High-starch diets increase behavioural reactivity, alter hindgut microbiota and brain neurochemistry in horses

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Abstract

The digestive tracts of animals are home to bacterial communities comprising trillions of individual microorganisms. The community of microorganisms residing in the gut is the gut microbiota. Studies have shown that a symbiotic gut microbiota is intrinsic to the overall health and wellbeing of the individual. As well as influencing an animal's overall health the gut microbiota affects central nervous system functioning and as such could play a role in influencing behaviour. The primary contribution of this thesis is to explain the role played by diet in altering behaviour through changes in gut microbiota and brain neurochemistry.

Anecdotal evidence suggests that horses receiving starch in their diets display increased behavioural reactivity. The addition of starch to the equine diet also results in changes to hindgut microbiota. This thesis aims to: investigate the effects of a high-starch diet on the behaviour of young, unhandled ponies, describe the microbial community composition of faecal samples and different regions of the hindgut in response to diet, and explore changes in serotonin and dopamine receptor densities in brain and gut tissues in relation to diet.

The use of young unhandled ponies is an important aspect of this study. Their inexperience meant that their responses to behavioural tests were natural, untrained reactions. The gut microbiota of the ponies had also not been exposed to the dietary changes experienced by mature horses. Therefore, this study gives a true reflection of the behavioural and hindgut microbial responses to a high-starch diet.

This thesis first looks at the behaviour of young, unhandled ponies when receiving a high-starch (HS) or high-fibre (HF) diet. 16S *rRNA* gene sequencing was then used to describe changes in the faecal microbial community composition in relation to diet. The ponies were found to be behaviourally more reactive on the HS diet compared to the HF diet. There were also differences in faecal microbial community structure related to diet. A reduction in Ruminococcaceae bacteria and an increase in *Streptococcus* bacteria was evident in the faecal microbiota of ponies fed the HS diet.

The microbial community composition of each hindgut region was then explored in relation to diet using 16S *rRNA* gene sequencing. Comparisons were made between faecal samples collected post-mortem and faecal samples collected during behavioural testing. For the HS and HF diets, the microbial community composition differed significantly in the ventral colon, dorsal colon and small colon regions of the hindgut. The HS diet resulted in a decrease in Ruminococcaceae-*Oscillospira* bacteria and an increase in *Streptococcus* in the dorsal and small colon regions. These changes were also observed in the faecal samples collected post-mortem and the faecal samples collected during behavioural testing.

The effects of diet on the relative expression of serotonin and dopamine receptor densities in the gut and brain tissues were explored using real-time polymerase chain reaction (qPCR). There was a greater relative expression of dopamine D2-like receptor densities in the caudate region of the brain for the HS diet compared to the HF diet and for the right-hand side of the brain compared to the left. There was lower relative expression of dopamine D2-like receptor densities for the nucleus accumbens region of the brain for the HS diet and for the right-hand side of the brain.

This study has shown that feeding horses starch results in increased behavioural reactivity. The addition of starch to the diet also alters the microbial community composition of more caudal regions of the hindgut and is associated with neurochemical changes in specific regions of the brain. These results lead us to question whether we should feed starch-based feeds to the horses in our care. Particularly when the energy demands of working horses could be met by other food sources.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature

Printed Name

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Abbreviations

5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytyptophan
ACTH	Adrenocorticotropic hormone
ANOSIM	Analysis of Similarity
BW	Body weight
CA	Caecum
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
DC	Dorsal colon
FAPM	Faecal post-mortem
FCP	Free-choice profiling
FWS	Faecal water syndrome
GABA	Gamma-aminobutyric acid
gDNA	Genomic deoxyribonucleic acid
GLM	Generalised linear model
GLMM	Generalised mixed effects model
GPA	Generalised Procrustes Analysis
HF	High-fibre
HPA	Hypothalamic pituitary adrenal
HS	High-starch
IBS	Irritable bowel syndrome
IFN-γ	Interferon-*Gamma*
NGS	Next generation sequencing
NMDS	Non-metric multidimensional scaling
NTC	Non-template control
OTUs	Operational taxonomic units
PCA	Principle component analysis
PM	Post-mortem

QBA	Qualitative behavioural analysis	**SSRI**	Selective serotonin reuptake inhibitor
qPCR	Real-time polymerase chain reaction	**Tm**	Primer melting temperature
rRNA	Ribosomal ribonucleic acid	**TNF-α**	Tumour necrosis factor-*Alpha*
s.d.	Standard deviation	**VC**	Ventral colon
SC	Small colon	**VFAs**	Volatile fatty acids
SCFAs	Short-chain fatty acids		
SERT	Serotonin reuptake transporter		

Chapter 1

1.1 Gut microbiota

In higher vertebrates, the majority of cells found in the body are not actually those belonging to the host. For example, in humans approximately 90% of the cells found in the body are prokaryotic. Most of these prokaryotic, bacterial cells reside in the gut, with the human gastrointestinal tract estimated to contain over 10 trillion individual microorganisms (Cryan and Dinan, 2012; Clarke *et al.*, 2014). The community of microorganisms residing in the gut is the gut microbiota and the genetic material that makes up that microbial community is the microbiome (Cryan and Dinan, 2012; Evans *et al.*, 2013).

Research into gut microbiota and the microbiome has developed into an area of significant scientific interest. Studies have investigated the wider physiological influence that gut microbiota has on the overall health and well-being of the individual (Sudo *et al.*, 2004; Kaakoush *et al.*, 2012; Sonnenburg and Sonnenburg, 2014; Bruce-Keller *et al.*, 2015; Tanaka *et al.*, 2017). For example, specific bacterial taxa have been associated with the development of the hypothalamic-pituitary-adrenal axis, and gut dysbiosis has been associated with the pathogenesis of Crohn's disease (Sudo *et al.*, 2004; Kaakoush *et al.*, 2012). It is becoming evident that the gut microbiota play a significant role in physiological function and are far more influential than first realised (Evans *et al.*, 2013).

The microbial community composition of an individual's gut microbiota is influenced by several intrinsic and extrinsic factors including, but not limited to; birthing mode, genetics, age, antibiotic treatments and diet (Palmer *et al.*, 2007; Kashyap *et al.*, 2013; Maukonen and Saarela, 2015; Tidjani Alou *et al.*, 2016; Angelakis and Raoult, 2018). These factors shape the microbial community structure of the gut and lead to differences in microbiota between individuals of the same species. Like other healthy ecosystems, the communities of microorganisms inhabiting the gut form stable, symbiotic relationships with each other and with their host. Although variation between individuals exists, the composition of the gut microbiota in healthy animals is generally balanced and there are commonalities, particularly within host species (Scott *et al.*, 2013;

Marques *et al.*, 2014). For example, in human adults the microbial populations of the gut predominantly comprise bacterial taxa from two main phyla; Bacteroidetes and Firmicutes (Evans *et al.*, 2013). There are also core groups of taxa that tend to be present or absent depending on an individual's diet and environment (Tidjani Alou *et al.*, 2016). For example, the gut microbiota of humans consuming high-fibre diets show an increase in *Roseburia* and *Blautia* bacterial taxa compared to those consuming diets lower in fibre (Tidjani Alou *et al.*, 2016).

There has been a long-term awareness of the relationship between diet and health. Research is now showing that the gut microbiota is at the centre of that diet-health relationship. While many extrinsic factors affect the specific composition of an individual's gut microbiota, in adults it is largely influenced by diet (Cryan and Dinan, 2012; Luna and Foster, 2015). In humans, the type of macronutrient that dominates a diet has a direct effect on which bacterial populations make up the gut microbiota (Scott *et al.*, 2013; Tidjani Alou *et al.*, 2016). For example, a diet of "processed food", commonly lacking in fibre, results in reduced gut microbial diversity (Sonnenburg and Sonnenburg, 2014; Sonnenburg *et al.*, 2016). Research has shown that dietary fibre is essential for gastrointestinal health in humans (Brownawell *et al.*, 2012). In humans, better overall health and a highly diverse gut microbiota are positively linked to increased dietary fibre intake (Sonnenburg and Sonnenburg, 2014).

While a stable, diverse gut microbiota is associated with good health, disruption to the composition of an individual's gut microbiota is associated with chronic conditions such as obesity, diabetes and inflammatory bowel diseases (Kaakoush *et al.*, 2012; Nyangale *et al.*, 2012; Mancabelli *et al.*, 2017). Gut dysbiosis is a breakdown of the symbiotic balance of bacterial communities residing in the gut (Kaakoush *et al.*, 2012). Modern human diets which include "processed foods" have been implicated in the increased incidence of several health conditions (Sonnenburg and Sonnenburg, 2014). Moreover, the effects of diet on the gut microbiota and consequently on an individual's health extend beyond dietary related health conditions to neurological dysfunction.

There are often associations between gastrointestinal health conditions and mental health conditions, including anxiety and depression (Holzer et al., 2012).

Research has found associations between diet, gut dysbiosis and neurological dysfunction (Bruce-Keller *et al.*, 2015). A high-fat diet, which is becoming more commonplace in human society, has been shown to increase neural oxidative stress and inflammation, thereby increasing the risk of dementia (Zhang *et al.*, 2005; Ribeiro *et al.*, 2009). Consumption of trans-saturated fats has been found to increase the risk of Alzheimer's disease (Morris *et al.*, 2003). However, some aspects of diet have been shown to counteract the negative effects of dietary-related cognitive conditions. The administration of vitamin E to rats receiving a high-fat, high-carbohydrate diet prevented memory impairment and reduced oxidative stress in the brain (Alzoubi *et al.*, 2013). It was suggested that these benefits may be as a direct result of the strong antioxidant properties of vitamin E (Alzoubi *et al.*, 2013). Coffee has also been shown to have positive neurological effects, with coffee-supplemented rats performing better in psychomotor and memory tasks (Shukitt-Hale *et al.*, 2013). It was suggested that the bioactive compounds found in coffee, rather than the caffeine, may help to reduce the cognitive deficits associated with ageing (Shukitt-Hale *et al.*, 2013).

1.2 Gut-brain axis

Given the strong links between diet, the gut microbiota and an individual's overall health and wellbeing it is not surprising that growing scientific interest is focusing on the relationship between the gut and brain. A two-way communication system exists between the gut and the brain and it is commonly referred to as the gut-brain axis. The gut-brain axis communication system is comprised of several communication pathways (Figure 1.1). These include direct communication via neural pathways and, indirect communication pathways that include the neuroendocrine and immune systems (Cryan and Dinan, 2012; Evans *et al.*, 2013; Clarke *et al.*, 2014; O'Mahony *et al.*, 2015). The organs and systems associating gut-brain axis communication pathways are thought to work together as a multidirectional network in order to maintain homeostasis (El Aidy *et al.*, 2014). Increasing evidence indicates that the microbial populations residing in the gut play a pivotal role in influencing gut-brain axis communication pathways (Cryan and Dinan, 2012). This has led to the emerging concept of the microbiota-gut-brain axis (Cryan and Dinan, 2012).

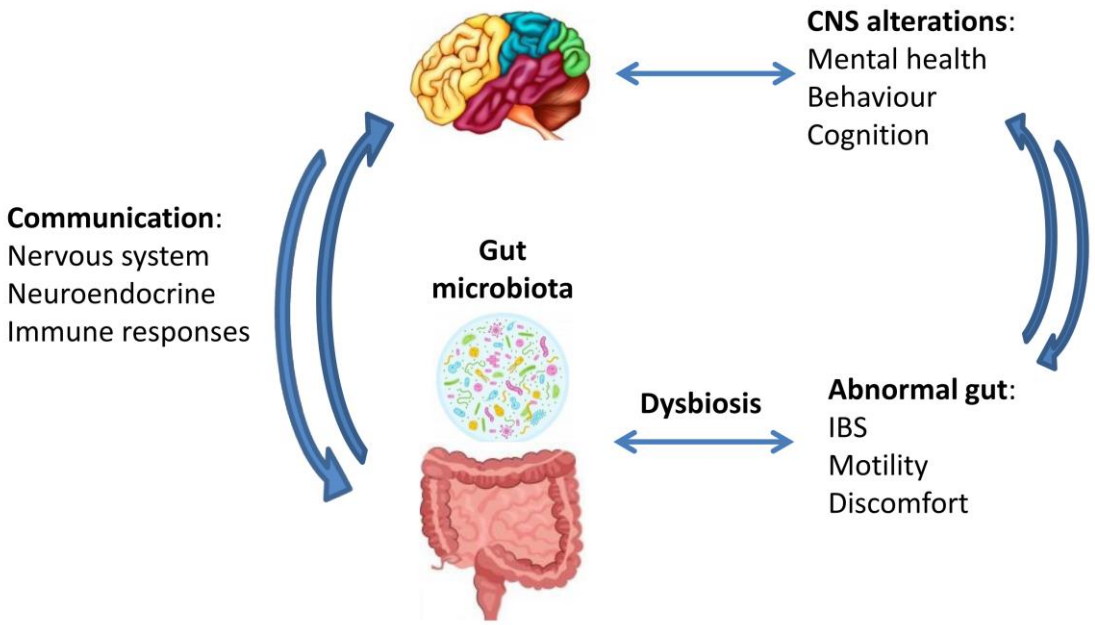

Figure 1.1 - Gut brain axis communication pathways

The influence of the gut microbiota is thought to extend to the functions of the central nervous system and influence an individual's behaviour (Cryan and Dinan, 2012; Clarke *et al.*, 2014; Williams *et al.*, 2014). The role of gut microbiota in influencing behaviour through gut-brain axis communication pathways is developing into an area of significant scientific interest. The ways in which the gut microbiota influences behaviour involves several mechanisms and is yet to be fully understood (Cryan and Dinan, 2012). The vagus nerve is a major nerve of the autonomic nervous system and a direct communication link between the gut and brain. The vagus nerve regulates the function of several organs as well as conveying sensory information from the organs back to the CNS (Cryan and Dinan, 2012). As such, the vagus nerve would appear to be the primary route for gut-brain axis communication. However, a study using vagotomised mice found that the gut microbiota worked independently of the autonomic nervous system to influence brain chemistry and behaviour (Bercik *et al.*, 2011). This confirms that a network of communication pathways is involved in gut-brain axis communication.

Research has shown associations between behaviour and an increased immune response (Cryan and Dinan, 2012). Mice with parasitic induced gut inflammation showed increased anxiety-like behaviour (Bercik *et al.*, 2010). Results showed increased levels of the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Bercik *et al.*, 2010). The administration of

anti-inflammatory agents reversed the behavioural response, as did inoculating the gut with *Bifidobacterium longum*. Although both interventions reversed the behavioural response, only the anti-inflammatory agents reduced the pro-inflammatory cytokine levels. These results suggest that the behavioural response of the mice was potentially being influenced through more than one communication route and may be related to the change in gut microbiota.

Research has also shown associations between gut microbiota and neuroendocrine communication pathways. Studies using rodents have found associations between gut microbiota and early behavioural development (Sudo *et al.*, 2004). When germ-free mice were exposed to stress, they released increased levels of adrenocorticotrophic and corticosterone hormones compared to mice with conventionally colonised gut microbiota (Sudo *et al.*, 2004). This enhanced stress response was reversed following colonisation of the gut with *Bifidobacterium infantis* (Sudo *et al.*, 2004). These results highlight the importance of gut microbiota in the early development of the hypothalamic-pituitary-adrenal axis and an individual's ability to cope with stress. In germ-free rats the absence of gut microbiota was also shown to exacerbate the rats' response to stress (Crumeyrolle-Arias *et al.*, 2014). Following the stress of post-natal maternal separation, colonising the guts of rats with *B. infantis* was shown to reduce the behavioural and biochemical stress responses (Desbonnet *et al.*, 2010).

The gastrointestinal tract produces more than 20 different hormones making it the largest endocrine organ in the body (Murphy and Bloom, 2006). Hormones released from the gut communicate with the brain, stimulate and suppress the release of numerous other hormones and thereby influence other organs and systems (Murphy and Bloom, 2006; Clarke *et al.*, 2014). Several hormones that can be produced by the gut microbiota also act as neurotransmitters, including serotonin and dopamine (Cryan and Dinan, 2012; Clarke *et al.*, 2014). These neurotransmitters are involved in several physiological functions including, locomotion, gastrointestinal motility, platelet function and vascular tone, and play a significant role in the behavioural responses of an individual.

Studies have demonstrated that the biosynthesis of neurotransmitters can be modulated by gut microbiota (Williams *et al.*, 2014; Reigstad *et al.*, 2015; Yano

et al., 2015). In rats, *B. infantis* bacteria were shown to elevate peripheral levels of the amino-acid tryptophan which is involved in the biosynthesis of serotonin (Desbonnet *et al.*, 2009). *B. infantis* has potential antidepressant properties and has been shown to be more effective than a commercially available antidepressant drug at reducing stress-related behaviours in mice (Desbonnet *et al.*, 2009; Savignac *et al.*, 2014).

Other bacterial taxa have also been shown to influence behaviour. Following colonisation of the gut with *Lactobacillus rhamnosus,* mice showed alterations in γ-aminobutyric acid (GABA) receptor expression in the brain and reduced anxiety and depression (Bravo *et al.*, 2011). Overall, the research undertaken in rodents has increased our understanding of the association between gut microbiota and neurological conditions. Studies using germ-free mice have allowed the wider influence of the microbiota on the individual to be explored. They allow experimental control for exploring the effects of specific bacterial populations on behaviour and gut-brain axis communication.

While it is clear that diet has a significant role to play in both cognitive function and behaviour, the precise mechanisms associating these changes are not always clear. The overflow of undigested carbohydrates into the large intestine has been associated with increased anxiety and aggression in rats (Hanstock *et al.*, 2004). These changes in behaviour were thought to be related to digestive discomfort. However, a study using mice found that the composition of the microbiota had an influence on the development of behaviour (Neufeld *et al.*, 2011a). Another study demonstrated that when the microbiota associated with a high-fat diet was transplanted into mice, it was found to be capable of disrupting the physiology and function of the brain (Bruce-Keller *et al.*, 2015). It is likely that the alterations to gut microbiota and the associated cognitive and behavioural changes outlined in rodents will occur in other species. Horse owners have anecdotally suggested that high-starch diets result in increased behavioural reactivity. However, there is only a small body of scientific evidence to substantiate this and the mechanisms behind dietary induced behavioural changes are yet to be understood (Bulmer *et al.*, 2015).

1.3 The equine diet

Horses have evolved to browse and graze and are well suited to high-fibre, low-starch diets (Harris, 2005; Henderson, 2007; Hill, 2007; Benhajali *et al.*, 2009). As a non-ruminant herbivore, the horse relies largely on the bacterial fermentation of structural carbohydrates in the large intestine to provide energy (Costa and Weese, 2012; Daly *et al.*, 2012; Dougal *et al.*, 2012; Steelman *et al.*, 2012). The equine digestive tract has evolved to digest high-fibre diets ingested through prolonged grazing (Willing *et al.*, 2009). The equine large intestine makes up approximately two thirds of the digestive tract and contains an abundance of microorganisms (Daly *et al.*, 2001; Biddle *et al.*, 2013).

The fermentation of structural carbohydrates in the large intestine yields volatile fatty acids (VFAs), referred to in humans as short-chain fatty acids (SCFAs). In the wild, VFAs would provide horses with the majority of their energy (Daly *et al.*, 2001). Therefore, the complex microbial community of the equine hindgut is of great importance to the health and wellbeing of the horse (Daly *et al.*, 2012; Steelman *et al.*, 2012). Horses have evolved to trickle feed on high-fibre diets. However, the dietary management of domestic horses is often far removed from that of their wild counterparts. Domestic horses commonly have limited foraging opportunities and the fibre component of their diet is often restricted.

In the domestic horse's diet the reduction in forage is substituted with concentrates, often in the form of high-starch cereal grains. Horse owners generally believe that these changes are necessary to provide the additional energy required for work. However, horses have a limited capacity for digesting starch. Evolution has shaped the horse's digestive system for digestion of fibre and has resulted in a reduced transit time through the small intestine (Julliand *et al.*, 2006). The horse also has relatively low concentrations of α-amylase in the gut which greatly reduces their capacity to digest starch (Al Jassim *et al.*, 2005; Julliand *et al.*, 2006).

A high-starch diet is unnatural for horses' systems and not what they have evolved to digest. Feeding high-starch diets to horses may have become more common over time. Historically, when horses were used as work animals there would have been limited opportunities for grazing. Horses were likely to have

been working for most of the day. Therefore, there will have been a need to provide them with energy dense meals that could be eaten quickly. It is possible that we still associate the idea of horses that work requiring this type of diet. However, very few horses in today's society will be working for these extended periods and grazing opportunities are generally plentiful. A trickle-fed, high-fibre diet would likely provide adequate nutrition and enough energy to fulfil the modern, leisure horse's requirements.

1.4 Equine hindgut microbiota

Having not evolved to consume high-starch meals, there is a risk that some undigested starch may overflow into the hindgut (Julliand *et al.*, 2006). Once in the hindgut, starch is fermented and reduces pH (de Fombelle *et al.*, 2003; Al Jassim *et al.*, 2005; Julliand *et al.*, 2006). To reduce the risk of undigested starch entering the hindgut a starch intake of no more than 2g/kg of bodyweight (BW) per meal is recommended (National Research Council, 2007). The overflow of starch into the hindgut has been shown to alter the microbial environment by reducing pH (Medina *et al.*, 2002; de Fombelle *et al.*, 2003; Al Jassim *et al.*, 2005; Julliand *et al.*, 2006; Vervuert *et al.*, 2009). Additionally, the hindgut of horses fed high-starch diets is less efficient at digesting hay compared to horses consuming mostly hay because of the alterations in microbiota (Julliand *et al.*, 2006). A reduction in fibrolytic bacteria reduces the ability of the gut to breakdown structural carbohydrates and therefore in theory may increase the risk of impaction colic. Forage diets that are similar to natural feeding allow the microbial populations of the hindgut to maintain a more natural balance (Willing *et al.*, 2009; Santos *et al.*, 2011).

Understanding of the structure and role of equine hindgut bacterial communities still remains limited (Costa and Weese, 2012). However, with greater access to modern genomic sequencing platforms an increasing number of studies are characterising the microbial populations of the equine hindgut (Daly *et al.*, 2001; Willing *et al.*, 2009; Daly *et al.*, 2012; Dougal *et al.*, 2012; Steelman *et al.*, 2012; Biddle *et al.*, 2013; Schoster *et al.*, 2013; van den Berg *et al.*, 2013; Dougal *et al.*, 2014). The characterisation of the microbiota in both symbiotic and dysbiotic states helps to develop a greater understanding of its role in health and disease. By understanding which bacterial taxa are associated with

health or disease we can reduce the incidence of ill health and promote higher welfare standards in our domestic horses.

Firmicutes is the most dominant phylum among healthy horses (Costa *et al.*, 2012; Shepherd *et al.*, 2012; Steelman *et al.*, 2012; Biddle *et al.*, 2013). It has been reported to dominate as much as 72% of the sequenced samples in grass-fed horses (Daly *et al.*, 2001), 73% in forage fed horses (Willing *et al.*, 2009), as well as 68.1% (Costa *et al.*, 2012) and 69.2% in healthy horses (Steelman *et al.*, 2012). However, the proportion of the bacterial population dominated by the Firmicutes phylum is significantly reduced when the microbiota of horses with certain health conditions or unnatural diets is explored. Firmicutes has been reported to comprise 56.72% of the hindgut microbial populations in horses with chronic laminitis (Steelman *et al.*, 2012) and only 30.3% in horses with colitis (Costa *et al.*, 2012). It is clear that Firmicutes makes up a much lower percentage of the overall hindgut microbial population in horses with certain health conditions compared to healthy horses. Firmicutes has also been reported to be reduced in horses fed high-starch diets (Willing *et al.*, 2009). This suggests that the microbiota of horses fed high-starch diets shows more similarity to those with certain health conditions rather than healthy horses.

When there is a reduction of one particular phylum, other bacterial taxa may start to account for a greater proportion of the population. This results in an overall shift in the balance of the bacterial populations of the hindgut. In horses with colitis, Bacteroidetes was found to be the most dominant phylum comprising 40% of the bacterial community composition compared to 14% in healthy horses (Costa *et al.*, 2012). This shift in population balance can also be observed related to dietary changes. Bacteroidetes has been reported to comprise 24.7% of the bacterial community composition in concentrate fed horses compared to 13.5% in grass-fed horses (Daly *et al.*, 2012). What is clear is that the balance of the bacterial populations of the hindgut in horses fed concentrate diets appears to be more similar to that of unhealthy horses. This is of particular concern because feeding horses high-starch diets is common practice. However, further investigation is required to understand whether these changes are just microbial adaptations to a new diet with little wider effect, or if they are having a negative effect on the overall health and wellbeing of the animal.

Investigating the microbial populations of the hindgut can be problematic due to the difficulty in obtaining samples. Obtaining samples directly from the hindgut requires euthanased or fistulated horses, both of which raise ethical implications. This can often limit sample sizes of studies which may make them less representative of the wider population. Therefore, it is not surprising that due to ease of sampling, a large number of studies have used faecal samples as a proxy to describe the microbial populations of the equine hindgut (Endo *et al.*, 2009; Willing *et al.*, 2009; Dougal *et al.*, 2012; Shepherd *et al.*, 2012; Steelman *et al.*, 2012; O'Donnell *et al.*, 2013; van den Berg *et al.*, 2013; Dougal *et al.*, 2014). While faecal samples are easy to obtain and appear to be an ideal platform for microbial studies, they are not truly representative of the different regions of the hindgut. Faeces have shown limited microbial similarity when compared to other regions of the digestive tract. One study found the greatest similarity occurring between the faeces and the caecum (Schoster *et al.*, 2013). Another study found that faeces were most similar to the right dorsal colon (Dougal *et al.*, 2012). Although faeces may not indicate where in the hindgut the microbial changes are occurring, it may be enough from a health perspective just to know that there are changes occurring.

Changes in hindgut microbiota have been shown to be directly associated with health in horses. One study induced laminitis through hind-gut acidosis resulting from oral dosing of oligo-fructose (Al Jassim *et al.*, 2005). The characterisation of lactic-acid producing bacterial populations in the aforementioned study provides an insight into the mechanisms behind hindgut lactic acidosis and its pathogenesis in laminitis (Al Jassim *et al.*, 2005). The alterations in hindgut microbiota with oligo-fructose-induced laminitis have also been explored using caecally cannulated horses (Milinovich *et al.*, 2008). This approach allowed regular sampling over a 36 hour period so the onset of the acidosis, and consequent laminitis, could be explored throughout rather than just from an end-point measurement (Milinovich *et al.*, 2008). Both of these studies have offered valuable insights into the effects of hindgut bacterial alterations on the overall health of the host. They also highlight specific bacterial population shifts associated with changes in the health of an animal. However, the laminitis induced in both of these studies was the result of extreme changes in hindgut microbiota. Both studies also raise ethical implications and as a result the samples sizes were limited.

1.5 The equine diet and behaviour

In line with the research conducted in rodents it is likely that, if diet can affect the health of a horse, it may also affect their behaviour. Research has found associations between the domestic equine diet and stereotypic behaviours in horses (Waters *et al.*, 2002; Thorne *et al.*, 2005; Hemmings *et al.*, 2007; Freire *et al.*, 2009; Hothersall and Nicol, 2009; Wickens and Heleski, 2010). One aspect of the domestic horse's diet that has been associated with the development of oral stereotypies is a reduction in forage (Wickens and Heleski, 2010). Horses have a psychological desire to forage which may go unfulfilled in horses receiving a restricted forage ration. As the digestibility of fibre decreases horses have been found to increase their intake (Edouard *et al.*, 2008). They also demonstrate more searching behaviours when fed a reduced fibre diet compared to horses receiving more hay (Elia *et al.*, 2010). Stabled horses are also often seen eating straw bedding when access to forage is restricted (Thorne *et al.*, 2005). Results from these studies suggest that horses will seek out more forage when this component of their diet is restricted. Abnormal behaviours may be redirected from a persistent motivation to forage (Hothersall and Casey, 2012). More natural diets that allow horses to carry out foraging behaviour will clearly be beneficial to the welfare of domestic horses.

A restriction in forage is often accompanied by an increase in concentrate feeds. This dietary change is often associated with working horses and thought to be required as a means of meeting the increased energy requirements (Medina *et al.*, 2002; Julliand *et al.*, 2006; Durham, 2009; Harris and Geor, 2009; Rosenfeld and Austbø, 2009; Willing *et al.*, 2009). While the drive to forage may be one factor behind the development of stereotypic behaviours, these behaviours may also be associated with digestive discomfort (Hemmings *et al.*, 2007; Moeller *et al.*, 2008; Wickens and Heleski, 2010; Hothersall and Casey, 2012). The domestic diet, which often includes meal feeding of concentrates combined with a reduced forage ration, may induce digestive discomfort. Additionally, reduced fibre and increased concentrate diets are associated with an increased risk of gastric ulcers owing to reduced chewing time and less saliva production (Murray *et al.*, 1996; Moeller *et al.*, 2008; Reese and Andrews, 2009; Elia *et al.*, 2010). While there are a number of influential factors associated with the development of oral stereotypies, it is clear from the studies to date that there is a strong

association with the high-starch, reduced forage diet often fed to domestic horses and the development of stereotypies.

The effects of the high-starch, reduced fibre domestic diet on handling and reactivity behaviours in horses are less well documented. A study investigating the effects of diet on recurrent exertional rhabdomyolysis found horses became more reactive and demonstrated increased nervousness and excitability when they were fed a high-starch diet (MacLeay *et al.*, 1999). Horses have also been shown to have higher heart rates during behavioural tests when fed high-starch diets compared to high-fibre diets even when both diets contained the same amount of energy (Bulmer *et al.*, 2015). Diet has also been shown to have positive effects on horse behaviour. Behaviour was quieter and aggression reduced at feeding time when horses' diets contained more hay (Zeyner *et al.*, 2004). Other studies have also found that a reduction in aggression was evident when there was a greater proportion of hay in the diet (Benhajali *et al.*, 2009). It is not only alterations to forage content in diets that have been shown to influence behaviour. High-fat diets have been show to result in horses that were less stressed and calmer than those fed high-starch diets (Redondo *et al.*, 2009). It is possible that the quieter behaviour in these studies may be the result of a digestive system free from discomfort, or from having satisfied the psychological desire to forage.

As previously mentioned, equine research shows that high-starch diets result in changes to hindgut microbiota. The changes in equine behaviour may be the result of dietary induced changes to hindgut microbiota. Studies in humans have found specific bacteria from the Firmicutes phylum could interfere with the biosynthesis of serotonin by reducing tryptophan availability (Williams *et al.*, 2014). While this is considered to be a rare enzymatic activity among bacteria, it is thought that over 10% of the human population carry at least one gut bacterial species that can produce this effect by decarboxylating tryptophan (Williams *et al.*, 2014). If there is a reduction in the availability of tryptophan, this may lead to a decrease in the production of serotonin (Williams *et al.*, 2014). Firmicutes is the most dominant phylum in the equine hindgut so it is possible that species with the ability to interfere with neurotransmitter biosynthesis also exist within the equine gastrointestinal tract.

The bacteria in the gut are intrinsic to the production of serotonin in the enterochromaffin cells (Williams *et al.*, 2014; Reigstad *et al.*, 2015; Yano *et al.*, 2015). Germ-free mice were found to have a 2.8 fold decrease in plasma serotonin levels compared to the control mice which was also accompanied by increased levels of tryptophan (Wikoff *et al.*, 2009). This suggests that there was disruption in the biochemical pathways leading to serotonin production from tryptophan which may be owing to the lack of a conventionally colonised gut microbiota. Alterations in the biosynthesis of neurotransmitters could be one example of how gut microbiota may be exerting influence on behaviour.

The production of serotonin in the CNS relies on peripheral tryptophan crossing the blood-brain barrier (O'Mahony *et al.*, 2015). Dietary tryptophan is absorbed through the gut into the circulatory system from where it is then available to cross the blood-brain barrier via a competitive transport carrier for serotonin synthesis in the CNS (Ruddick *et al.*, 2006). Because of the competitiveness of this transport carrier, the concentrations of circulating tryptophan do not necessarily directly correspond with the quantities of tryptophan that are transported across the blood-brain barrier (Ruddick *et al.*, 2006). However, serotonin production within the CNS depends on the there being peripheral tryptophan available (O'Mahony *et al.*, 2015). Alterations in the availability of peripheral tryptophan could result in behavioural changes. Whole egg administered orally to rats resulted in an anti-depressant like effect during a forced swimming test (Nagasawa *et al.*, 2014). This result was thought to be related to increased levels of tryptophan, which was elevated in the pre-frontal cortex of the brain in the rats receiving the whole egg treatment (Nagasawa *et al.*, 2014).

