THE MICROBIOME AND STALLION FERTILITY

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Photo of miniature stallion and mare recruited for intervention study by Anna Baer

DEDICATION

To the couples who were my patients, who sought to find answers to their fertility problems when conventional medicine could provide neither explanations nor solutions for their infertility.

Declaration

Apart from the assistance mentioned in the acknowledgements, the studies contained within this thesis were designed and executed by the author and have not been previously submitted for any degree to any other university or institution.

Carolyn Giselle Cooke, MB, BS

Authorship attribution statement

Appendix B1 of this thesis is published as Cooke CG, Gibb Z, Harnett JE. The Safety, Tolerability and Efficacy of Probiotic Bacteria for Equine Use. J Equine Vet Sci. 2021 Apr;99:103407. doi: 10.1016/j.jevs.2021.103407. Epub 2021 Feb 11. PMID: 33781424.

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I designed the studies, contributed to data analyses, and wrote the drafts of the manuscripts. I am designated corresponding author.

Carolyn Giselle Cooke
July 4, 2023

As a supervisor for the candidature upon this thesis is based, I can confirm that the authorship attribution statements above are correct.

Joanna Harnett
July 5, 2023

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ABSTRACT

Equine health and fertility and their relationship to the microbiome are topics of emerging scientific interest. In the sphere of human infertility research, the influence of the semen and gastrointestinal microbiomes on sperm quality and fecundity, as well as the potential for probiotic supplementation to improve male fertility outcomes, have been reported. The overarching aim of this thesis was to contribute to the body of knowledge regarding associations of these two microbiomes in horses (semen and gastrointestinal) with biomarkers of equine fertility, as well as the potential role of prebiotics and probiotics to influence the composition of these microbiomes and sperm quality.

Two literature reviews, which included studies involving equines, were conducted to elucidate what is known about the efficacy and safety of prebiotic and probiotic feed supplements on the general health of and disease in horses. These reviews revealed a gap in the knowledge with respect to the influence of probiotics and prebiotics on the semen microbiome composition and their associations with markers of stallion fertility. No studies had reported on the safety of prebiotic and probiotic supplementation in relation to biomarkers of fertility, to either sperm quality or conception rates. In addition to the general review of the literature, these two reviews subsequently informed the probiotic and prebiotic study that forms part of this thesis.

Across the three studies reported here, the compositions of the equine semen and gastrointestinal microbiomes were characterised and the influence of prebiotic and probiotic interventions on the composition of these microbiomes and their relation to sperm quality were explored. In addition, associations between the composition of the gastrointestinal microbiome, systemic inflammation, stud management and per cycle conception rates (PCCRs) are described.

Study 1: The composition of the semen microbiomes of four miniature stallions is described by taxonomic classification of phylum, order, class, family, and genus.

Study 2: In this study, the microbial diversity of the taxa detected in the semen and intestinal microbiomes, along with six sperm quality parameters, of four miniature pony

stallions are described in response to pre-, pro- and synbiotic (a combination of pre- and probiotic) supplementation, at four time points over a period of one year.

Study 3: The associations between the alpha-diversity, evenness, and richness of the faecal microbiome; stud location and management practices; and serum inflammatory cytokine concentrations of 19 commercial Thoroughbred stallions are described in relation to PCCRs.

The results of Study 1 provide, for the first time, a comprehensive description of the composition of the semen microbiome of healthy miniature stallions across multiple taxonomical classifications. These preliminary data can be used to inform larger scale research exploring equine reproductive health.

The results of Study 2 describe, for the first time, the impact of prebiotic, probiotic and synbiotic interventions on the diversity of the semen and intestinal microbiomes and the sperm quality of miniature pony stallions. There were no adverse effects of supplementation observed on either pony health or sperm quality; neither did any intervention significantly alter the composition of the semen microbiome, although some evidence was found for an impact of prebiotic supplementation on the faecal microbiome in relation to the Shannon's diversity index, a calculation of the measures of the abundance and evenness of the microbiota present.

The results of Study 3 found no associations between the microbial diversity of the faecal microbiome, serum inflammatory cytokine concentrations and PCCR in our cohort of commercial Thoroughbred stallions, although there was a significant stud farm effect on PCCR. This is the first study to have investigated a possible relationship between these three measures in these equines.

Conclusion

The studies conducted for this thesis revealed several new findings regarding the equine gastrointestinal and semen microbiomes. Our evaluation of the semen microbiome composition of miniature pony stallions showed an inter-individual variation in the bacterial composition of this microbiome in ponies that were stabled under the same

conditions and fed the same diet and supplements prior to any additional interventions. Pre-, pro- and synbiotic treatments administered to the same small cohort of fertile miniature pony stallions did not alter sperm quality parameters, nor did they significantly alter semen or intestinal microbiome profiles; however, safety and tolerability for these supplements was demonstrated, as they did not adversely impact the general health, digestion, or sperm parameters of these stallions. Only prebiotic treatment of these horses showed a positive effect on the diversity and richness measures of the intestinal microbiome, which warrants further investigation.

In a different breed, the Thoroughbred, we explored the associations of faecal microbial richness, evenness, and diversity with circulating TNF-α concentrations in relation to PCCR in a cohort of 19 commercial Thoroughbred stallions at stud. While no statistically significant relationships were observed between these criteria, by examining stud management practices we were able to ascertain an effect of stud on PCCR and a trend towards an effect of L-carnitine supplementation on the same fertility outcome. Finally, these findings suggest that there is more that could be explored about the effect of nutrition, prebiotics and probiotics on the semen and intestinal microbiomes of horses of various breeds, with the potential to improve stallion fertility and overall health.

LIST OF ABBREVIATIONS

16S-rRNA gene	16S ribosomal ribonucleic acid gene
1M	1 molar concentration
4HNE	4-hydroxynonenal
AOS	acidic oligosaccharides
ART	artificial or assisted reproductive technology
ASV	amplicon sequence variants
ATP	adenosine triphosphate
AV	artificial vagina
BTB	blood-testis barrier
CASA	computer-assisted sperm analysis
CCS	circular consensus sequencing
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGUS	equine gastric ulcer syndrome
EMS	equine metabolic syndrome
FITC	fluorescein isothiocyanate
FOS	fructo-oligosaccharides
FSH	follicle stimulating hormone
GI	gastrointestinal
GOS	galacto-oligosaccharides
HPC	high performance computing
IL	interleukin
IL-6	interleukin-6
IVF	*in vitro* fertilisation
JAI	Jerusalem artichoke inulin
LPS	lipopolysaccharide

MALDI-TOF	Matrix Assisted Laser Desorption Ionisation-Time Of Flight
MOS	mannan-oligosaccharides
MOSI	male oxidative stress infertility
MSR	MitoSOX Red
N	number
NA	taxon undescribed
NSAID	non-steroidal anti-inflammatory drug
OTU	operational taxonomic unit
PBMC	peripheral blood mononuclear cells
PCCR	per cycle conception rate
PCR	polymerase chain reaction
PO	per os (orally)
PUFA	polyunsaturated fatty acid
R2	coefficient of determination
RNA	ribonucleic acid
ROS	reactive oxygen species
SCFA	short chain fatty acid
SCSA	sperm chromatin structure assay
SD	standard deviation
SMRT	single molecule real-time
SNP	single nucleotide polymorphism
SPSS	statistical analysis software
STROBE	STrengthening Reporting of OBservational studies in Epidemiology
TMS	trimethoprim sulfadiazine
TNF-α	tumour necrosis factor alpha
VAP	average path velocity
VFA	volatile fatty acid
VSL	sperm cell velocity

WHO	World Health Organisation

GLOSSARY

ad libitum	freely, as desired
archaea	non-nucleated micro-organism
bioinformatics	collection and analysis of genetic codes for organism identification
cytokine	immunoregulatory proteins secreted by immune and other cells
DADA2	software for recovering copies of 16S gene from microbiome
demultiplexing	separating a multiplex signal into component parts electronically
dysbiosis	altered microbiome composition
enterocolitis	inflammation of the intestines and colon
fastq file	encoded biological (nucelotide) sequence and corresponding quality score stored in text format
FastQC	quality control analysis tool
forage	grass, hay grazed for food
gamete	haploid germ cell (sperm, ovum) containing a half complement of chromosomes of other biological cells
genetic polymorphism	variation in types of individuals of a species produced by two or more genetic expressions (discontinuous genotypes)
idiopathic	unexplained aetiology/cause
L-carnitine	an amino acid derivative which transports fatty acids intracellularly for energy production
laminitis	inflammation of the laminae (layers) of the hoof
leucocytospermia	appearance of white blood cells in semen
MAGPIX	fluorescent multiplexing instrument
microbiome	the collective genetic material belonging to the microorganisms in a specific environment or region

microbiota	the microorganisms inhabiting a specific biological region, niche, site or habitat
microflora	microbial residents (bacteria, viruses, fungi, yeasts, algae) of a specific biological region, niche, site or habitat
mitochondria	organelle located in cytoplasm of cells responsible for biochemical respiration and energy production (ATP)
polygamous	mate with more than one other animal of opposite sex in same species
prebiotic	nutrient promoting growth of beneficial microflora
probiotic	live micro-organisms which convey health benefits to host when consumed; supports microbiome composition
QIIME, QIIME2	microbiome bioinformatics platform
scouring	diarrhoea in foals
Shannon's Diversity Index	a measure of species diversity in a microbial community
spermatocyte	sperm cell (gamete) at second stage of development
spermatogenesis	process of development and maturation of male gametes
spermatozoa	motile male gametes formed in seminiferous tubules of testis and released into semen for ejaculation
stud	residence for stallions engaged in breeding
synbiotic	combination of prebiotic with probiotic
taxonomy genus	organism classification into phylum, order, class, family,
Thoroughbred	equine racing breed originally derived from English mares mated with Arab stallions
V1-V9	regions on 16SrRNA gene

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Figure 6.3: Relative abundance of phyla in faecal samples of 19 Thoroughbred stallions (A-S) by stud in an observational study to identify factors associated with per cycle conception rate and TNF-α.

LIST OF PUBLICATIONS

The work described in this thesis has resulted in, or contributed to, the following publications and presentations.

Journal Publications:

Cooke, C. G., Gibb, Z., Harnett, J. E., The Safety, Tolerability and Efficacy of Probiotic Bacteria for Equine Use, *Journal of Equine Veterinary Science* Vol 99 (2021) 103407,

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C Giselle Cooke, Zamira Gibb, Christopher G Grupen, Joanna E Harnett, "Prebiotics and synbiotics in equine health and disease – a review", *International Journal of Equine Science*, accepted for publication October 21, 2023 (DOI pending).

C Giselle Cooke, Zamira Gibb, Christopher G. Grupen, Kathrin Schemann, Nandan Deshpande, Joanna E Harnett, "The semen microbiome of miniature pony stallions". CSIRO journal Reproduction, *Fertility and Development*, accepted for publication following revisions, November 3, 2023.

Manuscript submitted to peer reviewed journals (currently under review):

C Giselle Cooke, Zamira Gibb, Christopher Grupen, Kathrin Schemann, Nandan Deshpande, Joanna E Harnett, "The impact of probiotics and prebiotics on the composition of the equine faecal and seminal microbiomes and sperm quality – a pilot study". Submitted to the Journal of Equine Veterinary Science, August 2, 2023.

Posters:

C Giselle Cooke, Zamira Gibb, Christopher G. Grupen, Joanna E Harnett, "The effect of a prebiotic and a probiotic protocol on the composition of the intestinal and semen microbiomes and sperm quality of stallions". International Human Microbiome Conference 2022, Kobe, Japan, November 8-10, 2022. Recorded commentary: file:///Users/gisellecooke/Downloads/Recording%2015%20Oct%202022,%2011_53%20pm.webm

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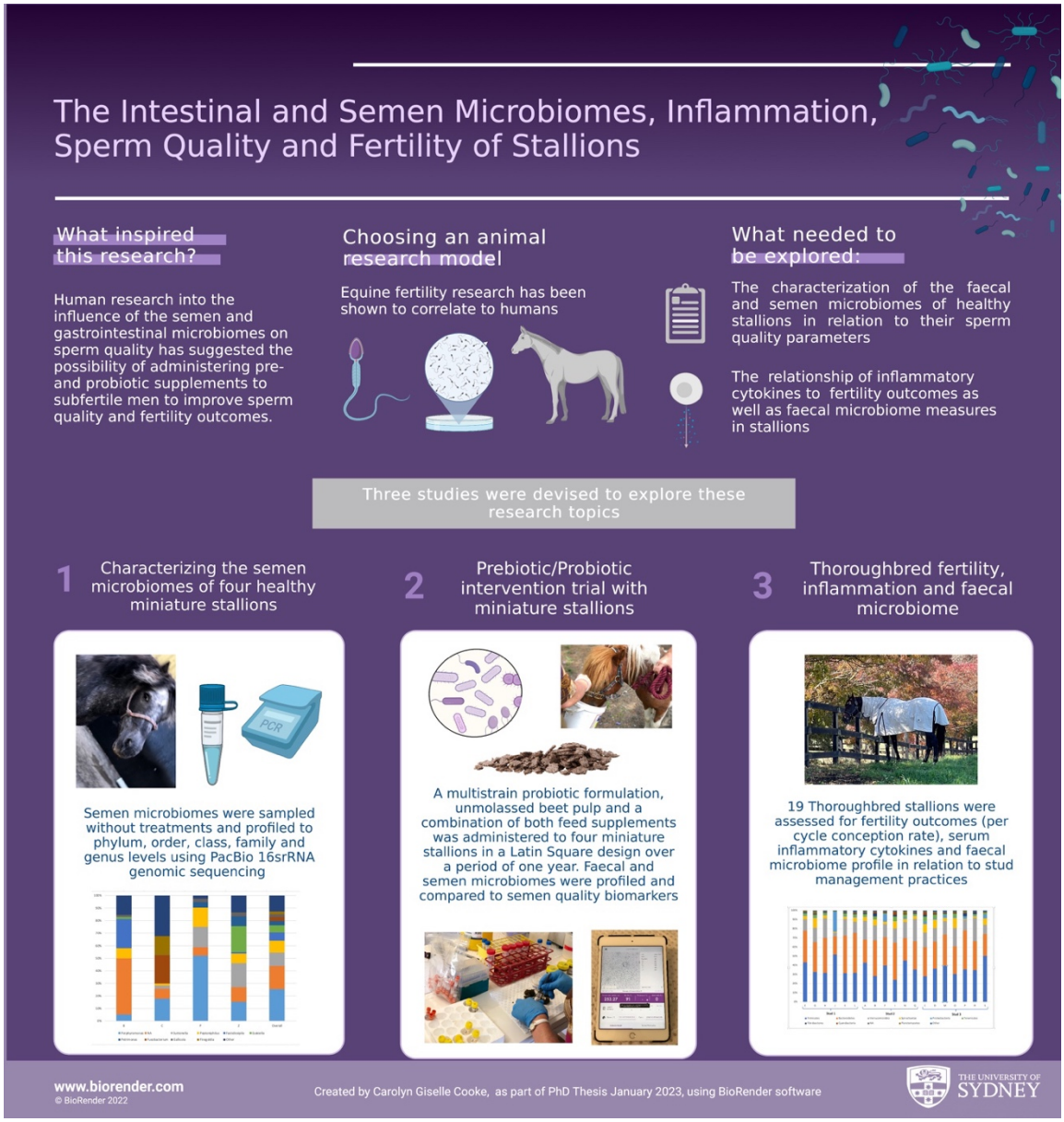

Figure 1: Graphical abstract of thesis

A general review of the literature opens this thesis, exploring the current phenomenon of declining male fertility witnessed in many animal species, including humans. The key research question for this thesis is whether male fertility, specifically sperm quality, is impacted by the semen and intestinal microbiomes, and furthering positing the hypothesis that altering the bacterial composition of these microbiomes by supplementation with prebiotics and probiotics could enhance fertility. Our chosen animal model for exploration of these research questions is the equine (Figure 1).

The literature pertaining to the general health and fertility of stallions is presented, in which breeding practices are discussed for different equine breeds. Factors affecting sperm quality and fertility in stallions, such as season, environment, nutrition, herbs, oxidative stress and microbial contamination are described. Current knowledge is explored in the literature pertaining to equine microbiomes: what is known about stallion microbiomes and more generally the equine intestinal microbiome and its role in health and disease; differing microbiomes in foals, mares and different breeds and by intestinal region; the impact of feed and pasture type, as well as antimicrobials and stress; equine diseases and systemic inflammation in relation to the microbiome; and the equine semen microbiome, the role of infection and how both affect fertility. The practice of prebiotic and probiotic feed supplementation in horses for improving digestion, as well as in the management of various diseases, is addressed.

Two narrative reviews have been conducted and published (see Appendix B1 and B2, Publications Arising from this Thesis). The first reports what is known (up to 2020) about the safety, tolerability, and efficacy of bacterial probiotic use in equines, and the second reports the same regarding prebiotics. These reviews were conducted to inform a pre- and probiotic intervention trial and to establish what is currently known about the efficacy and safety of probiotic and prebiotic use in the equine population, and to identify if any studies had been conducted in relation to equine fertility, specifically sperm quality.

In Chapter 2 the characterisation of the composition of the semen microbiome of a cohort of miniature stallions is reported. It reports the microbiota present in the semen of healthy, fertile, miniature pony stallions, identified to five taxonomical levels (ranks) using

metagenomic sampling and full-length 16S amplification. The main objective of this study was to identify the microbiota present in this breed to a depth that has not previously been reported in the literature for the stallion semen microbiome. This study also provides baseline data about this specific cohort, which we recruited for a prebiotic and probiotic intervention trial (Chapter 3).

Chapter 3 presents the main data chapter of this thesis, an intervention study which assesses the impact of a probiotic, prebiotic and a synbiotic regimen on the faecal and semen microbiomes, as well as sperm quality biomarkers, of a small cohort of miniature pony stallions. These animals were enrolled in the 12-month intervention period by employing a Latin square crossover trial design. The dual aspects of this study were, firstly, to explore any influence that pre-, pro- and synbiotics might have on the composition of the faecal and semen microbiomes of the miniature pony stallions over the year, and, secondly, any influence that these nutritional treatments might have on six biomarkers of sperm quality.

In Chapter 4 a retrospective study is presented, conducted with a cohort of elite Thoroughbred stallions recruited from three stud farms located in the Upper Hunter Valley, New South Wales, Australia. Data regarding stud farm management practices, nutritional supplements (including probiotics), stallion age, presence of inflammatory disease and per cycle conception rates (PCCRs: the percentage of mares pregnant of all mares mated by the stallion in that season) for the breeding season 2020 were collected; blood was collected for serum cytokine levels and faecal specimens were collected for metagenomic profiling from each stallion. Fertility, measured as PCCR, was assessed for any association with certain faecal microbiome characteristics, specifically phyla present and diversity measures of the faecal microbiota, as well as with serum inflammatory cytokine TNF-α.

A General Discussion follows in Chapter 5. A summary of the key findings of each of the data chapters and reviews is then discussed, taking into consideration the strengths and limitations of the studies and suggesting their potential to stimulate and inform future research in their domain. Finally, conclusions are drawn from the overall body of work for

this thesis, which highlight new knowledge that has been gained and recommendations for further research.

CHAPTER 1: REVIEW OF THE LITERATURE

1.1 GENERAL INTRODUCTION

The phenomenon of declining male fertility is currently evident across many species in many regions of the globe (1, 2) and several contributing factors have been identified, such as genes, nutrition, and the environment (3, 4). The purpose of this doctorate is to investigate specific factors that might impact male fertility, in particular sperm quality, and to primarily explore their relationship to the semen and intestinal microbiomes. To explore what is currently known about the influence of human microbiomes on male fertility, a review of the literature was conducted (see Appendix C). Novel approaches to managing male fertility are required to address known disruptors to spermatogenesis and gamete functionality, especially those adversely impacting sperm DNA integrity. Inflammation arising from local injury or infection to the testis or epididymis is known to damage developing spermatocytes (5, 6), but it may also be possible that inflammation arising from a more distant tissue or system, such as the gastrointestinal tract (GIT), may have the potential to damage gametes and impact fertility outcomes (6, 7). Central to the background of this thesis is the intersect between the composition and diversity of the intestinal and semen microbiomes and biomarkers of fertility. Dysbiosis (alterations to the composition of microbiomes with negative health impacts) of the GIT is associated with greater numbers of gram-negative bacteria colonising the gut, and releasing lipopolysaccharides from their outer membranes, which in turn increase intestinal permeability and passage into systemic circulation and an inflammatory response within target organs (8). Gut microbes play an integral role in immune responses, with dysbiosis being associated with inflammosome activation and the release of various cytokines (9). The question arises as to whether the intestinal microbiome, particularly in a dysbiotic state, may negatively influence sperm quality via inflammatory mediator cytokines and, additionally, whether the administration of probiotics to subfertile men could restore an altered gut microbiome and reduce inflammation to improve their sperm quality.

Restoration of the GIT microbiome's composition and diversity has been proposed via administration of diet and nutritional interventions such as pro-, pre- and synbiotics (10).

An equine model was chosen for the research outlined in this thesis for its increased feasibility compared to a human-based study; however, it became evident upon reviewing the literature that the topic of equine fertility and the microbiome was even less understood than that of the human. The equine intestinal microbiome has been studied more than any other anatomical region for this species; however, not in relation to stallions specifically, nor to their fertility. The area of prebiotic and probiotic supplementation for horses was scoped out as part of this literature search and a need to survey current use, and the efficacy and safety of these nutritional supplements in equines, became apparent.

1.1.1 FACTORS INFLUENCING MALE FERTILITY

Declining fertility has been witnessed in many animals, with 20–30% of livestock estimated to be culled – or more commonly in the case of horses, simply removed from the breeding population – due to low fertility (11, 12). Nutritional, environmental, genetic and stress factors have been identified as contributing to impairment of sperm quality and function in many species (13-18), with multiple exposures having the capacity to produce a compound effect.

Traits for reproductive fitness are heritable (19) and the increasing utilisation of assisted reproduction technologies (ART) may be perpetuating male infertility (1). Stallion sperm quality may be compromised by cryopreservation prior to artificial insemination (20, 21) and fertility is impacted by ageing (22) and genetic factors (17, 23), which affects the economic success of the equine breeding industry. Hazardous environmental chemical exposure has been linked to declining fertility in many species, largely attributable to decreasing sperm function (15); however, the evidence for chemical toxicity contributing specifically to stallion subfertility is currently weak (24, 25). Genetic polymorphisms linked to male infertility in humans and mice have also been identified in infertile stallions, suggesting more research into the links between the equine genome and fertility is warranted (17).

1.1.2 DECLINING MALE FERTILITY – APPLYING AN EQUINE MODEL

A growing body of research utilising the equine model as a proxy for human studies (26-31) is emerging, and fertility studies are among these (32, 33). Horses and humans have been shown to share similar biological processes, including allergic inflammatory responses to airborne allergens (31). Comparatively, the murine model, which requires artificial immune provocation or genetic manipulation to induce a similar response, thus providing a less translatable research model. The presence of leucocytospermia in humans and genital infections in stallions have both been shown to negatively impact sperm function (34), due to reactive oxygen species' generation in response to infection (35). Extensive investigations have been carried out on stallion sperm to assess the impact of oxidative stress on sperm membranes and mitochondria when exposed to different hazardous chemicals. These studies also explore strategies to mitigate the detrimental effects caused by oxidative stress (21, 36-38). These findings may be extrapolated to human sperm function and inform male infertility research, which has revealed that oxidative damage to sperm membranes, DNA and capacitation potential may account for up to 80% of idiopathic infertility in men (39). In the rapidly developing research domain of the microbiome, the effects of seminal microbiota on fertility, although limited, are beginning to be explored in humans (40-42) and more recently on stallions as well (43). Although probiotic supplementation has recently been trialled in infertile men to improve sperm parameters (44), similar studies in fertile men have yet to be conducted and none have been conducted in stallions to date.

1.1.3 GENERAL STALLION HEALTH AND FERTILITY

1.1.3.1 Stallion management

Access to clean water from a dam or trough and a mineral salt lick in the stallion's paddock will assist water and electrolyte intake in warmer climates (45). Regular daily paddock exercise allowing the stallion to run is essential for his fitness and general wellbeing, stepping up to more intensive exercise in July each year in preparation for the annual breeding season (45), which occurs from August to December in Australia. Hoof care is managed by a farrier every 6–8 weeks to protect against lameness, which can

hamper a stallion's breeding fitness, and dental health maintenance is provided by twice yearly dental checks (45). Preventing infections in stallions is important, as only a few days of a febrile illness can cause a prolonged negative impact on semen quality (45). Regular vaccination against tetanus, strangles and Hendra virus and worming to eliminate helminths from the stomach and intestines is carried out for Thoroughbred stallions at stud, according to a 2020 survey of stud managers at three Upper Hunter studs (see Chapter 6).

1.1.3.2 Factors impacting stallion fertility

1.1.3.2.1 Season

Stallions exhibit seasonal variations in sexual behaviour, hormones and fertility biomarkers in semen, with spring being the commencement of the annual equine breeding season. Testosterone, luteinizing hormone and Sertoli cell numbers increase in the spring/summer period (46) in line with semen volume and sperm count, quality and motility (47, 48), while sperm DNA fragmentation is lower (48). The effect of season is particularly prevalent when comparing fertile and subfertile stallions; subfertile stallions may have half the percentage of morphologically normal sperm as fertile stallions during the breeding season (49). In one study, sperm morphology, progressive motility, and kinematics were higher in the semen of fertile stallions (80–90% pregnancy rate) compared to subfertile stallions (40–60% pregnancy rate), although there was no difference between these cohorts during the non-breeding season (49).

1.1.3.2.2 Nutrition

The stallion at stud has greater nutritional requirements than the mare or gelding (castrated male horse), similar to that of working horses, requiring a 10% increase in feed of hay, oats or lucerne and pasture grass access ad libitum (45). The stallion's feed may also be supplemented with additional nutrients, such as vitamins (A and E), minerals (calcium, phosphorus, magnesium and selenium), essential fatty acids (seed oils) and amino acids (lysine, carnitine), to enhance fertility (45, 50). Having access to plenty of good quality pasture with additional hay and chaff feed supplements will optimise the

body condition of horses, and often stallions at stud are maintained slightly overweight to project the healthiest image to gain prospective business from the owners wishing to breed their mares. Additional body fat reserves are necessary during the breeding season to supply the stallion's energy requirements for the intense physical exertion of mating with mares; however, obese stallions may have lower libidos or be at fatal risk while in service (45). Ideally the stallion's body condition score should sit in the range 2–4 (51).

Nutrition plays a critical role in equine performance, by supporting both general wellbeing and long-term health (52). Hormones modulate nutrient supply to tissues, particularly myocytes during exercise (52), and, conversely, nutrition may also support the balance of hormones, such as insulin, progesterone and growth hormone in horses (53). Horses kept as companions may not require dietary management beyond meeting minimum requirements; however, equine athletes are given enhanced nutritional feeding programs suited to their discipline. One of the most challenging equestrian disciplines is endurance racing, requiring high level cardiometabolic fitness and musculoskeletal stamina and fitness (54), along with Thoroughbred track racing, which demands explosive strength and cardiorespiratory capacity from the horse as well as mental fitness for competition. With regard to stallion fertility, there is a growing body of work investigating the use of nutritional supplements to improve sperm quality and fertility in horses (50); however, there is currently no published literature which links semen quality to the composition of either the equine intestinal or semen microbiome, nor the potential of probiotic supplementation to influence sperm quality.

1.1.3.2.3 Nutritional, dietary and herbal supplements

Key nutrients are essential for healthy formation and function of equine spermatozoa. Essential fatty acids, in particular docosahexaenoic acid (DHA), are incorporated into the lipid bilayer of sperm outer membranes, enabling them to be flexible, compressible, deformable and elastic (55), as well as increasing progressive motility and velocity (56). Linseed oil supplementation of the diet improves sperm acrosome integrity, osmotic tolerance and viability (57), while selenium and vitamin E given with DHA significantly increase total sperm count and progressive motility (58). However, if dietary enrichment

was implemented with essential fatty-acid-containing whole foods, rather than with oils, no improvement was seen in sperm motility or morphology (59). Combined supplementation with vitamin E, selenium, L-carnitine, and omega-3 and -6 fatty acids produced beneficial effects on oxidative stress, acrosome integrity, motility and sperm longevity during *in vitro* storage (60), highlighting the potential for synergistic effects obtained with specific combinations of nutritional supplements.

Other nutritional supplements have been found to impact upon stallion sperm quality and function. L-carnitine, found in epididymal secretions (61, 62), is considered to support spermatogenesis, sperm maturation and mitochondrial activity, as well as protect germ cells from apoptosis (62). Raw semen acetyl- and L-carnitine levels correlate positively with both sperm concentration and progressive motility and have been suggested as potential biomarkers of sperm quality in stallions (63). L-carnitine supplementation has been shown to increase sperm quality and motility in sub-fertile stallions (63); hence, it is used by some thoroughbred studs as an oral supplement to enhance fertility. The limited capacity of horses to synthesise carnitine from methionine and lysine renders carnitine a conditionally essential amino acid, according to some nutritional scientists (64).

Vitamin E deficiency in stallions has been linked to the development of a higher number of abnormal sperm with poorer motility (50) and vitamin E supplementation of stallions improves both the total and progressive sperm motility of semen post-cooling (65). Both horses and cattle require vitamin A and carotene for healthy spermatogenesis, as these nutrients protect against the development of abnormal sperm and assist their motility (66). Dietary content of the antioxidant vitamins C, E and β-carotene correlate with sperm concentration and total progressive motility in humans (67), although supplementary antioxidants given to pony stallions did not improve sperm quality parameters above a well-balanced diet (68). Although the addition of a nutraceutical enriched with fatty acid DHA to the diets of breeding stallions tripled semen DHA concentration, no improvement was observed in sperm motility (56). Zinc and selenium in combination with alpha-tocopherol increased progressive motility and viability of sperm, as well as improved morphology, in fresh semen after 60 days' supplementation in fertile stallions (69).

Very few botanicals/herbal products have been used to address and support equine fertility. Stallion sperm quality can be enhanced by supplementation with the herb maca (Lepidium meyenii), in terms of higher sperm concentration and functionality, while protecting sperm against DNA fragmentation (70). The addition of 20 g of maca powder to the daily diet of stallions improved sperm count, concentration, motility and acrosome integrity of cooled semen (71).

1.1.3.2.4 Oxidative stress

By definition, oxidative stress is a biochemical state which results from the accumulation of reactive oxygen species (ROS) in biological tissues, generated by cellular respiration predominantly in mitochondria (72). Organisms possess protective cellular mechanisms and enzymatic processes that give them the ability to detoxify ROS; however, these oxidation by-products also have necessary roles to play in regulating systems via cell signalling (72), and also specifically regulate the functionality (73) and energy metabolism (37) of spermatozoa. Fertility in humans and stallions can be adversely impacted by disruptions to cellular redox balance (73), and ROS play important roles in controlling acrosome reaction, capacitation and oocyte binding ability of stallion sperm under conditions of intense mitochondrial activity (37, 74). In the stallion, unlike those of other animal species, there is a paradoxical relationship between *in vivo* fertility (following natural breeding) and *in vitro* oxidative stress parameters following a period of storage; Gibb et al. purport that the generation of cellular ATP, which fuels motility of equine spermatozoa, relies almost exclusively on oxidative phosphorylation (37), and the downstream effect of that intense metabolic activity is a state of oxidative stress.

If a state of oxidative stress arises, ATP production is compromised due to damage to the mitochondrial proteins associated with oxidative phosphorylation. At this point, spermatozoa rapidly lose their functionality (75) and suffer DNA strand breaks, leading to apoptosis (36). Seasonal variations in seminal ROS production have been observed in stallions, reflected in higher ROS concentrations and lipid peroxidation with lower chromatin fragmentation in winter when compared to summer specimens; however, interestingly, spermatozoa collected during winter appear to have improved

cryotolerance (76) as a result of altered lipid membrane composition (77). Levels of lipid and protein oxidation in spermatozoa vary seasonally and between fertile and subfertile stallions as well, acting as predictors of fertility, particularly during the non-breeding season (78).

1.1.3.2.5 Microbial contamination

A variety of non-pathogenic bacterial species, such as *Streptococcus sp., Staphylococcus sp.* and *Escherichia coli*, have been found to be commensal in semen at different sites of the genitourinary tract of healthy stallions, with environmental fungal and yeast colonisation evident in the urethra, being situated closer to the external environment (79). *Candida sp., Aspergillus sp.,* and *Cryptococcus sp*. have been isolated from up to one-third of frozen semen specimens and are known to become pathogenic in susceptible mares following artificial insemination (79). Contamination of stored stallion semen prior to freezing for ART is a concern also, because the presence of opportunistic pathogens in semen can affect sperm quality. Pathogenic bacteria, such as *Salmonella sp., Klebsiella sp.* and *Pseudomonas sp*., when present in semen, have been associated with equine infertility (80). During natural breeding the reproductive tract of mares is affected by transient post-coital uterine inflammation, which serves to reduce the bacterial load of the deposited semen and in some cases leads to persistent endometritis, a major contributor to subfertility in broodmares (81). An organism of concern that can be transmitted to mares from stallions during breeding and has been associated with endometritis is *Taylorella equigenitalis*. Historically, this highly contagious infection mostly affected the breeding of Thoroughbreds, but with the increasing use of artificial insemination for breeding other species of horse around the world*, T. Equigenitalis* has posed more of a concern as it can be transmitted via frozen-thawed semen (82).

1.1.3.2.6 Stallion microbiomes

The semen microbiome of men has recently been evaluated for association with sperm quality and hence fertility Lundy

(83, 84), while the semen and vaginal microbiomes of couples seeking to conceive by *in vitro* fertilisation contain bacterial phyla and species associated with both positive and negative pregnancy outcomes (85). By comparison, very little has been published in the equine domain regarding either the characterisation or the influence of the mare's vaginal and stallion's semen microbiomes on fertility. One equine study explored the relationship between bacterial families in semen and five measures of sperm quality, demonstrating an association between increased sperm motility and *Peptoniphilaceae,* while progressive motility was reduced in the presence of *Clostridiales incertae sedis XI* (43). One stallion, identified as a carrier of the pathogen *Taylorella equigenitalis*, demonstrated a specific semen microbiome profile which featured *Corynebacteriaceae* and *Peptoniphilaceae* bacterial families in the carrier state; however, this microbiome shifted predominantly towards the families *Bacteroidaceae, Porphyromonadaceae* and *Peptoniphilaceae* when the stallion was in the non-carrier state (86).

1.1.4 INVESTIGATING STALLION SUBFERTILITY

An important aspect of stallion reproductive management is identifying and investigating factors contributing to sub- or infertility. Difficulties in predicting stallion fertility based on sperm characteristics coincide with a wide range of fertility rates in many breeds of stallion (87). Subfertility has been declared in stallions with PCCR of less than 30%, or a seasonal pregnancy rate less than 10%, as compared to the average PCCR of fertile Thoroughbred stallions which has been reported to be approximately 68% (88, 89). Mare fertility factors actually have the greatest influence on breeding success, accounting for most of the variation in conceptions, while the semen quality and coital success of the stallion influenced only one-third of fertility outcomes (90).

Clinical fertility investigations generally consist of a thorough physical examination, diagnostic imaging and a basic semen analysis, with more advanced tests, such as computer assisted sperm analysis (CASA), DNA integrity, and oxidative stress assays being employed should the on-farm clinical examination be insufficient to identify causes of sub- or infertility. Testicular biopsy may be performed if testicular degeneration is suspected (91), and further endocrine testing for hormonal causes of infertility arising

from the hypothalamus, pituitary or testis, or metabolic screening for conditions such as insulin resistance, are added to blood screening in these subfertile stallions. More recently, genomic sequencing for mutations or single nucleotide polymorphisms (SNPs) which predict equine infertility is being considered as a clinical tool as research provides clues to genetic causes of this condition (91).

1.1.5 EQUINE BREEDING PRACTICES

Natural breeding or the use of artificial reproduction technologies (ART) are the two options for equine breeding, dependent upon the horse breed. Thoroughbred racehorse breeding is conducted naturally (i.e., without the use of any assisted reproductive technologies), as is the required practice by Thoroughbred breed societies globally. In Australia, the official breeding season begins in September each year, with the value of each successful cover (or mating) and resultant live foal birth reaching well into the hundreds of thousands of dollars for the most highly credentialled stallions, so the fertility of these athletes is paramount. Prized thoroughbred stallions may cover in excess of 200 mares during any breeding season; therefore, maintaining their semen quality is critical for success. Following a stallion's first season, a standard semen analysis (sperm count, motility and morphology) will allow them to be classified as satisfactory, questionable, or unsatisfactory for breeding (92). Pregnancy rates in the mares covered and successful births of healthy live young are recorded, rating the fertility success of stallions, the majority of which can produce high PCCR even with a full book of mares to cover per season (93).

Stallion fertility basically equates to the health of the spermatozoa; however, it is adversely impacted by a diverse array of factors, such as injury, lameness, disease, poor libido and behavioural disorders (94). The most fertile stallions have >60% morphologically normal sperm and it is generally recommended that mares receive 100–500 million progressively motile sperm by artificial insemination, or approximately one billion sperm per ejaculate for natural covers, to result in pregnancy (95). In breeds other than Thoroughbreds, artificial insemination may be employed to eliminate some of the mating obstacles, particularly if fresh semen is used in preference to frozen, with optimal sperm

concentration being obtained when semen collections are conducted during the breeding season, spaced by 48-hour intervals (96).

Stallion fertility deteriorates with age and varies between breeds (97). Selective breeding of horses has produced poorer fertility outcomes, in terms of lower PCCRs, than in many other species (19, 32, 33), with Thoroughbreds having lower fertility outcomes than most other breeds, been genetically selected for performance and not fertility (94). Inbreeding has been a concern for Thoroughbred horses (98), having all originated in England from a gene pool of only three founding Arabian stallions (99) bred with British mares. Intensive breeding management of Thoroughbreds may have inadvertently corrected for this inbreeding factor; industry practices have led to the selection of older mares which have shorter pregnancies and higher fecundity (98), and stallions with higher fertility will also produce more offspring per year (90).

1.1.6 PRIORITIES FOR EQUINE REPRODUCTION MANAGEMENT

For optimal fertility, ovulation in the female (mare) must be synchronised with effective deposition of ejaculated semen from the male (stallion) into the uterus at the mare's time of peak hormonal and uterine receptivity, and delivery of sufficient quantities of good quality semen into the mare's genital tract is required for best fertility outcomes (100).

Priorities for achieving fertility in mares are nurturing a healthy oestrus cycle, maintaining reliable ovulation, and achieving a healthy pregnancy for foaling success (101). Foetal development may be disrupted during pregnancy by maternal, nutritional or environmental factors, leading to birth defects or prenatal death, and complications of birthing may add additional risk (101). In the case of stallions, breeding soundness depends upon both physical and mental fitness factors (101), as well as their sexual behaviour, which may be influenced by domestication or have its origins in the wild (102). Key issues for optimal natural breeding are both quality sperm production and ability to cover (mate with) the mare, which may be affected by physical impediments or lack of libido (101). In terms of assisted reproduction techniques, best practices for semen collection, transport and *in vitro* storage are required to ensure the stallion's highest

conception rate (103), with, more recently, sperm selection techniques being employed to achieve better pregnancy rates and live foal births (104).

1.2 EQUINE MICROBIOMES

1.2.1 ASSESSMENT FOR GENERAL EQUINE HEALTH

Billions of commensal microorganisms (bacteria, fungi, archaea and viruses) and their genomic elements, inhabiting a biological or environmental domain, have become to be collectively known as a 'microbiome' (105), inhabiting regional domains of animals (including humans) and influencing many aspects of their physiology (106-112). High-throughput bacterial genomic sequencing technology, particularly sequencing of the full length of the 16S-rRNA bacterial gene, allows greater identification of microbiota compared to older technologies such as culture and microscopy and sequencing shorter regions of 16S rRNA genes. (113). Such technology expands the scope of the field of microbiome research significantly, revealing far more detail about the organisms residing in the host (114). The most widely researched anatomical region of the microbiome in horses is that of the gastrointestinal tract, where it is understood that the key roles for intestinal microbiota are in assisting feed digestibility, nutrient uptake and supporting the general health and wellbeing of the horse (110). The equine hindgut provides up to 80% of a horse's energy requirements from volatile fatty acids (VFA) (115) produced by fermentation of dietary fibre and the gastrointestinal microbiome, which is cultivated by ingesta, also supports immunity and metabolism in general (114, 116). The metabolome, being the collection of metabolites such as VFA's, produced by microbial cells, is the sum of biochemical reactions resulting from proteomic catalysis (117) For example, reduced populations of butyrate-producing gut bacterial species have been associated with inflammatory conditions such as laminitis (110, 118), attesting to the importance of metabolites produced by specific bacteria. It has been suggested that future roles for microbiome profiling and metabolome analysis might exist in preventive veterinary care, understanding the role of the microbiota in the development of certain equine diseases, enabling screening for predictive biomarkers before their onset, and facilitating selection of therapeutic interventions (114, 119).

1.2.2 Equine microbiomes, breeding

Other equine microbial communities that have been characterised and studied for their role in the protection against, and drivers of, various diseases are the uterine (120), respiratory (121), oral (122-124), skin (125) and ocular (126) microbiomes. The uterus of the healthy mare, once previously believed to be a sterile organ, displays a microbiome of microbial richness, evenness and alpha diversity reflecting the geographical location of the mare, perhaps influenced by local climate and feed (120). The vaginal microbiome of the mare has been studied as a potential source of probiotic bacteria to be administered for their antimicrobial activity, as an alternative to antibiotics, for managing infections of the female genital tract that can threaten the mare's fertility (127). The influence of the oral, faecal and vaginal microbiomes of the mare on the rectal and faecal colonies of her foal have also been explored and although some bacteria may travel from the placenta to the foal in utero, the majority of intestinal microbiome colonisation occurs at birth, with the microbial profile changing in the foal progressively over time (128).

Research has been conducted to analyse the intestinal microbiome of healthy horses, attempting to identify the core species (129), as well as its profile in intestinal diseases such as colitis (130). Pathogenic bacteria are more commonly cited as contributing to infertility in mares (131) and stallions (132), however the presence of intestinal dysbiosis, meaning the an alteration in the microbial composition of the normal intestinal microbiota, which can potentially cause mucosal inflammation, has not yet been explored in regards to equine infertility. One human study has examined the relationship between the intestinal microbiome, sperm quality and hence fertility (133); however, this concept has not yet been investigated in horses. This provided the motivation for one aspect of our research: to explore the relationship between the composition of the intestinal microbiome, inflammation and fertility outcomes in Thoroughbred stallions.

The relationship between sperm quality and the semen microbiome in humans has revealed that bacterial species that inhabit the semen can exert both positive and negative effects on sperm quality and function (40, 41, 84). Further, in mice, the testicular microbiome may play a role in maintaining a healthy functional blood-testis barrier (BTB),

with the microbial metabolome impacting the permeability of the BTB (134), with similarities to the observed effect of increased mucosal permeability evident with intestinal dysbiosis (135). The function of the reproductive microbiome has been explored in a variety of mammalian species (136) including mares (137), and a recent study has characterised the stallion semen microbiome (138); however, in contrast the intestinal microbiome of stallions and potential associations with semen parameters have yet to be examined.

1.2.3 The equine intestinal microbiome

Bacteria, viruses, archaea, fungi, parasites and protozoa comprise the diverse microbial populations (microbiota) that inhabit the equine intestinal tract, the entire genetic content of which is termed the 'microbiome'. Current research is exploring the microbial composition and metabolic activity of the intestinal tracts of many species, particularly that of humans, but also domestic and farm animals (139-141). Over the last decade, research exploring the microbiome of equids has emerged, with the highest number (six papers) published in 2021 (PubMed search, June 2013 to 2023), and to date there has been no consensus developed regarding the ideal composition of the healthy equine intestinal microbiome. The importance of the intestinal microbiome to equids lies in its constant production of essential nutritional, immune and metabolic compounds (metabolome) that support equine health and function (129).

1.2.3.1 THE ROLE OF THE INTESTINAL MICROBIOME IN EQUINE HEALTH

The majority of intestinal bacteria reside in the caecum and colon of the horse, processing ingesta for extraction of nutrients and fermenting non-digestible cellulose-rich forage for the release of short chain fatty acids which deliver energy-producing substrates to cells for optimal performance (142). Fermentation of feed commences in the non-glandular region of the equine stomach, where non-structural carbohydrates are processed by gastric microbes (143). Due to the relatively small size of the equine stomach (approximately 7.5–15.0 L, which comprises 8–10% of the volume of the gastrointestinal tract) (143), horses must continually graze to maintain their food intake for energy and nutritional requirements. This increases the importance of the work of the intestinal microbiota in

assisting digestion in horses. The small intestine is the primary location for chemical processing, digestion and absorption of ingesta, aided by bile and pancreatic secretions that are constantly supplied to the intestinal lumen, which emulsify and buffer the contents, respectively. Most of the essential nutrients are thus extracted from the ingesta before the remainder enters the caecum for further enzymatic processing and fermentation in the colon, which comprises approximately 64% of the volume of the entire gastrointestinal tract of the horse and houses a complex population of bacteria and other microbes (143).

1.2.3.2 REGIONS OF THE EQUINE GUT AND LOCAL MICROBIOTA

Anaerobes resident in the equine stomach number around 4.3×10^8; a similar bacterial count has been found in the caecum (4.2×10^8), while comparatively less are housed in the proximal colon (1.0×10^8), with the distal colon having around 6.7×10^7 organisms and the rectum 1.7×10^8 (143). Typical bacterial species found in the equine stomach are *Streptococcus equinis* and *bovis, Lactobacillus salivarius, mucosae* and *delbruekii,* and *Mitsuokella jalaludinii* (143). Faecal samples from horses have been shown to predominate in the phyla Firmicutes and Bacteroidetes (130). Faecal samples are widely used and considered representative of the microbial composition of the distal colon, but less so of the caecum in horses (144). Five regions of equine intestinal tracts were sampled for bacterial genetic sequencing in one study of 11 mature horses undergoing euthanasia for post-mortem collection of ingesta, revealing Firmicutes to be the major coloniser of all compartments; studies that used faecal samples have had similar findings (144). The stomach samples also contained mostly *Lactobacillus spp*, as well as *Sarcinia spp*, the duodenum had a higher quantity of *Streptococcus spp*, the ileum housed mostly *Actinobacillus* and *Clostridium spp*., and in the colon/faeces *Verrucomicrobium 5 genus incertae sedis* was identified as the predominant organism (144).

1.2.3.3 DEVELOPMENT OF THE INTESTINAL MICROBIOME

1.2.3.3.1 INTESTINAL MICROBIOTA IN FOALS

Microbial communities in the intestines of foals develop over time, rapidly cultivating at first and clustering by age, with Firmicutes-dominant microbiomes increasing in richness

over time (145). Transient species that are in greater abundance in early weeks of the foals' life, such as *Akkermansia sp*., are substituted by more established commensals, until their colonies stabilise around 60 days post-partum (145). By 90 months of age foals' intestinal microbiomes resemble the composition of their dam (perhaps influenced by pasture characteristics) and following weaning develop an abundance of *Fibrobacteres* (145).

1.2.3.3.2 COMPOSITION OF THE INTESTINAL MICROBIOME IN DIFFERENT BREEDS OF HORSES

The faecal microbiota of 189 healthy horses, representing six different sporting horse breeds and controlled for sex, age, equitation discipline and environmental factors, were profiled using 16SRNA gene sequencing, demonstrating that the abundance of 27 genera showed significant variation across breeds (146). The microbial alpha-diversity was found to be highest in Hanoverian and lowest in Lusitano horses, with more subtle findings in beta-diversity variation between several breeds (Lusitano, Anglo Arab and Central European). The overall composition of these microbiomes differed between breeds by microbial diversity, abundance and stability of bacterial communities; however, the breed of the host did not demonstrate a unique faecal microbial profile (146).

In another breed, the intestinal microbiome characteristics of Przewalski's mares and stallions were compared. The researchers found that Przewalski's mares had a higher Shannon diversity index and different beta-diversity in comparison to the stallions, which carried more pathogenic bacteria in their intestines (147).

1.2.3.4 FACTORS AFFECTING FAECAL MICROBIAL COMPOSITION

1.2.3.4.1 FEED, SUPPLEMENTS AND PASTURE TYPE

Factors influencing the composition and function of the equine intestinal microbiome extend from nutrition and supplements to age, exercise, season, stress, illness states and medical treatments (110). The composition of the equine intestinal microbiome can alter quite rapidly after a dietary change (148) or supplementation (149), some studies reporting this change occurring within four to six days (150, 151). Significant variation has

been observed in proportions of bacterial genera between groups of horses grazed on forage only (greater numbers of the fibrolytic genus *Fibrobacteria*) in comparison to those receiving concentrated feed supplements (predominantly *Bacteroidetes*) (152). The prebiotic characteristics of various types of pasture and feed impact their digestibility, thus the composition of the intestinal microbiome of the horse, which responds uniquely to variations in the amount of lignin and other plant fibre in their feed (153). When the lignin content of alfalfa feed is reduced, for example, an unequal shift in microbiome composition occurs across horses receiving this feed type, as did the beta diversity of intestinal microbiota of horses fed hay lignin (153). Fermentation of carbohydrates from ingested grass and hay by caecal microbial enzymes produces volatile fatty acids, which, apart from their main purpose of supplying the horse with an essential energy source, buffer the caecal region to support its colonisation by probiotic species (143).

Microbiome composition is stabilised by the presence of a greater diversity of micro-organisms; however, these intestinal conditions do not necessarily ensure the highest nutrient uptake. A blended diet of hay and oats can reduce microbial diversity in the hindgut, encouraging a predominance of the bacterial family *Porphyromonadaceae*, while producing better nutrient extraction from this feed when compared to a diet of hay only, that latter which supports the development of greater microbial diversity (154).

Equine athletes have additional energy requirements, and therefore are often given high-carbohydrate feed, the fermentation of which can be supported by the addition of supplemental yeasts to promote feed digestibility (155, 156). Probiotic yeasts, such as *Saccharomyces cerevisiae*, stabilise the pH of the caecum and balance hindgut microbial populations (157), preventing dysbiosis and supporting the growth of cellulolytic bacteria which assist in utilising and extracting nutrients from feed (155). While supplementation of the equine diet with either probiotic bacteria or yeasts to improve digestion will result in temporary colonisation only of the intestines with these species, they will not be adopted into the core microbiome of the horse (158) and hence will exhibit a transient presence. A need exists for more extensive investigation into the impact of the current practice of probiotic supplementation on the equine microbiome, to include the virome and mycobiome (110).

Season and weather conditions, especially high ambient temperatures and rainfall, influence the growth, quality and prebiotic characteristics of pasture grasses and thereby alter the faecal microbiome of horses grazing upon them, without producing any digestive disorders, such as colic. The faecal microbial phyla of horses grazed on pasture grass are predominantly Firmicutes and Bacteroidetes, regardless of season, the microbiome composition shifting towards more Fibrobacters and Spirochaetes phyla when the diet is supplemented with haylage (159).

1.2.3.4.2 AGE

Advancing age impacts faecal microbial composition in horses, specifically reducing species richness and both alpha- and beta-diversity (160). Amongst mature horses, advanced age has been observed to reduce microbial diversity, although a small core community of bacterial species persists throughout adult life (148). No specific differences in faecal microbiome profiles were found to be determined by sex (160).

1.2.3.4.3 ANTIMICROBIALS

Antimicrobials, including antibiotic and antiparasitic medications, have the potential to significantly disrupt the composition of the intestinal microbiome in horses (161, 162). A selection of antibiotic drugs – procaine penicillin (IM), ceftiofur sodium (IM) and trimethoprim sulfadiazine (oral) – were administered to 24 healthy mares in one study, for five consecutive days (161). Faecal samples were collected pre-treatment and on Days 5 and 14 and analysed using an Illumina MiSeq sequencer for high throughput sequencing of the V4 region of the 16S rRNA gene. Profile changes were noted in both community membership and population structure, most particularly after Day 5 of antimicrobial treatment. This finding concurs with human research on the same topic (163). The drug that showed the strongest impact on bacterial species richness and diversity, as well as population structure, was the sulpha drug trimethoprim sulfadiazine (TMS) (161), perhaps as its action is broader spectrum than both the penicillin and cephalosporin drugs. *Verrucomicrobium 5 genus incertae sedis* populations reduced dramatically with administration of TMS, allowing repopulation of the intestine with several genera of the Firmicutes phylum by Day 5, suggesting an innate microbial resilience and recovery from

antibiotic disruption (161). Disturbance to faecal microbiome composition was observed nine days following drug administration, with approximately full species recovery, similar to baseline, evident by Day 30 of this study.

The route of antimicrobial drug administration and its impact on microbial composition has been assessed. Oral administration may expose intestinal microbes to a higher concentration of active drug; however, high drug concentrations have also been observed in the intestines via parenteral administration (161). The authors recommended further studies should be conducted on this topic to elucidate the effects of the route of administration of antimicrobials on the equine intestinal microbiota (161).

The administration of the antiparasitic drug metronidazole to healthy horses has been shown to dramatically reduce the richness and evenness of caecal and faecal microbiome indices and adversely impact the production of metabolites of the faecal microbiota; the resultant shift in intestinal microbiota caused by administration of this drug corresponded with clinical gastrointestinal adverse events in all five subjects, severe enough for the investigators to halt the study after only three days (164). Twenty-one faecal metabolites were impacted by metronidazole treatment: those related to nucleic and amino acid metabolism, as well as carbohydrate and lipid metabolism. Each category of metabolites was elevated in Day 3 faecal samples, while metabolic co-factors and vitamin levels dropped significantly on Day 3 (164).

1.2.3.4.4 STRESS

Equine behaviour response to stressful circumstances occurs via the instruction of hormones and neurotransmitters (165) and gut microbial composition is known to influence host behaviour via the bidirectional gut-brain axis (166). In professional human athletes, gut microbiome composition can alter under conditions of intense physical exercise (167). In equine counterparts lipid metabolism and glycolysis, which the liver and muscles use to generate energy during intense physical exertion, produce temporary shifts in the intestinal microbiome and metabolome (168, 169).

Fasting, anaesthesia and transport of horses are stressful events that can impact faecal microbiome composition in most aspects except alpha diversity, with fasting producing a reduction in the abundance of Rickettsiales and transport reducing populations of Clostridiales. The effect of anaethesia on horses has been shown to impact the microbiome Jaccard Index (community membership) (170). Captivity of wild horses is another stressor to consider, which can affect gut microbial composition, greatly reducing faecal microbial diversity, as observed in a group of herded Przewalski's horses in comparison to wild controls resident on European nature reserves. This microbiome impact will be significantly affected by feed access and options, causing concern that human intervention is reshaping the intestinal microbiomes of equids (171).

1.2.3.5 EQUINE DISEASE AND THE INTESTINAL MICROBIOME

1.2.3.5.1 DISEASES OF THE GASTROINTESTINAL TRACT – AND THE INTESTINAL MICROBIOME

In general, gastrointestinal disease is more prevalent in equids, which house fewer intestinal microbial species and exhibit reduced microbiome richness and diversity (172). Intestinal microbial richness (numbers/abundance) is decreased in horses that experience diarrhoea (173). Horses with intestinal colic had fewer bacterial species with lower diversity when compared to healthy subjects admitted for elective surgical procedures and were also found to have an increased presence of the opportunistic pathogens Christenellaceae, Streptococcus and Sphaerochaeta (174). The predominant phyla, reported by one group of authors, in horses presenting with colic were Firmicutes, Bacteroidetes, Spirochaetes, Fibrobacteres and Proteobacteria when compared to healthy controls (10). Conversely, Firmicutes were reported to be generally depleted in horses with colitis by other investigators (173).

1.2.3.5.2 POSTPARTUM COLIC IN MARES

Suspecting perturbations of intestinal microbiota in pregnant mares may contribute to the development of postpartum colic, a team of researchers screened the faeces of a group of 31 mares (172). They found the faecal microbiomes of late-term pregnant mares to differ from non-pregnant controls and that foaling did not alter faecal microbial profiles

significantly. An abundance of Proteobacteria >4% and Firmicutes ≤50% was discovered in the faeces of mares that later developed colic episodes. Mares that were diagnosed with colon volvulus had differing faecal microbial membership to non-colicky controls (172).

1.2.3.5.3 METABOLIC SYNDROME

Equine Metabolic Syndrome (EMS) has been associated with intense feeding management of domesticated horses and a connection between this condition and the composition of the intestinal microbiome has been recognised (110). In the spring of 2014, one group of researchers in Kentucky, USA, were the first to characterise the gut microbiome of twenty horses of mixed breed and sex, all stabled at the university's veterinary science facility and fed a similar all-forage diet, comparing those with EMS to healthy controls (175). Their findings described that microbial diversity and general community structure were different in the EMS group; however, the membership of bacterial communities was similar in both groups. Reduced microbial diversity in the human intestine has been linked to the presence of obesity (176). One organism which predominated in the EMS group was an unclassified *Verrucomicrobium 5 genus incertae sedis*. Verrucomicrobia have been linked to obesity and metabolic disease in humans, their abundance having been suggested as a biomarker of increasing glucose intolerance (177). A predominance of *Fibrobacter spp*. was found in the control group of horses, being cellulolytic bacteria, and recognised as a core species of the healthy equine intestinal microbiome, particularly of that of equine athletes (178).

A relevant comment made in the study by Elzinga et al. regarding the faecal microbiome of horses with EMS was that this syndrome has been found to be a clinical causative factor for laminitis, a condition which produces significant morbidity for some horses (175) and could suggest potential to treat this condition by correcting intestinal dysbiosis in affected horses.

1.2.3.5.4 SYSTEMIC INFLAMMATION

Volatile fatty acids, enzymes and certain bacterial components are known to regulate inflammation in the gut milieu (179). The presence of bacterial lipopolysaccharides and

endotoxins in the intestinal lumen may alter the permeability of the intestinal mucosal epithelium, permitting microbial metabolites to escape into the systemic circulation, triggering a host immune response in which a cascade of inflammatory mediator molecules, such as cytokines and leukotrienes, are produced (180, 181). The testes of ageing stallions exhibit tissue degeneration associated with upregulation of inflammatory cytokine activity (182). Chronic seminal vesiculitis is a local inflammatory condition of the genitalia that can affect stallions, which may relapse after antibiotic treatment and interfere with fertility (183). Local inflammatory conditions in equines, such as infectious colitis, have the potential to become systemic, increasing circulating peripheral blood leucocytes and inflammatory cytokines (184). Mechanisms for the impact of the intestinal microbiome upon systemic inflammation have been explored in human medicine (185) to a greater extent than they have been in equine medicine; thus, the latter deserves further investigation, with only one study exploring the microbiome of horses with asthma (186).

A potential role exists for probiotic supplementation to manage inflammatory disorders in horses and thereby recover stallion fertility impacted by systemic inflammation. Probiotics have been studied in both animal and human models, *in vitro* as well as *in vivo,* and found to significantly reduce circulating inflammatory cytokine concentrations and also to improve the microbial composition of the gastrointestinal tract (187). Specific bacterial probiotics, namely *Lactobacillus acidophilus, L. plantarum* and *L. rhamnosus*, (species included in commercially available multistrain probiotic formulas promoted for equine use) have been shown to suppress TNF-α and IL-1 production and to improve the ratio of anti-inflammatory cytokines to inflammatory cytokines in human *in vitro* experiments (187). Yeast probiotics such as *Saccharomyces boulardii* have demonstrated similar anti-inflammatory effects, reducing secretion of IL-6 and TNF-α (a common probiotic used in equine feed supplementation), while increasing concentrations of the anti-inflammatory cytokine IL-10 (188). The role of probiotics for equine health and fertility is discussed below in this review of the literature, as well as their current use in equids in the review article which forms Chapter 3, the publication of which is attached in Appendix B1.

1.2.4 THE EQUINE SEMEN MICROBIOME

The semen microbiome has only recently been described in healthy stallions (138) and a relationship has been explored between semen characteristics and the presence of specific bacterial species that demonstrate an association with altered fertility (43). The bacterial phyla profiled in the semen of all the stallions sampled were Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. *Peptoniphilaceae* were present in the semen of stallions with better sperm motility, whereas *Clostridiales Incertae Sedis XI* was isolated from the semen of stallions with impaired progressive motility. No yeasts or fungi have been identified in stallion semen (79). A high level of individual variability of phyla has been noted by some researchers to be present in equine semen (138), and indeed variation of bacterial genera between stallions from the same stud farms (189); however, no association has been noted between bacterial genera and stallion age (35). To date, these findings have not been replicated in other studies.

Stallion semen may be contaminated with environmental and opportunistic bacteria as it passes along the genital tract, depending upon the husbandry practices impacting the animal's hygiene. There is a concern that commensal and contaminant bacteria present in semen may affect semen storage for later artificial insemination and that not all bacteria can be identified with current techniques including traditional MALDI-TOF (Matrix Assisted Laser Desorption Ionisation-Time Of Flight) analysis (80), hence the emergence of next generation DNA sequencing in microbiome research to assist accurate and comprehensive species identification. Genital microflora may contaminate semen during ejaculation; however, they have not been shown to alter semen parameters (190).

Further semen microbiome studies on healthy stallions are needed as most previous research has focussed on identifying urogenital pathogens in infected stallions (191). Bacteria may contaminate stallion semen during passage along the urogenital tract, from organisms resident on the skin of the horse's genitals and abdomen, as well as from the environment. Fifty percent of the bacteria isolated from stallion semen originated from skin and mucous membranes and 100% of semen specimens were found to be contaminated by *Staphlococcus* spp., in one study involving six Swedish stallions (80). In extended semen samples 50% were found to contain bacteria that originated from

environmental water and soil (80), and, surprisingly, the practice of penis washing prior to semen collection has been found to *increase* the level of bacterial contamination of the semen (192). Bacteria of the *Petrimonas* genus, which have been identified in stallion semen, normally reside in soil, where this 'electroactive' bacterium is able to effectively degrade hazardous compounds; hence, it is used for microbial pollutant removal (193). Potentially, soil contamination of the penis of stallions might occur, in particular for miniature stallions with their short stature, thereby introducing *Petrimonas* to their semen.

1.2.5 STALLION FERTILITY AND MICROBIOMES

Optimal reproductive outcomes are impacted by intrinsic factors in both the mare and stallion. Evidence for bacterial disruption of equine fertility of male origin focusses on pathogenic species leading to endometritis in the mare, which can adversely impact artificial reproduction technology outcomes. A single case report of a *Taylorella equigenitalis* carrier stallion linked to endometritis in the receiving mare has raised both concern and interest in the potential for flora transplant solution to protect the mare against infection (86).

The scope of this thesis is to examine reproduction in stallions only and the numerous factors that can affect their breeding success, mostly around sperm quality. Although equine reproduction has attracted a great deal of interest and research in the past, there remains a significant number of cases of stallion infertility due to poor sperm quality. Research has recently revealed underlying seminal microbiome aberrations that link to idiopathic infertility in men (41); however, only one such study has been conducted on stallions to examine seminal microbiota and any relationship to sperm quality (43).

1.2.6 Pre/probiotic supplementation in horses

The two reviews that form part of this thesis in Chapters 3 and 4 (see publications in Appendices B1 and B2) provide a detailed summary of literature on probiotic and prebiotics. Briefly here, probiotic and synbiotic administration to equines, both *in vitro* and *in vitro*, has been the topic of research only as recently as the past decade (194). Researchers have explored the use of probiotic supplementation to improve digestion in

horses by altering the intestinal microbial composition (195), with goals of reducing the incidence of scouring in foals (196), treating acute enterocolitis in hospitalised horses (197), managing other equine gastrointestinal disorders (158) and improving athletic performance (198, 199). The use of prebiotics to improve reproductive outcomes has been investigated in pigs, with the fertility of breeding sows enhanced by the addition of Enterococcus faecium DSM 7134 to their feed, increasing feed intake and litter size, thus preventing 'starvation sterility' (200). Furthermore, supplementing the feed of laying hens with *Clostridium butyricum* improved egg quality and feed conversion for better laying performance, related to the observed effect in this study by Xiang et al. of reduction of stress-induced reactive oxygen species (ROS) arising from intestinal lipid peroxidation (201).

The two reviews (Appendix B1 and B2) revealed no studies have been conducted to date investigating the impact of probiotic and/or prebiotic supplementation on the microbiome and sperm quality of stallions. Given the gaps in knowledge regarding the relationships between the equine gastrointestinal and semen microbiomes, probiotic and prebiotic interventions and their role in equine fertility, here we explored these associations.

1.3 HYPOTHESIS AND OBJECTIVES OF THIS THESIS

The hypothesis posited in the present study is that there is an association between the composition of the intestinal and semen microbiomes, inflammation, and biomarkers of fertility – specifically, sperm quality and conception rates.

This research explores a potential relationship between two regional microbiomes and biomarkers of fertility in an equine model with three main objectives:

1. To characterise the composition of the equine semen microbiome.

2. To evaluate the impact of a probiotic and prebiotic regimen on the composition of the semen and gastrointestinal microbiome and sperm quality.

3. To explore associations between the composition of the gastrointestinal microbiome, inflammatory markers and per cycle conception rates.

The content of this chapter forms a manuscript accepted for publication with revisions by the CSIRO journal Reproduction, Fertility and Development on the November 3, 2023.

Manuscript ID: RD23117. The submitted version is attached in Appendix B3.

Simple Summary

Little is currently known about the bacteria that comprise the equine semen microbiome (the microbiota that inhabit semen and their genetic content). Conducting an evaluation of the stallion semen microbiome will provide important data that can be used to inform fertility research. In this study we collected semen specimens during the Australian breeding season from four miniature pony stallions, who were stabled together and fed the same diet. Genomic profiling of their bacterial DNA content was conducted to identify microbial genes present in the semen and grouped according to a biological classification system of phyla, order, class, family, and genera. The bacteria present in the semen of the stallions studied differed between horses, some more abundant and common to all ponies, while others were found to be more predominant in individuals. Overall, the broadest bacterial classification, phylum, was mostly represented by Firmicutes and Bacteroidetes (76%), followed by Proteobacteria (15%). At the next level of classification, order, Bacteroidales, Clostridiales and Cardiobacteriales had the greatest representation (86%); at class level, Bacteroidia, Clostridia and Gammaproteobacteria (87%) collectively ranked the highest in number; at family level, *Porphyromonadaceae*, *Family_XI* and *Cardiobacteriaceae* (62%) were the largest groups; and at the level of genera, 80% of the abundance was comprised of seven genera – *Porphyromonas, Suttonella, Peptoniphilus, Fastidiosipila, Ezakiella, Petrimonas* and unknown taxonomy – microbes yet to be formally classified and named. Noteworthy is the use of full-length metagenomic sequencing in this study, which has expanded upon the current knowledge of this microbial domain in miniature stallions and further studies are encouraged, to

explore semen microbiome characteristics of stallions with varying health and disease status.

Graphical abstract

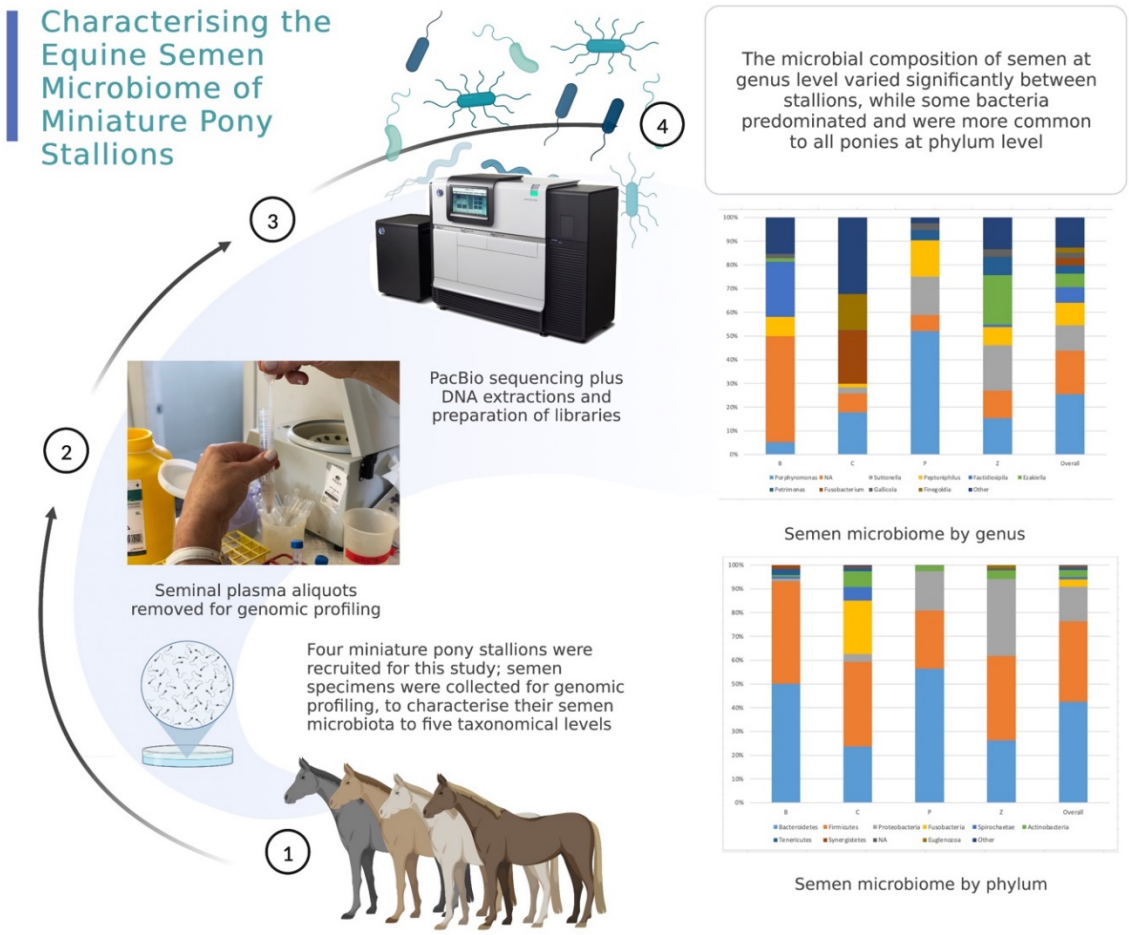

Figure 2.1: A graphical abstract of this study, characterising the semen microbiome of four miniature pony stallions to five taxonomical ranks.

2.1 INTRODUCTION

Exploring the composition of microbial communities that inhabit anatomical regions is an important area of research that seeks to understand the role of microbiota in the prevention of disease and support of normal health and function. More specifically, interest in the microbial communities that inhabit the reproductive tract of humans (40, 41) and animals (136, 202, 203), and the microbial associations with reproductive health and fertility has increased. Within the context of equine reproduction, genitourinary infections in the stallion and contamination of semen with bacteria, yeasts and fungi from passage along the distal urethra (79, 204) potentially affect sperm quality (189) and increase the risk of endometritis for the mare (205), impairing chances of conception and live foal birth outcomes. Bacterial contamination of stored equine semen poses significant challenges for breeders, requiring the addition of antibiotics to extended semen samples to prevent bacterial growth and transmission via artificial insemination, a practice which can also result in the development of antibiotic-resistant organisms and compromised sperm quality (206). With this concern, and in the context of equine fertility, pathogens have been studied more extensively by veterinarian researchers than the commensal bacteria that inhabit the stallion semen.

Over the past decade the equine intestinal microbiome has been characterised in both healthy and diseased horses and foals (129, 143-145); however, it is only very recently that the semen microbiome of stallions has been described (138, 189). To date, impaired progressive motility of stallion sperm has been correlated with the presence of the bacterium family *Clostridiales Incertae Sedis XI* (43), while improved total sperm motility was observed in the presence of the *Peptoniphilaceae* family (43). In the case of stallion semen, a predominance of *Porphyromonadaceae*, *Peptoniphilaceae*, *Corynebacteriaceae* and *Prevotellaceae* families characterised the microbiome profile of healthy, fertile individuals (138).

