, lactate abundance did not differ when compared to any of the other sampling points within the treatment group. The findings of de Fombelle *et al.*, (2003) suggest that lactate production is not always detected as it is converted by lactate utilizing microbiota to propionate. Nor were any significant changes in bacterial composition identified, e.g. *Lactobacilli* spp. when profiling the microbiota in chapter 6. In the fermentations when comparing the treatment and control groups 16 hours after moxidectin administration there was a difference in the abundance of alanine, a product of lactate fermentation, in the moxidectin treated group. This was not seen when comparing within treatment groups, the moxidectin treated horses 16 hours following administration to 160 hours post moxidectin administration, where the abundance of formate and ethanol differed.
Interestingly 5-HT has been associated with the onset of laminitis (Bailey and Elliott, 1998a), however a significant amount of 5-HT would be required in the blood stream to act as a vasoconstrictor and increase the risk of the onset of laminitis (Bailey and Elliott, 1998b). Previous studies have noted that some horses have had bouts of laminitis after anthelmintic treatment (Perymans et al., 1991; Hood, 1999; Heymering, 2010). Anthelmintic treatment was also identified by Wylie et al. (2013) as a risk factor for laminitis within four weeks of administration. No signs of colic or laminitis were identified in the horses used in the present study, however metabolites associated with laminitis were detected in the in vitro fermentations. The findings of this present study warrant further investigation of the link between moxidectin administration and a significant increase in metabolites that are also associated with intestinal disease. This could be achieved readily with the in vitro gut model described by Leng et al. (2017).

Inter-horse variability has previously been identified as a problem for microbiota profiling. Escalona et al. (2015) found that when profiling the Thoroughbred racehorse metabolome that there was little inter-horse variability within urine samples; the present study confirms this observation in a polo pony population. Inter-horse variability was more likely in faecal samples than other biofluids (Escalona et al., 2015). In the present study there was some inter-horse variability within the faecal samples, which is consistent with previous reports.

Urine was the primary biofluid selected for this experiment, faecal and fermentation sample sizes were small as they only reflected the in vitro fermentation element. Some of the faecal samples failed to generate data, thus limiting analysis of $^1$H NMR for faecal water.

$^1$H NMR is not a truly quantitative method of determining metabolite concentrations. It is possible that the sample sizes for faeces and fermentations within this experiment were too
small to determine significant differences in metabolite concentrations that reflect the difference in abundances detected within the OPLS-DA models.

The differences in metabolite abundances in the hay fermentations 16 hours post moxidectin administration suggest some alterations in bacterial metabolism in these fermentations. As there were no differences in metabolite abundances within the control group over any of the sampling time points it is reasonable to infer that the changes seen here were associated in some way with moxidectin administration.

7.5. Conclusions

The findings of this study suggest that moxidectin administration did not alter the faecal or urinary metabolome of treated horses at the time of treatment or over 160 hours after treatment. *In vitro* hay fermentations, inoculated with faeces collected from animals 16 hours post treatment did show significant differences compared to models inoculated with faeces of untreated animals. These *in vitro* findings suggest that moxidectin treatment may have a significant functional effect on the equine faecal microbiome.
Chapter 8.0

General Discussion

The first two aims of this thesis were to characterise aspects of efficacy of two of the three anthelmintic classes licensed for equine nematode treatment. Specifically, egg reappearance times for the macrocyclic lactone anthelmintics and to identify if ovicidal activity of BZs may still be useful in the face of resistance. Previous reports by Lester et al. (2013), Stratford et al. (2013) and Relf et al. (2014) suggested that anthelmintic resistance was not present in cyathostomins to the macrocyclic lactone group in both Thoroughbreds and leisure horses. However these studies did report a reduction in the expected egg reappearance period. More recently Tzelos et al. (2017) also reported a reduction in the egg reappearance times following macrocyclic lactone treatment. All of these findings support the findings of the study reported here. There appears to be a shortened egg reappearance time for the macrocyclic lactones when treating horse strongyles in the UK.

The reports by Lester et al. (2013), Stratford et al. (2013) Relf et al. (2014) and Tzelos et al. (2017) are all comparable as they all use the same study design. However many other studies that have previously evaluated anthelmintic efficacy are difficult to compare due to the lack of standardisation for its measurement. One of the fundamental problems with efficacy testing in equine parasitology is that all of the guidance to date has been adapted from ruminant models. This can be problematic when applying to premises with very few animals, which is often how horses are kept. Both Coles et al. (2006) and Nielsen et al. (2013a) have identified in the WAAVP and AAEP guidelines that efficacy testing should be on a minimum of six horses. The requirements for efficacy testing reflect the statistical power of the calculation used. This should not be problematic for farms of horses with high stocking densities similar to livestock farming. However this is much more difficult on premises with very few horses. For the
purposes of identifying shortened egg reappearance times the findings in chapter 3 for groups of horses over six were comparable to groups made up of less than six. While this model would lack statistical power for detecting resistance it does provide a useful tool for assessing the egg reappearance time in horses on premises with less than six animals.