Commercially available tryptophan supplements are marketed internationally as a calming agent for horses. Results from studies indicate a limited efficacy for tryptophan supplements calming excitable horses. Horses receiving low levels of tryptophan showed mild excitation when their behaviour was analysed during isolation tests and they demonstrated increased activity and heart rates compared to controls (Bagshaw *et al.*, 1994). Supplementation with a commercial tryptophan product has been considered unreliable in its calming influence on horses (Malmkvist and Christensen, 2007). Results indicated that

there was no significant difference during behavioural tests between the horses receiving tryptophan and control horses (Malmkvist and Christensen, 2007). The efficacy of supplementary tryptophan on any increases in the levels of serotonin would need to be investigated further. If gut microbiota disrupts the biochemical pathways of serotonin production, it could mean that calming products may only be effective in certain horses and this may be directly related to the microbiome of the individual.

It is clear that the role of gut microbiota in health and behaviour is far-reaching and more influential than it first appears. This is demonstrated in the quantity of literature from across a range of species. Studies indicate that there can be much variation between individuals in the composition of the bacterial communities of their gut microbiota (Cryan and Dinan, 2012). Although diverse and distinct at different stages of life and displaying great individual variation, these populations are generally of a balanced composition in healthy individuals (Cryan and Dinan, 2012; Moloney *et al.*, 2014; Sonnenburg and Sonnenburg, 2014; O'Mahony *et al.*, 2015). However, this balanced composition is sensitive and can be affected by a number of different influences including diet (Cryan and Dinan, 2012; Moloney *et al.*, 2014; Sonnenburg and Sonnenburg, 2014; O'Mahony *et al.*, 2015). Alterations in diet play a significant role in the stability of the hindgut microbial populations in horses and it has been demonstrated that equine diets high in starch can disrupt the balance of these populations (Medina *et al.*, 2002; de Fombelle *et al.*, 2003; Julliand *et al.*, 2006). If symbiosis of the gut microbiota is compromised, it is possible that a cascade of other reactions may occur including imbalance of neurotransmitter production. These reactions or imbalances may have a direct influence on the behaviour of horses and may ultimately originate from the horse receiving an unnatural diet.

1.6 Aims and objectives

Research has shown that gut microbiota play an influential role in the health and wellbeing of an individual. The influential role of gut microbiota is now thought to extend beyond the health of the individual to also include behavioural influences. Research has shown that gut microbiota can affect the biosynthesis of neurotransmitters which may in turn influence behaviour. The microbial community composition of gut microbiota is shaped largely by diet.

Horses have evolved to browse and graze and their digestive systems are ideally suited to digesting a high-fibre diet. The diets of domestic horses often combine a reduced forage ration which is substituted with concentrate feeds, often in the form of high-starch cereal grains. Horses have a limited capacity to digest starch and feeding high-starch diets have been shown to alter the microbial community composition of the hindgut. Changes to the hindgut microbiota have been associated with the onset of health conditions such as laminitis.

Anecdotally horse owners have suggested that the inclusion of starch in a horse's diet may cause increased behavioural reactivity. There is still only a small amount of scientific research that has been carried out to substantiate this. The overall aim of this thesis is to investigate the relationship between diet, hindgut microbiota, neurochemical changes and behaviour. Horses have evolved to eat a specific type of diet that is high in fibre. This makes them ideally suited for use in investigating the wider effects of dietary change which may be applicable to other species.

The use of naïve animals will form the basis of this thesis and they are an important aspect of the study. The ponies were defined as naïve as they were unaccustomed to human handling, had not undergone any training and from a dietary perspective had only received a forage diet. Behaviourally, the ponies were unhandled so their responses to behavioural tests were natural responses that could not have been shaped by the influences of regular human handling. Mature animals, through life experience and regular handling, may learn to react less in certain situations. Therefore, a natural response to behavioural tests from a mature animal may be less likely.

The use of naïve animals is also important from a gut microbiota perspective. The ponies had not been exposed to the dietary changes or variations in husbandry practices that mature animals may have been. Therefore, the gut microbiota of the naïve animals used for this thesis could be said to be in a more natural state. This makes them ideally suited for investigating the effects of dietary change on the hindgut microbial community composition.

The final contribution of this thesis is to develop and demonstrate the use of primers for quantifying the relative expression of specific serotonin and dopamine receptor densities in gut and brain tissues using real-time polymerase chain reaction (qPCR). This will further develop our understanding about the wider effects of diet and contribute to the increasing body of research exploring gut-brain axis communication pathways.

1.7 Structure of the thesis

This thesis uses naïve ponies to develop knowledge of the relationship between diet, behaviour, gut microbiota, and gut and brain tissue neurochemistry. The behaviour of the ponies in relation to diet will be described and the microbial community composition of the faeces and different regions of the hindgut will be detailed in relation to diet. Real-time PCR primers will be developed and used to quantify the relative expression of serotonin and dopamine receptor densities in brain and gut tissues in relation to diet. These studies are documented in Chapters 2 to 4 following this introduction. The overall thesis is concluded with a discussion (Chapter 5) detailing the implications of this work and offering suggestions for future research.

Chapter 2 uses a cross-over design incorporating two diets (high-starch (HS) and high-fibre (HF)) fed in two experimental periods to investigate the behaviour of naïve ponies. Quantitative and qualitative methods were used to describe the behaviour of the ponies in relation to diet. Faecal samples were collected during both experimental periods and 16S *rRNA* gene sequencing using the Illumina MiSeq platform was used to describe the faecal microbial community composition for both diets. A multi-variate modelling approach was used to examine which operational taxonomic units (OTUs) differed in relation to diet. This chapter has been published: Bulmer, L. S., Murray, J-A., Burns N. M.,

Garber A., Wemelsfelder, F., McEwan N., Hastie, P. 2019. High-starch diets alter equine faecal microbiota and increase behavioural reactivity. Scientific Reports 9, 18621.

Chapter 3 examines the effects of HS or HF diets on the microbial community composition of the hindgut. Following euthanasia, digesta samples were collected from different regions of the hindgut and 16S *rRNA* gene sequencing was used to explore the microbial community composition. Post-mortem faecal samples were also sequenced and compared to the faecal samples collected in Chapter 2 to ascertain if the faecal microbial community composition was consistent across both sampling times. A Bayesian modelling approach was used to describe the differences in microbial community composition for both diets in each hindgut region. Key patterns of changes in bacterial taxa consistent across faecal samples and hindgut regions were identified.

Chapter 4 explores the effects of diet on the neurochemistry of gut and brain tissues from the ponies. This chapter develops and demonstrates the use of real-time PCR primers to quantify the relative expression of specific serotonin and dopamine receptor densities in gut and brain tissues. The relative expression of neurotransmitter receptor densities were explored in relation to diet for the gut and brain tissues.

Chapter 5 summarises the combined findings from Chapters 2, 3 and 4 and ties together the effects of diet on behaviour, gut microbiota and neurochemistry. The implications of these findings are discussed in relation to common feeding practices and suggestions are made for how these practices could be improved. Ideas for the future development of the work undertaken in this thesis are discussed along with suggestions for future research.

Chapter 2 High-starch diets alter equine faecal microbiota and increase behavioural reactivity[1]

2.1 Abstract

Gut microbiota have been associated with health, disease and behaviour in several species and are an important link in gut-brain axis communication. Diet plays a key role in affecting the composition of gut microbiota. In horses, high-starch diets alter the hindgut microbiota. This type of diet is also associated with increased behavioural reactivity in horses. These changes in microbiota and behaviour may be associated, and this could have a detrimental impact on health and welfare. This chapter compares the faecal microbiota and behaviour of 10 naïve ponies. A cross-over design was used with experimental groups being fed a high-starch (HS) or high-fibre (HF) diet. The results showed that ponies were more reactive and less settled when being fed the HS diet compared to the HF diet. Multivariate analysis of 16S *rRNA* gene sequencing data showed diet affected faecal microbial community structure. The abundance of 85 OTUs differed significantly related to diet. Correlative relationships exist between dietary induced alterations to the faecal microbial community composition and behaviour. Results demonstrate a clear link between diet, faecal microbial community composition and behaviour. Dietary induced alterations to gut microbiota play a role in affecting the behaviour of the host.

[1] Bulmer, L. S., Murray, J-A., Burns N. M., Garber A., Wemelsfelder, F., McEwan N., Hastie, P. 2019. High-starch diets alter equine faecal microbiota and increase behavioural reactivity. Scientific Reports 9, 18621.

2.2 Introduction

In recent years, gut microbiota and their association with behaviour, health and disease has developed into a major area of scientific interest. The communication system between the gut and brain is commonly referred to as the gut-brain axis. Growing evidence is accumulating to indicate that communication exists between gut microbiota and the brain (Cryan and Dinan, 2012). Early studies using infant rodent models found strong associations between the early colonisation of the gut and the development of the hypothalamic-pituitary-adrenal (HPA) axis (Sudo *et al.*, 2004). The germ-free mice exhibited elevated levels of plasma adrenocorticotropic hormone (ACTH) and corticosterone in response to stress, which was reversed following colonisation of the gut with the bacterium *Bifidobacterium infantis* (Sudo *et al.*, 2004). *B. infantis* has also been shown to reduce the behavioural and biochemical changes in rats that occurred following a postnatal maternal separation experimental model (Desbonnet *et al.*, 2010).

In humans, high co-morbidity between gastrointestinal disorders and stress-related mental health conditions, like anxiety and depression, are often reported (Cryan and Dinan, 2012; Holzer *et al.*, 2012; Foster and McVey Neufeld, 2013; Crumeyrolle-Arias *et al.*, 2014). It is possible that the gut microbiota play a pivotal role in the connection between these conditions. The gastrointestinal tract releases over 20 different hormones (Murphy and Bloom, 2006). Several of these can be produced by the microbiota and are neurotransmitters, including serotonin and dopamine (Cryan and Dinan, 2012; Clarke *et al.*, 2014). These neurotransmitters, which are involved in several physiological functions, also play a significant role in behaviour. Therefore, disruption or dysregulation in the production of these neurotransmitters may have an influence on the overall behaviour of the host.

Studies have demonstrated that neurotransmitters can be modulated by gut bacteria. In rats, the bacterium *B. infantis* was shown to elevate peripheral levels of the amino acid, tryptophan (Desbonnet *et al.*, 2009). One of the roles of tryptophan is as a pre-cursor to the production of serotonin, and therefore *B. infantis* was suggested to have potential antidepressant properties (Desbonnet *et al.*, 2009). *Bifidobacteria* species have also been shown to be more effective

than a commercially available antidepressant drug at reducing stress-related behaviours in mice (Savignac *et al.*, 2014). Other bacterial taxa have also been found to influence behaviour. Mice administered with the bacterium *Lactobacillus rhamnosus* showed alterations in γ-aminobutyric acid (GABA) receptor expression in the brain coupled with reduced anxiety and depression related behaviours (Bravo *et al.*, 2011). Research undertaken in rodents has greatly increased our understanding of the associations between the gut microbiota and stress-related neurological conditions.

Diet is a key factor influencing the composition of gut microbiota (Cryan and Dinan, 2012). In rats, a high-fat diet was shown to induce oxidative stress and inflammation in the cerebral cortex which may be indicative of an increased risk of dementia (Zhang *et al.*, 2005). Unfortunately, this study did not investigate if there were changes in gut microbiota. However, another study did find that physiological alterations associated with a high-fat diet were present at both gut and brain level. Mice transplanted with gut microbiota associated with a high-fat diet showed increased intestinal barrier permeability along with disruptions in brain physiology and function (Bruce-Keller *et al.*, 2015). The overflow of undigested carbohydrates into the hindgut has also been associated with increased anxiety and depression in rats (Hanstock *et al.*, 2004). It is possible that dietary alterations to gut microbiota may have a wide-ranging influence over the behaviour of the host.

In equids, there has been a longstanding anecdotal association between high-starch diets and increased behavioural reactivity. The horse has evolved to browse and graze and has a digestive tract that is well suited to high-fibre, low-starch diets (Henderson, 2007; Hill, 2007; Willing *et al.*, 2009). The large intestine of the horse comprises approximately two thirds of the digestive tract and is abundant in microorganisms (Daly *et al.*, 2001; Biddle *et al.*, 2013). As such, the fermentation of structural carbohydrates is thought to provide the majority of a wild horse's energy (Daly *et al.*, 2012; Steelman *et al.*, 2012). However, the forage component of the domestic equine diet is often reduced and substituted for high-starch cereal grain-based feeds. It is perceived by many horse owners that the addition of starch to the diet is required as a means of meeting the energy requirements to fuel athletic performance. With the horse

having evolved to eat a specific diet, it makes it an ideal model for studying the effects of dietary changes on gut microbiota and behaviour.

A number of studies in horses have documented associations between the domestic diet and an increased risk of developing stereotypic behaviours (Nicol *et al.*, 2005; Hothersall and Nicol, 2009; Wickens and Heleski, 2010). However, there are often management factors that should be taken into consideration and therefore diet may not be the only contributory factor influencing the development of stereotypies. What is clear is that diet does have some role to play. If diet can have a contributory influence on the development of stereotypies it may also have an influence on the wider behaviour of the horse, in particular, their day-to-day handling and reactivity. However, until recently there has been very little scientific research undertaken to substantiate the anecdotal association between increased behavioural reactivity and diet.

A recent study using mature horses receiving isoenergetic diets found that when they were fed a high-starch diet they had significantly increased heart rates during behavioural tests compared to when they were receiving a high-fibre diet (Bulmer *et al.*, 2015). It was suggested that the increase in heart rates may have developed into an increased behavioural response had younger, or less experienced, horses been used. It is possible that the changes recorded in the aforementioned study may have been related to microbiota changes occurring in the hindgut as a result of diet.

Equine studies have shown that a high-starch diet can alter the hindgut microbial community composition (Medina *et al.*, 2002; de Fombelle *et al.*, 2003; Al Jassim *et al.*, 2005; Julliand *et al.*, 2006; Daly *et al.*, 2012; Dougal *et al.*, 2014). Alterations to this finely balanced microbial community have been studied in relation to health and disease and a shift in the population balance at phylum level has been recorded in horses with laminitis (Steelman *et al.*, 2012) and colitis (Costa *et al.*, 2012). However, community differences are not only seen in horses with health conditions. Differences have also been recorded in healthy horses related to diet. Changes in the hindgut microbiota have been recorded in horses fed high-starch diets (Willing *et al.*, 2009; Daly *et al.*, 2012; Dougal *et al.*, 2014).

It is possible that alterations to the hindgut microbiota in horses may be influencing behavioural changes via gut-brain axis communication pathways. However, very little research has been undertaken in equids to investigate if there is an association between behaviour and changes in gut microbiota as a result of diet. If there is an association between diet, behaviour and changes in microbiota then this could have strong implications for welfare. Horses that are more reactive are more difficult to handle. If addressing the domestic equine diet could help to reduce reactivity during handling, then this in turn could go a long way to improving equine welfare.

Recently a study using fistulated horses found that an increased stress response was associated with changes in the hindgut microbial profile (Destrez *et al.*, 2015). The quantity of starch that resulted in the increased stress response recorded in the study by Destrez *et al.*, (2015) exceeded the current recommended daily maximum levels of the National Research Council (2007). This meant that starch could pass into the hindgut undigested. It is possible that the increased stress response might have been related to digestive discomfort caused by undigested starch entering the hindgut. It is also unusual for leisure horses to be fed a diet that comprises such a high proportion of starch-based concentrates. Therefore, it would be worthwhile investigating if, with lower levels of starch in the diet, behavioural changes are still observed. The changes in microbiota recorded in the study by Destrez *et al.* (2015) focused on specific groups of bacteria using culture-based methods for the analysis. While the results are informative, they do not offer a broader view of the microbiota in terms of the changes to community structure that may be occurring. A modern sequencing approach will allow for a more in-depth understanding. The 16S *rRNA* gene sequencing method used in this chapter provides a broader analysis of microbial community structure related to diet.

Next generation sequencing (NGS) is a term that encompasses a number of different modern sequencing technologies. These modern sequencing tools are becoming more accessible and are starting to be used more often in equine hindgut microbiota studies (Costa *et al.*, 2012; Shepherd *et al.*, 2012; Steelman *et al.*, 2012; Dougal *et al.*, 2014). A number of other studies still elect to use more traditional culture-based methods (de Fombelle *et al.*, 2003; Respondek *et*

al., 2008; Muhonen *et al.*, 2009; van den Berg *et al.*, 2013). Not all bacteria are currently able to be cultured and these studies have naturally tended to focus on exploring specific groups of culturable bacteria. While the results from culture-based studies have greatly developed our understanding of specific bacterial groups, there is still a great deal more to understand, particularly in relation to the overall microbial community composition.

Results from NGS studies have reported large proportions of bacteria that were unassigned, particularly at genus level. For example, one study indicated that 57% of the bacterial populations were reported as being unassigned (O'Donnell *et al.*, 2013). Another study reported that 12 out of the 20 most abundant genera were unassigned (Steelman *et al.*, 2012). This highlights that what has been found so far, even though it has greatly advanced our understanding, is still only the tip of the iceberg. As sequencing technologies continue to develop, they allow the opportunity to advance our understanding of the complexities of the equine hindgut microbiota even further. Therefore, the Illumina 16S *rRNA* gene sequencing platform used in this chapter will provide a detailed and up to date addition to the pool of literature exploring the equine faecal microbiota.

The aim of this study was first: to investigate the behaviour of naïve ponies when being fed a high-starch and a high-fibre diet in a cross-over design study, and second, to use 16S *rRNA* gene sequencing of faecal samples to determine if there were any changes in faecal microbiota associated with diet.

2.3 Methods

2.3.1 Ethical approval

The experimental protocols in this study were approved by the University of Glasgow's School of Veterinary Medicine Veterinary Ethics & Welfare Committee (Equine hindgut health, nutrition and microbiota – Ref. 05a/14).

2.3.2 Ponies and experimental design

Ten unhandled 18-month-old Welsh section A ponies were used in a 2 x 2 cross-over design consisting of two 14 day periods and two experimental diets; high-fibre (HF) and high-starch (HS). The ponies were randomly divided into two groups but with each group comprising two fillies and three geldings. In experimental period one, five ponies received the HS diet and five received the HF diet. At the end of the first experimental period the ponies were transitioned to the alternative diet by graduating four feeds over two days. Ponies were housed in individual pens in an indoor barn and bedded on wood shavings. Prior to the study the ponies were fed hay with no additional feed supplementation.

The ponies are defined as naïve as they were unaccustomed to human handling, had not undergone any training and from a dietary perspective had only received a forage diet. Additional background information is included in Appendix A.

2.3.3 Diets

All ponies were fed according to National Research Council (2007) recommendations for growth using the estimated mature bodyweight (BW) of 250 kg. The HF diet consisted of hay plus high-temperature dried Lucerne with ponies receiving 0.46 g starch/kg of BW per meal (Table 2.1). The HS diet consisted of hay and a compound mix with ponies receiving 0.96 g starch/kg of BW per meal (Table 2.1). Both diets were fed in two daily meals and the daily ration was divided into 80% hay and 20% concentrates. All ponies received 40.2 MJ/DE (Mega joules of digestible energy) per day. Additional dietary information is included in Appendix A.

Table 2.1 - Energy and starch composition of feeds.

Feed	MJ/DE per kg	Starch %
Hay	7.5	3.6
Compound mix	10.0	23.0
Lucerne	10.0	5.0

2.3.4 Behavioural tests

Two behavioural tests were conducted at the end of each experimental period in a testing area (length 10 m x width 7 m x height 3 m) constructed from straw bales in an indoor barn. The floor had a covering of wood shavings. The ponies were familiarised with the testing area prior to the study. A video camera was positioned on top of the bales at one end of the testing area. Both tests lasted five minutes and were recorded for later analysis using Observer XT Software (version 12.5) (Noldus, 1991).

Freshly voided faeces were collected on both behavioural testing days. Care was taken to collect samples from the centre of the faeces that had not been in contact with the floor. Samples were immediately frozen at -20˚C and then stored at -80˚C prior to genomic DNA (gDNA) extraction.

2.3.4.1 Passive human test

The passive human test (Lansade and Bouissou, 2008) was carried out using a person unfamiliar to the ponies stood centrally 1 m in from the right hand wall (when viewed from camera) of the testing area. A different person was used for each behavioural testing period to ensure that the ponies did not become familiar with the person. The pony was released into the testing area and their behavioural responses to the motionless human were recorded (Table 2.2).

2.3.4.2 Novelty test

For the novelty test (Lansade *et al.*, 2008), the pony was released into the testing area which contained a black rubber feed bowl and a novel object. The feed bowl contained a mixture of Lucerne and compound mix and was positioned centrally 1.6 m in from the end wall. A novel object (1st period = large box (86 cm x 44 cm x 20 cm) wrapped in foil, and 2nd period = triangular road sign) was placed between the feed bowl and the entrance at 1.6 m from the feed bowl.

The pony needed to pass the novel object to reach the feed bowl. The behavioural responses of the ponies were recorded (Table 2.2).

Table 2.2 - Behavioural ethogram showing variables measured during the passive human and novelty tests.
Key: f = frequency, d = duration, l = latency. (References: [a] = (Lansade and Bouissou, 2008), [b] = (Lansade *et al.*, 2008), [c] = (Christensen *et al.*, 2005), [e] = (Bulmer *et al.*, 2015)

Behaviour	Passive human test	Novelty test
Alertness:		
Glances at human [a,b] (f)	**x**	
Glances at stimulus [b,c] (f)		**x**
Glances at feed bowl [b,c] (f)		**x**
Alert other [c] (f,d) (as with glancing but ears orientated in other directions)	**x**	**x**
Interaction:		
Investigate human [a,b] (f,d)	**x**	
Investigate stimulus [a,b] (f,d)		**x**
Investigate other [c] (f,d) (includes sniffing, touching and manipulating walls, gate or floor)	**x**	**x**
Feeding:		
Time to approach feed bowl [e] (l)		**x**
Sniff food [c] (l,d)		**x**
Time eating [c] (d) (head may be lifted away from feed bowl for short periods while chewing)		**x**
Locomotion:		
Stand (d)	**x**	**x**
Walking [c] (d)	**x**	**x**
Trotting [a,b,c] (d)	**x**	**x**
Cantering [a,b,c] (d)	**x**	**x**
Pace change (f)	**x**	**x**

2.3.5 Quantitative behaviour measures

Data from the behavioural variables (Table 2.2) were extracted from the video recordings using Observer XT Software (version 12.5) (Noldus, 1991) by one person who at this stage of the study was blinded to which ponies were receiving which diet. The locomotory variables were categorised as continuous and mutually exclusive. The other behavioural categories were categorised as mutually exclusive. From the locomotory variables, the frequency counts

recorded for each pace were combined to give 'frequency of pace-change', so that the overall number of times each pony changed pace during each test could also be analysed.

2.3.6 Qualitative behavioural analysis

Qualitative behavioural analysis (QBA) was used in addition to the quantitative measures to assess the animals' expressive demeanour as interpreted by a group of observers. Observer descriptions can include terms like bold, curious or nervous (Wemelsfelder *et al.*, 2001; Wemelsfelder, 2007; Napolitano *et al.*, 2008; Minero *et al.*, 2009; Wemelsfelder *et al.*, 2012; Fleming *et al.*, 2013). QBA allows another dimension of the behavioural responses to be explored and can give a detailed picture of the individual animal when assessed alongside quantitative measures.

Two-minute sections of the behavioural video recordings were used. For the passive human test, minutes 1.5 – 3.5 were used and for the novelty test the first 2 minutes were used; ensuring that the element of surprise at the novel object was captured. Ten undergraduate equine students participated as observers. The students were all horse owners and had experience working with horses. They had not specifically studied equine behaviour as part of their course but all had a basic understanding of equine behaviour prior to participating as observers. The students were unaware of the aim of the study and that different dietary treatments had been used. Free-choice profiling methodology (FCP) was used in two phases (Wemelsfelder *et al.*, 2001). In the first phase the observers each generated their own descriptive vocabularies. The second phase involved the quantification by each observer of their own descriptive terms (Wemelsfelder *et al.*, 2001). The observers watched the two-minute video clips in a random order. Following each clip, the observers used their own list of descriptive terms to score the observed pony using an unstructured visual analogue scale measuring 125mm. The extreme left (0 mm) was marked as 'minimum' and the extreme right (125 mm) as 'maximum'. Observers were instructed to use the distance between these points to mark the intensity of expression for each behavioural term (Wemelsfelder *et al.*, 2001).

2.3.7 DNA extraction and 16S *rRNA* gene sequencing

Genomic DNA (gDNA) was extracted from faecal samples using a Qiagen QIAamp® Fast DNA Stool Mini Kit. The standard protocol was followed but with slight adaptations (Appendix B, Protocol 1). The adapted protocol included the samples being immediately homogenised in the InhibitEx® Buffer using a homogeniser. Proteinase K® and Buffer AL® were added to the supernatant and the samples were then incubated at 70˚C for five minutes prior to centrifugation at full speed in a micro-centrifuge (14,000 x g) and pipetting into tubes containing Proteinase K®. 100% ethanol was added and the lysate was passed through the QIAamp® spin columns by centrifuging, discarding any filtrate. This was repeated using all of the lysate. The AW1® and AW2® buffers were added and passed through the spin columns and discarded. Finally, ATE® Buffer was added directly onto the QIAamp® membrane and incubated at room temperature for five minutes, instead of one minute, then centrifuged. The DNA samples were transferred into labelled microcentrifuge tubes. Extracted gDNA was assayed for concentration, quality and protein contamination using a nanodrop to measure A_{260} and A_{280} values prior to freezing at -20^{o}C. Samples were briefly thawed and aliquoted according to gDNA concentration, then frozen at -20^{o}C prior to 16S *rRNA* sequencing.

Library preparation and sequencing were undertaken by Glasgow Polyomics, University of Glasgow, using the Illumina protocol. Sequencing was run on an Illumina MiSeq using 2 x 300bp paired end reads. The library preparation used was based upon Illumina's 16S library preparation. Primers (forward 5'-CTTACGGGNGGCWGCAG-3' and reverse 5'-GACTACHVGGGTATCTAATCC-3') were used to amplify the V3 and V4 regions of the 16S *rRNA* gene including specific overhangs to allow the addition of Nextera XT V2 indices and adapters during the second round of PCR. The slight overlap improves the quality of the reads and reduces the risk of error. These libraries were quality controlled and the equimolar pooled and sequenced. 3.5% PhiX spike-in was added to the run as a sequencing control. Bioinformatic analysis of raw sequencing data were processed by Glasgow Polyomics using QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso *et al.*, 2010). Reads were clustered into operational taxonomic units (OTUs) based upon 97% identity using the default alignment

method ‘uclust’. OTUs were taxonomically identified using the Greengenes 16S *rRNA* gene database. All samples were rarefied to 9000 reads/sample.

2.3.8 Statistical analyses

2.3.8.1 Quantitative behavioural analysis

Data analyses were carried out using R (version 3.4.3) (R core team, 2017). A Generalised Linear Mixed Model (GLMM) with random intercept was selected using AIC to confirm the optimal random effects structure. Backward stepwise model selection was used to select the optimal fixed effects (Zuur *et al.*, 2009). Differences in behavioural response were modelled as a function of diet and experimental period using the ‘nlme’ package (Pinheiro *et al.*, 2017) for Gaussian data (e.g. time spent investigating) and ‘lme4’ (Bates *et al.*, 2015) for Poisson distributed data (e.g. frequency of pace change).

2.3.8.2 Qualitative behavioural analysis

The score for each pony from an observer’s behavioural terms was measured in millimetres. The level of agreement between the observer data matrices was analysed for each test using Generalised Procrustes Analysis (GPA), run by a specialist GenStat software programme developed for F.Wemelsfelder. GPA does not require pre-defined variables; a series of randomised iterative rotations finds a consensus profile or ‘best fit’ for the different observer data configurations (Wemelsfelder *et al.*, 2001; Wemelsfelder *et al.*, 2012). A one-tailed t-test was used to determine whether the percentage of variation explained by the ‘true’ consensus differed significantly from the mean of 100 randomised profiles, with a p-value <0.001 indicating a ‘true’ consensus. Principal Component Analysis (PCA) was then used to identify the main dimensions of the consensus profile explaining the majority of variation between observed animals. These dimensions were interpreted by correlating the individual scoring patterns to the main dimensions of the consensus profile and then collating terms for all observers that correlated with these dimensions ($r < -0.5$ and $r > 0.5$).

Analysis of the effect of diet and experimental period on GPA consensus scores for each behavioural test was carried out in R (version 3.4.3) (R core team,

2017) using a GLMM with pony as the random intercept. Spearman's rank correlation coefficient was then used to determine if any significant correlations were present between quantitative and qualitative behavioural outcomes related to diet.

2.3.8.3 16S *rRNA* gene sequencing data analyses

Statistical analyses on the microbial dataset were performed in R (version 3.4.3) (R core team, 2017). Diversity and richness for both diets were evaluated using Shannon diversity (vegan package (Oksanen *et al.*, 2017)) and Chao1 richness indices (fossil package (Vavrek, 2011)). Rarefied data were visualised according to diet and experimental period using non-metric multidimensional scaling (NMDS) plots. To investigate any associations between microbial community structure and behaviour, key behavioural variables were fitted over the microbiota NMDS ordination plots for both experimental periods using the 'envfit' function in the vegan package (Oksanen *et al.*, 2017).

The multivariate modelling of 16S *rRNA* gene data was undertaken using the mvabund package (Wang *et al.*, 2017). This package is a powerful tool with greater power properties than distance-based methods (Warton *et al.*, 2012). The 'manyglm' function with a negative-binomial distribution with log-link was used allowing a generalised linear model (GLM) to be fitted to each OTU (Wang *et al.*, 2012). To account for possible correlation between variables, ridge regularisation was applied to shrink the correlation matrix. A full model was fitted with 'diet', 'experimental period' and the associated interaction. Wald test statistics were used in backwards stepwise model selection. A robust approach was adopted to verify model accuracy using Monte Carlo cross-validation (Xu and Liang, 2001). 10,000 iterations of an 80:20 (training:test) data split were used to compare test set predictions with the true results using a Spearman's rank correlation coefficient.

16S *rRNA* sequencing using Illumina MiSeq does not use a long enough read length to accurately sequence bacteria down to species level. Therefore, for reporting purposes, any OTUs found to be significantly different related to diet were tracked back to their corresponding genera.

2.4 Results

2.4.1 Quantitative behavioural analysis

2.4.1.1 Passive human test results

During the passive human test there was a significant increase in the number of times the ponies changed pace when fed the HS diet compared to the HF diet (z = 4.22, p < 0.01). Additionally, ponies spent less time standing (t = -3.63, p = 0.01), and less time investigating their surroundings when fed the HS diet (t = -2.57, p < 0.05). There was no effect of experimental period on the behavioural variables for this test.

2.4.1.2 Novelty test results

During the novelty test ponies fed the HS diet showed an increase in the number of times they demonstrated a heightened alert state (z = 3.49, p < 0.01). In contrast to the passive human test there was an effect of experimental period on being alert. Ponies demonstrated increased frequencies of being alert during the second period compared to the first (z = -3.32, p < 0.01). A significant interaction between diet and experimental period was also retained in this model (z = -2.39, p < 0.05). The interaction between these terms showed ponies that received the HF diet in period two displayed a reduced frequency of alert behaviours but to a lesser extent than those that received the HF diet in period one.

2.4.2 Qualitative behavioural analysis

2.4.2.1 Passive human test results

The percentage of variation between observer scoring configurations explained by the GPA consensus profile was 74.1%. There was a significant difference between the GPA consensus profile and the variation explained by the mean randomised profile (t_{99} = 36.83, p < 0.001). Dimension one explained 56% of the variation. This dimension was defined by strongly correlating, frequently used terms such as relaxed/laid-back/settled at the positive end and nervous/tense/unsure at the negative end (Table 2.3). This suggests that dimension one characterised how relaxed and settled, or, how nervous and tense

the ponies were. There was a significant effect of diet on dimension one (t = -4.22, p < 0.01). Ponies were perceived as more tense/nervous/unsure when fed the HS diet.

Dimension two explained 15.5% of the variation and ranged from bold/confident/brave to wary/scared/hesitant, suggesting that this dimension characterised how 'confident' ponies were in responding to the test. There was no effect of diet on dimension two and no effect of period on either dimension for this test.

Table 2.3 - Passive human test qualitative behavioural analysis (QBA) dimensions. Observer terms and the significance of the dimension related to diet and experimental period are shown. Included are observer terms correlating strongly with those dimensions at the negative end (r<-0.5) and positive end (r>0.5). Values in brackets following terms indicate the number of observers using that term if greater than 1. The terms in bold were used to label the dimensions.