A range of technologies are used to characterise microbial communities and the genes that inhabit them. Advances in metagenomic 16S rRNA sequencing technology have provided insights into the microbial genes present in stallion semen (189) and enabled identification of microbiota that have not been identified via culture-based techniques or

MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization-Time Of Flight mass spectrometry) technology (80), which rapidly identifies microorganisms using an accurate high-throughput methodology. The adoption of polymerase chain reaction (PCR) genomic sequencing and 16S rRNA sequencing has associated advantages in identifying the presence of viable and non-viable bacteria – anaerobes and aerobes (207) – thus enabling the identification of lesser known microbes and those which may be difficult to culture (208). Microbial technologies using 16S rRNA have been estimated to identify up to 99% of microbes present in a sample to genus level (209), providing more specific detail about the microbial taxa that inhabit different regions. The PacBio (Pacific Biosciences) DNA processing, which was used in this study, can identify bacteria with 99.9% accuracy by employing circular consensus sequencing (CCS); this single molecule real-time genomic sequencing provides longer (HiFi) read lengths than comparative second-generation sequencing to achieve this level of accuracy.

Environmental factors and nutrition have been shown to influence the intestinal microbiome composition in horses (110, 160, 210-213) and their general health. The environment and nutrition are also known to impact fertility, yet little is known about the composition of the semen microbiota of healthy stallions and in different breeds. To date, no studies have reported the semen microbiome profiles of miniature pony stallions. The aim of this study was to add to the existing knowledge base regarding the composition of the equine seminal microbiota, by identifying bacteria in more detail than previously reported, from phylum to genus, in four miniature stallions (see Figure 2.1).

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS

Four miniature crossbred pony stallions of proven fertility, aged between 2 and 15 years and stabled at an equestrian centre in New South Wales, Australia, were recruited for this study in October 2019. They comprised a cohort of horses that routinely provide semen specimens for scientific purposes. All procedures undertaken in this study were approved by the University of Newcastle Animal Ethics Committee (AEC) and adhere to the

Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (AEC approval number A-2011-122).

The ponies grazed on native grasses in adjacent paddocks and lucerne hay was added to their diet throughout the year. No exercise regime was undertaken other than free paddock roaming, and the ponies were stabled overnight. The body condition score of the ponies was 6–7 on the US Body Condition Score Chart (51).

2.2.2 STUDY DESIGN

Semen samples were collected on the same morning from each pony after a minimum two days' rest. Seminal specimens were prepared and subjected to genomic profiling analysis of bacterial microbial composition, classified by phylum, order, class, family, and genera, for a descriptive analysis.

2.2.3 COLLECTION OF SEMEN SAMPLES

After careful washing of each stallion's prepuce and penis with warm water to remove smegma and other residues, semen collections were undertaken. Semen was collected from the pony stallions using a Missouri artificial vagina (Minitube Australia, Ballarat, VIC) with an in-line gel filter, and a phantom mount. Following semen collection, 250 µL was immediately removed and diluted with 20 µL of DNA Microshield, after which it was stored at −30 °C until analyses.

2.2.4 MICROBIAL DNA PROFILING OF SEMEN MICROBIOTA

ZymoBiomics DNA Miniprep kit (Zymo Research) was used to extract DNA from 150 µL specimens of seminal fluid, having been firstly digested with 5% (v/v) of Proteinase K (Cat. No. D3001-2-5) at 55 °C for 30 min following the manufacturer's instructions. Full-length 16S amplification was performed in preparation of the PacBio semen samples libraries using SMRTBell® Library Preparation and Sequencing Procedure and Checklist (Pacific Biosciences) with 15 ng of the extracted DNA. Initially, PCR was conducted with KAPA HiFi HotStart ReadyMix (Roche), with 20 cycles in 25 µL reactions following protocol cycling conditions. Amplified DNA was indexed using the barcoded F/R Universal Primers Plate

96 v2 (Pacific Biosciences) and PCR products were purified by using AMPure PB (Agencourt Bioscience), in accordance with PacBio's protocol.

Verification of amplicon sizes was carried out with a Fragment Analyser 5200 (Agilent Technologies) and Qubit 4.0 Fluorometer (ThermoFisher Scientific) using the High Sensitivity dsDNA assay. Pooling of barcoded amplicons was performed before the library construction process with the SMRTBell® Express Template Prep Kit 2.0 (Pacific Biosciences). Semen specimens were pooled in groups of 20, then sequenced on separate chips. A PacBio sequel (Pacific Biosciences) sequencing process was used with v3 sequencing chemistry in CCS (Circular Consensus Sequence) collection mode for 20 h on a 1M v3 LR SMRT Cell. Following this, CCS analysis was run prior to demultiplexing and fastq conversion. The CCS analysis algorithm is applied to processes that require closely related molecules of DNA to be distinguished.

2.2.5 TAXONOMICAL CLASSIFICATIONS OF MICROBIOTA

The semen microbiota of the subjects were categorised according to taxonomical classifications of phylum, order, class, family and genus (Figure 2.2).

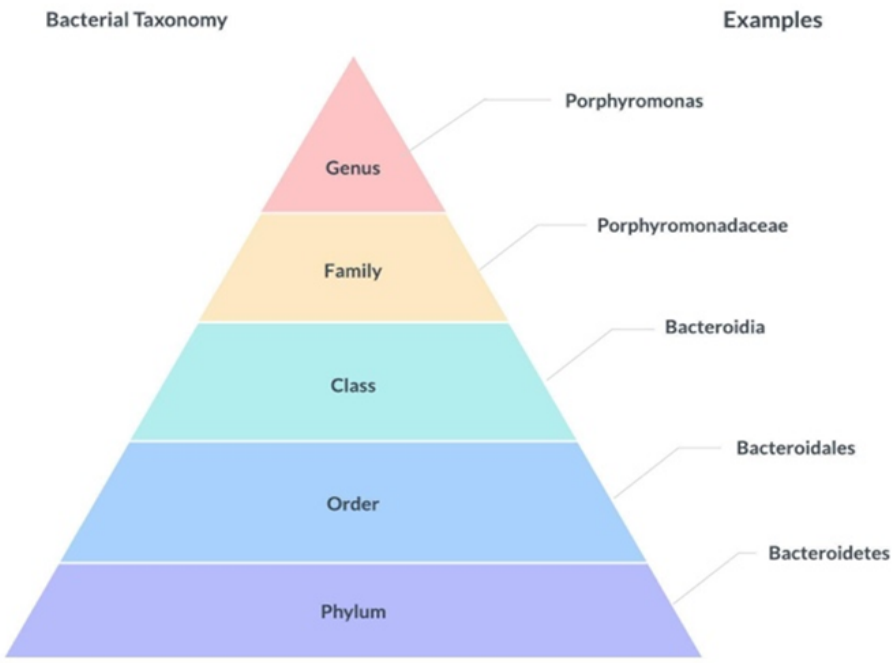

Figure 2.2. Taxonomical ranking of bacteria.

2.2.6 STATISTICAL METHODS

2.2.6.1 BIOINFORMATICS FOR MICROBIOME PROFILING

Prior to data analysis, the quality of the raw reads was checked using the package FastQC (Version 0.11.9). The next-generation microbiome bioinformatics platform QIIME2 (version q2cli version 2020.8.0) was used to analyse the amplicon data and further generation of Amplicon Sequence Variants (ASVs) was conducted from the raw amplicon data, using the algorithm DADA2 (version 1.19.2). Initially, the primer sequences using PacBio primers, targeting full-length 16S rRNA's, were removed . An average of 80-95 % of the raw reads were retained across the samples. This was followed by inspection of the length distribution of the reads. Most sequences were found to be in the expected range of approximately 1400 to 1550 bp. A filter was applied to retain the sequences between 1000 to 1600 bp length. This was followed by identification of ASVs using the DADA2 algorithm. An additional filter was then applied, which retained only those ASVs found in a minimum of two samples in the analysis. This assisted in identifying and removing potential false positives due sequencing issues or other factors in the ASV identification process.The ASVs display advantages over the more traditional approach using operational taxonomic units (OTUs) as they represent the exact amplicon sequences without coarse graining of taxa into OTUs and can resolve even single nucleotide base differences. DADA2-formatted training fasta files were derived from the Silva Project's version 128 release and used for taxonomy assignment. Three output files were generated using DADA2. These included the sequence feature count tables, the taxonomy identification file per ASV and a fasta file containing sequences of all ASVs. These files were exported in a phyloseq format for further analysis using QIIME2. These files along with the sample metadata files were used as input for different modules in the QIIME2 workflow.

Data produced by QIIME 2 exists as QIIME 2 artifacts, which contain data and metadata. The feature tables imported from DADA2 were filtered to retain the features, which were observed in at least two samples.

2.2.6.2 DATA ANALYSIS

This cross-sectional data sample was prepared in Microsoft Excel v16.67 (2022). ASV count data was aggregated first at the classification of phylum and sorted from highest to lowest frequency. This method was applied again for each of the remaining taxonomical ranks, namely order, class, family, and genera. The top ten organisms identified in each taxon for each pony were reported, with the addition of an overall microbial profile.

2.3 RESULTS

The principal results of this study indicate specific microbiota predominate in the semen of miniature stallion ponies within each taxonomical ranking, with some inter-individual variations.

The microbiota identified in the semen of the four equine subjects (Pony B, C, P, Z) are presented in Figures 2.3 to 2.7 by percentage abundance and taxonomical classifications of phylum, order, class, family, and genera. Overall, Firmicutes and Bacteroidetes phyla predominated (76%), followed by Proteobacteria (15%). Bacteroidales, Clostridiales and Cardiobacteriales predominated in the microbial rank of order (86%). For class, Bacteroidia, Clostridia and Gammaproteobacteria (87%) predominated, and for family, *Porphyromonadaceae*, *Family_XI* and *Cardiobacteriaceae* (62%) predominated. At the level of genus, 80% of the abundance comprised seven genera – *Porphyromonas*, *Suttonella*, *Peptoniphilus*, *Fastidiosipila*, *Ezakiella*, *Petrimonas* and taxon undescribed (NA) that are yet to named.

2.3.1 Phylum

The nine phyla predominating in the semen samples of four miniature stallions were Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, Spirochaete, Actinobacteria, Tenericutes, Synergistetes, and Euglenozoa, and those less frequently represented were categorised as other (Figure 2.3). Overall, the phyla Bacteroidetes and Firmicutes were most represented in this small cohort, comprising 76%, and Proteobacteria was the third

most abundant phylum, comprising another 15% of the phyla identified. Less than 1% of those present were taxon undescribed (NA).

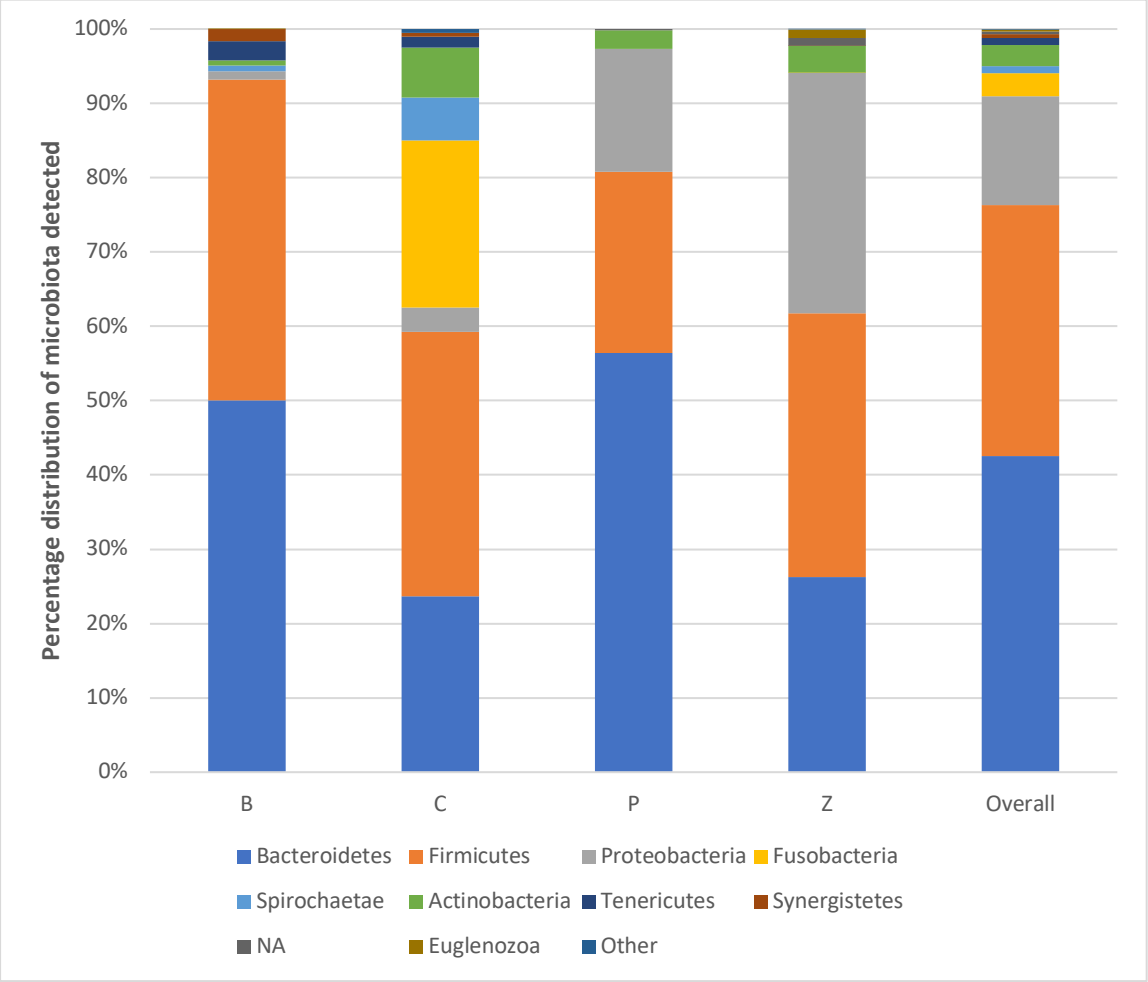

Figure 2.3. Composition of semen microbiota by taxonomical classification of phylum for the four miniature pony stallions individually and overall. (NA - Taxon undescribed)

2.3.2 Order

Within the taxonomical classification of order, Bacteroidales, Clostridiales, Cardiobacteriales, Fusobacteriales, Bradymonadales, Corynebacteriales, Propionibacteriales, Spirochaetales, and Mycoplasmatales were most abundant (Fig. 2.4). The three predominating order were Bacteroidales, Clostridiales and Cardiobacteriales, accounting for 86% of the abundance distribution.

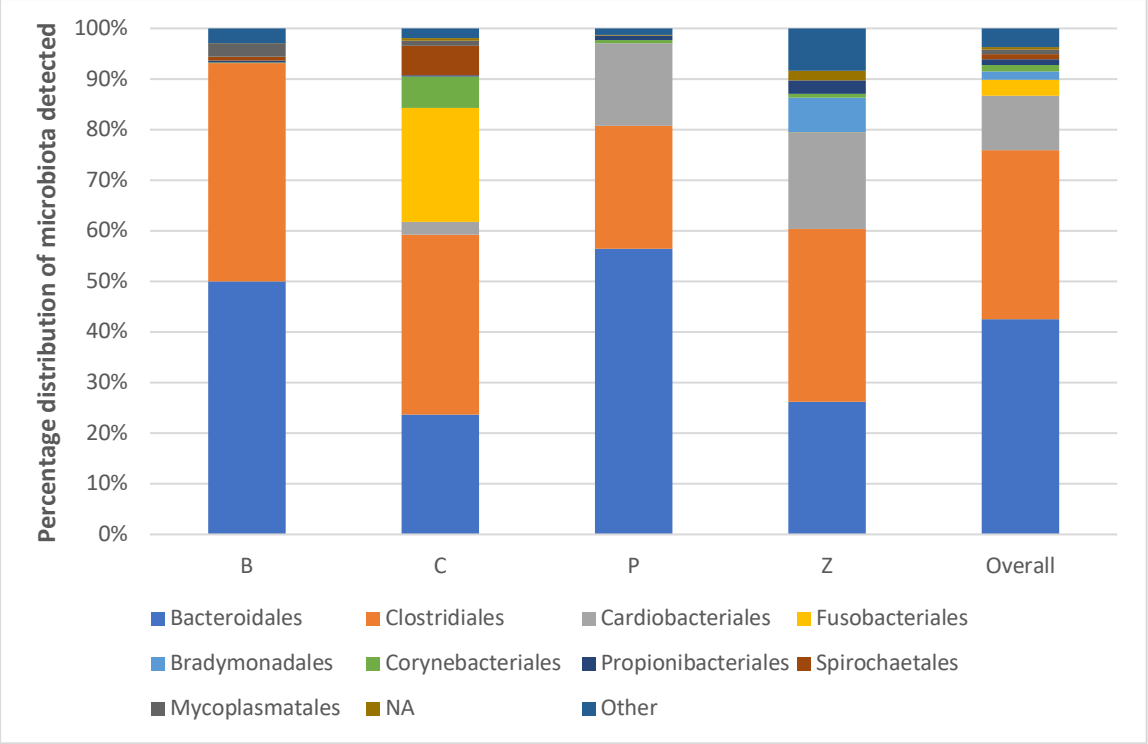

Figure 2.4. Composition of semen microbiota by taxonomical classification of order for the four miniature pony stallions individually and overall. (NA - Taxon undescribed)

2.3.3 Class

Within the taxonomic classification of microbial class, Bacteroidia, Clostridia, Gammaproteobacteria, Fusobacteriia, Actinobacteria, Deltaproteobacteria, Spirochaetes, Mollicutes, Betaproteobacteria and Alphaproteobacteria were the most abundant, with 87% comprised by Bacteroidia, Clostridia and Gammaproteobacteria (Figure 2.5).

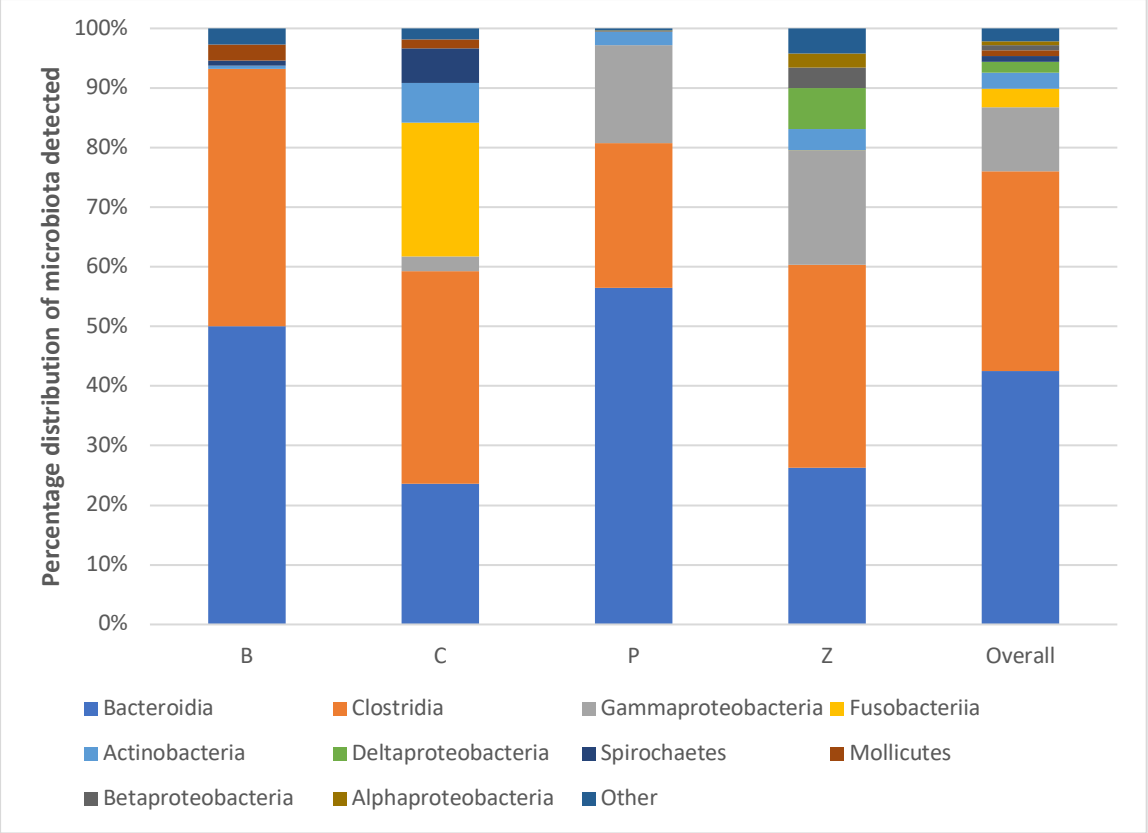

Figure 2.5. Composition of semen microbiota by taxonomical classification of class for the four miniature pony stallions individually and overall.

2.3.4 Family

The most abundant microbial families were *Porphyromonadaceae, Family_XI, Cardiobacteriaceae, Bacteroidales_BS11_gut_group, Ruminococcaceae, Fusobacteriaceae, Peptostreptococcaceae, Rikenellaceae,* and *Corynebacteriaceae*. Of these, *Porphyromonadaceae*, *Family_XI* (Family XI *Incertae sedis*) and *Cardiobacteriaceae* predominated, comprising 62% (Figure 2.6).

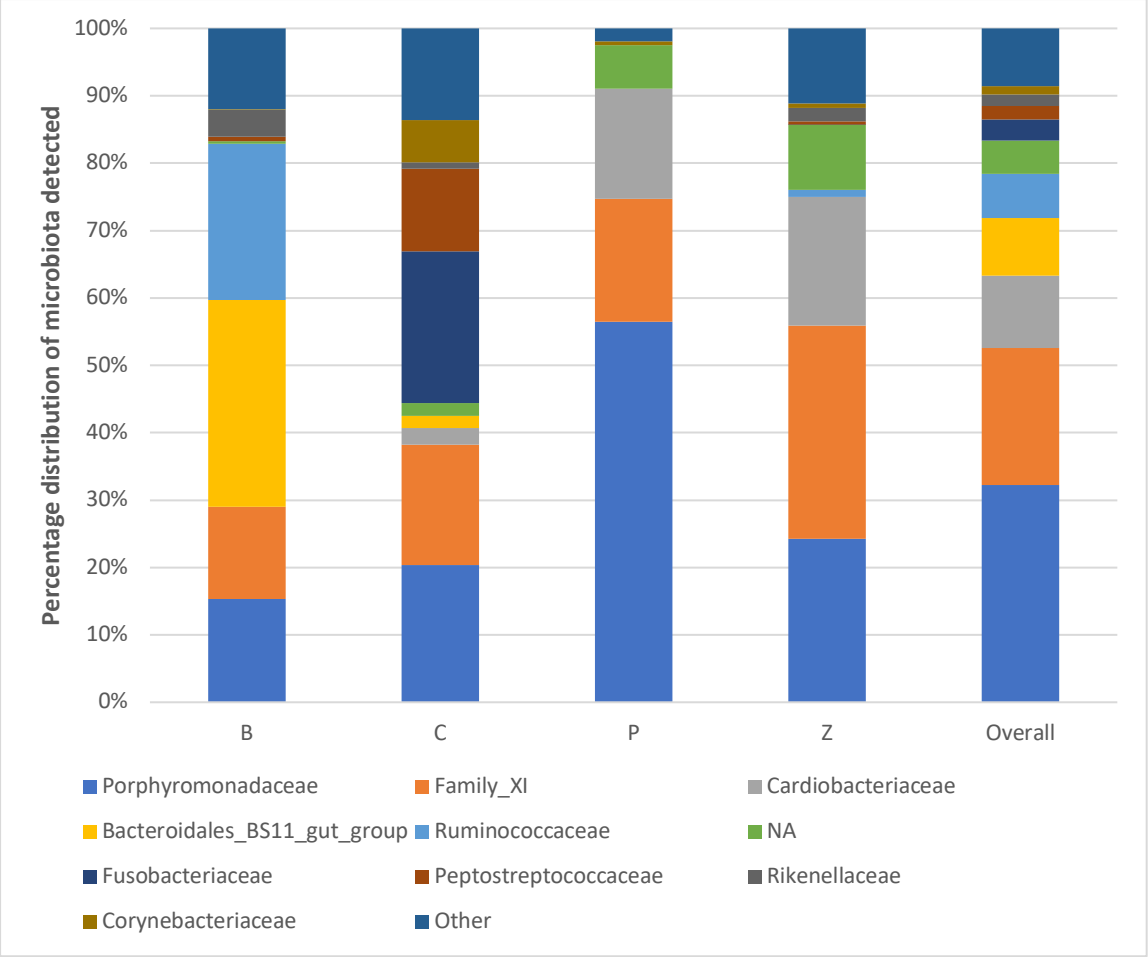

Figure 2.6. Composition of semen microbiota by taxonomical classification of family for the four miniature pony stallions individually and combined. (NA - Taxon undescribed)

2.3.5 Genus

Within the taxonomical classification of microbial genera, *Porphyromonas, Suttonella, Peptoniphilus, Fastidiosipila, Ezakiella, Petrimonas, Fusobacterium, Gallicola,* and *Finegoldia* were the most abundant genera identified in the semen. Of these, *Porphyromonas, Suttonella, Peptoniphilus, Fastidiosipila, Ezakiella, Petrimonas* and undescribed taxon (NA) predominated (Figure 2.7). In terms of microbiota represented in individual ponies, some differences were observed in the abundance at the family and genus level classifications. For example, Pony B had greater abundance of *Fastidiosipila* than the other ponies, Pony C had greater abundance of *Fusobacterium*, Pony P had greater abundance of *Suttonella* and *Pony Z* had greater abundance of *Ezakiella* genus.

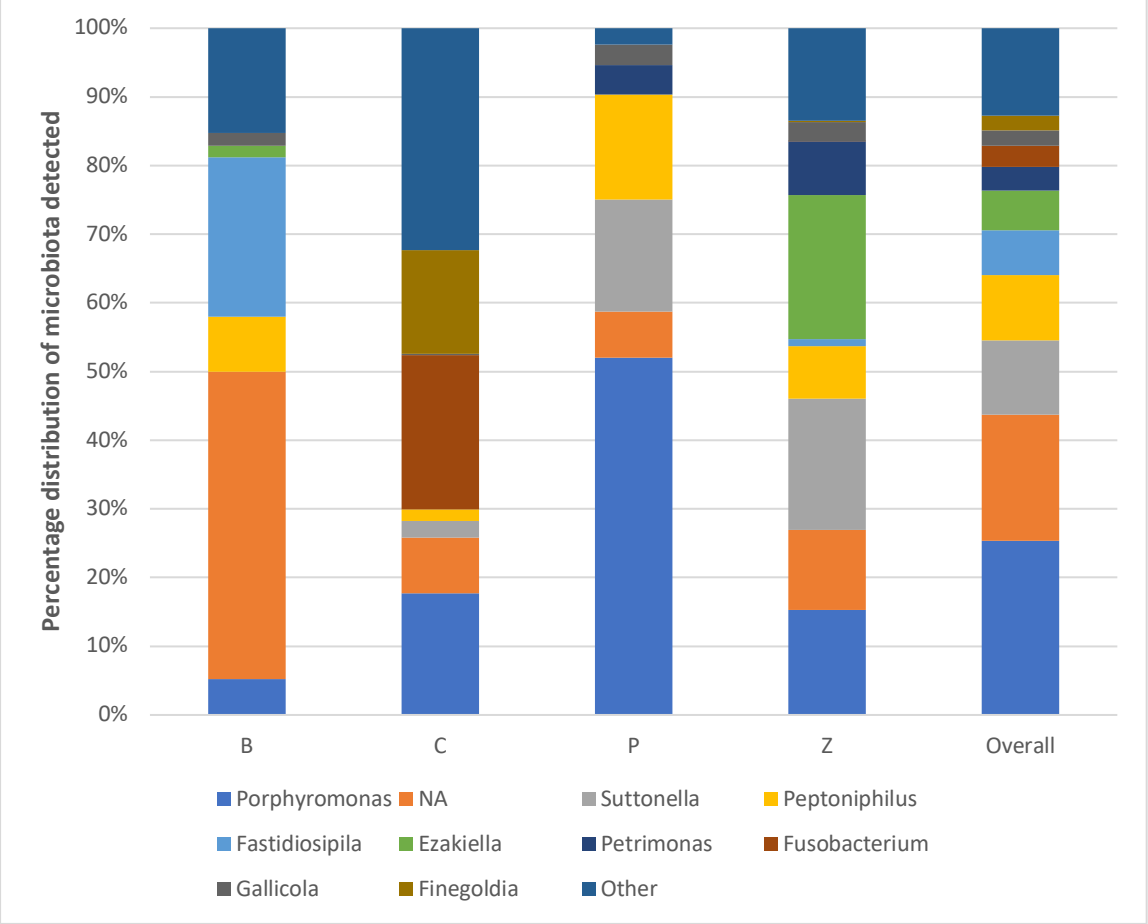

Figure 2.7. Composition of semen microbiota by taxonomical classification of genus for the four miniature pony stallions individually and combined. (NA - Taxon undescribed)

2.3.6 SPERM PARAMETERS IN RELATION TO SEMEN MICROBIOME CHARACTERISTICS

Table 2.1 presents a biomarkers of semen quality, including total sperm count, motility and morphology, as well as key microbiome measures. Due to the small sample size, comparison between the semen microbiome and microbial data was not conducted.

PONY	Total Count x10^6	% Motile	% Progressive Motility	% Normal Morphology	% DNA Intact	% Total 4HNE Positive	% Live MSR Positive	Faith_pd/ Richness	Evenness	Shannon Diversity Index
B	1.84	3.88	17.9	53	82.7	48.4	10.3	13.50	0.66	3.89
C	5.05	69.05	22.6	34.5	93.85	66.7	12.3	15.87	0.72	4.39
P	4.675	70.05	37.15	59.5	79.25	62.7	17.2	31.79	0.73	3.92
Z	17.3	74.8	26.95	62	88.75	35.8	15.5	79.92	0.70	4.40

Table 2.1 Six semen quality biomarkers and three semen microbiome measures of four miniature pony stallions

2.4 DISCUSSION

This exploratory study has described the microbial composition of the semen microbiome of healthy, fertile, miniature stallion ponies within five taxonomical classifications. In this study nine variable regions of the 16S rRNA were analysed gene across different taxa, using ZymoBiomics DNA Miniprep kit, a process reported to increase the accuracy of identification by 100-fold over single region or short reads of hypervariable regions (214). By comparison, Quiñones-Pérez et al (138) and Al-Kass et al (189) used the Ion 16STM Metagenomics Kit to examine only five hypervariable regions of the same gene to construct their seminal bacterial libraries. Specifically, the determination of Amplicon Sequence Variants (ASV's) via the use of the QIIME2 bioinformatics pipeline in this study, in preference to less accurate operational taxonomic units (OTU's), was important for achieving the best resolution for bacterial identification (215). Also, importantly, QIIME2 is considered a gold standard in bioinformatic analysis, hence its choice for this thesis project (216). The overall findings indicate that specific microbiota predominate in the semen of healthy miniature stallion ponies within each taxonomical ranking, with some inter-individual variations. The microbial phylum and families present in equine semen

have previously been reported (138, 189) and here we present all five taxonomical levels. Abundance of certain genera of bacteria present in human semen have been observed to correlate with abnormal sperm motility and morphology (40). Table 2.1 presents a biomarkers of semen quality, including total sperm count, motility and morphology, as well as key microbiome measures. Due to the small sample size, comparison between the semen microbiome and microbial data was not conducted. To date, not enough is known about the influence the semen microbiome of stallions might exert over sperm quality, such as the impact of pro-inflammatory bacteria, and since recognising and managing factors impacting stallion fertility is essential for optimising breeding outcomes, we believe it is important to explore this ecological niche. It must also be stated that sperm parameters of healthy miniature pony stallions are comparative to those of full-size stallions, rendering this study relevant to other breeds.

2.4.1 SEMEN MICROBIAL PHYLA AND FAMILIES

Research conducted by Quiñones-Pérez and colleagues evaluated the phylum and families present in semen in a cohort of 12 healthy Arab, Anglo-Arab and Andalusian stallions (aged 7–24 years) from the Spanish Army's breeding centre in Seville, Spain (43). Like Quiñones-Pérez et al. (217), we found that Bacteroidetes and Firmicutes predominated in the equine semen. However, Actinobacteria was the third most abundant phyla present in the semen of the Spanish stallions, whereas Proteobacteria was the third most abundant phyla in the miniature ponies' semen. Understanding the significance of the dominant representation of the Proteobacteria in our semen specimens is difficult, given that it has not been previously reported. However, what is known is that these Gram negative bacteria play an important metabolic, energy-generating role in the equine intestinal microbiome (218). Further, Proteobacteria have also been reported as being more represented in the gastrointestinal tract of horses with equine grass sickness (110) and colitis (219). Somewhat contrary to this, in a separate study, Proteobacteria colonies were reported to be less represented in the faeces of horses affected by diarrhoea (218). The presence of Proteobacteria in the semen was not associated with these conditions in the miniature ponies studied, which were of good health prior to the specimen collection date, with no reports of diarrhoea or other illness.

Interestingly, many of the less abundant phyla including Fusobacteria, Spirochaetes, Synergestes, Tenericutes and Chloroflexi were present in similar amounts in both studies. Such similarities signal there may be a characteristic semen microbiome of stallions.

At the taxonomic classification of family, Quiñones-Pérez et al. identified 69 families in their stallions' semen, of which only 22 families were present in proportions greater than 1% and only nine were common to all samples (138). The predominate microbial family represented in the Spanish stallion's semen was *Porphyromonadaceae*, which was similar in preponderance to that in our miniature ponies. Of interest to fertility research, *Porphyromonadaceae* is reported as a predominant family in fertile stallion semen (138), where it is found to be more dominant than in human semen (41). In contrast, the Spanish stallions had a much greater abundance of *Corynebacteriaceae* in the semen compared to the miniatures, who had more *Family XI* present. The *Corynebacteriaceae* family, which has also been associated with fertility in stallions, was not highly represented in our cohort of miniature ponies, although they had proven normal fertility status. In addition, *Corynebacteriaceae* family are found in low abundance on equine skin (125), as well as in semen, which might suggest a degree of cross-contamination of this bacterium that occurs during specimen collection.

The differences identified in phylum and family between the studies are not readily attributable to the chosen methods, as in many regards the methods were similar, employing similar collection processes during the spring season to obtain one semen specimen. However, the genomic sequencing techniques and bio-informatics pipelines employed by the studies were different, which may have influenced the respective findings. Differences between the findings of the Spanish study and those reported here are more likely to relate to breed, diet, geographical location and associated environmental factors. The stallions studied by Quiñones-Pérez et al. in Spain were fed alfalfa hay and oats, rather than lucerne hay, which may have produced a different semen microbial profile to that of our stallions, although it could be argued that dietary variations are more likely to be associated with differences observed in the gastrointestinal rather than extraintestinal microbial communities. The faecal microbiome of ponies and horses in good health is known to be impacted by environmental factors, such as air exposure,

farm location (region and country), season and access to pasture (160). Despite the anatomical proximity, there is minimal overlap reported in the literature between the bacterial phyla found in equine faeces and those identified in equine semen.

2.4.2 SEMEN MICROBIAL GENERA

The aforementioned factors may also explain the differences in genera identified in equine semen by Al-Kass et al. compared to our findings (189). These researchers examined the microbial composition of semen specimens from seven healthy stallions (aged 7 to 17 years) of unspecified breed on a commercial stud in Stockholm, Sweden, characterising the microbiota from one ejaculate per animal to genus level (189). They identified almost twice as many genera in the semen of their stallions in comparison to those discovered in our cohort, with only *Porphyromonas* and *Peptoniphilus* being common to both cohorts. The four most common genera found in the Swedish study were *Porphyromonas, Corynebacterium, Finegoldia* and *Peptoniphilus*, followed by *Mobiluncus, Chondromyces, Suttonella, Treponema, Actinetobacter, Campylobacter* and a group of 'other'. In comparison, the four most abundant genera in the semen of our miniature ponies were *Porphyromonas, Suttonella* and *Peptoniphilus* followed by *Fastidiosipila, Ezakiella, Petrimonas, Fusobacterium, Gallicola, Finegoldia* and 'other'. No *Ureaplasma, Spirochaeta, Prevotellaceae, Fibrobacter, Acinetobacter, Chondromyces,* or *Clostridium* were identified in our miniature ponies. Like the Al-Kass study, our miniature ponies displayed predominantly *Porphyromonas* genus, an organism also common to healthy human semen (41, 220). Consistent with other equine studies, we did not find any *Lactobacillus* genus in our samples, a bacterium which has been associated with good sperm quality in humans (40).

The methodology and technology (16S rRNA) employed in these two studies were similar in many regards; that is, specimens were collected in early spring from one ejaculate, and Al-Kass et al. used the same regular collection routine and manner (using a Missouri AV without mare contact) as we did for the ponies in our study. Although the stallions of the Swedish study were engaged in an artificial insemination program, the procedure for collecting and storing each aliquot of semen before its transport to the laboratory for

genomic sequencing was identical to that employed by our Australian stallion handlers, hence one would not expect to find an effect of stallion management on semen microbiome composition to influence the results of these two studies.

Soil bacteria may contact the stallion's urogenital tract from its environment; considering that a miniature pony stallion's erect penis can more easily contact the ground than that of a full-sized stallion, this can give rise to extrinsic contamination and differences in microbes detected. *Petrimonas* genus, found in the semen of our cohort of miniature pony stallions, resides in soil, where it is able to effectively degrade hazardous compounds (193) to innocuous acetic acid and carbon dioxide (221). *Fastidiosipila*, a Gram-positive anaerobe previously unrecognised in equine semen, identified in the miniature stallions, has also been identified in stored waste of landfill (222), hence it may have been acquired from the soil by these ponies. These soil-dwelling organisms were not isolated by Al-Kass et al. or Guo et al. from their full-size breeds. *Gallicola* genus was also unique in the semen of the miniature stallions and about which very little exists in the literature, other than they are Gram-positive, strictly anaerobic cocci associated with dark fermentation of food waste (223) and may be a faecal contaminant of ground water sourced from chicken manure (224).

2.4.3 THE SEMINOVAGINAL MICROBIOME

The influence of the vaginal microbiome on the composition of the semen microbiome is important to consider. The stallion urethral and possibly semen microbiomes may be contaminated by the mare's vaginal microbiota following mating, and although there are no detailed descriptions of a shared complementary equine seminovaginal microbiome, one study has found microbiome network links between semi-feral Welsh Mountain pony stallions and mares (225). *Porphyromonas* is the most abundant genus in Arabian mares' vaginal microbiome (approx. 14–17%) and Bacteroidales is the highest abundance by order (approx. 31%) (137). By comparison, our stallions' semen contained 25.4% *Porphyromonas* and 42.5% Bacteroidales, although they had not had genital contact with mares, so their microbiome could not have been influenced by a mare's. At phylum level the core species of the vaginal microbiome of the mare are comparative in ranking to

those phyla found in miniature stallion semen (Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria) (137).

2.4.4 FUTURE RESEARCH CONSIDERATION: CRYOPRESERVATION OF EQUINE SEMEN

It could be speculated that by optimising the microbial composition of semen with stallion management and nutrition, there exists potential to improve the results of cryopreservation of semen, protecting sperm against oxidative damage from the freezing-thawing process. In human research into the semen microbiome, the presence of bacteria such as *Brevundimonas* have been observed to reduce sperm oxidative stress, while status of chromatic protamination and appearance of double-stranded breaks in sperm DNA were reduced in the presence of *Paenibacillus, Actinomycetaceae* and *Ralstonia* bacteria (226). The authors of this mentioned study postulate that enzymatic and metabolomic activity of these bacterial genera might potentially conserve sperm DNA integrity, hence it may be possible that similar sperm DNA protection may be conferred by seminal probiotics upon frozen sperm during the thawing process.

2.4.5 LIMITATIONS OF THIS STUDY

Only one breed of stallion was recruited for this study, with single semen samples collected and profiled for each miniature stallion, thus reducing the generalisability of the findings. Due to the sample size, detailed statistical analysis and comparison with various measures of sperm quality were not possible, thus associations between the composition of the semen microbiome and sperm quality could not be made. The detailed characteristics of the semen microbiome in miniature stallions may not be generalisable but provides preliminary data to inform such research.

2.5 CONCLUSION

This comprehensive analysis of the semen microbiome of a cohort of healthy, fertile, miniature pony stallions, using full-length metagenomic sequencing, has expanded upon the current knowledge of this domain in this breed. The microbial composition of the

semen of the stallions studied varied between horses, with some bacteria predominating and common to all ponies, shared at various taxonomic classification ranks. The role of shared and unique microbiota in relation to the general health of the horse and fertility warrants further research and these findings could assist other researchers exploring the semen microbiome of miniature stallions. More specifically, the findings of this study can be used to inform future research, exploring the relationship between semen microbial composition and fertility of stallions particularly the richness, evenness or diversity of semen microbiome profiles and the potential for these characteristics to influence sperm quality biomarkers. Future studies are encouraged to also explore semen microbiome characteristics of stallions with disorders and diseases, such as insulin resistance and obesity, those receiving antibiotic therapy and those with inflammatory diseases.

CHAPTER 3: REVIEW ARTICLE "THE SAFETY, TOLERABILITY AND EFFICACY OF PROBIOTIC BACTERIA FOR EQUINE USE"

Review published in the Journal of Equine Veterinary Science on February 11, 2021.

Title: "The Safety, Tolerability and Efficacy of Probiotic Bacteria for Equine Use"

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Abstract

Background

Probiotic bacteria are used widely as nutritional supplements and treatment interventions in the management of livestock and companion animals. The aim of this review is to summarise the current evidence reporting on the safety, tolerability and efficacy of probiotic bacteria use in horses.

Methods

An online search of five databases for studies reporting on the use of probiotic bacteria use in horses which were either healthy or had a gastrointestinal or extra-intestinal disease was conducted.

Results

A total of 18 articles were eligible for full review. No clear benefits were identified to support supplementation of equids with probiotic bacteria to improve starch and fibre digestion, nor for the treatment of colic or prevention of salmonellosis. Conflicting results were seen with the management of scouring in neonatal foals. Exacerbation of diarrhoea and additional adverse events were reported in response to the administration of high doses of novel probiotic bacterial species. Probiotic bacteria given to exercising horses, improved aerobic fitness and stamina. The majority of probiotic bacterial species used in equine studies are bacterial species commonly used for human consumption and indigenous to the human gastrointestinal microbiota.

Conclusions

There is a paucity of evidence to support the use of probiotic bacteria in the health maintenance and disease management of horses. While there are unclear and conflicting results associated with probiotic bacteria use for gastrointestinal conditions in both horses and foals, the administration of multi-strain bacterial formulations to increase stamina in exercising horses shows promise.

3.1 INTRODUCTION

In humans, the composition and diversity of the gastrointestinal [GI] microbiota plays an important role in intestinal and extra-intestinal health and disease (227), including chronic inflammation (185), cardiovascular disease (228, 229), metabolic syndrome (230-232), depression and anxiety (233) and hypersensitivity conditions (234) . Much less is known about the role of the microbiome in the equine species. In horses with colitis, the composition of the faecal microbiome has been found to differ to that of healthy horses (219), though the literature pertaining to extra-intestinal conditions associated with the composition and diversity of the GI equine microbiome is otherwise limited (129, 235).

Functions attributed to the indigenous gastrointestinal microbiota of humans include enzyme and vitamin synthesis, hormone metabolism, competitive inhibition of pathogens for intestinal receptor sites, secretion of the antimicrobial factors bacteriocin and defensin, and immuno-modulation (236-238). The knowledge of the functions of these indigenous bacteria has led to an exponential growth in research exploring their possible therapeutic roles as probiotic medicines (239). Probiotics are defined by the World Health Organisation [WHO] as *'live microorganisms which when administered in adequate amounts confer a health benefit on the host'* (240). To date, an extensive number of bacterial species and sub-species from the *lactobacillus* and *bifidobacterium* genera have been evaluated (241). *Streptococcus thermophilus* and *Escherichia coli* strains have also received research attention in human health (240). The probiotic yeast, *Saccharomyces cerevisiae and boulardii* have been used to supplement feed in horses with beneficial outcomes, such as improved fibre digestibility (242) and reducing the duration of hospitalisation for horses with acute enterocolitis (197) respectively. However, in Australia, commercial probiotic yeast supplements for horses are not widely available.

Publication dates would indicate microbiome and probiotic research in equine health and disease is lagging approximately a decade behind that of human microbiome and probiotic research. Veterinary medicine has predominantly focussed on research using probiotics in companion and livestock animals (243, 244), the objective of the latter being to improve food conversion ratios by microbiome modification (141, 244, 245).

Equestrian breeding and sports organisations across the world are seeking solutions for common equine health problems. Scouring [diarrhoea] affects almost 80% of neonatal foals (196), threatening healthy growth and development, as well as survival of these young horses. Thoroughbred horses appear to have complex susceptibilities to developing diarrhoea in response to mental and physical stressors that can lead to inflammatory intestinal conditions, including colitis (196) and gastritis (246). Intestinal inflammation most commonly affects horses following periods of prolonged stress, as experienced with racing, transportation, surgical treatment or administration of antibiotics or anthelmintic agents (247). As the use of pharmaceuticals to manage diarrhoea in the general horse population is associated with adverse events, from mild reactions such as anorexia, skin wheals and oedema, to serious ones such as autoimmune blood dyscrasias and nephrotoxicity, there is a need for the development of safer and more natural treatment alternatives (196).

Athletic performance is the primary metric for the success of thoroughbred horses, and in addition to using selective breeding to optimise performance traits (248), the interest in enhancing stamina and racing performance via probiotic supplementation is increasing (198, 199). Indeed, it has been proposed that many of the current equine pathologies that veterinarians face, such as laminitis, colic, gastric ulcer syndrome, colitis, and neonatal diarrhoea may in future be addressed by pre- and probiotic therapy to optimise intestinal microbial balance and both prevent and treat such conditions (110). Research into the microbiome of the reproductive tract of the mare and how these organisms may impact fertility outcomes (249) and probiotic potential (123) is currently gaining interest, however equivalent research has not been conducted for the reproductive tract of the stallion, nor has the potential application of probiotics for enhancing equine fertility been explored in either sex. Despite the widespread use of probiotic bacteria supplements in veterinary practice, a comprehensive summary of the research reporting on the efficacy and safety of probiotic use in the equine population has not been conducted to date.

The aim of this review is to summarise the current research reporting on the use of probiotic bacteria supplementation in horses, with particular emphasis upon their safety [no significant adverse effects], tolerability [minimal side effects] and efficacy, to enable

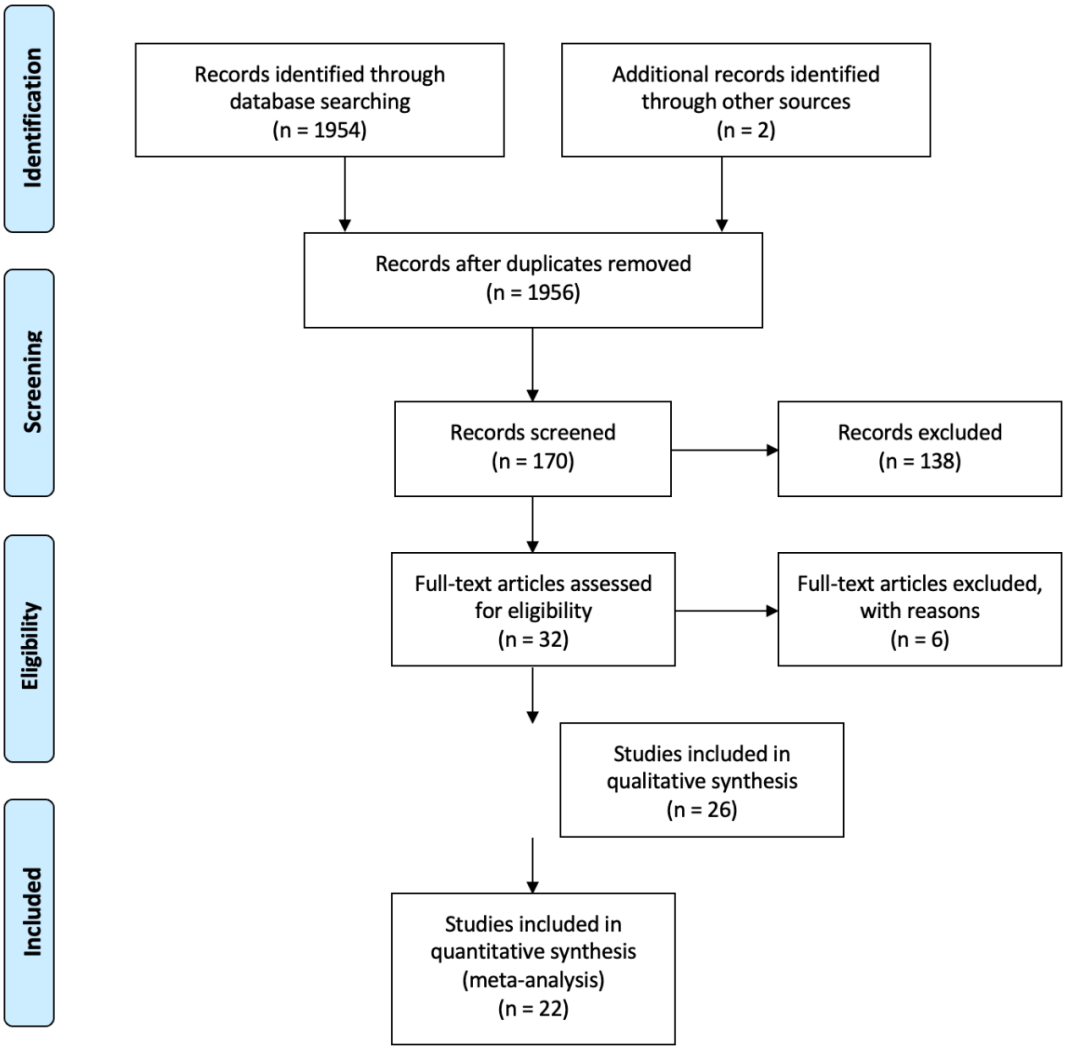
veterinarians and horse owners to carefully consider their use for both preventative and therapeutic purposes.

3.2 METHODOLOGY

3.2.1 LITERATURE SEARCH

To identify relevant literature published from 1954 to 2020, a search was conducted in Medline, Pubmed, CINAHL, Embase and Web of Science using the MESH terms 'probiotics' and 'horses'. The search was conducted in consultation with an academic librarian to ensure the MESH headings ‘Horses’ and ‘Probiotics’ captured all types of horses and probiotic bacteria. A search of Google Scholar and citation chaining was also undertaken to identify additional studies that may have been relevant to our objectives. The search was conducted between 13/1/2020 and 10/2/2020.

Figure 3.1: PRISMA flow diagram of selection process for publications for this review



3.2.2 STUDY SELECTION CRITERIA

The inclusion criteria applied for the selection of studies, included being published in English, available as full-text article, reported on the use of probiotic bacteria supplementation in both male and female horses of any breed and age which were either healthy or had a gastrointestinal or extra intestinal condition. Review articles and studies reporting on probiotic bacteria supplement use in other animals were not included nor were studies reporting on single probiotic yeast formulations. Studies reporting on the equine microbiome outside of the context of a probiotic intervention were not included.

3.2.3 DATA EXTRACTION

One author screened the articles for suitability for inclusion [GC]. Two authors agreed on the final papers for inclusion [GC and JH]. Studies that met the search criteria were downloaded into EndNote X9 citation software [Clarivate Analytics], and the data tabulated: first author, publication year, country in which conducted, type of horses studied, probiotic used, duration of study, and results [safety and tolerability, efficacy].

3.3 RESULTS AND DISCUSSION

A total of 1955 articles were identified from the five databases and 2 articles were identified from other sources after the removal of duplicates. After screening 1957 abstracts and titles, 171 records were selected. Of these, 33 full-text articles were further assessed for eligibility, 6 articles were excluded due to not meeting the inclusion criteria, then from 27 studies included in a qualitative synthesis, a total of 18 articles were selected for full review (Figure 3.1).

Of the final 18 included articles, all studied probiotic bacteria interventions in horses, and none specifically evaluated the use of probiotics in stallions; 3 studies involved geldings and 2 male horses only; 9 studies involved foals; 3 studies involved thoroughbreds; 16 studies involved healthy horses, and 1 study involved horses diagnosed with gastrointestinal disease (colic) and 1 study focused on non-GI disease (See Table 3.1).

Multi-strain probiotic formulations were administered in 13 studies. *Lactobacillus* species were assessed for efficacy in all 18 trials, *Bifidobacteria sp*. in 5 studies, *Enterococcus sp.* in 3 studies, *Streptococcus sp.* in 2 trials, one *Bacillus species* in one paper and 2 yeast species were included in a multi-strain formulation in one study. Doses of organisms ranged from 1 x 10^{3} CFU/g for neonatal foals [daily dose not specified] to 1.8 x 10^{13} CFU/day to exercising male Arab horses. The duration of studies varied from a single dose to 58 days. Safety data was reported in 14 studies.

Table 3.1: Publications selected for review of bacterial probiotics for equine use

First author, publication year, country of origin	**Study cohort – sex, age, breed, health status**	**Probiotic species, strain, dose**	**Duration of Study**	**Safety & Tolerability**	**Efficacy**
Weese, 2003, Canada.	21 clinically healthy, adult standardbred horses. 14 clinically healthy, pony foals, ranging from 1 to 3 days of age.	*L. rhamnosus* GG at dosages of 1 x 10^9 CFU/50kg body weight (BW)/day (group 1, *n*=7), 1x10^{10} CFU/50kgBW/day (group2, *n*=7), or 5 x 10^{10} CFU/50kg BW/day (group 3, *n* = 7).	Treated 1 x daily for 5 days. Faecal samples collected for up to 15 days after initial dosing.	No adverse effects.	Low colonisation in adult horses, more efficacious in foals (colonisation up to 9 days post-treatment).
Ward, 2004, USA.	130 hospitalised horses with non-gastrointestinal disease.	Probios Equine One Oral Gel containing 10^6 CFU/ g of *viable L. plantarum, L. casei, L acidophilus* and *E. faecium* Dose: 30 x 10^6 CFU/day.	Treated on admission and on days 1, 3, and 5.	Safety data not reported.	Faecal *Salmonella* shedding reduced by 65%.
Urubschurov, 2019, Germany.	34 healthy newborn Warmblood foals.	*E. faecium DSM 7134* Dose: 1.05 × 10^9 CFU/day *L. rhamnosus DSM 7133*) Dose: 4.50 × 10^8 CFU/day.	Treated daily for 14 days.	Safety data not reported.	No immediate impact on faecal microbial composition.
Weese, 2005, Canada.	153 newborn foals.	*L pentosus WE7* Dose: 2 x 10^{11} CFU/day.	Treated orally for 7 days, monitored for 14 days.	Depression, anorexia, and colic requiring treatment. Probiotic-treated foals had more days of diarrhoea	Promising in vitro properties of *L pentosus WE7* did not translate to in vivo benefits.

				compared to control group.	
John, 2015, Germany.	25 healthy newborn foals.	*B. cereus var. toyoi,* dosages : 5 x 10^8 CFU/day (n=7) 2 x 10^9 CFU/day (n=10) Control: 10 ml isotonic saline (n=8).	Treated 1 x daily for 58 days.	88% foals in study developed diarrhoea (high dose 10/10, low dose 5/7, placebo 7/8).	No effect on incidence of diarrhoea and health status in the foals. No alteration of intestinal microbiome.
Weese, 2003, Canada.	21 heathy adult horses, 14 healthy foals.	*L. rhamnosus strain GG* Adult group 1,7: 1x10^9 CFU/50kg BW/day; group 2,7: 1x10^{10} CFU/50kg BW/day; group 3,7: 5x10^9 CFU/50kg BW/day. Foal group 1,7: 2x10^{10} CFU/50kg BW/day; group 2,7: 1x10^{11} CFU/50kg BW/day.	Treated daily for 5 days.	No adverse effects were observed in any animal.	*L. rhamnosus* GG appeared transiently in faeces of adult horses; Intestinal *LGG* levels were higher in both foal groups compared to adult horses.
Laghi, 2018, Italy.	10 healthy exercising Standardbred horses (4 male, 6 female, median 6.5 years age).	Slab51 probiotic mixture (Sivoy®) 8 live strains: *L. acidophilus* DSM32241, *L. plantarum* DSM32244, *L casei* DSM 32243, *L. helveticus* DSM 32242, *L. brevis* DSM27961, *Strep. thermophilus* DSM 32245, *B. lactis* DSM 32246. DOSE: 2×10^{11}CFU/dose = 4×10^{11}CFU/day.	Oral probiotic formula supplementation given twice daily 21 days x 4 training periods.	No adverse reaction was observed during the trial.	Reduced post-exercise blood lactate concentration.

Zavistanaviciute, 2019, Lithuania.	34 healthy exercising male Arab horses.	*L. plantarum LUHS135* and *L. paracasei LUHS244* strains 8.0 – 9.0 x 10^{10} CFU/ml) DOSE: 1.6 – 1.8 x 10^{12} CFU/day.	Dosed with 200 mls probiotic mixture for 30 days.	No adverse reaction was observed during the trial. All horses maintained body weight and condition.	Increased HgO2 and blood O2 saturation, decreased blood lactate concentrations.
Schoster, 2015, Denmark.	72 healthy neonatal foals 94% Thoroughbreds 6% Standardbreds 36% female, 64% male.	*L. rhamnosus SP1, L. rhamnosus LRH19, L. plantarum LPAL, L. plantarum BG112,* dose 1 x 10^{9} CFU/day of each and *B. animalis lactis* strain 1 x 10^{10} CFU/day.	Treated orally once daily for 21 days starting Day 3 of life.	Treated foals more likely to develop diarrhoea requiring veterinary intervention (highest incidence at 8–15 days age).	No reduction in either incidence nor duration of diarrhoea and possibly aggravated condition.
Kim, 2001, USA.	246 equine colic patients.	51 horses treated with probiotic containing *L. lactis* and *E. faecium*, dose 5 x10^{9} CFU's of each, and live yeast cells, dose 1 x10^{8} with vitamins in 4 ml formula.	Dose: 4 ml, q 24 h for up to 5 days.	No difference in prevalence of fever, diarrhoea, or leukopenia during hospitalization.	No detectable differences in faecal *Salmonella* shedding between treatment and control groups.
Weese, 2004, Canada.	8 healthy mature Standardbred horses; 9 foals (2 days old).	*L. pentosus WE7*, Adults: 7 x 10^{11} CFU/day. Foals: 1 x 10^{11} CFU/day.	Treated orally daily for 5 days.	Safety data not reported.	Lactic acid bacterium of equine origin identified, able to survive gastrointestinal transit in mature horses and foals. Potentially beneficial *in*

					vitro properties.
Ishizaka, 2014, Japan.	10 healthy exercising adult geldings 8 Thoroughbreds and 2 Dutch Warmbloods Mean age 14.8 years.	Fermented liquid supplement producing *L. acidophilus* 5.6 x10^8 CFU/g, *S. cerevisiae* and *S. boulardii* 2.6 x 10^4CFU/g. Daily doses= 1.15 x10^{11} CFU *L. acidophilus* + 5.32 x 10^6 CFU yeast.	Treatment group received 200mls daily (n=5); control group given 200ml 0.9% saline (n=5) Duration: 28 days.	No adverse events.	Improved digestion of the probiotic-treated horses by stabilisation of neutral faecal pH and reduction of 4-methylphenol in faeces, suggesting reduced faecal enteric pathogen load.
Schoster, 2016, Switzerland.	38 healthy neonatal foals.	Four *Lactobacillus spp.* 3–4 x 10^3 CFU/g each, with *B. animalis spp. lactis*, 1x10 $^{3-4}$ CFU/g. *Daily dosage not provided.*	Daily treatment for 3 weeks.	59% foals in both arms of the study experienced diarrhoea – no statistical difference between the groups.	Limited potential for therapeutic modification of the gastrointestinal microbiota.
Harlow, 2017, USA.	7 healthy mature geldings (5 - 17 years).	*Ex vivo* experiment: *L. acidophilus* (ATCC # 4356), *L. buchneri* (ATCC # 4005) and *L. reuteri* (ATCC # 23272 from fresh suspension of horses' faeces, treated with additional probiotics:	*Lactobacilli* treatments added to faecal cell suspensions, dispensed into serum bottles containing ground corn, oats or wheat at 1.6% w/v starch concentration, incubated for 24 hours.	N/A	Exogenous *lactobacilli*, in particular 10^8 L. Reuteri, mitigated against pH decline and microbial changes associated with grain fermentation; increased short

					chain fatty acid production; decreased lactate levels up to 85%. Authors suggest potential application for management of hindgut acidosis.
Swyers, 2008, USA.	15 healthy mature Thoroughbred geldings.	Single species *L. acidophilus* (LAC1), dose 10^8 CFU/50 kg BW/day with grass hay + pelleted concentrate Commercial multi strain mixture of *Lactobacilli* (LAC4), dose 10^8 CFU/ 50 kg BW/day with grass hay + pelleted concentrate with Control diet: grass hay + pelleted concentrate	Treated once daily for 26 days.	Affect on iron digestibility; no other adverse events reported.	Limited effects on nutrient digestibility; no protection demonstrated against acidosis associated with concentrated starch intake.
Parraga, 1997, USA.	200 adult horses with colic, postoperative	Quick Fix® gel preparation (Colostrum Technologies, Inc) , dose 30 X $l0^7$ CFU/day (*L. plantarum, L. casei, L. acidophilus, Strep. jejunum*) Probiocin® Paste (Pioneer Laboratories): Dose of 41.75 X 10^8 CFU/day (*L. acidophilus, Strep. faecium, B.*	186 horses treated daily for 7 days.	Preliminary trial of probiotics on 18 healthy horses produced no adverse effects at up to 3 times manufacturers' recommended dose. No deaths among horses	Probiotic formulations administered at manufacturers' recommended dose had no effect on faecal *Salmonella* shedding; no reduction in postoperative diarrhea, requirement

		thermophilum, B. longum).		with other gastrointestinal disorders.	for antimicrobial therapy or period of hospitalization.
Yuyama, 2004, Japan.	54 healthy neonatal foals.	S5M™ probiotic *(L.salivarius YIT 0479, L.reuteri YIT 0480, L.johnsonii YIT 0482 and L.equi YIT 0483*), Dose: 5g *Daily CFU not provided*	5 grams daily for 7 days	No clinical side effects or adverse reactions	Body weight of foals increased significantly overall (P=0.008) Diarrhoea was 14.8% less in treated group at 2-3 weeks (P<0.05) No improvement on faecal short chain fatty acid content.
Tanabe, 2014, Japan.	130 healthy neonatal Thoroughbreds at ages birth to 20 weeks of age.	LacFi™, *(L. ruminis KK14, L. equi KK 15, L. reuteri KK18, L. johnsonii KK21*, and *Bifidobacterium boum HU)* Dose: 8.6×10^9 CFU/day	Single daily dose LacFi™-administered in 50 ml skim milk on Days 2-5 after birth, up to 20 weeks age group (n=101)	No weight reduction, nor signs of toxicity, no deaths	Diarrhea incidence reduced from 75.9% in the control group to 30.7% in the LacFi™ - administered group. Diarrhea prevented at 4 weeks and 10 - 16 weeks. Duration shorter in the LacFi™ group.

The results of this review demonstrate that limited research has been published to date on probiotic bacteria efficacy, safety and tolerability in equids. Perhaps the lack of research attention to horses is due to extrapolation of results obtained from human studies, where a range of probiotic bacterial species and strains have been shown to be both effective and safe under a range of conditions (250). Appropriating human probiotic bacterial strains for equine use seems to be widely conducted, without clear rationale regarding whether these organisms are either safe or tolerable in an equine host, especially in light of the different anatomy of the human and equine gastrointestinal tracts. It would appear that human strains are well tolerated and safe for use in horses but this has not been studied extensively and there are many unanswered questions regarding whether they can be efficacious in similar conditions across the human and equine species. Commercially available multi-strain products are currently being employed to treat a limited number of conditions afflicting horses, particularly in the management of scouring, with limited safety data and minimal efficacy reports. No studies were identified that explored the effects of probiotics on extra-intestinal markers of health, such as fertility.

3.3.1 SAFETY AND TOLERABILITY

While probiotic bacteria have traditionally been used to improve the health of animals who are sick or fail to thrive, the safety and efficacy of probiotics in normal, healthy horses has also been investigated.

Probiotic supplements of various species were well tolerated by adult horses and foals in several studies. Weese et al. [2003] administered *L. rhamnosus* GG at dosages up to 5 x 10^{10} CFU/50kg body weight to 21 clinically healthy adult Standardbred horses and 14 clinically healthy pony foals, once daily for five days, without any adverse effect being observed in any animal (251). Similarly, Laghi et al. [2018] demonstrated that 10 Standardbred horses could be supplemented with a commercial probiotic formula [Slab 51], containing 8 live bacterial strains, twice daily for 21 days without any adverse reaction being observed during this trial (198).

Of the studies included in this review, limited safety data or concerns were reported in adult horses, while substantial safety concerns were reported for foals (250, 252, 253). Foals did not respond well to being supplemented with high doses of multi-strain probiotics, developing or having aggravated diarrhoea when the administered formula containing probiotic bacteria species consumed in humans, such as 1×10^9 CFU of each of *L. rhamnosus SP1*, *L. rhamnosus LRH19, L. plantarum LPAL, L. plantarum BG112*, and 1×10^{10} CFU of *Bifidobacterium animalis lactis* strain (252). However, if the probiotic organisms selected were more closely related to equine commensals, as with the formulation *LacFi*^{TM} [*Lactobacillus ruminis KK14, L. equi KK 15, L. reuteri KK18, L. johnsonii KK21,* and *Bifidobacterium boum HU,* dose = 8.6×10^9 CFU/50 ml], the incidence of neonatal diarrhoea more than halved (196).

Schoster et al. [2015], evaluated 72 neonatal foals treated with a blend of *L. rhamnosus, L.plantarum* and *B. animalis* bacteria for 21 days from birth, these probiotic-supplemented newborns were more likely to develop more severe diarrhoea than the controls, requiring veterinary intervention, particularly from 8-15 days of the study (254). There may be several explanations for this adverse outcome, such as the daily administration of high doses of probiotics [1×10^9 CFU of each bacterium], the long durations of the intervention [21 days] and the use of probiotic species that are typically selected for human use. In another study, John et al. [2015] supplemented 25 neonatal foals with a less known *Bacillus* species [*B. cereus var. toyoi*] with low [5×10^8 CFU] and high [2×10^9 CFU] dose regimens for 58 days; compared to placebo, this intervention had no effect on the occurrence of diarrhoea nor the health status of the foals, possibly because this probiotic did not colonise the intestines nor alter the microbiota of the treated foals (252). The most significant adverse events reported by any probiotic intervention in this review were reported by Weese et al. [2005]; after dosing 153 neonatal foals with 2×10^{11} CFU of freeze-dried *Lactobacillus pentosus WE7* orally once daily for only seven days, these foals developed significant signs of anorexia, colic and depression during the study, requiring veterinary intervention. Furthermore, the cases of diarrhoea which occurred in the treated group lasted longer than for the foals in the control group without

altering the composition of their intestinal microbiota (250). Considering that foals affected by diarrhoea demonstrate a less diverse faecal microbiome composition when compared to healthy foals (253), one might query the rationale for this particular study design by Weese et al., with introducing a novel, non-commensal species to the delicate immune and digestive systems of the newborn.

3.3.2 EFFICACY

The overall results of the studies included in this review suggest that single probiotic intervention trials are less likely to be efficacious in the treatment of the chosen condition compared to multi-species probiotics. In addition, some multi-strain formulations were associated with favourable shifts in the composition of the horses' intestinal microbiomes, improved athletic performance and reduced grain-induced hindgut acidosis (198, 199, 255). Increasing the diversity of microbiome species in the intestinal milieu is associated with a positive homeostatic effect on many physiological functions, in particular immunoregulation; for example, a diverse array of host-defence peptides are produced by gut mucosa and immune cells to maintain communication between the host and its microbiota for the purpose of achieving a healthy homodynamic immune balance (256). The composition of the intestinal microbiome is thought to influence nutritional status via its effect on digestion, absorption and assimilation (257). In addition, the microbial composition of the intestine plays an important role in intestinal immune function and regulation of pro-inflammatory cytokines such as Il-6 (258).

While probiotic yeasts were not considered within the scope of this review, it is important to note that others have identified the *Saccharomyces* genus are more efficacious than single strains of bacteria for improving the digestion of cellulose and the treatment of acute enterocolitis. With respect to digestion, Jouany et al. [2008] supplemented 8 crossbred male horses with *Saccharomyces cerevisiae* and found that this yeast significantly improved cellulose digestibility, irrespective of diet (242). Garber et al. [2020] applied this same yeast species to a high-starch and a high-fibre diet, by supplementing a group of ponies on a grass hay and alfalfa diet with a 4%

Saccharomyces cerevisiae product, resulting in increased fibre fermentation and nutrient uptake by the ponies (259). Desrochers et al. [2005] administered *Saccharomyces boulardii* [10 x 10^9 yeast cells orally every 12 hours for 10 days] to 14 horses with acute enterocolitis, significantly decreasing the severity and duration of intestinal disease during hospitalisation, compared to horses receiving the placebo (197). In contrast, neither single nor multispecies probiotic supplementation has been shown to be efficacious in treating equine salmonellosis.

3.3.2.1 MULTISPECIES VERSUS SINGLE SPECIES FORMULATIONS

Overall, studies using multiple species of probiotic bacteria supplements reported being effective in treating gastrointestinal symptoms and pathology in both foals and adult horses, and/or had no adverse impact on the subjects (260-262), compared to the one study which used single species-strain formulations to treat foals (250). Almost 80% of neonatal foals are affected by diarrhoea in the first weeks of life, which is of great concern to breeders (254). Yuyama et al. [2004] supplemented 54 neonatal foals with S5M probiotic [*L.salivarius YIT 0479, L.reuteri YIT 0480, L.johnsonii YIT 0482* and *L.equi YIT 0483*] during their first week of life, resulting in a reduced incidence of diarrhoea and increased body weight at 2-4 weeks of age (263). Similarly Tanabe et al. [2014] gave 130 healthy neonatal thoroughbreds a daily dose of a multistrain probiotic preparation, LacFi™, containing *Lactobacillus ruminis KK14, L. equi KK 15, L. reuteri KK18, L. johnsonii KK21,* and *Bifidobacterium boum HU*, for the first 20 days of life, decreasing the incidence of diarrhoea in the treated foals to 30.7% (260). The duration of diarrhoea was also shorter in foals which were administered the probiotics, which protected them from further nutritional losses (260). As discussed earlier, native equine probiotic species such as *L.equi* are associated with fewer adverse effects (260).

3.3.2.2 DOSAGE, VIABILITY, DURATION, SPECIES SYNERGISM AND QUALITY OF PRODUCTS

The total dose of colony forming units [CFU] administered across the studies included in this review varied greatly, from 10^3 to 10^{11} CFU/day, with no obvious association between efficacy outcomes and the dose provided. Factors other than dose - such as

the multiple unique mechanisms of action specific to the different species/strains in a formulation - may play a more important role in efficacy outcomes than the number of CFU alone. Unsurprisingly, the viability of the supplemented organisms at the time of administration also influences trial outcomes (264). Actual viable CFU dosages using commercially prepared probiotic bacteria formulations are highly variable due to low survival rates of certain bacterial strains, suggesting the importance of conducting an independent analysis of the viable [live] CFU in products used in trials to ensure accurate dosage delivery and potentially improving outcomes (265). The variability in study outcomes may also have been influenced by study durations, with shorter intervention periods employed in many of the studies; the duration of studies where improvements were observed ranged from 21-30 days, which may have allowed time for any metabolomic effects of the probiotic bacteria to have an effect (198).

Some consideration must be given to the appropriateness of species, strains and dosages of probiotic bacteria for the putative treatment of various diseases. With regard to effective dosage regimens, Parraga et al. [1997] suggested that the ineffective outcomes in their study were due to their adherence to the manufacturers' dosage recommendations, offering the proviso that higher doses may have achieved better outcomes for the hospitalised horses involved in the study. A separate study evaluating tolerability at three times the recommended dosage did not produce any adverse events in 18 of the horses (266). The period of treatment in studies which examined *Salmonella* shedding was 5-7 days, with conflicting results not associated with the duration of treatment.

A concern about the quality and consistency of viable CFU in commercially available animal probiotic formulations was raised by Schoster et al. [2016], who suggested that this factor is likely to have contributed to variable outcomes across studies. The authors reported that administration of a multi-strain probiotic, containing 4 *Lactobacillus* spp. and *Bifidobacterium animalis* subsp. *lactis*, to 38 healthy neonatal foals did not alter the composition of the gastrointestinal microbiota (265). They suggested that the inclusion of product certificates of analysis specifying an

independent laboratory evaluation would contribute to raising the quality and enhancing the interpretation of probiotic studies (265).