Updated WAAVP guidelines are awaited, to date there is no provision in the WAAVP guidelines for egg reappearance testing. The AAEP guidelines (Nielsen et al., 2013a) identify expected egg reappearance times and suggest the use of a modification of the FECRT. In chapter 3 of this thesis multiple metrics were used for the same data to calculate a more robust measure of egg reappearance times. Further work would be required in an experimental setting to validate the use of multiple measures of calculating egg reappearance times, however the approach described in chapter 3 does provide a more robust approach than some of the previously reported methods (Little et al., 2003; Lyons et al., 2008; Molento et al., 2008).

The use of combination anthelmintics in sheep has been modelled and trialled to preserve efficacy and slow down the speed of resistance (Bartram et al., 2012; Leathwick et al., 2012). The experiment in chapter 4 was designed to identify whether the novel ovicidal activity of the benzimidazoles was retained in populations of BZ resistant cyathostomins. If the ovicidal efficacy remained, there would be the potential to use BZ as part of a combination anthelmintic to target cyathostomins. One of the limitations to the interpretation of the findings in chapter 4 is the lack of data on ovicidal efficacy and how long this is retained after administering fenbendazole. However when compared to previously reported cattle findings (Miller and Morrison, 1992) the data from chapter 4 suggests there is a reduced ovicidal effect in BZ resistant cyathostomin populations. In a recent report Lyons et al. (2016) identified combination of BZ, THP and piperazine we unsuccessful for strongyle control. Taken together the findings of chapter 4 and Lyons et al. (2016) suggest that it is unlikely that the ovicidal element of BZ can be incorporated into combination anthelmintics for horses.
The findings of our investigation into egg reappearance times for the ML group of anthelmintics is concerning with regards to their efficacy. This group of anthelmintic has played an important part of parasite control in horses for three decades. Recent findings of Peachey *et al.* (2017c) have identified that P-glycoproteins play a role in macrocyclic lactone resistance in cyathostomins. The findings from Peachey *et al.* (2017c) also suggest that P-GP inhibitors maybe a useful tool to increase drug sensitivity when combined with ivermectin administration.

The increase in the availability and decreased cost of next generation ‘omics technologies has facilitated our understanding of the horses’ hindgut microbiota. Older studies reliant on culture work and bacterial counts identified that changes e.g. to diet can lead to changes in the composition of microbiota which can result in intestinal disease. Similarly changes in the composition of hindgut microbiota can reflect alterations in fermentation, as strict fibrolytic species only function in conditions greater than pH 6. Changes in bacterial composition are often linked to pH shifts, specifically a reduced pH leads to proliferation of gram positive *Bacillus-Lactobacillus-Streptococcus* group of bacteria. A previous report on the effect of anthelmintic administration on equine hindgut microbiota identified changes in pH and composition from bacterial counts following treatment (Jacotot *et al.*, 2004).

The finding from chapters 5, 6, and 7, collectively suggest that moxidectin administration briefly altered bacterial metabolism reflected in fermentation changes and metabolite abundances from the *in vitro* model. However there were no changes in faecal microbiota community profile associated with moxidectin administration.

One of the problems with the metataxonomics approach is that by profiling the 16S rRNA gene, function of bacterial communities is not measured. A systems biology approach would be to apply full metagenomics and map gene expression. This approach would allow identification
of altered bacterial function following moxidectin administration as suggested by the fermentation kinetics and the metabolite abundances presented in this thesis.

The next generation ‘omics approach is still in its infancy. Interesting findings reported by Taxis et al. (2015) suggest that while individuals may have differing metataxonomic profiles, when gene expression was mapped the functional output was the same. Thus a focus on changes in community profile might not always reflect changes in the microbiome.