GPA dimension (variance explained)	Low value terms r< -0.5	High value terms r>0.5	Significance related to diet	Significance related to experimental period
1 (56%)	**Nervous** (6), **Tense** (7), **Unsure** (6), Stressed (5), Anxious (5), Alert (4), Excitable (4), Scared (4), Worried (3), Spooked (3), Flighty (3), Jumpy (2), On-Edge (2), Restless (2), Shy (2), Energetic (2), Agitated, Confused, Annoyed, Uptight, Terrified, Upset, Panicked, Frightened, Agitated, Skittish, Cautious, Dominant, Sharp, Skatty, Afraid, Aware	**Relaxed** (10), **Laid-back** (8), **Settled** (7), Calm (6), Happy (6), Easy-going (2), Content (2), Chilled (2), Gentle, Comfortable, Unphased, Bored, Quiet, Carefree	p < 0.01	n.s.
2 (15.5%)	**Wary** (4), **Scared** (2), **Hesitant** (2), Nervous (2), Unsure (2), Shy (2), Anxious, Cautious, Apprehensive, Interactive	**Bold** (8), **Confident** (6), **Brave** (4), Curious (2), Playful (3), Inquisitive, Nosey, Excited, Interested, Determined	n.s.	n.s.

Spearman's rank correlation coefficient showed a moderate and positive correlation between dimension one and the quantitative variable time spent investigating (n = 20, $\rho = 0.56$, $p < 0.05$). This indicates that ponies that explored more were perceived as relaxed/laid-back/settled. There was also a moderate negative correlation between dimension one and the quantitative variable frequency of pace-change (n = 20, $\rho = -0.51$, $p < 0.05$) indicating that ponies that changed pace more frequently were perceived as nervous/tense/unsure.

2.4.2.2 Novelty test results

The percentage of variation between observer scoring configurations explained by the GPA consensus profile was 70.2%. The GPA consensus profile differed significantly from the mean randomised profile ($t_{99} = 31.33$, $p < 0.001$). Dimension one explained 54.1% of the variation and characterised behaviours ranging from relaxed/laid-back/settled to nervous/unsure/tense (Table 2.4). Dimension two explained 13.3% of the variation and characterised behaviours like bold/curious/confident at the positive end. "Shy" was the only term at the negative end. There was no significant effect of diet on either dimension for the novelty test. However, there was an effect of experimental period on dimension one. Ponies were perceived as more nervous/unsure/tense in response to the foil-covered box used in experimental period one, than in response to the triangular road sign used in experimental period two.

Table 2.4 - Novelty test qualitative behavioural analysis (QBA) dimensions. Observer terms and the significance of the dimensions in relation to diet and experimental period are shown. Included are observer terms correlating strongly with those dimensions at the negative end (r<-0.5) and positive end (r>0.5). Values in brackets following terms indicate the number of observers using that term if greater than 1. The terms in bold were used to label the dimensions.

GPA dimension (variance explained)	Low value terms r< -0.5	High value terms r>0.5	Significance related to diet	Significance related to experimental period
1 (54.1%)	**Nervous** (8), **Unsure** (7), **Tense** (6), Scared (6), Anxious (6), Restless (5), Wary (5), Spooked (4), Alert (4), Stressed (4), Worried (3), On-edge (3), Hesitant (2), Agitated (2), Confused (2), Annoyed, Shy (2), Cautious (2), Uptight, Terrified, Upset, Panicked, Frightened, Excited, Skittish, Jumpy, Careful, Flighty, Sharp, Skatty, Afraid, Uncertain	**Relaxed** (10), **Laid-back** (8), **Settled** (7), Happy (7), Calm (6), Bold (3), Confident (2), Brave (2), Content (2), Chilled (2), Easy-going, Bored, Carefree, Comfortable, Interactive, Pre-occupied, Nosey, Unphased	n.s.	$p < 0.01$
2 (13.3%)	Shy	**Bold** (7), **Curious** (5), **Confident** (5), Jumpy (3), Brave (4), Inquisitive (4), Flighty (2), Spooky (2), Alert, Energetic, Courageous, Determined	n.s.	n.s.

2.4.3 16S *rRNA* gene sequencing

Results from the 20 faecal samples sequenced gave a total count of 1,251,244 reads, with an average of 65,026 ± 12,095 (mean ± s.d.) reads per sample for the HF diet and 60,098 ± 17,780 (mean ± s.d.) reads per sample for the HS diet. Shannon diversity results showed similar values for the HF and HS diets (Figure 2.1a), but overall diversity was lower and showed greater variance for the HS diet compared to the HF diet. The Chao1 richness estimator also showed similar values for both diets but overall richness was lower for the HS diet compared to the HF diet (Figure 2.1b).

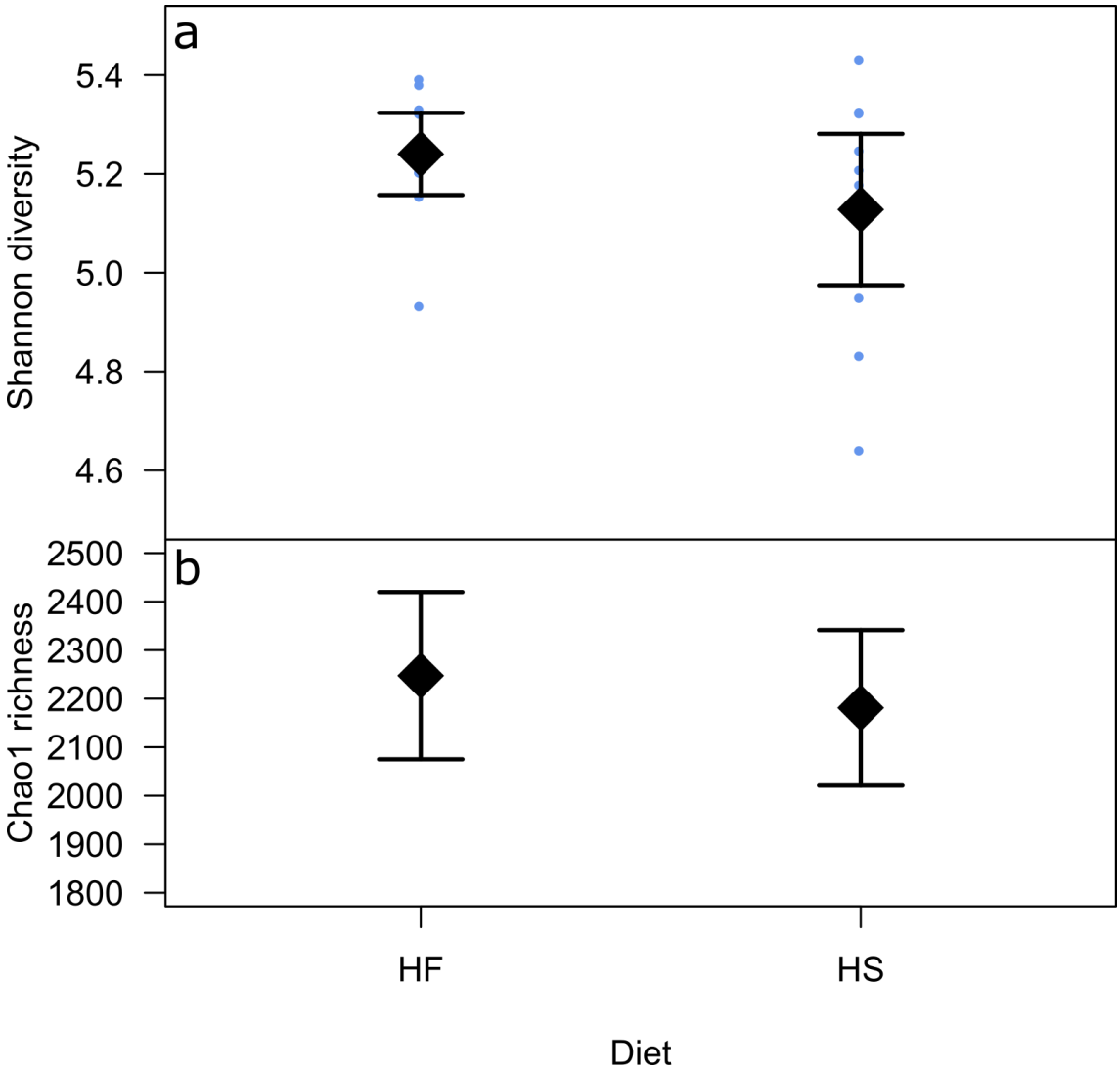

Figure 2.1 - Comparison of Shannon (a) and Chao (b) diversity indices for the high-starch (HS) and high-fibre (HF) diets.
Error bars show 95% confidence interval. Points on the Shannon plot denote individual Shannon diversity measures for each pony with the measures for the HS diet showing greater variance.

2.4.3.1 Phylum level relative abundance

Taxonomy results from the 16S *rRNA* gene sequencing data identified a total of five main phyla, which in terms of abundance, represented >97% of the overall microbial profile from the faecal samples (Figure 2.2). Each of the five phyla represented >1% of the total population. The overall profile was dominated by two main phyla, Firmicutes and Bacteriodetes, which represented >65% overall. These main phyla were followed by Verrucomicrobia, Fibrobacteres, and Spirochaetes.

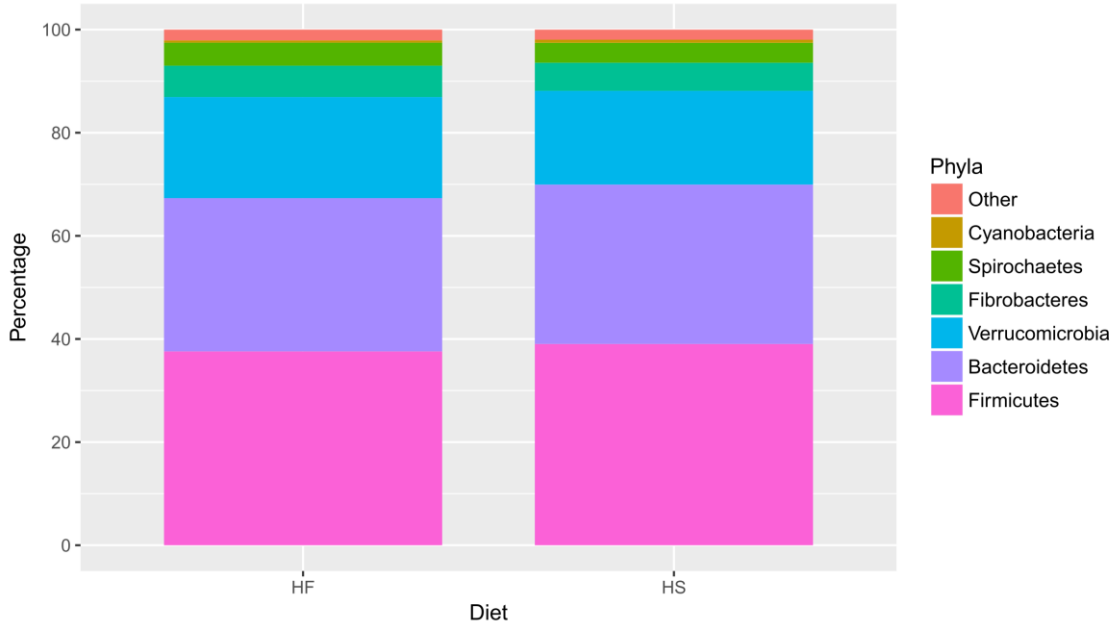

Figure 2.2. Bacterial taxa plot showing phyla level relative abundance for the high-starch (HS) and high-fibre (HF) diets.

2.4.3.2 Family level relative abundance

The Firmicutes phylum primarily comprised family level bacteria from the Clostridiales order and included Ruminococcaceae, Lachnospiraceae, Clostridiaceae and Veillonellaceae along with another bacterial taxa unassigned at family level (Figure 2.3). Other family level bacteria from the Firmicutes phylum were from the Lactobacillales order and were present at lower abundance levels. These included Lactobacillaceae and Streptococcaceae. All family level bacteria from the Bacteroidetes phylum were from the Bacteroidales order with the predominant bacterial taxa from this phylum being unassigned at family level. Other family level bacteria from the Bacteroidetes phylum included BS11, Prevotellaceae, RF16 and [Paraprevotellaceae]. Bacteroidaceae and Porphyromonadaceae, also from the Bacteroidales order,

were present in lower levels of abundance. There were two family level bacteria from the Verrucomicrobia phylum which included Verrucomicrobiaceae from the Verrucomicrobiales order and RFP12 from the WCHB1-41 order. There was one family level bacterial taxa from the Fibrobacter phylum which was Fibrobacteraceae and one family level bacterial taxa from the Spriochaetes phylum which was Spriochaetaceae.

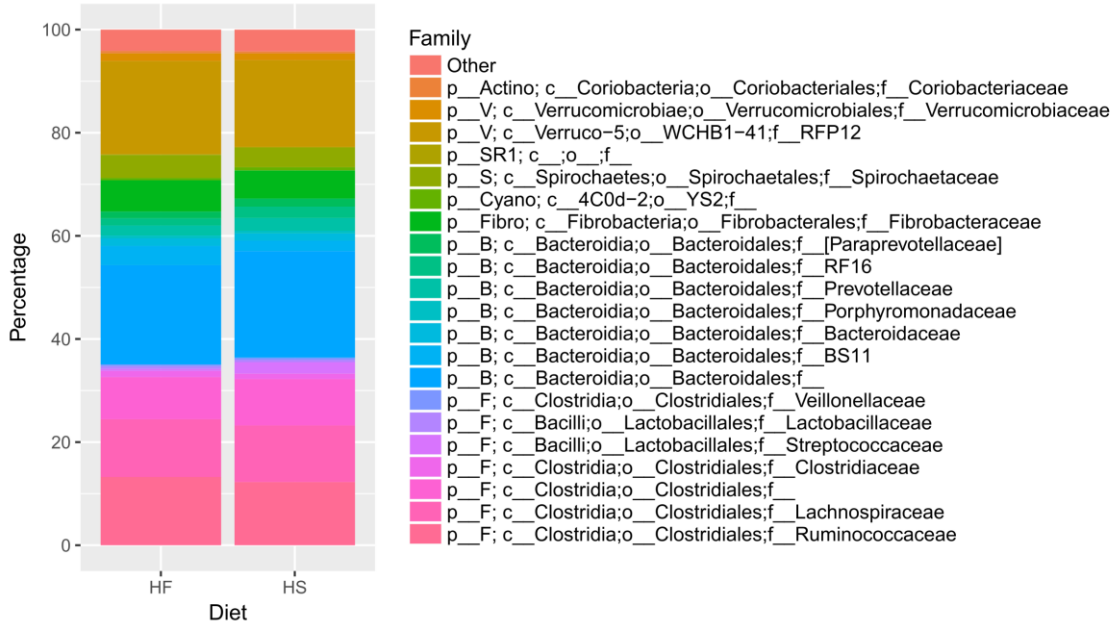

Figure 2.3 - Bacterial taxa plot showing family level relative abundance for the high-starch (HS) and high-fibre (HF) diets.
Legend includes abbreviated phyla level bacteria (p_), F = Firmicutes, B = Bacteroidetes, Fibro = Fibrobacteres, Cyano = Cyanobacteres, S = Spirochaetes, V = Verrucomicrobia, Actino = Actinobacteria.

2.4.3.3 Genus level relative abundance

At genus level, there were 14 bacterial taxa identified with a mean relative abundance >1% (Figure 2.4). Of the 14 bacterial taxa identified at genus level, five were from the Firmicutes phylum with three of these being from the Clostridiales order, one from the Lactobacillales order and one from the Erysipelotrichales order. There were also five genus level taxa from the Bacteroidetes phylum, all of which were from the Bacteroidales order. Seven of the bacterial taxa identified at genus level with a mean relative abundance of >1% were unassigned, including the most abundant genus overall. This predominant genus was from the Bacteroidales order and was also unassigned at family level. The second most abundant genus overall was also unassigned at this level and was from the WCHB1-41 order from the Verrucomicrobia phylum. The

third and fourth most abundant genera overall were from the Clostridiales order with both unassigned at genus level but originating from the Ruminococcaceae and Lachnospiraceae families. Bacterial taxa assigned to genus level also included *Fibrobacter* from the Fibrobacteres phylum, the genus *Treponema* from the Spirochaetes phylum, bacterial taxa from the *Prevotella* genus (Bacteroidetes phylum) and the *Streptococcus* genus (Firmicutes phylum).

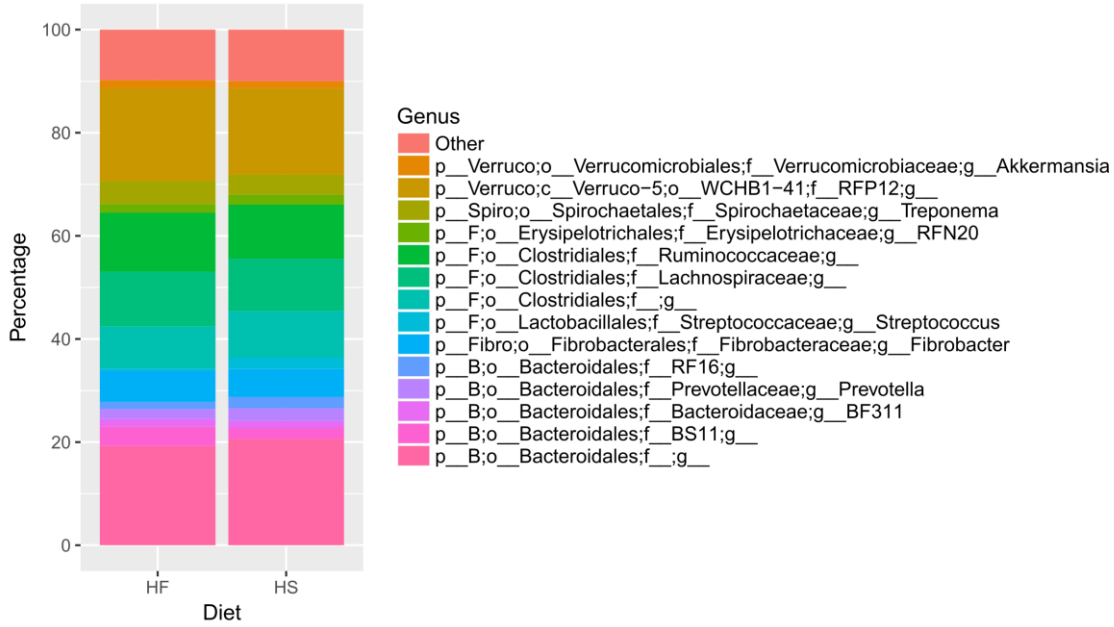

Figure 2.4 - Bacterial taxa plot showing genus level relative abundance for the high-starch (HS) and high-fibre (HF) diets.
Legend includes abbreviated phyla level bacteria (p_), F = Firmicutes, B = Bacteroidetes, Fibro = Fibrobacteres, Spiro = Spirochaetes, Verruco = Verrucomicrobia.

Results from NMDS ordination plots showed grouping of microbial community structure related to diet for both experimental periods (Figure 2.5). While some overlap was evident in the ordination plots, the community structure also showed differences related to diet. This was consistent for both testing periods; however, period one did show wider variation in community structure for the HS diet compared to the HF diet. A clear relationship was visible between the microbial community structure and the behaviour of the ponies when plotted on the ordination plots in both experimental periods. Time spent investigating increased in the direction of the HF diet and the frequency of pace-change increased in the direction of the HS diet (Figure 2.5). Results showed strong correlations for each of the behavioural variables with faecal microbial community composition.

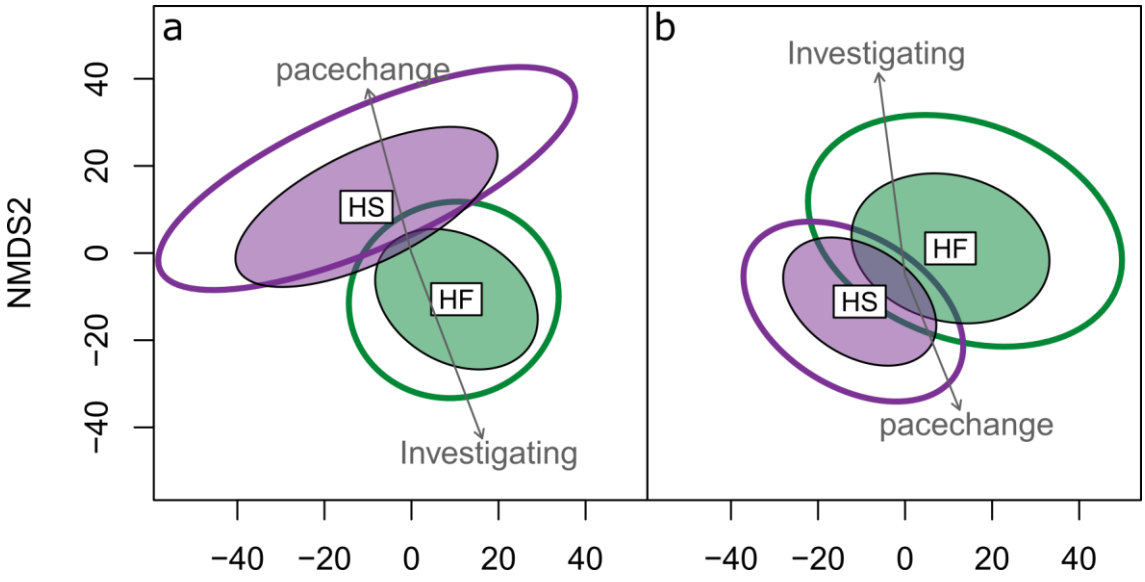

Figure 2.5 - NMDS plots comparing faecal microbial community structure for high-starch (HS) and high-fibre (HF) diets in experimental period 1 (a) and 2 (b).
Shaded ellipses show the diet grouping of microbial taxa to one standard deviation of point scores. Coloured rings display ellipses encircling all points in the group. The significant behavioural results from the passive human test (time spent investigating and frequency of pace-change) have been plotted over the top of the diets showing the direction towards which these behaviours are seen to increase. The arrows point towards the direction of increasing gradient with the length of the arrows indicating the proportion of correlation. The frequency of pace-change increases in the direction of the HS diet while the time spent investigating increases in the direction of the HF diet.

For the faecal microbial community composition analysis, model selection resulted in an optimal multivariate model which included the explanatory variables, ‘diet’, ‘experimental period’ and the interaction between diet and period. This model was significantly better than the simpler nested model which did not include the interaction ($p < 0.01$, Wald = 66.68). Cross validation, comparing the optimal model predictions of OTU abundance with true OTU abundance showed a strong and significant ($\rho = 0.75$, $p < 0.001$) correlation between the prediction and true values.

Multivariate GLMs showed that the abundance of several OTUs was significantly affected by diet (Table 2.5). Results also showed an effect of the interaction between diet and period on the abundance of OTUs. Diet had a significant effect on the abundance of 85 OTUs regardless of experimental period ($p < 0.05$). Figure 2.6 displays the 20 OTUs which show a significant difference depending on diet at $p < 0.001$. Of these 20 OTUs 18 were from the Firmicutes phylum, one was from the Bacteroidetes phylum and one from the Proteobacteria phylum. Proteobacteria represented less than 1% of the overall relative abundance. From the OTUs of the Firmicutes phylum, 17 were from the Clostridia class and

Clostridiales order, with the remaining OTU coming from the Bacilli class and Lactobacilli order.

Table 2.5 - Model output from 16S rRNA gene sequencing multivariate model. Results show a significant effect of diet on community composition and a significant interaction between diet and experimental period on community composition.

Test statistics	Wald value	P-value
(Intercept)	191.36	< 0.001
DietHS	40.38	0.015
Period2	32.64	0.261
DietHS:Period2	39.18	0.013

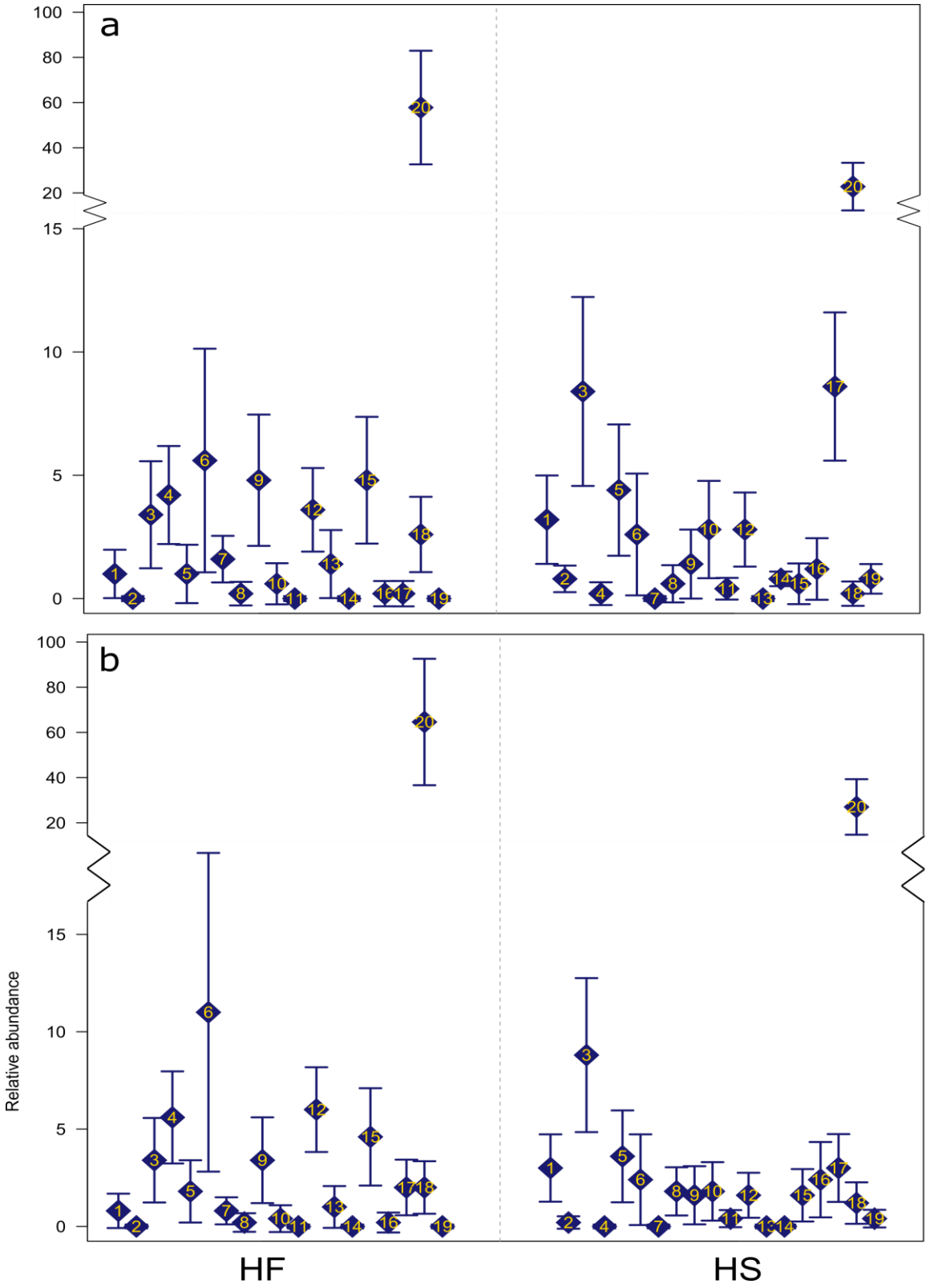

Figure 2.6 – Differences in relative abundance of Operational Taxonomic Units (OTUs) related to diet.
Plots show significantly different ($p < 0.001$) OTUs depending on diet in experimental period 1 (a) and experimental period 2 (b). HF = High-fibre and HS = High-starch. OTU identification: 1 = Firmicutes-Lachnospiraceae, 2 = Proteobacteria-Alphaproteobacteria, 3 = Firmicutes-Lachnospiraceae, 4 = Firmicutes-*Oscillospira*, 5 = Bacteroidetes-Bacteroidales, 6 = Firmicutes-Ruminococcaceae, 7 = Firmicutes-Ruminococcaceae, 8 = Firmicutes-Clostridiales, 9 – 12 = Firmicutes-Ruminooccaceae, 13 = Firmicutes–Lachnospiraceae, 14 & 15 = Firmicutes-Clostridiales, 16 = Firmicutes-*Streptococcus*, 17 = Firmicutes-*Oscillospira*, 18 = Firmicutes-Christensenallaceae, 19 = Firmicutes-*Oscillospira*, 20 = Firmicutes-Lachnospiraceae.

From the 17 OTUs of the Clostridiales order, three were from the Lachnospiraceae family and were unassigned at genus level, and ten were from the Ruminococcaceae family with three of these assigned to the *Oscillospira* genus with the remainder unassigned at genus level. Of the four remaining OTUs from the Clostridiales order, three were unassigned at family level and one was from the Christensenellaceae family. Christensenallaceae was significantly decreased with the HS diet compared to the HF diet regardless of experimental period. The remaining OTU from the Firmicutes phylum (Bacilli class and Lactobacillales order) was identified to genus level as *Streptococcus* and showed greater abundance in the HS diet than the HF diet regardless of experimental period. The OTU from the Bacteroidetes phylum was from the Bacteroidia class and Bacteroidales order but was unassigned below this level. The OTU from the Proteobacteria phylum was from the Alphaproteobacteria class and was unassigned below this level.

2.5 Discussion

These results indicate a clear effect of diet on the faecal microbiota and behaviour of naïve ponies. They also showed a strong association between behaviour and faecal microbial profile related to diet. An increase in the frequency of pace-change was closely associated with a HS diet. An increase in time spent investigating was closely associated with a HF diet. The faecal microbial profile from the HS diet is associated with increased behavioural reactivity, while the faecal microbial profile from the HF diet is associated with more settled behaviours. The increase in reactive behaviours seen here with the addition of starch in the diet may make horses more difficult to handle.

When fed the HS diet ponies were more alert and reactive. Conversely when they were fed the HF diet the ponies were more settled. The increased frequency of pace-change with the HS diet shows that ponies were more unsettled and reactive. The QBA assessment also supports this, with observers perceiving the ponies to be more nervous/tense/unsure on the HS diet. It has been reported previously that horses with lower stress levels display less time in locomotion (Benhajali *et al.*, 2009). Conversely, increased "flightiness" has been associated with horses that were most fearful (Visser *et al.*, 2001). The young ponies used in the current study may have found the presence of a person in the testing enclosure potentially threatening. Increased locomotory behaviour has been seen previously in young horses fed a starch diet compared to youngsters which received an alternative fat and fibre diet (Nicol *et al.*, 2005). Increased behavioural reactivity may generally be more obvious when younger horses are studied, with mature horses, over time, learning to become less reactive. This does not mean that mature horses will not react but that it just may not be so obviously displayed and could therefore be more difficult to quantify in a behavioural testing situation.

The results also showed that ponies spent significantly less time investigating their surroundings when fed the HS diet compared to the HF diet. This, like the increase in pace-change results, suggests that ponies were less at ease and unsettled. The QBA assessment also supports this, with observers perceiving the ponies to be more nervous/tense/unsure. The correlation between the quantitative and qualitative measures indicates an overall picture of increased

reactivity and unease when ponies were receiving the HS diet compared to the HF diet; as the time spent investigating decreased the QBA assessment of the ponies was perceived as being more nervous/tense/unsure. Changes in investigative behaviours have been observed previously in relation to diet. One study found that youngsters receiving a fat and fibre diet appeared to be less distressed and were more attentive to their environment than youngsters receiving a starch-sugar diet (Nicol *et al.*, 2005). A reduction in investigative behaviours was also seen in mature horses; when they received a high grain diet they spent less time exploring their stabled environment (Freire *et al.*, 2009). However, results from the current study not only show that changes in behaviour related to diet could be measured quantitatively, they also show that the behavioural differences were displayed clearly enough for observers to see.

In the novelty test, ponies showed an increased frequency of being alert when they were fed the HS diet compared to the HF diet. Again, this suggests that ponies were less at ease. In the current study, some food in a bucket was in place during the novelty test to motivate ponies to approach the novel object. This motivation to approach a novel object is important so that the animal's response to it can be interpreted (Christensen *et al.*, 2005). There was no significant effect of diet on the frequency with which the ponies glanced at the feed bowl suggesting that there was no difference in their motivation to eat. These result contrasts with an earlier study that found weanlings being fed a fat and fibre based diet spent significantly more time investigating a novel object compared to those fed a starch-sugar diet (Nicol *et al.*, 2005). As a flight animal, a horse's response to an unfamiliar object cannot always be anticipated and the difference in results may be due to differences in the novel objects used. QBA analysis found that ponies were more nervous/tense/unsure in experimental period one. This could be due to the different novel objects used for the two experimental periods. It is possible that the different objects were not equally alarming to the ponies. There was no effect of diet, however, on the ponies' responses to either object.