Researchers in the area of probiotics and equine microbiome are now exploring the presence and therapeutic potential of horse-specific bacterial strains (267) and precipitate (250, 262, 268). While novel equine probiotic species have been identified, neither *in vitro* nor *in vivo* studies have shown them to be beneficial (250, 262). Morotomi et al. [2002] are credited with discovering *Lactobacillus equi*, isolating this species from the faeces of 50% of horses sampled. Being a lactic acid-producing bacterium of equine origin, able to survive gastrointestinal transit with potentially beneficial *in vitro* properties, it was expected to have therapeutic value, but this is yet to be determined (267). Novel native strains such as *Lactobacillus pentosus* WE7 (269) and *B. cereus var. toyoi* (252) did not improve the intestinal health of trial subjects and indeed were more likely to cause diarrhoea in foals, particularly at higher doses, hence were unsuitable for use as therapeutic probiotics in this cohort.

3.3.3 THE IMPACT OF PROBIOTIC BACTERIA SUPPLEMENTS ON THE EQUINE MICROBIOME

As has been previously stated, intestinal microbial populations which shift in favour of a dominant *Firmicutes : Bacteroidetes* balance provide a key determinant for a healthier equine microbiome. Interestingly, in some studies, the reverse phylum ratio appears to confer health in humans, with the healthiest enterotypes carrying an abundance of *Bacteroidetes* amongst their faecal microbiota (270, 271). The results of the studies included in this review suggest that the composition of the equine faecal microbiome does not appear to be altered by probiotic supplementation in any lasting way, in either foals (265) or adult horses (251), but rather that certain species have been shown to survive transit though the equine gut (251). It is generally held that host-native strains of probiotic bacteria are more successful and persistent colonisers of the equine gastrointestinal tract and that although several studies have attempted to introduce probiotic bacterial species typically used in humans to both foals and adult horses, such as *L. rhamnosus LGG,* they have not succeeded in prolonging intestinal survival of these species following cessation of their

supplementation (272). Thus, it is recommended that to produce any clinical benefit with bacterial probiotic therapy in horses these supplements must be delivered either long term or via repeated courses (272).

An acidic faecal pH in horses can indicate the presence of dysbiosis that can interfere with the function of important fibre-degrading bacteria, which are acid-sensitive; fermented feed containing multiple probiotic species and prebiotics has been shown to both restore a neutral faecal pH in exercising horses, as well as to reduce the production of volatile organic compounds in the equine gut by enteric pathogens such as *Escherichia. coli* and *Clostridium perfringens* (273).

3.3.3.1 BIOMARKERS OF INFLAMMATION AND MICROBIAL METABOLISM.

Intestinal dysbiosis contributes to the development of enteral disorders in horses (130) and the metabolome of the equine intestine plays a significant role in regulating inflammatory disease of that region (274). Multi-strain probiotic formulations that were successful in reducing the incidence of scouring in foals and hindgut acidosis in adult horses have been shown to reduce inflammatory dysbiosis and improve levels of anti-inflammatory cytokines in mice (260). In human studies, for example, Polysaccharide A, a regulatory molecule produced by the human symbiont *Bacteroides fragilis*, has the capability of suppressing pro-inflammatory interleukin-17 release from gut immune cells, while upregulating anti-inflammatory interleukin-10 secreted by CD4+ T-lymphocytes (396). In ruminants, excessive grain intake can result in elevations of serum D-lactate and dropping intestinal pH, designating D-lactate as a subclinical biomarker of sepsis (275).

The commercial formulation LacFiTM used by Tanabe et al. [2014] contains *Lactobacillus reuteri* which, in combination with other probiotic species, has been shown to improve the production of IL-10 in a colitis mouse model. The authors report that "the four-species probiotic cocktail (including *L. reuteri*) was more effective than the species individually and anti-inflammatory drugs in repairing the dysbiosis of mucosal microbial ecology and reducing intestinal inflammation" (276).

3.3.3.2 EQUINE DIET, DYSBIOSIS AND PROBIOTIC BACTERIA

Grain-dominant diets fed to horses to enhance athletic performance are associated with an increased risk for developing colic, hindgut acidosis and laminitis (277). This feeding regimen reduces faecal microbial diversity when comparing these animals' intestinal microbiota to that of pasture-grazed horses (212). Respondek et al. [2008] proposed that such disorders could be prevented by the addition of fructooligosaccharides (FOS) to grain-based feed and produce the desired healthier microbiome balance (278) (400). Ishizaka et al. [2014] gave 10 healthy exercising adult geldings a liquid extract of a multi-grain fermentation, which contained *L. acidophilus*, *S. cerevisiae* and *S. boulardii* as well as FOS, for 28 days and speculated that this probiotic preparation may have improved the microbiome and metabolome of these horses, hence improving their overall digestive function and health without producing adverse effects (273).

By contrast, Swyers et al. [2008] supplemented the diets of 15 healthy mature thoroughbred geldings with either a single or mixed strain direct-fed lactic acid bacteria only [without prebiotic]; the intervention had limited effect on the absorption of nutrients and was not effective in reducing the risk of acidosis associated with feeding high-starch concentrates (279). Such differences in outcomes suggests a role for more synergistic approaches to interventions that include both pre- and probiotic approaches.

The potential to offset hindgut acidosis in grain-fed horses by administration of probiotics was further explored by Harlow et al. [2017] in an *ex vivo* experiment. After fermenting separate bottles each containing ground corn, wheat and oat feed with freshly processed and extracted faecal bacterial cell suspensions for 24 hours, the researchers applied two species of *Lactobacillus* as treatments to these fermented solutions *in vitro*, resulting in an increase in short chain fatty acid and a decrease in lactic acid content. *L. acidophilus* and particularly *L. reuteri* reduced lactate concentrations by as much as 85%, leading the researchers to proffer the suggestion that targeted probiotic therapy may reduce the rate of intestinal starch processing

and allow hindgut microbiota more time to recover and to stabilise, hence minimising colic in treated horses (255).

3.3.3.3 EXTRA-INTESTINAL EFFECTS OF PROBIOTIC BACTERIA

We were unable to identify studies that investigated the extra-intestinal effects (effects on other organs outside the gastrointestinal tract) of probiotic bacteria supplementation on the inflammatory condition laminitis or on infertility. However, in terms of performance, supplementing racehorses with *Lactobacillus* resulted in a beneficial supplementary source of energy production and energy conservation via a metabolomic mechanism (198, 248). Laghi et al. [2018] and Zavistanaviciute et al. [2019] examined the benefits of probiotics on physical endurance of horses [Trotters and Thoroughbreds]. Their studies revealed a reduction in post-exercise blood lactate levels in supplemented horses, hence a potential for application in the racing industry. Supplementation of these exercising horses with "Slab51", a probiotic mixture containing 8 live bacterial strains *(L. acidophilus DSM32241, L. plantarum DSM32244, L.casei DSM 32243 , L. helveticus DSM 32242, L. brevis DSM27961, Strep. thermophilus DSM 32245 , B. lactis DSM 32246* and *B. lactis DSM32247*), significantly reduced post-exercise blood lactate concentration, suggesting an effective switch of energy source in muscle from carbohydrates to short-chain fatty acids (198). The *L. plantarum* and *L. paracasei* mixture increased HgO2 and blood O2 saturation, while significantly decreasing blood lactate concentrations (198).

Regarding equine fertility, research characterising the equine vaginal microbiome has commenced, with a view to selecting specific probiotic bacteria species for the management of the reproductive health of mares (127). If human research is a reliable indicator, the vaginal microbiome most likely has an influence on foal health (276), and for this reason alone it is a worthy area of research. Likewise, based on human studies evaluating reproductive microbiomes of men (280-282), exploring the relationship between the stallion semen microbiome and altered sperm quality is a worthy pursuit.

3.3.4 PROBIOTIC BACTERIA FOR TREATING EQUINE DISEASE STATES

3.3.4.1 SALMONELLOSIS

The healthy equine intestinal microbiome provides defence against nosocomial infectious agents such as *Salmonella* , and for this reason, it has been postulated that probiotic supplementation of surgical patients might offer some protection against faecal *Salmonella* shedding (262); however, conflicting study results debate the proposed benefit of probiotic bacteria supplementation for reducing the incidence of *Salmonella* outbreaks in hospitalised horses (261).

According to James et al., 17% of an apparently healthy horse population was identified as having *Salmonella spp* in their faeces by use of a PCR-based assay (283). A matter for greater concern is hospitalised equine populations and the possibility of outbreaks of nosocomial infections such as salmonellosis, as most of this cohort of horses asymptomatically shed this bacterium in their faeces [74%] (266). *Salmonella* shedding often affects stressed horses as well as those in recovery from colic surgery; this condition significantly increases the morbidity of patients [up to 45%] (284), as it leads to potentially fatal surgical complications.

Veterinary researchers seeking a probiotic supplement solution to this problem have produced inconclusive results to date. Ward et al. [2004], studied 130 horses hospitalised with non-gastrointestinal related diseases; they administered four doses of the multistrain probiotic Probios Equine One Oral Gel, containing 10^6 CFU per gram of viable *L. plantarum, L. casei, L. acidophilus* and *Enterococcus faecium*, on admission and then over 5 days, with the result that the risk of *Salmonella* faecal shedding in these animals was reduced by 65% (262). Although they commented that these results were not statistically significant, the researchers recommended dosing horses which were to be exposed to stressors, such as transportation, antibiotic therapy and surgery, with a probiotic formulation in early anticipation of the events, to minimise their risk of diarrhoea and *Salmonella* shedding and consequent salmonellosis outbreaks (266).

Parraga et al. [1997] studied a large cohort of 200 adult horses undergoing surgery for colic and reported that neither placebo nor multi-strain probiotic treatments reduced the prevalence of *Salmonella* faecal shedding, incidence of postoperative diarrhoea, length of antimicrobial therapy, or length of hospitalisation in these animals (266). Kim et al. [2001] found that 51 equine colic patients treated with a two-species probiotic formulation did not differ from control horses in regard to their likelihood of faecal *Salmonella* shedding (261).

Regarding the selection of probiotic species in each of the studies reviewed in this paper, there have been no optimal formulations which have been put forward for managing specific equine conditions, particularly as their findings have been conflicting. The 3 *Lactobacillus* species chosen by Ward et al. [2004] which resulted in improvements when administered to patients admitted to hospital with non-gastrointestinal diseases, were the same *Lactobacilli* as those in the probiotic gel employed in the Parraga [1997] study which did not demonstrate any benefit for managing the same condition. *E. faecium* was present in the Probios oral gel employed by Ward et al. [2004], which indeed was one of the probiotics used in the study of Kim et al. [2001], who reported no benefit compared to controls, with respect to prophylactic probiotic supplementation in reducing *Salmonella* shedding amongst horses admitted to hospital suffering with colic; hence it is difficult to discern the optimal probiotic combination to address *Salmonellosis* from these limited studies (263, 266, 284). It is possible that efficacy lies in the correct microbial combination given at the ideal dose, which may not necessarily be the most complex formulation, nor the highest CFU dose. It is also possible that the confounding factor of stress and inadequate intake of nutrients, which may be experienced by ill and hospitalised horses, may also have unfavourably influenced the composition and metabolic activity of the intestinal microbial balance of these animals (241).

3.4. CONCLUSION

The field of research into the equine microbiome is currently in a state of emergence, as new studies are being published and questions are being posed about the future

therapeutic potential for probiotic therapy to manipulate equine gut microbiota and provide prophylactic and therapeutic benefits. Currently some promising benefits for bacterial probiotic supplementation in horses have been identified in the area of equine performance and nutrient status for stamina and endurance, which could offer opportunity for future applications in racing, eventing, polo and other equine sports. Prevention of neonatal diarrhoea with multi-strain probiotic bacteria may offer some potentially safe and effective applications for veterinarians. More specific research is necessary to characterise the equine microbiome of various tissue sites, along with their effects on the health of those sites. Perhaps the extent to which probiotic supplementation has developed following two decades of clinical research, might herald future applications for evidence-based, species-specific probiotic therapy for the management of both healthy and diseased equids, for optimal conditioning, healthy reproduction and peak performance.

CHAPTER 4: REVIEW ARTICLE "PREBIOTICS AND SYNBIOTICS IN EQUINE HEALTH AND DISEASE"

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Title: "Prebiotics and synbiotics in equine health and disease – Review"

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Abstract

Prebiotics are non-digestible food ingredients that promote the growth of probiotic microorganisms in the intestines and are marketed for use in supporting equine digestion, metabolism, growth and immunity. Synbiotics are supplements that contain combinations of prebiotics and probiotic bacteria and/or yeasts. Both are commercially available and promoted for use in supporting equine athletic

performance and reducing morbidity associated with intestinal disease. The aim of this narrative review was to summarise the literature reporting on the use of prebiotics and synbiotics, in equine nutritional practice. Fifteen papers were identified that report on the use of prebiotic or synbiotic supplementation in horses. The literature presented here indicates that prebiotics may play a role in equine health and disease prevention. Prebiotics have been studied for their effects on athletic performance; increasing production of volatile fatty acids associated with hindgut fibre fermentation; insulin resistance; carbohydrate metabolism associated with reduction in the development of gastric mucositis, and hindgut acidosis and laminitis. Prebiotic fibre is thought to have an entero-protective effect by improving the composition and diversity of the intestinal microbiota, that in turn impacts immune function via metabolomic effects. Synbiotic supplements containing psyllium seed husks have been shown to improve intestinal sand clearance and relieve sand colic. Prebiotic yeast extracts have been associated with reducing intestinal pathobionts and accelerating healing in acute enterocolitis. Overall, the current evidence to support the use of prebiotics and synbiotics in equine health and disease is not extensive but promising.

Keywords: equine, prebiotics, synbiotics, fibre, microbiome, digestion

List of Abbreviations

AOS	acidic-oligosaccharides
EGUS	equine gastric ulcer syndrome
FOS	fructo-oligosaccharides
GOS	galacto-oligosaccharides
LPS	lipopolysaccharide
IgA, IgG	immunoglobin A, G
IMOS	isomalto-oligosacharrides
MOS	mannan-oligosaccharides
PBMC	peripheral blood mononuclear cells
SCFA	short chain fatty acids
TNF-α	tumour necrosis factor alpha
VFA	volatile fatty acids
XOS	xylo-oligosaccharides

4.1 INTRODUCTION

Equine veterinarians play an important role in advising on interventions that prevent and treat gastrointestinal disorders in horses. Equine gastrointestinal disorders include scouring in neonates (285), colitis and fatal colic events (286), parasitic gastrointestinal infections (Pfister (287), gastric ulceration (288), salmonellosis associated inflammatory bowel disease (289), coronavirus infection with necrotizing enteritis and eosinophilic enterocolitis (289), sand-accumulation enteropathy (290) and caecal disorders (291) and dysbiosis of the intestinal microbiome may predispose horses to many of these gastrointestinal disorders (173). Interestingly, there is evidence to suggest that equid type (size, breed, or conformation) may influence predisposition to the development of various gastrointestinal lesions; for example, larger breeds such as Clydesdales experience more caecal disorders, whereas smaller breeds and miniature horses are less likely to be affected by colon displacements (292). Furthermore, exacerbations in asthma (186), obesity (293) and impaired athletic performance (294) have all been associated with alterations to the composition and diversity of the gastrointestinal microbiome. Therefore, the equine microbiome has gained increasing attention in veterinary research (129), as reflected by the initiation of the Equine Gut Microbiome Project in 2015 (295).

Popular interventions thought to alter the composition and diversity the gastrointestinal microbiome include supplementation with probiotics and prebiotics. Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (296) and prebiotics are defined as "selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (297). Prebiotics are widely used in equine management regimens to support intestinal processing of a variety of feedstuffs, based on the belief that they improve the diversity and richness of the species that inhabit the intestinal microbiome (195). When a feeding regimen is suddenly changed, this affects digestion; research conducted with Irish thoroughbreds suggested that sensitivity to altered dietary

regimens could be increased by carrying a limited abundance of hindgut core bacterial species (298). However, preventing the signs and symptoms of digestive disturbance associated with changes to diet has been shown to be ineffective in response to supplementation with the yeast organism, *Saccharomyces cerevisiae CBS 493.94 (299).* Adding prebiotics containing fructo-oligosaccharides or mannan-oligosaccharides to high fibre diets increases feed digestibility, supporting energy production and has been shown to improve the general fitness and health of the horse (195). When probiotic bacteria and/or yeasts are combined with prebiotics, the formulation is referred to as a 'synbiotic'. These combinations have been used in human studies to promote the growth of beneficial microbes with a view to increasing the diversity and richness of the intestinal milieu (300).

While there are limited studies in the equid, the potential role of pro- and prebiotics in human health and disease has been explored far more extensively, with the current PubMed citations exceeding five thousand. No summary of studies that report on the nutritional and therapeutic role of prebiotics and synbiotics in equine health and disease has been conducted to date. Therefore, the aim of this review was to provide an overview of the literature reporting on the use of prebiotics and synbiotics, in equine nutritional practice. An online search of the of Embase and PubMed database identified fifteen papers reporting on the effects of prebiotic or synbiotic supplementation in horses. The findings of those studies are discussed and evaluated here. This review does not address the use of probiotics in equine populations, which was the topic of a previous review published by the authors of the present manuscript (194).

4.2 TYPES OF PREBIOTICS

In the horse, prebiotics can constitute part of the regular feeding regimen, supporting potential colonisation of the intestinal environment with bacteria that are thought to confer a benefit. Prebiotics are predominantly oligosaccharides or disaccharides of which there are several types: oligofructose, inulins, isomalto-oligosacharrides (IMOS), lacto-sucrose, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS),

lactulose, pyrodextrins and xylo-oligosaccharides (XOS), and mannan-oligosaccharides (MOS) (301). Certain prebiotic types are compounded dietary sugars, for example FOS is composed of short chains of fructose molecules and GOS is composed of a chain of galactose molecules; IMOS is derived from the disaccharide maltose (sourced from honey, for example) following enzymatic activity, which produces a mixture of short-chain carbohydrates; XOS is a natural prebiotic fibre extracted from sugar cane fibre and Inulin belong to a class of polysaccharides known as "fructans" and are found in vegetables such as Jerusalem artichoke, plantains and chicory root. Soluble arabinogalactans are classified as non-carbohydrate sources of prebiotic fibre derived from larch tree (*Larix sp*.) (302). From the literature, equine prebiotics most studied are inulins, FOS, GOS and MOS.

Different prebiotic classes supply fuel to resident microbes in various regions of the equine gut. The caecum and colon comprise 66% of the digestive tract volume and contain large populations of anerobic bacteria; these probiotic species process pectin, cellulose, hemicellulose and starch, producing short chain fatty acids (SCFA) through the process of fermentation, to be utilised as a metabolic energy source (Shirazi-Beechey, 2008). A more complex microbiome resides in the hindgut (colon and caecum), composed of mostly bacteria but also viruses, fungi, protozoa, archaebacteria and phages; these microorganisms ferment the residual indigestible cellulose and lignin plant fibre which has not been unprocessed proximally, to produce volatile fatty acids that serve as a fuel source for the host (142). Nutrients including protein and B-group vitamins are also rendered more digestible by microbial fermentation in the hindgut (195).

4.3 PREBIOTIC USE IN HINDGUT FERMENTERS

Given the limited literature relating to prebiotic use in horses, it is of interest to consider what is known about other species that are hindgut fermenters. Hindgut fermentation is a digestive process seen in animals with a simple, single-chambered stomach, which include equids, rhinoceros, koalas, rodents and rabbits; in these mono-gastric herbivores, cellulose from plant foods is digested with the support of

bacteria (303). Researchers have explored the effects of adding fermentable sugars, such as prebiotics, to the diets of other hindgut fermenting animals, specifically rodents and rabbits, and associated improvements were found in lipid metabolism, composition of the intestinal microbiome and nutrient absorption, in particular minerals (303). Hindgut fermentation, cell-mediated immunity and various blood parameters improved in rats fed pulverised Jerusalem artichoke, rich in prebiotic inulin and FOS for twelve weeks (304). Further, in these rats, skin indurations induced by a mitogen reduced, while CD4 lymphocyte populations increased and blood levels of glucose, urea and haemoglobin in these rats adjusted favourably (304). Yeast-derived MOS supplementation enhanced SCFA concentrations and lower pH in the cecal region of rabbit intestines and was associated with longer intestinal villi when compared to controls. In addition, the weight gain observed with the addition of MOS to the diets of the study animals was similar to that which could be expected from antibiotic treatment for promoting growth (305). Although evidence supporting the use of prebiotics in the dietary management of horses is rudimentary, there are many commercial prebiotics and synbiotic supplementary feed formulations, currently available in several countries, which are purported to enhance the intestinal microbiome, digestion, energy production and immunity.

4.4 FORAGE, PREBIOTICS AND CARBOHYDRATE DIGESTION

Forage of various types supplies energy, nutrients and prebiotics to equids, the latter assisting in the cultivation of microbiota thought to confer a benefit. Mature grasses are mostly composed of less digestible fibre than young fresh grasses, which protects the host against infection with pathogens and reduces the risk of hindgut acidosis (306). Fructans are long-chain sugar molecules found in a variety of pasture grasses; the fructan content of forage varies, from nutrient dense verdant grasses higher in fructan content to low-fructan containing native grass species, prevalent in Australia (307). Grass varieties such as Timothy and some types of Perennial Rye are either processed rapidly in the equine intestine or have a significant proportion of their carbohydrate pass undigested into the hindgut for fermentation by resident microbiota, generating lactic acid as a by-product (307). High fructan intake is

thought to contribute to the development of hindgut acidosis, increasing the risk of laminitis (308), and may be ameliorated by the addition of different prebiotic fibres to the feed which degrade more slowly (309). From a management perspective, grazing horses on native grasses is preferable to administering prebiotic extracts, as native grasses are more fully processed and do not contribute to increased intestinal gas production which might arise from unfermented fructans reaching the hindgut (307). The starch component of an ideal equine prebiotic feed should predominantly degrade in the intestine, proximal to the hindgut. Jerusalem artichoke is a source of the prebiotic FOS and inulin fibre, which can play a role in the metabolism of hindgut microbiota by modulating fermentation of ingesta (195). However, according to one study in which carbohydrate processing from stomach to transverse colon was evaluated, Jerusalem artichoke may also contribute to increased gas production in the hindgut as a result of fermentation of undigested fructans (309). Importantly, inulin derived from Jerusalem artichoke is fermented differently to that present in grasses and may not support development of beneficial *Lactobacillus* colonies as effectively as does the inulin from forage. The rapid fermentation of high fructan grasses favours hindgut cultivation of lactate-producing bacterial species, such as *Streptococcus bovis* and *S. equinus*, overwhelming colonies of predominantly lactate-utilizing species, such as *Veillonella sp.* and *Megapshera sp.*, the latter which metabolise lactate to propionate (308, 310, 311). Therefore, with ingestion of Jerusalem artichoke inulin, it is likely the composition and function of the equine intestinal microbiome may not be as protective against the development of systemic inflammation and conditions such as laminitis (308). If carbohydrates ferment early in their passage through the equine foregut (oesophagus, stomach, and small intestine), the resulting production of organic acids may accumulate and produce mucosal injury (ulceration) (309). For example, short-chain fatty acids, particularly butyrate, resulting from bacterial fermentation of FOS, may irritate and injure the gastric mucosa, increasing the risk of equine gastric ulcer syndrome (EGUS) (274).

4.5 THE EFFECTS OF PREBIOTICS ON THE EQUINE MICROBIOME

There is a paucity of studies evaluating how the addition of prebiotics to commercial horse feeds impacts the microbiome. Most attention has been given to the modulation of intestinal carbohydrate processing by specific prebiotics to reduce the incidence of laminitis, which can be experimentally induced by administering large doses (10g/kg BW) of fructans to horses (312, 313). Such carbohydrate loading causes acidification of the intestinal milieu, producing a shift from eubiotic Gram-positive bacteria to lactate-producing species, such as *Streptococcus bovis/equinus* in the gut microbiome (308, 314); it is believed that these bacterial strains are responsible for causing the development of laminitis in horses (313).

Prebiotic sugars and fibres provide energy and nutrients to intestinal microbiota to fuel their metabolic activity. Berg et al (314) evaluated the addition of FOS to the diet of yearling quarter horses, finding that this intervention led to alterations in the microbiome and metabolome, with particular respect to faecal pH and concentrations of volatile fatty acids (VFAs) (314). These researchers reported a quadratic effect on faecal *Eschericia coli* populations following FOS supplementation without any apparent alteration in the numbers of *Lactobacillus sp*., and faecal propionate, butyrate, acetate and lactate concentrations increased linearly with total VFAs in this study (314). Such short chain fatty acids generated by intestinal microbiota can provide an additional energy source additional to carbohydrates for athletic performance in trotting horses. Hence, providing such prebiotics to generate fuel could be considered performance-enhancing supplementation.

In warmblood horses fed FOS and inulin daily for 3 weeks, the relative abundance of *Lactobacillus sp.* increased and *Streptococcus sp.* decreased in the gastric microbiome increased and decreased respectively, while higher alpha diversity and richness of the intestinal microbiome increased (315). Fructo-oligosaccharides are thought to support the colonisation of the equine intestine with *Bifidobacteria*, although the effect of this increased colonization is not fully understood (316). There is limited evidence for the function of *Bifidobacteria* in the equine gut microbiome, which contains a much larger proportion of *Lactobacilli*, even in relation to that found

in humans, however these indigenous probiotic bacteria do not typically utilise FOS as consistently as do *Bifidobacteria* (316).

The core intestinal equine microbiome is considered to be healthy if it contains a relative abundance of *Firmicutes* with respect to *Bacteroidetes* phyla (173). The faecal microbiome composition of ten healthy horses following supplementation with prebiotic cellobiose for 14 days was profiled by Paßlack et al.; the relative abundance of Firmicutes, Coriobacteriales and Clostridium increased in a dose-dependent way, with a dose-dependent decrease was measured in the relative abundance of Bacteroidetes (213). By association, these findings suggest an improvement in the composition of the faecal microbiomes of the study horses following administration of prebiotics.

Lastly, the entero-protective role of prebiotics, by promoting a healthy balance between species in both the major and minor phyla of the intestinal microbiome, supports the condition of the mucosa, enhancing nutrient absorption, health and athletic performance (317). Antibiotic use can be significantly reduced by supplementing livestock and companion animals with synbiotics (317), however such benefits have not yet been shown in equine management (316).

4.6 THE EFFECTS OF PREBIOTICS ON EQUINE HEALTH AND DISEASE

Table 4.1 Publications selected for review of prebiotics and synbiotics used in equine health and disease

Author - Date **Country**	**Horses studied - sex, age, breed, health status**	**Intervention**	**Duration of Study**	**Results**
Berg et al, 2005 USA	9 Quarter horses	Diets supplemented with 8 g of FOS/d (low),	Three 10-day feeding periods	Faecal pH decreased and total VFA's increased linearly from control to low-FOS, then high-FOS diets. FOS supplementation altered faecal microbiomes, with a

		or 24 g of FOS/d (high)		quadratic effect observed for *Escherichia coli* populations ($P<0.01$)
Respondek, 2011 France	8 obese mature Arab geldings (BCS = 8)	4 horses received maltodextrin 45 g/day/horse (control) and 4 horses received the same amount of short-chain fructo-oligosaccharides (scFOS)	Six weeks	Supplementation with scFOS increased insulin sensitivity and reduced acute insulin response to glucose and resting serum insulin in comparison with maltodextrin ($P<0.05$), without affecting body weight or body condition score. No changes were observed in plasma glucose, serum leptin or triglyceride levels ($P>0.05$)
Adams, 2015, USA	40 mixed breed and sex horses, age range 20-33	Four treatment groups: 1) Traditional grain mix; n=10 2) Control; n=10 3) Control + DHA; n=10 4) Control + ActivAge®prebiotic; n=10	Twice daily for 161 days All horses vaccinated Day 56 with equine influenza vaccine and a novel antigen (OVA) vaccination was boosted on day 70	Serum cytokines TNF-a, IL-6, and IFN-g measured prior to and 2 weeks post-vaccination. All inflammatory markers reduced significantly in prebiotic supplemented group compared to horses receiving traditional grain mix, control diet only and DHA
Czech, 2006, Poland	20 Thoroughbred mares	10g/d mannan oligosaccharides (Bio-Mos®)	20 days prepartum	Bio-Mos® improved blood antioxidant status but did not significantly alter nutritional composition of mare's milk other than slight protein increase. Treated mares had 20% higher RBC; 30% higher alkaline phosphatase and lactate dehydrogenase; 24% higher plasma superoxide dismutase than controls
Vendrig, 2014, The Netherlands	10 Warmblood pony foals	6 foals supplemented orally with 15g Vivinal® syrup (GOS 45%, lactose 16%, glucose 14%, and 25% water), 4 foals were controls	Twice daily for 28 days from birth	No differences observed between Vivinal® treated foals and the control group for the range of hematological parameters (haematocrit, white blood cell types, protein, albumin, various immunoglobulins). *Ex vivo* LPS-induced mRNA expression levels of IFN-γ and IL-6 were significantly lower in PBMCs derived from treated foals compared to the control group

Vendrig, 2013, The Netherlands	Peripheral blood mononuclear cells (PBMC) from 12 healthy Dutch Warmbloods	*In Vitro*	N/A	TNF-α production by PBMC's increased significantly at all oligosaccharide concentrations, compared to controls, with dose-dependent effects seen for all three fractions. Production of IL-10 in unchallenged PBMCs was not significantly influenced by GOS/FOS/AOS compared with blank controls. Several oligosaccharide fractions produced distinct direct immunomodulatory effects
Hassel, 2020 USA	10 horses with radiographic presence of large quantities of sand	Assure® 15g/d or Assure Plus® 226g/d containing custom blend of psyllium, prebiotics, probiotics, yeast and digestive enzymes	35 days	Reduction in sand accumulation was observed in all horses, however there were no significant differences between the treatment and control groups.
Niinistö, 2020 Finland	34 hospitalised horses	12 horses given psyllium 1 g/kg BW only; 10 horses given $MgSO_4$ 1 g/kg BW only; 12 horses given combined	Via nasogastric intubation daily for 4 days	Combination of psyllium and MgSO4 cleared areas of sand accumulation significantly more ($P<0.001$) than control horses
Landes, 2008 USA	8 clinically healthy equids (5 horses, 3 mules, 1 pony)	60g prebiotic/ probiotic, containing minimum 40 x 10^9 cells *Saccharomyces cerevisiae*, 2.25 x 10^9 CFUs *Lactobacillus acidophilus* and 1.55 x 10^9 CFUs *Enterococcus faecium* plus 0.5 g/kg psyllium	Daily for 35 days	Average faecal sand output for each horse was at least 2.5 times greater during treatment than in the pre-treatment period for all equids. Faecal sand output Days 1 – 3 was same as pre-treatment, then significantly increased and remained higher Day 4 - 31
Glatter, 2019 Germany	12 adult, healthy warmblood horses	FOS + inulin from Jerusalem artichoke meal (JAM) 0.2 g/kg bwt/d	Daily for 3 weeks, then euthanized 1 hour after final meal	Relative abundance of *Lactobacillus* increased and *Streptococcus* decreased predominantly in stomach of prebiotic-fed group. Higher alpha diversity and richness of microbiota seen in all regions of the GIT, particularly large intestine, for subjects fed JAM compared to controls ($P < 0.05$). Similar beta diversity seen in treated subjects and controls. More evenness of species seen in small and large intestines of JAM supplemented subjects.
Bachmann, 2020 Germany	12 adult, healthy	FOS + inulin from Jerusalem artichoke	Daily for 3 weeks, then euthanized	FOS and inulin were mostly fermented in stomach, not reaching hindgut in significant quantities, indicated by increased gastric gas production.

	warmblood horses	meal (JAM) 0.2 g/kg bwt/d *In vitro:* Fresh digesta removed from selected regions of GI tract, and incubated anaerobically to measure gas production	1 hour after final meal.	Stomach pH was lower and VFA concentrations higher in JAM-treated compared to control subjects, but similar in hindguts of both groups. Oxidation-reduction potential increased twofold from pre- to post-incubation with JAM
Cehak, 2019 Germany	Gastric mucosa samples obtained from 3 healthy horses, euthanized	*In vitro:* Mucosal tissue samples treated with four butyric acid concentrations of 10, 18, 24 and 32 mmol/l	N/A	Tissue samples *in vitro* produced histopathological changes consistent with those observed in horses fed prebiotic JAM. Severity of mucosal injury increased with higher concentrations of butyric acid.
Glatter, 2017 Germany	Six healthy, warm-blooded mares (age: 6–13 years)	Standard diet (crushed oat grains, 1 g starch/kg bwt/d with meadow hay 2 kg/100 kg bwt/d) plus either 0.15 g FOS and inulin /kg bwt/d via JAM or control (equal amount of maize cob meal)	2 x 21-day treatment periods	Feeding of JAM versus control resulted in a particularly rapid and definite peak of serum insulin, followed by a faster decline in both plasma glucose and serum insulin post prandial. Plasma glucose returned to baseline most completely with JAM but not control.
Bachmann, 2021 Germany	Twelve Warmblood horses (10 females, 2 males)	6 horses received 0.15 g FOS plus inulin/kg body weight/d via JAM 6 horses fed corncob meal without grains (placebo)	20 days, then euthanized	Simple sugars and fructans rapidly disappeared from gastric digesta at the postprandial state in prebiotic supplemented horses; elevated degradation to lactic acid and SCFA, especially n-butyric acid, may have gastric and metabolic health impacts.
Paßlack, 2020, Germany	8 healthy adult horses, 2 ponies	10 g cellobiose /d or 20 g cellobiose /d	14 days	Dose-dependent increase in relative abundance of Firmicutes ($P = .049$), Coriobacteriales ($P < .001$), and Clostridium ($P = .031$) detected. Dose-dependent decrease in relative abundance of Bacteroidetes ($P = .035$)

4.6.1 EQUINE PERFORMANCE

Exercising horses are often fed high carbohydrate, grain-dominant diets to deliver immediate energy for enhanced performance. This practice reduces natural forage intake, placing these athletes at risk of developing hindgut acidosis and possibly intestinal dysbiosis, as has been seen in cattle fed high carbohydrate (318). The prebiotic fibre found in sugar beet is thought to offer a glycogen-sparing effect when added to equine feed due to enhanced propionate production by intestinal microbiota, thereby assisting energy production for performance (319). During intense exercise in standardbred geldings, increased concentrations of muscle glycogen, lower muscle lactate levels and lower peak plasma lactate concentrations were found following the addition of sugar beet pulp to their diet. This effect was attributed to additional volatile fatty acids being utilised for aerobic energy production instead of glycogen (319).

High fibre diets supply equivalent amounts of energy via glycogen and propionate production compared to high-carbohydrate, grain-based diets, also delivering a beneficial alkalizing effect to protect against lactic acidosis generated by intense exercise (314). These findings could inform equine athlete management for protection against dietary complications and consequent illness, whilst improving performance in these horses

4.6.2 THE EFFECTS OF PREBIOTICS ON EQUINE GLUCOSE REGULATION AND INSULIN RESISTANCE

Dried sugar beet pulp fibre, commercially available as a prebiotic feed additive, is generally well-tolerated and effective without probiotic co-administration and is favoured as a supplement for athletes. Resting and post-exercise blood glucose levels were measured in two groups of intensively exercising thoroughbreds, which were fed standard diets with the addition of oats alone, or oats plus dried sugar beet pulp (320). Pre-exercise blood glucose levels were lower in the cohort receiving added prebiotic beet pulp as compared to the group fed an oats-based diet only, however there was no discrepancy found between the two groups in post-exercise

blood glucose levels and no adverse events were reported with the addition of beet pulp to the feed (320). Resting post-prandial plasma insulin was found to be lower in a cohort of Standardbred geldings whose hay and oat-based diet was supplemented with molassed sugar beet pulp and notably, following an exercise test, peak plasma muscle lactate was lower and muscle glycogen content higher than controls (319). Similarly, supplementing the standard crushed oat and meadow hay diet of six healthy warmblood mares with Jerusalem artichoke meal for 21 days produced a rapid serum insulin peak, followed by a faster decline of both glucose and insulin when compared to controls (321).These findings also suggest a potential protective effect against insulin resistance/metabolic syndrome and obesity in these animals. High carbohydrate equine feed (grains and lush spring grass) increases the prevalence of insulin resistance and obesity, by shifting the intestinal microbiome unfavourably (322). Supplementing the daily diet of eight obese mature Arab geldings with short-chain fructo-oligosaccharides over a period of six weeks optimised fibre processing which increased insulin sensitivity, while reducing acute insulin response to glucose and resting serum insulin, without altering body weight or body condition score (278). Further, senior horses experience a decline in digestive capacity and the risk of developing insulin resistance increases with advancing age. Supplementing the diet of older horses with FOS may protect the intestinal microbiome of these animals and consequently their metabolic condition (323).

4.6.3 THE EFFECTS OF PREBIOTICS ON EQUINE INTESTINAL INFLAMMATION

The discovery that prebiotic supplements may suppress intestinal inflammation has huge potential applications for veterinary medicine. Anti-inflammatory and immunomodulatory activity has been demonstrated in two equine studies Adams

(324, 325) and one human study (326) using prebiotics. Specific actions of microbial metabolites, such as butyrate, are believed to include reduced cytokine production by the mucosal cells lining the intestine (326). Anti-inflammatory and immunomodulatory activity of prebiotics were demonstrated in one *in vitro* study, in which equine peripheral blood mononuclear cells (PBMCs) were challenged with

lipopolysaccharide (LPS) to induce an inflammatory response (325). A protective effect was observed when PBMCs were pre-incubated with GOS/FOS/AOS (acidic oligosaccharides) prior to being challenged with LPS, producing a dose-dependent reduction in both TNF-α and interleukin-10 production. However, if PBMCs were pre-treated with GOS or GOS/FOS fractions alone, TNF- α production by the immune cells rose substantially (325). These oligosaccharide fractions demonstrated a dampening effect on allergic response while enhancing immune defences in PBMCs; pre-treatment incubation with glucose/lactose concentrations similar to 1% GOS, as a comparison control, significantly increased cell viability by 14%, while GOS/FOS/AOS pre-treatment increased cell viability by a range of 38-61% (325). The authors suggested that this protective effect on PBMCs was due to lower mitogenic potential (325).

4.7 PREBIOTIC YEAST EXTRACTS IN EQUINE HEALTH AND DISEASE

The beneficial effects of yeast supplementation in horses with respect to improved feed digestibility are disputed, with varying outcomes reported by studies employing a range of doses of supplemental yeast (327). Growth rate was significantly increased when weaned foals were fed yeast supplements, as a result of improved feed conversion efficiency (Van Saun, 2008). Twenty thoroughbred mares supplemented with MOS for 20 days prepartum exhibited improved blood antioxidant parameters, namely 24% higher plasma superoxide dismutase levels than controls, without alteration to the nutritional composition of their milk (328). Furthermore, there is evidence to suggest that mares' plasma and colostrum IgG levels may be boosted by adding MOS to their feed, protecting both mare and foal for 24 hours post-partum from serious infectious complications, such as diarrhea, sepsis and possibly death (329). MOS derived from *S. cerevisiae* cell walls protects against bacterial infection by competitively inhibiting the binding of bacterial lectins to intestinal enterocytes (330) and a dried yeast extract from mechanically ruptured *S. cerevisiae* cells containing beta glucan acts as a prebiotic substance, effectively absorbs mycotoxins from contaminated feed and thereby offering immune protection (331).

In terms of the impact of prebiotic yeast extracts on inflammation, horses exhibiting exercise-induced stress demonstrated more rapid recovery of cortisol and cytokine levels following eight weeks' supplementation with a fermented *S. cerevisiae* product as compared to controls (332). In relation to equine emergency medicine, *Saccharomyces boulardii* yeast administered to horses hospitalized with acute enterocolitis accelerated recovery when compared to the control, without altering the intestinal microbiome (197).

Yeast organisms such as *S. cerevisiae* have been utilized widely over the past decade as a nutritional supplement for livestock, as they assist with clearing intestinal pathogens by reducing intestinal membrane adhesion, effectively reducing the need for antibiotics, while improving feed digestibility, immunity, general condition and performance (327). In horses fed a high carbohydrate diet, live yeast culture supplementation increased the production of enzymes that assisted the intestinal digestion of nutrient fibre (333). Supplementation of live yeast culture was also found to support bacterial cellulolysis for energy extraction and utilization in horses fed both high-starch and high-fibre diets, as well as assisting in elimination of opportunistic bacteria by competitive exclusion (333).

With respect to composition of the equine intestinal microbiome, one study revealed increased relative abundance of families of *Lachnospiraceae* and *Dehalobacteriaceae*, which are associated with a healthy core microbiome in horses, following supplementation of a high-fibre diet with *S. cerevisiae* (110). In the same study bacterial fibrolysis of hindgut contents was found to improve following a high-fibre diet to which *S. cerevisiae* was added, producing higher numbers of ruminococci and a high-starch diet, also supplemented with the same yeast, resulted in a beneficial lower relative abundance of lactate-producing streptococci (110).

4.7.1 PREBIOTIC SAFETY

It is generally considered that prebiotics may be safely administered to horses, although this assumed safety has not yet been scientifically evaluated. Any alteration

in feed or supplementation which might adversely impact microbial diversity and species richness could disturb intestinal homeostasis (110). Intestinal dysbiosis, defined as "a loss of diversity, a bloom of potential pathobionts, and a loss of commensals" (334) often results from administration of antibiotics to horses and may render the animal susceptible to proliferation of pathogenic species such as *Clostridium difficile* and *Salmonella sp.* in the gut, causing antimicrobial-induced colitis and diarrhea (110). This being said, prebiotics have been shown to protect horses against intestinal dysbiosis, which can contribute to the development of colitis (130), by supporting the colonization of favorable probiotic species and dispelling enteric pathobionts (311). Soluble arabinogalactans, the prebiotic fiber derived from larch (*Larix sp.)* was administered to foals to treat scouring with some benefit and, although its specific mode of action is yet to be determined, its administration has proven to be safe in young horses (316). Another study supplemented seven mares and their foals with a soluble arabinogalactans preparation (LaraFeed AC9) for 2 weeks pre-foaling and until 14 days post-foaling, using five mares and their foals as controls. The results were a lower frequency of diarrhoea episodes with more normal faeces amongst the foals, requiring less veterinary intervention in comparison to controls. No treatment effects were found in the foals with respect to weight, blood parameters (complete blood count, IgA, IgG), or faecal *Salmonella* or *Rotavirus* cultures, demonstrating tolerance for the larch extract (335).

4.8 SYNBIOTICS IN EQUINE HEALTH AND DISEASE

A combination of a probiotic and a prebiotic, referred to as a synbiotic, can increase the survival time and colonization potential of the probiotic bacteria or yeast in the intestinal milieu (316). There is also some evidence that synbiotics may be beneficial for assisting intestinal sand clearance in horses, thereby playing a preventive role against the development of sand colic and enteropathy (336). The accidental ingestion of sand commonly occurs in horses as a result of daily feeding on pasture grasses (Landes et al., 2008), which may result in sand accumulation and lead to colic. Synbiotic treatments containing probiotics (*Lactobacillus acidophilus, Enterococcus faecium)*, prebiotics (derived from *Saccharomyces cerevisiae*) and up to 90% psyllium

seed husks have been effectively utilized to reduce and treat sand enteropathy and sand colic (336). While sand accumulation was significantly reduced with this treatment, radiographic evidence of clearance was not observed (337). It is uncertain, however, whether pre- and probiotics play a significant role in correcting this painful condition, or whether favourable results are achieved by dosing with high-fiber pulverized psyllium seeds alone. This concept, also, has recently been challenged, with one report of more effective removal of accumulated intestinal sand when psyllium seed husk therapy was supported by the addition of magnesium sulphate via nasogastric delivery (338).

The addition of probiotics to prebiotic supplementation has also been shown to assist in improving the composition of the equine microbiome (195) and provide protection against excess colonization of microbial pathogens, thereby reducing the requirement for antibiotic use (329). A recent review reported on the safety, tolerability and efficacy of probiotic bacteria currently in equine veterinary use, revealing conflicting evidence for the overall health benefits of probiotic bacterial treatments for horses, although some specific benefits for managing scouring in foals and improving athletic performance were reported (194). In general, recent publications are supporting the use of prebiotics and probiotics to improve animal health by manipulating the microbiome of many different domestic, livestock and wild species, without the need for antibiotic use, thus creating significant societal and economic impact (339).

4.9 CONCLUSION

Current evidence for the use of prebiotics and synbiotics in the management of equine health and disease is not extensive, although it could be considered promising and practical in many cases. Prebiotics and synbiotics, when added to the equine diet, have been shown to improve insulin sensitivity for obesity prevention, to reduce systemic inflammation and allergy markers and to correct intestinal dysbiosis. Reduction of hindgut acidosis and correcting intestinal dysbiosis by the

administration of prebiotic fibre to the equine diet may also confer protection against serious inflammatory conditions such as colitis and laminitis. In addition, prebiotic supplementation may protect gastric mucosa from ulceration by acidity resulting from rapid processing of ingested sugars in the equine stomach, thus reducing risk of developing equine gastric ulcer syndrome. There also exists potential for synbiotic supplements to reduce requirement for antibiotic use in horses by enabling recovery from dysbiosis and improving intestinal microbiome composition. Finally, the thoroughbred racing industry might consider adopting the safe and legal practice of administering prebiotics to their athletes, to assist energy production from volatile fatty acids resulting from microbial fermentation.

CHAPTER 5: THE IMPACT OF PROBIOTICS AND PREBIOTICS ON THE COMPOSITION OF THE EQUINE FAECAL AND SEMINAL MICROBIOMES AND SPERM QUALITY – A PILOT STUDY

This chapter forms the content of the manuscript submitted to the Journal of Equine Veterinary Science, August 2, 2023 (currently under review). The submitted version can be located in the Appendix B4.

Simple Summary

This study involved a small group of fertile miniature pony stallions, recruited for an intervention trial to explore the impact of three nutritional supplements – prebiotic, probiotic and a combination of these (synbiotic) – on the microbial composition of their faeces and semen, as well as their sperm quality. Stallions were assigned three treatments and controls on rotation over 12 months, with microbial DNA profiling conducted on faecal and semen specimens and a comprehensive analysis of six sperm quality biomarkers undertaken at each crossover point and at completion of the trial. Analysis was undertaken of microbiome characteristics and sperm quality resulting from each treatment. A significant variation in diversity of bacterial species was found in both faeces and semen across all ponies, while prebiotic fibre supplementation somewhat improved the diversity of faecal bacteria as compared to probiotics, synbiotics and control. No effect was measured on either semen microbiome characteristics or sperm biomarkers for any of the treatments, all of which were well tolerated by the ponies without adverse events. Further exploration of this novel research topic with larger numbers of stallions is recommended.

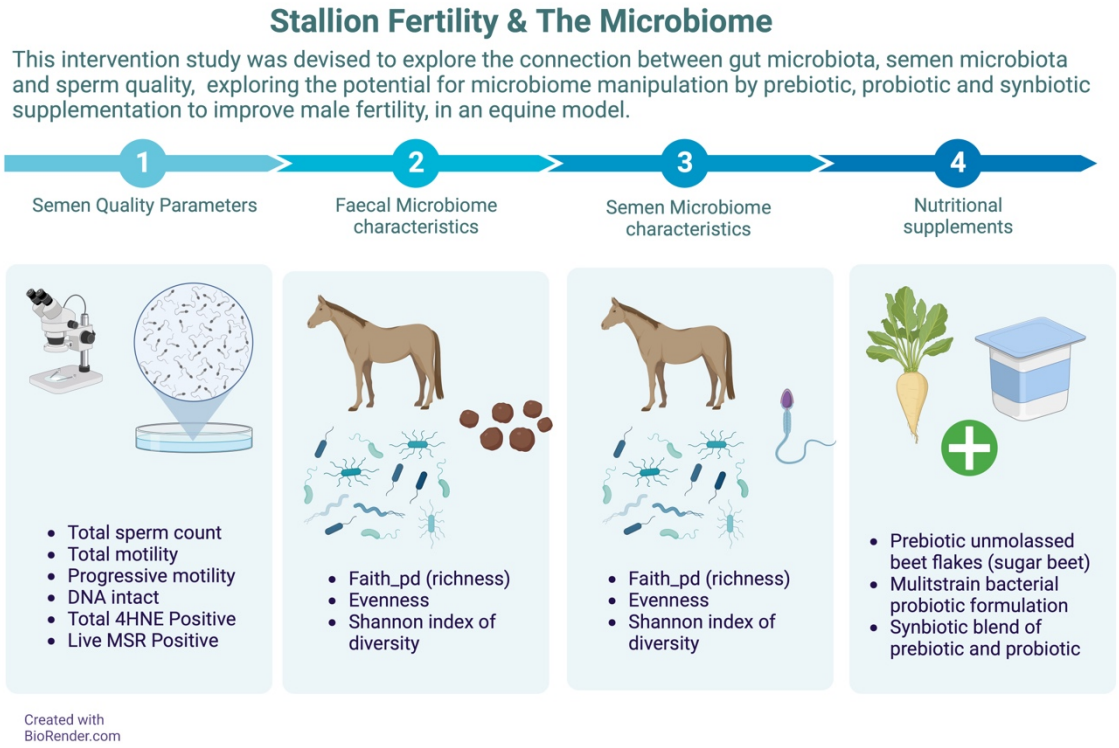

Figure 5.1: Graphical abstract of stallion fertility and the microbiome intervention trial.

5.1 INTRODUCTION

Genetics, age, nutritional and environmental factors are known to influence equine fertility and the composition of microbial communities (23, 24, 32, 69). During the equine breeding season, a standard semen analysis (sperm count, motility and morphology) is routinely conducted and stallions classified as satisfactory, questionable, or unsatisfactory for breeding (92). Husbandry practices that support the general health and therefore fertility of the horse, including nutritional interventions, are frequently implemented (340). Despite a growing body of research investigating the use of various nutritional supplements to improve sperm quality and fertility in horses (50), to date, there is no published literature reporting on the effects of pre-, pro- and synbiotic supplementation on either sperm quality or the composition of the equine semen and gastrointestinal microbiomes.

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (240). To date, research exploring their use in equine veterinary practices has mainly used human species and strains from the genera *Lactobacillus* and *Bifidobacterium* and the yeast *Saccharomyces* (194). Probiotic supplementation has predominantly been used to improve digestion in horses by altering the GI microbiota (195), with the goals of reducing the incidence of scouring in foals (196), treating acute enterocolitis in hospitalised horses (197), managing other equine gastrointestinal disorders (158), and for improving athletic performance (198, 199). The exact mechanisms by which these effects are conferred have not been fully elucidated but are thought to be via competitive inhibition, the production of antimicrobial proteins including bacteriocins, and metabolomic effects associated with the production of beneficial short chain fatty acids (195). However, a specific role for probiotics in influencing equine fertility has not been explored.

Prebiotics are non-digestible food ingredients that selectively promote the growth of probiotic microorganisms in the intestines and increase microbial diversity and richness (341, 342), and are marketed for use in supporting equine digestion, metabolism, growth and immunity (317). Synbiotics are "a mixture comprising live microorganisms and substrate(s) selectively utilised by host microorganisms that confers a health benefit on the host" (343), and thus they contain prebiotic substances and probiotic bacteria and/or yeasts. Their effects have been evaluated in athletic performance, increasing the production of volatile fatty acids associated with hindgut fibre fermentation, insulin resistance, ca26rbohydrate metabolism associated with reduction in the development of gastric mucositis, and hindgut acidosis and laminitis (unpublished data). Prebiotic fibre is thought to have an entero-protective effect by improving the composition and diversity of the intestinal microbiota, which in turn impacts immune function via metabolomic effects (344). While not specifically studied in horses, *Enterococcus faecium* DSM 7134 fed to sows as a probiotic was shown to prevent 'starvation sterility' and increase feed intake, thereby producing larger litters with less perinatal loss (200). In hens, supplementation of feed with *Clostridium butyricum* has been shown to improve egg quality (201).

Indirect and direct effects of pre-, pro- and synbiotic supplementation on the diversity, richness and evenness of the semen and GI microbiomes of stallions, and potential associations with parameters of sperm quality, are yet to be examined. However, the function of the reproductive microbiome has been explored in mares (137)and other mammalian species (133) and a recent study has characterised the stallion semen microbiome (138). The GI microbiome of healthy horses has also been evaluated to identify core species that are characteristic of general health (129); however, the majority of equine microbiome research has focussed on the role of the pathogen in equine diseases such as colitis (130), and the finding that pathogenic bacteria can contribute to infertility in mares (131) and stallions (132). The compositions of healthy GI and semen microbiomes have not been explored specifically in relation to sperm quality. To date there is only one report on the relationship between the intestinal microbiome of men, their sperm quality and hence their fertility (133).

Equine models are being increasingly used to pave the way in human research (26, 30, 31). Research in a murine model has shown the significant role of the blood-testis barrier (BTB) in spermatogenesis, protecting germ cells from toxins and immune attack by maintaining tight junctions between the Sertoli cells, the peritubular myoid cells and interstitial epithelial cells of the testis (345). Under the influence of testosterone and cytokines the permeability of the BTB can alter (346), weakening the selectivity of the BTB; it is possible that blood-borne bacterial toxins may also increase permeability of the BTB in the same way that endotoxins penetrate the blood-brain barrier (347); and that gastrointestinal endotoxins have the potential to damage sperm DNA during their development by driving up inflammation (348), resulting in poor sperm quality and impaired fertility. Remarkably, beneficial bacteria resident in the semen can offer protection by manipulating intercellular adhesion molecules occludin, zonulin-2 and E-cadherin to reduce BTB permeability (134) (See Figure 5.2).

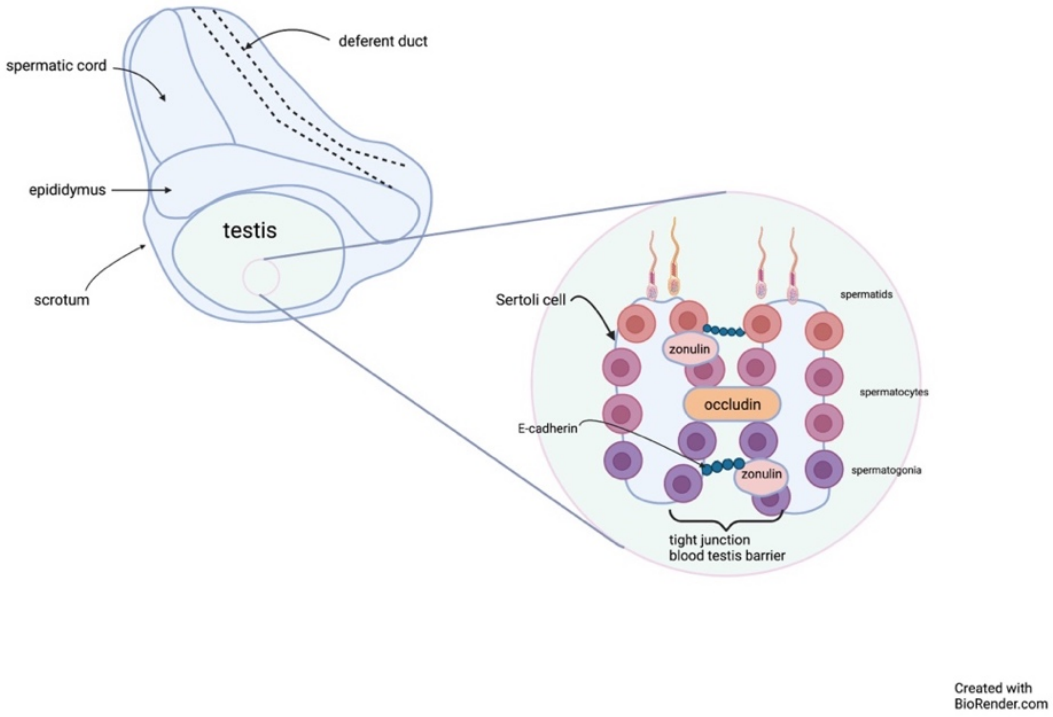

Figure 5.2: Structure of the stallion testis with detailed view of the blood-testis barrier, regulated by adhesion molecules zonulin, occludin and E-cadherin, which maintain tight junctions between Sertoli cells.

The hypothesis for this study was that pre-, pro- and/or synbiotics would increase the diversity, richness and evenness of the GI and seminal microbiomes, facilitating metabolomic crosstalk between the gut and testis, to assist healthy spermatogenesis and hence improve sperm quality. The aim of this study was to evaluate the impact of a multispecies probiotic, a prebiotic fibre, and a synbiotic combination of both on the composition of the faecal and semen microbiomes and sperm quality of stallions (see Figure 5.1).

5.2 MATERIALS AND METHODS

5.2.1 SUBJECTS

Four miniature pony stallions ranging in age between 4 and 14 years of age, housed in Williamtown, NSW, Australia, were recruited for this study, which ran from October 2019 to December 2020.

5.2.2 EXPERIMENTAL DESIGN

As presented in Figure 3.3, a Latin square crossover design without replicates was employed in this study. Faecal and semen specimens were collected at four different time points from October 2019 to November 2020 from four pony stallions in good health. Each pony received the same base diet and was rotated to receive each of the three supplemental treatments (a pre-, pro- or synbiotic combination) at different time points.

Semen and faecal samples were collected from the ponies following each treatment period. Each daily feed/supplementation cycle was 11 weeks to allow sufficient time for the microbiome to adjust to the new regimen (153) and completion of a full spermatogenic cycle of 57 days (349), at the end of which semen and faeces were collected from each pony. No supplementation was given to the ponies for four weeks following and prior to starting the next arm of the study. Treatments were rotated in a Latin square method for a total of four collection periods.

5.2.3 SUPPLEMENTAL FEEDING SCHEDULE PLUS INTERVENTION

Quantities of feed and supplements were calculated according to the body weight of each pony including their lucerne chaff. Canola oil (10 mL) was given as a placebo for the probiotic formulation, Protexin® Liquid (IAHP), (10 mL), for which the carrier is also vegetable oil. The prebiotic, Speedi-Beet® (Barastoc), was dosed at 200–300 g. Each pony received Equine Vit&Min Essentials (www.farmalogicglobal.com/equine-vitamin-supplements/evm-essentials/) and coarse salt as part of standard care. Ponies grazed on the same type and quality of pasture with supplemental lucerne chaff.

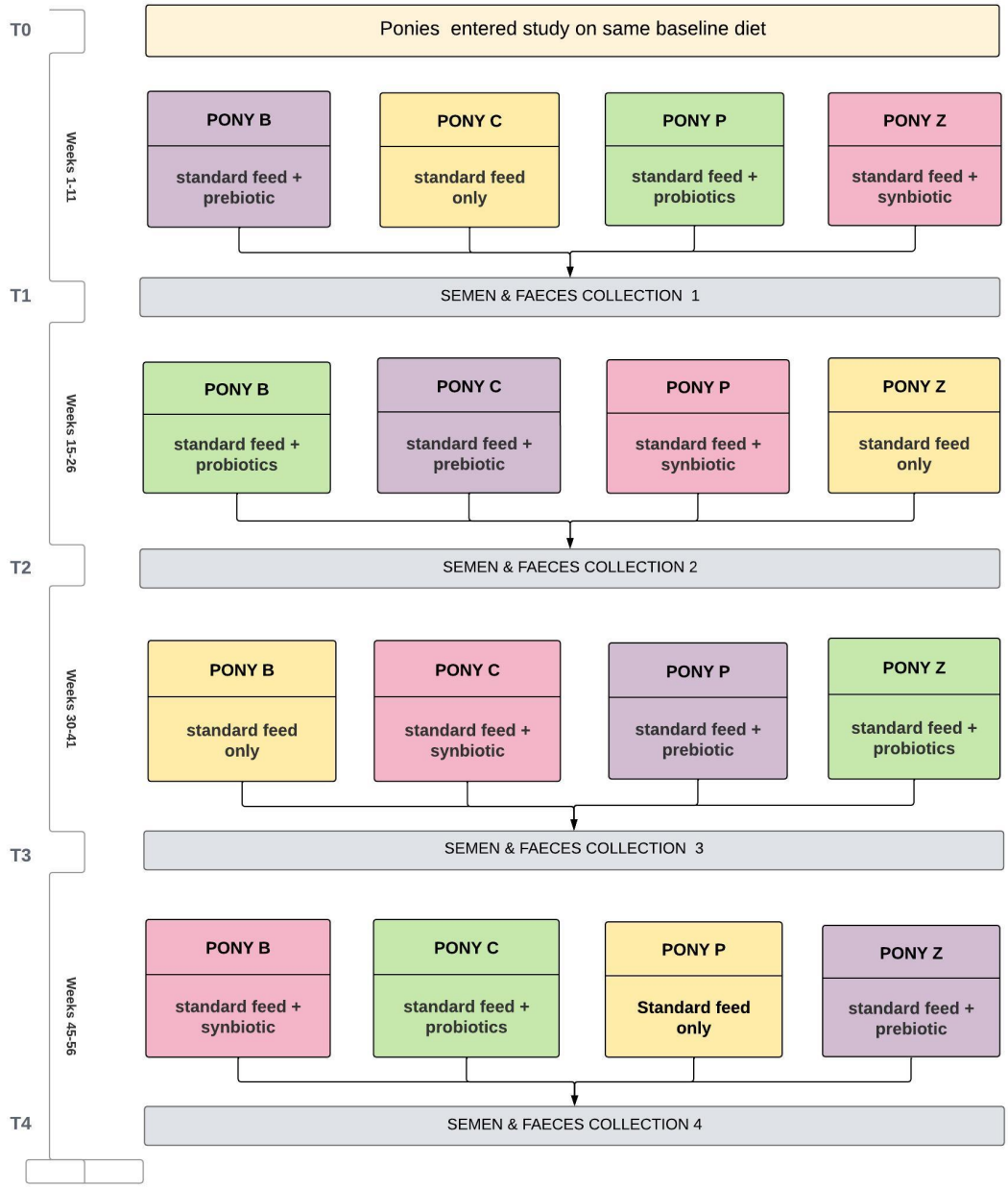

Figure 5.3: Design of experiment involving four miniature pony stallions receiving three different treatments on rotation, including control periods.

5.2.4 COLLECTION OF SEMEN AND FAECAL SAMPLES

Semen was collected from the pony stallions using a Missouri artificial vagina (Minitube Australia, Ballarat, VIC) with an in-line gel filter, and a phantom mount. Following semen collection, 250 µL was immediately removed and diluted with 20 µL of DNA Microshield after which it was stored at −30 °C until analyses. The remaining semen sample was diluted 2:1 (diluent:semen) with EquiPlus (Minitube), and stored at

room temperature (RT) in the dark until being transported to the laboratory for semen analyses (within 3 h of collection). All care was taken by the veterinary research assistants to ensure the comfort of the animal during semen collection, aiming to minimise any distress to the animal whilst being handled.

Faecal balls were collected within 1 h of defecation from each of the stallion's own paddocks for sampling. Faecal balls were opened and core contents, which had not contacted pasture, were extracted using forceps. From these faecal balls 250 mg of material was placed into three 5 mL specimen vials, each containing 3 mL DNA Microshield, and stored at ambient temperature for transport to the laboratory within 4–6 h. Faecal samples were then snap frozen in liquid nitrogen and stored at −80 °C until all samples were collected at the end of the trial for final assessment.

Faecal and semen samples were transported on dry ice to the Central Analytical Research Facility (CARF), Queensland University of Technology (QUT) for PacBio sequencing and DNA extraction. Specimens were analysed as one batch to eliminate any variations often associated with multiple batch analysis of 16S rRNA.

5.2.5 STANDARDS/REAGENTS FOR SEMEN ANALYSIS

Sigma-Aldrich supplied all chemicals used for analysis of semen samples, unless otherwise indicated.

Throughout this study a modified Biggers, Whitten, and Whittingham (BWW) medium (239-241) was used, which comprised 95 mM NaCl, 4.7 mM KCl, 1.7 mM CaCl2.2H2O, 1.2 mM KH2PO4, 1.2 mM MgSO4.7H2O, 25 mM NaHCO3, 5.6 mM D-glucose, 275 mM sodium pyruvate, 3.7 mL/mL 60% sodium lactate syrup, 50 U/mL penicillin, 50 mg/mL streptomycin, 20 mM HEPES, and 0.1% (w/v) polyvinyl alcohol, with an approximate osmolarity 310 mOsm/kg and pH of approximately 7.4.

5.2.6 SPERM PARAMETERS

For preparation of spermatozoa, upon arrival in the laboratory, extended semen was aliquoted into 15 mL conical tubes and centrifuged at 350 x *g* for 15 min to pellet the cells. The supernatant was removed, and the resulting pellet resuspended to 20 x 10^6

spermatozoa/mL in BWW, as determined by automated cell counter, NuceloCounter(R) SP-100 (Chemometec). This cell suspension was utilised for all proceeding sperm assays.

5.2.6.1 MORPHOLOGY

An aliquot of washed sperm suspension was fixed using 2% paraformaldehyde (5 min at 5 °C), after which it was washed in PBS via centrifugation and stored in 0.1 M glycine in PBS at 5 °C for up to one week until analysis. An assessment of morphology was made by directly visualising sperm via phase-contrast microscopy (Zeiss Axio Imager A1) under oil immersion at 1000x magnification. From a field of approximately 100 cells, spermatozoa were classified as being either normal, or with abnormalities of the head, midpiece, or tail, along with the presence of any distal or proximal cytoplasmic droplets (350).

5.2.6.2 VITALITY

Employing LIVE/DEAD Far Red Fixable Stain (Molecular Probes, Australia), sperm vitality was assessed. These cells were incubated at 37°C for 20 min, according to the manufacturer's instructions with reconstituted stain, at a concentration of 0.5 mL/mL. per cells. Following this staining process, spermatozoa were washed in BWW via centrifugation, and then fixed in paraformaldehyde (as for the morphological analyses). Samples were analysed using flow cytometry (FACSCanto, BD, USA), after which they were classified as being either vital (unstained) or nonvital (highly stained). A snap-frozen ('dead') positive control was utilised for gating purposes.

5.2.6.3 TOTAL CELL COUNT

Sperm concentration was determined using a NucleoCounter® SP-100 (Chemometec, Denmark) according to the manufacturer's instructions.

5.2.6.4 COMPUTER-ASSISTED SPERM ANALYSIS (CASA) OF MOTILITY

Using computer-assisted sperm analysis (CASA; IVOS, Hamilton Thorne, Danvers, MA) to determine sperm motility objectively, the following settings were employed: negative phase-contrast optics, recording rate of 60 frames/s, minimum contrast of 70, minimum cell size of 4 pixels, low size gate of 0.17, high size gate of 2.9, low-

intensity gate of 0.6, high-intensity gate of 1.74, nonmotile head size of 10 pixels, nonmotile head intensity of 135, progressive average path velocity (VAP) threshold of 50 mm/s, slow (static) cell VAP threshold of 20 mm/s, slow (static) cell velocity (VSL) threshold of 0 mm/s, and threshold straightness (STR) of 75%. Cells that exhibited a VAP of ≥50 mm/s and an STR of ≥75% were considered progressive. At a stage temperature of 37 °C and using 20 mm Leja standard count slides (Gytech, Australia), a minimum of 200 spermatozoa were assessed In at least five fields.

5.2.6.5 SPERM DNA INTEGRITY

For assessment of sperm DNA integrity, the sperm chromatin structure assay (SCSA) was performed according to the guidelines produced by Evenson and Jost (243). Briefly, aliquots of sperm suspension were further diluted to a of 10 x 10^6 cells/mL, then snap frozen in liquid nitrogen and stored at −80 °C until assessment. On the day of analyses, samples were thawed at 37 °C and then maintained on ice until analysis. Prior to flow cytometric analysis, 200 mL of cold acid detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton X-100, pH of 1.2) was added to 100 mL of sperm suspension and, exactly 30 s later, 600 mL of acridine orange staining solution (0.1 M citric acid, 0.2 M Na2PO4, 1 mM EDTA, 0.15 M NaCl, 22.6 mM acridine orange, pH 6.0) was added to this mixture. Samples were run on low flow rate using a FACSCanto flow cytometer with a standard argon laser (488 nm) and FACSDiva software. The sample was allowed to equilibrate for 3 min, after which data from 10,000 cells was recorded. Using a forward scatter/side scatter dot plot, cell debris were gated out by drawing a region around sperm cells. The percentage of cells revealing detectable DNA fragmentation (stained red), the DNA fragmentation index of cells (mean red fluorescence divided by the sum of mean red and mean green fluorescence, i.e., single-stranded DNA as a proportion of total DNA), and the percentage of poorly protaminated cells (high green fluorescence), were calculated and recorded.

5.2.6.6 SPERM LIPID MEMBRANE PEROXIDATION

Sperm lipid membrane peroxidation was evaluated by the presence of the 4-hydroxynonenal (4HNE) adduct using an anti-4HNE antibody (Jogmar Diagnostics, TX, USA). Approximately 2 x 10^6 spermatozoa were pelleted via centrifugation,

resuspended in a 1:50 dilution of antibody in BWW containing 1:2000 Far Red LIVE/DEAD Fixable stain as a vitality probe, and incubated for 30 min at 37 °C. Cells were washed twice in BWW following incubation, then resuspended in a 1:100 dilution of FITC-labelled secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG; Molecular Probes, Australia) in BWW, and further incubated for 15 min at 37 °C. Prior to flow cytometric analysis, sperm were washed twice and then resuspended in BWW. A secondary antibody-only control was used to set the gate between low (background fluorescence) and high levels

of lipid peroxidation.

5.2.6.7 MITOCHONDRIAL ROS PRODUCTION

Production of superoxide by mitochondria was measured by incubating sperm with 2 mM MitoSOX Red (MSR; Molecular Probes, Australia) and 5 nM Sytox Green vitality stain (Molecular Probes, Australia) for 15 min at 37 °C, after which they were washed once via centrifugation and resuspended in BWW for flow-cytometric analysis. Only live cell data were used for statistical analyses, as due to an artefact, all membrane damaged cells stain positive for MSR (as residual ethidium in the probe binds to DNA). Gating controls included snap-frozen (dead) cells stained with Sytox Green only, and a positive control, using 50 mM arachidonic acid with MSR alone.

5.2.7 MICROBIAL DNA PROFILING OF SEMEN AND FAECES

PacBio DNA extractions, library preparation and sequencing were performed as follows. DNA was extracted using the ZymoBiomics DNA Miniprep kit (Zymo Research) from a 150 mg sample of faeces, following the standard protocol for faecal extraction. For seminal fluid samples, 150 µL was first digested with 5% (v/v) of Proteinase K (Cat. No. D3001-2-5) at 55 °C for 30 min before following the standard protocol. PacBio libraries were prepared following the Full-Length 16S Amplification, SMRTBell® Library Preparation and Sequencing Procedure and Checklist (Pacific Biosciences), using 2.5 ng extracted DNA for faecal samples and 15 ng for semen samples. Initial PCR was carried out with KAPA HiFi HotStart ReadyMix (Roche), with 20 cycles in 25 µL reactions following protocol cycling conditions. Amplified DNA was

indexed using the barcoded F/R Universal Primers Plate 96 v2 (Pacific Biosciences) and PCR products were purified using AMPure PB (Agencourt Bioscience), according to PacBio's protocol.

Amplicon sizes were verified with a Fragment Analyser 5200 (Agilent Technologies) and Qubit 4.0 Fluorometer (ThermoFisher Scientific) using the High Sensitivity dsDNA assay. Barcoded amplicons were pooled before library construction with the SMRTBell® Express Template Prep Kit 2.0 (Pacific Biosciences). Faecal samples and semen samples were pooled separately, with 20 samples per pool, sequenced on separate chips. Sequencing was carried out on a PacBio sequel (Pacific Biosciences) with v3 sequencing chemistry in CCS collection mode for 20 h on a 1M v3 LR SMRT Cell. CCS analysis was then run before demultiplexing and fastq conversion.

5.2.7 MICROBIOME COMPOSITION MEASURES

Several measures of microbiome composition were evaluated in the faeces and semen of the study animals: faith_pd, evenness and Shannon diversity of bacterial species. Faith_pd is an abbreviated term referring to Faith's phylogenetic diversity, a measure of microbial species richness within an individual sample. Evenness refers to the abundance of species within a single sample with a numerical range of 0–1. The Shannon Index is a measure of species diversity within a single sample, with respect to the total unique species count, calculated as EH = H / ln(S), where H represents the Shannon Diversity Index and S represents the total number of unique species (351).