8.1. Parasites and microbiota

The complex relationship between microbiota and macrobiota may be key in our understanding of parasitic infection. Previous equine studies (Klei and Chapman, 1999) have identified Th-2-type response to cyathostomin burdens in horses. This response has been identified in other animal models (Helmby, 2009). It would appear helminth infections lead to a Th-2-type immune response that helminths are able to survive (Helmby, 2009). In mice Grencis (2001) reports that host genotypes which expressed a Th-1-type response to helminth burdens had increased susceptibility to parasitic infection. Thus a combination of immune response and novel immune evasion strategies allow parasite survival (Grencis, 2001). This response has been identified for both adult and immature strongylids (Cantacessi et al., 2012). Where the parasite infestation is chronic a TH1-type response is observed but with significant changes to the immune response to the helminths allowing their survival (Cantacessi et al., 2012).

In rodents, induced parasite burdens lead to changes in the composition of microbiota and alterations in carbohydrate metabolism. However anti-parasite treatment reversed these changes to reflect composition and metabolism prior to infection (Reynolds et al., 2015). Similar findings have also been reported in strongylid parasites of small ruminants (Li et al., 2016). In our study we identified that low parasite burdens did not influence the composition of microbiota. Recent findings of Peachey et al. (2017b) identified changes in the composition
of microbiota in horses harbouring high cyathostomin burdens. Collectively these findings suggest that high cyathostomin burdens may influence the composition of gut microbiota. This may be linked to the more complex immune responses that have been identified previously, as the microbiome plays a role within the immune system.

Microbiota and macrobiota have co-evolved to co-inhabit the horse’s hindgut. The compulsion of horse-owners to eradicate nematodes to reduce the risk of intestinal disease does not take into account the potential benefits of a low parasite burden. The hygiene hypothesis suggests that being exposed to potential pathogens may have health benefits. In western countries where hygiene is good, there are greater incidences of allergic diseases that are not seen in developing countries that have less well developed hygiene practices (Wills-Karp et al., 2001; Okada et al., 2010). In the horse a previous epidemiological study identifying risk factors for equine grass sickness identified horses with a low strongyle burden were at greater risk of the disease (McCarthey et al., 2004). Strongyle parasite infection appears to confer some resistance to equine grass sickness.

From all of the evidence to date it would appear the high burdens of cyathostomins are potentially pathogenic in the horse. However, scientists and vets are currently unable to answer the question: “what constitutes a high parasite burden.” Previous studies indicate that high burdens defined by FEC are “best guess” figures (Uhlinger, 2007). Until a quantitative, equine cyathostomin ELISA test is commercially available to identify pre-patent infection, the FEC remains the gold standard for estimating cyathostomin burdens in horses (Mitchell et al., 2016).
8.2. The future of equine parasite control?

Developments within diagnostic parasitology have been slow to emerge. However a recent study has identified that faecal egg counting via a mechanised system and smartphone application provided increased precision and comparable accuracy to the McMaster technique which is most commonly used in practice (Scare et al., 2017). This technology would allow field use of diagnostics but interpretation of these tests by owners is a barrier to implementing this technology. Furthermore this is an update of a historic approach to parasite diagnosis which lacks sensitivity and relies on patent infection to predict parasite burden.

To date the study presented in chapter 7 is the first to use an NMR-based metabolomics approach to investigate equine parasite control. In our study we were specifically looking for changes in microbial metabolism following anthelmintic treatment. This approach has recently been applied in humans in a study which identified differences in metabolite abundances of patients with parasite burdens when compared to uninfected controls (Kostidis et al., 2017).

The recent findings of Peachey et al. (2017b) support the theory that macrobiota do influence the composition of microbiota within the microbiome. The works of Reynolds et al. (2015) reported that parasitised mice showed increases in Firmicutes specifically Lactobacilli spp. As parasite burdens increased Lactobacillaceae also increased, suggesting a mutualistic relationship. The functional fermentation changes identified in chapter 5 may also be identified in untreated parasitised animals if parasites do alter microbiome composition, this has potential to alter forage fermentation if there are changes in Lactobacilli spp. abundances. This requires further investigation.
Much attention is currently being paid to the intestinal microbiome and effects of different conditions or treatments on it. However the next step is to consider the “nemabiome”. In 2015 Avremenko et al. used a next generation sequencing approach to amplify rDNA ITS-2 using NC1 and NC2 primers complementary to 5.8S and 28S coding sequences of nematodes of cattle. This was the first study to introduce the concept of the nemabiome to describe the parasitic community present within a host (Avremenko et al., 2015). This approach is complex due to large families of genes with similar genetic structures, large population sizes with high levels of sequence polymorphisms and the difficulty of accessing parasitic stages that occur within the host (Wit and Gilleard, 2017). However genome reference libraries are now available for referencing the nemabiome. The nemabiome has recently been used to identify the onset of anthelmintic resistance to macrocyclic lactones within cattle nematodes (Avremenko et al., 2017). Thus ‘omics technologies provide a new and valuable tool to allow a much greater understanding of parasite biology, host parasite interaction and markers for anthelmintic resistance.