The combination of 16S *rRNA* gene sequencing using the Illumina MiSeq platform and the multivariate modelling approach of the sequencing data used in this chapter has enabled in-depth analyses to be undertaken. Multivariate analyses

results showed a significant difference in microbial community composition related to diet even though richness and diversity measures showed similarity. Therefore, it is the composition of the bacterial taxa that contribute to the overall diversity which differed in relation to diet.

In this study, Firmicutes was the dominant phylum and remained dominant irrespective of diet, closely followed by Bacteroidetes. This concurs with results from other studies that used faecal samples from horses receiving different diets (Dougal *et al.*, 2014; Fernandes *et al.*, 2014). A shift in the relative abundance of the two dominant phyla has previously been reported from faecal samples obtained from horses with colitis (Costa *et al.*, 2012). In the current study, the ponies were healthy and received diets with a starch level below what is considered to be the recommended upper limit of intake (National Research Council, 2007). Therefore, such a dramatic shift in population at phylum level is not to be expected.

The OTUs that were identified as showing significant differences in abundance related to diet mostly originated from the Firmicutes-Clostridia-Clostridiales lineage, with the majority coming from the Ruminococcaceae family. Ruminococcaceae bacteria have previously been identified as fibrolytic bacteria (Daly *et al.*, 2012). The current study shows that even a small addition of starch to the diet is enough to reduce this bacterial population. An increase in lactic-acid producing bacteria has also been reported to be coupled with a corresponding decrease in fibrolytic bacterial abundance (Daly *et al.*, 2012). Another of the OTUs that was significantly different related to diet was *Streptococcus*, which showed a higher relative abundance for the HS diet compared to the HF diet. *Streptococcus*, from the Firmicutes-Bacilli-Lactobacillales taxonomic lineage, is a lactic-acid producing bacteria (de Fombelle *et al.*, 2003) and has been reported to be the predominant bacteria seen to increase prior to the onset of oligofructose-induced laminitis (Milinovich *et al.*, 2008).

Streptococcus bacteria have also been reported to progressively increase in response to dietary change (Daly *et al.*, 2012; van den Berg *et al.*, 2013), with lower increases when there was less starch in the diet (de Fombelle *et al.*, 2003). In the current study, starch was fed at a level of 1 g/kg of BW per meal

for the HS diet and the faecal microbiota still showed an increase in *Streptococcus* bacteria. This shows that even the addition of some starch to the diet, which should not pass through the small intestine undigested, can still result in an increase of *Streptococcus* bacteria. However, what is unclear is whether an increase in *Streptococcus* at these levels has any wider health implications for the host, or if this is a microbial adaptation brought about by the addition of starch to a mostly fibre-based diet.

Results show that a HS diet does have an effect on the behaviour of naïve ponies and also alters faecal microbiota. The NMDS plots showed strong correlations between behaviour and microbial profile related to diet. When key behaviours were plotted against microbial profile an increase in the frequency of pace-change was closely associated with a HS diet, whereas, an increase in time spent investigating was closely associated with a HF diet. The faecal microbial profile from the HS diet is associated with increased behavioural reactivity, while the faecal microbial profile from the HF diet is associated with more settled behaviours. These results are from feeding starch levels that are within what is currently recommended as an acceptable level (National Research Council, 2007).

A study using mice found that dietary induced alterations to microbiota resulted in significant differences in behaviour (Lyte *et al.*, 2016). In horses, a recent study using culture-based techniques found correlations between alterations in gut microbiota and an increased stress response (Destrez *et al.*, 2015). However, a greater quantity of starch was fed meaning that it may have passed undigested into the hindgut. It is therefore possible that the increased stress response might have been associated with digestive discomfort. In the current study it is unlikely that starch would enter the hindgut undigested as the feeding levels were within recommended daily maximum levels. Therefore, what remains to be determined are the mechanisms behind the associated behavioural and microbiota changes that were observed in this study.

What is clear from this study is that dietary change resulted in alterations in behaviour and faecal microbiota. These changes occurred by making a small dietary adjustment. It is likely that alterations to diet may also initiate similar effects in other species. The increased starch in this chapter had an undesirable

effect on behaviour and gut microbiota; it made the ponies more reactive in their behaviour and moved the microbial community composition of the gut towards dysbiosis. However, the opposite was true of the HF diet. This diet resulted in ponies displaying more settled behaviour and gut microbiota more in keeping with a healthy equine gut. Therefore, diet may also have a positive, beneficial effect to the host. As such the wider effects of diet on the host should not be underestimated.

The current study comprised a sample size of ten ponies. This is a similar size to other equine studies exploring alterations in microbiota related to diet; Medina *et al.,* (2002) used eight horses and Destrez *et al.,* (2015) used six horses. However, some studies have had access to larger samples sizes; Daly *et al.*, (2012) had a total of 18 horses divided into three groups of six and Dougal *et al.*, (2014) had a total of 17 horses. The study by Dougal *et al.,* (2014) used a cross-over design that maximised the use of the horses and gave a larger sample size. The cross-over design approach was also used in the current study bringing the overall sample size to 20. Using a cross-over design increased the sample size and meant that all ponies had received both diets thereby reducing the risk of individual variability influencing the results. For this type of study design to be effective robust statistical analyses like those used in the current study are required.

The gut-brain axis encompasses a number of different communication systems, including direct communication via neural pathways and also indirect communications via endocrine and inflammatory pathways (Cryan and Dinan, 2012). Behavioural and neurochemical changes have been observed in rats following antibiotic induced modulation of gut microbiota (Hoban *et al.*, 2016). It is possible that the changes in behaviour found in this study were triggered by neuroendocrine alterations brought about by dietary-induced changes to gut microbiota. Although the faecal samples from the current study provide a good indication that changes in gut microbiota did occur as a result of diet, what remains to be determined is where in the hindgut these changes originated.

Chapter 3 The addition of starch to equine diets shifts the microbial community in the hindgut towards dysbiosis

3.1 Abstract

Alterations in gut microbiota have been shown to be associated with behavioural changes in rodents. The previous chapter showed a high-starch (HS) diet was associated with increased reactivity and unsettled behaviour. There were also changes in faecal microbial community composition with the HS diet that accompanied the changes in behaviour. Although the microbial communities in the faecal samples differed significantly on high-starch (HS) and high-fibre (HF) diets, it was not possible to identify where in the gut these changes originated. Dietary alterations in faecal microbiota may be associated with specific regions of the hindgut or may be indicative of a more general hindgut wide alteration. In this chapter, the hindgut microbiota of 10 naïve ponies being fed either a HS or HF diet is compared using 16S *rRNA* gene sequencing on the Illumina MiSeq platform. Five ponies received a high-starch (HS) diet and five received a high-fibre (HF) diet. The ventral, dorsal and small colon regions were identified as having an overall community structure that was significantly different related to diet. Multivariate analysis found that the dorsal colon had the greatest number of operational taxonomic units (OTUs) that differed in relation to diet (n= 52). Similarities related to diet were also identified between the faecal samples collected as part of the behavioural study (Chapter 2) and the faecal samples collected post-mortem for this chapter. From both sets of faecal samples for the HS diet, Ruminococcaceae-*Oscillospira* bacteria decreased combined with an increase in *Streptococcus* bacteria. These changes were also seen in the dorsal and small colon regions of the hindgut. It is therefore possible that these key bacterial taxa may be indicative of a hindgut at risk of becoming dysbiotic as a result of dietary change.

3.2 Introduction

In the previous chapter, faecal samples collected from ponies on high-starch (HS) and high-fibre (HF) diets contained differences in faecal microbial communities. The ponies also displayed differences in behaviour depending on which diet they were being fed. However, what remains unclear is where in the hindgut the differences observed in the faecal samples originated and, if there are particular regions of the hindgut that are affected by dietary change more than others.

Faecal samples are often used to develop an understanding of alterations to gut microbiota owing to the ease of obtaining samples. Using faecal samples often results in larger sample sizes being available for analyses compared to studies where digesta samples are obtained directly from the gut. While faeces are not directly comparable to the different regions of the gut they provide a broad overview of any changes that may be occurring related to ill health or alterations in diet (Costa *et al.*, 2012; Dougal *et al.*, 2014). Human studies using faecal samples have found associations between faecal microbiota and health conditions. For example, faecal samples have been used to identify similarities in bacterial community structure in Japanese infants that developed allergies (Tanaka *et al.*, 2017). A meta-analysis study of patients presenting with different gastrointestinal diseases aimed to identify if there were universal biomarkers for gut disease (Mancabelli *et al.*, 2017). Their results showed that these patients exhibited a greater abundance of specific bacterial populations that differed from their control groups (Mancabelli *et al.*, 2017). The identification of such biomarkers aids diagnosis and prognosis of patients presenting with gastrointestinal diseases. Results from human studies have provided a greater understanding about the associations between gut microbiota and health.

Differences in faecal microbiota have also been observed in horses presenting with gastrointestinal related health conditions (Costa *et al.*, 2012). As a non-ruminant herbivore, the horse has a digestive tract that has evolved to trickle feed on high-fibre diets (Daly *et al.*, 2012). However, the domestic horse's diet and feeding patterns are often far removed from this. Modern feeding practices such as reduced fibre rations and meal feeding are often closely associated with

gastrointestinal conditions such as colic (Secombe and Lester, 2012). Differences in the faecal microbial populations between healthy horses and horses with colitis have been observed (Costa *et al.*, 2012). Alterations in faecal microbiota have also been observed in healthy horses related to dietary changes. For example, horses being fed a fibre diet had more stable faecal microbial populations compared to horses being fed a high-starch diet (Willing *et al.*, 2009). Horses have a limited capacity to digest starch and feeding a diet high-in starch can result in undigested starch entering the large intestine (de Fombelle *et al.*, 2003). This overflow of undigested starch can result in alterations in the microbial community composition of the hindgut. Undigested starch entering the hindgut has resulted in an increase in lactic-acid producing bacteria coupled with a decrease in fibrolytic bacteria (de Fombelle *et al.*, 2003; Daly *et al.*, 2012).

It is possible to detect changes to the microbial populations of the hindgut through the use of faecal samples. Although faecal samples provide a good indication that microbial alterations are occurring, they may not be truly representative of different regions of the digestive tract. Therefore, it is not clear which region of the digestive tract is being affected by a dietary change and hence what the physiological significance of these changes might be. Studies comparing faeces to different regions of the digestive tract have reported conflicting results. One study found that the faeces were closer in similarity to the right dorsal colon than the caecum (Dougal *et al.*, 2012). It is to be expected that samples taken from gut regions closer to the rectum would be more similar to faecal samples. These findings contrast with another study that compared a number of gut regions and found that the closest in similarity to the faeces was the caecum (Schoster *et al.*, 2013).

While faecal samples have proven to be useful indicators of microbial changes in the gut, they do not provide a clear indication where in the digestive tract the changes are occurring. The fact that alterations in microbial populations are detected in the faeces may be enough to indicate gut dysbiosis. However, to understand the effects that diet may have on gut microbiota a clearer understanding of which hindgut regions are affected is required. This allows the exploration of whether changes in microbiota are associated with the wider

physiology of the host, or, are a necessary microbial adaptation with no wider health implications.

To obtain a detailed understanding of the changes occurring in different hindgut regions, it is possible to sample directly from the digestive tract using fistulated animals. This approach has been used in various studies, allowing direct, and repeated, sampling (Milinovich *et al.*, 2008; Muhonen *et al.*, 2009; Hansen *et al.*, 2014; Destrez *et al.*, 2015; Grimm *et al.*, 2017; Warzecha *et al.*, 2017). Using fistulated animals, researchers have analysed the microbial changes occurring in specific regions of the digestive tract. However, there are restrictions as fistulas can only be fitted to one or two regions of the digestive tract. Therefore, although samples are being taken directly from specific regions of the digestive tract, the sampling sites are limited. In the studies by Destrez *et al.*, (2015) and Grimm *et al.*, (2015) two regions of the large intestine were sampled using fistulas fitted to the caecal and right-ventral colon regions, while the study by Muhonen *et al.*, (2009) only sampled from the ventral colon, and Milinovich *et al.*, (2008) and Hansen *et al.*, (2014) only sampled from the caecum.

Fitting fistulas also raises ethical concerns and as such limits the sample sizes that are used in each study. The studies by Destrez *et al.*, (2015) and Grimm *et al.*, (2009) each used six horses, Milinovich *et al.*, (2008) used five horses and the studies by Muhonen *et al.*, (2009) and Hansen *et al.*, (2014) both used four horses. While the results of these studies have provided understanding of specific gut regions, the small sample sizes mean that the results may not be truly representative of the wider population. It is also possible that fitting fistulas may induce an altered microbial profile in that region as it is not possible to ensure the area is completely sealed. The study by Hansen *et al.*, (2015) excluded some of their results from statistical analysis because oxygen was suspected to have leaked into the caecum through the plug. It is, therefore, likely that the microbial composition of fistulated horses will differ from those without fistulas as the hindgut environment cannot be guaranteed to be the same.

Whether studies have used faeces or direct sampling from fistulated animals they have shown that dietary changes can alter hindgut microbiota. What is often unclear is whether these changes have wider health impacts on the animal

or are just a necessary microbial adjustment to a different diet. The extent of the microbial changes may depend on the composition of the feed and how in keeping it is with the natural diet of the horse. The faecal bacterial communities of yearlings rapidly transitioned from a forage and grain-based diet to pasture became similar to the yearlings already at pasture within four days, despite there being significant differences at the start (Fernandes *et al.*, 2014). The hindgut microbiota of the yearlings in this study adapted quickly to the new diet with no ill effects to health being reported. This is perhaps because the dietary change moved from a less natural diet to a more natural pasture diet. The dietary transition may not have been so smooth had the abrupt change been the other way around and the yearlings went from pasture to the forage-grain diet. A study that transitioned fistulated horses from hay, to a hay and grain-based diet observed no ill effects on health in the study horses (Grimm *et al.*, 2017). However, it did take six days longer before microbial stability of the hindgut was established.

Changes to hindgut microbiota have had wider health impacts on the host. When horses received higher levels of barley, which exceeded the current National Research Council (2007) recommended maximum starch levels, white blood cell concentrations showed a significant increase in granulocytes indicative of an increased immunological response (Destrez *et al.*, 2015). The increase in granulocytes had not returned to normal 10 days after receiving the HS diet (Destrez *et al.*, 2015). This indicates that diet has a much wider physiological effect on the host and not only at the time of receiving the diet but also for a prolonged period afterwards. Increases in *Streptococcus* bacteria have been found in caecally fistulated horses prior to the onset of oligo-fructose induced laminitis (Milinovich *et al.*, 2008). The use of caecal fistulas enabled regular sampling of the hindgut microbial changes. However, what is unclear is how the changes observed in the caecum may then present throughout the remainder of the hindgut. For example, it is possible that the shift in microbial populations observed in the caecum may become more pronounced further along the hindgut.

The aims of this chapter are, to describe the effects of a high-starch (HS) and high-fibre (HF) diet on the microbial community of the different regions of the

hindgut, and to compare the faecal samples collected during the behaviour chapter to those collected post-mortem for this chapter to ascertain if any similarities exist that could indicate the hindgut environment may also have been similar in the previous chapter.

3.3 Methods

3.3.1 Experimental design

The ten ponies underwent a two-week dietary wash-out period after the feeding and behaviour experimental procedures described in Chapter 2. During the wash-out period all ponies received hay *ad-libitum* and no concentrate ration. At the end of the wash-out period, the ponies were returned to the diets they were receiving at the end of the experimental trials from Chapter 2; ponies 1-5 received high-fibre (HF) and ponies 6-10 received high-starch (HS). They remained on this diet, receiving two feeds per day for a minimum of 14 days, following which the ponies were euthanased. Euthanasia took place on days 14, 16, 18, 21 and 23 with two ponies at random, with one pony from each sex and each diet, being euthanased on each occasion (Table 3.1). Additional background information on the ponies is included in Appendix A.

Table 3.1 - Pony euthanasia order and diet received.
'Day' indicates the day of euthanasia and corresponds to the number of days each pony received their allocated diet for prior to this. HF = high-fibre, HS = high-starch.

Day	Pony (Diet - HF)	Pony (Diet - HS)
14	3	10
16	4	9
18	2	8
21	5	7
23	1	6

3.3.2 Diets

The diets received by the ponies were the same as those described in Chapter 2. Briefly, all ponies were fed according to the National Research Council (2007) recommendations for growth using the estimated mature bodyweight (BW) of 250 kg, with each pony receiving 2.5% BW in feed per day. The HF diet consisted of hay plus high-temperature dried Lucerne with ponies receiving 0.46 g/kg BW

of starch per meal. The HS diet consisted of hay plus a compound mix with ponies receiving 0.96 g/kg BW starch per meal. Both the HF and HS diets were designed to provide equivalent energy with all ponies receiving 40.2 MJ/DE per day. Additional dietary information is included in Appendix A.

3.3.3 Euthanasia and sample collection

The ponies were transported by trailer to the euthanasia site in pairs; a short journey of approximately 5-10 minutes. Ponies were sedated prior to transportation with Domidine® (Detomidine hydrochloride 10.0 mg corresponding to 8.36 mg detomidine base) which was administered via intramuscular injection once the ponies were secured on the trailer. Ponies were euthanased individually, with the second pony remaining sedated on the trailer. All ponies were euthanased with 25 ml of Somulose® (Secobarbital sodium 400 mg and Cinchocaine hydrochloride 25 mg) administered intravenously via a pre-placed jugular catheter.

3.3.4 Gastrointestinal tract sampling

Digesta samples were collected from the gastrointestinal tract of the ten ponies. The entire gastrointestinal tract was removed immediately following euthanasia. The different regions of the gastrointestinal tract were identified and sampled one section at a time. Digesta samples were collected from the caecum, the ventral, dorsal and small colon regions as well as a rectal faecal sample. All digesta samples were collected within 60 minutes following euthanasia, placed on dry ice in a polystyrene container immediately following collection and transferred and stored at -80 ^{o}C until analysis (Daly *et al.*, 2012; Schoster *et al.*, 2013; Chen *et al.*, 2014; Moreau *et al.*, 2014).

3.3.5 DNA extraction and *16S rRNA* gene sequencing

The protocol for genomic DNA (gDNA) extraction used for the digesta samples followed the same protocol as for the faecal samples and is described in more detail in Chapter 2 (Appendix B, Protocol 1). The Qiagen QIAamp® Fast DNA Stool Mini Kit that was used in Chapter 2 for the faecal samples was also used for the digesta samples. The standard human protocol was revised as described in Chapter 2 (Appendix B, Protocol 1).

Extracted gDNA was assayed for concentration, quality and protein contamination using a nanodrop to measure A_{260} and A_{280} values prior to freezing at -20 ^{o}C. Samples were briefly thawed and aliquoted according to gDNA concentration, then frozen at -20 ^{o}C prior to 16S *rRNA* gene sequencing at Glasgow Polyomics, University of Glasgow. Library preparation and sequencing were undertaken by Glasgow Polyomics using the same protocol described in detail in Chapter 2. Sequencing was run on an Illumina MiSeq using 2 x 300bp paired end reads as described in more detail in Chapter 2.

3.4 Statistical analyses

All statistical analyses on the 16S *rRNA* gene sequencing data were performed in R (version 3.4.3) (R core team, 2017). Shannon diversity was measured using the Vegan package (Oksanen *et al.*, 2017) and Chao1 richness was measured using the Fossil package (Vavrek, 2011) to evaluate the alpha diversity and richness of the hindgut. To compare alpha diversities for the hindgut in relation to diet a Linear Mixed Model (LMM) with pony as the random effect (intercept) was used. Backwards stepwise model selection was used to determine the optimal model (Zuur *et al.*, 2009). The residuals from the diversity models were checked for a normal distribution. An ANOVA (analysis of variance) and post-hoc Tukey HSD (honest significant difference) test was then used to compare alpha diversity in the different hindgut regions for each diet. Any regional differences within the same diet could then be analysed before dietary effects on each region were investigated. Prior to this, a Generalised Linear Model (GLM) was used to check for the effect of 'euthanasia day' on the diversity measures, for the hindgut overall and for the individual regions.

Non-metric multidimensional scaling (NMDS) plots were used to visualise the community structure for each region of the hindgut for both diets. Analysis of similarity (ANOSIM) was then used to compare the community structures for each hindgut region related to diet. For regions identified as hosting significantly different microbial communities related to diet, a Bayesian multivariate modelling approach was used to analyse the effect of diet on operational taxonomic unit (OTU) abundances. The multivariate models were constructed using the BORAL (Bayesian Ordination and Regression Analysis of Multivariate Abundance Data in R) package (Hui and Poisot, 2016) in R. This approach to

analysing multivariate abundance data allows for correlations between the OTUs to be accounted for by incorporating latent variables (Hui and Poisot, 2016). A correlated response negative binomial model was fitted with pony as a random effect. Each region was modelled individually with diet as the explanatory variable and included two latent variables. This method was also used to re-analyse the faecal samples collected during the second experimental period from Chapter 2 to compare them to the post-mortem faecal samples from this chapter. This allowed the comparison of samples collected at different times and also enabled any patterns between the two sets of faecal samples and the other regions of the hindgut to be investigated.

3.5 Results

3.5.1 Hindgut digesta samples 16S *rRNA* gene sequencing results

Results from the 50 large intestine (five hindgut regions from ten ponies) digesta samples sequenced gave a total count of 2,159,033 reads; with a total of 1,118,247 of the reads coming from the HF diet and 1,040,786 coming from the HS diet. The mean number of reads for each hindgut region and for both diets are shown in Table 3.2.

Table 3.2 - Mean read numbers for each hindgut region on HS and HF diets.
Mean number of sequencing reads from 16s *rRNA* gene sequencing are displayed with one standard deviation for high-starch (HS) and high-fibre (HF) diets in all sampled hindgut regions.

Region	Mean reads (± s.d.) HF diet	Mean reads (± s.d.) HS diet
Caecum	28,772 (9934)	29,540 (3879)
Ventral colon	42,217 (12,386)	30,999 (14,778)
Dorsal colon	44,877 (23,930)	52,938 (8116)
Small colon	55,309 (14,316)	47,844 (13,699)
Faeces (post-mortem)	52,474 (17,598)	46,836 (16,160)

Results showed that there was no significant difference in Shannon diversity or Chao 1 richness measures for the overall hindgut (all regions combined) related to ‘euthanasia day’. The individual regions of the hindgut also showed no

significant difference in diversity or richness measures related to 'euthanasia day'. Therefore, there was no difference in diversity or richness measures related to the amount of time the ponies spent on the diets.

The results comparing diversity measures for the overall hindgut related to diet showed a significant difference in Shannon diversity measures (t = -4.7, p < 0.001) and Chao1 richness values (t = -3.5, p < 0.001). Microbial diversity and richness were lower in ponies being fed the HS diet compared to the HF diet.

Significant differences related to hindgut region (f = 8.86, p < 0.001) were observed in Shannon diversity measures for the HF diet. Differences between regions were seen between the caecum and the dorsal colon, the caecum and the faeces, and the caecum and the small colon (Table 3.3). Significant differences related to hindgut region (f = 6.96, p < 0.005) were also observed in Shannon diversity measures for the HS diet. Differences between regions were observed between the caecum and the dorsal colon, the caecum and the faeces, the caecum and the small colon, the ventral and the dorsal colon, and the ventral and the small colon (Table 3.3).

Table 3.3 - Results from post hoc analysis of Shannon diversity measures comparing gut regions.
Tukey HSD (honest significant difference) test for the high-fibre (HF) and high-starch (HS) diet in the caecum (CA), ventral colon (VC), dorsal colon (DC), small colon (SC) and the faeces collected post-mortem (FAPM). Significantly different regions are indicated in bold along with the difference. Level of significance is 95%.

Hindgut regions	Diversity difference HF	P- value	Diversity difference HS	P - value
DC-CA	**0.61**	**< 0.001**	**0.58**	**< 0.05**
FAPM-CA	**0.54**	**< 0.01**	**0.54**	**< 0.05**
SC-CA	**0.62**	**< 0.001**	**0.64**	**< 0.01**
VC-CA	0.33	0.09	0.09	0.98
FAPM-DC	-0.07	0.98	0.04	0.99
SC-DC	0.02	0.99	0.06	0.99
VC-DC	-0.27	0.22	**-0.49**	**< 0.05**
SC-FAPM	0.08	0.96	0.10	0.97
VC-FAPM	-0.20	0.49	-0.46	0.07
VC-SC	-0.29	0.18	**-0.55**	**< 0.05**

Significant differences related to hindgut region (f = 8.92, p < 0.001) were observed in Chao1 richness estimator measures for the HF diet. Differences between regions were seen between the caecum and the dorsal colon, the caecum and the faeces, the caecum and the small colon, and the ventral and the dorsal colon (Table 3.4). Significant differences related to hindgut region (f = 4.84, p < 0.01) were also observed in Chao1 richness estimator measures for the HS diet. Differences between regions were observed between the ventral and the dorsal colon, and the ventral and the small colon (Table 3.4).

Table 3.4 - Results from post hoc analysis of Chao1 richness estimator measures comparing gut regions.
Tukey HSD (honest significant difference) test for the high-fibre (HF) and high-starch (HS) diet in the caecum (CA), ventral colon (VC), dorsal colon (DC), small colon (SC) and the faeces collected post-mortem (FAPM). Significantly different regions are indicated in bold along with the difference. Level of significance is 95%.

Hindgut regions	Richness difference HF	P - value	Richness difference HS	P - value
DC-CA	**239.78**	**< 0.01**	73.03	0.42
FAPM-CA	**168.81**	**< 0.01**	18.78	0.99
SC-CA	**189.62**	**< 0.01**	77.35	0.37
VC-CA	88.53	0.31	-82.05	0.32
FAPM-DC	-70.97	0.52	-54.25	0.70
SC-DC	-50.16	0.79	4.32	0.99
VC-DC	**-151.25**	**< 0.05**	**-155.08**	**< 0.05**
SC-FAPM	20.81	0.99	58.57	0.63
VC-FAPM	-80.28	0.40	-100.83	0.15
VC-SC	-101.09	0.20	**-159.40**	**< 0.01**

3.5.1.1 Phylum level relative abundance

Results from the 16S *rRNA* gene sequencing identified a total of five main phyla, which represented over 97% of the overall microbial abundance (Figure 3.1). Firmicutes was the dominant phylum overall regardless of sampling region or diet, followed by Bacteroidetes, Verrucomicrobia, Fibrobacteres and Spirochaetes. The Cyanobacteria phylum showed a relative abundance of <1% for all sampled regions with the exception of the caecum.

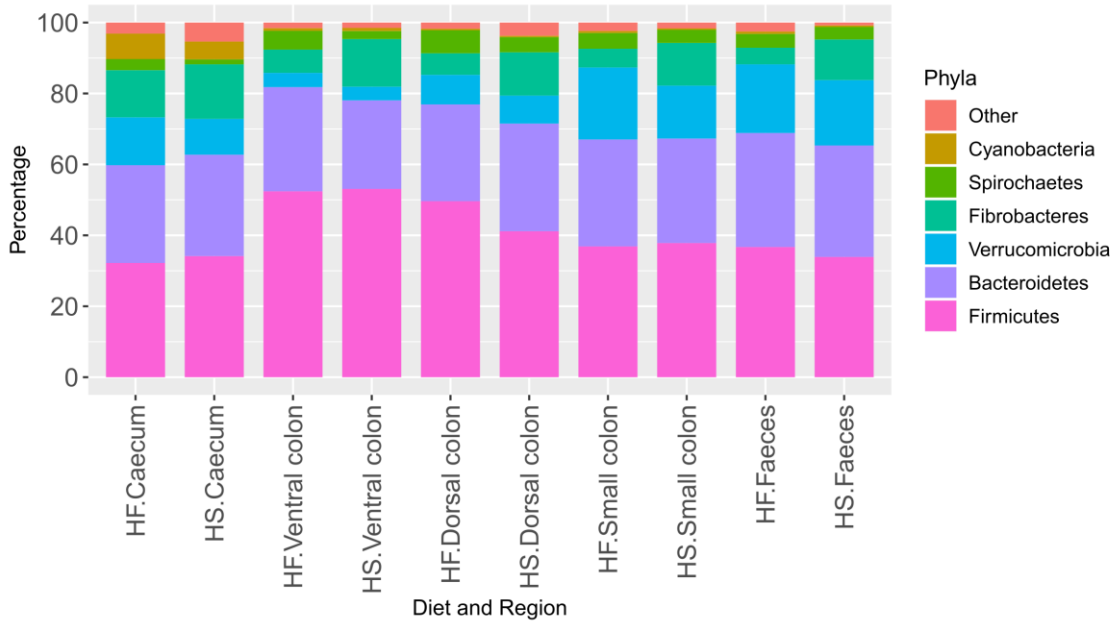

Figure 3.1 - Bacterial taxa plot showing phylum level relative abundance for high-starch (HS) and high-fibre (HF) diets in all sampled gut regions and faeces.

3.5.1.2 Family level relative abundance

The Firmicutes phylum comprised of family level bacteria primarily from the Clostridiales order with overall relative abundance from this order being dominated by Ruminococcaceae, closely followed by Lachnospiraceae. The Bacteroidetes phylum was dominated with family level bacteria from the Bacteroidales order with the dominant bacteria from this lineage being unassigned at family level. Bacteria from the Verrucomicrobia phylum was dominated by the family level bacteria RFP12 originating the Verruco-5 - WCHB1-41 lineage. The Fibrobacteres phylum included one family level lineage, Fibrobacteraceae. The Spirochaetes phylum was also dominated by one main family level lineage and this was Spirochaetaceae (Figure 3.2).

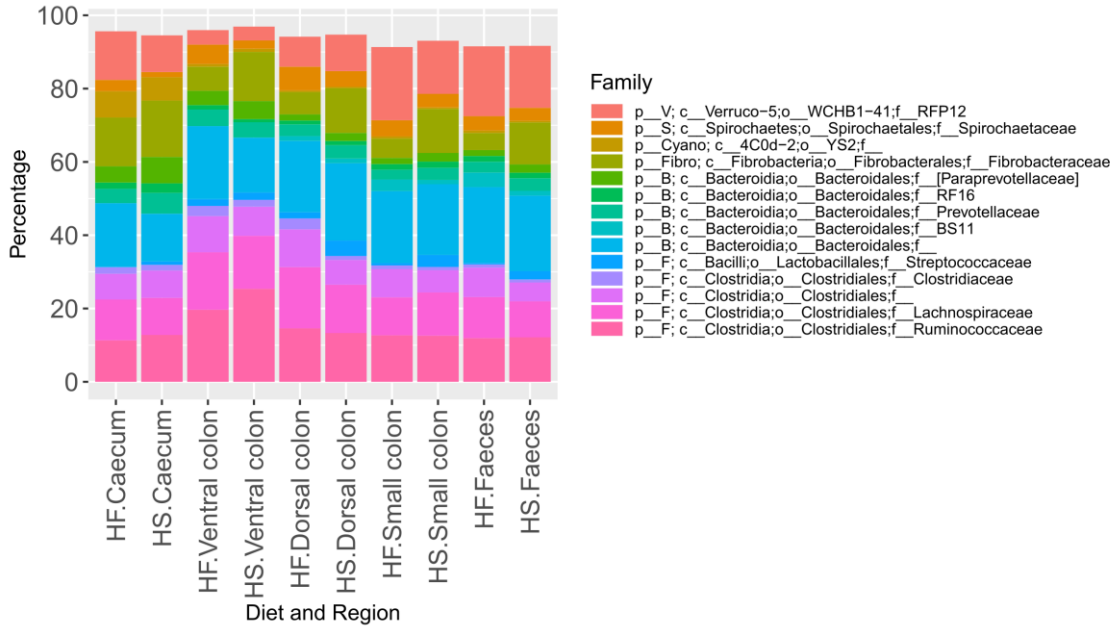

Figure 3.2 - Bacterial taxa plot showing family level relative abundance for high-starch (HS) and high-fibre (HF) diets in all sampled gut regions and faeces.
Legend includes abbreviated phyla level bacteria (p_), F = Firmicutes, B = Bacteroidetes, Fibro = Fibrobacteres, Cyano = Cyanobacteres, S = Spirochaetes, V = Verrucomicrobia.

3.5.1.3 Genus level relative abundance

At genus level, there were 16 bacterial taxa identified with a mean relative abundance >1%. Of the 16 bacterial taxa identified at genus level, seven were from the Firmicutes phylum. Five of these bacterial taxa were from the Clostridiales order, with only two being assigned to genus level which included *Clostridium* and *Ruminococcus*. Further bacterial taxa from the Firmicutes lineage included one from the Lactobacillales order and one from the Erysipelotrichales order with both being assigned at genus level and included *Streptococcus* and *RFN20* respectively. There were five bacterial taxa from the Bacteroidetes phylum all of which were from the Bacteroidales order. Only two of these were assigned at genus level; *Prevotella* from the Prevotellaceae family and *YRC22* from the [Paraprevotellaceae] family (Figure 3.3).