5.2.8 STATISTICAL METHODS

Prior to data analysis, bioinformatics analysis for this work was performed on the HPC server 'Artemis' at the University of Sydney. The quality of the raw reads were checked using the package FastQC (Version 0.11.9) (352). The next-generation microbiome bioinformatics platform QIIME2 (version q2cli version 2020.8.0 https://qiime2.org/) (216) was used for analysing the amplicon data. The first step involved generation of Amplicon Sequence Variants (ASVs) from the raw amplicon data, using the algorithm DADA2 (353). Since the available version of QIIME2 did not include the DADA2

capability to handle Pacbio reads, we used the DADA2 package externally (version 1.19.2). The ASVs display advantages over the more traditional approach (OTUs) as they represent the exact amplicon sequences without coarse graining into OTUs and can resolve even single nucleotide base differences. DADA2-formatted training fasta files (354) were derived from the Silva Projec''s version 128 release and used for taxonomy assignment. Three output files were generated using DADA2. These included the sequence feature count tables, the taxonomy identification file per ASV and a fasta file containing sequences of all ASVs. These files were exported in a phyloseq (355) format for further analysis using QIIME2. These files along with the sample metadata files were used as input for different modules in the QIIME2 workflow.

Data produced by QIIME2 exists as QIIME2 artifacts, which contain data and metadata. The feature tables imported from DADA2 were filtered to retain the features that were observed in at least two samples. A rooted phylogenetic tree was generated using the filtered feature table for downstream diversity analysis. The next steps in the QIIME2 analysis involved computation of alpha diversity indices.

5.2.9 DATA ANALYSIS

The data was prepared in Microsoft Excel (2021). The distribution of each variable in the data set was checked using summary statistics and frequency tables in SPSS software (v28, 2021). The distribution of the twelve outcome variables by the categories of the treatment variable were summarised and plotted. A general linear model for each of the twelve outcome measures (six semen parameters, three faecal microbiome and three semen microbiome measures) was run adjusting for the blocking variables 'Individual' and 'Timepoint'. The predicted means and 95% confidence intervals from these models were reported. Model fit was checked by creating a scatter plot of predicted values versus residuals. Additionally, the means of the paired faecal and semen sample variables for faith_pd (richness), evenness and Shannon Index (diversity) were compared using a paired t-test. ASV count data was aggregated at the Phyla level and sorted from highest to lowest frequency. The top

10 phyla were plotted and phyla with low frequencies were combined to an "Other" category for plotting.

5.3 RESULTS

In this healthy cohort of four miniature pony stallions, no significant effect on parameters of sperm quality was seen from any of the treatments, nor was there any statistically significant change to the composition of either the faecal or semen microbiomes associated with these interventions when compared to controls. However, significant differences between the bacterial composition of the semen and that of the GI microbiomes were found, with strong evidence that the means of faecal and semen evenness and Shannon's diversity were not equal ($t=10.5$, $df=15$, $p<0.001$ and $t=20.4$, $df=15$, $p<0.001$, respectively). There was no evidence that the means for the faith_pd samples from faecal and semen samples were different (t statistic=1.2, $df=15$, $p=0.245$). Bacteroidetes and Firmicutes comprised the predominant phyla in both control microbiomes, together accounting for up to 56% of faecal bacteria detected (Figure 3.4) and up to 90% of semen bacteria detected (Figure 5.5). Verrucomicrobia, Spirochaetae, Fibrobacteres, Lentisphaerae, Planctomycetes and Cyanobacteria were in the top ten phyla present in faecal samples (Figure 5.4) but these phyla were not detected in semen profiles (Figure 5.5); Fusobacteria, Actinobactera, Synergistetes and Fibrobacteres phyla were detected in semen samples but not in faecal profiles. Proteobacteria and Tenericutes phyla were present in both faeces and semen and variations in their abundances were generally observed when comparing faecal profiles with semen profiles.

5.3.1 EFFECT OF INTERVENTIONS ON PARAMETERS OF SPERM QUALITY

Table 5.1 presents the mean values and SD for the six parameters of sperm quality by treatment group. The results of the general linear model used in this analysis showed there was no significant effect on the mean values of the total sperm count, total motility percentage, progressive motility percentage, DNA intact %, total 4HNE

positive % and live MSR positive % observed for any of the interventions when compared to the control.

Table 5.1: Effect of pre-, pro- and synbiotic interventions on parameters of sperm quality.

Sperm Parameter	**Control** **Mean (SD)**	**Prebiotics** **Mean (SD)**	**Probiotics** **Mean (SD)**	**Synbiotic** **Mean (SD)**	**P-value**
Total sperm count (x109)	8.1 (± 5.1)	14.7 (± 19.2)	6.4 (± 4.6)	5.1 (± 3.7)	0.631
Total motility %	61.8 (± 11.7)	62.0 (± 17.7)	63.7 (± 9.0)	71.7 (± 13.5)	0.481
Progressive motility %	30.3 (± 13.6)	30.5 (± 14.8)	29.1 (± 11.7)	32.7 (± 10.8)	0.906
DNA intact %	93.0 (± 3.3)	91.0 (± 4.2)	93.3 (± 3.1)	90.5 (± 7.9)	0.288
Total 4HNE Positive %	30.9 (± 17.1)	41.6 (± 13.3)	33.7 (± 10.5)	35.5 (± 9.6)	0.430
Live MSR Positive %	13.2 (± 10.6)	13.1 (± 12.6)	10.9 (± 8.8)	21.6 (± 19.6)	0.210

5.3.2 EFFECT OF INTERVENTIONS ON GI MICROBIOME COMPOSITION MEASURES

Table 5.2 presents the mean value and standard deviation (SD) for the three GI microbiome measures in response to the three treatments. Increases in the GI microbiome faith_pd (p=0.09) and Shannon diversity (p=0.07) measures provided some evidence of a response to the prebiotic supplementation. No statistically significant differences in the faith_pd, evenness or diversity measures were found in response to the pre-, pro- or synbiotic interventions.

Table 5.2: Faecal microbiome measures in four miniature pony stallions resulting from pre-, pro- and synbiotic interventions.

Microbial composition	Control Mean (SD)	Prebiotics Mean (SD)	Probiotics Mead (SD)	Synbiotic Mean (SD)	*p*-value
Faith_pd/ richness	37.6 (± 2.9)	39.9 (± 3.2)	36.3 (± 1.8)	37.9 (± 1.5)	0.094
Evenness	0.83 (± 0.03)	0.88 (± 0.02)	0.84 (± 0.02)	0.85 (± 0.04)	0.105
Shannon diversity	7.4 (± 0.5)	8.0 (± 0.3)	7.4 (± 0.1)	7.5 (± 0.4)	0.073

Regarding the effects on the abundance of bacterial phyla within faecal specimens, Figure 5.4 presents the relative ASV abundance by percentage distribution of the predominant phyla identified, by treatment groups, showing Firmicutes, Bacteroidetes and Verrucomicrobia were the most abundant in the faeces of all miniature pony stallions for all treatments and time points.

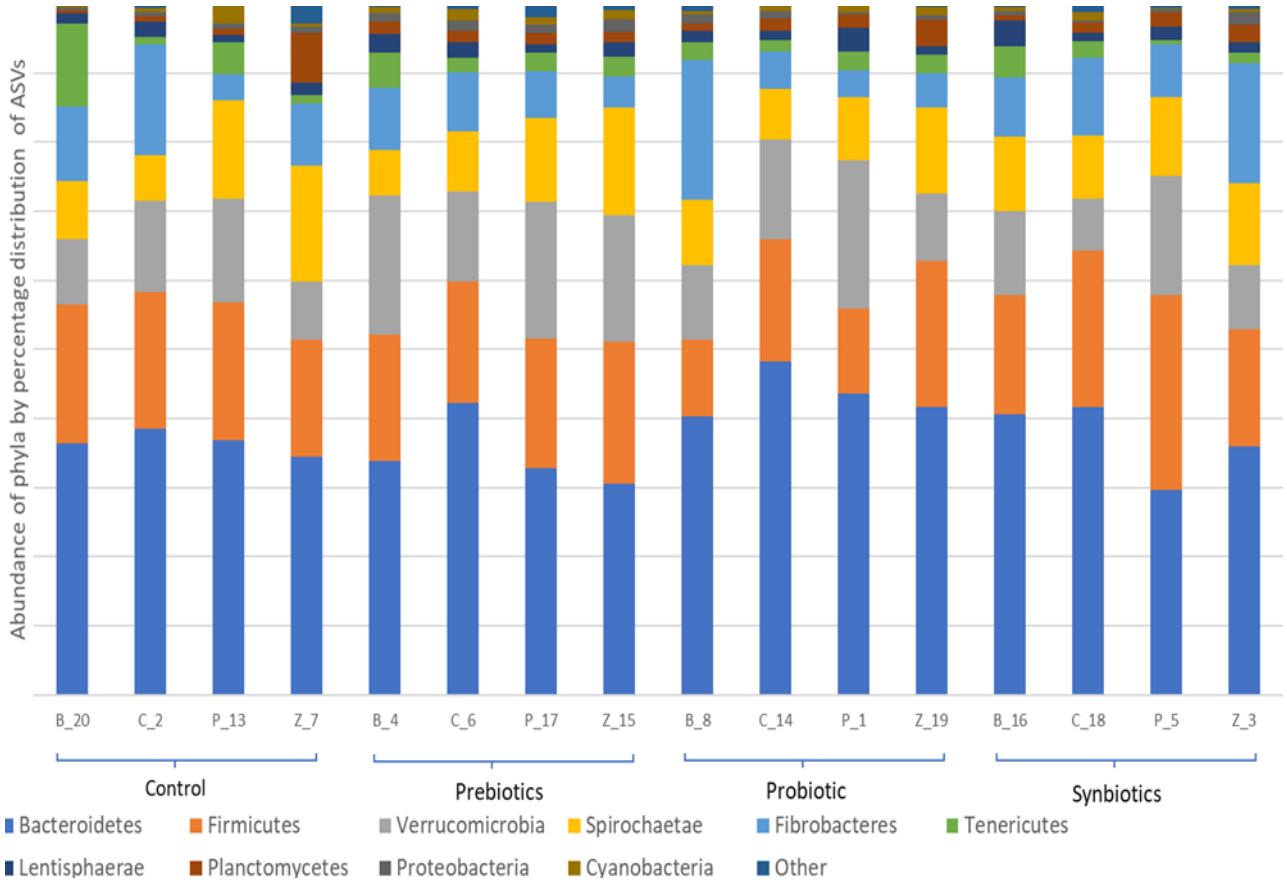

Figure 5.4: Abundance and distribution of phyla in faecal samples of four miniature pony stallions by treatment group.

5.3.3 EFFECT OF INTERVENTIONS ON SEMEN MICROBIOME MEASURES

The effect of the interventions on the semen microbiome are presented in Table 5.3, showing that no differences were found for the mean values of the microbial measures of faith_pd/richness, evenness or diversity ($p>0.05$).

Table 5.3: Semen microbiome measures in four miniature pony stallions resulting from pre-, pro- and synbiotic interventions.

Treatment **Outcome**	**Control**	**Prebiotics**	**Probiotics**	**Synbiotic**	***p*-value**
Faith_pd/ richness	37.3 (± 16.3)	25.9 (± 24.9)	40.4 (± 9.0)	27.5 (± 19.3)	0.235
Evenness	0.67 (± 0.08)	0.68 (± 0.09)	0.67 (± 0.06)	0.66 (± 0.06)	0.931
Shannon diversity	4.0 (± 0.7)	3.9 (± 0.7)	3.9 (± 0.6)	3.8 (± 0.7)	0.870

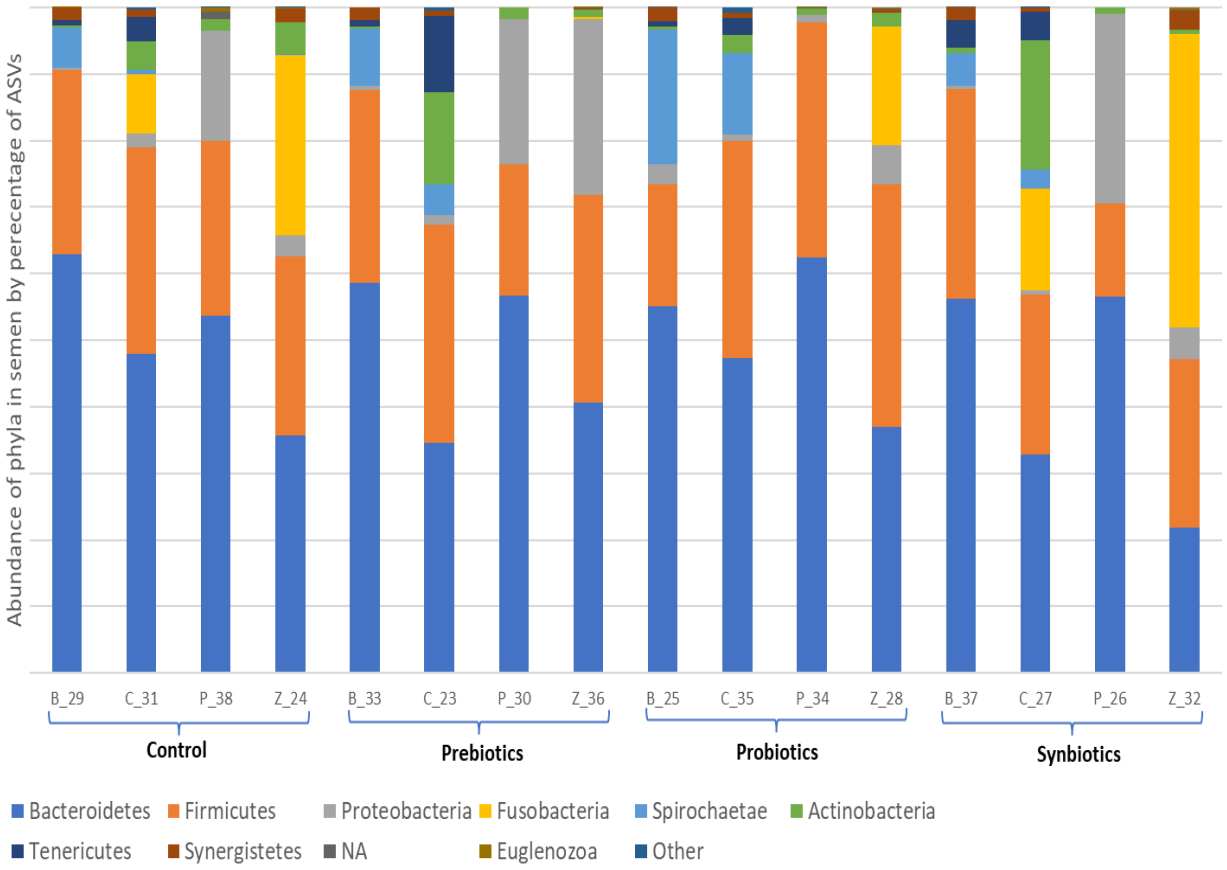

Figure 5.5: Composition of phyla in semen samples of four miniature pony stallions by treatment group.

5.4 DISCUSSION

This study has examined and reported for the first time the effects of pre-, pro- and synbiotics on semen quality markers, in addition to comparing the composition of faecal and semen microbiomes, in miniature pony stallions. Our hypothesis that pre-, pro- and/or synbiotics would increase the diversity, richness and evenness of the GI and seminal microbiomes, facilitating metabolomic crosstalk between the gut and testis to assist healthy spermatogenesis and hence improve sperm quality, was not supported. However, significant differences in the diversity and evenness of the microbiomes of the stallion faeces compared to those of semen were found. Although the interventions did not result in any statistically significant differences in the composition of these microbiomes, some evidence was found for the effects of prebiotics on the diversity and faith_pd (richness) of the GI microbiome. A greater standard deviation is evident in the results of several semen parameters (total count, total motility and progressive motility), as well as the Faith_pd/ richness of both the faecal and semen microbiomes, following prebiotic supplementation, suggesting a higher inter-individual response to this treatment. A higher proportion of Tenericutes phylum was measured in the semen of Pony C following prebiotic supplementation in comparison to the other ponies; this same intervention caused an apparent elimination of Fusobacteria from the semen of all subjects, while synbiotic supplementation produced an increase in Fusobacteria in the semen of Pony Z. In relation to faecal microbial composition following all interventions, the amount of Planctomycetes in the faeces of Pony Z was reduced. Whilst the sample size for this study is small, it is, however, consistent with that of similar studies conducted in the emerging research domain of the equine microbiome (153, 189, 213, 356-360). Importantly, the treatments were well-tolerated by the stallions without any adverse outcomes, such as digestive or metabolic complications, nor were there any negative effects on the ponies' sperm quality throughout the treatment periods.

From a biological plausibility perspective, if the general health and nutrition of the horse is improved, physiological function will improve. Diet has a major impact on the richness and diversity of the equine hindgut microbiome (212), for which reason the four pony stallions recruited for this study were fed the same basic diet of lucerne chaff and pasture

grass, with additional vitamins, minerals and salts, to ensure that no nutritional deficiencies existed and thereby create effective control conditions. Theoretically, had we examined the prebiotic effects on intestinal microbiota in a larger cohort, we may have found stronger (statistically significant) evidence of alterations in the GI microbial composition. The commercially available prebiotic studied, Speedi-Beet® unmolassed beet pulp, is rapidly degraded by ponies (361) and commonly fed to horses of all types as a low-sugar fibre supplement to support digestibility of feeds and hindgut fermentation, and assist in short chain fatty acid and energy production (362). Prebiotic fibre has been shown to alter the microbial composition of equine faeces, with a dose-related shift in favour of the Firmicutes:Bacteroidetes ratio (213). Contrary to this, following supplementation of healthy Thoroughbreds with an amylase-rich malt extract, the proportion of Bacteroidetes populations increased in the faeces and the proportion of Firmicutes reduced (149); this effect of an increased Bacteroidetes:Firmicutes ratio was not measured in the present study for comparison. Although some concerns about the safety of prebiotic use in equids has been raised (316), this concern was not supported by our study, nor in the findings of a recent review (363). Our finding that the prebiotic trialled was not associated with adverse effects supports the proposal that these fibrous feed substances are safe to administer in horses, although larger studies are required to confirm this.

In terms of the commercial probiotic supplement selected for this study, its administration was associated with no effect on the composition of either the faecal or seminal microbiome, nor was there any effect on sperm quality. Although Protexin® is commercially manufactured for use in many domestic animals, this product contains seven bacterial species that are not commonly found either in the equine diet nor resident in the gastrointestinal tract: *Lactobacillus acidophilus*, *L. delbruekii subspecies bulgaricus*, *L. plantarum*, *L. rhamnosus*, *Bifidobacterium bifidum*, *Enterococcus faecium* and *Streptococcus salivarius subspecies thermophilus*. While common practice, to some degree, the use of human probiotic strains (as opposed to those found in the equid) may have contributed to the results of this study. The probiotic species used all belong to the Firmicutes phylum, hence it would be expected to see greater representation of this

phylum in the faecal microbiome profiles of the subjects fed either probiotics alone or synbiotics; however, this was not the case. Consistent with findings in humans, probiotic supplementation may support temporary colonisation of the gastrointestinal tract with commensal species; however, it is thought extrinsic probiotic species do not themselves establish sustainable colonies, with any effect they might exert on the region not persisting beyond the period of administration (364, 365). In our study, following each treatment crossover when the diet is altered to either include or exclude prebiotic supplementation, no persistent increase of Firmicutes populations was observed.

In addition to prebiotic fibre, probiotics may benefit the digestion of horses by increasing their capacity to process fibre in the hindgut, conveying protection upon the diversity of their intestinal microbiome and stabilising bacterial populations, particularly under conditions of abrupt dietary change (366). Other microbial species, for example the nutritional yeast *Saccharomyces cerevisiae*, have been administered to horses to evaluate resultant changes occurring in faecal microbiomes; a commercial formulation containing 4% *S. cerevisiae* was administered in a similar crossover treatment protocol, employing both high-starch and high-fibre diets, demonstrating benefits to hindgut acidosis and fibre digestibility (356). It should be noted that the probiotic formulation employed in that particular study contrasts with the commercial multi-strain probiotic product chosen for our study, which contains only bacterial species.

Synbiotics, specifically the combination of unmolassed beet flakes and the multistrain bacterial probiotic used in this study, were expected to have the most impact on the composition of the faecal microbiomes due to potential compound effects, but no such lasting impact was observed. Based on limited evidence to date, the combination of the pro- and prebiotic to provide a synbiotic formulation would exert an effect on the composition of the intestinal microbiome by increasing the probiotic bacteria's viability and reducing the production of unfavourable bacterial metabolites (367). For this reason, it was expected that the effect of the synbiotic intervention on the faecal and perhaps also the semen microbiome composition of our subjects would be greater richness and diversity. However, this was not the case. While not statistically significant, the synbiotic intervention tended to alter the composition of the ponies' semen microbiomes by

increasing the proportion of Actinobacteria, Proteobacteria, Fusobacteria and Firmicutes while reducing the proportion of Firmicutes and Bacteroidetes. This observation suggests that the majority of shifts in the distribution of the phyla of the semen microbiomes were associated with synbiotic treatments rather than prebiotics and probiotics alone. Interestingly, human research has equated a lower semen Firmicutes:Bacteroidetes ratio to poorer fertility outcomes (85). No such correlation was found in the present study.

The significant differences between the bacterial composition (diversity and evenness) of the semen and that of the GI microbiomes (as determined by the microbiota present in faeces) found in this study are consistent with other studies showing that the type, abundance and composition of microbes differ between regional microbiome communities (114, 120, 368). It is important to note that faecal microbiota are not necessarily representative of the entire equine GI microbiome, which has been shown to exhibit significant profile variation at different sites of the tract (144). A study by Costa et al. (144) found that Firmicutes was the predominant phylum in each of the equine gastrointestinal regions .

The composition of the GI microbiome is known to be influenced by a range of environmental, diet and husbandry practices, and breed of horse (145, 149, 153, 160, 161, 164, 167, 171, 210, 356, 366), which may account for the similarities and differences observed in the predominant phyla identified in the faeces of the miniature pony stallions and those reported in other studies. The predominant phyla in the faeces of our miniature pony stallions were Bacteroidetes, followed by Firmicutes, Verrucomicrobia, Spirochaetae and Fibrobacteres, which is consistent with a stable hindgut microbial composition reported in other equine cohorts (369). The percentage distribution of phyla differed from the faecal microbial compositions identified in eight healthy active Thoroughbred geldings studied by Proudman et al., where the highest abundance of phyla was Firmicutes followed by Bacteroidetes (149). Additionally, an increased Firmicutes: Bacteroidetes ratio resulted from prebiotic supplementation of ten racing Thoroughbreds when compared to controls (370). Perhaps the fact that these two studies involved active, racing horses has produced a contrasting outcome in phylum ratios to that of our relatively inactive miniature stallions, as it is known that exercise induces changes in the

gut microbiome of horses (371) . Another cohort of five 12-month-old Irish Thoroughbreds of unspecified sex underwent faecal microbial sequencing following a course of antiparasitic drug treatments; the key phyla present amongst these individuals were similar to those in our study, namely Bacteroidetes, Firmicutes, Proteobacteria and Spirochaetae, along with many novel phyla (over 100 new species) not previously identified in equine faeces (359). It is possible that the antiparasitic treatment enabled a greater diversity of commensals to colonise the hindgut of these horses by clearing pathobionts, however this is an area which requires further research. In a study of five healthy pregnant mares from the same farm, the faecal microbial profile of these horses demonstrated similar proportions of bacterial phyla to the miniature pony stallions in our study, with a predominance of Bacteroidetes and Firmicutes, followed by Spirochaetae, Fibrobacteres and Verrucomicrobia, with this faecal microbial composition remaining relatively consistent into the postpartum period (372). In a cohort of seven healthy horses from the same English paddock, which were sampled over a 12-month period on a standard diet with seasonal feed variation for faecal microbiome profiling, the dominant phyla found were Firmicutes and Bacteroidetes, followed by lesser populations of Fibrobacteres, Spirochaetes, Verrucomicrobia and Proteobacteria, demonstrating some seasonal variation in the phyla of lower abundance (159). The faecal microbiota of the English horses, which were also managed on paddock grass and some lucerne chaff, were similar to those of the miniature pony stallions in the present study, which were fed the same basic diet.

The present study identified nine main phyla in the semen of miniature pony stallions, predominantly Bacteroidetes, Firmicutes and Proteobacteria; this is a similar finding to Quiñones-Pérez et al., who reported similar bacterial phyla in the semen of a cohort of Arabian and Andalusian stallions but with slightly different percentage distributions, which may be accounted for by the factors previously discussed (138). Quiñones-Pérez et al. also found a high inter-individual species variability, which is consistent with our findings, as well as those of Al-Kass et al. (189). In comparison to semen microbiome profiling in other species, these equine findings were different in regard to the percentage distribution of these predominant phyla (41, 373-376). For example, the

murine semen microbiome (wild type) was found to contain predominantly Firmicutes, Proteobacteria and Actinobacteria (375). Perhaps the technology chosen by Quiñones-Pérez et al., focussing on the hypervariable region of V3 location, rather than full length 16S rRNA hypervariable regions V1- V9 used in our study or V1–V2 (37) or V4 (373) of 16S rRNA in human studies, may have affected phyla detected. Notably, Hou et al. discovered that there was no typical semen microbial profile characteristic of infertile men and that amongst the diverse taxa identified in human semen were included organisms previously not identified in this fluid (41).

In summary, the pre-, pro- and synbiotic supplements used in this study did not show any statistically significantly impact on the composition of either the equine faecal or semen microbiomes of healthy miniature pony stallions when compared to controls. This study is not without limitations. While the study was considered exploratory and a pilot to establish a proof-of-concept, the sample size was small, resulting in underpowered statistical analyses. Secondly, analysis of Latin square design without replicates assumes that the design variables 'individual' and 'timepoint' are nuisance or blocking variables, preventing an assessment of the interaction between treatment and timepoint. Veterinarians would expect season to confound the effect of treatment on sperm parameters, as the horse is a seasonal breeder; this study ran over 12 months. However, regarding the microbiome, seasons may be less of a confounder as the diet was consistent throughout the year. As a pilot study, however, results such as the means and standard deviations could be used for sample size calculation for larger studies. Finally, metabolomic assays and descriptions of the postbiotic effect of the interventions administered to these stallions was outside the scope of this study due to financial feasibility; however, we would encourage other authors to explore the metabolomic effect of pre-, pro- and synbiotic supplementation of stallions with respect to fertility outcomes.

Notwithstanding these shortcomings, the results of this pilot intervention do provide preliminary efficacy and safety data that can be used to inform the design of larger equine studies that seek to understand the connection between the microbiome composition of faeces and semen and associations with stallion fertility. Future research in this domain could consider different equine populations, such as various breeds, wild versus

domesticated and athletes of different disciplines. Other probiotic organisms which have shown to improve equine digestion, such as the yeasts *Saccharomyces cerevisiae* (242) and *S. boulardii* (197), could be evaluated for specific impact on the semen microbiome, a concept which has not yet been studied, either by single yeast supplementation or as part of a multi-strain formulation with probiotic bacteria. Equine-specific probiotic species have been investigated for therapeutic application (250, 267) and may in the future be administered to stallions for more impact upon the equine faecal and semen microbiomes. Researchers interested in dietary modification of the faecal or semen microbiota of stallions may find the results of our research beneficial for planning other intervention studies.

5.5 CONCLUSION

Treatment of a small cohort of miniature pony stallions with pre-, pro- and synbiotic supplements showed no benefit nor adverse impact on sperm quality and did not adversely impact the general health or digestion, nor significantly alter the semen or intestinal microbiome profiles, of these stallions. However, increases in the diversity and richness measures of the GI microbiome provided some evidence of a prebiotic treatment effect requiring further investigation. As such, the findings of this study can be used to inform the design of future research into the impact of pre -, pro- and synbiotic supplementation on equine GI and semen microbiomes and sperm quality.

CHAPTER 6: INVESTIGATION OF THE RELATIONSHIP OF INTESTINAL MICROBIOME ALPHA DIVERSITY INDICES WITH PER CYCLE CONCEPTION RATES AND SERUM CYTOKINE TNF-ALPHA IN COMMERCIAL THOROUGHBRED STALLIONS

Simple summary

This retrospective observational study investigated the relationships between the intestinal microbiome and inflammation with fertility in commercial Thoroughbred stallions. The key objectives of this research were, firstly, to investigate the associations between faecal microbiome measures (richness, evenness and diversity), cytokine TNF-α, stallion age, stud, certain dietary, nutritional and medical treatments on per cycle conception rate (PCCR); and, secondly, the association of these microbiome indices and age with cytokine TNF-α. Retrospective stud management data were obtained from clinical histories of nineteen Australian Thoroughbred stallions to assess a range of factors thought to impact intestinal microbial composition and stallion fertility data (PCCR). A serum cytokine assay for TNF-α was performed and 16S rRNA faecal microbial profiling was conducted. Univariable linear regression models were used to test association of age, stud, and microbiome indices with PCCR and TNF-α. Microbiome data were aggregated at phylum level and grouped by stud. A significant effect of stud was observed on PCCR, although no association was found between any microbial measure nor stallion age with PCCR and TNF-α levels (the mean TNF-α of this cohort of stallions was found to be low). While not statistically significant, an observation that stallions receiving carnitine supplementation demonstrated slightly higher PCCRs. The top bacterial phyla and α-diversity were measured in the faeces of these stallions. Faecal microbiome composition was consistent across samples in terms of microbial evenness, with *Firmicutes, Bacteroidetes* and *Verrucomicrobia* forming majority of phyla for each horse, however a significant variation in microbial richness was observed between stallions. This study provides baseline data about the faecal microbiome, inflammatory cytokines, and fertility status of a cohort of Australian Thoroughbreds to potentially inform future research into stallion health and fertility. The association between stud farm

management practices and stallion fertility outcomes requires further investigation, particularly with respect to the influence of specific nutritional supplementation regimens.

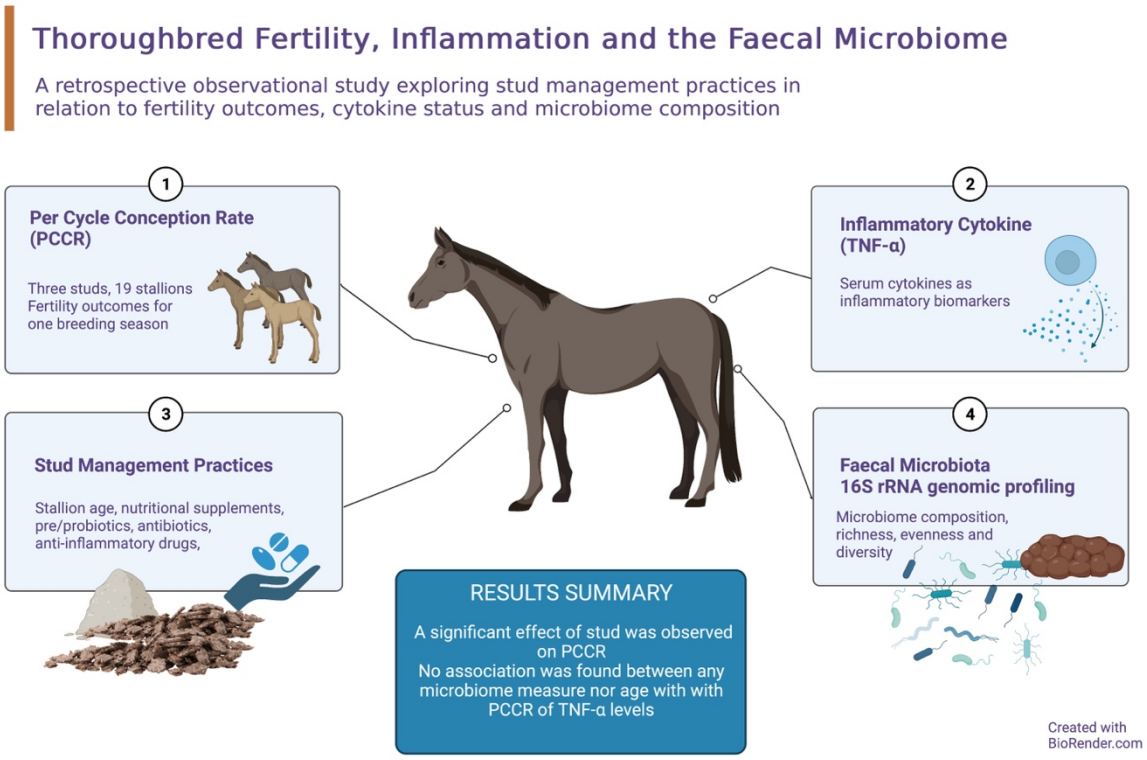

Figure 6.1: A graphical abstract of this study, exploring the relationship between per cycle conception rate (PCCR), an inflammatory biomarker (TNF-α) and the intestinal microbiome (faecal microbial profile) of 19 Thoroughbred stallions, while exploring the effect of stud management practices on these measures.

6.1 INTRODUCTION

Thoroughbred stallion stud practices in Australia are informed by dedicated equine reproduction research, forming a collaboration between industry and academia, seeking to achieve optimal breeding outcomes (377). Thoroughbred stallion health and breeding performance are of paramount importance to owners and stud managers who, each season, invest in feeding and management strategies that aim to maximise fertility outcomes through improving the horses' general health and sperm quality.

Supplementing feed with antioxidants, carnitine and polyunsaturated fatty acids (50, 69) and the herb maca (Lepidium meyenii) (70) have all been shown to improve parameters of sperm quality. The addition of probiotic and prebiotics to feed are widely used in general equine nutrition and are thought to influence the composition and function of the gastrointestinal microbiome (194). However, the composition of the equine gastrointestinal microbiome and effects on systemic health, including associations with inflammation and fertility, have yet to be described.

Research to date has shown there are a range of factors that influence the microbial composition of the equine gastrointestinal tract (110, 129). These factors include different feeding regimens (378, 379), intense exercise (380), and health status (173), including metabolic disorders (293). While the uterine, vaginal (249) and faecal (372) microbiomes of mares have been examined with respect to their impact upon pregnancy outcomes, there remains a gap in veterinary knowledge with respect to the potential influence that the faecal microbiome might have upon sperm quality, fertility and seasonal breeding success in Thoroughbred stallions.

Multiple factors affect semen quality and breeding success in Thoroughbred stallions, such as season (381), number of mares covered (89, 93), age (89, 382), obesity (238), illness or infection (204, 383), medications (384-386) and nutritional supplements (69), especially when administered to older stallions (387). As in other species, the age of the mare has been shown to be more significantly associated with reproductive outcomes than the age of the stallion (89). In regard to physical constitution, a moderate to 'fleshy' body condition of around 6–7 on the US Body Condition Score Chart (51) favours breeding stamina. Equine sperm quality may be improved via supplementation with specific nutrients (50) and endogenous antioxidants (388) and adversely affected by local inflammation (183, 389). The relationship between inflammation and fertility has been demonstrated in men, with an increased expression of interleukin 6 (IL-6) receptor (found at the midpiece of sperm) in subfertile patients with an associated lower sperm count (390). As cytokine receptors have been identified on the equine spermatozoon (391), it is reasonable to hypothesize that the same relationship may exist in the equine species. However, linking the gut microbiota with systemic inflammation and fertility is relatively

new. Dietary prebiotic intake has been shown to improve the composition of the human intestinal microbiome and increase serum anti-inflammatory cytokine expression, whilst suppressing expression of pro-inflammatory cytokines (392). Further, intestinal microbiota can influence the permeability of the blood-testis barrier, which may further impact upon spermatogenesis (345). In consideration of these factors, it is plausible that low-grade inflammation caused by disruption of the composition of the intestinal microbiome (dysbiosis) and injury to testicular germ cells is associated with reduced sperm count, morphology, and function.

Therefore, the aim of this observational study was to investigate factors associated with per cycle conception rates (PCCRs) and the cytokine, tumour necrosis factor (TNF-α), in commercial Thoroughbred stallions. The specific objectives of the study were to (1) investigate any associations between three microbiome summary measures (richness, evenness, and diversity), inflammation (TNF-α), age, stud management (diet and nutrition prebiotics, probiotics and carnitine supplements), as well as treatments (antibiotic and anti-inflammatory drugs) on fertility outcomes (PCCR); (2) investigate any associations between faecal microbiome measures and age on inflammatory status (TNF-α) and (3) to describe the relative abundance of phyla in faecal specimens. (see Figure 6.1)

6.2 MATERIALS AND METHODS

6.2.1 ANIMAL WELFARE STATEMENT

All procedures undertaken in this study were reviewed by the University of Newcastle Animal Ethics Committee and approved under the Australian Code for the Care and Use of Animals for Scientific Purposes (approval number A-2011-122). The Australian Code for the Care and Use of Animals for Scientific Purposes adheres to the EU standards for the protection of animals used for scientific purposes. Ethics approval for this study: Animal Research Authority No. A-2011-122

6.2.2 STUDY DESIGN

This observational study collected semen, serum, and faecal specimens, in addition to retrospective data from the clinical histories of 19 Thoroughbred stallions, to investigate factors associated with PCCR and TNF-α The study is reported in accordance with the STROBE checklist for reporting observation studies (393).

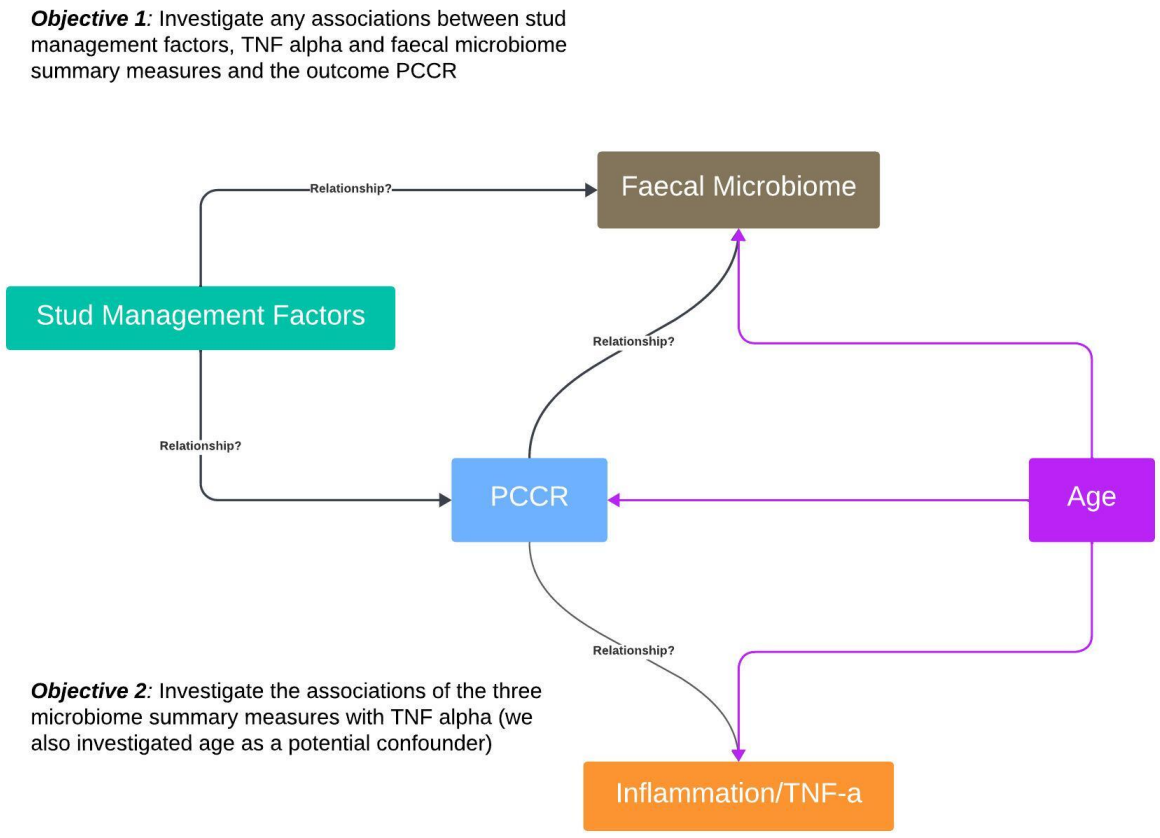

Figure 6.2: Conceptual framework for predictor variables (PCCR and TNF-α) and confounder (age), exploring the relationship between PCCR, inflammatory biomarker (TNF-α) and the faecal microbial composition of 19 Thoroughbred stallions, as well as the effect of various stud management practices on these measures.

6.2.3 ANIMALS

The stallions selected for this study were 19 commercial Thoroughbred stallions housed on three stud farms located in the Upper Hunter Valley of New South Wales, Australia (Stud 1: N = 6, Stud 2: N = 6, and Stud 3: N = 7 stallions). All stallions are engaged in annual breeding programs and considered to be in excellent general health. Stallion ages ranged from 4 to 22 years, with a mean age of 10.5 and a median age of 8.0. There were no 'shuttle stallions' (stallions that travel between the northern and southern hemispheres to cover both breeding seasons) involved in this study.

6.2.4 STALLION MANAGEMENT DATA COLLECTION

Stud managers at each of the three participating studs were interviewed over two days in May 2021. Criteria assessed for each stallion included age, nutritional supplements (prebiotics, probiotics, synbiotics, carnitine) and treatments (antibiotics, anti-inflammatories). Breeding outcomes were declared by the relevant stud managers for August – December 2020 season, specifically November 16 – December 18, 2020, as weekly PCCR. The PCCR is calculated as the percentage of the pregnant mares of the total number of mares covered during the period in question (4–5 weeks).

6.2.5 COLLECTION OF FAECAL AND BLOOD SAMPLES

Blood and faecal samples were collected from the 19 Thoroughbred stallions between December 16–18, 2020 and stored at −80 °C until analyses were conducted.

6.2.6 SAMPLE PREPARATION

6.2.6.1 PREPARATION OF FAECAL SAMPLES

Faecal balls were collected within 1 h of defecation from each of the stallion's own paddocks for sampling. Intact faecal balls were opened and core contents, which had not contacted pasture, were extracted using forceps. From these faecal balls 250 mg of material was placed into three 5 mL specimen vials, each containing 3 mL DNA Microshield, and stored at ambient temperature for transport to the laboratory within 4–6 h. Faecal samples were then snap frozen in liquid nitrogen and stored at −80 °C until all samples were collected at the end of the trial for final assessment.

6.2.6.2 PREPARATION OF SERUM SAMPLES

Approximately 5 mL of venous blood was collected from each stallion into a serum Vacutainer (Becton Dickinson) and allowed to sit at room temperature (RT) for 30 min to allow a clot to form. The tubes were then centrifuged at 1000 x *g* for 10 min, the serum aspirated, aliquoted into cryovials, snap frozen in liquid nitrogen, and stored at −80 °C until analysis.

6.2.7 FAECAL MICROBIAL GENOMIC PROCESSING

PacBio DNA extractions, library preparation and sequencing of faecal samples were conducted at Central Analytical Research Facility (CARF), Queensland University of Technology, Brisbane. DNA was extracted using the ZymoBiomics DNA Miniprep kit (Zymo Research) from 150 mg samples of faeces, following the protocol for faecal extraction outlined on page 6-7 of this protocol brochure: https://files.zymoresearch.com/protocols/_d4300t_d4300_d4304_zymobiomics_dna_miniprep_kit.pdf

PacBio libraries were prepared following the Full-Length 16S Amplification, SMRTBell® Library Preparation and Sequencing Procedure and Checklist (Pacific Biosciences) from 2.5 ng samples of extracted DNA. Initial PCR was carried out with KAPA HiFi HotStart ReadyMix (Roche), with 20 cycles in 25 µL reactions following protocol cycling conditions. Amplified DNA was indexed using the barcoded F/R Universal Primers Plate 96 v2 (Pacific Biosciences) and PCR products purified using AMPure PB (Agencourt Bioscience) according to PacBio's protocol. Amplicon sizes were verified with a Fragment Analyser 5200 (Agilent Technologies) and Qubit 4.0 Fluorometer (ThermoFisher Scientific) using the High Sensitivity dsDNA assay. Barcoded amplicons were pooled before library construction with the SMRTbell® Express Template Prep Kit 2.0 (Pacific Biosciences). Samples were pooled, with 20 samples per pool, and sequenced on separate chips. Sequencing was carried out on a PacBio sequel (Pacific Biosciences) with v3 sequencing chemistry in circular consensus sequencing (CCS) collection mode for 20 hours on a 1M v3 LR SMRT Cell. CCS analysis was then run before demultiplexing and fastq conversion.

6.2.8 ANALYSIS OF SERUM CYTOKINE LEVELS

Serum levels of the cytokine TNF-α were measured using the Milliplex MAP Equine Cytokine Magnetic Bead Panel Immunology Multiplex Assay kit (Merck Millipore; catalogue no. EQCYTMAG-93K), MAGPIX instrument system, and xPONENT software, in accordance with manufacturer instructions: https://www.sigmaaldrich.com/AU/en/product/mm/eqcytmg93kpx23

Median fluorescent intensity data were processed using Belysa analysis software (version 1.1.0, Merck); 4-parameter logistic regression was used to generate standard curves, from which the sample cytokine s were determined.

6.2.9 BIOINFORMATICS FOR MICROBIOME PROFILING

The bioinformatics analysis for this work was performed on the HPC server 'Artemis' at the University of Sydney.

The quality of the raw reads were checked using the package FastQC (Version 0.11.9) (245). The next-generation microbiome bioinformatics platform QIIME2 (version q2cli version 2020.8.0 https://qiime2.org/) (246) was used for analysing the amplicon data. The first step involved generation of Amplicon Sequence Variants (ASVs) from the raw amplicon data, using the algorithm DADA2 (247). Since the available version of QIIME2 did not include the DADA2 capability to handle Pacbio reads, we used the DADA2 package externally (version 1.19.2). The ASVs display advantages over the more traditional approach of operational taxonomical units as they represent the exact amplicon sequences without coarse graining into OTUs and can resolve even single nucleotide base differences. DADA2-formatted training fasta files (248) were derived from the Silva Project's version 128 release and used for taxonomy assignment. Three output files were generated using DADA2. These included the sequence feature count tables, the taxonomy identification file per ASV and a fasta file containing sequences of all ASVs. These files were exported in a phyloseq (249) format for further analysis using QIIME2. These files along with the sample metadata files were used as input for different modules in the QIIME2 workflow.

Data produced by QIIME 2 exists as QIIME 2 artifacts, which contain data and metadata. The feature tables imported from DADA2 were filtered to retain the features that were observed in at least two samples. A rooted phylogenetic tree was generated using the filtered feature table for downstream diversity analysis. The next steps in the QIIME2 analysis involved computation of alpha diversity indices (richness, evenness, and Shannon's diversity).

6.2.10 STATISTICAL METHODS

During data processing, binary variables were created for 'any oral anti-inflammatory drug use' (stallion received at least one dose of phenylbutazone, flunixin meglumine or pentoxifylline), 'any antibiotic drug use' (stallion received at least one dose of penicillin, gentamicin, sulfadimidine, trimethoprim or bromhexine hydrochloride) and 'pre-/pro- or synbiotic use' (stallion received at least one of grain-free fibre, rice bran, linseed, a multi-species probiotic/yeast mixture or a yeast culture).

The distribution of each variable in the data set was checked using summary statistics and frequency tables for numeric and categorical variables, respectively. Two separate outcome measures were considered in this study: PCCR and TNF-α. The relationships of ten predictor variables with the outcome PCCR were assessed: demographics (age, stud), treatments (anti-inflammatory use, antibiotic use, pre-/pro-/synbiotics and carnitine), TNF-α concentration and three faecal microbiome summary measures. Similarly, the relationships of the three microbiome summary measures (species richness (faith_pd), evenness and Shannon's diversity) and age with the TNF-α outcome measure were described. For numeric predictors, the relationship with the numeric outcome was assessed with a scatter plot. For categorical predictors, the outcome was summarised by category and plotted using dot plots and side-by-side boxplots.

Simple linear regression models were used to formally test the association of numeric predictor variables with the outcome variables. The model assumptions of linearity, normally distributed residuals and equality of variance were assessed with scatter plots of the raw data as well as of predicted values versus residuals and plotting of the residuals. Outliers and influential values were assessed using Cook's distance and leverage. A

sensitivity analysis for an influential value with large residual was performed by excluding the data point from analysis and comparing the results to analysis of the complete data set.

The associations of binary and nominal predictor variables with the outcome PCCR were tested using univariable linear models.

ASV count data were aggregated at the phyla level and sorted from highest to lowest frequency. The top 10 phyla were plotted and phyla with low frequencies were combined to an 'Other' category for plotting. The data was prepared in Microsoft Excel (2021) and all statistical analyses were conducted in SPSS software (v28, 2021).

6.3 RESULTS

Overall, the average PCCR for the 19 stallions in this study was 54.6 ± 7.1%. A significant effect of stud was observed on PCCR, although no association was found between any microbial measure nor age with PCCR and TNF-α levels. Ages of the stallions ranged from 4 to 22 years, with an average age of 10.5 years. The average TNF-α (18.4 pg/mL) was within the normal range for this cytokine (4–4,000 pg/mL, Milliplex multiplex cytokine assays). With respect to the faecal microbiome measures evaluated in this study, microbial evenness was consistent across samples (0.92 with SD ± 0.05), while microbial richness (faith_pd) varied more greatly between stallions (344.5 ± 49.0). The Shannon index for these samples ranged from 9.2 to 11.5 with a mean of 10.7.

The average TNF-α of this cohort of stallions was low (normal) at 18.4 pg/mL, suggesting that the presence of significant local or systemic inflammatory conditions would be unlikely in these subjects. The distribution of TNF-α was positively skewed by one elevated result, while the other numeric variables in the study had an approximately normal distribution. The distributions of the numeric variables age, TNF-α, evenness, faith_pd (richness) and Shannon's diversity are presented in Table 6.1.

Table 6.1: Summary statistics for the numeric variables TNF-α, age, evenness, faith_pd and Shannon's diversity in a study of 19 Thoroughbred stallions.

Variable	Minimum	Q1	Median	Q3	Maximum	Mean	SD
TNF-α (pg/mL)	0.0	0.0	0.4	17.0	210.6	18.4	47.8
Age (years)	4.0	6.0	8.0	17.0	22.0	10.5	5.9
Evenness	0.82	0.89	0.94	0.96	0.97	0.92	0.05
Faith_pd	227.8	305.8	351.8	385.7	409.7	344.5	49.0
Shannon's index of diversity	9.2	10.3	10.9	11.1	11.5	10.7	0.7

6.3.1 PER CYCLE CONCEPTION RATE

The distribution of PCCR overall and by categories of the predictors is shown in Table 6.2. Table 6.3 presents the results of the univariable regression analyses for categorical predictors of PCCR. Stud was significantly associated with PCCR, with Stud 3 having a significantly higher predicted PCCR compared to Stud 1 (59.9% (95% CI: 55.5–64.3%) versus 48.5% (95% CI: 43.8–53.3%); p = 0.007). On average, the four stallions that were supplemented with carnitine had an almost 5% higher PCCR than those without carnitine supplementation. Three of those four stallions were housed at Stud 3, which had the highest average PCCR, hence it is difficult to assert that the effect of stud was not confounded by the factor of carnitine supplementation. No impact of antibiotic or anti-inflammatory drug was demonstrated on PCCR, nor was any effect seen with pre/pro/synbiotic supplementation on this measure.

Table 6.2: Summary statistics for PCCR overall and by the categories of the nominal predictor variables stud, antibiotic, anti-inflammatory, pre-/pro-/synbiotics and carnitine.

		N (%)	Minimum	Q1	Median	Q3	Maximum	Mean	SD
PCCR		19 (100)	34.2	50.0	56.4	60.9	63.9	54.6	7.1
Stud	1	6 (32)	34.2	44.5	49.0	54.9	56.6	48.5	7.8
	2	6 (32)	48.7	53.0	55.6	56.7	56.9	54.6	3.1
	3	7 (36)	50.0	59.0	61.5	61.9	63.9	59.9	4.6
Antibiotic	No	16 (84)	48.0	50.0	55.7	60.4	63.9	55.3	5.3
	Yes	3 (16)	34.2	34.2	56.4	59.2	61.9	50.8	14.7
Anti-inflammatory	No	6 (32)	50.0	53.3	55.7	61.0	61.5	56.4	4.3
	Yes	13 (68)	34.2	48.4	56.4	60.5	63.9	53.8	8.1
Pre-/pro-/synbiotics	No	7 (37)	48.7	50.0	56.4	56.9	61.9	55.0	4.5
	Yes	12 (63)	34.2	48.5	55.7	61.4	63.9	54.4	8.4
Carnitine	No	14 (74)	34.2	48.5	55.4	57.4	61.9	53.3	7.2
	Yes	5 (26)	50.0	52.4	61.5	62.9	63.9	58.4	5.8

The results of univariable linear model regression analyses for numeric predictors of PCCR are shown in Table 6.4 and show there was no effect of TNF-α, stallion age and three microbiome measures on the fertility outcome, PCCR.

Table 6.3: Results of five univariable linear models for categorical predictor variables of PCCR.

Variable		**Predicted Mean**	**95% CI**	**p-value**
Stud	1	48.5	43.8 – 53.3	0.007
	2	54.6	49.9 – 59.4	
	3	59.9	55.5 – 64.3	
Carnitine	No	53.3	49.4 – 57.1	0.17
	Yes	58.4	51.9 – 64.9	
Anti-inflammatory	No	55.4	51.6 – 59.1	0.32
	Yes	50.8	42.2 – 59.4	
Antibiotics	No	56.4	50.2 – 62.5	0.48
	Yes	53.8	49.6 – 58.0	
Pre-/pro-/synbiotics	No	55.0	49.2 – 60.8	0.87
	Yes	54.4	50.0 – 58.9	

Table 6.4: Results of five simple linear regression analyses for numeric predictor variables of PCCR.

	Regression estimate β	**Standard error (β)**	**95% CI**	**p-value**	**R2**
Constant	53.9	1.7			
TNF-α (pg/mL)	0.04	0.035	−0.04 – 0.11	0.28	0.07
Constant	51.6	3.4			
Age (years)	0.29	0.28	−0.30 – 0.89	0.31	0.06
Constant	85.5	33.9			
Evenness	−33.4	36.7	−110.7 – 44.0	0.38	0.05
Constant	75.0	27.9			
Shannon's index of diversity	−1.9	2.6	−7.4 – 3.6	0.47	0.03
Constant	57.4	12.1			
Faith_pd/Richness	−0.008	0.035	−0.82 – 0.066	0.82	<0.01

6.3.2 TNF-α ANALYSES

The results of univariable linear model regression analyses for TNF-α are presented in Table 6.5. There was no evidence of an effect of stallion age and three microbiome measures on serum concentrations of the inflammatory cytokine, TNF-α. The predictors only explained between 0–5% of variation in the TNF-α outcome variable (R2, Table 6.5).

One stallion had an extreme outlier value for TNF-α, which resulted in a skewed distribution and large residual value with high influence on model fitting. A sensitivity analysis excluding this value revealed no change to the interpretation compared to the models using the whole dataset, which are presented here.

Table 6.5: Results for four simple linear regression analyses for numeric predictor variables of TNF-α.

Parameter	Regression estimate β	Standard error (β)	95% CI	p-value	R2
Constant	−9.3	22.3			
Age (years)	2.6	1.9	−1.3 – 6.6	0.17	0.05
Constant	93.0	190.8			
Shannon's Index of diversity	−6.9	17.7	−44.4 – 30.5	0.70	0.01
Constant	99.6	234.3			
Evenness	−87.9	253.4	−622.5 – 446.7	0.73	0.01
Constant	43.5	82.1			
Faith_pd/Richness	−0.07	0.24	−0.57 – 0.43	0.76	<0.01

6.3.3 FAECAL MICROBIAL COMPOSITION

The results of the faecal microbiome profiling are presented to phylum level for each stallion and compared between studs and between individuals in Figure 6.3. Overall, microbial profiles were similar across studs and individuals. Firmicutes and Bacteroidetes comprised the bulk of phyla for each horse (approximately 60–78% of profile), along with Verrucomicrobia up to approximately 90% of composition. Only one horse (J) showed a preponderance of Proteobacteria in its profile.

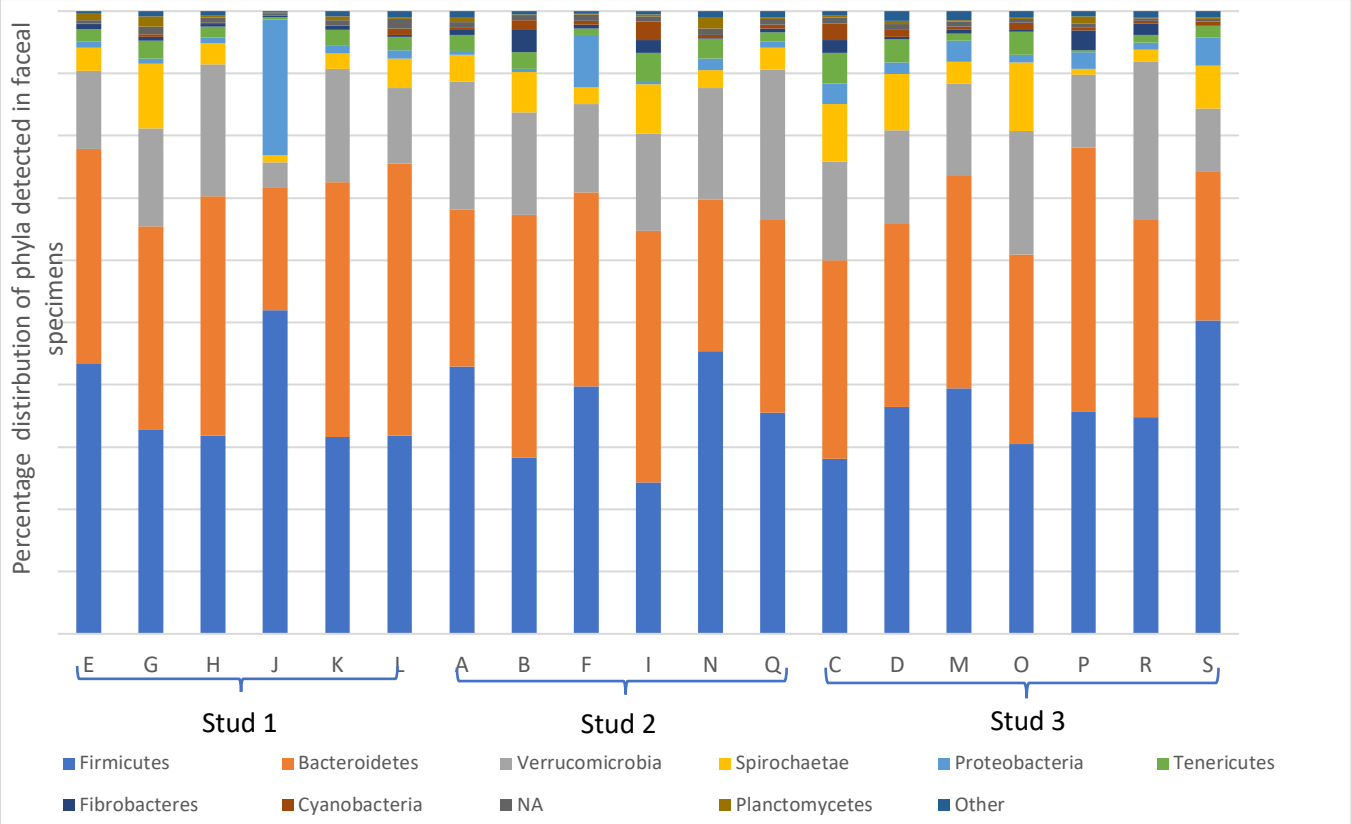

Figure 6.3: Relative abundance of phyla in faecal samples of 19 Thoroughbred stallions (A–S) by stud in an observational study to identify factors associated with PCCR and TNF-α. 'Other' includes Actinobacteria, Lentisphaerae, Saccharibacteria, Synergistetes, Euryarchaeota, Chloroflexi, Elusimicrobia, Euglenozoa, Armatimonadetes and SR1_(Absconditabacteria)

6.4 DISCUSSION

To our knowledge, this is the first study to investigate the association between the composition of the faecal microbiomes of Thoroughbred stallions and PCCR. In addition, we examined the associations between nutrition, dietary supplements, medical treatments, and serum inflammatory cytokine levels with PCCR. Except for differences in PCCR by stud farm, we did not identify any other statistically significant associations with the other factors measured; however, a small positive effect of carnitine supplementation observed on PCCR may have clinical relevance to stallion breeding.

Factors impacting sperm quality and functionality are of primary concern to stallion managers, keen to achieve peak fertility for their horses each breeding season. Human research is beginning to explore the role of the intestinal microbiome on sperm quality (133). Moreover, correcting intestinal dysbiosis to attenuate pro-inflammatory states in infertile men by supplementation with probiotics has resulted in improvement in several sperm quality parameters (394).

In this study we characterised the faecal microbial profiles of 19 commercial stallions and collected details of the specialist stud management practices and nutritional supplementation supplied, as well as fertility outcomes for the spring breeding season of 2020. These valuable horses were stabled in prime conditions, grazed on ideal pastures, and exercised or rested daily according to their specific requirements for the number of covers they were required to perform; they were fully vaccinated according to Australian veterinary standards and treated immediately for any health concerns by specialist equine veterinarians. For these reasons it could be expected that their general health, digestion, and fitness to breed were optimal during this season.

6.4.1 PER CYCLE CONCEPTION RATE

Our results demonstrated that the range of PCCR for the three stud farms varied, with an average of 54.6% for all 19 stallions. Three stallions on Stud 3 received L-carnitine supplementation during the study period and one stallion on Stud 2 received this amino acid supplement, comprising 26% of the cohort. The results of this study identified some effect of L-carnitine on PCCR ($p = 0.17$). Carnitine, an amino acid that is either synthesised in the liver or kidneys or nutritionally sourced, may be a conditionally essential amino acid under certain conditions (395) and is more concentrated in spermatozoa than any other mammalian cell (396, 397). Furthermore, according to *in vitro* research, L-carnitine is considered a 'prosurvival' nutrient for stallion sperm, increasing sperm motility and ATP levels while protecting sperm mitochondria against oxidation by reactive oxidant species (21), generated by oxidative phosphorylation resulting from high level mitochondrial activity (37, 377).

Prebiotics and probiotic yeast supplements were incorporated in the feed mixes of all horses on Stud 3, most horses on Stud 2 and only one stallion on Stud 1; this nutritional supplementation factor is the closest correlate with PCCR outcomes for this study, as Stud 3 had the best average PCCR result, Stud 2 the median PCCR and Stud 1 the lowest average PCCR result for this breeding season. Synbiotic supplementation of feed improves the microbial composition of the faeces, so it is expected that stallions receiving these supplements will possess better microbiome diversity indices, which translates to improved digestion and nutrient uptake. In relation to omega-3 oils, which exert an anti-inflammatory effect on vital tissues and delicate cells such as the spermatozoon, five horses on Stud 1 were given this supplement, all six horses on Stud 2 received omega-3 oils and all seven horses on Stud 3 received at least two types of anti-inflammatory oils in their feed. Since all but one stallion in this study returned low (normal) TNF-α results, it might be inferred that nutritional supplementation of these horses could have dampened any potential inflammatory tissue responses, for example from an injury or arthritis, which could have otherwise elevated levels of this cytokine. Numerous other vitamins, minerals, trace elements, antioxidants and herbals were administered to all stallions across all three studs, many of which are known to improve sperm quality, such as vitamin E, zinc and selenium, as well as polyunsaturated fatty acids (PUFA's) found in seeds, which protect spermatozoal membranes against oxidative damage.

Successful conception not only depends upon a healthy stallion libido, breeding performance and sperm quality (95), but also the mare's age (398), reproductive fitness and number of matings (399). Nath et al. demonstrated that the highest fertility outcomes were with the youngest mares (age 2–8 years), as older mares tend to conceive less efficiently and experience more pregnancy loss (88). Mares covered only once during oestrus had higher conception rates in comparison to mares mated multiple times over several oestrus periods in one season (399). No clinical data were collected pertaining to the breeding fitness of the mares covered during our study, hence it was not possible to evaluate any negative effect on PCCR for these stallions that might indeed be attributed to the mares. Also, PCCR is not a clear predictor of fertility outcomes for stallions, as those with a PCCR as low as 45% have achieved 90% seasonal pregnancy rates (400). Another

unpredictable aspect of stallion fertility was highlighted in a study conducted during the 1998 breeding season with 1393 Thoroughbred mares covered by 41 stallions on 22 stud farms in Newmarket, United Kingdom; a wide range of PCCRs resulted, with each stallion that covered more than 10 mares achieving pregnancy rates as low as 37% and as high as 90% (400).

Thoroughbred stallions have lower PCCRs than other breeds of horse (88), hence this was reflected in our findings; these horses are primarily selected and bred for their athletic performance and track winnings rather than their fertility status. By comparison, PCCRs are significantly higher amongst domestic livestock animals, for example rams (80–90%) and boars (85–90%) as reproductive efficiency is paramount for these food-producing species (401, 402). The environmental conditions to which the stallions were exposed at each stud were optimal in terms of pasture quality, paddock area and stabling accommodation.

6.4.2 INFLAMMATORY CYTOKINES

In the human and mouse, a possible connection exists between intestinal microbiome disruption, systemic inflammation, testicular injury and impaired fertility (403). In the present study the cytokine TNF-α was chosen for measurement as a biomarker of inflammation. All TNF-α results were within the normal range for the specific cytokine kit used, indicating that no abnormal levels of inflammation were present in any of the stallions at time of specimen collection.

Our results showed no correlation between TNF-α and the parameters of age, or faecal microbial richness, evenness, or diversity. This result was not entirely unexpected, as the cohort of stallions we studied was in excellent health when specimens were taken (404, 405). Animals exhibiting tissue inflammation, for example in lung, may produce elevated serum TNF-α, which can be modulated by the beneficial influence of eubiotic intestinal flora regulating barrier function (406). Bacteria that assist in maintaining a healthy intestinal biofilm, such as *Akkermansia muciniphila*, have been shown to downregulate proinflammatory cytokines in human patients with ulcerative colitis (407). Horse O returned the highest TNF-α result among the cohort, whilst within the refence range;

however, this stallion also had one of the highest PCCRs, hence we could not conclude that any level of inflammation present in this stallion affected his fertility. It is important also to note that 68% of the stallions in this study cohort were treated with anti-inflammatory drugs at some time during the relevant prior breeding season, mostly 'bute' (phenylbutazone) for managing musculoskeletal injuries and pain, which may have also suppressed inflammatory cytokine production.

6.4.3 INTESTINAL MICROBIOMES

The average Shannon index of diversity was 10.7 for the 19 horses in the present study, which is high in comparison to average human faecal diversity (1.66) (351). Consistent with other similar studies, the faecal microbial profiles of these stallions showed the highest abundance of the phyla Firmicutes and Bacteroidetes (164, 408, 409), followed by Verrucomicrobia (129), Spirochaetae, Proteobacteria (144), Tenericutes, Fibrobacteres (410) and Cyanobacteria (408). Another ten bacterial phyla were categorised as 'Other' and were in the lowest preponderance. Our stallion cohort carried higher abundances of Firmicutes and Bacteroidetes, a microbiome composition which is considered healthy, which may be explained by their nutritional supplementation. Diets rich in protein increase intestinal Firmicutes colonisation, while a high intake of plant fibre increases Bacteroidetes numbers to enable fermentation of cellulose and xylan (148). These nutrient categories were well supplied to the stallions in this study.

Although the intestinal microbiome profiles of equids is impacted by breed (411), feed supplementation and weather (159), domestication (171), medications and illness (129), the core bacterial taxa are thought to be common to all equids. A survey of the core faecal bacterial phyla in a similar cohort of six Irish Thoroughbred racehorses was conducted by employing 16S rRNA gene (V4 region) amplicon pyrosequencing (178). The key findings were a preponderance of Firmicutes and Bacteroidetes, which comprised about three-quarters of the reads. Proteobacteria, Verrucomicrobia, Actinobacteria, Euryarchaeota, Fibrobacteres, Spirochaetes and another group comprised the remaining one-quarter of phyla discovered. These findings correlate with our results of faecal microbiota in stallions, for which 60–78% of their phyla were combined Firmicutes and Bacteroidetes.

The Irish Thoroughbred cohort included three geldings, two fillies and a mare, but no stallions, with ages ranging less than one month to four years, whereas our stallions ranged in age from 4 to 22 years. Perhaps immaturity explains the difference in median Shannon Index (8.58) and evenness (0.75) of these young horses in comparison to our study findings of Shannon Index (10.9) and evenness (0.94), in horses with a median age of eight.

The bacterial phyla Actinobacteria, Euryarchaeota were not represented amongst the core species of our cohort, but Tenericutes and Cyanobacteria were. A relative abundance of Tenericutes has been associated with obesity in a study of Welsh mountain pony mares, with a reduction of both Firmicutes and Tenericutes phyla observed following dietary restriction of these horses (412). The body condition of our study stallions was relatively heavy, scoring 6–7 on the Body Condition Score Chart (51), which may explain the presence of Tenericutes amongst their core phyla.

The presence of Cyanobacteria in stallions who foraged on lush pastures suggests that these bacteria may transit through the equine intestinal microbiome as passengers from ingested plants, rather than being core host species. Cyanobacteria are obligate phototrophs that have harnessed a circadian clock mechanism (413); they are known to possess circadian oscillators (414). This oxygenic phylum performs a role in plant photosynthesis and corresponds to chlorophyll sequences observed in foraging horses; however, no role has been identified for Cyanobacteria in equids (408).

Antibiotics were administered to 21% (four) of the study stallions, at some time during this particular breeding season, prior to faecal specimen collection and 63% (12) of the stallions received pre- and/or probiotic supplementation in their feed over this same period, which may have recovered any loss of microbial diversity following antibiotic administration.

6.4.4 STUDY LIMITATIONS

Our PCCR results were limited to one breeding season in one region of Australia; thus, research conducted over more seasons in different geographical locations may produce varying results. We were unable to assess any seasonal variation in either cytokines or

microbiome measures due to collection of specimens at one single timepoint. With respect to TNF-α and the microbiome results, the subject cohort were healthy, fertile Thoroughbred stallions in peak condition, managed under premium stud farm conditions, with no inflammatory diseases or intestinal disorders. Detailed information regarding the administration of anti-inflammatory and antibiotic drugs to this cohort was not always made available by the stallion handlers at each stud farm during the retrospective collection of these study data, which may be considered a limitation for the interpretation of serum cytokines and faecal microbiota present in the samples. Faecal microbiome measures did not appear to influence fertility in this study. Analysing the semen microbiomes of this cohort may have produced evidence of a PCCR effect in relation to microbial composition of this more proximate site, rather than the bacteria present in the rectum. Also, the small sample size of this study did not allow for multivariable analyses to be performed on the data; for example, carnitine supplementation was given to only five stallions. This could also have created a confounding effect for stud variations of PCCR, so further conclusions were unable to be drawn about the value of carnitine for improving stallion fertility in this study, with a small cohort during one breeding season. The opportunistic nature of this sample of healthy, fertile Thoroughbred stallions, which were given similar diets, must be considered when evaluating these data. Very little difference exists in stud practices with respect to feed and nutrition – all commercial equine feeds are fortified with vitamins, minerals, essential fatty acids, prebiotics and some probiotics, hence these predictors cannot be very sensitively measured. Although the sample size for this study was small in some respects, it comprises an adequately sized sample for the purpose of description of the faecal microbiome composition of these animals, a homogenous group of healthy stallions with proven fertility, which has provided category-specific data on the equine microbiome.

6.5 CONCLUSIONS

In this study of 19 Thoroughbred stallions, in peak health and condition, no relationship was found between per cycle conception rate (PCCR) and measures of age, faecal

microbial richness, evenness or diversity, nor to circulating TNF-α levels. While PCCR varied between studs, we did not observe any specific effect due to the stud management practices; however, the observation that the nutritional supplement carnitine showed some evidence for an effect in increasing PCCR requires further research attention. Given the limitations outlined in this study, further research is encouraged to clarify the relationship between the equine intestinal microbiome, systemic inflammation, and fertility, particularly in subfertile stallions, as understanding of any impact that these factors may have on equine sperm quality could potentially inform future animal stud farm management practices.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

7.1 PURPOSE OF THIS RESEARCH

The fundamental aim of this thesis was to explore the relationship between male fertility and the gastrointestinal and semen microbiomes, hoping to understand any influence that the composition and diversity of microbiota might exert on sperm quality, which may in turn affect the fertility of that animal. The initial impetus for this work was the rising global incidence of human infertility over the past six decades and how little is known about the male factor that contributes to cases of idiopathic infertility. In studying the stallion, we hoped to discover what influence the microbiota occupying these two anatomical regions might exert on sperm quality and per cycle conception rates of horses, with the potential to also find future application to human infertility research, the equine now being accepted as an appropriate model for informing human research in reproductive medicine (26, 415).