The interaction with parasites and the microbiome leaves many further questions to be answered. In this thesis we demonstrated that while moxidectin did not alter the community profile of faecal microbiota it did influence the metabolism of faecal microbiota for a short period reflected by metabolite abundances and fermentation kinetics in vitro. Both Nielsen et al. (2013b) and Betancourt et al. (2015) suggest that the macrocyclic lactones appear to promote an anti-inflammatory effect within the intestine, an effect previously identified in mice (Yan et al., 2011). Macrocyclic lactones have also been identified as risk factors for the onset of colic (Hillyer et al., 2002) and the onset of equine grass sickness (McCarthey et al., 2004). These findings, taken together, suggest a complex relationship between macrobiota, microbiota, host, and drug treatment that may be possible to explore further in the future using these next-generation ‘omics technologies.
8.3. Parasitism, anthelmintic treatment and intestinal disease

Studies to date provide a wealth of evidence that cyathostomin burdens, especially large burdens are associated with colic in horses (Duncan and Love, 1991; Love, 1992; Kaenne et al., 1997; Love et al., 1999; Cohen et al.; 1999; Hillyer et al., 2002; Matthews, 2008). The mechanism of the link between anthelmintic treatment and colic that has previously been identified (Proudman 1991; Cohen et al., 1999; Hillyer et al., 2002) remains poorly characterised. The mechanism leading to colic following anthelmintic treatment is likely to be multifactorial. The previous mechanisms proposed including changes in gut motility and parasite death may all play a role (Bueno et al., 1979; Love, 1992; Proudman, 1999).

8.4. Future study

The findings of the in vitro fermentation kinetics and metabolomics studies require further investigation with a larger sample size to confirm whether anthelmintic administration has a significant effect on microbial metabolism. Ideally this should be implemented using full metagenomics rather than metataxonomics. Furthermore the findings of Peachey et al. (2017b) and Kostidis et al. (2017) suggest that cyathostomin burdens do modify the hindgut microbiome and that changes in the metabolome associated with parasitism can be identified using NMR. Thus the next stage in this research should be to identify the effects of anthelmintic treatment on cyathostomin burdens of different magnitude through faecal and urinary metabolomics and fermentation kinetics. This will help to determine the associations between, parasitism, anthelmintic treatment and intestinal disease.
9.0 Conclusions

The role of cyathostomins in equine colic is likely to be multifactorial, however the evidence to date suggests that it is important to prevent large cyathostomin burdens in horses to reduce the risk of intestinal disease.

The egg reappearance time, therefore efficacy, of the macrocyclic lactones in horses is decreasing. This is concerning as moxidectin is one of the two anthelmintics which is effective against the most pathogenic stage of cyathostomins. Benzimidazole resistance has been reported as widespread for some time, the conclusion of chapter 4 is that a novel element of this anthelmintic, its ovicidal effect, is short lived in the face of BZ resistant strongyles.

The conclusion of chapters 6 and 7 is that anthelmintic treatment did not alter the composition of faecal microbiota, nor did it alter the urinary metabolome. However in vitro gas production was used to identify significant changes in feed fermentation kinetics, cumulative gas pool and the rate of fermentation, directly following moxidectin administration (chapter 5). From the fermentations, metabolomics identified differences in abundance of metabolites previously identified as associated with colic and laminitis. Whilst no clinical symptoms were seen in any of the horses in these trials this data suggests functional changes in the metabolome and fermentation activity directly following moxidectin administration. Previous reports have speculated that changes associated with parasite death and changes in gut motility may play a role in colic following anthelmintic treatment. Studies reported in this thesis suggest that changes in the metabolome may also play a role.

The findings from these studies provide progress in unravelling the complex relationship between parasite, host and microbiota in intestinal disease in horses. Further development of ‘omics technologies will allow further exploration of this complex relationship.
List of References


Central European Institute of Technology. www.ceitec.eu/ [accessed 15/10/17].


Github. www.github.com [accessed 14/10/17].


Human Metabolome Database www.hmdb.ca/

Institute of Chemistry www.chem.ch.huji.ac.il/ [accessed 15/10/17]


Sketch map, www.sketchmap.co.uk. [accessed 20/07/13]


Appendix

Research outputs from this thesis:

**Peer reviewed papers**


These papers are enclosed on the following pages.

**Peer reviewed conference – oral presentations**