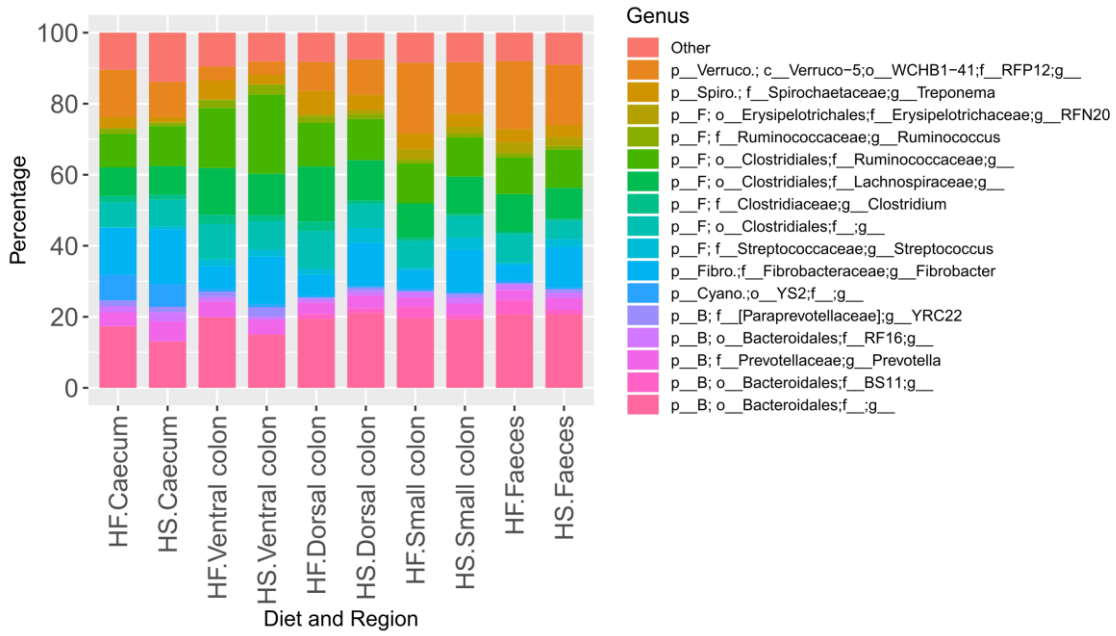

Figure 3.3 - Bacterial taxa plot showing genus level relative abundance for high-starch (HS) and high-fibre (HF) diets in all sampled gut regions and faeces.
Legend includes abbreviated phyla level bacteria (p_), F = Firmicutes, B = Bacteroidetes, Fibro = Fibrobacteres, Cyano = Cyanobacteres, Spiro = Spirochaetes, Verruco = Verrucomicrobia.

The remaining bacterial taxa at genus level with a mean relative abundance of >1% included bacterial taxa from the following phyla; Cyanobacteria, Fibrobacteres, Spirochaetes and Verrucomicrobia. The bacterial taxa from the Fibrobacteres phylum assigned to genus level included, *Fibrobacter*, along with genus level taxa from the Spirochaetes phylum, *Treponema*.

3.5.2 Microbial community composition

The non-metric multidimensional scaling (NMDS) plots displayed a visual grouping of community structure related to diet for each region of the hindgut. ANOSIM results showed no significant dissimilarity in community structure related to diet for the caecum (Figure 3.4a). ANOSIM results showed significant dissimilarities in community structure related to diet for the ventral colon ($R = 0.424$, $p < 0.05$ (Figure 3.4b)), the dorsal colon ($R = 0.416$, $p < 0.01$ (Figure 3.4c)) and the small colon ($R = 0.40$, $p < 0.05$ (Figure 3.4d)) regions. There was no significant dissimilarity for the faeces collected post-mortem (Figure 3.4e).

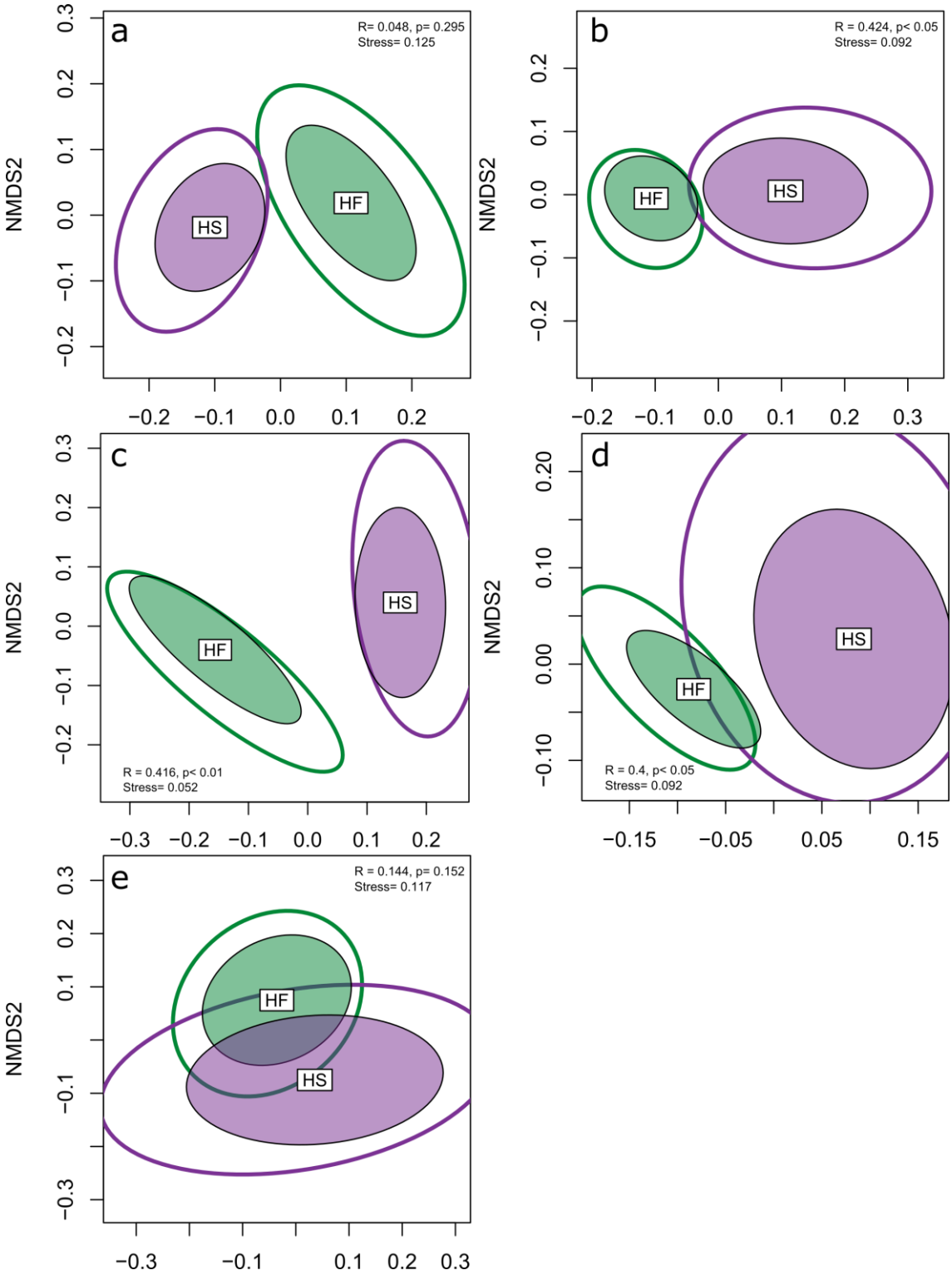

Figure 3.4 - NMDS plots showing microbial community structure and ANOSIM results for high-starch (HS) and high-fibre (HF) diets.
Plots show the sampled regions of the hindgut; (a) – caecum, (b) – ventral colon, (c) – dorsal colon, (d) – small colon, (e) – faeces collected post-mortem.

Bayesian multivariate analysis of community structure showed that there were 36 OTUs which differed significantly in abundance related to diet in the ventral colon. These significantly different OTUs were dominated by bacterial taxa from the Firmicutes phylum (24 OTUs). The remaining OTUs comprised of bacterial taxa from a range of phyla: Bacteroidetes (3 OTUs), Spirochaetes (4 OTUs), Fibrobacteres (3 OTUs), SRI (1 OTU) and Cyanobacteria (1 OTU).

The OTUs from the Firmicutes phylum showing significant differences related to diet in the ventral colon were predominantly from the Clostridia class (23 of 24 OTUs). This class comprised 11 OTUs from the Lachnospiraceae family of which 10 showed greater abundance for the HS diet. Only three OTUs from this lineage were assigned to genus level; *Coprococcus*, *Dorea* and *Blautia*. The Clostridia class also comprised six OTUs from the Ruminococcaceae family with three of these being more abundant for the HS diet including the genus level bacterial taxa, *Ruminococcus*. There were three OTUs from this lineage that showed a lower abundance for the HS diet including the genus level bacterial taxa, Ruminococcaceae-*Oscillospira*. The Clostridia class also included one OTU from the Clostridiaceae family which showed a lower abundance for the HS diet. The remaining five OTUs from the Clostridia class were unassigned below this level. The one Firmicutes phylum OTU not following the Clostridia class lineage was from the Erysipelotrichi class and included the genus level bacterial taxa *RFN20* at lower abundance for the HS diet in the ventral colon.

Two out of the three OTUs originating from the Bacteroidetes phylum were from the Bacteroidales order and unassigned below this level. The remaining OTU, also from the same order, originated from the Prevotellaceae family – *Prevotella* genus and showed greater abundance for the HS diet in the ventral colon. The four OTUs originating from the Spirochaetes phylum were all assigned to genus level, *Treponema*. Three of these showed lower abundance for the HS diet. The 3 OTUs from the Fibrobacteres phylum were all assigned to the same genus, *Fibrobacter* and showed greater abundance for the HS diet in the ventral colon.

Bayesian multivariate analysis of community structure showed a greater effect of diet on the dorsal colon region with 52 OTUs that differed significantly. These OTUs were dominated by bacterial taxa from the Firmicutes phylum (29 OTUs). The remaining bacterial taxa originated from a range of phyla; Bacteroidetes (10

OTUs), Verrucomicrobia (8), Spirochaetes (2), Fibrobacteres (2) and the remaining OTU originated from the Proteobacteria phylum.

The OTUs from the Firmicutes phylum showing significant differences related to diet in the dorsal colon were predominantly from the Clostridia class (27 of 29 OTUs). This class comprised 11 OTUs from the Lachnospiraceae family with eight of these OTUs being more abundant for the HS diet including the genus level bacterial taxa, *Anaerostipes* (1 OTU) and *Coprococcus* (2 OTUs). The Clostridia class also comprised nine OTUs from the Ruminococcaceae family with seven showing greater abundance for the HS diet and unassigned below family level. The remaining two OTUs from the Ruminococcaceae family – *Oscillospira* genus and showed lower abundance for the HS diet. The remaining seven OTUs from the Clostridia class were unassigned to family level. The two OTUs from the Firmicutes phylum that did not originate from the Clostridia class lineage both showed greater abundance for the HS diet in the dorsal colon. One was from the Erysipelotichi class and was assigned to genus level, *RFN20*. The remaining OTU was from the Bacilli class and was assigned to genus level, *Streptococcus*. This bacterial genus level taxa also showed greater abundance for the HS diet in the dorsal colon.

All ten of the Bacteroidetes phylum OTUs were from the Bacteroidales order with five of these unassigned to a lower taxonomic level and four out of the five showing greater abundance for the HS diet in the dorsal colon. Four of the bacterial taxa from the Bacteroidales order were assigned to genus level, including *Paludibacter*, *Prevotella* and two OTUs from the *YRC22* genus, all of which were more abundant for the HS diet. The remaining OTU was from the RF16 family and was also more abundant for the HS diet. The eight OTUs originating from the Verrucomicrobia phylum were all from the same lineage; class – Verruco-5, order – WCHB1-41, family – RFP12 and all were more abundant for the HS diet in the dorsal colon. The two OTUs from the Spirochaetes phylum were both assigned to genus level, *Treponema*, one showed greater abundance for the HS diet while the other was less. Both OTUs from the Fibrobacteres phylum were also both from the same genus, *Fibrobacter*, with both showing greater abundance for the HS diet. The remaining OTU originated from the

Proteobacteria phylum was only assigned to order level, Tremblayales and showed greater abundance for the HS diet in the dorsal colon.

Bayesian multivariate analysis of community structure showed that there were 28 OTUs that differed significantly related to diet for the small colon. Again, these were dominated by bacterial taxa originating from the Firmicutes phylum (15 OTUs). The remaining bacterial taxa originated from a range of different phyla; Bacteroidetes (9 OTUs), Fibrobacteres (2), Spirochaetes (1) and the remaining OTU originating from the Lentisphaerae phylum.

The 15 Firmicutes OTUs that differed related to the diet in the small colon regions were again dominated by bacterial taxa from the Clostridia class (13 OTUs). This class comprised five OTUs from the Lachnospiraceae family and all showed greater abundance for the HS diet with two of these OTUs assigned to genus level; *Coprococcus* and *Epulopiscium*. The Clostridia class also comprised five OTUs from the Ruminococcaceae family with four showing greater abundance for the HS diet. The remaining OTU, *Oscillospira*, showed less abundance for the HS diet in the small colon. There was one OTU from the Clostridia class - Veillonellaceae family and this showed greater abundance for the HS diet. The remaining two OTUs from the Clostridia class were unassigned below order level and also showed greater abundance for the HS diet. The remaining two OTUs originating from the Firmicutes phylum but not from the Clostridia class lineage included the genus level bacterial taxa, *p-75-a5* from the Erysipelotrichi class and bacterial taxa from the Bacilli class, *Streptococcus*. Both bacterial taxa showed greater abundance for the HS diet in the small colon region.

All nine of the OTUs originating from the Bacteroidetes phylum were from the Bacteroidales order with six unassigned below this level and five of these showing a greater abundance for the HS diet in the small colon. Two of the remaining bacteria from this lineage were assigned to genus level; *Paludibacter* and*YRC22* with both showing greater abundance for the HS diet. The remaining OTU belonged to the [Paraprevotellaceae] family and also showed greater abundance for the HS diet in the small colon. The two OTUs from the Fibrobacteres phylum were also both from the same genus, *Fibrobacter* and both showed greater abundance for the HS diet in the small colon. The one OTU from

the Spirochaetes phylum, *Treponema* showed less abundance for the HS diet in the small colon as did the remaining OTU from the Lentisphaerae phylum - Victivallaceae family.

3.5.2.1 Faecal sample comparison

Bayesian multivariate analysis of community structure showed that there were 44 OTUs that differed significantly related to diet for the faecal samples obtained post-mortem. There were 24 OTUs also identified as significantly different related to diet for the faecal samples collected during experimental period 2 (Chapter 2). Several similarities were observed between the taxonomic lineages from these two sets of results. These similarities were dominated by bacterial taxa originating from the Firmicutes plylum and Clostridiales order. For the period 2 faecal samples (Chapter 2), 13 out of 24 OTUs were from this lineage with the remaining OTU being from the Bacilli order. The post-mortem samples (this chapter) comprised 20 OTUs from the Firmicutes phylum with 18 originating from the Clostridiales order, one from the Bacilli order and the other from the Erysipelotrichales order.

Both sets of faecal samples showed the same pattern in abundance for Lachnospiraceae family level bacterial taxa originating from the Clostidiales order with both sets of faecal samples containing seven OTUs from this family with six showing greater abundance for the HS diet. There were also similarities for bacterial taxa from the Ruminococcaceae family with the *Oscillospira* genus showing decreased abundance across both sets of faecal samples for the HS diet. The genus *Streptococcus*, from the Firmicutes-Bacilli lineage showed increased abundance for both sets of faecal samples for the HS diet.

Similar patterns were also seen across both sets of faecal samples for bacterial taxa originating from the Bacteroidetes phylum and Bacteroidales order with all showing increased abundance for the HS diet. Not all of these bacterial taxa were assigned below order level but those that were included the genera *Prevotella, Paludibacter, YRC22* and *GF231*. Bacterial taxa originating from the Spirochaetes phylum included the genus level taxa, *Treponema* which showed greater abundance for the HS diet across both sets of faecal samples. This was also the case for the genus, *Fibrobacter* from the Fibrobacteres phylum.

While the majority of OTUs from the two sets of faecal samples showed similarity in their taxonomic lineages and abundance, there were eight individual OTUs from the behaviour period 2 faecal samples (Chapter 2) that exactly matched OTUs from the post-mortem faecal samples that were identified as being significantly different related to diet (Table 3.5). The abundance levels for these OTUs also showed the same pattern across both sets of faecal samples.

Table 3.5 - Taxonomic lineages of the OTUs that were identified in both sets of faecal samples as being significantly different related to diet.
HS = high-starch diet. Arrows indicate if OTU abundance was increased (↑) or decreased (↓) in the HS diet for each set of faecal samples. HS (P2) = behaviour period 2 faecal samples (Chapter 2). HS (PM) = faecal samples collected post-mortem.

OTU taxonomy (phylum-class-order-family-genus)	HS (P2)	HS (PM)
Firmicutes-Clostridia-Clostridiales-Ruminococcaceae-*Oscillospira*	↓	↓
Firmicutes-Clostridia-Clostridiales-Lachnospiraceae-*g_*	↑	↑
Firmicutes-Clostridia-Clostridiales-Lachnospiraceae-*g_*	↓	↓
Firmicutes-Bacilli-Lactobacilales-Streptococcaceae-*Streptococcus*	↑	↑
Bacteroidetes-Bacteroidia-Bacteroidales-RF16-*g_*	↑	↑
Bacteroidetes-Bacteroidia-Bacteroidales-f_*g_*	↑	↑
Bacteroidetes-Bacteroidia-Bacteroidales-f_*g_*	↑	↑
Fibrobacteres-Fibrobacteria-Fibrobacterales-Fibrobacteraceae-*Fibrobacter*	↑	↑

Both sets of faecal samples showed a great deal of similarity not only in the taxonomic lineages but also in terms of abundance. However, there were a small number of dissimilarities observed between the two sets of faecal samples. The first was the presence of six OTUs from the Verrucomicrobia phylum – genus *RFP12* identified in the post-mortem faecal samples and all showed increased abundance for the HS diet. These bacterial taxa were not identified for the Chapter 2 faecal samples as being significantly different related to diet. Secondly, one OTU from the Lentispiraceae phylum – Victivallaceae family showed lower abundance for the HS diet from the post-mortem faecal samples but not for the faecal samples from Chapter 2.

The eight OTUs that were identified as being significantly different related to diet from both sets of faecal samples also showed a similar pattern for specific regions of the hindgut. There were six out of eight OTUs identified in the small and dorsal colon regions of the hindgut that showed the same pattern related to

diet as both sets of faecal samples (Table 3.6). This pattern was not observed in the ventral colon or the caecum.

Table 3.6 - Taxonomic lineages of the 8 OTUs that were identified as significantly different related to diet in both sets of faecal samples and the pattern for the small and dorsal colon regions.
HS = high-starch diet. Arrows indicate if OTU abundance was increased (↑) or decreased (↓) in the HS diet for each set of samples. HS faeces (both) = behaviour period 2 faecal samples (Chapter 2) + faecal samples taken post-mortem (this chapter). HS (SC) = small colon. HS (DC) = dorsal colon. X = not identified in this region as significantly different related to diet.

OTU taxonomy (phylum-class-order-family-genus)	HS Faeces (both)	HS (SC)	HS (DC)
Firmicutes-Clostridia-Clostridiales-Ruminococcaceae-*Oscillospira*	↓	↓	↓
Firmicutes-Clostridia-Clostridiales-Lachnospiraceae-*g_*	↑	↑	**x**
Firmicutes-Clostridia-Clostridiales-Lachnospiraceae-*g_*	↓	**x**	↓
Firmicutes-Bacilli-Lactobacilales-Streptococcaceae-*Streptococcus*	↑	↑	↑
Bacteroidetes-Bacteroidia-Bacteroidales-RF16-*g_*	↑	**x**	↑
Bacteroidetes-Bacteroidia-Bacteroidales-f*_g_*	↑	↑	**x**
Bacteroidetes-Bacteroidia-Bacteroidales-f*_g_*	↑	↑	↑
Fibrobacteres-Fibrobacteria-Fibrobacterales-Fibrobacteraceae-*Fibrobacter*	↑	↑	↑

3.6 Discussion

Shannon diversity and Chao1 richness measures differed for the hindgut overall related to diet. There was lower diversity and richness for the HS diet compared to the HF diet. In Chapter 2, faecal samples showed no significant difference in Shannon diversity measures or Chao1 richness estimator measures related to the same diets. This highlights that faecal samples do not necessarily provide an accurate picture about the extent of the changes occurring in the hindgut. There have been conflicting results from some studies using faecal samples. For example, there was no significant difference for the Shannon diversity indices of faecal samples from horses fed different diets (Dougal *et al.*, 2014). Willing *et al.*, (2009) used Simpson's index to measure faecal diversity and also found no difference related to diet. However, the faecal samples of yearlings fed a diet containing hay and grain had lower Simpson diversity measures compared to yearlings at pasture (Fernandes *et al.*, 2014).

Some studies have highlighted differences in diversity measures between individual horses within the same study (Schoster *et al.*, 2013; Dougal *et al.*, 2014). In the current study a mixed effects model, with pony included as the random effect, was used to compare diversity and richness measures related to diet. Therefore, the effect of individual pony was taken into account when analysing these measures. Given the wide diversity of microbes that colonise the equine hindgut it is likely that some studies will show individual variability between study animals. There is often wide variability in the horses that studies use in terms of their age, breed and management. However, the ponies used in the current study were all the same breed, age and were managed in the same way so inter-animal variation will have been less likely. The young age of the ponies also meant they had not been exposed to the range of different dietary changes and husbandry techniques of older horses that would contribute towards shaping individual variability in gut microbiota. The use of young, truly naïve ponies combined with samples having been obtained from each region of the hindgut makes this a particularly robust and original study.

In this chapter differences in diversity between hindgut regions were found irrespective of diet. For both the HS and HF diets, the caecum showed lower diversity than the dorsal colon, small colon and faeces. Previous studies have

also found that diversity measures differed between intestinal regions (Dougal *et al.*, 2012; Schoster *et al.*, 2013). When Dougal *et al.*, (2012) compared samples from the caecum, right dorsal colon and the faeces they found greater diversity in the right dorsal colon and faeces compared to the caecum. However, Schoster *et al.*, (2013) found closer similarity between the caecum and the faeces. In this chapter and for the HS diet only the ventral colon was less diverse than the dorsal and small colon regions. There were also differences in richness between regions. For the HF diet, the caecum showed lower richness than the dorsal colon, small colon and the faeces and showed a similar pattern to the diversity measures for the HF diet in the same regions. Overall, the hindgut of ponies irrespective of diet is richer and more diverse towards more caudal regions of the hindgut.

In terms of community structure, NMDS plots showed a community structure grouping related to diet for all sampled regions. Three of these regions were identified as differing significantly related to diet; the ventral, dorsal and small colon regions. Modelling results allowed identification of which bacterial taxa differed related to diet for each region of the hindgut. Irrespective of region, the phylum that contained the majority of significantly different OTUs related to diet was the Firmicutes phylum. As the overall dominant phylum it is unsurprising that the majority of changes occurred to OTUs originating from that phylum.

There were also some regional differences observed for bacterial taxa originating from the Firmicutes phylum. For example, the relative abundance of *Streptococcus* bacteria did not differ significantly in the ventral colon but was significantly increased for the HS diet in the dorsal and small colon regions. *Streptococcus* is classed as lactic-acid producing bacteria (de Fombelle *et al.*, 2003) and has been seen to increase in the caecum prior to the onset of oligofructose-induced laminitis (Milinovich *et al.*, 2008). Caecally fistulated horses were used for the study by Milinovich *et al.*, (2008) and samples were not obtained from other regions of the hindgut. In this chapter, the increase in *Streptococcus* abundance occurred in more caudal regions of the hindgut and did so in response to starch quantities that were within the current maximum recommendations (National Research Council, 2007).

The regions that showed significant differences in microbial community composition related to diet were the ventral, dorsal and small colon regions. Additional regions may also have been affected by diet had a greater quantity of starch been fed. The amount of starch fed in this study was within the recommended maximum intake (National Research Council, 2007). Therefore, substantial microbial changes throughout the hindgut would not necessarily have been expected. However, the fact that changes have occurred with the starch levels that were fed in this study highlights that the inclusion of some starch in the diet has the potential to alter the microbial composition of the hindgut. The region that showed the greatest number of OTUs differing significantly related to diet was the dorsal colon. The ventral and small colon regions, although still significantly different related to diet, had fewer OTUs that were different. This suggests that the dorsal colon was the region most affected by the different diets. The microbial changes that started in the ventral colon, peaked in the dorsal colon, and were then seen to become less pronounced again in the small colon. This suggests that the dietary induced changes in gut microbiota may have started to become more stable again.

Bacterial taxa from the Verrucomicrobia phylum were found to be significantly different related to diet for some sampled regions, in particular the dorsal colon and the faecal samples obtained post-mortem. All of the OTUs from this phylum that differed related to diet showed increased abundance for the HS diet. The abundance of this particular phylum has shown variation in the results of some studies that have used faecal samples. It has been reported with lower levels of abundance (Dougal *et al.*, 2014; Fernandes *et al.*, 2014) but also found to have similar levels of abundance (Steelman *et al.*, 2012) to those found in this chapter. The higher levels of Verrucomicrobia abundance in the study by Steelman *et al.*, (2012) were found in the faceal samples of horses with chronic laminitis.

Not only does the abundance of Verrucomicrobia vary from one study to another but also from one hindgut region to another. The abundance of Verrucomicrobia ranged from <5% in the ventral colon, irrespective of diet, to >18% for the HF diet in the faeces. Although Verrucomicrobia showed variation in abundance across the sampled regions it only showed differences related to diet in the

dorsal colon and post-mortem faecal samples, with eight OTUs from the dorsal colon showing greater abundance for the HS diet. This shows that the abundance of Verrucomicrobia is widely varied and therefore, levels of abundance obtained from faecal samples cannot give clear indications for the rest of the hindgut. However, what is clear from this study is that Verrucomicrobia increases in abundance in the dorsal colon of the hindgut when a HS diet is fed.

The Fibrobacteres phylum also showed variation related to diet and was more abundant for the HS diet compared to the HF diet for all sampled regions. The dissimilarity in relative abundance for this phylum related to diet was seen to increase further towards more caudal regions of the hindgut. With only one taxonomic branch to family and genus level this pattern continued to lower taxonomic levels with significant differences related to diet for the ventral, dorsal and small colon regions along with both sets of faecal samples. The results seen here are in contrast with findings from another study. Daly *et al.*, (2012) found that *Fibrobacter* spp. were lower for concentrate fed horses compared to those that were grass fed. As fibrolytic bacterial taxa, *Fibrobacter* would be expected to be more abundant in ponies receiving the HF diet. However, one species from this taxonomic lineage, *Fibrobacter succinogenes* has been shown to peak in abundance in caecally fistulated horses for at least six hours after receiving a barley diet (Kristoffersen *et al.*, 2016). The time series plots from that study show great variability in abundance over time for *F. succinogenes* for the barley supplemented diet compared to more consistent abundance for the hay only diet (Kristoffersen *et al.*, 2016). Therefore, the increase in abundance of *Fibrobacter* seen in this chapter with the HS diet could be more related to the timing of euthanasia in relation to feeding times.

There was a great deal of similarity in the bacterial taxa that was present related to diet when comparing the faecal samples collected post-mortem with the faecal samples collected during behavioural testing (Chapter 2). Along with these similarities in bacterial taxa there were eight exact OTU matches present in both sets of faecal samples that showed the same pattern of abundance related to diet. These samples were taken a minimum of four weeks apart and therefore this suggests consistency in the changes that occurred related to diet over time. The consistency of these bacterial taxa not only indicates a stable

pattern over time but also suggests a direct association between these OTUs and the HS diet. Six of the OTUs found in both sets of faecal samples also showed the same pattern of abundance for the small colon and the dorsal colon regions related to diet. These findings indicate that faecal samples most closely represent more caudal regions of the hindgut.

The consistency of these results allows the opportunity to identify specific bacterial lineages as key faecal biomarkers that are indicative of hindgut microbial alterations specific to a HS diet. There were two key bacterial lineages both from the Firmicutes phylum shown to be consistently different in abundance that could be selected; Ruminococcaceae-*Oscillospira* and *Streptococcus*. *Oscillospira* bacteria, from the Ruminococcaceae family, showed significantly lower abundance for the HS diet across both sets of faecal samples as well as the small and dorsal colon regions. Bacteria from the Ruminococcaceae family have previously been identified as a fibrolytic bacterial population (Daly *et al.*, 2012). A higher abundance of the *Oscillospira* genus has also been positively associated with good health in humans while a reduced abundance has been associated with inflammatory gastrointestinal diseases (Gophna *et al.*, 2017). Children presenting with Crone's disease have a lower abundance of *Oscillospira* compared to healthy controls (Kaakoush *et al.*, 2012). Therefore, if these bacterial taxa serve a similar purpose in the equine gut, the lower abundance of *Oscillospira* seen with the HS diet may be associated with a hindgut that is heading towards dysbiosis and inflammation.

This decrease in Ruminococcaceae-*Oscillospria* associated with the HS diet was also combined with an increase in *Streptococcus* bacteria. This increase in *Streptococcus* abundance with the HS diet was consistent across both sets of faecal samples along with the dorsal and small colon regions of the hindgut. This genus has already been identified as a lactic-acid producing bacteria (de Fombelle *et al.*, 2003) seen to increase prior to the onset of acute laminitis (Milinovich *et al.*, 2008). *Streptococcus* has also been found to be more abundant in the faecal samples of horses with chronic laminitis (Steelman *et al.*, 2012). A study using caecally fistulated horses and feeding higher levels of starch found a similar trend associated with diet and also reported a decrease in *Ruminococcus* populations combined with an increase in *Streptococcus* (Warzecha *et al.*, 2017).

Therefore, the shift in abundance of these bacterial taxa may be indicative of a hindgut that is heading towards dysbiosis. It is concerning that these changes in bacterial taxa, which are usually associated with serious health conditions in horses, were also found in the ponies being fed the HS diet.

Fibrobacter was also seen to increase in association with the HS diet and consistently so across both sets of faecal samples as well as the small and dorsal colon regions. However, this genus has already been identified as showing wide variability in abundance related to feeding time and showed high peaks in abundance following a HS meal (Kristoffersen *et al.*, 2016). Therefore, this level of variation makes *Fibrobacter* unsuitable as a possible measure of hindgut dysbiosis.

The close association between the faecal samples obtained post-mortem and the faecal samples taken four weeks previously (from Chapter 2) show that some of the dietary-induced changes to microbiota are stable over time. In Chapter 2, behavioural tests showed that ponies were more reactive and less settled in their behaviour when they were fed the HS diet compared to the HF diet. The microbial community composition of their faeces also differed related to diet, in particular, the HS diet faecal samples showed lower abundance for the family level bacteria, Ruminococcaceae, combined with an increase in the genus level bacteria, *Streptococcus*. With these same trends also being seen in the faecal, small colon and dorsal colon samples in this chapter it is possible that the changes in microbiota associated with the HS diet also underpin the increased behavioural reactivity associated with the HS diet. However, the mechanisms behind these associations are unclear and require further investigation. Dietary induced changes to the gut microbiota of rodents have been associated with behavioural changes that were suggested to be related to alterations in serotonin and dopamine (Hanstock *et al.*, 2004). Therefore, it is possible that the dietary-induced changes in gut microbiota and behaviour observed in this chapter and Chapter 2 may be associated with alterations in serotonin and dopamine biosynthesis.

Chapter 4 Neurochemical changes in the equine brain and increased behavioural reactivity are linked to increased dietary starch

4.1 Abstract

Studies in other species have shown associations between gut dysbiosis and neurological alterations. Diet is often the key factor linking these changes. High-fat diets have been shown to induce oxidative stress in the brains of rodents. Alterations to specific bacterial populations of the gut have been shown to influence an animal's ability to cope with stress. In Chapter 2, when ponies were fed a high-starch (HS) diet it resulted in increased behavioural reactivity. There were also differences in the faecal microbial community composition related to diet. In Chapter 3, the microbial community composition of more caudal aspects of the hindgut showed similarities to the faecal samples obtained post-mortem and those collected during behavioural testing (Chapter 2). The increased behavioural reactivity brought about by the HS diet may be associated with neurological changes resulting from dietary-induced alterations in hindgut microbiota. Ten 18-month-old naïve ponies were divided into two groups and fed either a high-starch (HS) or high-fibre (HF) diet for a minimum period of two weeks. The ponies were euthanased and their gut and brain tissues were collected. Total RNA was extracted from the jejunum, caecum, ventral colon, dorsal colon and small colon regions of the gut tissues and was also extracted bilaterally from the caudate, putamen, nucleus accumbens, substantia nigra and ventral tegmental area of each brain. Real-time polymerase chain reaction (qPCR) primers were designed and used to quantify the relative expression of serotonin and dopamine receptor densities in the sampled gut and brain tissues. Results showed the caudate region of the brain had greater relative expression of dopamine D2-like receptor densities for the HS diet compared to the HF diet and for the right-hand side of the brain compared to the left. The nucleus accumbens region of the brain showed lower relative expression of dopamine D2-like receptor densities for the HS diet compared to the HF diet and for the right-hand side of the brain compared to the left. There was also lower relative expression of dopamine D1-like receptor densities for the HS diet and the small

colon tissue. This chapter shows that HS diets induce neurochemical changes in the brains of ponies.