7.2 THE SEMEN MICROBIOME

The first study of this thesis (Chapter 2) described the microbiomes of four healthy fertile miniature stallion ponies, all receiving the same feed and stabled in the same environment. In the domain of equine fertility research there is very little known about this topic and only two other studies published to date have explored the semen microbiomes of stallions, albeit to only three levels (phylum, family and genus) (43, 189). This was also considered as a standalone study for future research to explore factors that impact the semen microbiome.

Compared to the other studies that identified stallion semen microbiota by the V3 hypervariable region of the 16S ribosomal RNA from bacterial DNA (43, 189), amplification was carried out on the full length of the 16S DNA in our study from metagenomic samples (113). As the relative abundance of dominant bacterial genera is similar between full-length and short-read sequencing, comparison at the genus level was

possible, thus adding to the body of knowledge regarding the composition of the equine semen microbiota (416).

Results of this study revealed that, overall, Bacteroidetes and Firmicutes phyla were the most prominent in these stallions and Proteobacteria was the third most represented phylum; only a very small number of bacteria (< 1%) are as yet unclassified. These results are comparable to the semen microbiome profiles at phylum level as reported in the literature of other horses (Arab, Anglo-Arab and Andalusian full-size stallions) of unknown fertility status (43), with Bacteroidetes and Firmicutes featuring as the most abundant phyla, along with other less abundant phyla. It would seem in this case that stallion breed does not significantly impact the composition of the semen microbiome at the rank of phylum. Some consistency in composition was evident across all subjects in our miniature pony stallions at phylum level, which was observed to diversify as the microbiota classifications refined from class through to genus, at which level the microbial profiles of each stallion appeared unique. The Quiñones-Pérez et al. (2021) study of fertile Spanish stallions found a high level of bacterial family variation in semen microbiomes between subjects (138), also suggesting that microbial diversification may be a common finding in different stallion breeds.

At the level of family, the most abundant bacteria in the miniature pony stallions were *Porphyromonadaceae,* and to a lesser degree *Corynebacteriaceae*, families which have been identified in the semen of other fertile stallions (138). The most abundant eleven genera present in our cohort were *Porphyromonas, Suttonella, Peptoniphilus, Fastidiosipila, Ezakiella, Petrimonas, Fusobacterium, Gallicola, Finegoldia,'* Other' and 'unnamed'. At genus level, the semen microbiomes of a cohort of Swedish stallions of unknown breed show significant differences to our cohort; the authors reported double the genera of those present in our miniature stallions, with only two genera*, Porphyromonas*, and *Peptoniphilus,* in common between the two cohorts (189). It is possible to postulate, on the basis of these findings at the level of genus, that equine breeds may have different semen microbiome signatures; however, data is presently too limited to make a confident prediction of this postulate. Three genera found in the semen of our pony stallions have also been identified in human semen, namely

Peptoniphilus, *Ezakiella* and *Finegoldia* (226), the significance of which is not yet understood; however, it is worth mentioning that men share some seminal bacterial types with stallions.

Only one study to date explored correlations between the composition of the stallion semen microbiota and sperm quality biomarkers (43), hence it is not possible at this stage to confidently posit a microbial profile of stallion semen that might predict their fertility. Nevertheless, our analysis reporting the seminal microbiomes of four fertile miniature stallions, along with that of twelve mixed breed, full-size fertile stallions reported by Quiñones-Pérez et al. (138), provide baseline semen microbiome data for further stallion fertility research. Quiñones-Pérez et al. compared five sperm quality parameters with semen microbiome profiles of their cohort of 12 stallions, identifying alterations in sperm motility with *Peptoniphilaceae* family (increased) and *Clostridiales Incertae Sedis XI* (reduced). They concluded from their study findings that sperm quality was similar amongst their subjects and that further research was necessary to explore the impact of bacterial commensals in semen on sperm quality parameters. Our later survey in Chapter 3 of the semen microbiomes of this cohort of miniature pony stallions in relation to six sperm quality measures also failed to distinguish clear correlations between the presence of specific microbiota and six parameters of sperm quality.

The uniqueness of our analysis of semen microbiota in pony stallions is the depth of the classification, to five taxonomical ranks, as well as being conducted in a previously unexamined equine breed. As six semen quality parameters were evaluated in the same four miniature stallions that participated in this study during control periods of the following intervention study, these data now provide novel baseline information in relation to the composition of the semen microbiome profiles of miniature stallions that can inform other research exploring correlations between fertility and the semen microbiota in the future. As more research will no doubt be conducted in this arena, it may become possible in the future to compare these findings with those of fertile stallions of other breeds.

As mentioned, our next project was an intervention study (Chapter 3), in which pre-, pro- and synbiotic supplements were administered to the same subjects over a period of one year and their semen and faecal microbiomes were profiled, along with six sperm quality parameters, at the crossover point of each intervention period. To inform this study, two reviews were conducted of the literature pertaining to the current use of these nutritional supplements in equines (194, 363).

7.3 PREBIOTICS, PROBIOTICS AND SYNBIOTICS - IMPACTS ON EQUINE HEALTH, DISEASE, MICROBIOMES AND FERTILITY

Chapters 3 and 4 contain two literature reviews that were conducted to explore applications of pre, pro- and synbiotics for equine health and disease management. The findings of those reviews identified that limited data is currently available on the safety and tolerability of probiotics for use in horses, let alone the efficacy of such supplementation, which is concerning as commercially produced probiotic supplements are currently marketed for the purpose of improving general equine health and digestion. Probiotics were not found to improve digestion of the starch and fibre components of feed, for which there is more proven benefit obtained from prebiotic nutrients (195); however, multi-strain preparations of live probiotic bacteria were found to improve the composition of the intestinal microbiome, to reduce hindgut acidosis and to enhance athletic performance (194), the latter which is also an important consideration for attaining breeding fitness in stallions.

No research to date has investigated any benefits of administering pre- or probiotics to either stallions or mares for the purpose of improving fertility outcomes, nor the safety of such supplementation on the equine reproductive microbiome. One human study has applied this thinking by administering probiotics to a cohort of asthenozoospermic men, improving their sperm motility and reducing sperm DNA fragmentation (44); another review explored the potential for treating male infertility by modifying the intestinal microbiome of infertile men with pre- and probiotic supplementation and faecal transplants (403). It had been hoped by conducting these reviews that pre-, pro- and synbiotic research had already been conducted in equines in relation to microbiomes and fertility, however intervention studies with subfertile stallions are yet to emerge.

Many microbiome researchers consider the metabolome (the biochemical products of the microbiome) to hold the key to the function of probiotic organisms. *Lactobacillus sp.* fermentation of ingested fibre produces short chain fatty acids (SCFA), which act as an additional energy source for racing horses (199). With particular importance to animal fertility, gut microbiota have been shown to influence gonadotrophin and testosterone regulation in mice and the intestinal production of the SCFA butyrate by *Clostridium Tyrobutyricum*, found to restore the integrity of the blood-testis barrier in these research animals (134), the disruption of which negatively impacts spermatogenesis and hence fertility (417). Although a metabolomics study was outside the financial scope of this research, with respect to stallion fertility one might postulate from these research findings that supporting the intestinal metabolome, with either pre- or probiotic supplementation for beneficial SCFA production, might potentially support healthy spermatogenesis.

Probiotics play an important role in supporting healthy immune function while downregulating both local and systemic inflammation (272, 418). Evidence for prebiotic and synbiotic supplementation in equines reveals their ability to reduce insulin resistance (419), systemic inflammation (325) and intestinal dysbiosis (297). Paradoxically, *Lactobacillus*, *Bifidobacterium and Enterococcus sp.,* which are included in many equine multi-strain probiotic formulations, are not currently documented to be part of the core intestinal microbiome of horses (409); however, their application as supplements of human strains has produced some benefits for particular conditions in both adult horses and foals (194). It is questionable, therefore, whether *Lactobacillus* supplementation of stallions during the breeding season could improve sperm quality in a comparable way to the effect observed when subfertile men were treated with *Lactobacillus* and *Bifidobacterium* supplements (44), or when an abundance of *Lactobacillus sp.* is present in human semen (40).

Prebiotics are currently incorporated into in many specialist equine nutritional regimens for the purpose of supporting feed digestibility and nutrient uptake (420) and, as our intervention study found, this addition to the equine diet also improves the microbial diversity and richness of the gastrointestinal microbiome, which in turn supports and sustains the overall health of the horse (195). For these reasons prebiotic feed

supplementation might be considered for stallions at stud, to enhance their nutritional status and thereby sperm quality; in fact, prebiotic yeasts, such as *Saccharomyces cerevisiae*, were administered routinely to many of the Thoroughbred stallions recruited for our retrospective stud management study (Chapter 6).

To explore the role of pre-, pro- and synbiotics on stallion sperm quality and the composition of two microbiomes, we conducted an intervention study, which comprises the main data chapter of this thesis (Chapter 5). The aims of this pilot study are central to the research question directing this thesis and notably this is the first study to explore pre-, pro- and synbiotic interventions in relation to sperm quality, as well as faecal and semen microbiomes, in either stallions or humans. Key findings of this study were that the composition of the faecal and semen microbiomes of this small cohort of cohabitating miniature stallions were significantly different, both between anatomical regions and individuals; that dietary supplementation with prebiotic unmolassed beet flakes increased the Shannon's diversity index of the stallions' GI microbiome; and also that neither probiotic nor synbiotic supplementation produced any significant impacts on the composition of either the faecal or semen microbiomes. As prebiotic supplementation increased the faecal microbial alpha-diversity of these stallions, one could expect this outcome to improve nutrient uptake from feed (420); however, such potentially enhanced absorption of dietary nutrients, such as antioxidant vitamins and minerals that could improve stallion fertility (50), was not reflected in any significant improvements in sperm quality in this study. The results of our intervention trial demonstrated no impact of pre-, pro- or synbiotic treatments on any of six sperm quality biomarkers, bearing in mind that the subjects treated had proven fertility.

Overall, it was found that the treatments in this intervention study were well tolerated with no reports of any adverse events, which is reassuring for consideration of the use of pre- and probiotic supplementation for breeding stallions. No beneficial effects were observed on either the semen microbiome or sperm quality; however, it must be noted that the research stallions recruited for this study had normal sperm parameters and were of good health and proven fertility, hence improvements may not have been possible with these subjects. Hypothetically, if these treatments were to be administered to a cohort of

subfertile stallions, it may be possible to observe changes toward an improvement in our selected biomarkers of sperm quality resulting from pre- and synbiotic supplementation, as has been demonstrated in humans (394). By improving microbiome composition and microbial metabolism in these regions and thereby potentially reducing systemic inflammation (185), which can in turn stabilise spermatozoon membranes against oxidative stress for prosurvival (421), better fertility outcomes may be achieved.

7.4 THOROUGHBRED FERTILITY, INFLAMMATION, AND THE INTESTINAL MICROBIOME

To further explore associations between intestinal microbiota, nutrition and stallion fertility, an exploratory study was conducted involving 19 Thoroughbred stallions, evaluating associations between PCCR, one biomarker of inflammation and three microbiome summary measures (richness, evenness and diversity). This study also explored associations between PCCR and age, stud management by medications, antibiotics and anti-inflammatory drugs; prebiotics, probiotics and carnitine nutritional supplements; and described the relative abundance of phyla in faeces.

This retrospective observational study is reported in Chapter 6, entitled "Investigation of the relationship of intestinal microbiome alpha diversity indices with per cycle conception rates and serum cytokine TNF-α in commercial Thoroughbred stallions". No publication to date has sought to link factors of stallion fertility, inflammatory status, and faecal microbiome characteristics per se, while also making observations of current nutritional supplementation practices at stud farms and their possible influence on breeding outcomes. Information about PCCR, health status and management strategies that may influence inflammatory status and intestinal microbiome composition were collected on one day in December (summer) 2020.

The results of this study revealed that PCCR was not impacted by age, cytokine status, nor any of the characteristics of the faecal microbiome (richness, evenness or diversity), nor could an association be drawn between cytokine TNF-α and stallion age or any microbiome measure. Measurement of serum concentrations of the inflammatory cytokines IL-1a, IL-6 and TNF-α were made in this cohort of Thoroughbreds; however,

following exploratory statistical analysis, only TNF-α was included in the statistical model for associations, as IL-1a and IL-6 were not detected. Intestinal microbiome measures of richness, evenness and diversity were not associated with TNF-α concentrations nor PCCR , most probably because TNF-α results for this cohort were in the normal range. No other study exists, to our knowledge, that also evaluated the associations of inflammatory biomarkers with faecal microbiome measures and PCCR in equines.

One significant effect in this study was observed: that of stud farm on PCCR, with Stud 1 reporting the lowest PCCR and Stud 3 the highest PCCR. Different feed and nutritional supplementation regimens were reported across the stud farms: Stud Farm 1 supplemented all six horses with commercial pre- and probiotics; Stud Farm 2 provided pre- and probiotics to only two horses and one was given carnitine daily throughout the breeding season; Stud Farm 3 supplemented three of their seven horses with carnitine and all horses received a much more comprehensive nutritional supplementation regimen of vitamins, minerals, antioxidants, omega-3 and -6 oils and yeast probiotics; all farms administered omega-3 oils, vitamins and minerals to their stallions. Carnitine has been shown to improve sperm survival and motility (422), while omega-3 oils can suppress tissue inflammation in horses with regular ingestion (423) and all supplementation had the potential to improve the sperm quality, breeding fitness and general health of these horses (50).

It could be suggested that the minor variation of nutritional practices of the stud farms might have impacted fertility outcomes for their respective stallions, particularly noting the addition of carnitine to the feed of more individuals (three stallions) at Stud Farm 3, which had the highest PCCR, in comparison to Stud Farm 1 and Stud Farm 2 (which supplemented 0 and 1 stallion with carnitine, respectively), producing an effect nearing significance. However, details regarding specific carnitine dosages were not available for evaluation against PCCR in this cohort. Stud Farm 1 added synbiotics to the feed of all their horses, while Stud Farm 3 supplemented their stallions with additional prebiotic and anti-inflammatory nutrients, the latter known to protect sperm against oxidative stress (50).

In this study, an analysis of the specific effect of supplemental syn- and prebiotics on either the PCCR or faecal microbiome measures of these 19 stallions was not conducted due to the heterogenous formulations and dosages used for each stallion. Another uncontrolled factor, which could explain a variation in conception rates between stud farms, is the fertility of the mares covered, as no data was collected about the mares' breeding fitness. Veterinary management, although quality could be expected to be similar at each of the prominent stud farms, may have been another determinant of fertility outcomes for this cohort in this breeding season.

The composition of the faecal microbiota of the 19 Thoroughbred horses was consistent across all stud farms, suggesting effects from similar management routine and grazing on similar seasonal paddock grasses (150, 424), as well as similar health status (425). Similarity of faecal microbiome profiles of these stallions could be accounted for by their location in the same geographical region of the Upper Hunter Valley of NSW, experiencing similar climate and possibly also a similar pasture type and soil microbiome, along with comparable stabling conditions (426). A more contentious suggestion would be that our subjects, belonging to the same breed, could impact the composition of their faecal microbiota (146, 149, 411, 427).

Characterisation of the intestinal microbiomes from faecal specimens of these stallions provides just the second such report, one other having been conducted in only six, younger, mixed sex, Irish Thoroughbreds, profiled from phyla to species level (427), and none specifically in stallions, limiting comparison. Nevertheless, the Irish Thoroughbreds demonstrated six of the nine most abundant phyla in their faeces that were found in our Australian Thoroughbred faecal specimens; the two leading phyla, namely Firmicutes and Bacteroidetes, are also known to contribute to the composition of a healthy equine intestinal microbiome (218). The other key phyla identified in our cohort were Verrucomicrobia, Spirochaetae, Proteobacteria, Tenericutes, Fibrobacteres, Cyanobacteria, Planctomycetes and a group of other lesser bacteria, including Actinobacteria, which were found in comparatively greater abundance by O'Donnell et al. in their study. It could be argued that differences in location, climate, pasture and local plant and soil microbiomes might account for the variation between the microbial

composition of the faeces of Australian and Irish horses, as well as differences in the specific nutritional supplementation regimens of each farm.

Research into the core equine intestinal microbiome has revealed very few dissimilarities between different equids (411) and levels of domesticity (171), although the abundance of microbial populations varies under these conditions (171). With respect to stallion age, microbiome diversity declines around age 20 (428); however, this phenomenon was not observed in our stallion cohort, perhaps due to protective effects by the nutritional prebiotic fibre which was routinely administered to these animals, known to stabilise microbial populations and to improve general health of equines (195).

7.5 SUMMARY OF STRENGTHS AND LIMITATIONS AND FUTURE DIRECTIONS

The miniature pony stallions selected for the semen microbiome characterization study are regularly tested with weekly semen analyses and have proven fertility. Although sperm quality was not assessed in this study for evaluation against semen microbiome characteristics, which could be considered a limitation in this study, it was evaluated in detail for the intervention study we conducted with the same cohort of stallions, presented in Chapter 3. Nevertheless, the strengths of this first study lie in the fact of its being the first to characterise the equine semen microbiome to five taxonomical ranks (phylum, order, class, family and genus), while also the first to examine the semen microbiome profiles of this specific breed.

With respect to conducting the nutritional intervention study with only four subjects, it is not uncommon to see studies published on the topic of the equine microbiome that have based their findings on cohorts of less than ten subjects (153, 189, 356-360). Using each pony stallion as their own control, this second study was likely underpowered and, as such, could be considered a 'proof of concept' study. It is recommended that larger studies, involving more stallions of different breeds, with varying fertility status, could be designed to test the hypothesis that manipulating the semen and/or faecal microbiome with probiotics or prebiotics may positively impact sperm biomarkers.

It can be stated that the third, retrospective Thoroughbred study is the first to explore stud management, nutritional supplementation, and fertility outcomes in Thoroughbred stallions in relation to their inflammatory status and faecal microbiome composition. Due to financial limitations and those imposed by the global pandemic, it was unfortunate that sperm quality biomarkers and semen microbiome measures were not conducted on this breed to increase the data acquired from this study for comparison with the pony nutritional intervention trial. Based on both the pony and Thoroughbred studies we conducted, it may be possible for future research to address pre- and/or probiotic supplementation and fertility outcomes with another cohort of subfertile Thoroughbreds, to explore the potential for these microbiome modifiers to reduce systemic inflammation and enhance sperm parameters, as there are currently very little in the way of safe and well-tolerated treatments available to improve the fertility of these valuable horses whilst at stud. Examining other similar cohorts of equine athletes, there are publications characterising the faecal microbiome (427) or exploring the effect of pre- and probiotic supplementation on enhancing racing performance (199, 332); however, there is no existing research that explores these criteria in relation to Thoroughbred fertility, for which we consider this particular study a significant development in the exploration of the influence of the equine intestinal microbiome on fertility outcomes.

By way of informing both the pony and Thoroughbred studies, two reviews were conducted discussing published literature on these topics, offering context for the current use of prebiotics and probiotics in equines. While conducting these two reviews, no reference was found in the literature to any influence of either prebiotics or probiotics on equine reproductive health. To date, no pre-, pro- or synbiotic intervention has been explored for effects either generally on stallion health, or specifically on sperm quality or fertility outcomes. The reproductive microbiomes of mares and stallions are beginning to be characterised; however, no studies have been published to date that describe modification of these microbiomes to improve fertility outcomes. Further research is recommended to investigate the effect of pre-, pro- or synbiotic supplementation in a cohort of subfertile stallions, evaluating both semen quality biomarkers and PCCR, to gain

a better understanding of the potential for improvement of the composition of semen and faecal microbiomes to improve stallion fertility.

Future directions in equine reproduction research, which could be informed by the findings of this thesis, might include developing safe and sustainable synbiotic formulations for improving sperm quality in subfertile stallions and foaling outcomes for the mares they cover. Potential exists for future research to be designed based on the scope and outcomes of the retrospective study, exploring stallion fertility in relation to nutritional status, medical treatments, inflammatory conditions and digestive disorders, by providing useful baseline observations for healthy Thoroughbreds at stud. For human research in reproductive medicine and the management of the subfertile or infertile male, the concepts raised in this thesis might inform the design of studies that could explore the relationship of sperm quality to both the semen and faecal microbiomes, integrating factors of nutrition, digestion, inflammation and ageing, with the potential to improve fertility status and reproductive outcomes of men by prescribing synbiotic supplementation.

7.6 CONCLUSION

This PhD project investigated the relationship between stallion semen and faecal microbiomes, the possible influence of these microbes on sperm quality and per cycle conception rates, and the potential for adopting safe prebiotic and probiotic supplementation to enhance fertility outcomes. The conclusions drawn from this project are that pre-, pro- and synbiotic supplementation of healthy, fertile stallions was safe and well-tolerated, but did not influence either sperm quality or semen microbiome profiles, although prebiotic supplementation alone improved the richness and diversity of the composition of the faecal microbiome. The discovery that the semen microbiome composition of miniature pony stallions shared some similarities with other breeds, while also exhibiting uniqueness, which could be attributed to breed, regional location and stud farm management differences, was made in the process of this research. Our work in characterising stallion semen microbiota for the first time to five taxonomical levels might provide a healthy baseline to inform future studies exploring nutritional and beneficial

microbial interventions in stallions. The finding that Thoroughbred stallion PCCR was influenced by stud farm management practices and possibly also L-carnitine supplementation bears significance for the Thoroughbred breeding industry. No correlation was identified between the faecal microbiomes of these stallions, nor inflammatory status, and their fertility outcomes; however, the findings of this retrospective study may inform others seeking to explore safe ways to improve sperm quality and fertility of subfertile Thoroughbreds through pre- and probiotic supplementation, considering the additional performance benefits of these products. Some insights gained in the process of undertaking this thesis include the discovery that the semen and faecal microbiomes of stallions, particularly in relation to their fertility status, are relatively unchartered domains and that there may be future potential to improve breeding outcomes for horses by administration of pre- and probiotics, if one can extrapolate from the findings of human interventional studies with subfertile men. Much is still to be explored about the potential for identifying and restoring a dysbiotic intestinal microbiome with pro-, pre-, and synbiotic supplementation, for the purpose of improving reproductive health in the equine population.

8. REFERENCES

1. Aitken RJ. The changing tide of human fertility. Human reproduction (Oxford, England). 2022;37(4):629-38.
2. Bernanke J, Köhler HR. The impact of environmental chemicals on wildlife vertebrates. Rev Environ Contam Toxicol. 2009;198:1-47.
3. Benatta M, Kettache R, Buchholz N, Trinchieri A. The impact of nutrition and lifestyle on male fertility. Arch Ital Urol Androl. 2020;92(2).
4. Krausz C, Riera-Escamilla A. Genetics of male infertility. Nature reviews Urology. 2018;15(6):369-84.
5. Jacobsen FM, Rudlang TM, Fode M, Østergren PB, Sønksen J, Ohl DA, et al. The Impact of Testicular Torsion on Testicular Function. World J Mens Health. 2020;38(3):298-307.
6. Hedger MP. Immunophysiology and pathology of inflammation in the testis and epididymis. J Androl. 2011;32(6):625-40.
7. Collodel G, Castellini C, del Vecchio MT, Cardinali R, Geminiani M, Rossi B, et al. Effect of a bacterial lipopolysaccharide treatment on rabbit testis and ejaculated sperm. Reprod Domest Anim. 2012;47(3):372-8.
8. Fuke N, Nagata N, Suganuma H, Ota T. Regulation of Gut Microbiota and Metabolic Endotoxemia with Dietary Factors. Nutrients. 2019;11(10).
9. Belizário JE, Faintuch J, Garay-Malpartida M. Gut Microbiome Dysbiosis and Immunometabolism: New Frontiers for Treatment of Metabolic Diseases. Mediators of inflammation. 2018;2018:2037838.
10. Tsai Y-L, Lin T-L, Chang C-J, Wu T-R, Lai W-F, Lu C-C, et al. Probiotics, prebiotics and amelioration of diseases. Journal of biomedical science. 2019;26(1):3-.
11. Archana SS, Selvaraju S, Binsila BK, Arangasamy A, Krawetz SA. Immune regulatory molecules as modifiers of semen and fertility: A review. Molecular Reproduction and Development. 2019;86(11):1485-504.
12. Tani S, Piñeiro C, Koketsu Y. Culling in served females and farrowed sows at consecutive parities in Spanish pig herds. Porcine Health Manag. 2018;4:3.
13. Audet I, Laforest J-P, Martineau G, Matte J. Effect of vitamin supplements on some aspects of performance, vitamin status, and semen quality in boars. Journal of animal science. 2004;82(2):626-33.
14. Lea RG, Byers AS, Sumner RN, Rhind SM, Zhang Z, Freeman SL, et al. Environmental chemicals impact dog semen quality in vitro and may be associated with a temporal decline in sperm motility and increased cryptorchidism. Scientific Reports. 2016;6(1):31281.
15. Mima M, Greenwald D, Ohlander S. Environmental Toxins and Male Fertility. Curr Urol Rep. 2018;19(7):50.
16. Shokri S, Soltani A, Kazemi M, Sardari D, Mofrad FB. Effects of Wi-Fi (2.45 GHz) Exposure on Apoptosis, Sperm Parameters and Testicular Histomorphometry in Rats: A Time Course Study. Cell J. 2015;17(2):322-31.
17. Leeb T, Sieme H, Töpfer-Petersen E. Genetic markers for stallion fertility—lessons from humans and mice. Animal Reproduction Science. 2005;89(1):21-9.

18. Eskiocak S, Gozen AS, Taskiran A, Kilic AS, Eskiocak M, Gulen S. Effect of psychological stress on the L-arginine-nitric oxide pathway and semen quality. Braz J Med Biol Res. 2006;39(5):581-8.
19. Griffin RA, Swegen A, Baker MA, Ogle RA, Smith N, Aitken RJ, et al. Proteomic analysis of spermatozoa reveals caseins play a pivotal role in preventing short-term periods of subfertility in stallions†. Biol Reprod. 2022;106(4):741-55.
20. Meyers S, Bulkeley E, Foutouhi A. Sperm mitochondrial regulation in motility and fertility in horses. Reproduction in Domestic Animals. 2019;54(S3):22-8.
21. Gibb Z, Lambourne SR, Quadrelli J, Smith ND, Aitken RJ. L-carnitine and pyruvate are prosurvival factors during the storage of stallion spermatozoa at room temperature. Biol Reprod. 2015;104:1-9.
22. Turner RM. Declining testicular function in the aging stallion: Management options and future therapies. Animal Reproduction Science. 2019;207:171-9.
23. Laseca N, Anaya G, Peña Z, Pirosanto Y, Molina A, Demyda Peyrás S. Impaired Reproductive Function in Equines: From Genetics to Genomics. Animals (Basel). 2021;11(2).
24. Perrett J, Harris IT, Maddock C, Farnworth M, Pyatt AZ, Sumner RN. Systematic Analysis of Breed, Methodological, and Geographical Impact on Equine Sperm Progressive Motility. Animals (Basel). 2021;11(11).
25. Spinaci M, Nerozzi C, Mislei B, Blanco-Prieto O, Mari G, Galeati G, et al. Impact of glyphosate and its formulation Roundup® on stallion spermatozoa. Theriogenology. 2022;179:197-203.
26. Hillmann A, Ahrberg AB, Brehm W, Heller S, Josten C, Paebst F, et al. Comparative Characterization of Human and Equine Mesenchymal Stromal Cells: A Basis for Translational Studies in the Equine Model. Cell Transplant. 2016;25(1):109-24.
27. Harman RM, Theoret CL, Van de Walle GR. The Horse as a Model for the Study of Cutaneous Wound Healing. Adv Wound Care (New Rochelle). 2021;10(7):381-99.
28. Karagianni AE, Lisowski ZM, Hume DA, Scott Pirie R. The equine mononuclear phagocyte system: The relevance of the horse as a model for understanding human innate immunity. Equine veterinary journal. 2021;53(2):231-49.
29. Witkowski L, Cywinska A, Paschalis-Trela K, Crisman M, Kita J. Multiple etiologies of equine recurrent uveitis--A natural model for human autoimmune uveitis: A brief review. Comp Immunol Microbiol Infect Dis. 2016;44:14-20.
30. Horohov DW. The equine immune responses to infectious and allergic disease: a model for humans? Mol Immunol. 2015;66(1):89-96.
31. Bullone M, Lavoie JP. The equine asthma model of airway remodeling: from a veterinary to a human perspective. Cell Tissue Res. 2020;380(2):223-36.
32. Carnevale EM, Catandi GD, Fresa K. Equine Aging and the Oocyte: A Potential Model for Reproductive Aging in Women. Journal of Equine Veterinary Science. 2020;89:103022.
33. Gibb Z, Aitken RJ. The Impact of Sperm Metabolism during In Vitro Storage: The Stallion as a Model. Biomed Res Int. 2016;2016:9380609.
34. Baumber J, Vo A, Sabeur K, Ball BA. Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. Theriogenology. 2002;57(3):1025-33.

35. Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl. 2000;21(6):895-902.
36. Medica AJ, Aitken RJ, Nicolson GL, Sheridan AR, Swegen A, De Iuliis GN, et al. Glycerophospholipids protect stallion spermatozoa from oxidative damage in vitro. Reprod Fertil. 2021;2(3):199-209.
37. Gibb Z, Lambourne SR, Aitken RJ. The paradoxical relationship between stallion fertility and oxidative stress. Biol Reprod. 2014;91(3):77.
38. Aitken RJ. Impact of oxidative stress on male and female germ cells: implications for fertility. Reproduction. 2020;159(4):R189-r201.
39. Agarwal A, Parekh N, Panner Selvam MK, Henkel R, Shah R, Homa ST, et al. Male Oxidative Stress Infertility (MOSI): Proposed Terminology and Clinical Practice Guidelines for Management of Idiopathic Male Infertility. World J Mens Health. 2019;37(3):296-312.
40. Baud, Pattaroni C, Vulliemoz N, Castella V, Marsland BJ, Stojanov M. Sperm Microbiota and Its Impact on Semen Parameters. Frontiers in Microbiology. 2019;10(234).
41. Hou D, Zhou X, Zhong X, Settles ML, Herring J, Wang L, et al. Microbiota of the seminal fluid from healthy and infertile men. Fertility & Sterility. 2013;100(5):1261-9.
42. Morawiec E, Czerwiński M, Czerwińska AB-, Wiczkowski A. Semen dysbiosis—just a male problem? Frontiers in Cellular and Infection Microbiology. 2022;12.
43. Quiñones-Pérez C, Martínez A, Ortiz I, Crespo F, Vega-Pla JL. The Semen Microbiome and Semen Parameters in Healthy Stallions. Animals (Basel). 2022;12(5).
44. Valcarce DG, Genovés S, Riesco MF, Martorell P, Herráez MP, Ramón D, et al. Probiotic administration improves sperm quality in asthenozoospermic human donors. Benef Microbes. 2017;8(2):193-206.
45. Huntington P, Myers, J., Owens, E. Horse Sense. 2004:157-9.
46. Johnson L, Thompson DL, Jr. Age-related and seasonal variation in the Sertoli cell population, daily sperm production and serum concentrations of follicle-stimulating hormone, luteinizing hormone and testosterone in stallions. Biol Reprod. 1983;29(3):777-89.
47. Roser JF, Hughes JP. Seasonal effects on seminal quality, plasma hormone concentrations, and GnRH-induced LH response in fertile and subfertile stallions. J Androl. 1992;13(3):214-23.
48. Crespo F, Wilson R, Díaz-Jimenez M, Consuegra C, Dorado J, Barrado BG, et al. Effect of season on individual stallion semen characteristics. Anim Reprod Sci. 2020;223:106641.
49. Suliman Y, Becker F, Tuchscherer A, Wimmers K. Seasonal variations in quantitative and qualitative sperm characteristics in fertile and subfertile stallions. Arch Anim Breed. 2020;63(1):145-54.
50. Bazzano M, Laus F, Spaterna A, Marchegiani A. Use of nutraceuticals in the stallion: Effects on semen quality and preservation. Reprod Domest Anim. 2021;56(7):951-7.
51. Henneke DR, Potter GD, Kreider JL, Yeates BF. Relationship between condition score, physical measurements and body fat percentage in mares. Equine veterinary journal. 1983;15(4):371-2.
52. Frape DL. Nutrition and the growth and racing performance of thoroughbred horses. Proceedings of the Nutrition Society. 1989;48(1):141-52.

53. Salazar-Ortiz J, Monget P, Guillaume D. The influence of nutrition on the insulin-like growth factor system and the concentrations of growth hormone, glucose, insulin, gonadotropins and progesterone in ovarian follicular fluid and plasma from adult female horses (Equus caballus). Reprod Biol Endocrinol. 2014;12:72.
54. Fraipont A, Van Erck E, Ramery E, Fortier G, Lekeux P, Art T. Assessing fitness in endurance horses. The Canadian veterinary journal = La revue veterinaire canadienne. 2012;53(3):311-4.
55. Parks JE, Graham JK. Effects of cryopreservation procedures on sperm membranes. Theriogenology. 1992;38(2):209-22.
56. Brinsko SP, Varner DD, Love CC, Blanchard TL, Day BC, Wilson ME. Effect of feeding a DHA-enriched nutriceutical on the quality of fresh, cooled and frozen stallion semen. Theriogenology. 2005;63(5):1519-27.
57. Rodrigues PG, de Moura RS, Rocha LGP, Bottino MP, Nichi M, Maculan R, et al. Dietary polyunsaturated fatty acid supplementation improves the quality of stallion cryopreserved semen. Journal of Equine Veterinary Science. 2017;54:18-23.
58. Goedde LD. Effects of Feeding a Yeast-based Supplement Containing Docosahexaenoic Acid (DHA) from a Heterotrophically Grown Microalgae, Vitamin E, and Selenium on Stallion Sperm Motion Characteristics. Thesis for: Master of Science. 2016.
59. Grady S, Cavinder C, Brinsko S, Forrest D, Sawyer J, Scott B. Dietary supplementation of two varying sources of n-3 fatty acids and subsequent effects on fresh, cooled, and frozen seminal characteristics of stallions. The Professional Animal Scientist. 2009;25(6):768-73.
60. Freitas ML, Bouéres CS, Pignataro TA, de Oliveira FJG, de Oliveira Viu MA, de Oliveira RA. Quality of fresh, cooled, and frozen semen from stallions supplemented with antioxidants and fatty acids. Journal of Equine Veterinary Science. 2016;46:1-6.
61. Magistrini M, Seguin F, Beau P, Akoka S, Le Pape A, Palmer E. 1H nuclear magnetic resonance analysis of stallion genital tract fluids and seminal plasma: contribution of the accessory sex glands to the ejaculate. Biology of Reproduction. 1995;52(monograph_series1):599-607.
62. Mongioi L, Calogero AE, Vicari E, Condorelli RA, Russo GI, Privitera S, et al. The role of carnitine in male infertility. Andrology. 2016;4(5):800-7.
63. Stradaioli G, Sylla L, Zelli R, Chiodi P, Monaci M. Effect of L-carnitine administration on the seminal characteristics of oligoasthenospermic stallions. Theriogenology. 2004;62(3-4):761-77.
64. Cuddeford D. Equine nutrition: Crowood Press; 1996.
65. Gee E, Bruemmer J, Siciliano P, McCue P, Squires E. Effects of dietary vitamin E supplementation on spermatozoal quality in stallions with suboptimal post-thaw motility. Animal Reproduction Science. 2008;3(107):324-5.
66. Ralston S, Rich G, Jackson S, Squires E. The effect of vitamin A supplementation on seminal characteristics and vitamin a absorption in stallions. Journal of Equine Veterinary Science. 1986;6(4):203-7.
67. Eskenazi B, Kidd S, Marks A, Sloter E, Block G, Wyrobek A. Antioxidant intake is associated with semen quality in healthy men. Human reproduction. 2005;20(4):1006-12.
68. Deichsel K, Palm F, Koblischke P, Budik S, Aurich C. Effect of a dietary antioxidant supplementation on semen quality in pony stallions. Theriogenology. 2008;69(8):940-5.

69. Contri A, De Amicis I, Molinari A, Faustini M, Gramenzi A, Robbe D, et al. Effect of dietary antioxidant supplementation on fresh semen quality in stallion. Theriogenology. 2011;75(7):1319-26.
70. D'Anza E, Albarella S, Galdiero G, Tafuri S, Del Prete C, Cocchia N, et al. DNA fragmentation and morphometric studies in sperm of stallions supplemented with maca (Lepidium meyenii). Zygote. 2021;29(4):325-30.
71. Del Prete C, Tafuri S, Ciani F, Pasolini M, Ciotola F, Albarella S, et al. Influences of dietary supplementation with Lepidium meyenii (Maca) on stallion sperm production and on preservation of sperm quality during storage at 5 C. Andrology. 2018;6(2):351-61.
72. Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcoraci V, et al. Oxidative Stress: Harms and Benefits for Human Health. Oxidative medicine and cellular longevity. 2017;2017:8416763-.
73. Peña FJ, O'Flaherty C, Ortiz Rodríguez JM, Martín Cano FE, Gaitskell-Phillips GL, Gil MC, et al. Redox regulation and oxidative stress: The particular case of the stallion spermatozoa. Antioxidants. 2019;8(11):567.
74. Swegen A, Lambourne SR, Aitken RJ, Gibb Z. Rosiglitazone improves stallion sperm motility, ATP content, and mitochondrial function. Biology of Reproduction. 2016;95(5):107, 1-12.
75. Aitken RJ, Gibb Z, Mitchell LA, Lambourne SR, Connaughton HS, De Iuliis GN. Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. Biol Reprod. 2012;87(5):110.
76. Mislei B, Bucci D, Malama E, Bollwein H, Mari G. Seasonal changes in ROS concentrations and sperm quality in unfrozen and frozen-thawed stallion semen. Theriogenology. 2020;144:89-97.
77. Aurich C, Ortega Ferrusola C, Peña Vega FJ, Schrammel N, Morcuende D, Aurich J. Seasonal changes in the sperm fatty acid composition of Shetland pony stallions. Theriogenology. 2018;107:149-53.
78. Morte MI, Rodrigues AM, Soares D, Rodrigues AS, Gamboa S, Ramalho-Santos J. The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: Seasonal distinctions and correlations with DNA strand breaks, classical seminal parameters and stallion fertility. Animal Reproduction Science. 2008;106(1):36-47.
79. Rota A, Calicchio E, Nardoni S, Fratini F, Ebani VV, Sgorbini M, et al. Presence and distribution of fungi and bacteria in the reproductive tract of healthy stallions. Theriogenology. 2011;76(3):464-70.
80. Al-Kass, Eriksson E, Bagge E, Wallgren M, Morrell JM. Microbiota of semen from stallions in Sweden identified by MALDI-TOF. Vet Anim Sci. 2020;10:100143-.
81. Christoffersen M, Troedsson M. Inflammation and fertility in the mare. Reproduction in Domestic Animals. 2017;52(S3):14-20.
82. Schulman ML, May CE, Keys B, Guthrie AJ. Contagious equine metritis: Artificial reproduction changes the epidemiologic paradigm. Veterinary Microbiology. 2013;167(1):2-8.
83. Lundy SD, Vij SC, Rezk AH, Cohen JA, Bajic P, Ramasamy R. The microbiome of the infertile male. Curr Opin Urol. 2020;30(3):355-62.

84. Farahani L, Tharakan T, Yap T, Ramsay JW, Jayasena CN, Minhas S. The semen microbiome and its impact on sperm function and male fertility: A systematic review and meta-analysis. Andrology. 2021;9(1):115-44.
85. Okwelogu SI, Ikechebelu JI, Agbakoba NR, Anukam KC. Microbiome Compositions From Infertile Couples Seeking In Vitro Fertilization, Using 16S rRNA Gene Sequencing Methods: Any Correlation to Clinical Outcomes? Front Cell Infect Microbiol. 2021;11:709372.
86. Quinones-Perez C, Martinez A, Crespo F, Vega-Pla JL. Comparative Semen Microbiota Composition of a Stallion in a Taylorella equigenitalis Carrier and Non-Carrier State. Animals (Basel). 2020;10(5):868.
87. Jasko DJ. Evaluation of Stallion Semen. Veterinary Clinics of North America: Equine Practice. 1992;8(1):129-48.
88. Nath L, Anderson G, McKinnon A. Reproductive efficiency of Thoroughbred and Standardbred horses in north-east Victoria. Australian Veterinary Journal. 2010;88(5):169-75.
89. Lane EA, Bijnen MLJ, Osborne M, More SJ, Henderson ISF, Duffy P, et al. Key Factors Affecting Reproductive Success of Thoroughbred Mares and Stallions on a Commercial Stud Farm. Reproduction in domestic animals. 2016;51(2):181-7.
90. Blanchard TL, Thompson JA, Brinsko SP, Varner DD, Love CC, Ramsey J, et al. Some Factors Associated With Fertility of Thoroughbred Stallions. Journal of Equine Veterinary Science. 2010;30(8):407-18.
91. Ball BA. Diagnostic methods for evaluation of stallion subfertility: a review. Journal of Equine Veterinary Science. 2008;28(11):650-65.
92. Blanchard TL DD, Varner, MS, James Schumacher, Charles C.Love, Steven P. Brinsko, Sherri L. Rigby. Examination of the Stallion for Breeding Soundness. Manual of Equine Reproduction (Second Edition). 3rd ed: Mosby Elsevier; 2011. p. 205.
93. Allen WR, Wilsher S. The influence of mare numbers, ejaculation frequency and month on the fertility of Thoroughbred stallions. Equine veterinary journal. 2012;44(5):535-41.
94. Varner DD, Gibb Z, Aitken RJ. Stallion fertility: a focus on the spermatozoon. Equine veterinary journal. 2015;47(1):16-24.
95. Hurtgen JP. Evaluation of the stallion for breeding soundness. Vet Clin North Am Equine Pract. 1992;8(1):149-65.
96. Sieme H, Katila T, Klug E. Effect of semen collection practices on sperm characteristics before and after storage and on fertility of stallions. Theriogenology. 2004;61(4):769-84.
97. Dowsett KF, Knott LM. The influence of age and breed on stallion semen. Theriogenology. 1996;46(3):397-412.
98. Todd ET, Hamilton NA, Velie BD, Thomson PC. The effects of inbreeding on covering success, gestation length and foal sex ratio in Australian thoroughbred horses. BMC genetics. 2020;21(1):41-.
99. Todd ET, Ho SYW, Thomson PC, Ang RA, Velie BD, Hamilton NA. Founder-specific inbreeding depression affects racing performance in Thoroughbred horses. Scientific Reports. 2018;8(1):6167.
100. Sertich PL. Infertility in Horses. MSD Veterinary Manual. 2019.

101. Brinsko S, Blanchard, TL, Varner, DD, Schumacher, J, Love, CC, Hinrichs, K, Hartman, D. Manual of Equine Reproduction. 2011(3rd Edition):118-20.
102. McDonnell SM. Revisiting Clinical Stallion Sexual Behavior: Applying Ethology in the Breeding Shed. Journal of Equine Veterinary Science. 2016;43:S18-S22.
103. Katila T. Procedures for handling fresh stallion semen. Theriogenology. 1997;48(7):1217-27.
104. Morrell JM. Stallion Sperm Selection: Past, Present, and Future Trends. Journal of Equine Veterinary Science. 2012;32(8):436-40.
105. Ho H-e, Bunyavanich S. Role of the Microbiome in Food Allergy. Current Allergy and Asthma Reports. 2018;18(4):27.
106. Fan Y, Pedersen O. Gut microbiota in human metabolic health and disease. Nat Rev Microbiol. 2021;19(1):55-71.
107. Pham VT, Dold S, Rehman A, Bird JK, Steinert RE. Vitamins, the gut microbiome and gastrointestinal health in humans. Nutr Res. 2021;95:35-53.
108. Järbrink-Sehgal E, Andreasson A. The gut microbiota and mental health in adults. Curr Opin Neurobiol. 2020;62:102-14.
109. Matson V, Chervin CS, Gajewski TF. Cancer and the Microbiome-Influence of the Commensal Microbiota on Cancer, Immune Responses, and Immunotherapy. Gastroenterology. 2021;160(2):600-13.
110. Garber A, Hastie PM, Murray J-A. Factors Influencing Equine Gut Microbiota: Current Knowledge. Journal of Equine Veterinary Science. 2020;88:102943.
111. Nagpal J, Cryan JF. Microbiota-brain interactions: Moving toward mechanisms in model organisms. Neuron. 2021;109(24):3930-53.
112. Williams CL, Garcia-Reyero N, Martyniuk CJ, Tubbs CW, Bisesi JH, Jr. Regulation of endocrine systems by the microbiome: Perspectives from comparative animal models. Gen Comp Endocrinol. 2020;292:113437.
113. Jeong J, Yun K, Mun S, Chung W-H, Choi S-Y, Nam Y-d, et al. The effect of taxonomic classification by full-length 16S rRNA sequencing with a synthetic long-read technology. Scientific Reports. 2021;11(1):1727.
114. Murcia PR. Clinical insights: The equine microbiome. Equine veterinary journal. 2019;51(6):714-5.
115. Vermorel M, Martin-Rosset W. Concepts, scientific bases, structure and validation of the French horse net energy system (UFC). Livestock Production Science. 1997;47(3):261-75.
116. Dicks LMT, Botha M, Dicks E, Botes M. The equine gastro-intestinal tract: An overview of the microbiota, disease and treatment. Livestock Science. 2014;160(1):69-81.
117. Færgestad EM, Langsrud Ø, Høy M, Hollung K, Sæbø S, Liland KH, et al. 4.08.2.4 Metabolome. In: Brown SD, Tauler R, Walczak B, editors. Comprehensive Chemometrics. Oxford: Elsevier; 2009. p. 221-78.
118. Milinovich GJ, Klieve AV, Pollitt CC, Trott DJ. Microbial events in the hindgut during carbohydrate-induced equine laminitis. Vet Clin North Am Equine Pract. 2010;26(1):79-94.
119. Sanz MG. Science-in-brief: Equine microbiomics makes its way into equine veterinary medicine. Equine veterinary journal. 2022;54(2):453-4.

120. Holyoak GR, Premathilake HU, Lyman CC, Sones JL, Gunn A, Wieneke X, et al. The healthy equine uterus harbors a distinct core microbiome plus a rich and diverse microbiome that varies with geographical location. Sci Rep. 2022;12(1):14790.
121. Lowman ME, Tipton CD, Labordère AL, Brown JA. Equine sinusitis aetiology is linked to sinus microbiome by amplicon sequencing. Equine veterinary journal. 2022.
122. Kennedy R, Lappin DF, Dixon PM, Buijs MJ, Zaura E, Crielaard W, et al. The microbiome associated with equine periodontitis and oral health. Vet Res. 2016;47:49.
123. Gao W, Chan Y, You M, Lacap-Bugler DC, Leung WK, Watt RM. In-depth snapshot of the equine subgingival microbiome. Microb Pathog. 2016;94:76-89.
124. Borkent D, Reardon RJM, Mc LG, Glendinning L, Dixon PM. A microbiome analysis of equine peripheral dental caries using next generation sequencing. Equine veterinary journal. 2020;52(1):67-75.
125. Kaiser-Thom S, Hilty M, Axiak S, Gerber V. The skin microbiota in equine pastern dermatitis: a case-control study of horses in Switzerland. Veterinary Dermatology. 2021;32(6):646-e172.
126. Martin de Bustamante M, Gomez D, MacNicol J, Hamor R, Plummer C. The Fecal Bacterial Microbiota in Horses with Equine Recurrent Uveitis. Animals (Basel). 2021;11(3).
127. Fraga M, Perelmuter K, Delucchi L, Cidade E, Zunino P. Vaginal lactic acid bacteria in the mare: evaluation of the probiotic potential of native Lactobacillus spp. and Enterococcus spp. strains. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology. 2008;93(1-2):71-8.
128. Husso A, Jalanka J, Alipour MJ, Huhti P, Kareskoski M, Pessa-Morikawa T, et al. The composition of the perinatal intestinal microbiota in horse. Scientific reports. 2020;10(1):441-.
129. Kauter A, Epping L, Semmler T, Antao E-M, Kannapin D, Stoeckle SD, et al. The gut microbiome of horses: current research on equine enteral microbiota and future perspectives. Animal Microbiome. 2019;1(1):14.
130. Costa MC, Arroyo LG, Allen-Vercoe E, Stampfli HR, Kim PT, Sturgeon A, et al. Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16S rRNA gene. PloS one. 2012;7(7):e41484.
131. Nocera FP, D'Eletto E, Ambrosio M, Fiorito F, Pagnini U, De Martino L. Occurrence and Antimicrobial Susceptibility Profiles of Streptococcus equi subsp. zooepidemicus Strains Isolated from Mares with Fertility Problems. Antibiotics (Basel). 2021;11(1).
132. Al-Kass Z, Eriksson E, Bagge E, Wallgren M, Morrell JM. Bacteria detected in the genital tract, semen or pre-ejaculatory fluid of Swedish stallions from 2007 to 2017. Acta Vet Scand. 2019;61(1):25.
133. Wang H, Xu A, Gong L, Chen Z, Zhang B, Li X. The Microbiome, an Important Factor That Is Easily Overlooked in Male Infertility. Front Microbiol. 2022;13:831272.
134. Al-Asmakh M, Stukenborg JB, Reda A, Anuar F, Strand ML, Hedin L, et al. The gut microbiota and developmental programming of the testis in mice. PloS one. 2014;9(8):e103809.
135. Thevaranjan N, Puchta A, Schulz C, Naidoo A, Szamosi JC, Verschoor CP, et al. Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. Cell Host Microbe. 2017;21(4):455-66.e4.

136. Rowe M, Veerus L, Trosvik P, Buckling A, Pizzari T. The Reproductive Microbiome: An Emerging Driver of Sexual Selection, Sexual Conflict, Mating Systems, and Reproductive Isolation. Trends in Ecology & Evolution. 2020;35(3):220-34.
137. Barba M, Martínez-Boví R, Quereda JJ, Mocé ML, Plaza-Dávila M, Jiménez-Trigos E, et al. Vaginal Microbiota Is Stable throughout the Estrous Cycle in Arabian Maress. Animals (Basel). 2020;10(11).
138. Quiñones-Pérez C, Hidalgo M, Ortiz I, Crespo F, Vega-Pla JL. Characterization of the seminal bacterial microbiome of healthy, fertile stallions using next-generation sequencing. Animal Reproduction. 2021;18.
139. Wernimont SM, Radosevich J, Jackson MI, Ephraim E, Badri DV, MacLeay JM, et al. The Effects of Nutrition on the Gastrointestinal Microbiome of Cats and Dogs: Impact on Health and Disease. Frontiers in microbiology. 2020;11:1266-.
140. Gomaa EZ. Human gut microbiota/microbiome in health and diseases: a review. Antonie Van Leeuwenhoek. 2020;113(12):2019-40.
141. Chang CH, Teng PY, Lee TT, Yu B. Effects of Multi-Strain Probiotics Combined with Gardeniae fructus on Intestinal Microbiota, Metabolites, and Morphology in Broilers. J Poult Sci. 2019;56(1):32-43.
142. Julliand V, Grimm P. Horse Species Symposium: The microbiome of the horse hindgut: History and current knowledge. J Anim Sci. 2016;94(6):2262-74.
143. Al Jassim RAM, Andrews FM. The Bacterial Community of the Horse Gastrointestinal Tract and Its Relation to Fermentative Acidosis, Laminitis, Colic, and Stomach Ulcers. Veterinary Clinics of North America: Equine Practice. 2009;25(2):199-215.
144. Costa MC, Silva G, Ramos RV, Staempfli HR, Arroyo LG, Kim P, et al. Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses. The Veterinary Journal. 2015;205(1):74-80.
145. Costa MC, Stämpfli HR, Allen-Vercoe E, Weese JS. Development of the faecal microbiota in foals. Equine Veterinary Jourrnal. 2016;48(6):681-8.
146. Massacci FR, Clark A, Ruet A, Lansade L, Costa M, Mach N. Inter-breed diversity and temporal dynamics of the faecal microbiota in healthy horses. J Anim Breed Genet. 2020;137(1):103-20.
147. Hu D, Chao Y, Li Y, Peng X, Wang C, Wang Z, et al. Effect of Gender Bias on Equine Fecal Microbiota. Journal of Equine Veterinary Science. 2021;97:103355.
148. Dougal K, de la Fuente G, Harris PA, Girdwood SE, Pinloche E, Geor RJ, et al. Characterisation of the Faecal Bacterial Community in Adult and Elderly Horses Fed a High Fibre, High Oil or High Starch Diet Using 454 Pyrosequencing. PloS one. 2014;9(2):e87424.
149. Proudman C, Hunter J, Darby A, Escalona E, Batty C, Turner C. Characterisation of the faecal metabolome and microbiome of Thoroughbred racehorses. Equine veterinary journal. 2015;47(5):580-6.
150. Fernandes KA, Kittelmann S, Rogers CW, Gee EK, Bolwell CF, Bermingham EN, et al. Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. PloS one. 2014;9(11):e112846.
151. van den Berg M, Hoskin SO, Rogers CW, Grinberg A. Fecal pH and Microbial Populations in Thoroughbred Horses During Transition from Pasture to Concentrate Feeding. Journal of Equine Veterinary Science. 2013;33(4):215-22.

152. Daly K, Proudman CJ, Duncan SH, Flint HJ, Dyer J, Shirazi-Beechey SP. Alterations in microbiota and fermentation products in equine large intestine in response to dietary variation and intestinal disease. British Journal of Nutrition. 2012;107(7):989-95.
153. Gomez A, Sharma AK, Grev A, Sheaffer C, Martinson K. The Horse Gut Microbiome Responds in a Highly Individualized Manner to Forage Lignification. Journal of Equine Veterinary Science. 2021;96:103306.
154. Hansen NC, Avershina E, Mydland LT, Næsset JA, Austbø D, Moen B, et al. High nutrient availability reduces the diversity and stability of the equine caecal microbiota. Microb Ecol Health Dis. 2015;26:27216.
155. Perricone V, Sandrini S, Irshad N, Comi M, Lecchi C, Savoini G, et al. The Role of Yeast Saccharomyces cerevisiae in Supporting Gut Health in Horses: An Updated Review on Its Effects on Digestibility and Intestinal and Fecal Microbiota. Animals (Basel). 2022;12(24).
156. Morgan L, Coverdale J, Froetschel M, Yoon I. Effect of yeast culture supplementation on digestibility of varying forage quality in mature horses. Journal of Equine Veterinary Science. 2007;27(6):260-5.
157. Medina B, Girard ID, Jacotot E, Julliand V. Effect of a preparation of Saccharomyces cerevisiae on microbial profiles and fermentation patterns in the large intestine of horses fed a high fiber or a high starch diet. Journal of Animal Science. 2002;80(10):2600-9.
158. Schoster A. Probiotic Use in Equine Gastrointestinal Disease. Veterinary Clinics of North America-Equine Practice. 2018;34(1):13-+.
159. Salem SE, Maddox TW, Berg A, Antczak P, Ketley JM, Williams NJ, et al. Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period. Scientific Reports. 2018;8(1):8510.
160. Theelen MJP, Luiken REC, Wagenaar JA, Sloet van Oldruitenborgh-Oosterbaan MM, Rossen JWA, Zomer AL. The Equine Faecal Microbiota of Healthy Horses and Ponies in The Netherlands: Impact of Host and Environmental Factors. Animals (Basel). 2021;11(6).
161. Costa MC, Stämpfli HR, Arroyo LG, Allen-Vercoe E, Gomes RG, Weese JS. Changes in the equine fecal microbiota associated with the use of systemic antimicrobial drugs. BMC Vet Res. 2015;11:19-.
162. Arnold C, Pilla R, Chaffin K, Lidbury J, Steiner J, Suchodolski J. Alterations in the fecal microbiome and metabolome of horses with antimicrobial-associated diarrhea compared to antibiotic-treated and non-treated healthy case controls. Animals. 2021;11(6):1807.
163. De La Cochetière M-F, Durand T, Lepage P, Bourreille A, Galmiche J, Dore J. Resilience of the dominant human fecal microbiota upon short-course antibiotic challenge. Journal of clinical microbiology. 2005;43(11):5588-92.
164. Arnold CE, Isaiah A, Pilla R, Lidbury J, Coverdale JS, Callaway TR, et al. The cecal and fecal microbiomes and metabolomes of horses before and after metronidazole administration. PloS one. 2020;15(5):e0232905.
165. Henshall C, Randle H, Francis N, Freire R. The effect of stress and exercise on the learning performance of horses. Sci Rep. 2022;12(1):1918.
166. Clark A, Mach N. Exercise-induced stress behavior, gut-microbiota-brain axis and diet: a systematic review for athletes. J Int Soc Sports Nutr. 2016;13:43.

167. Barton W, Penney NC, Cronin O, Garcia-Perez I, Molloy MG, Holmes E, et al. The microbiome of professional athletes differs from that of more sedentary subjects in composition and particularly at the functional metabolic level. Gut. 2018;67(4):625-33.
168. Plancade S, Clark A, Philippe C, Helbling J-C, Moisan M-P, Esquerré D, et al. Unraveling the effects of the gut microbiota composition and function on horse endurance physiology. Scientific reports. 2019;9(1):1-15.
169. Le Moyec L, Robert C, Triba MN, Billat VL, Mata X, Schibler L, et al. Protein catabolism and high lipid metabolism associated with long-distance exercise are revealed by plasma NMR metabolomics in endurance horses. PloS one. 2014;9(3):e90730.
170. Schoster A, Mosing M, Jalali M, Staempfli HR, Weese JS. Effects of transport, fasting and anaesthesia on the faecal microbiota of healthy adult horses. Equine veterinary journal. 2016;48(5):595-602.
171. Metcalf JL, Song SJ, Morton JT, Weiss S, Seguin-Orlando A, Joly F, et al. Evaluating the impact of domestication and captivity on the horse gut microbiome. Scientific reports. 2017;7(1):1-9.
172. Weese JS, Holcombe SJ, Embertson RM, Kurtz KA, Roessner HA, Jalali M, et al. Changes in the faecal microbiota of mares precede the development of post partum colic. Equine veterinary journal. 2015;47(6):641-9.
173. Costa MC, Weese JS. Understanding the Intestinal Microbiome in Health and Disease. Vet Clin North Am Equine Pract. 2018;34(1):1-12.
174. Stewart HL, Southwood LL, Indugu N, Vecchiarelli B, Engiles JB, Pitta DW. Differences in the equine faecal microbiota between horses presenting to a tertiary referral hospital for colic compared with an elective surgical procedure. Equine veterinary journal. 2019;51:33V 342.
175. Elzinga SE, Weese JS, Adams AA. Comparison of the Fecal Microbiota in Horses With Equine Metabolic Syndrome and Metabolically Normal Controls Fed a Similar All-Forage Diet. Journal of Equine Veterinary Science. 2016;44:9-16.
176. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. nature. 2009;457(7228):480-4.
177. Zhang X, Shen D, Fang Z, Jie Z, Qiu X, Zhang C, et al. Human gut microbiota changes reveal the progression of glucose intolerance. PloS one. 2013;8(8):e71108.
178. O'Donnell MM, Harris HMB, Jeffery IB, Claesson MJ, Younge B, O'Toole PW, et al. The core faecal bacterial microbiome of Irish Thoroughbred racehorses. Letters in Applied Microbiology. 2013;57(6):492-501.
179. Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. Nat Rev Immunol. 2016;16(6):341-52.
180. Al Bander Z, Nitert MD, Mousa A, Naderpoor N. The Gut Microbiota and Inflammation: An Overview. Int J Environ Res Public Health. 2020;17(20).
181. Brandsma E, Kloosterhuis NJ, Koster M, Dekker DC, Gijbels MJJ, Velden Svd, et al. A Proinflammatory Gut Microbiota Increases Systemic Inflammation and Accelerates Atherosclerosis. Circulation Research. 2019;124(1):94-100.
182. Woodward E, Schlingmann K, Tobias J, Turner R. Characterisation of the testicular transcriptome in stallions with age-related testicular degeneration. Equine veterinary journal. 2023;55(2):239-52.

183. Sancler-Silva YFR, Monteiro GA, Ramires-Neto C, Freitas-Dell'aqua CP, Crespilho AM, Franco MMJ, et al. Does semen quality change after local treatment of seminal vesiculitis in stallions? Theriogenology. 2020;144:139-45.
184. Vinther AM, Heegaard PM, Skovgaard K, Buhl R, Andreassen SM, Andersen PH. Characterization and differentiation of equine experimental local and early systemic inflammation by expression responses of inflammation-related genes in peripheral blood leukocytes. BMC Vet Res. 2016;12:83.
185. Clemente JC, Manasson J, Scher JU. The role of the gut microbiome in systemic inflammatory disease. BMJ (Clinical research ed). 2018;360:j5145.
186. Leclere M, Costa MC. Fecal microbiota in horses with asthma. Journal of veterinary internal medicine. 2020;34(2):996-1006.
187. Cristofori F, Dargenio VN, Dargenio C, Miniello VL, Barone M, Francavilla R. Anti-Inflammatory and Immunomodulatory Effects of Probiotics in Gut Inflammation: A Door to the Body. Front Immunol. 2021;12:578386.
188. Thomas S, Przesdzing I, Metzke D, Schmitz J, Radbruch A, Baumgart DC. Saccharomyces boulardii inhibits lipopolysaccharide-induced activation of human dendritic cells and T cell proliferation. Clin Exp Immunol. 2009;156(1):78-87.
189. Al-Kass, Guo Y, Vinnere Pettersson O, Niazi A, Morrell JM. Metagenomic analysis of bacteria in stallion semen. Animal Reproduction Science. 2020;221:106568.
190. Pasing SS, Aurich C, von Lewinski M, Wulf M, Krüger M, Aurich JE. Development of the genital microflora in stallions used for artificial insemination throughout the breeding season. Animal Reproduction Science. 2013;139(1):53-61.
191. Samper JC, Tibary A. Disease transmission in horses. Theriogenology. 2006;66(3):551-9.
192. Ramires Neto C, Sancler da Silva YFR, Resende HL, Guasti PN, Monteiro GA, Papa PM, et al. Control Methods and Evaluation of Bacterial Growth on Fresh and Cooled Stallion Semen. Journal of Equine Veterinary Science. 2015;35(4):277-82.
193. Zhao H, Zhang Q. Performance of electro-Fenton process coupling with microbial fuel cell for simultaneous removal of herbicide mesotrione. Bioresource Technology. 2021;319:124244.
194. Cooke CG, Gibb Z, Harnett JE. The Safety, Tolerability and Efficacy of Probiotic Bacteria for Equine Use. J Equine Vet Sci. 2021;99:103407.
195. Coverdale JA. Horse Species Symposium: Can the microbiome of the horse be altered to improve digestion? Journal of Animal Science. 2016;94(6):2275-81.
196. Tanabe S, Suzuki T, Wasano Y, Nakajima F, Kawasaki H, Tsuda T, et al. Anti-inflammatory and Intestinal Barrier-protective Activities of Commensal Lactobacilli and Bifidobacteria in Thoroughbreds: Role of Probiotics in Diarrhea Prevention in Neonatal Thoroughbreds. J Equine Sci. 2014;25(2):37-43.
197. Desrochers AM, Dolente BA, Roy MF, Boston R, Carlisle S. Efficacy of Saccharomyces boulardii for treatment of horses with acute enterocolitis. Journal of the American Veterinary Medical Association. 2005;227(6):954-9.
198. Laghi L, Zhu C, Campagna G, Rossi G, Bazzano M, Laus F. Probiotic supplementation in trained trotter horses: effect on blood clinical pathology data and urine metabolomic assessed in field. J Appl Physiol (1985). 2018;125(2):654-60.
199. Zavistanaviciute P, Poskiene I, Lele V, Antanaitis R, Kantautaite J, Bartkiene E. The influence of the newly isolated Lactobacillus plantarum LUHS135 and Lactobacillus

paracasei LUHS244 strains on blood and faeces parameters in endurance horses. Polish Journal of Veterinary Sciences. 2019;22(3):513-21.
200. Böhmer BM, Kramer W, Roth-Maier DA. Dietary probiotic supplementation and resulting effects on performance, health status, and microbial characteristics of primiparous sows. Journal of Animal Physiology and Animal Nutrition. 2006;90(7-8):309-15.
201. Xiang Q, Wang C, Zhang H, Lai W, Wei H, Peng J. Effects of Different Probiotics on Laying Performance, Egg Quality, Oxidative Status, and Gut Health in Laying Hens. Animals (Basel). 2019;9(12).
202. Heil BA, Paccamonti DL, Sones JL. Role for the mammalian female reproductive tract microbiome in pregnancy outcomes. Physiol Genomics. 2019;51(8):390-9.
203. Ong CT, Ross EM, Boe-Hansen G, Turni C, Hayes BJ, Fordyce G, et al. Adaptive sampling during sequencing reveals the origins of the bovine reproductive tract microbiome across reproductive stages and sexes. Scientific Reports. 2022;12(1):15075.
204. Hughes JP, Loy RG. The Relation of Infection to Infertility in the Mare and Stallion. Equine veterinary journal. 1975;7(3):155-9.
205. Madsen M, Christensen P. Bacterial flora of semen collected from Danish Warmblood stallions by artifical vagina. Acta Veterinaria Scandinavica. 1995;36(1):1-7.
206. Malaluang P, Wilén E, Lindahl J, Hansson I, Morrell JM. Antimicrobial Resistance in Equine Reproduction. Animals (Basel). 2021;11(11).
207. Böttger EC. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiology Letters. 1989;65(1):171-6.
208. Woo PCY, Lau SKP, Teng JLL, Tse H, Yuen KY. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clinical Microbiology and Infection. 2008;14(10):908-34.
209. Mignard SF, J.P. . 16S rRNA sequencing in routine bacterial identification: A 30-month experiment. Journal of Microbiological Methods 2006;67:574–81.
210. Bull K, Davies G, Jenkins TP, Peachey LE. The Equine Faecal Microbiota Undergoes Step-wise Compositional Changes in Accordance With Increasing Levels of Domestication. 2021.
211. Mach N, Ruet A, Clark A, Bars-Cortina D, Ramayo-Caldas Y, Crisci E, et al. Priming for welfare: gut microbiota is associated with equitation conditions and behavior in horse athletes. Scientific Reports. 2020;10(1):8311.
212. Julliand V, Pauline G. The Impact of Diet on the Hindgut Microbiome. Journal of equine veterinary science. 2017;52:23-8.
213. Paßlack N, Vahjen W, Zentek J. Impact of dietary cellobiose on the fecal microbiota of horses. Journal of Equine Veterinary Science. 2020:103106.
214. Fuks G, Elgart M, Amir A, Zeisel A, Turnbaugh PJ, Soen Y, et al. Combining 16S rRNA gene variable regions enables high-resolution microbial community profiling. Microbiome. 2018;6(1):17.
215. Jeske JT, Gallert C. Microbiome Analysis via OTU and ASV-Based Pipelines-A Comparative Interpretation of Ecological Data in WWTP Systems. Bioengineering (Basel). 2022;9(4).

216. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37(8):852-7.
217. Quinones-Perez C, Hidalgo M, Ortiz I, Crespo F, Vega-Pla JL. Characterization of the seminal bacterial microbiome of healthy, fertile stallions using next-generation sequencing. Anim Reprod. 2021;18(2):e20200052.
218. Li Y, Lan Y, Zhang S, Wang X. Comparative Analysis of Gut Microbiota Between Healthy and Diarrheic Horses. Frontiers in Veterinary Science. 2022;9:882423.
219. Ayoub C, Arroyo LG, MacNicol JL, Renaud D, Weese JS, Gomez DE. Fecal microbiota of horses with colitis and its association with laminitis and survival during hospitalization. Journal of Veterinary Internal Medicine. 2022;36(6):2213-23.
220. Liu CM, Osborne BJ, Hungate BA, Shahabi K, Huibner S, Lester R, et al. The semen microbiome and its relationship with local immunology and viral load in HIV infection. PLoS Pathogens. 2014;10(7):e1004262.
221. Grabowski A, Tindall BJ, Bardin V, Blanchet D, Jeanthon C. Petrimonas sulfuriphila gen. nov., sp. nov., a mesophilic fermentative bacterium isolated from a biodegraded oil reservoir. International journal of systematic and evolutionary microbiology. 2005;55(3):1113-21.
222. Wang X, Cao A, Zhao G, Zhou C, Xu R. Microbial community structure and diversity in a municipal solid waste landfill. Waste Management. 2017;66:79-87.
223. Shi Z, Zhang L, Yuan H, Li X, Chang Y, Zuo X. Oyster shells improve anaerobic dark fermentation performances of food waste: Hydrogen production, acidification performances, and microbial community characteristics. Bioresource Technology. 2021;335:125268.
224. Naphtali P, Mohiuddin MM, Paschos A, Schellhorn HE. Application of high-throughput 16S rRNA sequencing to identify fecal contamination sources and to complement the detection of fecal indicator bacteria in rural groundwater. Journal of Water and Health. 2019;17(3):393-403.
225. Antwis RE, Lea JMD, Unwin B, Shultz S. Gut microbiome composition is associated with spatial structuring and social interactions in semi-feral Welsh Mountain ponies. Microbiome. 2018;6(1):207.
226. Garcia-Segura S, Del Rey J, Closa L, Garcia-Martínez I, Hobeich C, Castel AB, et al. Seminal Microbiota of Idiopathic Infertile Patients and Its Relationship With Sperm DNA Integrity. Front Cell Dev Biol. 2022;10:937157.
227. Cho I, Blaser MJ. The human microbiome: at the interface of health and disease. Nature Reviews Genetics. 2012;13(4):260-70.
228. Ettinger G, MacDonald K, Reid G, Burton JP. The influence of the human microbiome and probiotics on cardiovascular health. Gut Microbes. 2014;5(6):719-28.
229. Koopen AM, Groen AK, Nieuwdorp M. Human microbiome as therapeutic intervention target to reduce cardiovascular disease risk. Current Opinion in Lipidology. 2016;27(6):615-22.
230. Madupu R, Szpakowski S, Nelson KE. Microbiome in Human Health and Disease. Science Progress. 2013;96(2):153-70.
231. Maruvada P, Leone V, Kaplan LM, Chang EB. The Human Microbiome and Obesity: Moving beyond Associations. Cell Host Microbe. 2017;22(5):589-99.

232. Mazidi M, Rezaie P, Kengne AP, Mobarhan MG, Ferns GA. Gut microbiome and metabolic syndrome. Diabetes Metab Syndr. 2016;10(2 Suppl 1):S150-7.
233. Foster JA, McVey Neufeld KA. Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci. 2013;36(5):305-12.
234. Pascal M, Perez-Gordo M, Caballero T, Escribese MM, Lopez Longo MN, Luengo O, et al. Microbiome and Allergic Diseases. Front Immunol. 2018;9:1584.
235. Bahrndorff S, Alemu T, Alemneh T, Lund Nielsen J. The Microbiome of Animals: Implications for Conservation Biology. Int J Genomics. 2016;2016:5304028.
236. Guarner F, Malagelada J-R. Gut flora in health and disease. The Lancet. 2003;361(9356):512-9.
237. Wehkamp J, Fellermann K, Herrlinger KR, Bevins CL, Stange EF. Mechanisms of Disease: defensins in gastrointestinal diseases. Nature Clinical Practice Gastroenterology & Hepatology. 2005;2(9):406-15.
238. Gomez-Arango LF, Barrett HL, McIntyre HD, Callaway LK, Morrison M, Dekker Nitert M. Connections Between the Gut Microbiome and Metabolic Hormones in Early Pregnancy in Overweight and Obese Women. Diabetes. 2016;65(8):2214.
239. Day RL, Harper AJ, Woods RM, Davies OG, Heaney LM. Probiotics: current landscape and future horizons. Future Sci OA. 2019;5(4):FSO391.
240. World Health Organisation W, editor Food and Agricultural Organization of the United Nations and World Health Organization. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. World Health Organization [online]; 2001.
241. McFarland LV, Evans CT, Goldstein EJC. Strain-Specificity and Disease-Specificity of Probiotic Efficacy: A Systematic Review and Meta-Analysis. Front Med (Lausanne). 2018;5:124.
242. Jouany JP, Medina B, Bertin G, Julliand V. Effect of live yeast culture supplementation on hindgut microbial communities and their polysaccharidase and glycoside hydrolase activities in horses fed a high-fiber or high-starch diet. Journal of Animal Science. 2009;87(9):2844-52.
243. Alessandri G, Milani C, Mancabelli L, Longhi G, Anzalone R, Lugli GA, et al. Deciphering the Bifidobacterial Populations within the Canine and Feline Gut Microbiota. Applied and Environmental Microbiology. 2020;86(7):e02875-19.
244. Quan J, Cai G, Ye J, Yang M, Ding R, Wang X, et al. A global comparison of the microbiome compositions of three gut locations in commercial pigs with extreme feed conversion ratios. Sci Rep. 2018;8(1):4536.
245. Kylie J, Weese JS, Turner PV. Comparison of the fecal microbiota of domestic commercial meat, laboratory, companion, and shelter rabbits (Oryctolagus cuniculi). BMC Vet Res. 2018;14(1):143.
246. Contreras M, Morales A, Garcia-Amado MA, De Vera M, Bermudez V, Gueneau P. Detection of Helicobacter-like DNA in the gastric mucosa of Thoroughbred horses. Lett Appl Microbiol. 2007;45(5):553-7.
247. Larsen J. Acute colitis in adult horses. A review with emphasis on aetiology and pathogenesis. Vet Q. 1997;19(2):72-80.
248. Han H, McGivney BA, Farries G, Katz LM, MacHugh DE, Randhawa IAS, et al. Selection in Australian Thoroughbred horses acts on a locus associated with early two-year old speed. PloS one. 2020;15(2):e0227212.