4.2 Introduction

The two-way communication system between the digestive tract and the brain is now commonly known as the 'gut-brain axis'. Several communication pathways exist between the gut and the brain. These pathways include direct communication via the enteric and central nervous system and indirect communication via neuroendocrine and neuro-immune pathways (Cryan and Dinan, 2012). The gut microbiota is now recognised as being influential in gut-brain axis communication (Cryan and Dinan, 2012). Several studies have investigated the wider role and influence that gut microbiota play, particularly in terms of health and behaviour (Sudo *et al.*, 2004; Neufeld *et al.*, 2011b; Marques *et al.*, 2014; Hoban *et al.*, 2016; Tidjani Alou *et al.*, 2016).

Dietary induced alterations to gut microbiota could be the cause behind behavioural changes. In Chapter 2, naïve ponies showed significant differences in behaviour related to the diet they were receiving. Overall, the ponies displayed increased behavioural reactivity when they were receiving the high-starch (HS) diet compared to the high-fibre (HF) diet. On the HS diet, ponies showed an increased frequency of changing pace and spent more time being alert. They also spent significantly less time investigating their surroundings. The qualitative behavioural analysis observers perceived the ponies to be more 'nervous', 'tense' and 'unsure' when receiving the HS diet compared to the HF diet. Overall, the ponies were less at ease and more unsettled in their behaviour when receiving the HS diet compared to the HF diet. It is unclear why these diets result in behavioural differences. Both diets contained the same energy so this rules out the suggestion that behavioural differences occurred as a result of different energy levels. It is therefore hypothesised that the dietary-induced changes observed in the microbial populations of the hindgut in Chapter 3 may in turn induce a wider physiological effect. Dietary induced changes to hindgut microbiota may be the link between the HS diet and increased behavioural reactivity.

Gut-brain axis studies using germ-free mice have developed a greater understanding of the wider role of gut microbiota (Sudo *et al.*, 2004; Desbonnet *et al.*, 2009). Early life-stage colonisation of the gut by specific microbes (*Bifidobacteria infantis*) has been shown to affect hypothalamic-pituitary-adrenal axis development and endocrine responses to stress (Sudo *et al.*, 2004). When *B. infantis* was administered to rats, it reduced the pro-inflammatory immune response and elevated the peripheral levels of the essential amino-acid, tryptophan (Desbonnet *et al.*, 2009). Tryptophan is a precursor for the biosynthesis of serotonin, so it was suggested that *B. infantis* may have antidepressant properties (Desbonnet *et al.*, 2009). The administration of *Bifiodobacteria* was found to be more efficient at reducing stress-related behaviours in mice than escitalopram; a commonly used selective serotonin reuptake inhibitor (SSRI) class of medication (Savignac *et al.*, 2014). These studies highlight a strong association between gut microbiota and behavioural development.

The research undertaken in rodents has helped to further advance our understanding of mental health conditions affecting humans. Studies have shown associations between gut dysbiosis and neurological alterations, and diet is often implicated as a key factor associating these conditions (Zhang *et al.*, 2005; Ribeiro *et al.*, 2009; Bruce-Keller *et al.*, 2015). High fat diets have been shown to induce oxidative stress in the brains of rats and this was associated with increased inflammation in the cerebral cortex (Zhang *et al.*, 2005; Ribeiro *et al.*, 2009). These neurological alterations may also be associated with an increased risk of dementia (Zhang *et al.*, 2005). These studies did not include gut microbiota investigations so what formed the link between the dietary changes and the brain is less well understood. However, recent studies investigating dietary induced behavioural changes have started to consider the role of gut microbiota (Bruce-Keller *et al.*, 2015). Behavioural changes in mice associated with a high-fat diet showed alterations in gut microbiota along with changes indicative of brain inflammation (Bruce-Keller *et al.*, 2015).

Soluble carbohydrates fermented in the hindgut of rats have also been shown to result in increased anxiety and aggression (Hanstock *et al.*, 2004). Findings indicated that dietary-induced changes causing hindgut fermentation and

increased lactic-acid production were associated with these behavioural changes. The authors suggested that lactic acid may alter the biosynthesis of the neurotransmitters; serotonin and dopamine (Hanstock *et al.*, 2004). Rats displaying depressive-like behaviours following depletion of their gut microbiota also showed reduced serotonin levels in the brain (Hoban *et al.*, 2016). A large number of human behavioural processes are modulated by serotonin and alterations to serotonin levels are implicated in several neurological conditions such as anxiety and depression (Berger *et al.*, 2009). Therefore, dysregulation in the biosynthesis of serotonin could have wide-reaching implications on the behaviour of the individual.

Although mostly associated with the central nervous system, serotonin operates at both ends of the gut-brain axis (O'Mahony *et al.*, 2015). The majority of the body's serotonin (5-hydroxytryptamine, 5-HT (>90%)) is found in the gut where it is produced by enterochromaffin cells (Reigstad *et al.*, 2015). Serotonin is intrinsic to central nervous system function and is involved in many important physiological functions, including regulating gastrointestinal motility, cardiovascular vasoconstriction and dilation, and platelet function (Gershon and Tack, 2007; Berger *et al.*, 2009; Yano *et al.*, 2015).

Biosynthesis of serotonin is a two–stage enzymatic process which starts with the hydroxylation of the essential amino-acid tryptophan by tryptophan hydroxylase, to produce 5-hydroxytyptophan (5-HTP) (Shajib and Khan, 2015). Following that, 5-hydroxytryptophan is then decarboxylated by aromatic L-amino acid decarboxylase (DOPA decarboxylase) to produce serotonin (5-HT) (Shajib and Khan, 2015). Gut microbiota play a regulatory role in the production of serotonin by the enterochromaffin cells (Reigstad *et al.*, 2015; Yano *et al.*, 2015). The biosynthesis of serotonin in the brain relies upon peripherally circulating tryptophan crossing the blood-brain barrier via large amino-acid transporters (O'Mahony *et al.*, 2015). It has been suggested that gut microbiota may influence tryptophan metabolism at gut level and simultaneously alter the amount available for serotonin biosynthesis in the brain (O'Mahony *et al.*, 2015). Although further work is still required to fully understand these processes, the findings indicate that alterations to the complex microbial communities of the gut could have a wider physiological effect on the individual.

Serotonin is implicated in the pathogenesis of a number of neurological conditions, including anxiety, depression and aggressive behaviours (Shirayama and Chaki, 2006; Berger *et al.*, 2009). Selective serotonin reuptake inhibitors (SSRIs) limit the reuptake of serotonin in the brain and are used to treat anxiety disorders in humans (Savignac *et al.*, 2014). A range of different behaviours are regulated by serotonin receptors which are expressed throughout the brain (Berger *et al.*, 2009). The serotonin system acts on over 14 receptor subtypes which are divided into seven classes or families (Mittal *et al.*, 2016). Some therapeutic drugs focus on targeting specific receptor families. However, because the serotonin receptor families regulate a range of different behaviours and physiological processes targeted treatments are not generally treating just one aspect of behaviour (Berger *et al.*, 2009). This does not indicate reduced efficacy for these treatments but that perhaps they are just not as targeted as is suggested. It has also been suggested that while SSRIs may be useful for the treatment of mental health conditions, there may also be associated negative side-effects (Mittal *et al.*, 2016). This may be because the treatments are also affecting other behavioural and physiological processes. Therefore, it may be worthwhile investing in future anti-depressant and anti-anxiety medications to treat these conditions by shifting the focus to a completely different starting point; the gut. Given that gut microbiota can have a direct effect on the biosynthesis of serotonin, treatments starting at gut level may be able to treat the starting point behind some anxiety and depressive behaviours. It may be that in some cases the expression of anxiety and depression in the brain could be the 'side-effects' associated with a dysbiotic gut-brain axis communication system.

In the gut, serotonin plays a crucial role in the neurotransmission of the enteric nervous system (Gershon and Tack, 2007). It is vital for the regulation of gastrointestinal functioning including, gut motility, secretion of fluids and sensation (Gershon and Tack, 2007; Giancola *et al.*, 2017). Specific serotonin receptors are expressed via the extrinsic primary afferent neurons of the gut and relay sensory information from the gut to the central nervous system (CNS) (Giancola *et al.*, 2017). Serotonin then binds to these different receptors resulting in a range of signalling responses (Mittal *et al.*, 2016). There are at least five receptor families (5-HT_{1-4}, 5-HT_7) that are involved in gastrointestinal function with each of these containing a number of sub-types (Prause *et al.*,

2009). Abnormalities in serotonin signalling have been associated with a range of gastrointestinal tract conditions, such as irritable bowel syndrome (IBS) (Gershon and Tack, 2007). As serotonin is continually secreted, inactivation is required and this is carried out both in the CNS and gut primarily by the serotonin reuptake transporter (SERT) molecule (Gershon and Tack, 2007). A study using SERT knockout mice found that they had increased gut motility and displayed irregular patterns of diarrhoea and constipation (Chen *et al.*, 2001). These irregular patterns and increased gut motility indicate that alterations in serotonin metabolism may contribute to the pathogenesis of IBS in humans (Chen *et al.*, 2001). It is possible that changes in gut microbiota may be behind the alterations in serotonin metabolism.

Alterations is gut serotonin production could be one factor contributing to free-faecal water in horses. Horses presenting with faecal water syndrome (FWS) usually pass faeces of normal consistency but have water that runs from the anus which can coincide with defecation or occur independently (Kienzle *et al.*, 2016). When surveyed, horse owners commonly perceived the cause of FWS to be related to parasite burden but no differences in parasite burden were detected between FWS horses and controls (Kienzle *et al.*, 2016). The authors suggested social stress as a possible contributory factor. One factor underlying the pathogenesis of FWS could be alterations in gut serotonin metabolism. These alterations may be the result of dietary induced changes to gut microbiota influencing the biosynthesis of serotonin and thereby altering gut motility.

Dopamine is another neurotransmitter that also operates at both ends of the gut brain axis and has the potential to be affected by alterations in gut microbiota. Dopamine is a centrally acting catecholamine neurotransmitter involved in a number of behavioural and physiological functions including, motor function, cognition, emotion, motivation and reward (Seamans and Yang, 2004; Rangel-Barajas *et al.*, 2015; Mittal *et al.*, 2016). Dopamine is mainly synthesised in the brain but is also involved in gut homeostasis (Mittal *et al.*, 2016). It is also the precursor to adrenaline and noradrenaline (Mittal *et al.*, 2016).

Dopamine receptors are expressed widely throughout the brain, and in mammals there are five receptor subtypes which can be divided into two families; the D1-like family which includes D_1 and D_5 receptors and the D2-like family which

includes D_2, D_3 and D_4 receptors (Rangel-Barajas *et al.*, 2015). The dopamine receptor family groupings are based upon their structure and biological response (Rangel-Barajas *et al.*, 2015). In the brain, there are three major dopaminergic pathways; the nigrostriatal, mesocorticolimbic and tuberinfundibular pathways (Rangel-Barajas *et al.*, 2015). The nigrostriatal pathway is associated with motor function and the mesocorticolimbic with motivation, cognition and emotion (Rangel-Barajas *et al.*, 2015).

A number of studies have investigated the effects of stress on the dopamine pathways within the brain (Cabib *et al.*, 1988; Cabib *et al.*, 1997; Cabib *et al.*, 1998; McBride and Hemmings, 2005; Pascucci *et al.*, 2007). The brain is the main organ at the centre of a stress response and the mesoaccumbens dopamine system, in particular, is a major mediator in the response to stress (Cabib and Puglisi-Allegra, 2012). Stress has been shown to promote significant changes in dopamine receptor densities in the mesoaccumbens and nigro-striatal regions (Cabib *et al.*, 1998). Until more recently, the mesoaccumbens dopamine involvement in the stress response was at odds with the strong association between mesoaccumbens dopamine and reward (Cabib and Puglisi-Allegra, 2012). However, while these experiences may appear to be opposite to one other, from a behavioural perspective they are very closely and strongly associated and are therefore also mediated by the same neural circuitry (Holly and Miczek, 2016).

Stress can present in many different forms and some stress is often required for an animal to make behavioural and physiological responses that are essential for survival. Coping strategies are required to deal with the challenge of stress and the mesocorticolimbic pathways contribute to the development and regulation of these strategies (Cabib and Puglisi-Allegra, 2012). Chronic forms of uncontrollable stress can lead animals to develop extreme behavioural coping strategies usually categorised as stereotypic behaviours. Stereotypies can present in animals being kept in sub-optimal living conditions and are identified as repetitive behavioural patterns that appear to serve no apparent function (Waters *et al.*, 2002; Fureix *et al.*, 2013). Neurochemical differences have been found in the brains of horses that display oral stereotypies compared to control horses (McBride and Hemmings, 2005). Horses displaying oral stereotypies had

increased D1-like and D2-like receptor densities in the nucleus accumbens and lower D1-like receptor densities in the caudate nucleus (McBride and Hemmings, 2005). Increased dopamine in the nucleus accumbens is usually associated with active coping strategies in response to controllable stressors, whereas inhibited dopamine in the nucleus accumbens region is more associated with uncontrollable stressors and is more related to a helplessness coping strategy (Cabib and Puglisi-Allegra, 2012). Stereotypies are extreme coping strategies displayed by domestic animals maintained in sub-optimal living conditions. These living conditions would generally be considered an uncontrollable stressor. However, it may be that by carrying out an active coping strategy the animal is able to exert some level of control over an uncontrollable stressor.

Prey species like horses often exhibit a flight response to stressful and threatening situations. Neurotransmitters, including dopamine and adrenaline, play an important role in this flight response (Mittal *et al.*, 2016). The alterations in behaviour seen in the ponies in Chapter 2 could be anxiety or stress related. In the behavioural study from Chapter 2, the presence of a person or novel object in the testing area could be classified as a 'stressor' as both had the potential to illicit a stress response in the ponies. Therefore, the increased behavioural reactivity in response to a person or novel object could be indicative of a stress response. However, this does not explain why there was increased behavioural reactivity when ponies received the HS diet compared to the HF diet unless diet was having a direct effect on the stress response of the ponies.

Diet may also have a direct influence on dopamine systems. As already highlighted, dopamine is involved in a number of different aspects of behaviour including appetitive and aversive motivation (Salamone *et al.*, 2016). Dopamine concentrations in the nucleus accumbens of rats have been shown to immediately increase when receiving an appetitive reward of sucrose but showed significantly decreased levels following the aversive taste of quinine (Roitman *et al.*, 2008). For animals to display the appropriate behavioural response, they need to be able to discriminate between different stimuli quickly. This implies that they must respond directly to whether the taste is rewarding or aversive. However, sucrose has been shown to elevate brain dopamine levels in mice with no taste receptor signalling (de Araujo *et al.*,

2008). Therefore, the elevated dopamine levels in these mice could not be attributed to the sweet taste but may have resulted from post-ingestive detection of increased sucrose levels.

The time between ingestion and dopamine response should also be considered. The dopamine response in the rats with taste receptors peaked at 4.4 seconds post-infusion (Roitman *et al.*, 2008). This quick dopamine response suggests that in this case it was more likely to be taste related. However, this does not mean that sucrose does not have more than one way of producing an increased dopamine response. A more recent study suggested that increased dopamine release was related to how a nutrient regulates glucose metabolism (Ren *et al.*, 2010). This is potentially the mechanism by which animals have evolved to seek out food sources that are more nutrient dense and thereby increase their chance of survival. The aforementioned studies highlighted different ways the body recognises an appetitive reward which can induce biochemical changes in the brain. None of these studies explored the role played by gut microbiota which may provide another link between gut-brain communications.

Dopamine receptors are also found in the gut and are located in the mucosal layer (D_4), the nerve ending layer (D_2) and some can be found in both (D_1, D_3 and D_5) (Mittal *et al.*, 2016). Dopamine helps to modulate gastrointestinal tract motility (Zizzo *et al.*, 2010). It is possible that microbial dysbiosis in the gut could affect dopamine receptor density and have a wider effect on the host. The distribution and role of receptor subtypes throughout the regions of the gastrointestinal tract is complex and some studies have found differing results. For example, dopamine increased the contractibility of the small intestine in mice and D1 receptors were suggested to be the mediators of these effects (Zizzo *et al.*, 2010). Gastrointestinal motility has been shown to abnormally increase in the absence of D2 receptors which have also been suggested to regulate motility (Li *et al.*, 2006). It is likely that both dopamine receptor types play a role in regulating gastrointestinal motility and that the overall balance of dopamine and its receptors is the important factor in regulating gastrointestinal motility. If the balance of dopamine receptor densities in the gut is altered, then the physiological function of the gut may in turn also be altered.

Serotonin and dopamine are neurotransmitters that function at both ends of the gut-brain axis. Changes in the biosynthesis of these neurotransmitters have been shown to have a wider effect on health and behaviour. Gut microbiota have been associated with alterations in the biosynthesis of dopamine and serotonin. The HS dietary induced behavioural reactivity seen in Chapter 2 may be a result of neurochemical changes in the brain. These changes may be associated with the dietary induced changes to gut microbiota seen in Chapter 3. Therefore, the aim of this chapter was to investigate the relative gene expression of serotonin and dopamine receptor densities in brain and gastrointestinal tissues related to diet.

4.3 Methods

4.3.1 Experimental design

The ten ponies underwent a two-week dietary wash-out period after the feeding and behaviour experimental procedures described in Chapter 2. During the wash-out period all ponies received hay *ad-libitum* and no concentrate ration. Following the wash-out period, the ponies were returned to the diet they were receiving at the end of the previous experimental trials; ponies 1-5 received HF and ponies 6-10 received HS. They remained on this diet, receiving two feeds per day for a minimum of 14 days, following which the ponies were euthanased.

4.3.2 Diets

All ponies were fed according to the National Research Council (2007) recommendations for growth using the estimated mature bodyweight of 250 kg, with each receiving 2.5% of bodyweight in food per day. Details of the diets are included in Chapter 2 and Appendix A.

4.3.3 Euthanasia and sample collection

The ponies were transported to the euthanasia site in pairs. All ponies were euthanased using the same protocol as described in Chapter 3. Ponies were euthanased one at a time, with the second pony remaining sedated on the trailer.

4.3.3.1 Brain tissue sampling

The head of each pony was removed immediately following euthanasia at the junction of the first cervical vertebra and the skull. The whole brain was removed by cutting an opening in the frontal bone in line with the frontal crest. After removal, each brain was placed on dry ice for approximately 10 minutes to freeze them and allow cleaner cutting. The fore and mid brain was cut into coronal sections approximately 15 mm wide (McBride and Hemmings, 2005). The brain slices were then wrapped in aluminium foil, with lab-film separating each slice to prevent sticking, and placed on dry-ice in a polystyrene container, prior to being transferred and stored at -80 ˚C.

4.3.3.2 Gastrointestinal tissue sampling

The entire gastrointestinal tract was removed immediately following euthanasia. The different regions of the gastrointestinal tract were separated and sampled one section at a time. All mucosal samples were collected within 60 minutes following euthanasia, placed on dry ice in a polystyrene container immediately following collection and then transferred and stored at -80 ºC until analysis (Daly *et al.*, 2012; Schoster *et al.*, 2013; Chen *et al.*, 2014; Moreau *et al.*, 2014). Gastrointestinal mucosal samples measuring 2 x 2 cm approximately (Chen *et al.*, 2014) were collected from the jejunum, caecum, ventral, dorsal and small colon regions. Mucosal samples were cleaned of digesta with water. Samples were then wrapped in lab-film prior to being wrapped in aluminium foil to prevent sticking during freezing at -80 ˚C.

4.3.4 Brain tissue preparation

The pony brains were removed from the -80 ˚C freezer individually and placed within a cryostat machine overnight with the temperature in the machine set to -14 ˚C. This allowed the tissue to remain frozen but brought it to a temperature that allowed biopsy punch sample collection. The brain tissue was kept frozen and RNA extracted immediately to reduce the risk of RNA degradation.

Once the areas to be sampled were identified for each brain, the appropriate coronal slice of brain was placed on a metal block lined with lab-film to prevent the tissue sticking to the metal block. The metal block was set on ice to ensure the tissue did not thaw during the sampling process. Each tissue sample was extracted from the identified sampling area using a 4 mm biopsy punch. With each coronal slice being approximately 15 mm thick, each tissue biopsy punch taken was then divided into three sections measuring approximately 5mm in thickness. The 5 mm segment used for further analysis was taken from either the caudal or more rostral segment based upon visual identification of the correct sampling area. Sample punches were extracted from the caudate, putamen and nucleus accumbens. All three structures are located within the striatum region of the basal forebrain near the internal capsule (Figure 4.1). The globus palladus was used to identify the caudal aspect of the internal capsule region. Samples were not taken from the globus palladus.

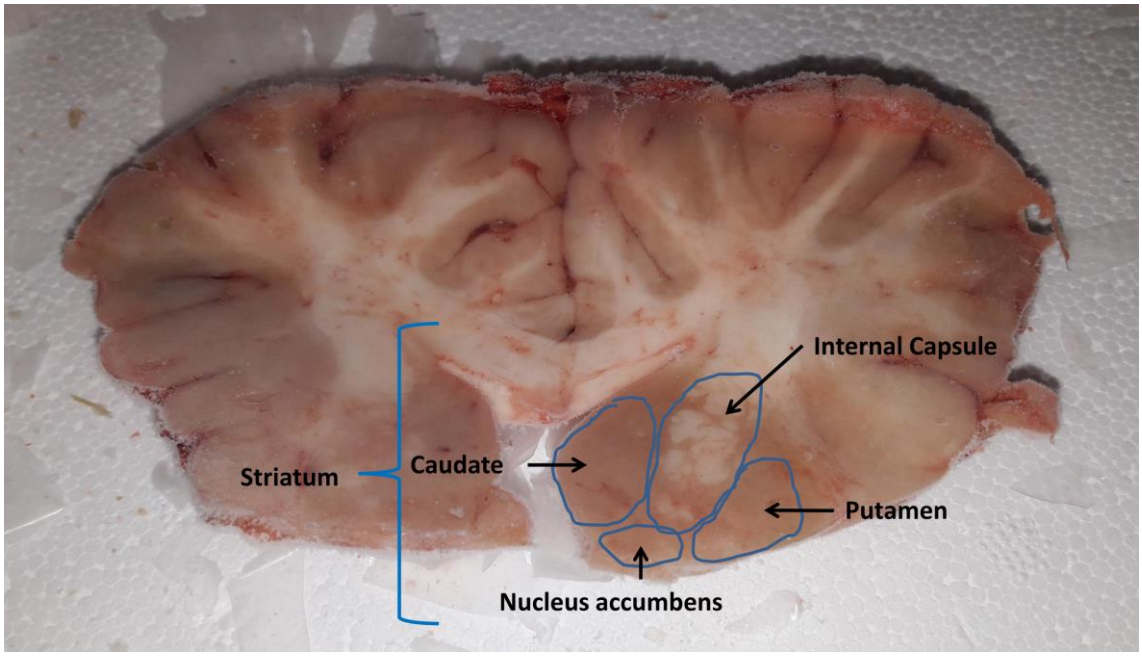

Figure 4.1 - Coronal slice of forebrain looking caudally and showing the sampling regions located within the striatum area.
Sampled regions included the caudate, putamen and the nucleus accumbens. All regions were sampled bilaterally. The internal capsule is indicated as a point of reference for locating sampling regions.

There were also two sampling areas from the mid-brain which included the substantia nigra and the ventral tegmental area (Figure 4.2). All structures were sampled bilaterally. Each sample identified for RNA extraction was placed immediately in an RNA-free ribolyser tube and sat on dry ice while the other samples from the same brain were extracted. The ribolyser tubes were labelled in advance of sampling and placed on dry ice so they were cold when the brain tissue samples were placed in them.

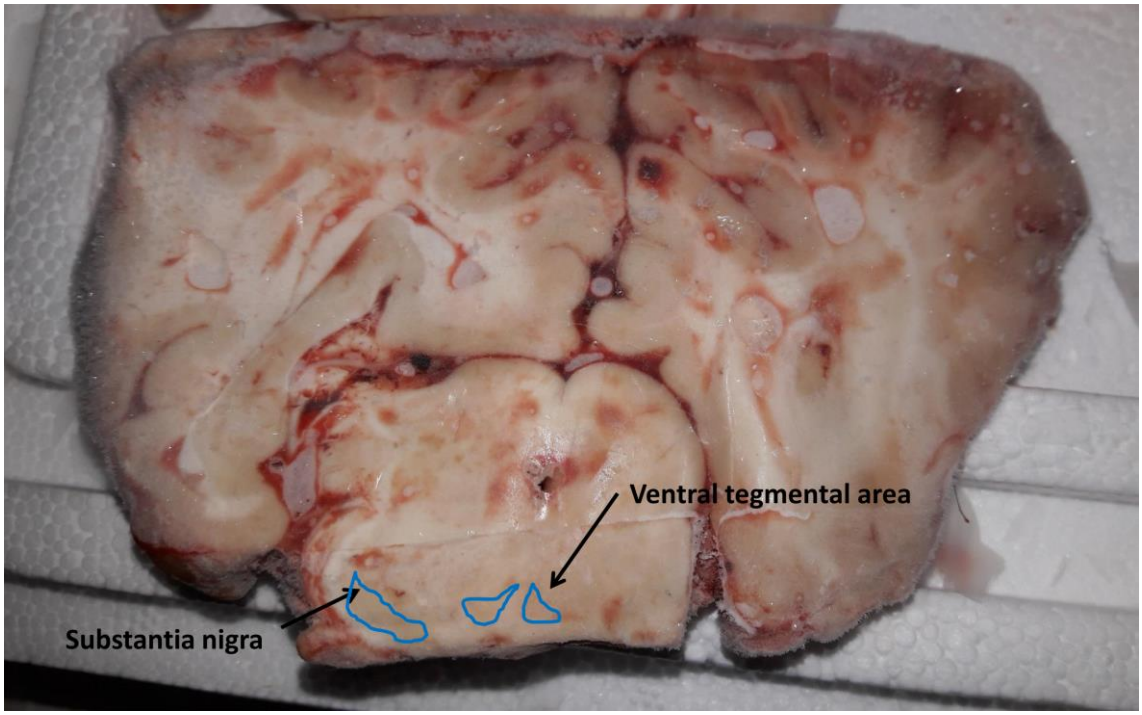

Figure 4.2 - Coronal slice of mid-brain looking caudally and showing sampled regions. Sampled regions included the ventral tegmental area and substantia nigra. Both regions were sampled bilaterally.

4.3.5 Gut tissue preparation

The gut tissues were sampled from one pony at a time and the RNA extracted immediately to reduce the chance of RNA degradation. For each pony, gut tissue samples from the jejunum, caecum, ventral, dorsal and small colon regions were removed from the -80 ˚C freezer and placed in a polystyrene container on dry ice. Each region was sampled individually. The selected sample of tissue was placed on a metal block which was set on ice and lined with lab-film to prevent the tissue sticking. A 4 mm biopsy punch was used to obtain a full thickness tissue sample from each gut region which was placed immediately into an RNA-free ribolyser tube. The samples were placed on dry ice until the remaining gut tissues samples from the same pony were taken and then RNA extraction was carried out immediately.

4.3.6 RNA extraction

Total RNA extraction for brain and gut tissues was carried out using the Qiagen RNeasy® Mini Kit. The standard kit protocol (Appendix C, Protocol 1) for tissue extraction was followed which started with 350µl of RLT buffer being added to the tissue in the ribolyser tubes. Samples were ribolysed for 20 seconds to ensure that the tissue was broken up. The tubes were centrifuged to move the

beads to the bottom, then 250 µl of lysate was pipetted into fresh collection tubes and 250 µl of 70% ethanol added to the lysate and mixed. The contents were pipetted into the spin columns provided in the kit and centrifuged. The lysate was discarded and 700 µl of RW1 was added to the spin columns and centrifuged. The wash through was discarded and 500 µl of RPE buffer was added to the spin columns. The spin columns were centrifuged, the wash through discarded and the RPE buffer step was repeated. This time the top section of the spin columns were transferred to fresh labelled RNase-free microcentrifuge tubes and 30 µl of RNase-free water was pipetted onto the spin columns. These were centrifuged and the top of the spin column was then discarded. The remaining RNA liquid samples were placed in ice, checked for concentration and purity using a nanodrop spectrophotometer machine and then immediately frozen at -80 ˚C.

4.3.6.1 DNase treatment and reverse transcription

To prepare the RNA samples for real-time polymerase chain reaction (qPCR), the samples first underwent DNase treatment (Appendix C, Protocol 2) followed by reverse transcription (Appendix C, Protocol 3). DNase treatment was carried out using the Invitrogen DNA-*free*™ Kit – DNase Treatment and Removal. This started with 30 µl of RNA being pipetted into labelled microcentrifuge tubes and 0.1 volume (3 µl) of 10X DNase I Buffer added. 1 µl of rDNase I was then added and mixed using the pipette. The samples were incubated in a water bath at 37˚C for 25 minutes. The DNase Inactivation Reagent was re-suspended and 0.1 volume (3 µl for 30 µl of RNA) was added and mixed by vortexing. Samples were incubated for 2 minutes at room temperature and vortexed occasionally prior to centrifuging. RNA was then transferred to new RNase-free microcentrifuge tubes taking care not to transfer any pelleted DNase Inactivation Reagent.

Immediately following DNase treatment, reverse transcription of RNA samples to complimentary DNA (cDNA) was carried out using Invitrogen SuperScript® III Reverse Transcriptase kit. The dNTP set was prepared by adding 150 µl of each dNTP to a labelled microcentrifuge tube plus 150 µl of purified H_2O (Milli-Q®). The mastermix to be used for reverse transcription was then prepared (Table 4.1). 6 µl of the mastermix was pipetted into new labelled microcentrifuge tubes (one for each RNA sample). 4 µl of RNA was then pipetted into the same tube.

This quantity was adjusted if required based upon the quantity of RNA in each sample so that 200 ng of RNA was used for reverse transcription. The mastermix and RNA were pipetted onto opposite sides of the tube so that all samples were mixed at the same time. The solution was mixed by pulsing on the centrifuge. The samples were incubated for three minutes at room temperature and then incubated in a water bath for one hour. The temperature of 42 ˚C was set for adding the samples and then turned up to 50 ˚C for the remaining time. Samples were then frozen and stored at -20 ˚C until PCR.

Table 4.1 - Reverse transcription mastermix

Mastermix components	Per sample volume (µl)
Buffer X5	2.00
DTT (10 mM)	1.00
Random Hexamers (c1181)	0.20
dNTP mix	0.20
RNase block	0.26
Rtase (SIII)	0.26
H_2O (Milli-Q®)	2.10

4.3.7 Primer design and testing

Reference gene primers were designed based upon genes that had previously been used in an equine study that investigated their suitability for use as reference genes (Cieslak *et al.*, 2015). The genetic sequence database, GenBank, at the National Centre for Biotechnology Information (NCBI) (2019) was used to explore dopamine and serotonin receptor gene sequences that had previously been used for equine tissues. Primers for the genes of interest were designed based upon dopamine and serotonin receptor primers for that had already been used in equine studies, albeit in different tissue types (King *et al.*, 2005; Momozawa *et al.*, 2007; King *et al.*, 2008; Hori *et al.*, 2016). Gene of interest primers and primers to be used as housekeeping genes were all designed using Primer-BLAST (NCBI, 2019). For primer design, the name of the selected gene was pasted into the PCR template window. The primer parameters were

set as PCR product size minimum at 70 and the maximum 160, the melting temperature settings were a minimum of 64 ^{o}C, an optimal of 65 $^{\circ}$C and a maximum of 66 $^{\circ}$C. The intron inclusion box was selected to ensure that the primer pair is separated by at least one intron. This step is important for cDNA primer design so that genomic DNA contamination can be accounted for. The output results were checked to ensure that the primer pairs were specific to the input template and that the total intron size was no more than 1000 base pairs (Czechowski *et al.*, 2004). The forward and reverse primers from the sequences were then synthesised from Eurofins Genomics (Eurofins Scientific, 2018).