249. Pugh G. The Role of Microbial Diversity in Female Reproductive Health: An Analysis of the Microbiome's Influence on Pregnancy Outcomes in Postparturient Mares: Eastern Kentucky University; 2018.
250. Weese JS, Rousseau J. Evaluation of Lactobacillus pentosus WE7 for prevention of diarrhea in neonatal foals. Javma-Journal of the American Veterinary Medical Association. 2005;226(12):2031-4.
251. Weese JS, Anderson MEC, Lowe A, Monteith GJ. Preliminary investigation of the probiotic potential of Lactobacillus rhamnosus strain GG in horses: fecal recovery following oral administration and safety. Canadian Veterinary Journal-Revue Veterinaire Canadienne. 2003;44(4):299-302.
252. John J, Roediger K, Schroedl W, Aldaher N, Vervuert I. Development of intestinal microflora and occurrence of diarrhoea in sucking foals: Effects of Bacillus cereus var. toyoi supplementation. BMC Vet Res. 2015;11(1).
253. Schoster A, Staempfli HR, Guardabassi LG, Jalali M, Weese JS. Comparison of the fecal bacterial microbiota of healthy and diarrheic foals at two and four weeks of life. BMC Vet Res. 2017;13(1):144.
254. Schoster A, Staempfli HR, Abrahams M, Jalali M, Weese JS, Guardabassi L. Effect of a Probiotic on Prevention of Diarrhea and Clostridium difficile and Clostridium perfringens Shedding in Foals. Journal of Veterinary Internal Medicine. 2015;29(3):925-31.
255. Harlow BE, Lawrence LM, Harris PA, Aiken GE, Flythe MD. Exogenous lactobacilli mitigate microbial changes associated with grain fermentation (corn, oats, and wheat) by equine fecal microflora ex vivo. PLoS ONE [Electronic Resource]. 2017;12(3):e0174059.
256. Meade KG, O'Farrelly C. β-Defensins: Farming the Microbiome for Homeostasis and Health. Front Immunol. 2018;9:3072.
257. Krajmalnik-Brown R, Ilhan Z-E, Kang D-W, DiBaise JK. Effects of gut microbes on nutrient absorption and energy regulation. Nutr Clin Pract. 2012;27(2):201-14.
258. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. Cell. 2014;157(1):121-41.
259. Garber A, Hastie PM, Farci V, Bulmer L, Alzahal O, Murray JMD. The effect of supplementing pony diets with yeast on 1. In vivo and in vitro digestibility, faecal pH and particle size. animal. 2020:1-12.
260. Aguilera M, Vergara P, Martinez V. Stress and antibiotics alter luminal and wall-adhered microbiota and enhance the local expression of visceral sensory-related systems in mice. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society. 2013;25(8):e515-29.
261. Kim L, Morley PS, Traub-Dargatz JL, Salman MD, Gentry-Weeks C. Factors associated with Salmonella shedding among equine colic patients at a veterinary teaching hospital. Journal of the American Veterinary Medical Association. 2001;218(5):740-8.
262. Ward MP, Alinovi CA, Couetil LL, Glickman LT, Wu CC. A Randomized clinical trial using probiotics to prevent Salmonella fecal shedding in hospitalized horses. Journal of Equine Veterinary Science. 2004;24(6):242-7.
263. Yuyama T YS, Takai S, Tsubaki S, Kado Y, Morotomi M. Evaluation of a Host-Specific Lactobacillus Probiotic in Neonatal Foals. International Journal of Applied Research In Veterinary Medicine. 2004(2):26-33.

264. Terpou A, Papadaki A, Lappa IK, Kachrimanidou V, Bosnea LA, Kopsahelis N. Probiotics in Food Systems: Significance and Emerging Strategies Towards Improved Viability and Delivery of Enhanced Beneficial Value. Nutrients. 2019;11(7):1591.
265. Schoster A, Guardabassi L, Staempfli HR, Abrahams M, Jalali M, Weese JS. The longitudinal effect of a multi-strain probiotic on the intestinal bacterial microbiota of neonatal foals. Equine veterinary journal. 2016;48(6):689-96.
266. Parraga ME, Spier SJ, Thurmond M, Hirsh D. A clinical trial of probiotic administration for prevention of Salmonella shedding in the postoperative period in horses with colic. Journal of Veterinary Internal Medicine. 1997;11(1):36-41.
267. Morotomi M, Yuki N, Kado Y, Kushiro A, Shimazaki T, Watanabe K, et al. Lactobacillus equi sp nov., a predominant intestinal Lactobacillus species of the horse isolated from faeces of healthy horses. International Journal of Systematic and Evolutionary Microbiology. 2002;52:211-4.
268. Laukova A, Stykova E, Kubasova I, Gancarcikova S, Placha I, Mudronova D, et al. Enterocin M and its Beneficial Effects in Horses-a Pilot Experiment. Probiotics Antimicrob Proteins. 2018;10(3):420-6.
269. Weese JS, Anderson MEC, Lowe A, Penno R, Da Costa TM, Button L, et al. Screening of the equine intestinal microflora for potential probiotic organisms. Equine veterinary journal. 2004;36(4):351-5.
270. Hills RD, Jr., Pontefract BA, Mishcon HR, Black CA, Sutton SC, Theberge CR. Gut Microbiome: Profound Implications for Diet and Disease. Nutrients. 2019;11(7):1613.
271. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. nature. 2006;444(7122):1027.
272. Schoster A, Weese JS, Guardabassi L. Probiotic Use in Horses – What is the Evidence for Their Clinical Efficacy? Journal of Veterinary Internal Medicine. 2014;28(6):1640-52.
273. Ishizaka S, Matsuda A, Amagai Y, Oida K, Jang H, Ueda Y, et al. Oral administration of fermented probiotics improves the condition of feces in adult horses. J Equine Sci. 2014;25(4):65-72.
274. Cehak A, Krageloh T, Zuraw A, Kershaw O, Brehm R, Breves G. Does prebiotic feeding affect equine gastric health? A study on the effects of prebiotic-induced gastric butyric acid production on mucosal integrity of the equine stomach. Res Vet Sci. 2019;124:303-9.
275. Ewaschuk JB, Naylor JM, Zello GA. D-Lactate in Human and Ruminant Metabolism. The Journal of nutrition. 2005;135(7):1619-25.
276. Wang Y, Xie Q, Zhang Y, Ma W, Ning K, Xiang JY, et al. Combination of probiotics with different functions alleviate DSS-induced colitis by regulating intestinal microbiota, IL-10, and barrier function. Appl Microbiol Biotechnol. 2020;104(1):335-49.
277. James., Rowe B, Michael J, Lees, Pethick DW, editors. nutrition and Performance Prevention of Acidosis and Laminitis Associated with Grain Feeding in Horses 12006.
278. Respondek F, Goachet AG, Julliand V. Effects of dietary short-chain fructooligosaccharides on the intestinal microflora of horses subjected to a sudden change in diet. Journal of Animal Science. 2008;86(2):316-23.

279. Swyers KL, Burk AO, Hartsock TG, Ungerfeld EM, Shelton JL. Effects of direct-fed microbial supplementation on digestibility and fermentation end-products in horses fed low- and high-starch concentrates. Journal of Animal Science. 2008;86(10):2596-608.
280. Craig LB, Peck JD, Xu J, Sankaranarayanan K, Warinner C, Hansen KR, et al. Characterizing the semen microbiome and associations with semen parameters: the chasm study. Fertility and sterility. 2015;104(3):e66.
281. Alfano M, Ferrarese R, Locatelli I, Ventimiglia E, Ippolito S, Gallina P, et al. Testicular microbiome in azoospermic men—first evidence of the impact of an altered microenvironment. Human Reproduction. 2018;33(7):1212-7.
282. Koedooder R, Mackens S, Budding A, Fares D, Blockeel C, Laven J, et al. Identification and evaluation of the microbiome in the female and male reproductive tracts. Human reproduction update. 2019;25(3):298-325.
283. Cohen ND, Martin LJ, Simpson RB, Wallis DE, Neibergs HL. Comparison of polymerase chain reaction and microbiological culture for detection of salmonellae in equine feces and environmental samples. Am J Vet Res. 1996;57(6):780-6.
284. Traub-Dargatz J, Salman M, Jones R. Epidemiologic study of salmonellae shedding in the feces of horses and potential risk factors for development of the infection in hospitalized horses. Journal of the American Veterinary Medical Association. 1990;196(10):1617-22.
285. Browning GF, Chalmers RM, Snodgrass DR, Batt RM, Hart CA, Ormarod SE, et al. The prevalence of enteric pathogens in diarrhoeic Thoroughbred foals in Britain and Ireland. Equine veterinary journal. 1991;23:405 - 9.
286. McConnico RS. Acute Colitis in Horses. Robinson's Current Therapy in Equine Medicine. 2015:297-301.
287. Pfister K, van Doorn D. New Perspectives in Equine Intestinal Parasitic Disease: Insights in Monitoring Helminth Infections. The Veterinary clinics of North America Equine practice. 2018;34(1):141-53.
288. Shawaf T, El-Deeb WM, Elgioushy M. The Contribution of Specific and Nonspecific Biomarkers in Diagnosis of Equine Gastric Ulcer Syndrome (EGUS) Under Field Condition. J Equine Vet Sci. 2020;84:102853.
289. Sanchez LC. Disorders of the Gastrointestinal System. Equine Internal Medicine. 2018:709-842.
290. Kilcoyne I, Dechant JE, Spier SJ, Spriet M, Nieto JE. Clinical findings and management of 153 horses with large colon sand accumulations. Vet Surg. 2017;46(6):860-7.
291. Dart A, Hodgson D, Snyder J. Caecal disease in equids. Australian Veterinary Journal. 1997;75(8):552-7.
292. Dunkel B, Buonpane A, Chang YM. Differences in gastrointestinal lesions in different horse types. Vet Rec. 2017;181(11):291.
293. Morrison PK, Newbold CJ, Jones E, Worgan HJ, Grove-White DH, Dugdale AH, et al. The Equine Gastrointestinal Microbiome: Impacts of Age and Obesity. Frontiers in microbiology. 2018;9:3017-.
294. Mach N, Moroldo M, Rau A, Lecardonnel J, Le Moyec L, Robert C, et al. Understanding the Holobiont: Crosstalk Between Gut Microbiota and Mitochondria During Long Exercise in Horse. Front Mol Biosci. 2021;8:656204.
295. Biddle A. The Equine Microbiome Project. 2021.

296. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology & Hepatology. 2014;11(8):506-14.
297. Valcheva R, Dieleman LA. Prebiotics: Definition and protective mechanisms. Best Practice & Research Clinical Gastroenterology. 2016;30(1):27-37.
298. M.M. O' Donnell HMBH, I.B. Jeffery , M.J. Claesson , B. Younge , P.W. O' Toole and, Ross1 RP. The core faecal bacterial microbiome of Irish Thorough bred racehorses. Letters in Applied Microbiology 2013;ISSN 0266-8254(57):492-501.
299. Grimm P, Julliand V, Philippeau C, Sadet-Bourgeteau S. Effect of yeast supplementation on hindgut microbiota and digestibility of horses subjected to an abrupt change of hays. Livestock Science. 2016;186:34-40.
300. van Zanten GC, Knudsen A, Röytiö H, Forssten S, Lawther M, Blennow A, et al. The effect of selected synbiotics on microbial composition and short-chain fatty acid production in a model system of the human colon. PloS one. 2012;7(10):e47212.
301. Davani-Davari D, Negahdaripour M, Karimzadeh I, Seifan M, Mohkam M, Masoumi SJ, et al. Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications. Foods. 2019;8(3).
302. Fitzpatrick A, Roberts A, Witherly S. Larch arabinogalactan: A novel and multifunctional natural product. Agro Food Industry Hi-Tech. 2004;15:30-2.
303. Xiao J, Metzler-Zebeli BU, Zebeli Q. Gut Function-Enhancing Properties and Metabolic Effects of Dietary Indigestible Sugars in Rodents and Rabbits. Nutrients. 2015;7(10):8348-65.
304. Samal L, Chaturvedi VB, Saikumar G, Somvanshi R, Pattanaik AK. Prebiotic potential of Jerusalem artichoke (Helianthus tuberosus L.) in Wistar rats: effects of levels of supplementation on hindgut fermentation, intestinal morphology, blood metabolites and immune response. J Sci Food Agric. 2015;95(8):1689-96.
305. Mourão JL, Pinheiro V, Alves A, Guedes C, Pinto L, Saavedra MJ, et al. Effect of mannan oligosaccharides on the performance, intestinal morphology and cecal fermentation of fattening rabbits. Animal Feed Science and Technology. 2006;126(1-2):107-20.
306. Daniels S. Fuel or filler?
What is the real nutrient content of grass for horses? The Journal for Equine Nutrition. 2021;1(1):7-10.
307. Saastamoinen M, Fradinho MJ, Santos AS, Miraglia N, editors. Forages and grazing in horse nutrition. Forages and grazing in horse nutrition; 2012.
308. Ince JC, Longland AC, Moore-Colyer MJS, Harris PA. In vitro degradation of grass fructan by equid gastrointestinal digesta. Grass and forage science. 2014;69(3):514-23.
309. Bachmann M, Glatter M, Bochnia M, Wensch-Dorendorf M, Greef JM, Breves G, et al. In Vitro Gas Production from Batch Cultures of Stomach and Hindgut Digesta of Horses Adapted to a Prebiotic Dose of Fructooligosaccharides and Inulin. Journal of Equine Veterinary Science. 2020;90:103020.
310. Al Jassim RA, Scott PT, Trebbin AL, Trott D, Pollitt CC. The genetic diversity of lactic acid producing bacteria in the equine gastrointestinal tract. FEMS Microbiol Lett. 2005;248(1):75-81.

311. Julliand V. Impact of nutrition on the microflora of the gastro-intestinal tract in horses. Applied Equine Nutrition Wageningen Academic Publishers, Netherlands. 2005:85-103.
312. French KR, Pollitt CC. Equine laminitis: loss of hemidesmosomes in hoof secondary epidermal lamellae correlates to dose in an oligofructose induction model: an ultrastructural study. Equine veterinary journal. 2004;36(3):230-5.
313. Milinovich GJ, Trott DJ, Burrell PC, van Eps AW, Thoefner MB, Blackall LL, et al. Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. Environ Microbiol. 2006;8(5):885-98.
314. Berg EL, Fu CJ, Porter JH, Kerley MS. Fructooligosaccharide supplementation in the yearling horse: Effects on fecal pH, microbial content, and volatile fatty acid concentrations. Journal of Animal Science. 2005;83(7):1549-53.
315. Glatter M, Borewicz K, van den Bogert B, Wensch-Dorendorf M, Bochnia M, Greef JM, et al. Modification of the equine gastrointestinal microbiota by Jerusalem artichoke meal supplementation. PloS one. 2019;14(8).
316. Weese JS. Probiotics, prebiotics, and synbiotics. Journal of Equine Veterinary Science. 2002;22(8):357-60.
317. Di Gioia D, Biavati B. Probiotics and Prebiotics in Animal Health and Food Safety: Conclusive Remarks and Future Perspectives. In: Di Gioia D, Biavati B, editors. Probiotics and Prebiotics in Animal Health and Food Safety. Cham: Springer International Publishing; 2018. p. 269-73.
318. Sanz-Fernandez MV, Daniel JB, Seymour DJ, Kvidera SK, Bester Z, Doelman J, et al. Targeting the Hindgut to Improve Health and Performance in Cattle. Animals (Basel). 2020;10(10).
319. Karlsson CP, Jansson A, Essen-Gustavsson B, LINDBERG JE. Effect of molassed sugar beet pulp on nutrient utilisation and metabolic parameters during exercise. Equine veterinary journal. 2002;34(S34):44-9.
320. Gurbuz E, Coskun B. Effect of Dried Sugar Beet Pulp on Some Blood Parameters and Heart Rate in Exercised Horses. KAFKAS UNIVERSITESI VETERINER FAKULTESI DERGISI. 2011;17(2):191-5.
321. Glatter M, Bochnia M, Goetz F, Gottschalk J, Koeller G, Mielenz N, et al. Glycaemic and insulinaemic responses of adult healthy warm-blooded mares following feeding with Jerusalem artichoke meal. J Anim Physiol Anim Nutr (Berl). 2017;101 Suppl 1:69-78.
322. Milinovich GJ, Burrell PC, Pollitt CC, Klieve AV, Blackall LL, Ouwerkerk D, et al. Microbial ecology of the equine hindgut during oligofructose-induced laminitis. Isme Journal. 2008;2(11):1089-100.
323. Heaton CP, Cavinder CA, Paz H, Rude B, Smith T, Memili E, et al. Are prebiotics beneficial for digestion in mature and senior horses. Journal of Equine Veterinary Science. 2019;76:87-8.
324. Adams AA, Vineyard KR, Gordon ME, Reedy S, Siard MH, Horohov DW. The effect of n-3 polyunsaturated fatty acids (DHA) and prebiotic supplementation on inflammatory cytokine production and immune responses to vaccination in old horses. Journal of Equine Veterinary Science. 2015;35(5):407-8.
325. Vendrig JC, Coffeng LE, Fink-Gremmels J. In vitro evaluation of defined oligosaccharide fractions in an equine model of inflammation. BMC Vet Res. 2013;9(1):147.

326. Sartor RB. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. Gastroenterology. 2004;126(6):1620-33.
327. Elghandour MMY, Tan ZL, Abu Hafsa SH, Adegbeye MJ, Greiner R, Ugbogu EA, et al. Saccharomyces cerevisiae as a probiotic feed additive to non and pseudo-ruminant feeding: a review. Journal of Applied Microbiology. 2019.
328. Czech A, Grela ER, Lyons TP, Jacques KA, Hower JM, editors. Influence of Bio-Mos® mannan oligosaccharides in mare diets on colostrum and milk composition and blood parameters2006.
329. Spearman K, editor Effect of Mannan Oligosaccharie (MOS) supplementation on the immune status of mares and their foals 2004: Semantic Scholar
330. Asadpoor M, Peeters C, Henricks PAJ, Varasteh S, Pieters RJ, Folkerts G, et al. Anti-Pathogenic Functions of Non-Digestible Oligosaccharides In Vitro. Nutrients. 2020;12(6):1789.
331. El-Naggar MA, Thabit TM. Evaluation of β-D-glucan biopolymer as a novel mycotoxin binder for fumonisin and deoxynivalenol in soybean feed. Foodborne Pathog Dis. 2014;11(6):433-8.
332. Valigura HC, Leatherwood JL, Martinez RE, Norton SA, White-Springer SH. Dietary supplementation of a Saccharomyces cerevisiae fermentation product attenuates exercise-induced stress markers in young horses. Journal of Animal Science. 2021;99(8).
333. Jouany JP, Gobert J, Medina B, Bertin G, Julliand V. Effect of live yeast culture supplementation on apparent digestibility and rate of passage in horses fed a high-fiber or high-starch diet. Journal of Animal Science. 2008;86(2):339-47.
334. Petersen C, Round JL. Defining dysbiosis and its influence on host immunity and disease. Cellular microbiology. 2014;16(7):1024-33.
335. Wemer HK LL, Bames T. Effects of administration of an oral arabinogalactan supplement on mares and foals.
.
336. Landes AD, Hassel DM, Funk JD, Hill A. Fecal sand clearance is enhanced with a product combining probiotics, prebiotics, and psyllium in clinically normal horses. Journal of equine veterinary science. 2008;2008 v.28 no.2(no. 2):pp. 79-84.
337. Hassel DM, Curley T, Hoaglund EL. Evaluation of Fecal Sand Clearance in Horses With Naturally Acquired Colonic Sand Accumulation With a Product Containing Probiotics, Prebiotics, and Psyllium. J Equine Vet Sci. 2020;90:102970.
338. Niinistö K. Naturally occurring large colon sand accumulations and their medical treatment in horses. 2020.
339. Jin Song S, Woodhams DC, Martino C, Allaband C, Mu A, Javorschi-Miller-Montgomery S, et al. Engineering the microbiome for animal health and conservation. Experimental Biology and Medicine. 2019;244(6):494-504.
340. Noble GK. Horse Husbandry-Nutrition, Management and Welfare. Animals. 2023;13(1):169.
341. Enam F, Mansell TJ. Prebiotics: tools to manipulate the gut microbiome and metabolome. J Ind Microbiol Biotechnol. 2019;46(9-10):1445-59.
342. Kisuse J, La-Ongkham O, Nakphaichit M, Therdtatha P, Momoda R, Tanaka M, et al. Urban Diets Linked to Gut Microbiome and Metabolome Alterations in Children: A Comparative Cross-Sectional Study in Thailand. Front Microbiol. 2018;9:1345.

343. Swanson KS, Gibson GR, Hutkins R, Reimer RA, Reid G, Verbeke K, et al. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. Nature Reviews Gastroenterology & Hepatology. 2020;17(11):687-701.
344. Schley PD, Field CJ. The immune-enhancing effects of dietary fibres and prebiotics. The British journal of nutrition. 2002;87 Suppl 2:S221-30.
345. Al-Asmakh M, Hedin L. Microbiota and the control of blood-tissue barriers. Tissue Barriers. 2015;3(3):e1039691.
346. Xia W, Mruk DD, Lee WM, Cheng CY. Cytokines and junction restructuring during spermatogenesis--a lesson to learn from the testis. Cytokine Growth Factor Rev. 2005;16(4-5):469-93.
347. Brown GC. The endotoxin hypothesis of neurodegeneration. J Neuroinflammation. 2019;16(1):180.
348. Rescigno M. The intestinal epithelial barrier in the control of homeostasis and immunity. Trends Immunol. 2011;32(6):256-64.
349. Johnson L, Blanchard TL, Varner DD, Scrutchfield WL. Factors affecting spermatogenesis in the stallion. Theriogenology. 1997;48(7):1199-216.
350. Sieme H, editor Chapter 6 – Semen Evaluation2009.
351. Li K, Bihan M, Yooseph S, Methe BA. Analyses of the microbial diversity across the human microbiome. PloS one. 2012;7(6):e32118.
352. Wingett SW, Andrews S. FastQ Screen: A tool for multi-genome mapping and quality control. F1000Res. 2018;7:1338.
353. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nature methods. 2016;13(7):581-3.
354. Callahan B. Silva taxonomic training data formatted for DADA2 (Silva version 128). Zenodo. 2017.
355. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one. 2013;8(4):e61217.
356. Garber A, Hastie PM, Farci V, McGuinness D, Bulmer L, Alzahal O, et al. The effect of supplementing pony diets with yeast on 2. The faecal microbiome. Animal. 2020;14(12):2493-502.
357. Godwin T, Webb G, Lancaster P. Effect of Donor Diet on In Vitro Digestibility of Forages by Fecal Inoculate. J Equine Vet Sci. 2021;105:103722.
358. Blackmore TM, Dugdale A, Argo CM, Curtis G, Pinloche E, Harris PA, et al. Strong stability and host specific bacterial community in faeces of ponies. PloS one. 2013;8(9):e75079-e.
359. Gilroy R, Leng J, Ravi A, Adriaenssens EM, Oren A, Baker D, et al. Metagenomic investigation of the equine faecal microbiome reveals extensive taxonomic diversity. PeerJ. 2022;10:e13084-e.
360. Hu D, Yang J, Qi Y, Li B, Li K, Mok KM. Metagenomic Analysis of Fecal Archaea, Bacteria, Eukaryota, and Virus in Przewalski's Horses Following Anthelmintic Treatment. Frontiers in Veterinary Science. 2021;8.
361. Moore-Colyer MJ, Hyslop JJ, Longland AC, Cuddeford D. The mobile bag technique as a method for determining the degradation of four botanically diverse fibrous

feedstuffs in the small intestine and total digestive tract of ponies. The British journal of nutrition. 2002;88(6):729-40.
362. Crandell KPD. Benefits of Beet Pulp for Horses. Equinews Nutrition & Health Daily. 2018.
363. Cooke CG GZ, Grupen CG, Harnett JE. Prebiotics and synbiotics in equine health and disease – Review. International Journal of Equine Science. 2023.
364. Sanders ME. Impact of probiotics on colonizing microbiota of the gut. J Clin Gastroenterol. 2011;45 Suppl:S115-9.
365. Han S, Lu Y, Xie J, Fei Y, Zheng G, Wang Z, et al. Probiotic Gastrointestinal Transit and Colonization After Oral Administration: A Long Journey. Front Cell Infect Microbiol. 2021;11:609722.
366. Collinet A, Grimm P, Julliand S, Julliand V. Sequential Modulation of the Equine Fecal Microbiota and Fibrolytic Capacity Following Two Consecutive Abrupt Dietary Changes and Bacterial Supplementation. Animals (Basel). 2021;11(5).
367. Markowiak P, Śliżewska K. The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut pathogens. 2018;10:21-.
368. Leng J, McNally S, Walton G, Swann J, Proudman C, Argo C, et al. Hay versus haylage: Forage type influences the equine urinary metabonome and faecal microbiota. Equine veterinary journal. 2022;54(3):614-25.
369. Fitzgerald DM, Spence RJ, Stewart ZK, Prentis PJ, Sillence MN, de Laat MA. The effect of diet change and insulin dysregulation on the faecal microbiome of ponies. J Exp Biol. 2020;223(Pt 7).
370. Adams VJ, LeBlanc N, Penell J. Results of a Clinical Trial Showing Changes to the Faecal Microbiome in Racing Thoroughbreds after Feeding a Nutritional Supplement. Vet Sci. 2022;10(1).
371. Almeida ML, Feringer WHJ, Carvalho JR, Rodrigues IM, Jordão LR, Fonseca MG, et al. Intense Exercise and Aerobic Conditioning Associated with Chromium or L-Carnitine Supplementation Modified the Fecal Microbiota of Fillies. PloS one. 2016;11(12):e0167108.
372. Salem SE, Hough R, Probert C, Maddox TW, Antczak P, Ketley JM, et al. A longitudinal study of the faecal microbiome and metabolome of periparturient mares. PeerJ. 2019;7:e6687.
373. Weng SL, Chiu CM, Lin FM, Huang WC, Liang C, Yang T, et al. Bacterial communities in semen from men of infertile couples: metagenomic sequencing reveals relationships of seminal microbiota to semen quality. PloS one. 2014;9(10):e110152.
374. Rosenfeld CS, Javurek AB, Johnson SA, Lei Z, Sumner LW, Hess RA. Seminal fluid metabolome and epididymal changes after antibiotic treatment in mice. Reproduction. 2018;156(1):1-10.
375. Javurek AB, Spollen WG, Ali AM, Johnson SA, Lubahn DB, Bivens NJ, et al. Discovery of a Novel Seminal Fluid Microbiome and Influence of Estrogen Receptor Alpha Genetic Status. Sci Rep. 2016;6:23027.
376. Serrano M, Climent E, Freire F, Martínez-Blanch JF, González C, Reyes L, et al. Influence of the Ovine Genital Tract Microbiota on the Species Artificial Insemination Outcome. A Pilot Study in Commercial Sheep Farms. High Throughput. 2020;9(3).
377. Griffin RA, Baker M, Aitken RJ, Swegen A, Gibb Z. What makes a fertile sperm? Unique molecular attributes of stallion fertility. Reproduction. 2019;158(4):R125-r37.

378. de Fombelle A, Julliand V, Drogoul C, Jacotot E. Feeding and microbial disorders in horses: 1 - Effects of an abrupt incorporation of two levels of barley in a hay diet on microbial profile and activities. Journal of Equine Veterinary Science. 2001;21(9):439-45.
379. Franzan BC, Coelho IDS, de Souza MT, Santos MMM, de Almeida FQ, Silva VP. Fecal Microbiome Responses to Sudden Diet Change in Mangalarga Marchador horses. J Equine Vet Sci. 2022;108:103803.
380. Górniak W, Cholewińska P, Szeligowska N, Wołoszyńska M, Soroko M, Czyż K. Effect of Intense Exercise on the Level of Bacteroidetes and Firmicutes Phyla in the Digestive System of Thoroughbred Racehorses. Animals (Basel). 2021;11(2).
381. Crespo F, Quiñones-Pérez C, Ortiz I, Diaz-Jimenez M, Consuegra C, Pereira B, et al. Seasonal variations in sperm DNA fragmentation and pregnancy rates obtained after artificial insemination with cooled-stored stallion sperm throughout the breeding season (spring and summer). Theriogenology. 2020;148:89-94.
382. Aouane N, Nasri A, Bekara MAA, Metref AK, Kaidi R. Retrospective study of the reproductive performance of Barb and Thoroughbred stallions in Algeria. Vet World. 2019;12(7):1132-9.
383. Card C. Reproductive problems of stallions (Proceedings). 2011.
384. Fioratti EG, Villaverde AISB, Melo CM, Tsunemi MH, Papa FO, Alvarenga MA. Influence of Steroidal Anti-Inflammatory Drugs on Viability and Fertility of Equine Semen. Journal of Equine Veterinary Science. 2012;32(12):771-5.
385. Aurich C, Spergser J. Influence of bacteria and gentamicin on cooled-stored stallion spermatozoa. Theriogenology. 2007;67(5):912-8.
386. Bedford SJ, McDonnell SM. Measurements of reproductive function in stallions treated with trimethoprim-sulfamethoxazole and pyrimethamine. J Am Vet Med Assoc. 1999;215(9):1317-9.
387. Cappai MG, Taras A, Cossu I, Cherchi R, Dimauro C, Accioni F, et al. Effects of Dietary Zn/Se and α-Tocopherol Supplementation on Metabolic Milieu, Haemogram and Semen Traits of Breeding Stallions. Biol Trace Elem Res. 2021;199(9):3287-96.
388. Gibb Z, Blanco-Prieto O, Bucci D. The role of endogenous antioxidants in male animal fertility. Research in Veterinary Science. 2021;136:495-502.
389. Scheeren VFC, Sancler-Silva YFR, El-Sheikh Ali H, Kastelic JP, Alvarenga MA, Papa FO. Update on Seminal Vesiculitis in Stallions. Journal of Equine Veterinary Science. 2020;94:103234.
390. Djourabchi Borojerdi AS, Welchowski T, Peng W, Buchen A, Novak N, Haidl G, et al. Human spermatozoa of male patients with subfertility express the interleukin-6 receptor. Andrologia. 2020;52(4):e13511.
391. Machado-Neves M, Gibb, Z., Curry, B., Lambourne, S. and Aitken, R. J. (The expression and function of stallion sperm cytokine receptors. 8th International Congress on Animal Reproduction, Tours, France. 2016:pp. 93-4.
392. Shokryazdan P, Faseleh Jahromi M, Navidshad B, Liang JB. Effects of prebiotics on immune system and cytokine expression. Med Microbiol Immunol. 2017;206(1):1-9.
393. STROBE. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies. https://wwwequator-networkorg/reporting-guidelines/strobe/. 2022.

394. Helli B, Kavianpour M, Ghaedi E, Dadfar M, Haghighian HK. Probiotic effects on sperm parameters, oxidative stress index, inflammatory factors and sex hormones in infertile men. Human fertility (Cambridge, England). 2022;25(3):499-507.
395. Borum PR, Bennett SG. Carnitine as an essential nutrient. Journal of the American College of Nutrition. 1986;5(2):177-82.
396. Gibb Z, Aitken RJ. Recent Developments in Stallion Semen Preservation. Journal of Equine Veterinary Science. 2016;43:S29-S36.
397. Jeulin C, Lewin LM. Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa. Human reproduction update. 1996;2(2):87-102.
398. Rizzo M, Ducheyne KD, Deelen C, Beitsma M, Cristarella S, Quartuccio M, et al. Advanced mare age impairs the ability of in vitro-matured oocytes to correctly align chromosomes on the metaphase plate. Equine veterinary journal. 2019;51(2):252-7.
399. Brück I, Anderson G, Hyland J. Reproductive performance of Thoroughbred mares on six commercial stud farms. Australian Veterinary Journal. 1993;70(8):299-303.
400. Morris LH, Allen W. Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. Equine veterinary journal. 2002;34(1):51-60.
401. Menzies PI. Reproductive health management programs. Current Therapy in Large Animal Theriogenology. 1999:pp 643-9.
402. Colenbrander B, Feitsma H, Grooten HJ. Optimizing semen production for artificial insemination in swine. J Reprod Fertil Suppl. 1993;48:207-15.
403. Wang Y, Xie Z. Exploring the role of gut microbiome in male reproduction. Andrology. 2022;10(3):441-50.
404. McFarlane D, Holbrook TC. Cytokine Dysregulation in Aged Horses and Horses with Pituitary Pars Intermedia Dysfunction. Journal of Veterinary Internal Medicine. 2008;22(2):436-42.
405. Singh P, Teal TK, Marsh TL, Tiedje JM, Mosci R, Jernigan K, et al. Intestinal microbial communities associated with acute enteric infections and disease recovery. Microbiome. 2015;3(1):45.
406. Tang J, Xu L, Zeng Y, Gong F. Effect of gut microbiota on LPS-induced acute lung injury by regulating the TLR4/NF-kB signaling pathway. International Immunopharmacology. 2021;91:107272.
407. Qu S, Fan L, Qi Y, Xu C, Hu Y, Chen S, et al. Akkermansia muciniphila Alleviates Dextran Sulfate Sodium (DSS)-Induced Acute Colitis by NLRP3 Activation. Microbiology spectrum. 2021;9(2):e0073021.
408. Shepherd ML, Swecker WS, Jr., Jensen RV, Ponder MA. Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. FEMS Microbiol Lett. 2012;326(1):62-8.
409. Dougal K, de la Fuente G, Harris PA, Girdwood SE, Pinloche E, Newbold CJ. Identification of a Core Bacterial Community within the Large Intestine of the Horse. PloS one. 2013;8(10):e77660.
410. Slater R, Frau A, Hodgkinson J, Archer D, Probert C. A Comparison of the Colonic Microbiome and Volatile Organic Compound Metabolome of Anoplocephala perfoliata Infected and Non-Infected Horses: A Pilot Study. Animals. 2021;11(3):755.
411. Edwards JE, Shetty SA, van den Berg P, Burden F, van Doorn DA, Pellikaan WF, et al. Multi-kingdom characterization of the core equine fecal microbiota based on multiple equine (sub)species. Anim Microbiome. 2020;2(1):6.

412. Morrison PK, Newbold CJ, Jones E, Worgan HJ, Grove-White DH, Dugdale AH, et al. The equine gastrointestinal microbiome: impacts of weight-loss. BMC Vet Res. 2020;16(1):78.
413. Williams SB. A circadian timing mechanism in the cyanobacteria. Adv Microb Physiol. 2007;52:229-96.
414. Mihalcescu I, Hsing W, Leibler S. Resilient circadian oscillator revealed in individual cyanobacteria. Nature. 2004;430(6995):81-5.
415. Benammar A, Derisoud E, Vialard F, Palmer E, Ayoubi JM, Poulain M, et al. The Mare: A Pertinent Model for Human Assisted Reproductive Technologies? Animals. 2021;11(8):2304.
416. Matsuo Y, Komiya S, Yasumizu Y, Yasuoka Y, Mizushima K, Takagi T, et al. Full-length 16S rRNA gene amplicon analysis of human gut microbiota using MinION™ nanopore sequencing confers species-level resolution. BMC Microbiol. 2021;21(1):35.
417. Griswold MD. Interactions between germ cells and Sertoli cells in the testis. Biol Reprod. 1995;52(2):211-6.
418. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The role of short-chain fatty acids in health and disease. Adv Immunol. 2014;121:91-119.
419. Respondek F, Myers K, Smith TL, Wagner A, Geor RJ. Dietary supplementation with short-chain fructo-oligosaccharides improves insulin sensitivity in obese horses. J Anim Sci. 2011;89(1):77-83.
420. Anadón A, Ares I, Martínez-Larrañaga MR, Martínez MA. Prebiotics and Probiotics in Feed and Animal Health. In: Gupta RC, Srivastava A, Lall R, editors. Nutraceuticals in Veterinary Medicine. Cham: Springer International Publishing; 2019. p. 261-85.
421. Sheweita SA, Tilmisany AM, Al-Sawaf H. Mechanisms of male infertility: role of antioxidants. Curr Drug Metab. 2005;6(5):495-501.
422. Agarwal A, Said TM. Carnitines and male infertility. Reproductive biomedicine online. 2004;8(4):376-84.
423. Dinnetz JM. Omega-3 fatty acid supplementation reduces basal TNFalpha but not toll-like receptor stimulated TNFalpha in full sized and miniature mares. 2009.
424. Willing B, Voros A, Roos S, Jones C, Jansson A, Lindberg JE. Changes in faecal bacteria associated with concentrate and forage-only diets fed to horses in training. Equine veterinary journal. 2009;41(9):908-14.
425. Costa MC, Weese JS. The equine intestinal microbiome. Animal Health Research Reviews. 2012;13(1):121-8.
426. Weinert-Nelson JR, Biddle AS, Williams CA. Fecal microbiome of horses transitioning between warm-season and cool-season grass pasture within integrated rotational grazing systems. Anim Microbiome. 2022;4(1):41.
427. O' Donnell MM, Harris HMB, Jeffery IB, Claesson MJ, Younge B, O' Toole PW, et al. The core faecal bacterial microbiome of Irish Thoroughbred racehorses. Letters in Applied Microbiology. 2013;57(6):492-501.
428. Ericsson AC, Johnson PJ, Lopes MA, Perry SC, Lanter HR. A Microbiological Map of the Healthy Equine Gastrointestinal Tract. PloS one. 2016;11(11):e0166523.

The Author

9. APPENDICES

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APPENDIX A1: CHAPTER 5

The distribution of the ten outcome variables individually and by treatment are presented in Appendix Tables 5.4 and 5.5.

Appendix Table 5.4: Summary statistics for four sperm quality outcome measures overall and by treatment categories

Parameter	Intervention	Minimum	Q1	Median	Q3	Maximum	Mean	Std. dev.
Total sperm count (x10^9)	Overall	1.9	3.5	4.7	11.9	43.3	8.6	10.0
	Control	2.7	3.3	8.7	12.5	12.5	8.1	5.1
	Prebiotics	3.4	3.5	4.1	34.5	43.4	14.7	19.2
	Probiotics	3.1	3.3	4.7	11.2	13.1	6.4	4.6
	Synbiotics	1.9	2.3	4.1	8.9	10.3	5.1	3.7
Progressive motility %	Overall	13.3	20.7	30.9	40.9	47.2	30.6	11.5
	Control	15.1	17.1	30.9	43.0	44.4	30.3	13.6
	Prebiotics	15.7	16.9	29.9	44.8	46.5	30.5	14.8
	Probiotics	13.3	17.3	30.9	39.1	41.3	29.1	11.7
	Synbiotics	21.6	23.4	31.0	43.7	47.2	32.7	10.8
DNA intact %	Overall	78.7	90.2	92.4	95.4	97.6	91.9	4.7
	Control	89.7	90.3	92.4	96.3	97.6	93.0	3.3
	Prebiotics	86.4	87.3	90.6	95.1	96.5	91.0	4.2
	Probiotics	90.4	90.5	93.2	96.2	96.5	93.3	3.1
	Synbiotics	78.7	82.2	93.9	95.3	95.4	90.5	7.9
Live 4HNE Positive %	Overall	1.7	2.3	9.8	18.9	25.9	10.8	9.0
	Control	1.7	4.0	12.3	22.4	25.3	12.9	9.7

	Prebiotics	1.9	2.0	12.8	25.2	25.9	13.4	12.9
	Probiotics	2.2	2.3	9.5	18.9	19.8	10.2	9.0
	Synbiotics	1.9	2.2	5.9	12.0	13.0	6.7	5.2

Appendix Table 5.5: Summary statistics for six microbiome summary measures overall and by treatment categories

Parameter	**Intervention**	**Minimum**	**Q1**	**Median**	**Q3**	**Maximum**	**Mean**	**Std. dev.**
Faecal								
Faith_pd	Overall	34.3	36.2	37.3	38.9	44.5	37.9	2.5
	Control	35.6	35.8	36.4	40.6	41.9	37.6	2.9
	Prebiotics	37.3	37.7	38.8	43.1	44.5	39.9	3.2
	Probiotics	34.3	34.6	36.4	38.0	38.2	36.3	1.8
	Pre- and probiotics	36.2	36.5	37.9	39.3	39.6	37.9	1.5
Evenness	Overall	0.81	0.83	0.85	0.88	0.90	0.85	0.03
	Control	0.81	0.81	0.82	0.86	0.87	0.83	0.03
	Prebiotics	0.86	0.86	0.89	0.90	0.90	0.88	0.02
	Probiotics	0.83	0.83	0.84	0.86	0.87	0.84	0.02
	Pre- and probiotics	0.81	0.82	0.85	0.88	089	0.85	0.04
Shannon's diversity	Overall	7.1	7.3	7.5	7.9	8.4	7.6	0.4
	Control	7.1	7.1	7.2	7.9	8.1	7.4	0.5
	Prebiotics	7.6	7.7	8.0	8.3	8.4	8.0	0.3
	Probiotics	7.3	7.3	7.4	7.5	7.5	7.4	0.1

	Pre- and probiotics	7.1	7.2	7.5	7.9	8.0	7.5	0.4
Semen								
Faith_pd	Overall	10.3	16.0	32.5	50.0	63.1	32.8	17.6
	Control	25.0	21.1	32.3	54.6	59.7	37.3	16.3
	Prebiotics	11.4	11.8	14.6	51.3	63.1	25.9	24.9
	Probiotics	34.8	35.1	36.5	49.6	53.8	40.4	9.0
	Pre- and probiotics	10.3	11.8	23.1	47.8	53.7	27.5	19.3
Evenness	Overall	0.55	0.61	0.69	0.73	0.76	0.67	0.07
	Control	0.55	0.59	0.71	0.73	0.73	0.67	0.08
	Prebiotics	0.56	0.59	0.70	0.75	0.76	0.68	0.09
	Probiotics	0.59	0.61	0.69	0.73	074	0.67	0.06
	Pre- and probiotics	0.59	0.61	0.65	0.72	0.74	0.66	0.06
Shannon's diversity	Overall	2.9	3.4	4.0	4.5	4.7	3.9	0.6
	Control	3.0	3.3	4.3	4.5	4.5	4.0	0.7
	Prebiotics	3.2	3.3	3.9	4.6	4.7	3.9	0.7
	Probiotics	3.4	3.4	3.9	4.5	4.6	3.9	0.6
	Pre- and probiotics	2.9	3.1	3.8	4.4	4.5	3.8	0.7

Protexin® Liquid was renamed PRON8URE® after the pony intervention trial was conducted, without changing the multistrain probiotic formula (see product brochure below).

PRON8URE®

PRON8URE® IS A MULTI-STRAIN PROBIOTIC CONTAINING LIVE BENEFICIAL BACTERIA.

PRESENTATION: There are different types of **PRON8URE®:**

PRON8URE® POWDER	- In feed formula containing 60 million colony forming units (CFU)/g
PRON8URE® SOLUBLE	- Water soluble formula containing 180 million CFU/g
PRON8URE® PASTE	- Oral paste formula containing 180 million CFU/g
PRON8URE® LIQUID	- Oil based formula containing 180 million CFU/mL

ACTIVE CONSTITUENTS: Each g or mL contains: *Lactobacillus acidophilus, Lactobacillus delbrueckii* subspecies *bulgaricus, Lactobacillus plantarum, Lactobacillus rhamnosus; Bifidobacterium bifidum; Enterococcus faecium; Streptococcus salivarius* subspecies *thermophilus.*

PROPERTIES: PRON8URE® is a blend of live viable microbes which benefits the host animal by improving the intestinal microbial balance. Everyday use of **PRON8URE®** helps maintain a balanced digestive system, thus optimising digestion of feed and enhancing your animals' health, naturally. These microbes are safe, non-toxic and residue free.

PRON8URE® may be used during periods of stress (stresses disrupt the normal intestinal balance), e.g. overcrowding, pregnancy and lactation, scouring and diarrhoea. temperature extremes, transportation, training and exercise, and medication; and following animal husbandry procedures or practices such as antibiotic use, birth, castration, debeaking/dehorning, diet changes, loss of appetite, surgery, vaccination, weaning and worming. **PRON8URE®** is compatible with feed ingredients including anthelmintics, coccidiostats, minerals and vitamins, and some antibiotics.

Indications for use (Australia):

- An aid during periods of intestinal dysfunction in all animals, including treatment and control of scouring or diarrhoea, exclusion and suppression of pathogens such as *E. coli, Salmonella* spp. and *Aeromonas* spp.
- Post-antibiotic therapy in all animals for the re-establishment of gut microflora following antibiotic usage.
- Improved weight gains and feed conversion in growing pigs and cattle.
- Productivity improvements and reduced mortality inpoultry.
- Aids in the establishment of gastrointestinal microflora of physiologically immature animals, e.g. day old chicks, neonatal calves, pigs, foals, lambs and kids, puppies and kittens.

Indications for use (New Zealand):

- An aid during periods of intestinal dysfunction in all animals.
- Post-antibiotic therapy in all animals for the re-establishment of gut microflora following antibiotic use.
- Improved weight gains and feed conversion in growing pigs and cattle.
- Productivity improvements and reduced mortality inpoultry.
- Aids in the establishment of gastrointestinal microflora of physiologically immature animals, e.g. day old chicks, neonatal calves, pigs, foals, lambs and kids, puppies and kittens.

DOSAGE AND ADMINISTRATION:

(Australia)
RESTRAINT: PRON8URE® should not be used in conjunction with Penicillins, Cephalosporins, Tetracyclines, Avoparcin, Gentomycin, Lincomycin, Streptomycin, Tiamulin or Tylosin, but may be used immediately following these antibiotics.

(New Zealand)
RESTRAINT: Using concurrently with some antibiotics may compromise beneficial effects. PRON8URE® efficacy may be reduced if used in conjunction with some antibiotics. Please contact International Animal Health Products for advice regarding the use of PRON8URE® in conjunction withantibiotics.

(Australia & New Zealand)
Individual Dose: Use at stress levels for the first 5 days then at the maintenance dose level. **In-Feed:** Refer to label for instructions.

PRODUCT ►	P. POWDER			P. SOLUBLE**		P. PASTE	P. LIQUID		
SPECIES	INDIVIDUAL DOSE (per head per day)		IN FEED*	INDIVIDUAL DOSE (per head per day)		INDIVIDUAL DOSE (per head per day)	INDIVIDUAL DOSE (per head per day)		IN FEED*
	Maintenance	Stress	(kg/tonne)	Maintenance	Stress	Stress	Maintenance	Stress	(mL/tonne)
HORSES:	10 g	20 g	1.0	10 g	20 g	10-20 g	10 mL	20 mL	335 mL
FOALS/PONIES:	5 g	10 g	2.0	5 g	10 g	5-10 g	5 mL	10 mL	665 mL
CATTLE:	2 g	4 g	0.5-1.0	2 g	4 g	2-4 g	2 mL	4 mL	165-335mL
CALVES:	1 g	2 g	0.4	1 g	2 g	1-2 g	1 mL	2 mL	135mL
SHEEP/GOATS:	2 g	4 g	1.2	2 g	4 g	2-4 g	2 mL	4 mL	400 mL
LAMBS/KIDS:	1 g	2 g	1.4	1 g	2 g	1-2 g	1 mL	2 mL	465 mL
PIGS:	1-2 g	2-4 g	0.5-0.6	2 g	4 g	2-4 g	2 mL	4 mL	200 mL
PIGLETS:	1 g	2 g	1.4	1 g	2 g	1-2 g	1 mL	2 mL	465 mL
DOGS:	1-3 g	2-6 g♦	1.5	1-3 g	2-6 g♦	1-3 g to 2-6 g	1-3 mL	2-6 mL♦	500 mL
PUPS/KITTENS:	1 g	2 g	1.0-3.0	1 g	2 g	1-2 g	1 mL	2 mL	335-1000 mL
CATS:	1 g	2 g	1.5	1 g	2 g	1-3 g to 2-6 g	1 mL	2 mL	500 mL
POULTRY:									
CHICKS				1g/L**					
BROILERS/LAYERS			0.5-1.5	500 g/500 L**					165-500 mL
CAGED BIRDS:				1 g/L**			1 mL/500 g feed		

*Use as a guide only. Rates may vary depending on daily feed intakes. ♦ (Small-2 g, Medium-4 g, Large-6 g). **Add P. Soluble to drinking water – make fresh daily.

For other animals contact International Animal Health Products. **NOTE:** A level measure holds around 2 g (small), 25 g (large) **(P. POWDER, P. SOLUBLE)** & the finger pump delivers 1.5 mL **(P. LIQUID)**.

WITHHOLDING PERIODS: MEAT: NIL; MILK: NIL; EGGS: NIL
TRADE ADVICE: EXPORT SLAUGHTER INTERVAL (ESI): ESI not required **(Australia)**
POISONS SCHEDULE: EXEMPT **(Australia)**

REGULATORY STATUS: **Australia:** AUSTRALIAN PESTICIDES & VETERINARY MEDICINES AUTHORITY
New Zealand: EXEMPT

PACK SIZES: **PRON8URE® POWDER:** 125 g, 250 g, 1 kg, 5 kg, 25 kg **PRON8URE® SOLUBLE:** 125 g, 250 g, 500 g, 2.5 kg, 25 kg
PRON8URE® PASTE: 30 g, 100 g (syringe) **PRON8URE® LIQUID:** 125 mL, 250 mL, 1 L, 5 L, 25 L

Australia-Wide Toll Free: 1800 801 201	New Zealand Toll Free: 0800 424 999 (0800 IAH 999)
Email: **info@iahp.com.au**	Website: **www.iahp.com.au**

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APPENDIX A2: CHAPTER 6

STUD FARM NUTRITIONAL SUPPLEMENTATION REGIMENS

SUPPLEMENT	STUD 1	STUD 2	STUD 3
EOS oil [a]	5		7
Omega-3 oil		6	
Linseed			4
Nano-E [a]			5
Carnitine	1		3
Equi-Jewel [a]			1
Gold Pellet [a]	5		
Sweet Feed No.4 [b]		5	
Inner Balance [b]		2	
Cool Command [c]	2		
Diamond V XPC [c]	1		6
Legend [c]			6
Balancer Pellets [d]			2
Gastro Pellets [g]		1	
Old Timer [e]		1	
4Cyte Joint Support [j]			1
Farrier's Formula [f]		1	
Hoof Gold [h]			1
Livamol [k]		6	
EVM [l]		6	

N = numbers of horses receiving supplement

[a] KER: Kentucky Equine Research, [b] Horse Power, [c] Barastoc, [d] Equine Vit&Min, [e] Johnsons, [f] Life Data Labs Inc, [g] Med-Vet Pharmaceuticals, [h] Scone Equine Group, [i] Hubbard, [j] Provet, [k] IAHP, [l] Farmalogic

STUD FARM PRODUCT INGREDIENTS

EOS oil, Omega-3 oil, Linseed – omega-3 fatty acids

Nano-E - highly bioavailable vitamin E

Equi-Jewel - marine-derived calcium, linoleic acid

Sweet Feed No.4 - triticale, micronised corn, micronised barley, micronised lupins, sunflower seeds, molafos, omega fish and canola oils, salt, dicalcium phosphate, limestone, Vitamin E, Agrimos prebiotic and chelated chromium

Gold Pellet - amino acids, vitamins, antioxidants, and trace minerals

Cool Command - controlled starch, optimal level of protein, with mineral and vitamin fortification

Legend - barley, corn, lupins, sunflower seeds, ProteniseTM Pellet, calcium carbonate, mono-di calcium phosphate, salt, magnesium oxide, omega rich vegetable oil, molasses, Barastoc *GastroliseTM* (red seaweed extract), lysine, methionine, *Diamond V® XPC* (prebiotic yeast), vitamin E, KER vitamin & mineral premix.

Diamond V® XPC - prebiotic yeast

Inner Balance - Zeolite volcanic clay mineral, equine B9, botanic extracts, Agrimos® mannanoligopolysaccharide (MOS) prebiotic

Carnitine – amino acid

Balancer Pellets – minerals, vitamins, lysine, trace elements, live yeast probiotic (*Saccharomyces cerevisiae*)

Old Timer - oaten hay, lucerne hay, lupins, faba beans, soybean and oil, vitamins, chelated minerals, prebiotics, probiotics, and mycotoxin binder

4Cyte Joint Support – cartilage repair nutrients, anti-inflammatory

Bio Bloom hoof and coat : biotin, organic zinc, lysine, methionine, iodine, lecithin, soybean meal

Farrier's Formula - phospholipids, omega fatty acids, vitamins, minerals, and amino acids

Gastro Pellets - antioxidants, probiotics (Levucell SC yeast) and herbals

Hoof Gold – biotin, methionine, methylsulfonylmethane (MSM), zinc, vitamins, other minerals, amino acids

Livamol - protein, essential fatty acids

EVM – vitamins, minerals and trace elements

APPENDIX B1: THE SAFETY, TOLERABILITY AND EFFICACY OF BACTERIA FOR EQUINE USE

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Review Article

The Safety, Tolerability and Efficacy of Probiotic Bacteria for Equine Use

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ABSTRACT

Probiotic bacteria are used widely as nutritional supplements and treatment interventions in the management of livestock and companion animals. The aim of this review is to summarize the current evidence reporting on the safety, tolerability and efficacy of probiotic bacteria use in horses. An online search of five databases for studies reporting on the use of probiotic bacteria use in horses which were either healthy or had a gastrointestinal or extraintestinal disease was conducted. A total of 18 articles were eligible for full review. No clear benefits were identified to support supplementation of equids with probiotic bacteria to improve starch and fiber digestion, nor for the treatment of colic or prevention of salmonellosis. Conflicting results were seen with the management of scouring in neonatal foals. Exacerbation of diarrhea and additional adverse events were reported in response to the administration of high doses of novel probiotic bacterial species. Probiotic bacteria given to exercising horses, improved aerobic fitness and stamina. The majority of probiotic bacterial species used in equine studies are bacterial species commonly used for human consumption and indigenous to the human gastrointestinal microbiota. There is a paucity of evidence to support the use of probiotic bacteria in the health maintenance and disease management of horses. While there are unclear and conflicting results associated with probiotic bacteria use for gastrointestinal conditions in both horses and foals, the administration of multistrain bacterial formulations to increase stamina in exercising horses shows promise.

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1. Introduction

In humans, the composition and diversity of the gastrointestinal (GI) microbiota plays an important role in intestinal and extraintestinal health and disease [1], including chronic inflammation [2], cardiovascular disease [3,4], metabolic syndrome [5–7] depression and anxiety [8], and hypersensitivity conditions [9]. Much less is known about the role of the microbiome in the equine species. In horses with colitis, the composition of the fecal microbiome has been found to differ to that of healthy horses [10], though the literature pertaining to extra-intestinal conditions associated with the composition and diversity of the GI equine microbiome is otherwise limited [11,12].

Functions attributed to the indigenous gastrointestinal microbiota of humans include enzyme and vitamin synthesis, hormone metabolism, competitive inhibition of pathogens for intestinal receptor sites, secretion of the antimicrobial factors bacteriocin and defensin, and immuno-modulation [13–15]. The knowledge of the functions of these indigenous bacteria has led to an exponential growth in research exploring their possible therapeutic roles as probiotic medicines [16]. Probiotics are defined by the World Health Organization (WHO) as '*live microorganisms which when administered in adequate amounts confer a health benefit on the host*' [17]. To date, an extensive number of bacterial species and sub-species from the *lactobacillus* and *bifidobacterium* genera have been evaluated [18]. *Streptococcus thermophilus* and *Escherichia coli* strains have also received research attention in human health [17]. The probiotic yeast, *Saccharomyces cerevisiae and boulardii* have been used to supplement feed in horses with beneficial outcomes, such as improved fiber digestibility [19] and reducing the duration

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APPENDIX B2: PREBIOTICS AND SYNBIOTICS IN EQUINE HEALTH AND DISEASE – A REVIEW

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Abstract

Prebiotics are non-digestible food ingredients that promote the growth of probiotic microorganisms in the intestines and are marketed as feed supplements for use in supporting equine digestion, metabolism, growth and immunity. Synbiotics are supplements that contain combinations of prebiotics and probiotic bacteria and/or yeasts. Both are commercially available and promoted for use in supporting equine digestion

and athletic performance, as well as reducing stress and morbidity associated with intestinal disease. The aim of this narrative review was to summarise the literature reporting on the use of prebiotics and synbiotic supplementation in equine nutritional practice. Sixteen papers were identified that report on the use of prebiotic or synbiotic supplementation in horses. The literature presented here suggests that prebiotics may play a role in equine health and disease prevention. Prebiotics have been studied for their effects on athletic performance; increasing production of volatile fatty acids (VFA's) associated with hindgut fibre fermentation; insulin resistance and carbohydrate metabolism associated with reduction in the development of gastric mucositis, and hindgut acidosis and laminitis. Prebiotic compounds are thought to have an entero-protective effect by improving the composition and diversity of the intestinal microbiota, that in turn impacts immune function via metabolomic effects. Prebiotics derived from yeasts, including mannan-oligosaccharides, have been shown to reduce colonies of intestinal pathobionts and accelerate healing in acute enterocolitis. Overall, the current evidence to support the use of prebiotics and synbiotics in equine health and disease is not extensive but promising.

Keywords: equine, prebiotics, fibre, synbiotics, microbiome, digestion

List of Abbreviations

EGUS	equine gastric ulcer syndrome
FOS	fructo-oligosaccharides
GOS	galacto-oligosaccharides
LPS	lipopolysaccharide
IgA, IgG	Immunoglobin A, G

IMOS	isomalto-oligosacharrides
MOS	mannan-oligosaccharides
PBMC	peripheral blood mononuclear cells
TNF-α	tumour necrosis factor alpha
VFA	volatile fatty acids
XOS	xylo-oligosaccharides

1. Introduction

Equine veterinarians play an important role in advising on interventions that prevent and treat gastrointestinal disorders in horses. Equine gastrointestinal disorders include scouring in neonates (Browning et al., 1991), colitis and fatal colic events (McConnico, 2015), parasitic gastrointestinal infections (Pfister & van Doorn, 2018), gastric ulceration (Shawaf et al., 2020), salmonellosis associated inflammatory bowel disease (Sanchez, 2018), coronavirus infection with necrotizing enteritis and eosinophilic enterocolitis (Sanchez, 2018), sand-accumulation enteropathy (Kilcoyne et al., 2017) and caecal disorders (Dart et al., 1997). Dysbiosis of the intestinal microbiome defined as "a loss of diversity, a bloom of potential pathobionts, and a loss of commensals" (Petersen & Round, 2014) may predispose horses to many of these gastrointestinal disorders (Costa & Weese, 2018). Interestingly, there is evidence to suggest that equid type (size, breed, or conformation) may influence predisposition to the development of various gastrointestinal lesions; for example, larger breeds such as Clydesdales experience more caecal disorders, whereas smaller breeds and miniature horses are less likely to be affected by colon displacements (Dunkel et al., 2017). Furthermore, extraintestinal conditions such as exacerbations in asthma (Leclere & Costa, 2020), obesity (Morrison et al., 2018), and impaired athletic performance (Mach et al., 2021) have all been associated

with alterations to the composition and diversity of the equine gastrointestinal microbiome. Therefore, the equine microbiome has gained increasing attention in veterinary research (Kauter et al., 2019) as reflected by the initiation of the Equine Gut Microbiome Project in 2015 (Biddle, 2021).

Hindgut fermentation, which fundamentally influences intestinal microbiome composition (Costa & Weese, 2012; Xu et al., 2021), is a digestive process seen in animals with a simple, single-chambered stomach, which include equids, rhinoceros, koalas, rodents and rabbits; in these mono-gastric herbivores, cellulose from plant foods is digested with the support of intestinal bacteria (Xiao et al., 2015). Popular interventions thought to alter the composition and diversity of the gastrointestinal microbiome include supplementation with probiotics and prebiotics. Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014) and prebiotics are defined as "selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Valcheva & Dieleman, 2016).

Prebiotics are widely used in equine management regimens to support intestinal processing of a variety of feedstuffs, based on the belief that they improve the diversity and richness of the species that inhabit the intestinal microbiome (Coverdale, 2016). Adding prebiotics containing fructo-oligosaccharides or mannan-oligosaccharides to high fibre diets increases feed digestibility, supporting energy production and has been shown to improve the general fitness and health of the horse (Coverdale, 2016). Research conducted with Irish thoroughbreds undergoing a sudden change in feeding regimen found that digestive sensitivity particularly affected individuals carrying a limited abundance of hindgut core bacterial species (O'Donnell et al., 2013).

When probiotic bacteria and/or yeasts are combined with prebiotics, the formulation is referred to as a 'synbiotic'. These combinations have been used in human studies to promote the growth of beneficial microbes with a view to increasing the diversity and richness of the intestinal milieu (van Zanten et al., 2012).

While there are limited studies in the equid, the potential role of pro- and prebiotics in human health and disease has been explored far more extensively, with the current PubMed citations exceeding five thousand. No summary of studies that report on the nutritional and therapeutic role of prebiotic and synbiotic supplementation in equine health and disease has been conducted to date. Therefore, the aim of this review was to provide an overview of the literature reporting on the use of prebiotic and synbiotic supplements, in equine nutritional practice. An online search of Embase and PubMed databases for papers published from inception to 2023, using the search terms 'prebiotics', 'synbiotics', 'horses' and 'equine', was conducted to identify original research evaluating the effects of prebiotics and synbiotics on the composition and/or metabolism of the equine intestinal microbiome, along with associated health outcomes.

Sixteen papers reporting on the effects of prebiotic or synbiotic supplementation in horses were subject to full text review. The findings of those studies are discussed and evaluated here. This review does not address the use of probiotics in equine populations, which was the topic of a previous review published by the authors of the present manuscript (Cooke et al., 2021).

2. Types of prebiotics

In the horse, naturally occurring prebiotic compounds can constitute part of the regular feed or supplementation regimen, supporting potential colonisation of the intestinal environment with bacteria that are thought to confer a benefit. Prebiotics include fermentable sugars, predominantly oligosaccharides or disaccharides, of which there are several types: oligofructose, inulins, isomalto-oligosacharrides (IMOS), lacto-sucrose, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), lactulose, pyrodextrins and xylo-oligosaccharides (XOS), and mannan-oligosaccharides (MOS) (Davani-Davari et al., 2019). Certain prebiotic types are compounded dietary sugars, for example FOS is composed of short chains of fructose molecules and GOS is composed of a chain of galactose molecules; IMOS is derived from the disaccharide maltose (sourced from

honey, for example) following enzymatic activity, which produces a mixture of short-chain carbohydrates; XOS, a natural prebiotic fibre extracted from sugar cane fibre, and inulin belong to a class of polysaccharides known as "fructans" and are found in vegetables such as Jerusalem artichoke, plantains and chicory root. MOS are complex carbohydrate molecules, derived from the outer cell wall of the yeast *Saccharomyces cerevisiae* - mainly β-glucans (mannoproteins). Soluble arabinogalactans are classified as non-carbohydrate sources of prebiotic fibre derived from larch tree (*Larix sp*.) (Fitzpatrick et al., 2004). From the literature, equine prebiotics most studied are inulins, FOS, GOS and MOS.

Different prebiotic classes supply fuel to resident microbes in various regions of the equine gut. The caecum and colon comprise 66% of the digestive tract volume and contain large populations of anerobic bacteria; these probiotic species process pectin, cellulose, hemicellulose and starch, producing volatile fatty acids (VFA's) through the process of fermentation, to be utilised as a metabolic energy source (Shirazi-Beechey, 2008). The hindgut (colon and caecum) houses a complex microbiome, composed of mostly bacteria but also viruses, fungi, protozoa, archaebacteria and phages; these microorganisms ferment the residual indigestible cellulose which has not been processed proximally, to produce VFA's that serve as a fuel source for the host (Julliand & Grimm, 2016). Nutrients including protein and B-group vitamins are also rendered more digestible by microbial fermentation in the hindgut (Coverdale, 2016). Equids digest a very limited amount of the lignin present in plants, and it is mostly excreted in faeces, as they lack the necessary enzymes, relying on microbially-generated enzymes to aid digestion (Wunderlich et al., 2023).

3. Prebiotics in forage and carbohydrate digestion

Forage of various types supplies energy, nutrients and prebiotics to equids; by definition, the prebiotic constituents of regular feed assist in promoting the growth of intestinal microbiota that confer a health benefit (Weese, 2002). Naturally occurring fructans are

long-chain sugar molecules found in a variety of pasture grasses, with nutrient dense verdant grasses being higher in fructan content and ranging to low-fructan native grass species, the latter being prevalent in Australia (Saastamoinen et al., 2012). While fructans are considered to act as prebiotics, grass varieties such as Timothy and some types of Perennial Rye are either processed rapidly in the equine intestine or have a significant proportion of their carbohydrate pass undigested into the hindgut for fermentation by resident microbiota, generating lactic acid as a by-product (Saastamoinen et al., 2012). Contrary to the definition of a prebiotic, high fructan intake is thought to contribute to the development of hindgut acidosis, increasing the risk of laminitis (Ince et al., 2014), however, this effect may be ameliorated by the addition of different prebiotic fibres to the feed which degrade more slowly (Bachmann, 2020). Mature grasses are composed of more indigestible fibre (Ermers et al., 2023) and less fructans than young fresh grasses, hence presumably would pose less risk of hindgut acidosis to horses grazing on these pastures. From a management perspective, grazing horses on native grasses is preferable to administering prebiotic extracts, as native grasses are more fully processed and do not contribute to increased intestinal gas production which might arise from unfermented fructans reaching the hindgut (Saastamoinen et al., 2012).

The starch component of an ideal equine prebiotic feed should predominantly degrade in the intestine, proximal to the hindgut. Jerusalem artichoke is a source of the prebiotic FOS and inulin, which can play a role in the metabolism of hindgut microbiota by modulating fermentation of ingesta (Coverdale, 2016). Additionally, according to one study in which carbohydrate processing from stomach to transverse colon was evaluated, Jerusalem artichoke can also contribute to increased gas production in the hindgut as a result of fermentation of undigested fructans (Bachmann, 2020). Importantly, inulin derived from Jerusalem artichoke is fermented differently to that present in grasses and may not support development of beneficial *Lactobacillus* colonies as effectively as does the inulin from forage. The rapid fermentation of high fructan grasses favours hindgut cultivation of lactate-producing bacterial species, such as *Streptococcus bovis* and *S. equinus*, overwhelming colonies of predominantly lactate-utilizing species, such as

Veillonella sp. and *Megapshera sp.,* the latter which metabolise lactate to propionate (Al Jassim et al., 2005; Ince et al., 2014; Julliand, 2005). If carbohydrates ferment early in their passage through the equine foregut (oesophagus, stomach, and small intestine), rather than having this process slowed by the presence of indigestible prebiotic fibre, the resultant production of organic acids may accumulate and produce mucosal injury (ulceration) (Bachmann, 2020). An *in vivo* experiment conducted on postmortem equine gastric mucosa tissue samples, applying various concentrations of butyrate to replicate those achieved by natural Jerusalem artichoke bacterial fermentation in the hindgut, showed evidence of injury to the gastric mucosa, postulating that this FOS-rich prebiotic supplement could increase the risk of developing equine gastric ulcer syndrome (EGUS) (Cehak et al., 2019).

4. Prebiotic supplementation in hindgut fermenters

Given the limited literature relating to the use of prebiotic supplementation in horses, it is of interest to consider what is known about other species that are hindgut fermenters. Researchers have explored the effects of supplementing the diets of other hindgut fermenting animals with prebiotics, specifically rodents and rabbits; associated improvements were found in lipid metabolism, composition of the intestinal microbiome and nutrient absorption, in particular minerals (Xiao et al., 2015). Hindgut fermentation, cell-mediated immunity and various blood parameters improved in rats supplemented with pulverised Jerusalem artichoke, rich in prebiotic inulin and FOS for twelve weeks (Samal et al., 2015). Further, in these rats, skin indurations induced by a mitogen reduced, while CD4 lymphocyte populations increased and blood levels of glucose, urea and haemoglobin in these rats adjusted favourably (Samal et al., 2015). Yeast-derived MOS supplementation enhanced VFA concentrations and lower pH in the cecal region of rabbit intestines and was associated with longer intestinal villi when compared to controls. In addition, the weight gain observed with the addition of MOS to the diets of the study animals was similar to that which could be expected from antibiotic treatment for

promoting growth (Mourão et al., 2006). By further comparison, koalas possess a proportionally large hindgut for processing their exclusive diet of Eucalyptus leaves and, while their core intestinal microbiome has been explored (Barker et al., 2013), no study has been published involving prebiotic supplementation of this marsupial species nor the other remaining hindgut fermenter, the rhinoceros. Although evidence supporting the use of prebiotics in the dietary management of horses is limited, there are many commercial prebiotics and synbiotic supplementary feed formulations, currently available in several countries, which are purported to enhance the intestinal microbiome, digestion, energy production and immunity.

5. The effects of prebiotics on the equine microbiome

There is a paucity of studies evaluating how the addition of prebiotic compounds to commercial horse feeds impacts the intestinal microbiome. Most attention has been given to the modulation of intestinal carbohydrate processing by specific prebiotics to reduce the incidence of laminitis, which can be experimentally induced by administering large doses (10g/kg BW) of fructans to horses (French & Pollitt, 2004; Milinovich et al., 2008). Such carbohydrate loading causes acidification of the intestinal milieu, producing a shift from eubiotic Gram-positive bacteria to lactate-producing species, such as *Streptococcus bovis/equinus* in the gut microbiome (Ince et al., 2014). It is believed that these bacterial species, via the generation of vasoactive amines and endotoxins which can permeate into the systemic circulation and cause ischaemia/reperfusion injury to the equine digit, contribute to the development of laminitis in horses (Milinovich et al., 2006). A balance needs to be maintained between the number of commensal bacterial species which are lactate-producers (e.g. *Lactobacillus salivarius, L.delbreukii, L. mucosae*) and those which are lactate-utilisers (e.g. *Veillonella sp., Megasphera sp.)* (Julliand, 2005); the latter metabolise lactate to propionate to support energy production, with propionate being a key precursor molecule for gluconeogenesis (Plancade et al., 2019).

Prebiotic sugars and fibres provide energy and nutrients to intestinal microbiota to fuel their metabolic activity. Berg et al evaluated supplementing the diet of yearling quarter horses with FOS, finding that this intervention led to alterations in the microbiome and metabolome, with respect to faecal pH and concentrations of VFA's (Berg et al., 2005). These researchers reported a quadratic effect on faecal *Eschericia coli* populations following FOS supplementation without any apparent alteration in the numbers of *Lactobacillus sp*.; faecal propionate, butyrate, acetate and lactate concentrations increased linearly with total VFAs in this study (Berg et al., 2005). Such VFA's generated by intestinal microbiota can provide an additional energy source additional to carbohydrates for athletic performance in trotting horses. Hence, providing such prebiotics to generate fuel could be considered performance-enhancing supplementation.

In Warmblood horses fed FOS and inulin supplements daily for 3 weeks, the abundance of *Lactobacillus sp.* increased in relation to that of *Streptococcus sp.,* which decreased in the gastric microbiome, while higher alpha diversity of the intestinal microbiome were observed (Glatter et al., 2019). Alpha diversity refers to the number of species (species richness) within that region. Fructo-oligosaccharides are thought to support the colonisation of the equine intestine with *Bifidobacteria*, although the effect of this increased colonisation is not fully understood (Weese, 2002). There is limited evidence for the function of *Bifidobacteria* in the equine gut microbiome, which contains a much larger proportion of *Lactobacilli*, even in relation to that found in humans, however these indigenous probiotic bacteria do not typically utilise FOS as consistently as do *Bifidobacteria* (Weese, 2002).

The core intestinal equine microbiome is considered to be healthy if it contains a relative abundance of *Firmicutes* with respect to *Bacteroidetes* phyla (Costa & Weese, 2018) and specifically it is the metabolic function of the intestinal microbes which determines equine health. The faecal microbiome composition of ten healthy horses following supplementation with prebiotic cellobiose for 14 days was profiled by Paßlack et al.; the relative abundance of FIRMICUTES, CORIOBACTERIALES, and CLOSTRIDIUM increased in a dose-dependent way, with a dose-dependent decrease was measured in the relative

abundance of BACTEROIDETES (Paßlack et al., 2020). By association, these findings suggest an improvement in the composition of the faecal microbiomes of the study horses following administration of prebiotics.

Yeast derived prebiotic and postbiotic compounds, derived from fermentation *S.cerevisiae* and administered as feed topdressing to a cohort of Quarter horses in a recent study, have been shown to stabilise the composition of the intestinal microbiome following a stress event (Ganda et al., 2023). These entero-protective supplements were also found to favourably modulate the early immune response of 11 English Thoroughbreds following vaccination against equine influenza (Lucassen et al., 2021).

By promoting a healthy balance between species in both the major and minor phyla of the intestinal microbiome, prebiotics support the condition of the gut mucosa, enhance nutrient absorption and athletic performance and overall equine health (Di Gioia & Biavati, 2018). Antibiotic use can be significantly reduced by supplementing livestock and companion animals with synbiotics (Di Gioia & Biavati, 2018), however such benefits have not yet been shown in equine management (Weese, 2002).

6. The effects of prebiotics on equine health and disease

Table 1. Publications selected for review concerning the effects of prebiotic or synbiotic supplementation in horses.

6.1 Equine performance

Exercising horses are often fed high carbohydrate, grain-dominant diets to deliver immediate energy for enhanced performance. This practice reduces natural forage intake, placing these athletes at risk of developing hindgut acidosis and possibly intestinal dysbiosis, a condition which may also be stress-induced (Collinet et al., 2022), and which has been observed in cattle fed high carbohydrate (Sanz-Fernandez et al., 2020). The

prebiotic fibre found in sugar beet is thought to offer a glycogen-sparing effect when added to such feed, due to enhanced propionate production by intestinal microbiota, thereby assisting energy production for performance (Karlsson et al., 2002). During intense exercise in standardbred geldings, increased concentrations of muscle glycogen, lower muscle lactate levels and lower peak plasma lactate concentrations were found following supplementation of their standard diet with sugar beet pulp. This effect was attributed to additional VFAs being utilised for aerobic energy production instead of glycogen (Karlsson et al., 2002). High prebiotic fibre-containing diets supply equivalent amounts of energy via glycogen and propionate production compared to lower prebiotic, high-carbohydrate, grain-based diets, also delivering a beneficial alkalizing effect to protect against lactic acidosis generated by intense exercise (Berg et al., 2005). These findings could inform equine athlete management for protection against dietary complications and consequent illness, whilst improving performance in these horses

6.2 The effects of prebiotics on equine glucose regulation and insulin resistance

Polyphenols and fibres with prebiotic properties have been proposed to reduce inflammation in senior horses possibly via improvements in insulin resistance (Siard et al., 2016). Improved glucose metabolism is thought to occur via the metabolomic effects including an increase butyrate (Portincasa et al., 2022). In humans, lower abundance of butyrate-producing intestinal bacteria has been associated clinically with a higher incidence of Type 2 diabetes, in comparison to healthy controls unaffected by intestinal dysbiosis (Qin et al., 2012).

Supplementing the standard crushed oat and meadow hay diet of six healthy Warmblood mares for 21 days with Jerusalem artichoke meal, providing prebiotic FOS and inulin, produced a rapid serum insulin peak, followed by a faster decline of both glucose and insulin when compared to controls; this effect was associated with the relative abundances of *Lactobacillus* increasing and *Streptococcus* decreasing in the stomach of the prebiotic-fed group (Glatter et al., 2017).

These findings also suggest a potential protective effect of this prebiotic (FOS and inulin) against insulin resistance/metabolic syndrome and obesity in these animals. High soluble carbohydrate in equine feed (grains and spring grass) increases the prevalence of insulin resistance and obesity, reducing the bacterial diversity of the intestinal microbiome and shifting its composition unfavourably towards potential pathogens such as *Streptococcus lutetiensis,* a species linked to the development of laminitis (Milinovich et al., 2008). Supplementing the daily diet of eight obese mature Arab geldings with short-chain FOS over a period of six weeks optimised fibre processing which increased insulin sensitivity, while reducing acute insulin response to glucose and resting serum insulin, without altering body weight or body condition score (Respondek et al., 2011). Further, senior horses experience a decline in digestive capacity and the risk of developing insulin resistance increases with advancing age; supplementing the diet of older horses with FOS may improve the composition of the intestinal microbiome of these animals, resulting in better metabolic condition (Heaton et al., 2019).

6.3 The effects of prebiotics on equine intestinal inflammation

The discovery that prebiotic supplements may modulate intestinal inflammatory and immune responses has potential applications for veterinary medicine. Anti-inflammatory and immunomodulatory activity have been demonstrated in two equine studies (Adams et al., 2015; Vendrig et al., 2013) and one human study (Sartor, 2004) using prebiotics. Specific actions of microbial metabolites, such as butyrate, are believed to include reduced cytokine production by the mucosal cells lining the intestine (Sartor, 2004). Anti-inflammatory and immunomodulatory activity of prebiotics were demonstrated in one *in vitro* study, in which equine peripheral blood mononuclear cells (PBMCs) were challenged with lipopolysaccharide (LPS) to induce an inflammatory response (Vendrig et al., 2013). A protective effect was observed when PBMCs were pre-incubated with GOS/FOS/AOS (acidic oligosaccharides) prior to being challenged with LPS, producing a dose-dependent reduction in both TNF-α and interleukin-10 production. However, if PBMCs were pre-treated with GOS or GOS/FOS fractions alone, TNF- α production by the

immune cells rose substantially (Vendrig et al., 2013). These oligosaccharide fractions demonstrated a dampening effect on allergic response while enhancing immune defences in PBMCs; pre-treatment incubation with glucose/lactose concentrations similar to 1% GOS, as a comparison control, significantly increased cell viability by 14%, while GOS/FOS/AOS pre-treatment increased cell viability by a range of 38-61% (Vendrig et al., 2013). The authors suggested that this protective effect on PBMCs was due to lower mitogenic potential (Vendrig et al., 2013).

7. Prebiotic yeast extracts in equine health and disease

Mannan-oligosaccharides, derived from proteins in *S. cerevisiae* cell walls, protects against bacterial infection by competitively inhibiting the binding of bacterial lectins to intestinal enterocytes (Asadpoor et al., 2020) and a dried yeast extract from mechanically ruptured *S. cerevisiae* cells containing beta glucan acts as a prebiotic substance, effectively absorbing mycotoxins from contaminated feed and thereby offering immune protection (El-Naggar & Thabit, 2014). In terms of the impact of prebiotic yeast extracts such as MOS on inflammation, horses exhibiting exercise-induced stress demonstrated more rapid recovery of cortisol and cytokine levels following eight weeks' supplementation with a fermented *S. cerevisiae* product as compared to controls (Valigura et al., 2021).

Twenty thoroughbred mares supplemented with MOS, for 20 days prepartum, exhibited improved blood antioxidant parameters, namely 24% higher plasma superoxide dismutase levels than controls, without alteration to the nutritional composition of their milk (Czech et al., 2006). Furthermore, there is evidence to suggest that mares' plasma and colostrum IgG levels may be boosted by adding MOS to their feed, protecting both mare and foal for 24 hours post-partum from serious infectious complications, such as diarrhea, sepsis and possibly death (Spearman, 2004)

7.1 Prebiotic safety

It is generally considered that prebiotics may be safely administered to horses, although this assumed safety has not yet been scientifically evaluated. Any alteration in feed or supplementation which might adversely impact microbial diversity and species richness could disturb intestinal homeostasis (Garber et al., 2020). Intestinal dysbiosis often results from administration of antibiotics to horses and may render the animal susceptible to proliferation of pathogenic species such as *Clostridium difficile* and *Salmonella sp.* in the gut, causing antimicrobial-induced colitis (Costa et al., 2012), and diarrhea (Garber et al., 2020). Prebiotics have been shown to protect horses against intestinal dysbiosis, by supporting the colonization of favorable probiotic species and dispelling enteric pathobionts (Julliand, 2005). Soluble arabinogalactans, the prebiotic fiber derived from larch (*Larix sp.)* was administered to foals to treat scouring with some benefit and, although its specific mode of action is yet to be determined, its administration has proven to be safe in young horses (Weese, 2002). Another study supplemented seven mares and their foals with a soluble arabinogalactan preparation (LaraFeed AC9) for 2 weeks pre-foaling and until 14 days post-foaling, using five mares and their foals as controls. The results were a lower frequency of diarrhoea episodes with more normal faeces amongst the treated foals, requiring less veterinary intervention in comparison to controls. No treatment effects were found in the foals with respect to weight, blood parameters (complete blood count, IgA, IgG), or faecal *Salmonella* or *Rotavirus* cultures, demonstrating tolerance for the larch extract (Wemer HK).

8. Synbiotics in equine health and disease

A combination of a probiotic and a prebiotic, referred to as a synbiotic, can increase the survival time and colonization potential of the probiotic bacteria or yeast in the intestinal milieu (Weese, 2002). There is also some evidence that synbiotics may be beneficial for assisting intestinal sand clearance in horses, thereby playing a preventive role against the

development of sand colic and enteropathy (Landes et al., 2008). The accidental ingestion of sand commonly occurs in horses as a result of daily feeding on pasture grasses (Landes et al., 2008), which may result in sand accumulation and lead to colic. Synbiotic treatments containing probiotics (*Lactobacillus acidophilus, Enterococcus faecium)*, prebiotics (derived from *S. cerevisiae*) and up to 90% psyllium seed husks have been effectively utilized to reduce and treat sand enteropathy and sand colic (Landes et al., 2008). While sand accumulation was significantly reduced with this treatment, radiographic evidence of clearance was not observed (Hassel et al., 2020). It is uncertain, however, whether pre- and probiotics play a significant role in correcting this painful condition, or whether favorable results are achieved by dosing with high-fiber pulverized psyllium seeds alone. A synbiotic-only effect on this condition cannot be confirmed with this study, as the treatment contained predominantly psyllium seed husks, however there may have been a therapeutic synergism delivered by the compound treatment formulation. Another report has been published of more effective removal of accumulated intestinal sand with psyllium seed husk therapy supported by the addition of cathartic magnesium sulphate via nasogastric delivery (Niinistö, 2020).

The addition of probiotics to prebiotic supplementation has also been shown to assist in improving the composition of the equine microbiome (Coverdale, 2016) and provide protection against excess colonization of microbial pathogens, thereby reducing the requirement for antibiotic use (Spearman, 2004). A recent review reported on the safety, tolerability and efficacy of probiotic bacteria currently in equine veterinary use, revealing conflicting evidence for the overall health benefits of probiotic bacterial treatments for horses, although some specific benefits for managing scouring in foals and improving athletic performance were reported (Cooke et al., 2021). In general, recent publications are supporting the use of prebiotics plus probiotics to improve animal health by manipulating the microbiome of many different domestic, livestock and wild species, without the need for antibiotic use, thus providing societal and economic benefits (Jin Song et al., 2019).

9. Conclusion

Current evidence for the use of prebiotics and synbiotics in the management of equine health and disease is not extensive, although it could be considered promising and practical in many cases. Prebiotics and synbiotics, when added to the equine diet, have been shown to influence insulin sensitivity for obesity prevention, to reduce markers of systemic inflammation and allergy, and intestinal dysbiosis. Reduction of hindgut acidosis and correcting intestinal dysbiosis by the administration of prebiotic containing fibre to the equine diet may also confer protection against inflammatory conditions such as colitis and laminitis. Finally, the thoroughbred racing industry might consider adopting the safe and legal practice of administering prebiotics to their athletes, to assist energy production from volatile fatty acids resulting from microbial fermentation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Ethical Approval

No ethics approval was necessary to conduct this review.

Data Availability

All data presented are published by the authors listed in this review.

Authors' Contributions

CGC conducted the review of the literature, compiled the table of publications, and drafted the manuscript; JH, ZG and CGC reviewed the findings, revised the manuscript and approved the final draft.

REFERENCES

Adams, A. A., Vineyard, K. R., Gordon, M. E., Reedy, S., Siard, M. H., & Horohov, D. W. (2015). 55 The effect of n-3 polyunsaturated fatty acids (DHA) and prebiotic supplementation on inflammatory cytokine production and immune responses to vaccination in old horses. *Journal of Equine Veterinary Science*, *35*(5), 407-408. https://doi.org/https://doi.org/10.1016/j.jevs.2015.03.065

Al Jassim, R. A., Scott, P. T., Trebbin, A. L., Trott, D., & Pollitt, C. C. (2005). The genetic diversity of lactic acid producing bacteria in the equine gastrointestinal tract. *FEMS Microbiol Lett*, *248*(1), 75-81. https://doi.org/10.1016/j.femsle.2005.05.023

Asadpoor, M., Peeters, C., Henricks, P. A. J., Varasteh, S., Pieters, R. J., Folkerts, G., & Braber, S. (2020). Anti-Pathogenic Functions of Non-Digestible Oligosaccharides In Vitro. *Nutrients*, *12*(6), 1789. https://doi.org/10.3390/nu12061789

Bachmann, M. (2020). In Vitro Gas Production from Batch Cultures of Stomach and Hindgut Digesta of Horses Adapted to a Prebiotic Dose of Fructooligosaccharides and Inulin [Original Research]. *Journal of Equine Veterinary Science*, *90*. https://doi.org/doi.org/10.1016/j.jevs.2020.103020

Barker, C. J., Gillett, A., Polkinghorne, A., & Timms, P. (2013). Investigation of the koala (Phascolarctos cinereus) hindgut microbiome via 16S pyrosequencing. *Veterinary Microbiology*, *167*(3), 554-564. https://doi.org/https://doi.org/10.1016/j.vetmic.2013.08.025

Berg, E. L., Fu, C. J., Porter, J. H., & Kerley, M. S. (2005). Fructooligosaccharide supplementation in the yearling horse: Effects on fecal pH, microbial content, and volatile fatty acid concentrations [Article]. *Journal of Animal Science*, *83*(7), 1549-1553. https://www.scopus.com/inward/record.uri?eid=2-s2.0-33645965589&partnerID=40&md5=77b1cbbd51cfcdb3dfafbf7a13348630

Biddle, A. (2021). The Equine Microbiome Project. https://sites.udel.edu/equine-microbiome/equine-microbiome-project/

Browning, G. F., Chalmers, R. M., Snodgrass, D. R., Batt, R. M., Hart, C. A., Ormarod, S. E., Leadon, D., Stoneham, S. J., & Rossdale, P. D. (1991). The prevalence of enteric pathogens in diarrhoeic thoroughbred foals in Britain and Ireland. *Equine Vet J*, *23*(6), 405-409. https://doi.org/10.1111/j.2042-3306.1991.tb03751.x

Cehak, A., Krageloh, T., Zuraw, A., Kershaw, O., Brehm, R., & Breves, G. (2019). Does prebiotic feeding affect equine gastric health? A study on the effects of prebiotic-induced gastric butyric acid production on mucosal integrity of the equine stomach. *Res Vet Sci*, *124*, 303-309. https://doi.org/10.1016/j.rvsc.2019.04.008

Collinet, A., Grimm, P., Jacotot, E., & Julliand, V. (2022). Biomarkers for monitoring the equine large intestinal inflammatory response to stress-induced dysbiosis and probiotic supplementation. *J Anim Sci*, *100*(10). https://doi.org/10.1093/jas/skac268

Cooke, C. G., Gibb, Z., & Harnett, J. E. (2021). The Safety, Tolerability and Efficacy of Probiotic Bacteria for Equine Use. *J Equine Vet Sci*, *99*, 103407. https://doi.org/10.1016/j.jevs.2021.103407

Costa, M. C., Arroyo, L. G., Allen-Vercoe, E., Stampfli, H. R., Kim, P. T., Sturgeon, A., & Weese, J. S. (2012). Comparison of the fecal microbiota of healthy horses and horses with colitis by high throughput sequencing of the V3-V5 region of the 16S rRNA gene. *PLoS One*, *7*(7), e41484. https://doi.org/10.1371/journal.pone.0041484

Costa, M. C., & Weese, J. S. (2012). The equine intestinal microbiome. *Animal health research reviews*, *13*(1), 121-128. https://doi.org/10.1017/S1466252312000035

Costa, M. C., & Weese, J. S. (2018). Understanding the Intestinal Microbiome in Health and Disease. *Vet Clin North Am Equine Pract*, *34*(1), 1-12. https://doi.org/10.1016/j.cveq.2017.11.005

Coverdale, J. A. (2016). Horse Species Symposium: Can the microbiome of the horse be altered to improve digestion? *Journal of Animal Science*, *94*(6), 2275-2281. https://doi.org/10.2527/jas.2015-0056

Czech, A., Grela, E. R., Lyons, T. P., Jacques, K. A., & Hower, J. M. (2006). Influence of Bio-Mos® mannan oligosaccharides in mare diets on colostrum and milk composition and blood parameters.

Dart, A., Hodgson, D., & Snyder, J. (1997). Caecal disease in equids. *Australian veterinary journal*, *75*(8), 552-557. https://doi.org/https://doi.org/10.1111/j.1751-0813.1997.tb14191.x

Davani-Davari, D., Negahdaripour, M., Karimzadeh, I., Seifan, M., Mohkam, M., Masoumi, S. J., Berenjian, A., & Ghasemi, Y. (2019). Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications. *Foods*, *8*(3). https://doi.org/10.3390/foods8030092

Di Gioia, D., & Biavati, B. (2018). Probiotics and Prebiotics in Animal Health and Food Safety: Conclusive Remarks and Future Perspectives. In D. Di Gioia & B. Biavati (Eds.), *Probiotics and Prebiotics in Animal Health and Food Safety* (pp. 269-273). Springer International Publishing. https://doi.org/10.1007/978-3-319-71950-4_11

Dunkel, B., Buonpane, A., & Chang, Y. M. (2017). Differences in gastrointestinal lesions in different horse types. *Vet Rec*, *181*(11), 291. https://doi.org/10.1136/vr.104098

El-Naggar, M. A., & Thabit, T. M. (2014). Evaluation of β-D-glucan biopolymer as a novel mycotoxin binder for fumonisin and deoxynivalenol in soybean feed. *Foodborne Pathog Dis*, *11*(6), 433-438. https://doi.org/10.1089/fpd.2013.1711

Ermers, C., McGilchrist, N., Fenner, K., Wilson, B., & McGreevy, P. (2023). The Fibre Requirements of Horses and the Consequences and Causes of Failure to Meet Them. *Animals : an open access journal from MDPI*, *13*(8). https://doi.org/10.3390/ani13081414

Fitzpatrick, A., Roberts, A., & Witherly, S. (2004). Larch arabinogalactan: A novel and multifunctional natural product. *Agro Food Industry Hi-Tech*, *15*, 30-32.

French, K. R., & Pollitt, C. C. (2004). Equine laminitis: loss of hemidesmosomes in hoof secondary epidermal lamellae correlates to dose in an oligofructose induction model: an ultrastructural study. *Equine Vet J*, *36*(3), 230-235. https://doi.org/10.2746/0425164044877125

Ganda, E., Chakrabarti, A., Sardi, M. I., Tench, M., Kozlowicz, B. K., Norton, S. A., Warren, L. K., & Khafipour, E. (2023). Saccharomyces cerevisiae fermentation product improves robustness of equine gut microbiome upon stress. *Front Vet Sci*, *10*, 1134092. https://doi.org/10.3389/fvets.2023.1134092

Garber, A., Hastie, P. M., & Murray, J.-A. (2020). Factors Influencing Equine Gut Microbiota: Current Knowledge. *Journal of Equine Veterinary Science*, *88*, 102943. https://doi.org/https://doi.org/10.1016/j.jevs.2020.102943

Glatter, M., Bochnia, M., Goetz, F., Gottschalk, J., Koeller, G., Mielenz, N., Hillegeist, D., Greef, J. M., Einspanier, A., & Zeyner, A. (2017). Glycaemic and insulinaemic responses of adult healthy warm-blooded mares following feeding with Jerusalem artichoke meal. *J Anim Physiol Anim Nutr (Berl)*, *101 Suppl 1*, 69-78. https://doi.org/10.1111/jpn.12669

Glatter, M., Borewicz, K., van den Bogert, B., Wensch-Dorendorf, M., Bochnia, M., Greef, J. M., Bachmann, M., Smidt, H., Breves, G., & Zeyner, A. (2019). Modification of the equine gastrointestinal microbiota by Jerusalem artichoke meal supplementation. *PLoS One*, *14*(8), Article e0220553. https://doi.org/10.1371/journal.pone.0220553

Hassel, D. M., Curley, T., & Hoaglund, E. L. (2020). Evaluation of Fecal Sand Clearance in Horses With Naturally Acquired Colonic Sand Accumulation With a Product Containing Probiotics, Prebiotics, and Psyllium. *J Equine Vet Sci*, *90*, 102970. https://doi.org/10.1016/j.jevs.2020.102970

Heaton, C. P., Cavinder, C. A., Paz, H., Rude, B., Smith, T., Memili, E., Harris, P., Liburt, N., & Krotky, A. (2019). Are prebiotics beneficial for digestion in mature and senior horses. *Journal of Equine Veterinary Science*, *76*, 87-88. https://doi.org/https://doi.org/10.1016/j.jevs.2019.03.116

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature reviews Gastroenterology & hepatology*, *11*(8), 506-514. https://doi.org/10.1038/nrgastro.2014.66

Ince, J. C., Longland, A. C., Moore-Colyer, M. J. S., & Harris, P. A. (2014). In vitro degradation of grass fructan by equid gastrointestinal digesta. *Grass and forage science*, *69*(3), 514-523. https://doi.org/10.1111/gfs.12061

Jin Song, S., Woodhams, D. C., Martino, C., Allaband, C., Mu, A., Javorschi-Miller-Montgomery, S., Suchodolski, J. S., & Knight, R. (2019). Engineering the microbiome for animal health and conservation [Article]. *Experimental Biology and Medicine*, *244*(6), 494-504. https://doi.org/10.1177/1535370219830075

Julliand, V. (2005). Impact of nutrition on the microflora of the gastro-intestinal tract in horses. *Applied Equine Nutrition. Wageningen Academic Publishers, Netherlands*, 85-103.

Julliand, V., & Grimm, P. (2016). HORSE SPECIES SYMPOSIUM: The microbiome of the horse hindgut: History and current knowledge. *J Anim Sci*, *94*(6), 2262-2274. https://doi.org/10.2527/jas.2015-0198

Karlsson, C. P., Jansson, A., Essén-Gustavsson, B., & Lindberg, J.-E. (2002). Effect of molassed sugar beet pulp on nutrient utilisation and metabolic parameters during exercise. *Equine Vet J*, *34*(S34), 44-49. https://doi.org/https://doi.org/10.1111/j.2042-3306.2002.tb05390.x

Kauter, A., Epping, L., Semmler, T., Antao, E.-M., Kannapin, D., Stoeckle, S. D., Gehlen, H., Lübke-Becker, A., Günther, S., Wieler, L. H., & Walther, B. (2019). The gut microbiome of horses: current research on equine enteral microbiota and future

perspectives. *Animal Microbiome*, *1*(1), 14. https://doi.org/10.1186/s42523-019-0013-3

Kilcoyne, I., Dechant, J. E., Spier, S. J., Spriet, M., & Nieto, J. E. (2017). Clinical findings and management of 153 horses with large colon sand accumulations. *Vet Surg*, *46*(6), 860-867. https://doi.org/10.1111/vsu.12679

Landes, A. D., Hassel, D. M., Funk, J. D., & Hill, A. (2008). Fecal Sand Clearance Is Enhanced with a Product Combining Probiotics, Prebiotics, and Psyllium in Clinically Normal Horses. *Journal of Equine Veterinary Science*, *28*(2), 79-84. https://doi.org/https://doi.org/10.1016/j.jevs.2008.01.004

Leclere, M., & Costa, M. C. (2020). Fecal microbiota in horses with asthma. *Journal of veterinary internal medicine*, *34*(2), 996-1006. https://doi.org/10.1111/jvim.15748

Lucassen, A., Finkler-Schade, C., & Schuberth, H. J. (2021). A Saccharomyces cerevisiae Fermentation Product (Olimond BB) Alters the Early Response after Influenza Vaccination in Racehorses. *Animals : an open access journal from MDPI*, *11*(9). https://doi.org/10.3390/ani11092726

Mach, N., Moroldo, M., Rau, A., Lecardonnel, J., Le Moyec, L., Robert, C., & Barrey, E. (2021). Understanding the Holobiont: Crosstalk Between Gut Microbiota and Mitochondria During Long Exercise in Horse. *Front Mol Biosci*, *8*, 656204. https://doi.org/10.3389/fmolb.2021.656204

McConnico, R. S. (2015). Acute Colitis in Horses. *Robinson's Current Therapy in Equine Medicine*, 297-301. https://doi.org/10.1016/B978-1-4557-4555-5.00068-6

Milinovich, G. J., Burrell, P. C., Pollitt, C. C., Klieve, A. V., Blackall, L. L., Ouwerkerk, D., Woodland, E., & Trott, D. J. (2008). Microbial ecology of the equine hindgut during oligofructose-induced laminitis. *Isme Journal*, *2*(11), 1089-1100. https://doi.org/10.1038/ismej.2008.67

Milinovich, G. J., Trott, D. J., Burrell, P. C., van Eps, A. W., Thoefner, M. B., Blackall, L. L., Al Jassim, R. A., Morton, J. M., & Pollitt, C. C. (2006). Changes in equine hindgut bacterial populations during oligofructose-induced laminitis. *Environ Microbiol*, *8*(5), 885-898. https://doi.org/10.1111/j.1462-2920.2005.00975.x

Morrison, P. K., Newbold, C. J., Jones, E., Worgan, H. J., Grove-White, D. H., Dugdale, A. H., Barfoot, C., Harris, P. A., & Argo, C. M. (2018). The Equine Gastrointestinal Microbiome: Impacts of Age and Obesity. *Front Microbiol*, *9*, 3017. https://doi.org/10.3389/fmicb.2018.03017

Mourão, J. L., Pinheiro, V., Alves, A., Guedes, C., Pinto, L., Saavedra, M. J., Spring, P., & Kocher, A. (2006). Effect of mannan oligosaccharides on the performance, intestinal morphology and cecal fermentation of fattening rabbits. *Animal Feed Science and Technology*, *126*(1-2), 107-120.

Niinistö, K. (2020). Naturally occurring large colon sand accumulations and their medical treatment in horses [Doctoral dissertation (article-based)]. (Helsingin yliopisto), http://urn.fi/URN:ISBN:978-951-51-6771-2

O'Donnell, M. M., Harris, H. M. B., Jeffery, I. B., Claesson, M. J., Younge, B., O'Toole, P. W., & Ross, R. P. (2013). The core faecal bacterial microbiome of Irish Thoroughbred racehorses [Article]. *Letters in Applied Microbiology*, *57*(6), 492-501. https://doi.org/10.1111/lam.12137

Paßlack, N., Vahjen, W., & Zentek, J. (2020). Impact of dietary cellobiose on the fecal microbiota of horses. *Journal of Equine Veterinary Science*, 103106.

Petersen, C., & Round, J. L. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cellular microbiology*, *16*(7), 1024-1033.

Pfister, K., & van Doorn, D. (2018). New Perspectives in Equine Intestinal Parasitic Disease: Insights in Monitoring Helminth Infections. *The Veterinary clinics of North America. Equine practice*, *34*(1), 141-153. https://doi.org/10.1016/j.cveq.2017.11.009

Plancade, S., Clark, A., Philippe, C., Helbling, J.-C., Moisan, M.-P., Esquerré, D., Le Moyec, L., Robert, C., Barrey, E., & Mach, N. (2019). Unraveling the effects of the gut microbiota composition and function on horse endurance physiology. *Scientific Reports*, *9*(1), 1-15.

Portincasa, P., Bonfrate, L., Vacca, M., De Angelis, M., Farella, I., Lanza, E., Khalil, M., Wang, D. Q., Sperandio, M., & Di Ciaula, A. (2022). Gut Microbiota and Short Chain Fatty Acids: Implications in Glucose Homeostasis. *Int J Mol Sci*, *23*(3). https://doi.org/10.3390/ijms23031105

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., Liang, S., Zhang, W., Guan, Y., Shen, D., Peng, Y., Zhang, D., Jie, Z., Wu, W., Qin, Y., Xue, W., Li, J., Han, L., Lu, D., . . . Wang, J. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, *490*(7418), 55-60. https://doi.org/10.1038/nature11450

Respondek, F., Myers, K., Smith, T. L., Wagner, A., & Geor, R. J. (2011). Dietary supplementation with short-chain fructo-oligosaccharides improves insulin sensitivity in obese horses. *J Anim Sci*, *89*(1), 77-83. https://doi.org/10.2527/jas.2010-3108

Saastamoinen, M., Fradinho, M. J., Santos, A. S., & Miraglia, N. (2012). Forages and grazing in horse nutrition. Forages and grazing in horse nutrition, https://doi.or EAAP Scientific , Volume 132, European Workshop on Equine Nutrition (EWEN) https://doi.org/10.3920/978-90-8686-755-4

Samal, L., Chaturvedi, V. B., Saikumar, G., Somvanshi, R., & Pattanaik, A. K. (2015). Prebiotic potential of Jerusalem artichoke (Helianthus tuberosus L.) in Wistar rats: effects of levels of supplementation on hindgut fermentation, intestinal morphology, blood metabolites and immune response. *J Sci Food Agric*, *95*(8), 1689-1696. https://doi.org/10.1002/jsfa.6873

Sanchez, L. C. (2018). Disorders of the Gastrointestinal System. *Equine Internal Medicine*, 709-842. https://doi.org/10.1016/B978-0-323-44329-6.00012-7

Sanz-Fernandez, M. V., Daniel, J. B., Seymour, D. J., Kvidera, S. K., Bester, Z., Doelman, J., & Martín-Tereso, J. (2020). Targeting the Hindgut to Improve Health and Performance in Cattle. *Animals : an open access journal from MDPI*, *10*(10). https://doi.org/10.3390/ani10101817

Sartor, R. B. (2004). Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology*, *126*(6), 1620-1633. https://doi.org/10.1053/j.gastro.2004.03.024

Shawaf, T., El-Deeb, W. M., & Elgioushy, M. (2020). The Contribution of Specific and Nonspecific Biomarkers in Diagnosis of Equine Gastric Ulcer Syndrome (EGUS) Under Field Condition. *J Equine Vet Sci*, *84*, 102853. https://doi.org/10.1016/j.jevs.2019.102853

Shirazi-Beechey, S. P. (2008). Molecular insights into dietary induced colic in the horse [Review article]. *Equine Vet J*, *Volume 40*(4), 414-421. https://doi.org/10.2746/042516408X314075

Siard, M. H., McMurry, K. E., & Adams, A. A. (2016). Effects of polyphenols including curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene on lymphocyte pro-inflammatory cytokine production of senior horses in vitro. *Veterinary Immunology and Immunopathology*, *173*, 50-59.

Spearman, K. (2004). Effect of Mannan Oligosaccharie (MOS) supplementation on the immune status of mares and their foals [Master of Science Thesis], Semantic Scholar, Corpus ID: 52992266

Valcheva, R., & Dieleman, L. A. (2016). Prebiotics: Definition and protective mechanisms. *Best Practice & Research Clinical Gastroenterology*, *30*(1), 27-37. https://doi.org/https://doi.org/10.1016/j.bpg.2016.02.008

Valigura, H. C., Leatherwood, J. L., Martinez, R. E., Norton, S. A., & White-Springer, S. H. (2021). Dietary supplementation of a Saccharomyces cerevisiae fermentation product attenuates exercise-induced stress markers in young horses. *Journal of Animal Science*, *99*(8). https://doi.org/10.1093/jas/skab199

van Zanten, G. C., Knudsen, A., Röytiö, H., Forssten, S., Lawther, M., Blennow, A., Lahtinen, S. J., Jakobsen, M., Svensson, B., & Jespersen, L. (2012). The effect of selected synbiotics on microbial composition and short-chain fatty acid production in a model system of the human colon. *PLoS One*, *7*(10), e47212. https://doi.org/10.1371/journal.pone.0047212

Vendrig, J. C., Coffeng, L. E., & Fink-Gremmels, J. (2013). In vitro evaluation of defined oligosaccharide fractions in an equine model of inflammation. *BMC Veterinary Research*, *9*(1), 147. https://doi.org/10.1186/1746-6148-9-147

Weese, J. S. (2002). Probiotics, prebiotics, and synbiotics. *Journal of Equine Veterinary Science*, *22*(8), 357-360. https://doi.org/https://doi.org/10.1016/S0737-0806(02)70006-3

Wemer HK, L. L., Bames T. Effects of administration of an oral arabinogalactan supplement on mares and foals, Equine Nutrition and Physiology Society (ENPS) Symposium Proceedings, https://www.vetassured.com/Arabinogalactan_Research.pdf

Wunderlich, G., Bull, M., Ross, T., Rose, M., & Chapman, B. (2023). Understanding the microbial fibre degrading communities & processes in the equine gut. *Anim Microbiome*, *5*(1), 3. https://doi.org/10.1186/s42523-022-00224-6

Xiao, J., Metzler-Zebeli, B. U., & Zebeli, Q. (2015). Gut Function-Enhancing Properties and Metabolic Effects of Dietary Indigestible Sugars in Rodents and Rabbits. *Nutrients*, *7*(10), 8348-8365. https://doi.org/10.3390/nu7105397

Xu, C., Liu, J., Gao, J., Wu, X., Cui, C., Wei, H., Peng, J., & Zheng, R. (2021). The Effect of Functional Fiber on Microbiota Composition in Different Intestinal Segments of Obese Mice. *Int J Mol Sci*, *22*(12). https://doi.org/10.3390/ijms22126525

APPENDIX B3: THE SEMEN MICROBIOME OF MINIATURE PONY STALLIONS

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Abstract

Context: Little is known about the microbial composition of stallion semen.

Aims: To describe the bacterial microbiota detected in the equine semen of healthy miniature stallions.

Methods: Semen specimens were collected using a Missouri Artificial Vagina (AV) at one single time point. PacBio genomic DNA sequencing of the 16S rRNA gene was performed on these specimens, following which next-generation microbiome bioinformatics platform QIIME2 was used to process fastq files and analyse the amplicon data. The data were categorised into genus, family, class, order, and phylum.

Key results: Firmicutes and Bacteroidetes phyla predominated (76%) followed by Proteobacteria (15%). Bacteroidales, Clostridiales and Cardiobacteriales predominated the microbial rank of order (86%). Class was mainly comprised of Bacteroidia, Clostridia and Gammaproteobacteria (87%), while family was mainly comprised of *Porphyromonadaceae*, *Family_XI* and *Cardiobacteriaceae* (62%). At the level of genera, 80% of the abundance was comprised of seven genera namely *Porphyromonas, Suttonella, Peptoniphilus, Fastidiosipila, Ezakiella, Petrimonas* and taxon unknown.

Conclusions: The findings indicate specific microbiota predominate the semen of healthy miniature stallion ponies with some inter-individual variations observed.

Implications: Larger equine or human studies involving both fertile and infertile subjects could be informed by this study, exploring the relationship of the semen microbiome to male fertility.

Key words semen, equine, pony, microbiome, stallion, microbiota

Graphical abstract

INSERT Figure 1*: A graphical abstract of this study, characterising the semen microbiome of four miniature pony stallions to five taxonomical ranks*

List of Abbreviations

16S rRNA - 16S ribosomal ribonucleic acid

AEC – Animal Ethics Committee

ATP - adenosine triphosphate

ASV - amplicon sequence variants

AV - artificial vagina

DNA - deoxyribonucleic acid

µL - microlitre

NA - taxon undescribed

PCR - polymerase chain reaction

1 | INTRODUCTION

Exploring the composition of microbial communities that inhabit anatomical regions is an important area of research that seeks to understand the role of microbiota in the prevention of disease and supporting normal health and function. More specifically, interest in the microbial communities that inhabit the reproductive tract of humans (Hou, Zhou et al. 2013, Baud, Pattaroni et al. 2019) and animals (Heil, Paccamonti et al. 2019, Rowe, Veerus et al. 2020, Ong, Ross et al. 2022), and the microbial associations with reproductive health and fertility has increased. Within the context of equine reproduction, genitourinary infections in the stallion and contamination of semen with bacteria, yeasts and fungi from passage along the distal urethra (Hughes and Loy 1975, Rota, Calicchio et al. 2011) potentially affect sperm quality (Al-Kass, Guo et al. 2020) and increase the risk of endometritis for the mare (Madsen and Christensen 1995), impairing chances of conception and live foal birth outcomes. Bacterial contamination of stored equine semen poses significant challenges for breeders, requiring the addition of antibiotics to extended semen samples to prevent bacterial growth and transmission via artificial insemination, a practice which can also result in the development of antibiotic-resistant organisms and compromised sperm quality (Malaluang, Wilén et al. 2021). With this concern, and in the context of equine fertility, pathogens have been studied more extensively by veterinarian researchers than the commensal bacteria that inhabit the stallion semen.

Over the past decade the equine intestinal microbiome has been characterised in both healthy and diseased horses and foals (Al Jassim and Andrews 2009, Costa, Silva et al. 2015, Costa, Stämpfli et al. 2016, Costa, Stämpfli et al. 2016, Costa, Stämpfli et al. 2016, Kauter, Epping et al. 2019), however it is only very recently that the semen microbiome of stallions has been described (Al-Kass, Guo et al. 2020, Quiñones-Pérez, Hidalgo et al. 2021). To date, impaired progressive motility of stallion sperm has been correlated with the presence of the bacterium Clostridiales Incertae Sedis XI (Quiñones-Pérez, Martínez et al. 2022), while improved total sperm motility was observed in the presence of Peptoniphilaceae family (Quiñones-Pérez, Martínez et al. 2022). In the case of stallion semen, a predominance of *Porphyromonadaceae*, *Peptoniphilaceae*, *Corynebacteriaceae*

and *Prevotellaceae* families characterised the microbiome profile of healthy, fertile individuals (Quiñones-Pérez, Hidalgo et al. 2021).

A range of technologies are used to characterise microbial communities and the genes that inhabit them. Advances in metagenomic 16s rRNA sequencing technology has provided insights into the microbial genes present in stallion semen (Al-Kass, Guo et al. 2020) and enabled identification of microbiota that have not been identified via culture-based techniques or MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization-Time Of Flight mass spectrometry) technology, (Al-Kass, Eriksson et al. 2020), which rapidly identifies microorganisms using an accurate high-throughput methodology. The adoption of polymerase chain reaction (PCR)metagenomic sampling and full length 16 rRNA sequencing is associated with strengths in identifying the presence of viable and non-viable bacteria – anaerobes and aerobes (Böttger 1989), thus enabling the identification of lesser known microbes and to reveal those which may be difficult to culture (Woo, Lau et al. 2008). Microbial technologies using 16s rRNA have been estimated to identify up to 99% of microbes present in a sample to genus level (Mignard 2006), hence increasing the scope and insight into the composition of microbial communities that inhabit different regions.

Environmental factors and nutrition have been shown to influence the intestinal microbiome composition in horses (Julliand and Pauline 2017, Garber, Hastie et al. 2020, Mach, Ruet et al. 2020, Paßlack, Vahjen et al. 2020, Bull, Davies et al. 2021, Theelen, Luiken et al. 2021) and their general health. The environment and nutrition are also known to impact fertility, yet little is known about the composition of the semen microbiota of healthy stallions and in different breeds. To date, no studies have reported the semen microbiome profiles of miniature pony stallions. The aim of this study was to add to the existing knowledge base regarding the composition of the equine seminal microbiota, by identifying bacteria in more detail than previously reported, from phylum to genus, in four miniature stallions. (See Figure 1).

2 | MATERIALS AND METHODS

2.1 | Subjects

Four miniature crossbred pony stallions, aged between 2 and 15 years and stabled at an equestrian centre in New South Wales Australia were recruited for this study in October 2019. They comprised a cohort of horses that routinely provide semen specimens for scientific purposes. All procedures undertaken in this study were approved by the University of Newcastle Animal Ethics Committee (AEC) and adhere to the Australian code for the care and use of animals for scientific purposes (AEC approval number A-2011-122).

The ponies grazed on native grasses in adjacent paddocks and lucerne hay was added to their diet throughout the year. No exercise regime was undertaken other than free paddock roaming, and the ponies were stabled overnight. The body condition score of the ponies was 6-7 in the range of 1 (very thin) to 9 (very fat) on the US body condition score chart (51).

2.2 | Study Design

Semen samples were collected on the same morning from each pony after a minimum two days' rest. Seminal specimens were prepared and subjected to genomic profiling analysis of bacterial microbial composition, classified by phylum, order, class, family, and genera, for a descriptive analysis.

2.3 | Collection of semen samples

After careful washing of each stallion's prepuce and penis with warm water to remove smegma and other residues, semen collections were undertaken. Semen was collected from the pony stallions using a Missouri artificial vagina (Minitube Australia, Ballarat, VIC) with an in-line gel filter, and a phantom mount. Following semen collection, 250 µL was immediately removed and diluted with 20 µL of DNA Microshield after which it was stored at -30 °C until analyses.

2.4 | Microbial DNA Profiling of semen microbiota

ZymoBiomics DNA Miniprep kit (Zymo Research) was used to extract DNA from 150µl specimens of seminal fluid, having been firstly digested with Proteinase K at 55°C for 30 minutes, following the manufacturer's instructions. Full-Length 16S Amplification (Hypervariable regions V1-9) was performed in preparation of the PacBio libraries, with SMRTBell® Library Preparation and Sequencing Procedure and Checklist (Pacific Biosciences) with 15ng extracted DNA input for the semen samples library preparation. Initially, PCR was conducted with KAPA HiFi HotStart ReadyMix (Roche), with 20 cycles in 25µl reactions following protocol cycling conditions. Amplified DNA was indexed using the barcoded F/R Universal Primers Plate 96 v2 - part number 100-466-100 (Pacific Biosciences) and PCR products were purified by using AMPure PB (Agencourt Bioscience), in accordance with PacBio's protocol.

Verification of amplicon sizes was carried out with a Fragment Analyser 5200 (Agilent Technologies) and Qubit 4.0 Fluorometer (ThermoFisher Scientific) using the High Sensitivity dsDNA assay. Pooling of barcoded amplicons was performed before the library construction process with the SMRTBell® Express Template Prep Kit 2.0 (Pacific Biosciences). Semen specimens were pooled in groups of 20, then sequenced on separate chips. A PacBio sequel (Pacific Biosciences) sequencing process was used with v3 sequencing chemistry in CCS collection mode for 20 h on a 1M v3 LR SMRT Cell. 16S used in this protocol is 1600 base pairs. Following this, CCS analysis was run prior to demultiplexing and fastq conversion. The CCS (Circular Consensus Sequence) analysis algorithm is applied to processes which require distinguishing of closely related molecules of DNA.

2.4.1 | Taxonomical classifications of microbiota

The semen microbiota of the subjects were categorised according to taxonomical classifications of phylum, order, class, family and genus.

2.5 | Statistical methods

2.5.1 | Bioinformatics for microbiome profiling

Prior to data analysis, the quality of the raw reads was checked using the package FastQC (Version 0.11.9). The next-generation microbiome bioinformatics platform QIIME2 (version q2cli version 2020.8.0) was used for analysing the amplicon data and further generation of Amplicon Sequence Variants (ASVs) was conducted from the raw amplicon data, using the algorithm DADA2 (version 1.19.2). The ASVs display advantages over the more traditional approach (OTUs) as they represent the exact amplicon sequences without coarse graining into OTUs and can resolve even single nucleotide base differences. DADA2-formatted training fasta files were derived from the Silva Project's version 128 release and used for taxonomy assignment. Three output files were generated using DADA2. These included the sequence feature count tables, the taxonomy identification file per ASV and a fasta file containing sequences of all ASVs. These files were exported in a phyloseq format for further analysis using QIIME2. These files along with the sample metadata files were used as input for different modules in the QIIME2 workflow.

Data produced by QIIME 2 exists as QIIME 2 artifacts, which contains data and metadata. The feature tables imported from DADA2 were filtered to retain the features, which were observed in at least two samples. A rooted phylogenetic tree was generated using the filtered feature table for downstream diversity analysis.

2.5.2 | Data analysis

This cross-sectional data sample was prepared in Microsoft Excel v16.67 (2022). ASV count data was aggregated first at the classification of phylum and sorted from highest to lowest frequency. This method was applied again for each of the remaining taxonomical ranks, namely order, class, family, and genus. The top ten organisms identified in each taxon for each pony, with the addition of an overall microbial profile were reported.

3 | RESULTS

The principal results of this study indicate specific microbiota predominate the semen of healthy miniature stallion ponies within each taxonomical ranking, with some inter-individual variations. The microbiota identified in the semen of the four equine subjects (Pony B, C, P, Z) are presented in Figures 3 to 7 by percentage abundance and taxonomical classifications of phylum, order, class, family, and genus. Overall, Firmicutes and Bacteroidetes phyla predominated (76%) followed by Proteobacteria (15%). Bacteroidales, Clostridiales and Cardiobacteriales predominated the microbial rank of order (86%). For class, Bacteroidia, Clostridia and Gammaproteobacteria (87%) predominated, and for family *Porphyromonadaceae*, *Family_XI* and *Cardiobacteriaceae* (62%) predominated. At the level of genus, 80% of the abundance comprised of seven genera – *Porphyromonas, Suttonella, Peptoniphilus*, *Fastidiosipila*, *Ezakiella*, *Petrimonas* and taxon undescribed (NA) that are yet to named.

3.1. Phylum

The nine phyla predominating the semen samples of four miniature stallions were Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, Spirochaete, Actinobacteria, Tenericutes, Synergistetes, Euglenozoa, and those less frequently represented were categorised as other (Fig.3). Overall, the phyla Bacteroidetes and Firmicutes were most represented in this small cohort, comprising 76%, and Proteobacteria was the third most abundant phylum, comprising another 15% of the phyla identified. Less than 1% of those detected were classified as taxon undescribed (NA)

Insert Figure 2

3.2 Order

Within the taxonomical classification of Order Bacteroidales, Clostridiales, Cardiobacteriales, Fusobacteriales, Bradymonadales, Corynebacteriales, Propionibacteriales, Spirochaetales, and Mycoplasmatales were most abundant (Fig. 4).

The three predominating Order were Bacteroidales, Clostridiales and Cardiobacteriales, accounting for 86% of the abundance distribution.

Insert Figure 3

3.3 Class

Within the taxonomic classification of microbial Class, Bacteroidia, Clostridia, Gammaproteobacteria, Fusobacteriia, Actinobacteria, Deltaproteobacteria, Spirochaetes, Mollicutes, Betaproteobacteria, and Alphaproteobacteria were the most abundant, with 87% comprised by Bacteroidia, Clostridia and Gammaproteobacteria (Fig.5).

INSERT Figure 4

3.4 Family

The most abundant microbial Families were *Porphyromonadaceae, Family_XI, Cardiobacteriaceae, Bacteroidales_BS11_gut_group, Ruminococcaceae, Fusobacteriaceae, Peptostreptococcaceae, Rikenellaceae,* and *Corynebacteriaceae*. Of these, *Porphyromonadaceae*, *Family_XI* (Family XI *Incertae sedis*) and *Cardiobacteriaceae* predominated, comprising 62% (Fig.6).

INSERT Figure 5

3.5 Genus

Within the taxonomical classification of microbial genera *Porphyromonas, Suttonella, Peptoniphilus, Fastidiosipila, Ezakiella, Petrimonas, Fusobacterium, Gallicola,* and *Finegoldia* were the most abundant genera identified in the semen. Of these, *Porphyromonas, Suttonella, Peptoniphilus, Fastidiosipila, Ezakiella, Petrimonas* and undescribed taxon (NA) predominated (Fig.7). In terms of microbiota represented in individual ponies, some differences were observed in the abundance of the family and genus level classification. For example, Pony B had greater abundance of *Fastidiosipila*

than the other ponies, Pony C had greater abundance of *Fusobacterium*, Pony P had greater abundance of *Suttonella and Pony Z* had greater abundance of *Ezakiella* genus.

INSERT Figure 6

4 | DISCUSSION

This exploratory study has described the microbial composition of the semen microbiome of healthy miniature stallion ponies. The overall findings indicate that common microbiota predominate the semen of healthy miniature stallion ponies within each taxonomical ranking, with some inter-individual variations. While the microbial phyla and families present in equine semen have previously been described (Al-Kass, Guo et al. 2020, Quiñones-Pérez, Hidalgo et al. 2021), here we have described additional taxonomical levels thus expanding this area of knowledge.

4.1. Semen microbial phyla and families

Quiñones-Pérez and colleagues, evaluated the phylum and families present in semen of 12 healthy Arab, Anglo-Arab and Andalusian stallions (aged 7-24 years) located at the Spanish Army's breeding centre in Seville, Spain (Quiñones-Pérez, Martínez et al. 2022). Like Quiñones-Pérez et al (Quiñones-Pérez, Hidalgo et al. 2021), we also found that Bacteroidetes and Firmicutes predominated the equine semen. However, Actinobacteria was the third most abundant phyla present in the semen of the Spanish stallions, whereas Proteobacteria was the third most abundant phyla in the miniature ponies' semen. These Gram negative bacteria play an important metabolic, energy-generating role in the equine intestinal microbiome (Li, Lan et al. 2022). Proteobacteria have also been reported as being more represented in the gastrointestinal tract of horses with equine grass sickness (Garber, Hastie et al. 2020) and colitis (Ayoub, Arroyo et al. 2022). Somewhat contrary to this, in a separate study, Proteobacteria colonies were reported to be less represented in the faeces of horses affected by diarrhoea (Li, Lan et al. 2022). The presence of Proteobacteria in the semen was not associated with these conditions in the

miniature ponies studied, which were of good health prior to and throughout the study period, with no reports of diarrhoea or other illness. Interestingly, many of the less abundant phyla including Fusobacteria, Spirochaetes, Synergestes, Tenericutes and Chloroflexi were present in similar amounts in both studies. Such similarities across demographic locations signal there may be a characteristic semen microbiome of stallions that is independent of environmental factors.

At the taxonomic classification of Family, Quiñones-Pérez et al identified 69 families in their stallions' semen, of which only 22 families were present in proportions greater than 1% and only nine were common to all samples (Quiñones-Pérez, Hidalgo et al. 2021). The microbial family represented in the Spanish stallion's semen were predominantly *Porphyromonadaceae*, which was similar in preponderance to our miniature ponies. Of interest to fertility research, *Porphyromonadaceae* is reported as a predominant family present in fertile stallion semen (Quiñones-Pérez, Hidalgo et al. 2021), where it is found to be more dominant than in human semen (Hou, Zhou et al. 2013). In contrast, the Spanish stallions had a much greater abundance of *Corynebacteriaceae* in the semen compared to the miniatures in this study, who had more *Family XI* present. *Corynebacteriaceae* family, which has also been associated with fertility in stallions but not highly represented in our cohort of miniature ponies.

Differences in phylum and family identification between the studies are not readily attributed to the chosen methods. In many regards, the methods in both studies were similar, involving similar collection processes during the spring season, to obtaining one semen specimen and using the 16S rRNA sequencing. One distinction which may account for the variations between the two studies was the Spanish study used the 16SrRNA hypervariable V3 region for microbial detection. However, in this study full length (hypervariable regions 1-9) 16S rRNA gene amplicon sequencing was used to detect microbial taxa. In addition, the bio-informatic pipelines employed by both studies were different, which may have influenced the respective findings. Differences between the Spanish study and the findings reported here are more likely to relate to breed, diet, geographical location and associated environmental factors (Theelen, Luiken et al. 2021). The stallions studied by Quiñones-Pérez et al in Spain were fed alfalfa hay and oats, rather

than lucerne hay, which may have produced a different semen microbial profile to that of our stallions. Although, it could be argued that dietary variations are more likely to be associated with differences observed in the gastrointestinal rather than extraintestinal microbial communities. The faecal microbiome of ponies and horses in good health is known to be impacted by environmental factors, such as air exposure, farm location (region and country), season and access to pasture (Theelen, Luiken et al. 2021), but these influences are yet to be demonstrated in relation to extraintestinal microbial communities such as semen.

4.2 Semen microbial genera

The aforementioned factors may also explain the differences in genera identified in equine semen by Al-Kass et al (Al-Kass, Guo et al. 2020) who examined the microbial composition of semen specimens from seven healthy stallions (aged 7 to 17 years) on a commercial stud in Stockholm, Sweden. Characterising the microbiota from one ejaculate per animal to genus level (Al-Kass, Guo et al. 2020), they identified almost twice as many genera in the semen in comparison to those discovered in our cohort, with only *Porphyromonas*, and *Peptoniphilus* being common to both cohorts. The four most common genera found in the Swedish study were *Porphyromonas, Corynebacterium, Finegoldia* and *Peptoniphilus followed by Mobiluncus, Chondromyces, Suttonella, Treponema, Actinetobacter, Campylobacter* and a group of "other". In comparison, the four most abundant genera in the semen of our miniature ponies were *Porphyromonas, Suttonella* and *Peptoniphilus* followed by *Fastidiosipila, Ezakiella, Petrimonas, Fusobacterium, Gallicola, Finegoldia* and "other". No *Ureaplasma, Spirochaeta, Prevotellaceae, Fibrobacter, Acinetobacter, Chondromyces,* or *Clostridium* were identified in our miniature ponies. Like the Al-Kass study, our miniature ponies displayed predominantly *Porphyromonas* genus, an organism also common to healthy human semen (Hou, Zhou et al. 2013, Liu, Osborne et al. 2014). Consistent with other equine studies, we did not find any *Lactobacillus* genus in our samples, a bacterium which has been associated with good sperm quality in humans (Baud, Pattaroni et al. 2019).

While the methodology and technology employed were similar in many regards i.e., specimens were collected in early spring from one ejaculate, and in the same regular collection routine and manner (using a Missouri AV without mare contact) as the ponies in our study. Although the stallions of the Swedish study were engaged in an artificial insemination program, the procedure for collecting and storing each aliquot of semen before its transport to the laboratory for genomic sequencing was identical to that employed by our Australian stallion handlers, hence one would not expect to find an effect of stallion management on semen microbiome composition to influence the results of these two studies.

Soil bacteria may contact the stallion's urogenital tract from its environment, considering that a miniature pony stallion's erect penis can more easily contact the ground than that of a full-sized stallion – thus giving rise to extrinsic contamination and differences in microbes detected. *Petrimonas* genus, found in the semen of our cohort of miniature pony stallions, resides in soil, where it is able to effectively degrade hazardous compounds (Zhao and Zhang 2021) to innocuous acetic acid and carbon dioxide (Grabowski, Tindall et al. 2005). *Fastidiosipila*, a Gram-positive anaerobe previously unrecognised in equine semen, identified in the miniature stallions, has also been identified in stored waste of landfill (Wang, Cao et al. 2017), hence may have been acquired from the soil by these ponies. These soil-dwelling organisms were not isolated by Al-Kass, Guo et al from their full-size breeds. *Gallicola* genus was also unique in the semen of the miniature stallions and about which very little exists in the literature, other than they are Gram-positive, strictly anaerobic cocci associated with dark fermentation of food waste (Shi, Zhang et al. 2021) and may be a faecal contaminant of ground water sourced from chicken manure (Naphtali, Mohiuddin et al. 2019).

4.3 The seminovaginal microbiome

The influence of the vaginal microbiome on the composition of the semen microbiome is important to consider. The stallion urethral and possibly semen microbiomes may be contaminated by the mare's vaginal microbiota following mating, and although there are no detailed descriptions of a shared complementary equine seminovaginal microbiome,

one study has found microbiome network links between semi-feral Welsh Mountain pony stallions and mares (Antwis, Lea et al. 2018). *Porphyromonas* is the most abundant genus in Arabian mares' vaginal microbiome (approx. 14-17%) and Bacteroidales is the highest abundance by order (approx. 31%) (Barba, Martínez-Boví et al. 2020). By comparison our stallions' semen contained 25.36% *Porphyromonas* and 42.5% *Bacteroidales,* although they had not had genital contact with mares, so perhaps the colonisation of the mare's vaginal microbiome arises from the stallion's penis, which harbours larger abundances of these genera. At phylum level the core species of the vaginal microbiome of the mare are comparative in ranking to those phyla found in miniature stallion semen (Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria) (Barba, Martínez-Boví et al. 2020). *Corynebacteriaceae* family are found in low abundance on equine skin (Costa, Stämpfli et al. 2016, Kaiser-Thom, Hilty et al. 2021) as well as in semen, which might suggest a degree of cross-contamination of this bacterium that occurs during specimen collection.

4.4 Limitations of this study

Only one breed of stallion was recruited for this study, with single semen samples collected and profiled for each miniature stallion thus reducing the generalizability of the findings. Due to the sample size detailed statistical analysis and comparison with various measures of sperm quality were not possible, thus associations between the composition of the semen microbiome and sperm quality could not be made. The detailed characteristics of the semen microbiome in miniature stallions may not be generalizable but provides preliminary data to inform such research.

5 | CONCLUSION

The microbial composition of the semen of the four miniature pony stallions studied varied between horses, with some bacteria predominating and common to all ponies, shared at various taxonomic classification ranks. The role of shared and unique microbiota in relation to the general health of the horse and fertility warrants further research. Future studies are encouraged to explore the richness, evenness or diversity of semen

microbiome profiles and the potential for these microbiome characteristics to influence sperm quality biomarkers. More specifically, the findings of this study can be used to inform future research exploring the relationship between semen microbial composition and fertility of horses.

CONFLICT OF INTEREST

The authors have declared no competing interests.

DECLARATION OF FUNDING

The authors received no funding support for this research project.

AUTHOR CONTRIBUTIONS

Study conceptualization and design, C.G.C., J.H., K.S., C.G.; methodology, J.H., K.S and C.G.C., project administration, Z.G., C.G.C; bioinformatics processing, ND; statistical analysis design and data analysis, KS; original draft manuscript preparation, C.G.C., manuscript revisions and editing, J.H., C.G., Z.G., K.S., project supervision, J.H., C.G., Z.G. All authors have read and agreed to the published version of the manuscript.

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ANIMAL WELFARE STATEMENT

All procedures undertaken in this study were approved by the University of Newcastle Animal Ethics Committee and adhere to the Australian code for the care and use of animals for scientific purposes (approval number A-2011-122). The Australian code for the

care and use of animals for scientific purposes adheres to the EU standards for the protection of animals used for scientific purposes.

DATA AVAILABILITY STATEMENT

All data collected for this study has been reported in this manuscript.

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APPENDIX B4: THE IMPACT OF PROBIOTICS AND PREBIOTICS ON THE COMPOSITION OF THE EQUINE FAECAL AND SEMINAL MICROBIOMES AND SPERM QUALITY - A PILOT STUDY

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Journal of Equine Veterinary Science

The impact of probiotics and prebiotics on the composition of the equine faecal and seminal microbiomes and sperm quality – a pilot study

--Manuscript Draft--

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Abstract:	Probiotic and prebiotic effects on equine semen and gastrointestinal microbiome composition and sperm quality are unknown. This study aimed to evaluate the effects of pre-, pro- or synbiotic supplementation on faecal and semen microbiome composition and sperm quality parameters of stallions. This Latin square crossover trial involved four miniature pony stallions receiving control diet only, or addition of a pro-, pre- or synbiotic formulation. Full-length 16S rRNA gene amplicon sequencing was used to measure diversity of semen and faecal microbiomes. Total sperm count, total motility, progressive motility, DNA integrity, lipid peroxidation and mitochondrial oxidative stress, biomarkers of sperm quality, were measured after each intervention. A general linear model was employed to analyse and compare microbiome diversity measures and sperm quality data across four time points. Shannon's diversity index (alpha-diversity), and evenness of semen and gastrointestinal microbiomes were significantly different ($p<0.001$). A trend towards significance was observed for

	prebiotic effects on the diversity indices of the GI microbiome (p= 0.073). No effects of treatments were observed on either semen microbiome or sperm quality. Prebiotic and probiotic supplements were well tolerated by healthy, fertile stallions with no negative effect on sperm quality parameters observed. This proof of concept provides preliminary data to inform future studies exploring the relationship between microbiomes and fertility.
Suggested Reviewers:	Marilena Bazzano University of Camerino marilena.bazzano@unicam.it Published review of nutritional supplementation for stallion fertility
	Carlota Quiñones-Pérez Cría Caballar de las Fuerzas Armadas, Córdoba, Espana lab.inv.aplic.simendef@mde.es Published research article on the semen microbiome of stallions
Opposed Reviewers:	

APPENDIX B5: INVESTIGATION OF THE RELATIONSHIP OF INTESTINAL MICROBIOME ALPHA DIVERSITY INDICES WITH PER CYCLE CONCEPTION RATES AND SERUM CYTOKINE TNF-α IN COMMERCIAL THOROUGHBRED STALLIONS

Prepared for journal submission

Running Title: microbiome, inflammation and fertility

Title: Investigation of the relationship of intestinal microbiome alpha diversity indices with per cycle conception rates and serum cytokine TNF-α in commercial Thoroughbred stallions

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Abstract

AIM: This retrospective observational study investigated the relationship between the faecal microbiota and inflammation with fertility in commercial Thoroughbred stallions. Key objectives of this research were firstly to investigate the association between intestinal microbiome measures of richness, evenness and diversity, stallion age, stud and certain dietary, nutritional and medical treatments on per cycle conception rate (PCCR); secondly to investigate the association of these microbiome indices and age on cytokine TNF-α.

METHODS: Retrospective stud management data were obtained from clinical histories of nineteen Australian Thoroughbred stallions to assess a range of factors thought to impact intestinal microbial composition and stallion fertility data (PCCR). A serum cytokine assay for TNF-α was performed and 16S rRNA faecal microbial profiling was conducted. Univariable linear regression models were used to test association of age, stud, and microbiome indices with PCCR and TNF-α. Microbiome data were aggregated at phylum level and grouped by stud.

RESULTS: A significant effect of stud was observed on PCCR, although no association was found between any microbial measure nor stallion age with PCCR and TNF-α concentration (the mean TNF-α of this cohort of stallions was found to be low). Stallions receiving carnitine supplementation demonstrated slightly better PCCRs. Faecal microbiome composition was consistent across samples in terms of microbial evenness, with *Firmicutes, Bacteroidetes* and *Verrucomicrobia* forming the large majority of phyla for each horse, however a significant variation in microbial richness was observed between stallions.

CONCLUSION: This study provides baseline data about the faecal microbiome, inflammatory cytokine, and fertility status of a cohort of Australian Thoroughbreds to potentially inform future research into stallion health and fertility.

CLINICAL RELEVANCE: Nutritional supplementation of stallions at stud with l-carnitine, antioxidants, pre- and probiotics may improve fertility outcomes.

Keywords: Equine, fertility, faecal microbiome, inflammation, nutrition, Thoroughbred

ABBREVIATIONS

16SrRNA	16S ribosomal ribonucleic acid
ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
IL-6	interleukin-6
µL	microlitre
N	number
NSAID	non-steroidal anti-inflammatory drugs
PCCR	per cycle conception rate
PCR	polymerase chain reaction
TNF-α	tumour necrosis factor alpha

INSERT GRAPHICAL ABSTRACT HERE

1 | INTRODUCTION

Thoroughbred stallion stud practices in Australia are informed by dedicated equine reproduction research, forming a collaboration between industry and academia, seeking to achieve optimal breeding outcomes (Griffin et al. 2019). Thoroughbred stallion health and breeding performance is of paramount importance to owners and stud managers who, each season, invest in feeding and management strategies that aim to maximise fertility outcomes through improving the horses' general health and sperm quality. Supplementing feed with antioxidants, carnitine and polyunsaturated fatty acids (Bazzano et al. 2021b; Contri et al. 2011) and the herb maca (*Lepidium meyenii*) (D'Anza et al. 2021) have all been shown to improve parameters of sperm quality. The addition of probiotic

and prebiotics to feed are widely used in general equine nutrition and are thought to influence the composition and function of the gastrointestinal microbiome (Cooke, Gibb, and Harnett 2021). However, the composition of the equine gastrointestinal microbiome and effects on systemic health, including associations with inflammation and fertility, have yet to be described.

Research to date has shown there are a range of factors that influence the microbial composition of the equine gastrointestinal tract (Garber, Hastie, and Murray 2020; Kauter et al. 2019). These factors include different feeding regimens (de Fombelle et al. 2001; Franzan et al. 2022), intense exercise (Górniak et al. 2021), and health status (Costa and Weese 2018), including metabolic disorders (Morrison et al. 2018). While the uterine, vaginal (Pugh 2018) and faecal (Salem et al. 2019) microbiomes of mares have been examined with respect to their impact upon pregnancy outcomes, there remains a gap in veterinary knowledge with respect to the potential influence that the faecal microbiome might have upon sperm quality, fertility and seasonal breeding success in Thoroughbred stallions. For feasibility reasons, faecal specimens are commonly analysed in equine studies to provide a valid representative proxy for the composition of the gastrointestinal microbiome (Gilroy et al. 2022).

Multiple factors affect semen quality and breeding success in Thoroughbred stallions, such as season (Crespo et al. 2020), number of mares covered (Lane et al. 2016b; Allen and Wilsher 2012), age (Aouane et al. 2019; Lane et al. 2016a), obesity (Gomez-Arango et al. 2016), illness or infection (Hughes and Loy 1975; Card 2011), medications (Fioratti et al. 2012; Aurich and Spergser 2007; Bedford and McDonnell 1999) and nutritional supplements (Contri et al. 2011), especially when administered to older stallions (Cappai et al. 2021). As in other species, the age of the mare has been shown to be more significantly associated with reproductive outcomes than the age of the stallion (Lane et al. 2016a). In regard to physical constitution, a moderate to "fleshy" body condition of around 6-7 on the US body condition score chart (University 2023) favours breeding stamina. Equine sperm quality may be improved via supplementation with specific nutrients (Ellis 2006) and endogenous antioxidants (Gibb, Blanco-Prieto, and Bucci 2021) and adversely affected by local inflammation (Scheeren et al. 2020; Sancler-Silva et al.

2020). The relationship between inflammation and fertility has been demonstrated in men, with an increased expression of interleukin 6 (IL-6) receptor activity (found at the midpiece of sperm) in sub fertile patients with an associated lower sperm count (Djourabchi Borojerdi et al. 2020). As cytokine receptors have been identified on the equine spermatozoon (Machado-Neves 2016), it is reasonable to hypothesize that the same relationship may exist in the equine species. However, linking the gut microbiota with systemic inflammation and fertility is relatively new. Dietary prebiotic intake has been shown to improve the composition of the human intestinal microbiome and increase serum anti-inflammatory cytokine expression, whilst suppressing expression of pro-inflammatory cytokines (Shokryazdan et al. 2017). Further, intestinal microbiota can influence the permeability of the blood-testis barrier, which may further impact upon spermatogenesis (Al-Asmakh and Hedin 2015). In consideration of these factors, it is plausible that low-grade inflammation caused by disruption of the composition of the intestinal microbiome (dysbiosis) and injury to testicular germ cells is associated with reduced sperm count, morphology, and function.

Therefore, the aim of this observational study was to investigate factors associated with per cycle conception rates (PCCR) and the cytokine, tumour necrosis factor (TNF-α), in commercial Thoroughbred stallions. The specific objectives of the study were to (1) investigate any associations between inflammation (TNF-α) and three microbiome summary measures (richness, evenness, and diversity) (2) between fertility outcomes (PCCR) and stud management by antibiotic and anti-inflammatory drugs, prebiotics, probiotics and carnitine supplements and (3) to describe the relative abundance of phyla in faecal specimens.

2 | MATERIALS AND METHODS

ANIMAL WELFARE STATEMENT

All procedures undertaken in this study were reviewed by the University of Newcastle Animal Ethics Committee and approved under the Australian code for the care and use of

animals for scientific purposes (approval number A-2011-122). The Australian code for the care and use of animals for scientific purposes adheres to the EU standards for the protection of animals used for scientific purposes. Ethics approval for this study: Animal Research Authority No. A-2011-122

2.1 Study design

This observational study collected semen, serum, and faecal specimens, in addition to retrospective data from the clinical histories of nineteen Thoroughbred stallions to investigate factors associated with PCCR and TNF-α The study is reported in accordance with the STROBE checklist for reporting observation studies (STROBE 2022).

2.2 Animals

The stallions selected for this study were 19 commercial Thoroughbred stallions housed on three stud farms located in the Upper Hunter Valley of New South Wales, Australia (Stud 1: N = 6, Stud 2: N = 6, and Stud 3: N = 7 stallions). All stallions are engaged in annual breeding programs and considered to be in excellent general health. There were no 'shuttle stallions' (stallions which travel between the northern and southern hemispheres to cover both breeding seasons) involved in this study. Ethics approval was secured for this study: Animal Research Authority No. A-2011-122

2.3 Stallion management data collection

Stud managers at each of the three participating studs were interviewed over two days in May 2021. Criteria assessed for each stallion included age, nutritional supplements (prebiotics, probiotics, synbiotics, carnitine) and treatments (antibiotics, anti-inflammatories). A schedule of the nutritional supplementation regimens at all three stud farms is attached in Appendix 3. All horses were given additional lucerne hay and grazed on similar pasture type and quality (rye, Timothy and fescue grasses).

Breeding outcomes were declared by the relevant stud managers for August – December 2020 season, specifically November 16 - December 18, 2020, as weekly PCCR. The PCCR is calculated as the percentage of the pregnant mares of the total number of mares covered during the period in question (4-5 weeks).

2.4 Collection of faecal and blood samples

Blood and faecal samples were collected from the jugular veins of 19 Thoroughbred stallions between December 16-18, 2020 and stored at -80 °C until analyses.

2.5 Sample preparation

2.5.1 Preparation of faecal samples

Faecal balls were collected within 1 hour of defecation from each of the stallion's own paddocks for sampling. Intact faecal balls were opened in the on-site laboratory using gloves and fresh green, moist, core contents, which had not contacted pasture, were extracted using forceps. Three grams (3 g) of this core faecal material was placed into 1.5 mL plastic tubes containing DNA Microshield* and stored at ambient temperature for transport to the laboratory within 4-6 h. Faecal samples were then snap frozen in liquid nitrogen (LN) and stored at -80 °C.

(**1:3 with liquids (200 µL semen + 600 µL Shield) and 1:9 with solids (1 g faeces + 9 mL* DNA Microshield)).

2.5.2 Preparation of serum samples

Approximately 5 mL of venous blood was collected from each stallion into a serum Vacutainer (Becton Dickinson) and allowed to sit at room temperature (RT) for 30 min to allow a clot to form. The tubes were then centrifuged at 1000 x g for 10 min, the serum aspirated, aliquoted into cryovials, snap frozen in LN, and stored at -80 °C until analysis.