A total of five housekeeping genes (Table 4.2) were purchased as potentially suitable reference genes for normalisation. The potential reference genes and the genes of interest were validated using a pooled mix of all brain cDNA samples from one pony. The template was checked to ensure that the primers were expressed stably. A non-template control (NTC) was also included on the plate to check for primer dimer and this comprised a SYBR and primer mixture with loTE buffer (3 mM Tris –HCl (pH 7.5), 0.2 mM EDTA (pH 7.5) in dH_2O) but no cDNA. A PCR plate containing duplicates of all DNase treated RNA samples (without reverse transcriptase) was used along with one of the checked primers to confirm that no genomic DNA was present in the RNA samples. Two NTCs were also run in duplicate on the same plate; 1 negative control containing no RNA and one positive control containing a sample of cDNA.

Table 4.2 - Primer information for reference genes and genes of interest. Suitability of the genes for use with equine brain and gut tissues is also included.

Gene symbol	Forward primer sequence (5' -> 3')	Reverse primer sequence (5' -> 3')	Tm (˚C)	Suitable Y/N
eKRT8	CAAAGGGGGCTGGGCAGGTAG	TAAGGCCCCCGAAGCTCGTC	65.7(F) 63.5(R)	N
eRPS9	CACCCTGGCCAAGATCCGCA	GTCTGCAGGCGCCTCTCCAA	63.5	Y
eB2M	TCCCCTGCTGCTGTGGTAGC	GGCGGATGGAACCCAGAGACA	63.5(F) 63.7(R)	N
eTop2b	AGGTAAAGGCCGAGGGGCAAA	TCCTAGCCCGACCAGTCCGT	61.8(F) 63.5(R)	Y
eYwhaz	AAACCCCGTGTCTGCGGAGC	TCAGCCTGCTCGGCCAGTTT	63.5(F) 61.4(R)	N
eCcn2	AGCTTTTCTGCTGTACCAGGACCC	TGCCACATTTCCAGTCGCAGTCA	64.4(F) 52.4(R)	N
eDRD1	GCCCTGGAAAGCTGTGGCTG	GGCTGCCTTGGGGGTCATCT	63.5	N
eDrd1(2)	TGTCGCTGCTCATCCTGTCCAC	AGGGCCAGAAGCCAGCAATCT	64.0(F) 61.8(R)	Y
eDRD2	GACCTCCTTGTGGCCACGCT	GGCCACGGCTGTGTACCTGT	63.5	Y
eHTR1A	ACGCTCATCTTCTGCGCGGT	GGTGACCTGTCCCAGCGTCC	61.4(F) 65.5(R)	N
eHTR1B	CATCTCGCTGCCGCCCTTCT	CGGGAGCGGGCTTCCACATA	63.5	Y

4.3.8 Real-time polymerase chain reaction (q-PCR)

The brain and gut tissue derived cDNA samples were amplified against each primer pair using real-time polymerase chain reaction (q-PCR) with SYBR green (Appendix C, Protocol 4). The PCR was carried out using an MX3000P cycler (Stratagene, Amsterdam, the Netherlands) real-time PCR system to quantify the relative level of gene expression. To prepare the samples for q-PCR, a 1 in 40 dilution of cDNA with loTE buffer was prepared. SYBR green (Brilliant II SYBR® Green qPCR Master Mix (Agilent Technologies)) was mixed with each selected primer using a ratio of 24:1 (SYBR:primer). 4.8 µl of the SYBR/primer mixture was pipetted into the left-hand side of each well on a 96 well PCR plate. The SYBR/primer mixture can bubble during pipetting so to ensure enough SYBR/primer mixture was available the calculation for each plate was worked out for 5 µl per well. 4.8 µl of cDNA/loTE buffer solution was then pipetted into the right-hand side of the appropriate wells with all samples being run in duplicate. A non-template control (NTC) to check for primer dimer and contamination was also included in duplicate for each primer; this included the SYBR/primer mixture plus loTE buffer but no cDNA. The cycling conditions for each PCR run were one cycle of denaturation at 95 ˚C for 7.5 minutes followed by 40 amplification cycles containing three segments: 95 ˚C for 25 seconds, 63 ˚C for 25 seconds and 72 ˚C for 25 seconds. The amplification cycles were followed by a melting cycle containing three segments: 95 ˚C for 60 seconds, 63 ˚C for 30 seconds and 95 ˚C for 30 seconds. Amplification plots were checked and melting curves were used for each primer to check for the presence of a single peak for each sample (Czechowski *et al.*, 2004). All samples were run in duplicate and the melting curve peaks were checked for similarity for each of the duplicates. The dissociation temperature (Tm) was also checked for similarity.

4.3.9 Statistical analyses

The genes of interest were normalised against one reference gene for the brain tissue samples and two reference genes for the gut tissue samples using the ΔCt method and based upon 90% efficiency (Czechowski *et al.*, 2004).

The substantia nigra region of the brain was removed from further analysis. During q-PCR some of the samples from this region failed to amplify sufficiently for the fluorescence emission to cross the C_T threshold, even when repeat samples were carried out.

Separate models were constructed for each neurotransmitter and tissue type (brain or gut). Each model was used to explore the effects of diet and tissue region on the relative expression of neurotransmitter receptor densities. Model selection started by comparing a Generalised Linear Mixed Model (GLMM) with 'pony' included as a random intercept to a Generalised Linear Model (GLM) using log-likelihood ratios and AIC (Zuur *et al.*, 2009). A backwards-forwards stepwise modelling approach was then used to determine the optimal fixed effects structure for each model (Zuur *et al.*, 2009).

4.4 Results

Two out of the five housekeeping genes were identified as suitable reference genes (Table 4.2). One of the reference genes (eTop2b) was removed from the analysis of the brain tissue. Both reference genes were expressed stably but one crossed the threshold earlier (eRPS9) than the genes of the interest while the other was later (eTop2b). Although both were stably expressed and therefore suitable as reference genes, both could not be used together to normalise the gene of interest to for the brain tissues as the reference gene Cts were either side of the genes of interest. Three of the six primers designed for the genes of interest were identified as suitable (Table 4.2). The suitable primers included two for dopamine-like receptors D1 and D2 and one primer for serotonin receptor 1b.

During model selection, the random effect of pony was not retained by any model and the inclusion of a random effect did not make any of the models significantly better ($p < 0.05$) when using log-likelihood ratio tests. The dropping of pony from all models means that there was no effect of individual pony on the relative expression of each neurotransmitter. GLMs were determined to be the optional model for both the brain and gut tissue samples and for each of the three neurotransmitters.

4.4.1 Brain tissue gene expression results

The optimal fixed effects GLM for dopamine D1-like receptor relative expression included the fixed effects of brain region, side of the brain and the interaction between these. Model output details are included in Appendix C, Table C.1. Results showed significant differences in relative expression related to the interaction between brain region and side of the brain (Figure 4.3). The caudate region showed greater relative expression of D1-like receptor densities for the right side of the brain compared to the left. The nucleus accumbens region showed lower relative expression for D1-like receptor densities for the right side of the brain compared to the left.

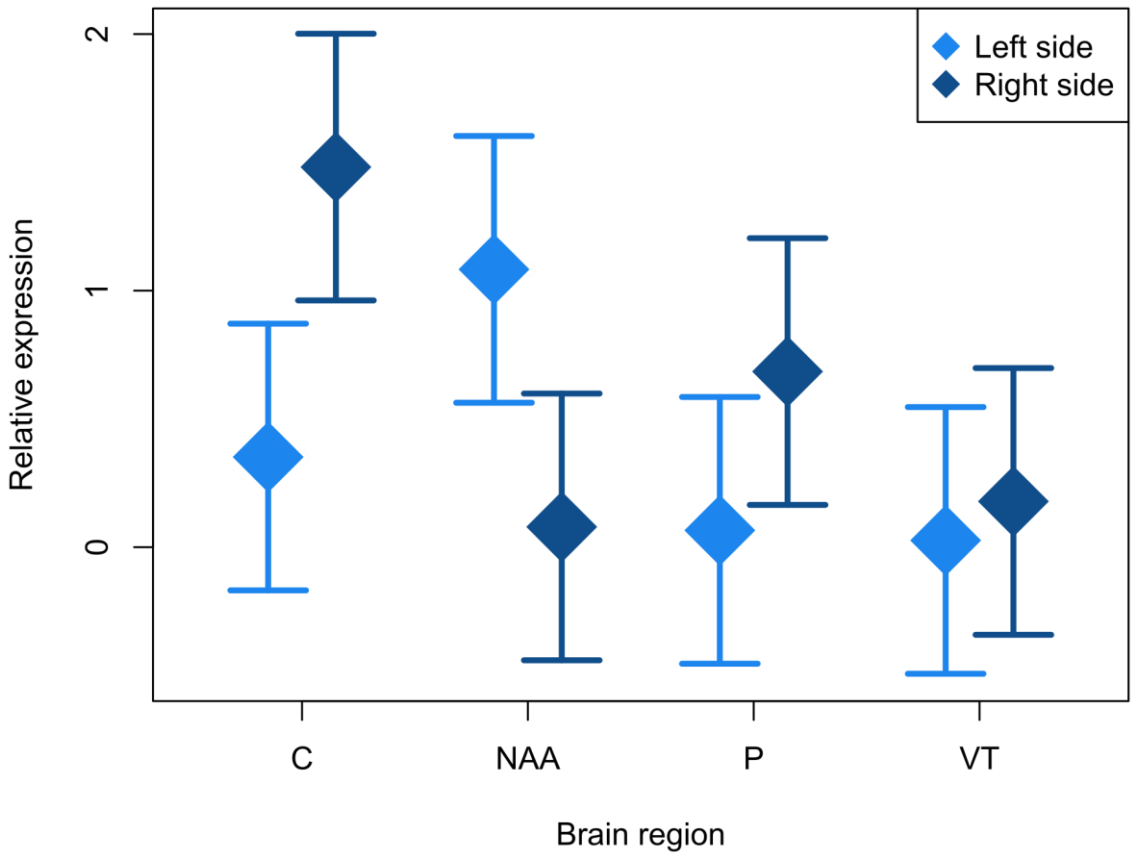

Figure 4.3 - Dopamine D1-like receptor relative expression related to brain regions and side. Each region displayed shows relative expression for the left and right side. Error bars show 95% CI. C = caudate region, NAA = nucleus accumbens area, P = putamen, VT = ventral tegmental area.

The optimal fixed effects GLM for dopamine D2-like receptor relative expression included the fixed effects of diet, brain region, side of the brain and the interaction between diet:region and region:side. Model output details are included in Appendix C, Table C.2.

Results showed significant differences in relative expression for dopamine D2-like receptor densities related to brain region and side of the brain. There were also significant differences in relative expression for some of the interactions between diet and brain region, and brain region and side of the brain (Figure 4.4). The caudate region of the brain showed greater relative expression of D2-like receptor densities for the HS diet compared to the HF diet and for the right-hand side of the brain compared to the left. The nucleus accumbens region of the brain showed lower relative expression of D2-like receptor densities for the HS diet compared to the HF diet and for the right-hand side of the brain compared to the left.

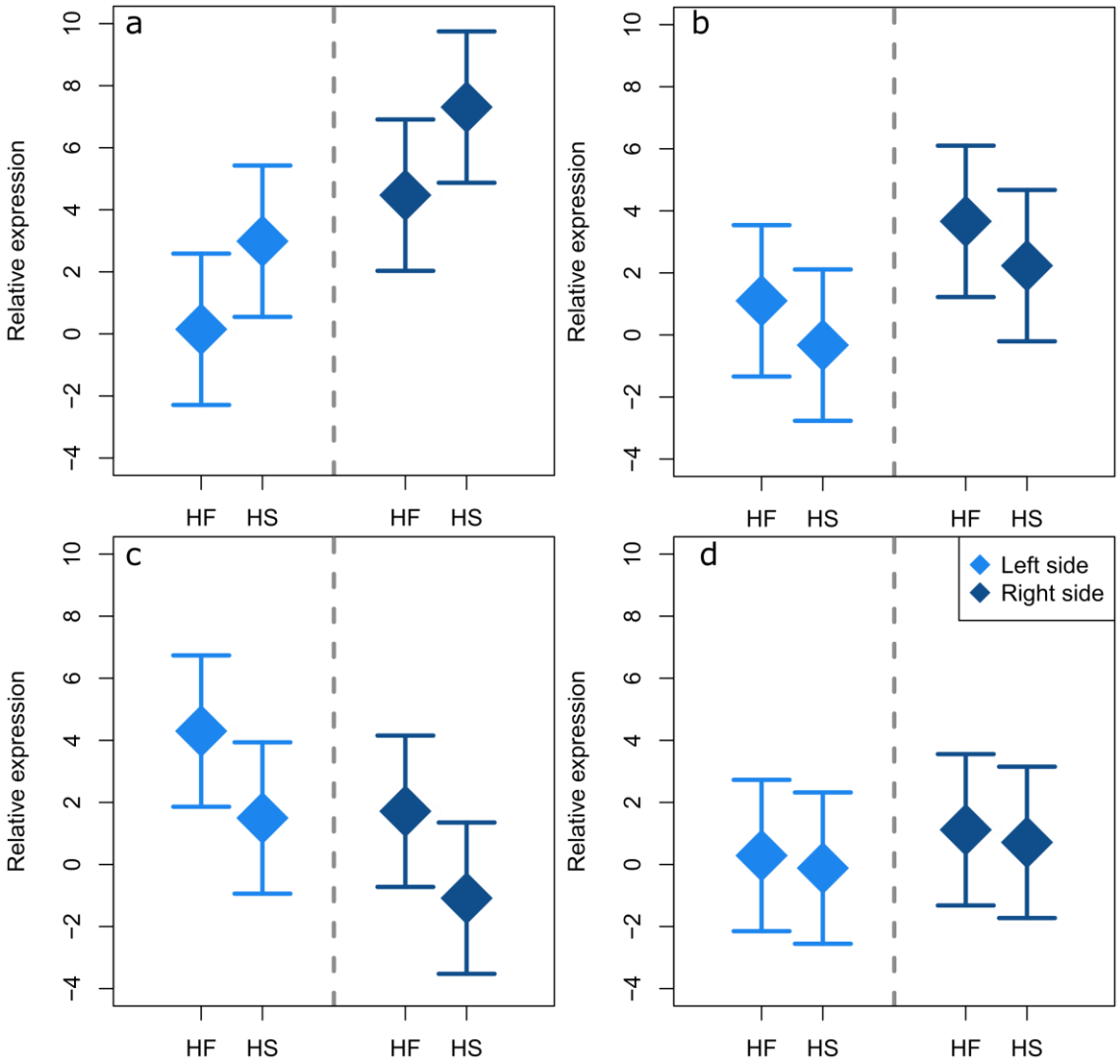

Figure 4.4 - Dopamine D2-like receptor relative expression related to diet, brain region and side of the brain and the interactions between diet:brain region and brain region:side of the brain.
The brain regions shown include the caudate (a), putamen (b), nucleus accumbens (c) and the ventral tegmental area (d). HF = high-fibre diet, HS = high-starch diet. Error bars show 95% confidence interval.

Model selection for serotonin receptor 1b expression in brain tissue retained no fixed effects. The fixed effects of diet, brain region, side of the brain or any interactions, were not significantly better than a 'null' model. The lack of a suitable optimal model indicates that none of the variables included in the models showed any significant differences for serotonin receptor expression.

4.4.2 Gut tissue gene expression results

The optimal fixed effects GLM for dopamine D1-like receptor relative expression included the fixed effects of diet, gut region and the interaction between them. Model output details are included in Appendix C, Table C.3. Results showed a significant difference related to the interaction between diet and one region of the gut. There was lower relative expression of D1-like receptor densities for the HS diet and the small colon.

There were no fixed effects retained by the D2-like receptor or serotonin 1b receptor expression models for the gut tissue. This indicates that there were no significant differences in D2-like receptor or serotonin 1b receptor expression related to diet or gut region. Neither of the fixed effects of diet or gut region were significantly better than a 'null' model.

4.5 Discussion

The wider effects of diet are often discussed in relation to health. Research is starting to further explore the two-way communication between the gut and the brain and the wider effects that diet may have. The results from this study showed differences in relative dopamine D2-like receptor density expression related to diet. The caudate region of the brain showed a relative increase in D2-like receptor expression with the high-starch (HS) diet compared to the high-fibre (HF) diet. In humans, increased D2-like receptor densities in the caudate region have been associated with mental health conditions. For example, an increased risk of schizophrenia along with poorer performance in cognitive tasks (Hirvonen *et al.*, 2005). Weaning rats in social isolation resulted in increases in D2-like receptor densities in the striatum region of the brain, these neurochemical changes are similar to those seen in humans with schizophrenia (King *et al.*, 2009). The rats also showed behavioural changes including hyper-reactivity and increased aggression (King *et al.*, 2009).

In the current study, no measures of aggressive behaviours were taken and the behavioural tests did not focus on aggressive behaviour so we are unable to say if the ponies displayed any differences. Behavioural testing results did show that increased reactivity occurred when ponies were receiving the HS diet compared to the HF diet. Ponies receiving the HS diet had significantly increased frequency of pace-change compared to ponies on the HF diet. They were also observed to be more nervous, tense and unsure during qualitative behavioural analysis. The behavioural changes recorded in Chapter 2 may result from neurochemical differences found in the caudate region of the brain related to the HS diet.

Differences were also found in the nucleus accumbens region of the brain related to diet with a relative decrease in D2-like receptor densities with the HS diet compared to the HF diet. The nucleus accumbens area of the brain is associated with reward and learning, and exposure to stress and drugs can impair its function and alter dopamine activity (Shirayama and Chaki, 2006). Mice exposed to a controllable stressor showed an increase in dopamine activity in the nucleus accumbens region (Cabib and Puglisi-Allegra, 1994). However, exposure to an uncontrollable stressor decreased activity in the same region (Cabib and Puglisi-Allegra, 1994). Therefore, whether or not an animal has the

ability to control a stressor can result in different dopamine responses in the nucleus accumbens region. In the behaviour tests from Chapter 2 the ponies had the ability to move a short distance away from the stressors. However, the ponies were confined to a limited space and as a flight animal the inability to flee may make this an uncontrollable stressor. Therefore, it could be that the neurochemical changes measured in the current study could be similar to the findings of Cabib and Puglisi-Allegra (1994).

The nucleus accumbens results of this chapter differ with results from horses displaying oral stereotypies (McBride and Hemmings, 2005). The study by McBride and Hemmings (2005) found increased D2-like receptor densities in the nucleus accumbens region of crib-biting horses compared to controls. The differing results observed here are likely to be due to the different characteristics of the behaviours being observed. Stereotypic behaviours are extreme behavioural coping strategies that are chronic in nature and therefore similar results would not be expected. The behavioural characteristics observed when ponies were receiving the HS diet were characteristics related to increased locomotory activity and unsettled behaviour. It is therefore to be expected that different dopaminergic changes were observed and this is attributable to the complexity of the dopaminergic signalling pathways and their relationship with behaviour. As already discussed subtle differences in stressors have previously produced differing dopaminergic responses within the same study (Cabib *et al.*, 1988; Cabib *et al.*, 2002).

Genetics also play a considerable role in dopaminergic responses. Two different strains of mice showed differences in D1 and D2-like receptor densities in response to repeated restraint stress (Cabib *et al.*, 1998). Studies in horses have also suggested there may be a genetic influence on whether an animal is more predisposed to developing stereotypic behaviours (Bachmann *et al.*, 2003; Albright *et al.*, 2009; Wickens and Heleski, 2010). Therefore, there may be an associative genetic factor with both the development of stereotypic behaviours and the neurochemical differences observed in animals that display such behaviours compared to controls. In this chapter, the effect of pony was not retained in any of the statistical models. This indicates that there was no effect

of individual pony on the relative expression on neurotransmitter receptor densities.

The increased behavioural reactivity observed in Chapter 2 could also be associated with a stress response brought about by behavioural testing. The brain dopaminergic systems are highly responsive to stress inducing stimuli with different behaviours being modulated by different dopamine receptors (Cabib *et al.*, 1988). The type of stress and whether that stress is acute or chronic is an important consideration, as is the type of behaviour being displayed. A study in mice found that different mobility behaviours had opposite responses to immobilisation stress with either an increase or a reduction in mobility being displayed depending on whether the stress was induced acutely or chronically (Cabib *et al.*, 1988). However, the dopaminergic alterations were still evident throughout the study regardless of the different physical responses suggesting that habituation to the immobility stress did not develop as the study continued. The observed alterations in behaviour continued to be displayed even after the stress inducing dopamine alterations had disappeared (Cabib *et al.*, 1988). This highlights the complexity of the dopamine - behaviour relationship and suggests that individual dopamine receptors combined with their CNS location play a slightly different role in the behavioural output depending on the situation. Therefore, it is important not to just focus on alterations in one CNS region but instead to consider how the overall working balance of the dopamine – behaviour relationship may be affected by any changes that occur.

The ponies' exposure to stressors during behavioural testing in Chapter 2 could have resulted in a dopaminergic response, although this does not explain why there were differences in behavioural response related to diet. It is possible that diet could play a role in an animals' ability to cope with a particular stressor and this may explain the behavioural and neurochemical differences related to diet observed in the current study. There were at least four weeks between the behavioural testing being carried out and when the ponies were euthanased. Measurements of dopamine and dopamine metabolites in mice found that 24 hours after the end of restraint stress the dopamine metabolic response to that stress had disappeared (Cabib *et al.*, 1988). It is unlikely that the ponies in the current study would have a continued neurochemical response lasting beyond

four weeks. Therefore, it is likely that the dopaminergic differences observed in the ponies were associated with diet rather than the stressors experienced during behavioural testing.

As previously mentioned, studies using rodents found elevated dopaminergic responses in relation to an appetitive reward of sweet-tasting food and the opposite to an aversive taste (Roitman *et al.*, 2008).This was also the case in mice bred without taste receptor signalling suggesting a wider physiological recognition and response in relation to the sugar-rich foods (de Araujo *et al.*, 2008). What should be considered here is the possibility that it could be the diets that are having a direct influence on the behaviour and dopaminergic pathways. It is possible that the two different diets may have had different effects on the dopaminergic reward systems of the ponies. The HS diet used in the current study was a coarse mix feed containing molasses. The appetitive reward nature of this diet may have provoked an altered dopamine reward response. The striatum region of the brain plays an important role in the reward pathways and has been linked to compulsive feeding behaviours in obese rats (Johnson and Kenny, 2010). The neurochemical changes which occur in conjunction with obesity have been shown to share many similarities with drug addiction (Volkow *et al.*, 2013). Both food and drug-addiction trigger similar reward pathways in the brain. The HS diet may be responsible for triggering dopaminergic reward system pathways and increase the ponies' motivation for that food.

The feed may not be the only factor triggering an altered dopamine response. The regularity and routine with which these concentrate feeds are fed to the animals in our care should also be considered. The ponies in the current study were fed two meals daily and at regular times each day. This is similar to common yard practices where concentrate meals are fed at regular times. Horses receiving this type of diet will develop anticipatory behaviours related to the appetitive reward of a starch-based sweetened feed. These anticipatory behaviours have been studied in horses and are characterised by an increase in activity and decrease in maintenance behaviours such as foraging and resting (Peters *et al.*, 2012). Horses on yards can often be observed leaving their hay and displaying excitable behaviours in anticipation of receiving a concentrate

feed. This behavioural response is unlikely to be hunger driven as the horses often have forage available. This is an area that requires further investigation as increased anticipatory behaviours are indicative of increased reward sensitivity and is therefore a potential indicator of welfare (Peters *et al.*, 2012).

There were no significant differences found in relative expression for the neurotransmitter serotonin sub-type 1B receptor densities in the gut or brain tissues related to diet or region. However, the serotonin system is complex and acts on over 14 different receptor sub-types (Mittal *et al.*, 2016). With such a wide range of receptor subtypes it is possible that a different serotonin receptor sub-type or even a different receptor family may produce different results to those found in the current study. With the behavioural and neurochemical changes already observed in the current study, further study investigating the expression of a range of serotonin families and sub-types is warranted. Particularly as serotonin regulates a wide range of behaviours and contributes to gastrointestinal motility (Berger *et al.*, 2009; O'Mahony *et al.*, 2015; Reigstad *et al.*, 2015). With multiple receptor sub-types, the full range of serotonin's physiological functions are yet to be elucidated (Gershon and Tack, 2007).

For the gut tissue, a significant difference was found for the expression of D1-like receptors for the interaction between diet and region; the HS diet and small colon interaction showed relatively lower expression of D1-like receptor densities. Along with serotonin, dopamine plays a significant role in the regulation of gut motility (Li *et al.*, 2006; Zizzo *et al.*, 2010). An *in vitro* study using tissue from mice found that D1-like receptors increased contractility in the ileum (Zizzo *et al.*, 2010). However, the aforementioned study did not examine other regions of the gastrointestinal tract so it is unclear as to whether D1-like receptors would produce the same effect in other regions. Another study found that the absence of D2-like receptors increased gastrointestinal motility which significantly reduced overall transit time and resulted in greater water content in the stools (Li *et al.*, 2006). These mice were also found to be smaller yet ate more than their wild counterparts. Therefore, there is also a wider physiological effect of altered motility related to nutrient absorption. In the current study it would be difficult to say whether gastrointestinal motility was altered in any way as this was not measured. Subjectively, the faecal samples of the ponies

remained similar throughout the study regardless of diet, so any difference in gastrointestinal motility was not enough to visibly alter the composition of the ponies' faeces. Therefore, it is unlikely that the increased behavioural reactivity recorded for the HS diet in Chapter 2 was associated with digestive discomfort related to alterations in gastrointestinal motility.

There is expression of serotonin 1b, dopamine D1-like and dopamine D2-like receptors throughout the sampled gastrointestinal tract regions. Until now, the majority of equine nutritional studies have focused on the luminal contents of the gastrointestinal tract. Our understanding of the microbiota of the equine hindgut is still developing. However, it is important that the relationship between luminal gut microbiota and gut tissue is given further consideration, particularly in relation to dietary changes. Investigating the luminal microbiota alone does not provide us with a complete picture. The inclusion of gut tissue in these studies will develop our understanding about the wider effects alterations in gut microbiota may have.

The development of modern molecular chemistry techniques for quantifying the relative expression of neurotransmitters in the brain and gut tissues of horses is an exciting development. While measurements of dopamine receptor densities have been measured in the horse previously, a ligand binding technique was used (McBride and Hemmings, 2005). The developments of the methods used in the current study will greatly open up opportunities for future research and are ideally suited to developing our understanding of gut-brain axis communication systems.

The routine feeding of starch-based concentrate feeds may result in increased anticipatory behaviours based upon the appetitive reward nature of the feed. This may have partially contributed towards the neurochemical differences associated with diet. However, it does not explain why there was an overall increase in behavioural reactivity associated with the HS diet in Chapter 2. It is more likely that a combination of factors contributed to the neurochemical changes observed here, which includes the appetitive reward nature of the HS diet, but also includes wider systemic changes brought about by the dietary induced alterations in gut microbiota.

Chapter 5 Discussion

Developing an understanding of the communication pathways between the gut and brain is essential to fully appreciate the wider effects of diet. Research focussing on gut microbiota has increased substantially with evidence building that these microbial communities may be pivotal to gut-brain axis communication. Gut microbiota influence, not only the physical health of an animal, but also their behaviour and diet plays a key role in influencing the community structure of gut microbiota. The gut-brain axis is a two-way communication system encompassing a number of different communication pathways (Figure 5.1). Direct communication pathways exist between the central and enteric nervous systems via the vagus nerve. Indirect communication pathways also exist and include the neuroendocrine and immune systems. Therefore, the gut microbiota has a number of pathways available to communicate with the brain and influence the behaviour of an animal.

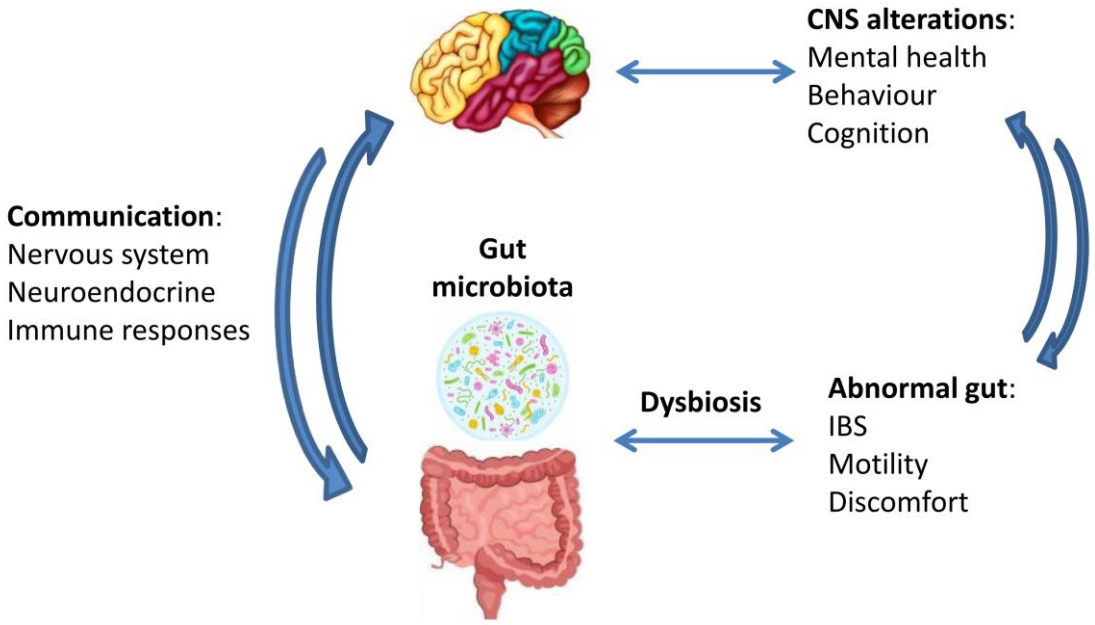

Figure 5.1 - Gut-brain axis communication pathways.

Gut-brain axis studies in rodents have highlighted the wider influence gut microbiota have in shaping animal behaviour by influencing physiological processes. Gut microbiota have been shown to play a pivotal role in the development of the hypothalamic-pituitary-adrenal stress axis during early life (Sudo *et al.*, 2004) and in altering the biosynthesis of serotonin which could influence behaviour (Williams *et al.*, 2014). Anecdotally, many horse owners

have also indicated that starch-based feeds may increase behavioural reactivity. If diet has the potential to influence behaviour, then it is indicative that a wider physiological change may be occurring as a result of diet. Therefore, developing a greater understanding of the mechanisms governing these behavioural changes is essential.

Several studies have investigated the microbial communities of the equine hindgut in response to dietary change. It is clear that the addition of starch to equine diets alters hindgut microbial community composition (de Fombelle *et al.*, 2003; Daly *et al.*, 2012; Dougal *et al.*, 2014; Grimm *et al.*, 2017). The gut-brain axis communication system could be the link between alterations in behaviour and hindgut microbiota resulting from dietary change. The hypothesis behind the motivation for this thesis was that dietary induced changes to hindgut microbiota influence behaviour through gut-brain axis communication pathways. The overall aims of the study were: (i) to investigate the behaviour of naïve ponies fed a high-starch (HS) and a high-fibre (HF) diet and describe the faecal microbial community composition associated with the two diets, (ii) to describe the microbial community composition from the different regions of the hindgut when fed HS or HF diets and to investigate similarities with the faecal samples from the previous chapter and, (iii) to use the relative expression of dopamine and serotonin receptor densities in gut and brain tissues to understand the physiological processes governing the dietary-induced behavioural changes.

There is only a small body of scientific research substantiating the effects of diet on behaviour in horses. There are also very few studies that have used modern genomic sequencing techniques to describe faecal microbial community composition associated with diet. Therefore, the rational for Chapter 2 was first, to conduct an extensive behavioural investigation using naïve ponies being fed HS and HF diets and, secondly, to use 16S *rRNA* gene sequencing to describe the faecal microbial community composition for both diets. These comparisons were made to ascertain if there were associations between the changes in microbiota and behaviour. Differences in behaviour related to diet were evident with the HS diet increasing behavioural reactivity. These differences in behaviour associated with diet were not only recorded quantitatively but were also clear to observers who were unaware of the aims of the study. The faecal microbial community

composition also differed related to diet. The microbial composition associated with the HS diet showed a reduction in fibrolytic bacterial taxa (Ruminococcaceae) combined with an increase in lactic-acid producing bacterial taxa (*Streptococcus*). Clear relationships were also evident between the dietary induced changes in faecal microbiota and behaviour. An increased frequency of pace-change was associated with the faecal microbial composition of the HS diet, and more time spent investigating was associated with the faecal microbial community composition of the HF diet.

After finding dietary induced differences in behaviour and faecal microbiota in Chapter 2, the main aim of Chapter 3 was to use 16S *rRNA* gene sequencing to describe the microbial community composition across the different regions of the hindgut for both diets. It also allowed the opportunity to ascertain if there were similarities between the microbiota of different hindgut regions and the faecal microbiota, including samples obtained post-mortem and those from Chapter 2. Several studies have investigated the microbiota of the equine hindgut, but very few have explored all regions of the hindgut. A modern, multivariate modelling approach allowed in-depth exploration of the data so that not only differences in regions could be explored but specifically which bacterial taxa differed in relation to diet. Differences in diversity and richness associated with diet were evident as were differences in microbial community composition for the ventral, dorsal and small colon regions. Differences in bacterial taxa related to diet from more caudal regions of the hindgut showed similarities with those found in post-mortem faecal samples as well as faecal samples from Chapter 2.

Chapter 4 aimed to explore connections between the gut and the brain to establish if the dietary induced changes in microbiota and behaviour were associated with neurochemical changes in gut and brain tissues. Chapter 4 included developing and validating primers suitable for use with real-time polymerase chain reaction (qPCR) to explore specific dopamine and serotonin receptor densities in gut and brain tissues. The development of the methods used in Chapter 4 makes a modern technique available for investigating neurochemical changes in these tissues in horses. Differences in the relative expression of D1-like receptor densities were evident related to brain region and

side of the brain. Differences in the relative expression of D2-like receptor densities were evident related to the interactions between diet and brain region, and brain region and side of the brain.

5.1 Research perspectives and future implications

When each stage of this study is viewed individually, they develop our understanding of the wider effects of diet on the animal. When Chapters 2, 3, and 4 are combined they further develop our understanding of the effect of diet on the animal and contribute to our understanding of the relationship between the gut and brain. Together this work has also opened a range of opportunities for further research. The use of naïve ponies has been of key importance to this study. From a behavioural perspective, the ponies being young and unhandled meant that their reactions to the behavioural tests were as natural as possible. Their responses had not been shaped by previous life experiences through human handling and husbandry influences. This does not mean that mature horses would not show dietary induced behavioural differences, just that their increased life experiences may mean that they learn over time to react less resulting in supressed reactions.

Quantitative methods of recording behaviour are important as they allow measurements of specific behaviours to be obtained in a controlled setting and greatly develop our understanding of how an animal behaves. However, it is also important that behavioural testing results are meaningful to horse owners and something they can relate to their own horses. The Qualitative Behavioural Analysis (QBA) used in Chapter 2 allowed horse owners to apply their own descriptive terms to the behaviours they observed and therefore potentially makes the behavioural results more accessible to horse owners.

Consideration should be given to the behavioural tests used and the behavioural responses they elicited. The passive human test elicited more behavioural differences compared to the novel object test. This could be because the ponies found the presence of a passive human more alarming than the novel object. This difference may be due to the ponies' limited exposure to humans. This also highlights the care researchers should give to selecting appropriate behavioural tests. The behavioural tests commonly used in equine studies often contain an

element of alarm or surprise to cause the horse to flee and react strongly. Perhaps by eliciting a stronger behavioural reaction these types of tests mask the subtleties in behavioural reactivity found in this study. Exposing horses to a less alarming behavioural testing situation may allow a wider range of behaviours to be displayed and recorded.

A physiological measure of behavioural reactivity would have been a useful addition to this study. With the ponies being unhandled the use of a heart rate monitor for obtaining heart rate measurements during behavioural testing was not possible. However, the use of faecal cortisol metabolites as a non-invasive measure of stress response would have been a useful addition to the behaviour section of Chapter 2. In the current study, daily faecal samples were collected throughout the two 14-day behavioural testing periods but unfortunately a storage issue meant that these became unusable. This type of non-invasive physiological measure of reactivity would be a useful addition to future studies investigating behavioural reactivity associated with diet.

The use of naïve ponies in this study is not only important from a behavioural perspective, it also plays an important role in the study of the hindgut microbiota. The use of naïve ponies meant that their digestive systems had not been exposed to the supplementary feeding of concentrates and dietary changes that most mature domestic horses experience. The ponies' digestive systems at the start of this study will be more similar to that of a wild horse. In comparison the digestive systems of more mature domesticated horses frequently used in microbiota studies will already have been influenced by concentrate feeding and dietary changes over prolonged periods. Therefore, the use of naïve ponies in this study gives a relatively unique and true reflection of the hindgut microbiota and its response to high-starch and high-fibre diets.

The use of 16S *rRNA* gene sequencing and robust statistical modelling in this study have allowed an in-depth investigation into the wider effects of diet and produced sound, reproducible results. The use of the *mvabund* package in R for the faecal samples in Chapter 2 not only offered greater power properties than distance-based analysis methods but also enabled a generalised linear model to be fitted to each bacterial taxa (Wang *et al.*, 2012; Warton *et al.*, 2012). This allowed the identification of which bacterial taxa differed in relation to diet. In

Chapter 3, using the Bayesian Ordination and Regression Analysis of Abundance Data (BORAL) package in R enabled analysis of specifically which bacterial taxa differed for each hindgut region related to diet, with the inclusion of pony as a random effect (Hui and Poisot, 2016). These modelling approaches for the analysis of 16S *rRNA* gene sequencing combined with the use of naïve ponies greatly enhance the current scientific knowledge about the effects of diet on the microbial communities of the equine hindgut.

The current study comprised a sample size of ten ponies. A larger sample size would always be more favourable as it would be more representative of a wider population and therefore lessen the chances of individual variability influencing the results. The cross-over study design approach used in Chapter 2 maximised the use of ponies and brought the overall sample size to 20. The risk of individual variability influencing the results was also reduced as all ponies received both diets in Chapter 2. The robust statistical analyses used in the current study were more powerful than the more traditional statistical tests that are sometimes used. The models used in the current study enabled the partitioning of variables and meant that the effect of 'pony' could be factored into the statistical models. The euthanasia of the ponies in this study should also be considered when sample size is discussed. The ponies used in the current study were already designated for slaughter. However, a power analysis to ascertain the sample size required should be carried out prior to future studies being undertaken, particularly if they are planning to use euthanased animals.

To date, this is the first study conducted in horses analysing the effects of diet on the enteric and central nervous systems. In Chapter 4, primers suitable for use in equine intestine and brain tissue were developed. These primers offer opportunities for research investigating gut-brain axis communication systems in horses and could also prove useful for other study areas including behavioural or gastrointestinal physiology studies. Previously, neurotransmitter receptor densities have been measured in horses using a ligand binding technique (McBride and Hemmings, 2005). The use of real-time PCR for analysing the relative expression of neurotransmitter receptor densities offers a more modern method of neurotransmitter quantification. In Chapter 4, differences in the relative expression for both D1-like and D2-like receptor densities were found

related to brain region and the side of the brain. This highlights the need for future studies to include these variables not only in their sampling but also in the statistical analyses. Taking into account the expression of neurotransmitter receptor density differences that already exist related to brain region and side of the brain enables a more accurate assessment of the changes that occur.

Although two serotonin receptor primers were developed, one of these was deemed to be unsuitable and as such only one receptor sub-type primer remained for analysis. Therefore, a useful addition to my work would have been the development of additional serotonin receptor primers. Developing these primers would have allowed me to undertake a more detailed exploration of the effects of diet on serotonin receptor densities. With over 14 serotonin receptor subtypes, the future development of additional serotonin primers will allow opportunities for further research.

The inclusion of additional regions of the brain would also have been a useful addition to this study. The substantia nigra region was removed from qPCR analysis as some of the samples failed to fluoresce sufficiently. This was unfortunate as the substantia nigra is a key region in the nigrostriatal dopaminergic pathway and the results from this area may have provided a more complete picture. It is unclear as to why the substantia nigra region failed to fluoresce. Although care was taken during sampling it is possible that sampling error occurred. It is also possible that RNA degradation occurred as this can be particularly sensitive.

The inclusion of the pre-frontal cortex would be a useful addition to future studies as it would enable the mesocortical dopaminergic pathway to be explored. Only a small body of research exists focussing on neurotransmitter expression in the central and enteric nervous systems of horses and as such this chapter makes a valid scientific contribution to this area.

5.2 Conclusions

The results of this study show the wide-reaching effects diet can have on the behaviour and physiology of the animal. In Chapter 2 the HS diet resulted in increased behavioural reactivity. The differences in behaviour recorded with the HS diet were obvious enough that not only were they recorded quantitatively but they were also recognisable to observers. The observers used terms like nervous, tense and unsure to describe the behaviours of ponies receiving the HS diet and relaxed, laid-back and settled when they were receiving the HF diet. These dietary induced behavioural differences could be viewed from two different perspectives; firstly, that the HS diet resulted in behaviours indicative of increased reactivity, or secondly, that the HF diet resulted in more relaxed and settled behaviours. Instead of just highlighting the increased behavioural reactivity associated with a HS diet, the behaviourally beneficial effects of a HF should also be considered. A HF diet may have a positive influence over a horse's behaviour and is therefore likely to be more effective than feeding commercially available calming supplements.

It has been demonstrated in this thesis that clear relationships existed between the dietary induced changes in faecal microbiota and behaviour. For example, when ponies were fed the HF diet they spent significantly longer investigating their surroundings and showed consistent trends in the microbial community composition of their faeces. In the same behavioural test, when ponies were fed the HS diet they changed pace more frequently, indicating they were more unsettled, and their faeces were characterised by a different microbial community composition. These changes in faecal microbial community structure and behaviour in response to diet, and the relationships between the microbial communities and behaviour were consistent in both experimental periods. This demonstrates a clear relationship between microbiota and behaviour.

The significant differences in microbial community composition related to diet identified in Chapter 3 in the ventral, dorsal and small colon regions indicated that the greatest dietary effect occurred in more caudal regions of the hindgut. These changes in microbiota were observed with the quantity of starch in the HS diet being within the current recommendations (National Research Council, 2007). These changes in microbiota indicate that even the addition of 2g/Kg of

bodyweight of starch per day to the equine diet can cause alterations to the microbial community composition and behaviour. Therefore, perhaps the current maximum recommendations should be reduced. The hindgut region showing the greatest number of bacterial taxa differing significantly in relative abundance related to diet was the dorsal colon. Dietary changes to hindgut microbiota began to develop in the ventral colon, peaked in the dorsal colon and then started to become less pronounced again by the small colon, indicating that the gut may have started to become more stable again. It is likely that this was only possible because the starch levels being fed did not exceed recommendations. Had greater quantities of starch been fed it is possible that all regions of the hindgut may have been affected, at which stage a 'tipping-point' in the symbiotic to dysbiotic balance of the gut may have been reached.

The decrease in Ruminococcaceae bacteria and increase in *Streptococcus* bacteria that was common to the behaviour faecal samples from Chapter 2 and the post-mortem faecal samples from Chapter 3 indicates that these changes remained consistent throughout the duration of the study. These changes in community structure were also evident in the more caudal regions of the hindgut. A decrease in Ruminococcaceae and increase in *Streptococcus* bacterial populations have been seen previously in the hindgut and faecal samples of horses with laminitis, horses with intestinal disease and those fed higher levels of starch (Milinovich *et al.*, 2008; Daly *et al.*, 2012; Steelman *et al.*, 2012; Warzecha *et al.*, 2017). The shift in relative abundance related to diet for Ruminococcaceae and *Streptococcus* bacterial taxa may be indicative of a hindgut moving towards a dysbiotic state. The consistency of these bacterial taxa and the fact that there was a trend in faecal and hindgut samples means that they could prove useful as faecal biomarkers for identifying dysbiotic alterations in hindgut microbiota. Although faecal samples cannot indicate which region of the digestive tract changes to microbiota are occurring, they do show that changes are occurring. Perhaps just knowing that these changes are occurring and that there is a risk of hindgut dysbiosis is enough. At this point changing the diet of the horse may be all that is required to initiate the return of the gut to a healthier state and prevent further problems.

This thesis showed the effects of the HS diet were wide reaching enough to not only alter gut microbiota and behaviour but also be associated with neurotransmitter changes in the central nervous system. The HS diet resulted in an increase in dopamine D2-like receptor densities in the caudate region of the brain while D2-like receptor densities decreased in the nucleus accumbens. Humans with an increased risk of developing schizophrenia and who show poorer performance in cognitive tasks also have increased D2-like receptor densities in the caudate region of the brain (Hirvonen *et al.*, 2005). Similar changes were also found in rats reared in isolation (King *et al.*, 2009). Exposure to stress has also been show to result in alterations in dopamine activity in the nucleus accumbens region in mice (Cabib and Puglisi-Allegra, 1994). These results from studies in humans and rodents show profound alterations to the CNS which are associated with complex mental-health conditions and stress. This thesis also found similar alterations to the central nervous system when ponies were fed a HS diet with starch levels within recommended feeding levels. These results, therefore, raise ethical questions about feeding diets that can induce this kind of extreme neurochemical change. Given that similar levels of energy can be achieved using diets low in starch recommendations should be made to reduce the starch component in equine diets. It also raises the question as to how the HS diet is having such a wide effect.

There are likely to be a combination of factors contributing to the behavioural and neurotransmitter changes associated with the HS diet. Firstly, there is the appetitive reward nature of the HS diet stimulating reward pathways in the brain. Secondly, the dietary induced changes to hindgut microbiota could be having a wider physiological effect. The reduction in Ruminococcaceae and increase in *Streptococcus* bacterial taxa between the faecal samples collected at different times shows that the changes in bacterial taxa remained relatively consistent over time. This indicates that these changes were not just an initial adjustment following a change of diet. The decrease in Ruminococcaceae and increase in *Streptococcus* bacterial taxa were also evident in more caudal regions of the hindgut. This consistency between samples indicates that these shifts in community structure probably also occurred in the hindgut during the behavioural testing periods. The reduction in Ruminococcaceae and increase in *Streptococcus* bacteria observed in the gut of ponies on the HS diet has

previously been associated with laminitis, intestinal disease, and high levels of starch in the diet (Milinovich *et al.*, 2008; Daly *et al.*, 2012; Steelman *et al.*, 2012; Warzecha *et al.*, 2017). It is possible that there is an association between the increased behavioural reactivity recorded with the HS diet and these alterations in gut microbiota. The consistent alterations in Ruminococcaceae and *Streptococcus* bacterial taxa may be responsible for wider systemic changes which result in increased behavioural reactivity. The link between these changes could be via the neuroendocrine communication pathways between the gut and the brain.

This thesis increases our knowledge of gut-brain axis communication systems and develops opportunities to increase our knowledge of the effects of diet even further. To evidence a specific link between gut microbiota and neuroendocrine communication pathways future work should include horses receiving HS diets containing different levels of starch. The faecal microbiota could be analysed for alterations in microbiota to understand the degree of alteration across a range of starch quantities in the diet. This thesis's contribution allows future studies to focus on key bacterial taxa including Ruminococcaceae and *Streptococcus* bacterial taxa. The gut tissue and brains of the horses could then be analysed for expression of dopamine receptor densities to assess the extent of expression alteration in relation to starch levels in the diet. This would require the euthanasia of the horses and could be improved, ethically, if there were animals available already designated for slaughter as was the case in the work contributing to this thesis.

It is likely that the neuroendocrine communication route is not the only gut-brain axis communication pathway being affected by dietary induced alterations to gut microbiota. Alterations in gut bacteria may also cause immune and inflammatory responses which may contribute to the behavioural and neurochemical changes reported in this thesis. Future research should include a wider investigation of the inflammatory and immune responses when horses are fed HS diets. This should again include horses being fed HS diets containing different levels of starch. The faecal microbiota could also be analysed to see if the degree of alteration corresponds with the increase in starch. Blood samples could be taken to check for circulating levels of pro-inflammatory cytokines

including tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Bercik *et al.*, 2010) to ascertain if there is an increase when the amount of starch in the diet increases. Post euthanasia, the gut tissue could be analysed for tight junction integrity by evaluating occludin protein concentrations to evaluate if changes in gut microbiota result in a 'leaky' gut. The brains could also be analysed for expression of dopamine receptor densities and inflammatory markers.

It has been established in this thesis that feeding a high-starch diet, in quantities commonly fed to domestic horses, resulted in alterations to gut microbiota that were indicative of a gut moving towards dysbiosis. The high-starch diet also caused increased behavioural reactivity and dopamine receptor density changes in the mesolimbic and nigrostriatal dopamine pathways. The behavioural and physiological changes were associated with ponies receiving a high-starch diet containing starch levels within the current recommended maximum levels (National Research Council, 2007). These results should inform a review of the current guidelines concerning daily starch intake by horses. Researchers and horse owners should also review whether a diet that can induce such a wide range of changes is required for the majority of leisure horses. In many cases leisure horses consuming high-starch diets could result in more problems that outweigh the perceived benefits. While horses in extreme levels of exercise are perceived to require starch-based feeds in order to meet their daily energy requirements it is unlikely that the majority of leisure horses are undertaking exercise that cannot be solely fuelled by fibre. Moreover, it is possible that alternative forms of energy-dense feeds may be more suitable for meeting the energy needs of performance horses. While some riders may feel that a more reactive horse may be desirable for certain equestrian disciplines, it does not mean that just because there is more energy available that it will necessarily result in a desired reactivity that is beneficial for the performance of the horse and rider. If the addition of some starch to the diet is enough to induce behavioural, microbial and neurochemical alterations then more consideration should be given as to the necessity of this type of diet before we feed it to the horses in our care.

Appendix A General supporting information

Additional pony information

Table outlining the passport information for the ten ponies used throughout this thesis.

Pony ID number	Date of birth (passport)	Sex
1	01/06/2013	Gelding
2	02/05/2013	Filly
3	16/05/2013	Gelding
4	01/06/2013	Filly
5	27/05/2013	Gelding
6	01/06/2013	Filly
7	06/06/2013	Gelding
8	26/04/2013	Gelding
9	28/04/2013	Gelding
10	01/06/2013	Filly

Background information

The passports for all the ponies show that their ownership was previously registered with another UK research institution; the date of ownership registration with that institute for all ponies was for June 2014. Prior to this the ponies were registered with their breeders. Ponies 1, 4, 6 and 10 had all come from the same breeder but had different sires. Ponies 2, 8 and 9 all came from another breeder and ponies 8 and 9 had the same sire. The remaining ponies came from different breeders.

The ponies had been purchased by another research institution for a research trial but were then deemed not to be required. They were then purchased and transported to the University of Glasgow, UK in November 2014. Upon arrival and prior to the start of the first experimental period the ponies were group housed in two large pens within an indoor barn for three weeks. One pen contained six ponies and the other contained 4 ponies. They were bedded on straw and had *ad libitum* access to water and hay. The hay was supplied in the form of a large

round bale situated in the centre of each pen. No supplementary feeding in the form of concentrates was provided.

The ponies received no handling during this time and were deemed to have not been handled previously. The ponies were not halter trained and were unaccustomed to being around humans and would flee if approached.

Health status

As handling of the ponies was not possible a visual observation to assess health status was made prior to the start of the first experimental period. All ponies were observed eating, drinking and passing droppings of normal consistency. None of the ponies showed any signs of nasal discharge, lethargy or patchy sweating. The ponies were deemed to be generally in a good and healthy condition and all had condition scores between 3 and 3.5 when using a condition scoring scale from 1 – 5 (Carroll and Huntington, 1988).

Faecal worm egg counts (FWEC) were carried out prior to the start of the first experimental period. A low to moderate count of strongyle eggs was recorded for all ponies. The ponies did not undergo any de-worming treatment at this stage as it was close to the start of the first experimental period. All ponies were then de-wormed at the start of the 14-day wash-out period using an oral solution of fenbendazole.

Additional feed information

Profile	Lucerne	Compound mix
Digestible energy	10 MJ/kg	10 MJ/kg
Starch	5%	23%
Protein	12%	10%
Fibre	27%	11%
Sugar	4.5%	7.5%

Compound mix main ingredients: Wheatfeed, oatfeed, barley, molasses, maize, peas, wheat, soya.

Appendix B Chapter 2 supporting information

Protocol 1 Isolation of gDNA Revised Protocol – Qiagen QIAamp® Fast DNA Stool Mini Kit

Preparation

- One side of water bath at 70 ^{o}C for samples – with polystyrene trays x2
- One side of water bath at 37 ^{o}C to re-dissolve buffers – AL and InhibitEX
- Bijoux tubes with distilled water for homogeniser cleaning in between – x3 per go
- Soak homogeniser for min 10 mins am & pm:
    - 250 ml hydrogen peroxide solution @ 3% final (30% original)
    - 225 ml distilled water & 25 ml 30% solution
    - Dry and run with distilled solution prior to first use
- Prepare tubes:
    - 1 set bijoux tubes for homogenisation
    - 1 set 2 ml tubes for transfer from bijoux tubes
    - 1 set 2 ml tubes for proteinase K
    - 1 set 1.5 ml tubes fully labelled for final DNA
- Prep AW1 and AW2 buffers as required with ethanol
- Dry ice for samples + spatula and distilled H_2O for cleaning in-between

Procedure

Run 6/8 samples per go but prepare only 2 at a time to prevent thawing

1. Add 1 ml of InhibitEX Buffer to each bijoux tube

2. Samples on dry ice - weigh 180 - 220 mg of sample in plastic tray and transfer to bijoux tube (fast) – Vortex quickly and then homogenise straight away (2 samples). Repeat with 6 samples

3. Samples to water bath for 5 min at 70 ^{o}C.

    a. Transfer sample to 2 ml tubes (pour in sample). Vortex 15 s

4. Centrifuge samples at full speed (14,000 x g) for 1 min to pellet samples (do not transfer any solids)

5. Pipette 25 ul proteinase K into new 2 ml tubes

6. Pipette 600 ul supernatant into the 2 ml tubes containing proteinase K (do not transfer bits)

7. Add 600 ul Buffer AL and vortex for 15 s

8. Incubate at 70 ^{o}C for 10 min (or 2 hours at 55 ^{o}C) -Centrifuge briefly to remove drops from lid

9. Add 600 ul of ethanol 100% to the lysate and mix by vortexing

10. Apply 600 ul of lysate to QIAamp spin column and centrifuge at 14,000 x g for 1 min. Empty filtrate and repeat step 10 until all lysate is used – once empty transfer column to new 2 ml tube (in kit)

11. Add 500 ul Buffer AW1 to spin column (reverse pipette) – centrifuge full speed for 1 min – discard filtrate

12. Add 500 ul Buffer AW2 to spin column (reverse pipette) – centrifuge 14,000 x g for 3 min – discard filtrate (note - may need to repeat this step if filtrate not clear, use 300 ul AW2 – sit for 1 min prior to centrifuge)

13. Centrifuge at full speed for 3 min (may need to repeat if spin column looks wet)

14. Remove spin column and place in new 2 ml collection tube. Pipette 200 ul ATE Buffer directly onto QIAamp membrane – sit for up to 5 min, then centrifuge 14,000 x g for 1 min

    a. Transfer DNA sample to labelled tubes and test at nanodrop machine (1.5 ul)

Appendix C Chapter 4 supporting information

Protocol 1 Brain Tissue Preparation and RNA Extraction

- Leave brains in cryostat overnight to bring up from -80˚C.
    - Brain tissue cuts best at -14˚C
- RNA free ribolyser tubes (with ceramic beads in) on dry ice
- Label micro-centrifuge tubes for freezer samples
- Metal block (on ice for cutting) – sheet of labfilm to prevent tissue sticking
- Biopsy punch (use 4 mm for horse) used to take tissue (bilateral samples taken) – 1 punch from each area (divide into 3)
- Put punched tissue sections in ribolyser tubes

RNA extraction

- Add 350 µl of RLT buffer to ribolyser tubes – use RNA pipette tips
    - Shake by hand to mix slightly
- Ribolyse tubes for 20s @ speed 4 – ensure tissue is broken up
- Centrifuge for 3 min @ max (13, 000 x g) to move beads to bottom
- Pipette lysate into collection tubes (250 µl) leaving beads behind
    - Pipette in 250 µl of EtOH (70%) and mix with pipette
- Pipette contents into spin columns and discard empty collection tubes
    - Centrifuge 1 min @ max

        - Take out and stand
        - Discard lysate

- Pipette 700µl of RW1 onto spin column
    - Centrifuge 15 sec @ max
    - Discard lysate
- Pipette 500 µl of RPE buffer onto spin column (may need to prep RPE buffer by adding 440 ml absolute EtoH)
    - Centrifuge 15 sec @ max
    - Discard lysate
- Pipette another 500 µl RPE buffer onto spin column
    - Centrifuge 1 min @ max
    - Discard lysate and collection tube, retain top section of spin column and place into a labelled micro-centrifuge tube with lid open
- Pipette 30 µl RNase-free water onto spin column
    - Centrifuge 1 min @ 8000 x g (lids open and facing down – avoid touching lid)
- Discard spin column and close lid on tubes (check liquid (RNA) has passed through the filter into bottom of tube)
- Test on nanodrop machine
    - Nucleic acid – ok, Sample type = RNA-40, run blank first.

Protocol 2 DNase Treatment

- RNA samples on ice
- Check water bath temp
- Label micro-centrifuge tubes
- Pipette 30/40 µl of RNA into labelled micro-centrifuge tubes for DNase treatment
- Add 0.1 vol (3 µl for 30 µl of RNA or 4 µl for 40 µl RNA) of 10X DNase l Buffer
- Add 1 µl rDNase l to the RNA and mix with pipette
- Incubate in water bath @ 37˚C for 25 min
- Label fresh micro-centrifuge tubes ready for end*

(Can prepare Reverse Transcription dNTP (if required) during this time. Details on Reverse Transcription protocol)

- Flick or vortex DNase Inactivation Reagent to re-suspend
- Add 0.1 vol (3 µl for 30 µl of RNA or 4 µl for 40 µl RNA) of DNase Inactivation Reagent and mix
- Incubate for 2 min at room temperature, mix (vortex) occasionally
- Centrifuge @ 10,000 x g for 1.5/2 min
- Transfer RNA to fresh tubes* (don’t transfer pelleted DNase Inactivation Reagent. Pipette amount set at 50 µl but leave some)
- Discard old tubes

Protocol 3 Superscript lll Reverse Transcription

- dNTP set preparation – 100 mM dilute 1/5 to 20 mM
    - Add 150 µl each dNTP + 150 µl H_20 (Milli-Q) (150 µl in 750 µl = 1/5)
- Label new micro-centrifuge tubes inc. RT on label. Check water bath temp.
- Prepare mastermix

Mastermix	**per sample vol (µl)**	**x12 (for 10 RNA samples)**
Buffer X5	2.0	24.00
DTT (10 mM)	1.0	12.00
Random Hexamers (c1181)	0.2	2.40
dNTP mix	0.2	2.40
RNase block	0.26	3.12
Rtase (Slll)	0.26	3.12
H_20 (Milli-Q)	2.1	25.20
		Total= 72.24/12 = 6.02 µl

- Pipette 6 µl mastermix into label tubes – add to one side of tube
- Pipette 4 µl RNA – add to opposite side of tube (approx. 200 ng - so adjust as required to make concentration more even)
    - Pulse on centrifuge to mix (>>)
- Incubate 3 min @ room temp

- Put in water bath at 42˚C then turn up to 50˚C and incubate for 1h

- Dry samples and freeze in polybag in -20˚C until PCR

Protocol 4 Primer Preparation and Real-Time Polymerase Chain Reaction

Primer preparation

- All primers have to be 100 pmol/µl
    - add H_20 (RNase free) as indicated on oligonucleotide synthesis report
    - vortex to mix

Primer mixture for PCR – final concentration. Label two tubes per primer

- In one tube for each primer add and vortex:
    - 60 µl H_20
    - 20 µl forward primer
    - 20 µl reverse primer
- Total = 100 µl. Remove 50 µl to second tube
    - 1 x 50 µl tube for storage (with primer stock) @ - 20˚C
    - 1 x 50 µl tube for using @ - 20˚C

Do checking run for all PCR primers – used a pooled mix of all pony 10 RT cDNA samples.

Dilute cDNA with LOTE buffer 1/40 dilution. Need to dilute cDNA with LoTE buffer as cDNA too concentrated for PCR

Made pooled mix in one tube (5 µl/100 µl) – usually 1/40 dilution

- 95 µl of LoTE buffer

- Add in 0.5 µl of each cDNA (x10) and pipette up and down to mix
    - Final concentration is usually 1/40

If doing individual samples do 1/40 dilution (1 µl cDNA to 39 µl LOTE) – scale up as required to ensure enough for each primer mix combination in duplicate

(Keep SYBR in fridge once defrosted)

Do SYBR green and primer mix - SYBR green should always be 24:1 with primer mix

- Label tubes 1 – 7 (1 for each primer)
- Add 30 µl SYBR to each tube (scale up if required)
- Add 1.25 µl of each primer mixture to corresponding tube (scale up as required)

Do calculation total with 5 µl of each for each well but use 4.8 µl so there is enough

- Draw plate plan onto sheet
- Pipette 4.8 µl SYBR and primer mix into plate in duplicate – into bottom of well
- Pipette 4.8 µl cDNA and LoTE buffer solution

Turn on plate seal machine to heat up. Seal plate – must be shiny side facing upwards otherwise sticks to machine. Place over plate (put other films back in box as light sensitive).

- Put plate in
- Press machine down and count to 5

- Lift lid and turn plate around
- Press machine down and count to 5
- Centrifuge - (large one) set to prog 1 = 10,000 x g for 1 min @ 20˚C (balance blank in opposite side)

Do PCR – book machine - use PCR machine two

- Switch machine on to warm up
- On computer:
    - Click MXp
    - RT2 machine – click ‘ok’
    - SYBR – click ‘ok’
    - Highlight whole plate in insert unknown well type for all, then change:
        - Not in use for empty wells
        - NTC – non-template control
        - Click SYBR for all wells
    - Thermal profile – import from first one
        - Seq 1 = 95˚C x 7.30 min for 1 cycle
        - Seq 2 = 95˚C x 25s for 40 cycles
        - = 63˚C x 25s
        - = 72˚C x 25s

- Seq 3 = 95˚C x 1 min for 1 cycle
- = 63˚C x 30s
- = 95˚C x 30s

- Saved as (RT1 horse pool 120717 etc)
- Open lid and place plate under lid
- Press run on pc
- ✓ turn lamp off at end of run
- Press start (RT2)

Chapter 4 model output tables

Table C.1 - The final GLM model output for dopamine D1-like receptor relative expression. The retained fixed effects following stepwise model selection included; region of the brain, side of the brain and the interaction between region:side. NAA = nucleus accumbens area, P = putamen, VTA = ventral tegmental area.

Fixed effects	Estimate	S.E.	t value	P value
Region - NAA	0.7315	0.3751	1.950	0.55
Region - P	-0.2858	0.3751	-0.762	0.45
Region - VTA	-0.3251	0.3751	-0.867	0.39
Region - Right	1.1302	0.3751	3.013	< 0.01
NAA:Right	-2.1341	0.5304	-4.023	< 0.001
P:Right	-0.5111	0.5304	-0.964	0.39
VTA:Right	-0.9782	0.5304	-1.844	0.07

Table C.2 - The final GLM model output for dopamine D2-like receptor relative expression. The retained fixed effects following stepwise model selection included; diet, brain region, side of the brain and the interactions between diet:region and region:side. HS = high-starch diet, NAA = nucleus accumbens area, P = putamen, VTA = ventral tegmental area.

Fixed effects	Estimate	S.E.	t value	P value
Diet - HS	2.8384	1.4366	1.976	0.52
Region – NAA	4.1494	1.7595	2.358	< 0.05
Region - P	0.9507	1.7595	0.540	0.59
Region – VTA	0.1389	1.7595	0.079	0.94
Side - Right	4.3219	1.4366	3.008	< 0.01
HS:NAA	-5.6401	2.0317	-2.776	< 0.01
HS:P	-4.2669	2.0317	-2.100	< 0.05
HS:VTA	-3.2440	2.0317	-1.597	0.11
NAA:Right	-6.9056	2.0317	-3.399	< 0.01
P:Right	-1.7607	2.0317	-0.867	0.39
VTA:Right	-3.4930	2.0317	-1.719	0.09

Table C.3 - The final GLM model output for dopamine D1-like receptor relative expression. The retained fixed effects following stepwise model selection included; diet, gut region and the interactions between diet:gut region. HS = high-starch diet, DC = dorsal colon, SC = small colon, SI = small intestine (jejunum), VC = ventral colon.

Fixed effects	Estimate	S.E.	t value	P value
Diet - HS	0.0066	0.005	1.456	0.15
Region – DC	0.0006	0.005	0.125	0.90
Region – SC	0.0067	0.005	1.481	0.15
Region – SI	-0.0016	0.005	-0.356	0.72
Region - VC	0.0068	0.005	1.504	0.14
HS:DC	-0.0079	0.006	-1.230	0.22
HS:SC	-0.0179	0.006	-2.781	< 0.01
HS:SI	-0.0061	0.006	-0.958	0.34
HS:VC	-0.0023	0.006	-0.363	0.71

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