2.6 Faecal microbial genomic processing

PacBio DNA extractions, library preparation and sequencing of faecal samples were conducted at Central Analytical Research Facility (CARF), Queensland University of Technology, Brisbane. DNA was extracted using the ZymoBiomics DNA Miniprep kit (Zymo Research) with 150 mg sample input for faeces following the protocol for faecal extraction outlined in Appendix 1. PacBio libraries were prepared following the Full-Length 16S Amplification, SMRTBell® Library Preparation and Sequencing Procedure and Checklist (Pacific Biosciences) with 2.5 ng extracted DNA input into the library preparation for samples. Initial PCR was carried out with KAPA HiFi HotStart ReadyMix (Roche), with 20 cycles in 25 µL reactions following protocol cycling conditions. Amplified DNA was indexed using the barcoded F/R Universal Primers Plate 96 v2 (Pacific Biosciences) and PCR products purified using AMPure PB (Agencourt Bioscience) according to PacBio's protocol. Amplicon sizes were verified with a Fragment Analyser 5200 (Agilent Technologies) and Qubit 4.0 Fluorometer (ThermoFisher Scientific) using the High Sensitivity dsDNA assay. Barcoded amplicons were pooled before library construction with the SMRTBell® Express Template Prep Kit 2.0 (Pacific Biosciences). Samples were pooled, with 20 samples per pool, and sequenced on separate chips. Sequencing was carried out on a PacBio sequel (Pacific Biosciences) with v3 sequencing chemistry in CCS collection mode for 20 hours on a 1M v3 LR SMRT Cell. CCS analysis was then run before demultiplexing and fastq conversion.

2.7 Analysis of serum cytokine levels

Serum levels of the cytokine TNF-α was measured using the Milliplex MAP Equine Cytokine Magnetic Bead Panel Immunology Multiplex Assay kit (Merck Millipore; catalogue no. EQCYTMAG-93K), MAGPIX instrument system, and xPONENT software, in accordance with manufacturer instructions (see Appendix 2). Median fluorescent intensity data were processed using Belysa analysis software (version 1.1.0, Merck); 4-parameter logistic regression was used to generate standard curves, from which the sample cytokine concentrations were determined.

2.8 Statistical analysis

2.8.1 Bioinformatics for microbiome profiling

The bioinformatics analysis for this work was performed on the HPC server 'Artemis' at the University of Sydney.

The quality of the raw reads were checked using the package FastQC (Version 0.11.9) (Wingett and Andrews 2018). The next-generation microbiome bioinformatics platform QIIME2 (version q2cli version 2020.8.0 https://qiime2.org/) (Bolyen et al. 2019) was used for analysing the amplicon data. The first step involved generation of Amplicon Sequence Variants (ASVs) from the raw amplicon data, using the algorithm DADA2 (Callahan et al. 2016). Since the available version of QIIME2 did not include the DADA2 capability to handle PacBio reads, we used the DADA2 package externally (version 1.19.2). The ASVs display advantages over the more traditional approach of operational taxonomical units as they represent the exact amplicon sequences without coarse graining into OTUs and can resolve even single nucleotide base differences. DADA2-formatted training fasta files (Callahan 2017) were derived from the Silva Project's version 128 release and used for taxonomy assignment. Three output files were generated using DADA2. These included the sequence feature count tables, the taxonomy identification file per ASV and a fasta file containing sequences of all ASVs. These files were exported in a phyloseq (McMurdie and Holmes 2013) format for further analysis using QIIME2. These files along with the sample metadata files were used as input for different modules in the QIIME2 workflow.

Data produced by QIIME 2 exists as QIIME 2 artifacts which contains data and metadata. The feature tables imported from DADA2 were filtered to retain the features which were observed in at least two samples. A rooted phylogenetic tree was generated using the filtered feature table for downstream diversity analysis. The next steps in the QIIME2 analysis involved computation of alpha diversity indices (richness, evenness, and Shannon's diversity).

2.8.2 Statistical methods

During data processing, binary variables were created for 'any oral anti-inflammatory drug use' (stallion received at least one dose of phenylbutazone, flunixin meglumine or pentoxifylline), 'any antibiotic drug use' (stallion received at least one dose of penicillin, gentamicin, sulfadimidine, trimethoprim or bromhexine hydrochloride) and 'pre-/pro- or synbiotic use' (stallion received at least one of grain-free fibre, rice bran, linseed, a multi-species probiotic/yeast mixture or a yeast culture).

The distribution of each variable in the data set was checked using summary statistics and frequency tables for numeric and categorical variables, respectively. Two separate outcome measures were considered in this study: PCCR and TNF-α. The relationship of ten predictor variables (demographics (age, stud), treatments (anti-inflammatory use, antibiotic use, pre-/pro-/synbiotics and carnitine), TNF-α concentration and three faecal microbiome summary measures) with the outcome PCCR was assessed. Similarly, the relationships of the three microbiome summary measures (species richness - faith_pd, evenness and Shannon's diversity) and age with the TNF-α outcome measure were described. Normality testing for the microbiome abundance data was carried out using a Kruskal-Wallis test for non-parametric analysis of the groups. For numeric predictors, the relationship with the numeric outcome was assessed with a scatter plot. For categorical predictors, the outcome was summarised by category and plotted using dot plots and side-by-side boxplots.

Simple linear regression models were used to formally test the association of numeric predictor variables with the outcome variables. The model assumptions of linearity, normally distributed residuals and equality of variance were assessed with scatter plots of the raw data as well as of predicted values versus residuals and plotting of the residuals. Outliers and influential values were assessed using Cook's distance and leverage. A sensitivity analysis for an influential value with large residual was performed by excluding the data point from analysis and comparing the results to analysis of the complete data set.

The associations of binary and nominal predictor variables with the outcome PCCR were tested using univariable linear models.

ASV count data were aggregated at the phyla level and sorted from highest to lowest frequency. The top 10 phyla were plotted and phyla with low frequencies were combined to an "Other" category for plotting. The data was prepared in Microsoft Excel (2021) and all statistical analyses were conducted in SPSS software (v28, 2021).

3 | RESULTS

Overall, the average PCCR for the 19 stallions in this study was 54.6 ± 7.1%. A significant effect of stud was observed on PCCR, although no association was found between any microbial measure nor age with PCCR and TNF-α levels. Ages of the stallions ranged from four to 22 years, with an average age of 10.5 years. The average TNF-α (18.4 pg/mL) was within the normal range for this cytokine (4 - 4,000 pg/mL, Milliplex multiplex cytokine assays). With respect to the faecal microbiome measures evaluated in this study, microbial evenness was consistent across samples (0.92 with SD ± 0.05), while microbial richness (faith_pd) varied more greatly between stallions (344.5 ± 49.0). The Shannon index for these samples ranged from 9.2 to 11.5 with a mean of 10.7.

The average TNF-α of this cohort of stallions was low (normal) at 18.4 pg/mL, indicating no inflammatory conditions were present in these subjects. The distribution of TNF-α was positively skewed by one elevated result, while the other numeric variables in the study had an approximately normal distribution. The distributions of the numeric variables age, TNF-α, evenness, faith_pd (richness) and Shannon's diversity are presented in Table 1.

Insert Table 1

3.1 Per cycle conception rate

The distribution of PCCR overall and by categories of the predictors is shown in Table 2. Table 3 presents the results of the univariable regression analyses for categorical predictors of PCCR. Stud was significantly associated with PCCR, with stud 3 having a significantly higher predicted PCCR compared to stud 1 (59.9% (95% CI: 55.5%-64.3%) versus 48.5% (95% CI: 43.8%-53.3%); p = 0.007). On average the five stallions which were supplemented with carnitine had an almost 5% higher PCCR than those without carnitine

supplementation. Four of those five stallions were housed at stud 3, which had the highest average PCCR. No impact of antibiotic or anti-inflammatory drug was demonstrated on PCCR, nor was any effect seen with pre/pro/synbiotic supplementation on this measure.

Insert Table 2

The results of univariable linear model regression analyses for numeric predictors of PCCR are shown in Table 4 and show there was no effect found for TNF-α, stallion age and three microbiome measures on the fertility outcome, PCCR.

Insert Table 3

Insert Table 4

3.2 TNF-α analyses

The results of univariable linear model regression analyses for TNF-α are presented in Table 5. There was no evidence of an effect of stallion age and three microbiome measures on serum levels of the inflammatory cytokine, TNF-α. The predictors only explained between 0-5% of variation in the TNF-α outcome variable (R^2, Table 5).
One stallion had an extreme outlier value for TNF-α, which resulted in a skewed distribution and large residual value with high influence on model fitting. A sensitivity analysis excluding this value revealed no change to the interpretation compared to the models using the whole dataset, which are presented here.

Insert Table 5

3.3 | Faecal microbial composition

The results of the faecal microbiome profiling are presented to phylum level for each stallion and compared between studs and between individuals. Overall, microbial profiles were similar across studs and individuals. *Firmicutes* and *Bacteroidetes* comprised the bulk of phyla for each horse (approximately 60-70% of profile), along with *Verrucomicrobia* up to approximately 90% of composition. Only one horse (J) showed a preponderance of *Proteobacteria* in its profile.

Insert Figure 1: Relative abundance of phyla in faecal samples of stallions (A-S) by stud. "Other" includes *Actinobacteria, Lentisphaerae*, *Saccharibacteria*, *Synergistetes*,

Euryarchaeota, *Chloroflexi*, *Elusimicrobia*, *Euglenozoa*, *Armatimonadetes* and *SR1_(Absconditabacteria)*

4 | DISCUSSION

To our knowledge, this is the first study to investigate the association between the composition of the faecal microbiomes of Thoroughbred stallions and PCCR. In addition, we examined the associations between nutrition, dietary supplements, medical treatments, and serum inflammatory cytokine levels with PCCR. Except for differences in PCCR by stud, we did not identify any other statistically significant associations with the other factors measured, however a small positive effect of carnitine supplementation observed on PCCR may have clinical relevance to stallion breeding.

Factors impacting sperm quality and functionality are of primary concern to stallion managers, keen to achieve peak fertility for their horses each breeding season. Human research is beginning to explore the role of the intestinal microbiome on sperm quality (Wang et al. 2022). Moreover, correcting intestinal dysbiosis to attenuate pro-inflammatory states in infertile men by supplementation with probiotics has resulted in improvement in several sperm quality parameters (Helli et al. 2020).

In this study we characterised the faecal microbial profiles of 19 commercial stallions, collected details of the specialist management practices and nutritional supplementations supplied, and the fertility outcomes for the spring breeding season of 2020. These valuable horses were stabled in prime conditions, grazed on ideal pastures, and exercised or rested daily according to their specific requirements for the number of covers they were required to perform; they were fully vaccinated according to Australian veterinary standards and treated immediately for any health concerns by specialist equine veterinarians. For these reasons it could be expected that their general health, digestion, and fitness to breed were optimal during this season.

4.1 Per cycle conception rate

Our results demonstrated that the range of PCCR for the three studs varied, with an average of 54.6% for all 19 stallions. Four stallions on Stud 3 received l-carnitine supplementation during the study period and one stallion on Stud 2 received this amino acid supplement, comprising 26% of the cohort. The results of this study provided some effect of l-carnitine on PCCR ($p = 0.17$). Carnitine, an amino acid which is either synthesised in the liver or kidneys or nutritionally sourced, may be a conditionally essential amino acid under certain conditions (Borum and Bennett 1986) and is more concentrated in spermatozoa than any other mammalian cell (Gibb and Aitken 2016; Jeulin and Lewin 1996) . Furthermore, according to *in vitro* research, l-carnitine is considered a "prosurvival" nutrient for stallion sperm, increasing sperm motility and ATP levels while protecting sperm mitochondria against oxidation by reactive oxidant species (Gibb et al. 2015), generated by oxidative phosphorylation resulting from high level mitochondrial activity (Griffin et al. 2019; Gibb, Lambourne, and Aitken 2014).

Successful conception not only depends upon a healthy stallion libido, breeding performance and sperm quality (Hurtgen 1992), but also the mare's age (Rizzo et al. 2019) reproductive fitness and number of matings (BRÜCK, ANDERSON, and HYLAND 1993). Nath et al demonstrated that the highest fertility outcomes were with the youngest mares (age 2-8 years), as older mares tend to conceive less efficiently and experience more pregnancy loss (Nath, Anderson, and McKinnon 2010). Mares covered only once during oestrus had higher conception rates in comparison to mares mated multiple times over several oestrus periods in one season (BRÜCK, ANDERSON, and HYLAND 1993). No clinical data were collected pertaining to the breeding fitness of the mares covered during our study, hence it was not possible to evaluate any negative effect on PCCR for these stallions which might indeed be attributed to the mares. Also, PCCR is not a clear predictor of fertility outcomes for stallions, as those with a PCCR as low as 45% have achieved 90% seasonal pregnancy rates (MORRIS and ALLEN 2002a). A study conducted during the 1998 breeding season with 1393 Thoroughbred mares covered by 41 stallions on 22 stud farms in Newmarket, United Kingdom, produced a wide range of PCCR results;

each stallion which covered more than 10 mares achieved pregnancy rates as low as 37% up to as high as 90% (Morris and Allen 2002b).

Stud selection of stallions for breeding capacity may have affected our results, as these horses are primarily selected and bred for their athletic performance and track winnings rather than their fertility status. By comparison, PCCRs are significantly higher amongst domestic livestock animals, for example rams (80-90%) and boars (85-90%) as reproductive efficiency is paramount for these food-producing species (Menzies 1999; Colenbrander, Feitsma, and Grooten 1993). The environmental conditions to which the stallions were exposed at each stud were optimal in terms of pasture quality, paddock area and stabling accommodation.

4.2 Inflammatory cytokines

In the human and mouse, a possible connection exists between intestinal microbiome disruption, systemic inflammation, testicular injury and impaired fertility (Wang and Xie 2022). In the present study the cytokine TNF-α was chosen for measurement as a biomarker of inflammation. All TNF-α results were within the normal range for the specific cytokine kit used, indicating no abnormal levels of inflammation were present in any of the stallions at time of specimen collection.

Our results showed no correlation between TNF-α and the parameters of age, or faecal microbial richness, evenness, or diversity. This result was not entirely unexpected, as the cohort of stallions we studied was in excellent health when specimens were taken (McFarlane and Holbrook 2008; Singh et al. 2015). Animals exhibiting tissue inflammation, for example in lung, may produce elevated serum TNF-α, which can be modulated by the beneficial influence of eubiotic intestinal flora regulating barrier function (Tang et al. 2021). Bacteria which assist in maintaining a healthy intestinal biofilm, such as *Akkermansia muciniphila,* have been shown to downregulate proinflammatory cytokines in human patients with ulcerative colitis (Qu et al. 2021). Horse O returned the highest TNF-α result among the cohort - while within the refence range, but also one of the highest PCCRs, hence we could not conclude that any level of inflammation present in this

stallion affected his fertility. Notably, this stallion had been supplemented with omega-3 oils, vitamin E, L-carnitine and synbiotics (prebiotic Diamond V®XPC yeast); this specialised breeding season nutritional regime may reduce inflammation and improve sperm quality and microbiome composition (Bazzano et al. 2021a; Coverdale 2016). It is important also to note that 68% of the stallions in this study cohort were treated with anti-inflammatory drugs during the relevant prior breeding season, mostly "bute" (phenylbutazone) for managing musculoskeletal injuries and pain, which may have also suppressed inflammatory cytokine production.

4.3 Intestinal/faecal microbiome

Colonies of faecal microbiota can be considered a proxy for the gastrointestinal microbiome, hence our use of the term "faecal microbiome"; although not specifically belonging to an anatomical region, faecal microbiota correlate with intestinal flora. Also, although faecal ball sampling may not be considered as accurate a methodology of specimen collection as faecal grabs, the care taken to extract core contents from each faecal ball ensured good quality specimens, particularly as the resultant microbial profiling did not revealed phyla which could be considered environmental contaminants.

The average Shannon index of diversity was 10.7 for the 19 horses in the present study, which is high in comparison to average human faecal diversity (1.66) (Li et al. 2012). Consistent with other similar studies, the faecal microbial profiles of these stallions showed the highest abundance of the phyla *Firmicutes* and *Bacteroidetes* (Shepherd et al. 2012; Arnold et al. 2020; Dougal et al. 2013), followed by *Verrucomicrobia* (Kauter et al. 2019)*, Spirochaetae, Proteobacteria* (Costa et al. 2015), *Tenericutes, Fibrobacteres* (Slater et al. 2021) and *Cyanobacteria* (Shepherd et al. 2012). Another ten bacterial phyla were categorised as "Other" and were in the lowest preponderance. Our stallion cohort carried higher abundances of *Firmicutes* and *Bacteroidetes*, a microbiome composition which is considered healthy, which may be explained by their nutritional management. Diets rich in protein increase intestinal *Firmicutes* colonisation, while a high intake of plant fibre increases *Bacteroidetes* numbers to enable fermentation of cellulose and xylan (Dougal et al. 2014). These nutrient categories were well supplied to the stallions in this study.

Although the intestinal microbiome profiles of equids is impacted by breed (Edwards et al. 2020), feed supplementation and weather (Salem et al. 2018), domestication (Metcalf et al. 2017), medications and illness (Kauter et al. 2019), the core bacterial taxa are thought to be common to all equids. A survey of the core faecal bacterial phyla in a similar cohort of six Irish Thoroughbred racehorses was conducted by employing 16S rRNA gene (V4 region) amplicon pyrosequencing (O'Donnell et al. 2013). The key findings were a preponderance of *Firmicutes* and *Bacteroidetes,* which comprised about three-quarters of the reads. *Proteobacteria, Verrucomicrobia, Actinobacteria, Euryarchaeota, Fibrobacteres, Spirochaetes* and another group comprised the remaining one-quarter of phyla discovered. These findings correlate with our results of faecal microbiota in stallions, for which 60-78% of their phyla were combined *Firmicutes* and *Bacteroidetes.* The Irish Thoroughbred cohort included three geldings, two fillies and a mare, but no stallions, with ages ranging less than one month to four years, whereas our stallions ranged in age from four to 18 years. Perhaps immaturity explains the difference in median Shannon Index (8.58) and evenness (0.75) of these young horses in comparison to our study findings of Shannon Index (10.9) and evenness (0.94), in horses with a median age of eight.

The bacterial phyla *Actinobacteria, Euryarchaeota* were not represented amongst the core species of our cohort, but *Tenericutes* and *Cyanobacteria* were. A relative abundance of *Tenericutes* has been associated with obesity in a study of Welsh mountain pony mares, with a reduction of both FIRMICUTES and *Tenericutes* phyla observed following dietary restriction of these horses (Morrison et al. 2020). The body condition of our study stallions was relatively heavy, scoring 6-7 on the Body Condition Score Chart (University 2023), which may explain the presence of *Tenericutes* amongst their core phyla.

The presence of *Cyanobacteria* in stallions who foraged on lush pastures suggests that these bacteria may transit through the equine intestinal microbiome as passengers from ingested plants, rather than being core host species. *Cyanobacteria* are obligate phototrophs which have harnessed a circadian clock mechanism (Williams 2007), known to possess circadian oscillators (Mihalcescu, Hsing, and Leibler 2004). This oxygenic phylum performs a role in plant photosynthesis and corresponds to chlorophyll

sequences observed in foraging horses, however no role has been identified for *Cyanobacteria* in equids (Shepherd et al. 2012).

In our study, the distinctive profile of one stallion (Horse J) had high *Proteobacteria* and low *Verrucomicrobia*. This horse was affected by chronic lameness, for which it was regularly administered a non-steroidal anti-inflammatory drug (phenylbutazone), plus intramuscular dexamethasone injections, for management of its pain, however NSAID are known to increase colonies of *Verrucomicrobia* in the equine intestine, as does diarrhoea (Li et al. 2022) Hence the microbiome profile for Horse J does not indicate an unhealthy stallion. Broad-spectrum antibiotics are also known to promote faecal populations of *Verrucomicrobia* (Dubourg et al. 2013), however four stallions in this study were administered antibiotics prior to faecal sample collection and none showed a predominance of *Verrucomicrobia* in their microbiome profiles.

Antibiotics were administered to 21% (four) of the study stallions in the period prior to faecal specimen collection and 63% (12) of the stallions received pre- and/or probiotic supplementation in their feed, which may have recovered any loss of microbial diversity following antibiotic administration.

4.4 Study limitations

Our PCCR results were limited to one breeding season in one region of Australia, thus research conducted over more seasons in different geographical locations may produce varying results. We were unable to assess any seasonal variation in either cytokines or microbiome measures due to collection of specimens at one single timepoint. With respect to TNF-α and the microbiome results, the subject cohort were healthy Thoroughbred stallions in peak condition with no inflammatory diseases or intestinal disorders. Faecal microbiome measures did not appear to influence fertility in this study. Analysing the semen microbiomes of this cohort may have produced evidence of a PCCR effect in relation to microbial composition of this more proximate site, rather than the bacteria present in the rectum. Also, the small sample size of this study did not allow for multivariable analyses to be performed on the data, for example carnitine

supplementation was given to only five stallions; this could also have created a confounding effect for stud variations of PCCR, so further conclusions were unable to be drawn about the value of carnitine for improving stallion fertility, with this small cohort during one breeding season. Although the sample size for this study was small, we utilised a homogenous group of healthy stallions with proven fertility, which has provided category-specific data on the equine microbiome.

5 | CONCLUSIONS

In this study of 19 Thoroughbred stallions, in peak health and condition, no relationship was found between per cycle conception rate (PCCR) and measures of faecal microbial richness, evenness or diversity, nor to circulating TNF-α levels. Similarly, PCCR was not affected by age, nor inflammatory cytokine status. While PCCR varied between studs, we did not observe any specific effect due to the stud management practices, however the observation that the nutritional supplement carnitine showed some evidence for an effect in increasing PCCR requires further research attention. Given the limitations outlined in this study, further research is encouraged to clarify the relationship between the intestinal microbiome, systemic inflammation, and fertility, particularly in sub fertile stallions, as understanding of any impact which these factors may have on equine sperm quality could potentially inform future animal husbandry practices.

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CONFLICT OF INTEREST

The authors have declared no competing interests

AUTHOR CONTRIBUTIONS

CGC, ZG, CG, and JH designed the study. CGC and ZG collected, co-ordinated shipping and evaluation of blood, semen, and faecal samples. AC conducted the cytokine analysis. KS and ND contributed the bioinformatics and statistical analysis. CGC drafted the manuscript. All authors contributed to the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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6 | REFERENCES

Al-Asmakh, Maha, and Lars Hedin. 2015. 'Microbiota and the control of blood-tissue barriers', *Tissue Barriers*, 3: e1039691.

Allen, W. R., and S. Wilsher. 2012. 'The influence of mare numbers, ejaculation frequency and month on the fertility of Thoroughbred stallions', *Equine Vet J*, 44: 535-41.

Aouane, N., A. Nasri, M. A. A. Bekara, A. K. Metref, and R. Kaidi. 2019. 'Retrospective study of the reproductive performance of Barb and Thoroughbred stallions in Algeria', *Vet World*, 12: 1132-39.

Arnold, C. E., A. Isaiah, R. Pilla, J. Lidbury, J. S. Coverdale, T. R. Callaway, S. D. Lawhon, J. Steiner, and J. S. Suchodolski. 2020. 'The cecal and fecal microbiomes and metabolomes of horses before and after metronidazole administration', *PLoS One*, 15: e0232905.

Aurich, C., and J. Spergser. 2007. 'Influence of bacteria and gentamicin on cooled-stored stallion spermatozoa', *Theriogenology*, 67: 912-8.

Bazzano, M., F. Laus, A. Spaterna, and A. Marchegiani. 2021a. 'Use of nutraceuticals in the stallion: Effects on semen quality and preservation', *Reprod Domest Anim*, 56: 951-57.

Bazzano, Marilena, Fulvio Laus, Andrea Spaterna, and Andrea Marchegiani. 2021b. 'Use of nutraceuticals in the stallion: Effects on semen quality and preservation', *Reproduction in domestic animals*, 56: 951-57.

Bedford, S. J., and S. M. McDonnell. 1999. 'Measurements of reproductive function in stallions treated with trimethoprim-sulfamethoxazole and pyrimethamine', *J Am Vet Med Assoc*, 215: 1317-9.

Bolyen, E., J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet, G. A. Al-Ghalith, H. Alexander, E. J. Alm, M. Arumugam, F. Asnicar, Y. Bai, J. E. Bisanz, K. Bittinger, A. Brejnrod, C. J. Brislawn, C. T. Brown, B. J. Callahan, A. M. Caraballo-Rodriguez, J. Chase, E. K. Cope, R. Da Silva, C. Diener, P. C. Dorrestein, G. M. Douglas, D. M. Durall, C. Duvallet, C. F. Edwardson, M. Ernst, M. Estaki, J. Fouquier, J. M. Gauglitz,

S. M. Gibbons, D. L. Gibson, A. Gonzalez, K. Gorlick, J. Guo, B. Hillmann, S. Holmes, H. Holste, C. Huttenhower, G. A. Huttley, S. Janssen, A. K. Jarmusch, L. Jiang, B. D. Kaehler, K. B. Kang, C. R. Keefe, P. Keim, S. T. Kelley, D. Knights, I. Koester, T. Kosciolek, J. Kreps, M. G. I. Langille, J. Lee, R. Ley, Y. X. Liu, E. Loftfield, C. Lozupone, M. Maher, C. Marotz, B. D. Martin, D. McDonald, L. J. McIver, A. V. Melnik, J. L. Metcalf, S. C. Morgan, J. T. Morton, A. T. Naimey, J. A. Navas-Molina, L. F. Nothias, S. B. Orchanian, T. Pearson, S. L. Peoples, D. Petras, M. L. Preuss, E. Pruesse, L. B. Rasmussen, A. Rivers, M. S. Robeson, 2nd, P. Rosenthal, N. Segata, M. Shaffer, A. Shiffer, R. Sinha, S. J. Song, J. R. Spear, A. D. Swafford, L. R. Thompson, P. J. Torres, P. Trinh, A. Tripathi, P. J. Turnbaugh, S. Ul-Hasan, J. J. J. van der Hooft, F. Vargas, Y. Vazquez-Baeza, E. Vogtmann, M. von Hippel, W. Walters, Y. Wan, M. Wang, J. Warren, K. C. Weber, C. H. D. Williamson, A. D. Willis, Z. Z. Xu, J. R. Zaneveld, Y. Zhang, Q. Zhu, R. Knight, and J. G. Caporaso. 2019. 'Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2', *Nat Biotechnol*, 37: 852-57.

Borum, P. R., and S. G. Bennett. 1986. 'Carnitine as an essential nutrient', *Journal of the American College of Nutrition*, 5: 177-82.

Brück, I, GA Anderson, and JH Hyland, 1993, 'Reproductive performance of Thoroughbred mares on six commercial stud farms', *Australian veterinary journal*, 70: 299-303.

Callahan, Benjamin. 2017. 'Silva taxonomic training data formatted for DADA2 (Silva version 128)', *Zenodo*.

Callahan, Benjamin J., Paul J. McMurdie, Michael J. Rosen, Andrew W. Han, Amy Jo A. Johnson, and Susan P. Holmes. 2016. 'DADA2: High-resolution sample inference from Illumina amplicon data', *Nature methods*, 13: 581-83.

Cappai, M. G., A. Taras, I. Cossu, R. Cherchi, C. Dimauro, F. Accioni, G. Boatto, M. Deroma, E. Spanu, D. Gatta, C. Dall'Aglio, and W. Pinna. 2021. 'Effects of Dietary Zn/Se and α-Tocopherol Supplementation on Metabolic Milieu, Haemogram and Semen Traits of Breeding Stallions', *Biol Trace Elem Res*, 199: 3287-96.

Card, Claire. 2011. 'Reproductive problems of stallions (Proceedings)'.

Colenbrander, B., H. Feitsma, and H. J. Grooten. 1993. 'Optimizing semen production for artificial insemination in swine', *J Reprod Fertil Suppl*, 48: 207-15.

Contri, A., I. De Amicis, A. Molinari, M. Faustini, A. Gramenzi, D. Robbe, and A. Carluccio. 2011. 'Effect of dietary antioxidant supplementation on fresh semen quality in stallion', *Theriogenology*, 75: 1319-26.

Cooke, C. G., Z. Gibb, and J. E. Harnett. 2021. 'The Safety, Tolerability and Efficacy of Probiotic Bacteria for Equine Use', *J Equine Vet Sci*, 99: 103407.

Costa, M. C., G. Silva, R. V. Ramos, H. R. Staempfli, L. G. Arroyo, P. Kim, and J. S. Weese. 2015. 'Characterization and comparison of the bacterial microbiota in different gastrointestinal tract compartments in horses', *The Veterinary Journal*, 205: 74-80.

Costa, M. C., and J. S. Weese. 2018. 'Understanding the Intestinal Microbiome in Health and Disease', *Vet Clin North Am Equine Pract*, 34: 1-12.

Coverdale, J. A. 2016. 'Horse Species Symposium: Can the microbiome of the horse be altered to improve digestion?', *Journal of Animal Science*, 94: 2275-81.

Crespo, F., C. Quiñones-Pérez, I. Ortiz, M. Diaz-Jimenez, C. Consuegra, B. Pereira, J. Dorado, and M. Hidalgo. 2020. 'Seasonal variations in sperm DNA fragmentation and pregnancy rates obtained after artificial insemination with cooled-stored stallion sperm throughout the breeding season (spring and summer)', *Theriogenology*, 148: 89-94.

D'Anza, E., S. Albarella, G. Galdiero, S. Tafuri, C. Del Prete, N. Cocchia, F. Ciani, V. Mastellone, M. P. Pasolini, D. Carotenuto, M. Selvaggi, F. Ciotola, and V. Peretti. 2021. 'DNA fragmentation and morphometric studies in sperm of stallions supplemented with maca (Lepidium meyenii)', *Zygote*, 29: 325-30.

de Fombelle, A., V. Julliand, C. Drogoul, and E. Jacotot. 2001. 'Feeding and microbial disorders in horses: 1 - Effects of an abrupt incorporation of two levels of barley in a hay diet on microbial profile and activities', *Journal of Equine Veterinary Science*, 21: 439-45.

Djourabchi Borojerdi, A. S., T. Welchowski, W. Peng, A. Buchen, N. Novak, G. Haidl, Y. G. Duan, and J. P. Allam. 2020. 'Human spermatozoa of male patients with subfertility express the interleukin-6 receptor', *Andrologia*, 52: e13511.

Dougal, Kirsty, Gabriel de la Fuente, Patricia A. Harris, Susan E. Girdwood, Eric Pinloche, Raymond J. Geor, Brian D. Nielsen, Harold C. Schott, II, Sarah Elzinga, and C. Jamie Newbold. 2014. 'Characterisation of the Faecal Bacterial Community in Adult and Elderly Horses Fed a High Fibre, High Oil or High Starch Diet Using 454 Pyrosequencing', *PLoS One*, 9: e87424.

Dougal, Kirsty, Gabriel de la Fuente, Patricia A. Harris, Susan E. Girdwood, Eric Pinloche, and C. Jamie Newbold. 2013. 'Identification of a Core Bacterial Community within the Large Intestine of the Horse', *PLoS One*, 8: e77660.

Dubourg, G., J. C. Lagier, F. Armougom, C. Robert, G. Audoly, L. Papazian, and D. Raoult. 2013. 'High-level colonisation of the human gut by Verrucomicrobia following broad-spectrum antibiotic treatment', *Int J Antimicrob Agents*, 41: 149-55.

Edwards, J. E., S. A. Shetty, P. van den Berg, F. Burden, D. A. van Doorn, W. F. Pellikaan, J. Dijkstra, and H. Smidt. 2020. 'Multi-kingdom characterization of the core equine fecal microbiota based on multiple equine (sub)species', *Anim Microbiome*, 2: 6.

Ellis, AD, Boekhoff, M, Bailoni, L, Mantovani, R. 2006. 'Nutrition and feeding of the broodmare': 341-62.

Fioratti, Eduardo Gorzoni, Ana Izabel S. Balbin Villaverde, Cely M. Melo, Miriam H. Tsunemi, Frederico O. Papa, and Marco Antonio Alvarenga. 2012. 'Influence of Steroidal Anti-Inflammatory Drugs on Viability and Fertility of Equine Semen', *Journal of Equine Veterinary Science*, 32: 771-75.

Franzan, B. C., I. D. S. Coelho, M. T. de Souza, M. M. M. Santos, F. Q. de Almeida, and V. P. Silva. 2022. 'Fecal Microbiome Responses to Sudden Diet Change in Mangalarga Marchador horses', *J Equine Vet Sci*, 108: 103803.

Garber, Anna, Peter Hastie, and Jo-Anne Murray. 2020. 'Factors Influencing Equine Gut Microbiota: Current Knowledge', *Journal of Equine Veterinary Science*, 88: 102943.

Gibb, Z., S. R. Lambourne, and R. J. Aitken. 2014. 'The paradoxical relationship between stallion fertility and oxidative stress', *Biol Reprod*, 91: 77.

Gibb, Z., S. R. Lambourne, J. Quadrelli, N. D. Smith, and R. J. Aitken. 2015. 'L-carnitine and pyruvate are prosurvival factors during the storage of stallion spermatozoa at room temperature', *Biol. Reprod.*, 104: 1-9.

Gibb, Zamira, and Robert J. Aitken. 2016. 'Recent Developments in Stallion Semen Preservation', *Journal of Equine Veterinary Science*, 43: S29-S36.

Gibb, Zamira, Olga Blanco-Prieto, and Diego Bucci. 2021. 'The role of endogenous antioxidants in male animal fertility', *Research in Veterinary Science*, 136: 495-502.

Gilroy, Rachel, Joy Leng, Anuradha Ravi, Evelien M. Adriaenssens, Aharon Oren, Dave Baker, Roberto M. La Ragione, Christopher Proudman, and Mark J. Pallen. 2022. 'Metagenomic investigation of the equine faecal microbiome reveals extensive taxonomic diversity', *PeerJ*, 10: e13084-e84.

Gomez-Arango, Luisa F., Helen L. Barrett, H. David McIntyre, Leonie K. Callaway, Mark Morrison, and Marloes Dekker Nitert. 2016. 'Connections Between the Gut Microbiome and Metabolic Hormones in Early Pregnancy in Overweight and Obese Women', *Diabetes*, 65: 2214.

Górniak, W., P. Cholewińska, N. Szeligowska, M. Wołoszyńska, M. Soroko, and K. Czyż. 2021. 'Effect of Intense Exercise on the Level of Bacteroidetes and Firmicutes Phyla in the Digestive System of Thoroughbred Racehorses', *Animals : an open access journal from MDPI*, 11.

Griffin, R. A., M. Baker, R. J. Aitken, A. Swegen, and Z. Gibb. 2019. 'What makes a fertile sperm? Unique molecular attributes of stallion fertility', *Reproduction*, 158: R125-r37.

Helli, B., M. Kavianpour, E. Ghaedi, M. Dadfar, and H. K. Haghighian. 2020. 'Probiotic effects on sperm parameters, oxidative stress index, inflammatory factors and sex hormones in infertile men', *Hum Fertil (Camb)*: 1-9.

Hughes, J. P., and R. G. Loy. 1975. 'The relation of infection to infertility in the mare and stallion', *Equine Vet J*, 7: 155-9.

Hurtgen, J. P. 1992. 'Evaluation of the stallion for breeding soundness', *Vet Clin North Am Equine Pract*, 8: 149-65.

Jeulin, C., and L. M. Lewin. 1996. 'Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa', *Hum Reprod Update*, 2: 87-102.

Kauter, Anne, Lennard Epping, Torsten Semmler, Esther-Maria Antao, Dania Kannapin, Sabita D. Stoeckle, Heidrun Gehlen, Antina Lübke-Becker, Sebastian Günther, Lothar H. Wieler, and Birgit Walther. 2019. 'The gut microbiome of horses: current

research on equine enteral microbiota and future perspectives', *Animal Microbiome*, 1: 14.

Lane, E. A., M. L. Bijnen, M. Osborne, S. J. More, I. S. Henderson, P. Duffy, and M. A. Crowe. 2016a. 'Key Factors Affecting Reproductive Success of Thoroughbred Mares and Stallions on a Commercial Stud Farm', *Reprod Domest Anim*, 51: 181-7.

Lane, EA, MLJ Bijnen, M Osborne, SJ More, ISF Henderson, P Duffy, and MA Crowe. 2016b. 'Key Factors Affecting Reproductive Success of Thoroughbred Mares and Stallions on a Commercial Stud Farm', *Reproduction in domestic animals*, 51: 181-87.

Li, Kelvin, Monika Bihan, Shibu Yooseph, and Barbara A. Methé. 2012. 'Analyses of the Microbial Diversity across the Human Microbiome', *PLoS One*, 7: e32118.

Li, Y., Y. Lan, S. Zhang, and X. Wang. 2022. 'Comparative Analysis of Gut Microbiota Between Healthy and Diarrheic Horses', *Frontiers in Veterinary Science*, 9: 882423.

Machado-Neves, M., Gibb, Z., Curry, B., Lambourne, S. and Aitken, R. J. (2016. 'The expression and function of stallion sperm cytokine receptors.', *8th International Congress on Animal Reproduction, Tours, France.*: pp. 93-94.

McFarlane, D., and T.C. Holbrook. 2008. 'Cytokine Dysregulation in Aged Horses and Horses with Pituitary Pars Intermedia Dysfunction', *Journal of Veterinary Internal Medicine*, 22: 436-42.

McMurdie, P. J., and S. Holmes. 2013. 'phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data', *PLoS One*, 8: e61217.

Menzies, P.I. 1999. 'Reproductive health management programs', *Current Therapy in Large Animal Theriogenology*: pp 643-49.

Metcalf, Jessica L, Se Jin Song, James T Morton, Sophie Weiss, Andaine Seguin-Orlando, Frédéric Joly, Claudia Feh, Pierre Taberlet, Eric Coissac, and Amnon Amir. 2017. 'Evaluating the impact of domestication and captivity on the horse gut microbiome', *Scientific Reports*, 7: 1-9.

Mihalcescu, I., W. Hsing, and S. Leibler. 2004. 'Resilient circadian oscillator revealed in individual cyanobacteria', *Nature*, 430: 81-5.

Morris, L. H. A., and W. R. Allen. 2002a. 'Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket', *Equine Vet J*, 34: 51-60.

Morris, Lee HA, and WR Allen. 2002b. 'Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket', *Equine Vet J*, 34: 51-60.

Morrison, P. K., C. J. Newbold, E. Jones, H. J. Worgan, D. H. Grove-White, A. H. Dugdale, C. Barfoot, P. A. Harris, and C. M. Argo. 2020. 'The equine gastrointestinal microbiome: impacts of weight-loss', *BMC Vet Res*, 16: 78.

Morrison, Philippa K., Charles J. Newbold, Eleanor Jones, Hilary J. Worgan, Dai H. Grove-White, Alexandra H. Dugdale, Clare Barfoot, Patricia A. Harris, and Caroline McG Argo. 2018. 'The Equine Gastrointestinal Microbiome: Impacts of Age and Obesity', *Frontiers in Microbiology*, 9: 3017-17.

Nath, LC, GA Anderson, and AO McKinnon. 2010. 'Reproductive efficiency of Thoroughbred and Standardbred horses in north-east Victoria', *Australian veterinary journal*, 88: 169-75.

O'Donnell, M. M., H. M. B. Harris, I. B. Jeffery, M. J. Claesson, B. Younge, P. W. O'Toole, and R. P. Ross. 2013. 'The core faecal bacterial microbiome of Irish Thoroughbred racehorses', *Letters in Applied Microbiology*, 57: 492-501.

Pugh, G. 2018. 'The Role of Microbial Diversity in Female Reproductive Health: An Analysis of the Microbiome's Inflfluence on Pregnancy Outcomes in Postparturient Mares', Eastern Kentucky University.

Qu, S., L. Fan, Y. Qi, C. Xu, Y. Hu, S. Chen, W. Liu, W. Liu, and J. Si. 2021. 'Akkermansia muciniphila Alleviates Dextran Sulfate Sodium (DSS)-Induced Acute Colitis by NLRP3 Activation', *Microbiol Spectr*, 9: e0073021.

Rizzo, M., K. D. Ducheyne, C. Deelen, M. Beitsma, S. Cristarella, M. Quartuccio, T. A. E. Stout, and M. de Ruijter-Villani. 2019. 'Advanced mare age impairs the ability of in vitro-matured oocytes to correctly align chromosomes on the metaphase plate', *Equine Vet J*, 51: 252-57.

Salem, S. E., R. Hough, C. Probert, T. W. Maddox, P. Antczak, J. M. Ketley, N. J. Williams, S. J. Stoneham, and D. C. Archer. 2019. 'A longitudinal study of the faecal microbiome and metabolome of periparturient mares', *PeerJ*, 7: e6687.

Salem, Shebl E., Thomas W. Maddox, Adam Berg, Philipp Antczak, Julian M. Ketley, Nicola J. Williams, and Debra C. Archer. 2018. 'Variation in faecal microbiota in a group of horses managed at pasture over a 12-month period', *Scientific Reports*, 8: 8510.

Sancler-Silva, Y. F. R., G. A. Monteiro, C. Ramires-Neto, C. P. Freitas-Dell'aqua, A. M. Crespilho, M. M. J. Franco, E. R. Silva-Junior, T. M. S. Cavalero, V. F. C. Scheeren, and F. O. Papa. 2020. 'Does semen quality change after local treatment of seminal vesiculitis in stallions?', *Theriogenology*, 144: 139-45.

Scheeren, Verônica F. C., Yame F. R. Sancler-Silva, Hossam El-Sheikh Ali, John P. Kastelic, Marco A. Alvarenga, and Frederico O. Papa. 2020. 'Update on Seminal Vesiculitis in Stallions', *Journal of Equine Veterinary Science*, 94: 103234.

Shepherd, M. L., W. S. Swecker, Jr., R. V. Jensen, and M. A. Ponder. 2012. 'Characterization of the fecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons', *FEMS Microbiol Lett*, 326: 62-8.

Shokryazdan, P., M. Faseleh Jahromi, B. Navidshad, and J. B. Liang. 2017. 'Effects of prebiotics on immune system and cytokine expression', *Med Microbiol Immunol*, 206: 1-9.

Singh, Pallavi, Tracy K. Teal, Terence L. Marsh, James M. Tiedje, Rebekah Mosci, Katherine Jernigan, Angela Zell, Duane W. Newton, Hossein Salimnia, Paul Lephart, Daniel Sundin, Walid Khalife, Robert A. Britton, James T. Rudrik, and Shannon D. Manning. 2015. 'Intestinal microbial communities associated with acute enteric infections and disease recovery', *Microbiome*, 3: 45.

Slater, Rachael, Alessandra Frau, Jane Hodgkinson, Debra Archer, and Chris Probert. 2021. 'A Comparison of the Colonic Microbiome and Volatile Organic Compound Metabolome of Anoplocephala perfoliata Infected and Non-Infected Horses: A Pilot Study', *Animals*, 11: 755.

STROBE 2022. 'The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies', *https://www.equator-network.org/reporting-guidelines/strobe/*.

Tang, Jia, Lingqi Xu, Yiwen Zeng, and Fang Gong. 2021. 'Effect of gut microbiota on LPS-induced acute lung injury by regulating the TLR4/NF-kB signaling pathway', *International Immunopharmacology*, 91: 107272.

University, Iowa State. 2023. 'Equine Body Condition Score Rating'.

Wang, H., A. Xu, L. Gong, Z. Chen, B. Zhang, and X. Li. 2022. 'The Microbiome, an Important Factor That Is Easily Overlooked in Male Infertility', *Front Microbiol*, 13: 831272.

Wang, Yan, and Zuogang Xie. 2022. 'Exploring the role of gut microbiome in male reproduction', *Andrology*, 10: 441-50.

Williams, S. B. 2007. 'A circadian timing mechanism in the cyanobacteria', *Adv Microb Physiol*, 52: 229-96.

Wingett, S. W., and S. Andrews. 2018. 'FastQ Screen: A tool for multi-genome mapping and quality control', *F1000Res*, 7: 1338.

APPENDIX

Figure A1-A6: Distributions of numeric variables

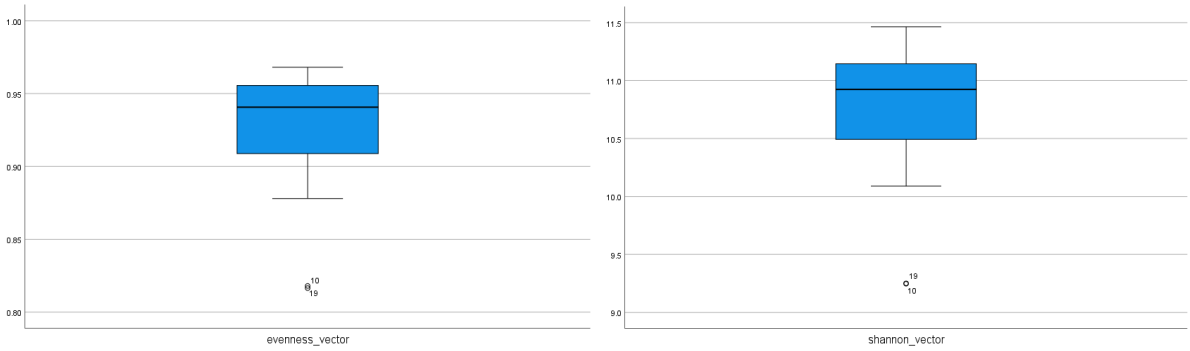

Table 1: Summary statistics for the numeric variables TNF-α, age, evenness, faith_pd and Shannon's diversity in a study of 19 Thoroughbred stallions

Variable	Minimum	Q1	Median	Q3	Maximum	Mean	SD
TNF-α (pg/mL)	0.0	0.0	0.4	17.0	210.6	18.4	47.8
Age (years)	4.0	6.0	8.0	17.0	22.0	10.5	5.9
Evenness	0.82	0.89	0.94	0.96	0.97	0.92	0.05
Faith_pd	227.8	305.8	351.8	385.7	409.7	344.5	49.0
Shannon's index of diversity	9.2	10.3	10.9	11.1	11.5	10.7	0.7

Table 2: Summary statistics for PCCR overall and by the categories of the nominal predictor variables stud, antibiotic, anti-inflammatory, pre-/pro-/synbiotics and carnitine

		N (%)	Minimum	Q1	Median	Q3	Maximum	Mean	SD
PCCR		19 (100)	34.2	50.0	56.4	60.9	63.9	54.6	7.1
Stud	1	6 (32)	34.2	44.5	49.0	54.9	56.6	48.5	7.8
	2	6 (32)	48.7	53.0	55.6	56.7	56.9	54.6	3.1
	3	7 (36)	50.0	59.0	61.5	61.9	63.9	59.9	4.6
Antibiotic	No	16 (84)	48.0	50.0	55.7	60.4	63.9	55.3	5.3
	Yes	3 (16)	34.2	34.2	56.4	59.2	61.9	50.8	14.7
Anti-inflammatory	No	6 (32)	50.0	53.3	55.7	61.0	61.5	56.4	4.3
	Yes	13 (68)	34.2	48.4	56.4	60.5	63.9	53.8	8.1
Pre-/pro-/synbiotics	No	7 (37)	48.7	50.0	56.4	56.9	61.9	55.0	4.5
	Yes	12 (63)	34.2	48.5	55.7	61.4	63.9	54.4	8.4

Carnitine	No	14 (74)	34.2	48.5	55.4	57.4	61.9	53.3	7.2
	Yes	5 (26)	50.0	52.4	61.5	62.9	63.9	58.4	5.8

Table 3: Results of five univariable linear models for categorical predictor variables of PCCR

Variable		**Predicted Mean**	**95% CI**	**p-value**
Stud	1	48.5	43.8 – 53.3	0.007
	2	54.6	49.9 – 59.4	
	3	59.9	55.5 – 64.3	
Carnitine	No	53.3	49.4 – 57.1	0.17
	Yes	58.4	51.9 – 64.9	
Anti-inflammatory	No	55.4	51.6 – 59.1	0.32
	Yes	50.8	42.2 – 59.4	
Antibiotics	No	56.4	50.2 – 62.5	0.48
	Yes	53.8	49.6 – 58.0	
Pre-/pro-/synbiotics	No	55.0	49.2 – 60.8	0.87
	Yes	54.4	50.0 – 58.9	

Table 4: Results of 5 simple linear regression analyses for numeric predictor variables of PCCR

Parameter	**Regression estimate β**	**Standard error (β)**	**95% CI**	**p-value**	**R^2**
Constant	53.9	1.7			
TNF-α (pg/mL)	0.04	0.035	-0.04 – 0.11	0.28	0.07
Constant	51.6	3.4			
Age (years)	0.29	0.28	-0.30 – 0.89	0.31	0.06
Constant	85.5	33.9			
Evenness	-33.4	36.7	-110.7 – 44.0	0.38	0.05
Constant	75.0	27.9			
Shannon's index of diversity	-1.9	2.6	-7.4 – 3.6	0.47	0.03
Constant	57.4	12.1			
Faith_pd/Richness	-0.008	0.035	-0.82 – 0.066	0.82	<0.01

Table 5: Results for four simple linear regression analyses for numeric predictor variables of TNF-α

Parameter	**Regression estimate β**	**Standard error (β)**	**95% CI**	**p-value**	**R^2**
Constant	-9.3	22.3			
Age (years)	2.6	1.9	-1.3 – 6.6	0.17	0.05
Constant	93.0	190.8			

Shannon's Index of diversity	-6.9	17.7	-44.4 – 30.5	0.70	0.01
Constant	99.6	234.3			
Evenness	-87.9	253.4	-622.5 – 446.7	0.73	0.01
Constant	43.5	82.1			
Faith_pd/Richness	-0.07	0.24	-0.57 – 0.43	0.76	<0.01

Appendix 5.1: Protocol for preparing faecal samples for DNA extraction

ZYMO RESEARCH

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DNA for microbiome or metagenome analyses

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- **Simple Workflow:** Simply bead-beat sample, purify via spin-column, and filter to remove PCR inhibitors. No precipitation or lengthy incubations!

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Appendix 5.2: Protocol for performing serum cytokine analysis

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User Guide

MILLIPLEX® Equine Cytokine/Chemokine Magnetic Bead Panel

96-Well Plate Assay

EQCYTMAG-93K
EQCYTMG-93KPX23
EQCTMG93KPX23BK

Appendix 5.3: Stud Farm Nutritional Supplementation Regimens

N = numbers of horses receiving supplement

Stud Farm Nutritional Supplementation Regimens

SUPPLEMENT	STUD 1	STUD 2	STUD 3
EOS oil [a]	5		7
Omega-3 oil		6	
Linseed			4
Nano-E [a]			5
Carnitine	1		3
Equi-Jewel [a]			1
Gold Pellet [a]	5		
Sweet Feed No.4 [b]		5	
Inner Balance [b]		2	
Cool Command [c]	2		
Diamond V XPC [c]	1		6
Legend [c]			6
Balancer Pellets [d]			2
Gastro Pellets [g]		1	
Old Timer [e]		1	
4Cyte Joint Support [j]			1
Farrier's Formula [f]		1	
Hoof Gold [h]			1
Livamol[k]		6	
EVM[l]		6	

[a] KER: Kentucky Equine Research, [b] Horse Power, [c] Barastoc, [d] Equine Vit&Min, [e] Johnsons, [f] Life Data Labs Inc, [g] Med-Vet Pharmaceuticals, [h] Scone Equine Group, [i] Hubbard, [j] Provet

PRODUCT INGREDIENTS

EOS oil, Omega-3 oil, Linseed – omega-3 fatty acids

Nano-E - highly bioavailable vitamin E

Equi-Jewel - marine-derived calcium, linoleic acid

Sweet Feed No.4 - triticale, micronized corn, micronized barley, micronized lupins, sunflower seeds, molafos, omega fish and canola oils, salt, dicalcium phosphate, limestone, Vitamin E, Agrimos prebiotic and chelated chromium

Gold Pellet - amino acids, vitamins, antioxidants, and trace minerals

Cool Command - controlled starch, optimal level of protein, with mineral and vitamin fortification

Legend - barley, corn, lupins, sunflower seeds, ProteniseTM Pellet, calcium carbonate, mono-di calcium phosphate, salt, magnesium oxide, omega rich vegetable oil, molasses, Barastoc *GastrolizeTM* (red seaweed extract), lysine, methionine, *Diamond V® XPC* (prebiotic yeast), vitamin E, KER vitamin & mineral premix.

Diamond V® XPC - prebiotic yeast

Inner Balance - Zeolite volcanic clay mineral, equine B9, botanic extracts, Agrimos® mannanoligopolysaccharide (MOS) prebiotic

Carnitine – amino acid

Balancer Pellets – minerals, vitamins, lysine, trace elements, live yeast probiotic (*Saccharomyces cerevisiae*)

Old Timer - oaten hay, lucerne hay, lupins, faba beans, soybean and oil, vitamins, chelated minerals, prebiotics, probiotics, and mycotoxin binder

4Cyte Joint Support – cartilage repair nutrients, anti-inflammatory

Bio Bloom hoof and coat : biotin, organic zinc, lysine, methionine, iodine, lecithin, soybean meal

Farrier's Formula - phospholipids, omega fatty acids, vitamins, minerals, and amino acids

Gastro Pellets - antioxidants, probiotics (Levucell SC yeast) and herbals

Hoof Gold – biotin, methionine, methylsulfonylmethane (MSM), zinc, vitamins, other minerals, amino acids

Human fertility – influence of the microbiome

To set the human context for this research a literature review was conducted of current publications relating to fertility in the healthy male and several microbiomes – seminal, sperm, testicular and gastrointestinal (See Appendix). Only seven publications were suitable for review, confirming how little is known about the influence of microbiota on spermatogenesis. It is apparent from these studies that it is not possible to clearly distinguish the semen microbiome profiles of fertile men from infertile counterparts, although some bacterial species predominated in the semen of those men with better quality sperm, in particular Lactobacillus and Gardnerella genera, while in those with poorer quality sperm *Prevotella* genus was more abundant. One author proposed a bacterial biomarker for male infertility to be the species *Anaerococcus*, which was associated with poorer sperm quality and moreover was identified in the semen of men reporting infertility (37). As was revealed by a comparative analysis of the semen and faecal microbiome profiles of our cohort of miniature pony stallions, the characteristic profile of the human semen microbiome exhibits a low abundance of organisms in comparison to the gastrointestinal microbiome, although the semen displays a high diversity.

Abstract

Introduction

According to the Human Microbiome Project, the urogenital tract of both males and females comprises approximately 9% of the mass of microbiota that inhabit humans; however, the microbiomes of the male urogenital tract have only featured in a few research publications to date, and consensus on what comprises a healthy male urogenital microbiome is lacking. The possible role of probiotic bacterial species in

influencing spermatogenesis has only recently been investigated and therapeutic solutions explored for subfertility.

Aim

The purpose of this review is to explore what is known about the microbiomes of the male genitourinary tract, and the connection between the intestinal and semen microbiomes and male fertility in healthy human subjects.

Methods

Three databases (Embase, PubMed and Scopus) were surveyed on 1/1/2022 for publications from 1954 to 2022, excluding systematic reviews and review articles, using the search terms male fertility and the microbiome. Publications referring to infertility, infections, diseases in men and all female and other species topics were excluded. A total of 83 papers were identified, of which 23 were screened for suitability, and after duplicates and papers unable to be retrieved were removed, a final 6 studies were included for review. Inclusion criteria were healthy human, male fertility and microbiomes.

Results

The semen microbiome demonstrates low abundance with high diversity in comparison to other body sites. Evaluating semen microbiota against several semen parameters, normal semen revealed higher proportions of *Lactobacillus* and *Gardnerella* genera, while *Prevotella* abundance was higher in lower quality semen samples. Alpha diversity of the sperm microbiomes of men presenting with idiopathic infertility was insignificantly lower than fertile comparators and there was no difference in Shannon diversity between the two groups of men. Similarly, alpha and beta diversity of semen microbiota showed no significant relationship to either subfertility or infertility, nor to fertility in a Chinese cohort. The microbiomes of fertile and infertile men were virtually indistinguishable. Microbiota sequenced from immature spermatozoa via testicular biopsy of both infertile men and controls were predominantly *Akkermansia*, *Faecalibacterium*, *Flavobacterium*, *Bacteroides* and *Alistipes* genera. The composition of the semen microbiome is transient and altered

by sexual intercourse, following which it contains more vaginal species, such as *Lactobacillus crispatus*.

Conclusion

Very few publications exist in the public domain regarding male genitourinary microbiomes and their relevance to spermatogenesis and male fertility. Although the semen microbiomes of fertile and subfertile men are not clearly distinguishable, one bacterial species, *Anaerococcus,* was identified as a possible biomarker of male infertility, while *Rhodopseudomonas*, *Oligotropha* and *Cutibacterium* genera were relatively abundant in men with idiopathic infertility. Normal semen contained higher proportions of *Lactobacillus* and *Gardnerella* genera, while *Prevotella* predominated the lower quality semen samples. Genitourinary microbiota are transient occupants of the lower urethra in men; hence, defining a healthy semen microbiome continues to defy researchers in this field.

INTRODUCTION

Human infertility rates globally have risen to approximately 10–15% of couples trying to conceive, with male factor accounting for 50% of cases (452). Extensive research has investigated environmental (453, 454) and toxic chemicals (15), radiation (455) and nutritional influences (456, 457, 458) on sperm count and motility. Genitourinary infections, diseases and malformations are commonly screened during the clinical work-up of male infertility, such as prostatitis, varicocele, cryptorchidism and hypospadias (459). More recently, impacts of various microbiomes on human fertility are being explored, searching for explanations for idiopathic infertility, particularly in men (36, 460, 461, 462, 463), but only recently has a relationship between sperm quality and the intestinal microbiome been explored in the scientific literature (319) and in another systematic review regarding sperm quality in relation to the semen microbiome (461).

A meta-analysis of 61 studies in 1992, including one study of 14,947 healthy men without a history of infertility, concluded that the average sperm count had declined by 1% per

annum from 1938 to 1990, a total reduction of 52% over that period (464). Following a data re-analysis of global trends in 1997, Swan et al. showed geographical variations of male fertility, producing evidence of an approximate 1.5% annual decline in sperm density in American men and 3% for European and Australian men (controlling for age, sexual activity and actual fertility) (465). In a robust study that evaluated declining male fertility in France from 1989 to 2005, Rolland et al. (2013) sampled 26,609 male partners of infertile women undergoing artificial reproduction technology (ART) procedures whilst attempting to conceive. They reported that men showed a progressive decrease in both sperm morphology and semen concentration of approximately 1.9% annually, without any effect on sperm motility (466).

The essential role of the intestinal microbiome in human health, as well as that of many other species, is becoming increasingly recognised as research emerges annually (136, 184, 351, 467, 468, 469). Microbial communities on our body surfaces influence most physiological systems, such as digestion (470), immunity (471), metabolism (472), cardiovascular (352) and cognitive functioning (357, 473) and hormonal balance (474, 475). In terms of the male genital tract, there is evidence for the role of microbiota affecting the outcome of spermatogenesis and fertility (36, 80, 461).

The individual human microbiome, representing the genetic content of microbial cells predominantly housed by the gastrointestinal tract, is comprised of 10 to 100 trillion organisms (476). The main biomass of these microbiota are bacteria, followed by eukaryotes, archaea, fungi and viruses in terms of content (477, 478, 479). The proportion of microbial to human cells in a human body of average male weight (70 kg) varies depending upon whether the comparison is made with nucleated cells only (10:1) or includes non-nucleated red blood cells (1:1) (480). What has been previously understood about the interaction between microbes and human fertility has been the role of pathogens producing infection in the genitourinary tract of men and women. Current research is revealing the epigenetic influence of the microbiota on human fertility during gametogenesis (460) as well as the interplay between the microbiome and the reproductive system (481).

According to the Human Microbiome Project, the urogenital tract of both males and females comprises approximately 9% of the microbiota that inhabit humans (482); however, the microbiomes of the male urogenital tract, including testes, have only featured in a few research publications to date, and consensus on what comprises a healthy male urogenital microbiome is lacking. The possible role of commensal microbiota in influencing spermatogenesis has only recently being investigated (461), and therapeutic solutions explored for subfertility (483, 484). The purpose of this review is to explore what is known about the connection between the intestinal and semen microbiomes and male fertility in healthy human subjects.

Genitourinary infections account for 15% of male infertility cases (485); specifically, reactive oxygen species released by leucocytes responding to seminal infection are implicated in damage to sperm integrity and function (486). Prior to genomic sequencing technology, semen from men presenting with subfertility was cultured to exclude infection, and found to be sterile in 48% of cases (487). However, semen is not sterile in any species (488, 489) and in humans contains a significant microbial load, predominantly bacterial, featuring *Lactobacillus* and *Prevotella* species (80). *In vitro* studies of seminal bacteria and sperm characteristics report negative impacts of these organisms on sperm motility and survival, with increased reactive oxygen species producing DNA damage and apoptosis; however, these findings have not translated into *in vivo* research (490).

The male urogenital microbiome has been characterised, but its purpose is not yet fully understood. The lower male urethra harbours a larger proportion of the bacterial load of this region in comparison to the proximal urethra and the prostate under healthy conditions (463). Bacteria found in the urethra and coronal sulcus of the penis are often similar to those resident in the vagina of the female sexual partner (37), especially if the woman experiences bacterial vaginosis (454). A shared semino-vaginal microbiome has been identified between heterosexual partners, containing 85% shared phylotypes; the diversity of bacterial communities in semen is greater than that of the vaginal microbiome, although the bacterial load of the vagina is much greater (491). The composition of the semen microbiome is altered by sexual intercourse, following which it contains more vaginal species, such as *Lactobacillus crispatus* (491), attesting to the transient nature of

the bacterial composition of the semen. It is postulated that the penile microflora may protect men against sexually transmitted infections (STIs), similar to the way the vaginal microbiome provides a barrier to STIs in women (492). A great deal of attention is paid to vaginal microflora in the literature, far less to penile microflora and almost none to the seminal microbiome.

Extensive evidence exists in the literature contributing male genitourinary infections to infertility; however, this is not the scope of this review. Complications of genitourinary infections in men include sterility, due to persistent inflammation associated with chronic orchitis and obstruction to the excurrent duct system of the testis. Certain phyla of bacteria are more consistently isolated from urine, ejaculate and urethral mucosa of men with infertility, namely Corynebacterium sp., coagulase-negative Staphylococci and Eubacterium species (492). A great deal of literature exists implicating genitourinary infections in both male and female infertility; however, this topic is beyond the scope of this review.

MATERIALS AND METHODS

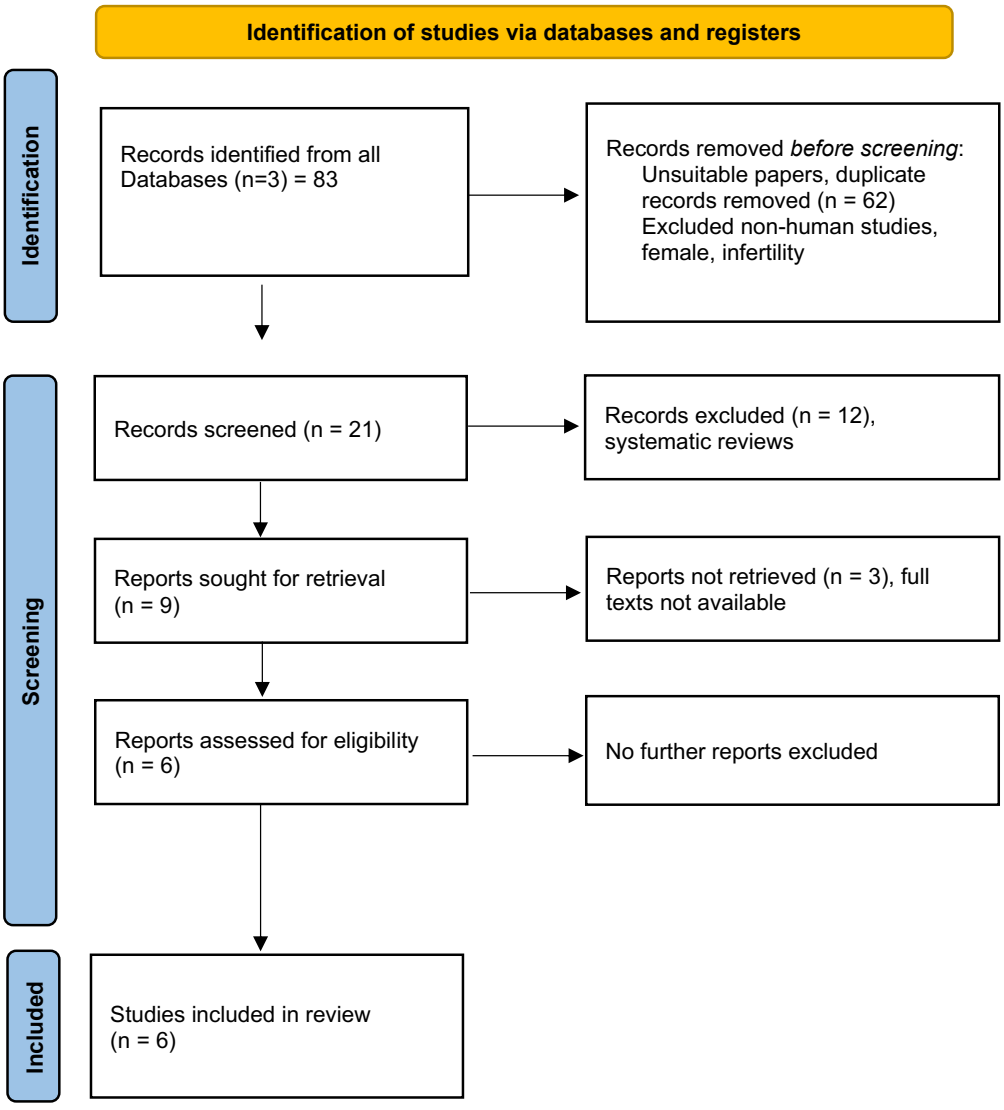
LITERATURE SEARCH

Three databases (Embase, PubMed and Scopus) were surveyed on 1/1/2022 for publications from 1954 to 2022, excluding systematic reviews and review articles, using the search terms male fertility and the microbiome (see Figure AC1).

STUDY SELECTION CRITERIA

A total of 83 papers were identified, of which 23 were screened for suitability after duplicates were removed. These papers were further screened by abstract reading, leaving 9 remaining for retrieval. Full texts were not able to be retrieved for three papers, hence a final number of 6 studies were included for review. Inclusion criteria were healthy human, male fertility and microbiomes. We excluded publications referring to infertility, infections, diseases in men and all female and other species topics.

Figure AC1: Prisma flow diagram of selection process for publications included in this review



DATA EXTRACTION

Articles were screened by one author for suitability for inclusion. Final papers were selected by two authors for inclusion. Studies that satisfied the search criteria were downloaded into EndNote X20 citation software (Clarivate Analytics). Data was tabulated accordingly: first author, publication year, country in which conducted, microbiome, findings and further implications (See Table 1).

Table AC1: List of selected publications for review

author, publication year, country	**microbiome**	**findings**	**further implications**
Molina, 2021, Spain	Testicular	Testicular biopsy samples revealed similar bacterial profiles to negative controls: 66 and 63 genera respectively. Only testicular samples contained *Prolixibacter, Delftia* and *Robinsoniella* genera. Sequencing of maturing spermatozoa identified *Akkermansia*, *Faecalibacterium*, *Bacteroides*, *Prevotella and Alistipes* and as the predominant genera. Testicular microbiome profiles were similar between individuals and when developing testicular spermatozoa were profiled at different stages of growth.	The human testicle contains a unique bacterial signature when decontaminated, which could contribute to the composition of the seminal microbiome, although in low abundance
Swanson, 2020, USA	Sperm	From a cohort of 84 couples with idiopathic infertility, male partners underwent semen analyses, 71 having healthy profiles according to WHO guidelines and 48 couples achieved successful live births. Alpha diversity of	*Streptococcus agalactiae* infection has been described in adults and neonates; non-targeted human sperm RNA-seq data may offer useful

		the sperm microbiomes were insignificantly lower from infertile couples than fertile couples; no difference was observed in Shannon diversity between the two cohorts. Microbial composition was typical of common genitourinary commensal organisms in consistent across all samples, characterised by four phyla and 11 genera. Two operational taxonomic units, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* were identified following multiple tests.	diagnostic testing for microbial as well as fertility status
Ma, 2022, China	Seminal	The results of a diversity-area relationship analysis demonstrated that fertility status did not influence semen microbiome biogeography significantly, particularly not in terms of alpha- and beta-diversity.	The authors postulated that their semen microbiome profiling could be considered characteristic of the Chinese male population from their findings, as no differences were demonstrated in profiles of fertile, subfertile or infertile men.
Hou, 2013, China	Seminal	No discrimination could be drawn between sperm donors and infertility patients on the basis of seminal microbiome profiles. Presence of *Anaerococcus sp.* was suggested as a biomarker for low sperm quality. Many bacterial species identified were considered commensals or contaminants of vaginal microflora.	Male fertility may be impacted by shared microflora from female sexual partners, particularly in the case of sexually transmitted infections and *Gardnerella sp*. from bacterial vaginosis. *Anaerococcus vaginalis* is present more significantly in semen of infertile men.
Baud, 2019, Switzerland	Seminal	*Lactobacillus sp*. in semen correlated with normal sperm morphology, while *Prevotella* was relatively more	Potential for probiotic correction of dysbiotic semen

		abundant in samples with impaired sperm motility. Alpha and beta diversity shifts did not correlate with semen parameters. Overall bacterial content of sperm might was not associated with male infertility status.	microbiome to improve fertility biomarkers.
Al-Asmakh, 2015, Qatar	Gastrointestinal	Blood -testis barrier (BTB) permeability is associated with weakened tight junction proteins (TJP) and lower expression of occludin and zonula occludens 2 (ZO-2). Butyrate produced by *Clostridium Tyrobutyricum* demonstrates a capacity for restoring TJP's to control level and also enhance histone acetylation *in vivo*.	Future research could be informed by these findings, in terms of potential for modulating the gastrointestinal microbiome via pre-, probiotic or antibiotic therapy to alter barrier permeability and thereby enhance reproductive health is of considerable importance. Identifying bacterial to host metabolite crosstalk and understanding signaling pathways which influence these selective membrane barriers could facilitate the development of novel clinical therapeutics .

RESULTS

THE TESTICULAR MICROBIOME

Eleven men with various infertility issues, presenting to a fertility clinic in Granada, Spain, between September 2014 and April 2016, underwent testicular biopsy, which produced 24 tissue samples that were analysed with 11 controls (493). The men had been diagnosed with either sperm DNA fragmentation and a history of five failed assisted

reproduction (ART) cycles, severe oligoasthenoteratozoospermia or azoospermia, and hence were a suitable cohort in which to explore the influence of microbiota on sperm quality. Only five controls were suitable for analysis and were found to be heavily contaminated. After decontamination of the tissue samples (comprising 50–70% of the reads), high-throughput sequencing that targets V3 and V4 regions of 16S rRNA gene was employed. Sixty-six genera were identified in the testicular samples of the infertile cohort and 63 genera were identified in the controls. Sequencing of bacteria associated with immature spermatozoa in this cohort produced profiles of *Faecalibacterium*, *Bacteroides*, *Prevotella*, *Alistipes*, and *Akkermansia* amongst the ten dominant genera in both cohorts (493). The authors note that *Prolixibacter*, *Delftia* and *Robinsoniella* genera were not present in the control samples. As the authors acknowledge, the analysis of sperm that had been cultured may have posed a limitation to this study, considering the potential for bacterial contamination of specimens using this method versus untreated spermatozoa. Additionally, as this study was conducted in oligo/astheno/terato/azoospermic males, the authors advise that their results do not reflect the microbiome of healthy testicular spermatozoa. Although the testis is a low-biomass organ, its microbiome could potentially contribute to that of the semen (493).

THE SPERM MICROBIOME

Sperm 16SRNA was isolated and identified by Illumina Next-Gen sequencing in one study (494). Swanson et al. identified four phyla with 11 genera associated with sperm collected from 85 American and Canadian males, with a mean age 34.73 years, who were experiencing idiopathic infertility with their female partners (494). Semen parameters were evaluated in this cohort of men and all found to significantly exceed the minimum values for volume, concentration, motility and percentage normal forms proposed by the WHO (494); hence, they were not technically infertile. Biobanked semen samples from these men were profiled on an Illumina Next-seq 500 machine for library quality evaluation, followed by Illumina Hi-seq 4000 for further depth of analysis. Alpha diversity of the sperm microbiomes of these men was insignificantly lower in the cohort that did not achieve live birth outcomes by the end of the study, as compared to the cohort that

successful conceived with live birth outcomes; there was no difference in Shannon diversity between the two groups of men (494). The sperm bacterial genera identified in this study, in order of abundance, were *Escherichia*, *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Riemerella*, *Cutibacterium*, *Baciullus*, *Corynebacterium*, *Acinetobacter*, *Burkholderia* and *Flavobacterium*, excluding the genera associated with the sperm of one extreme outlier (494). This outlier sample contained *Streptococcus agalactiae*, belonging to *Group B Streptococci* and a pathogenic species known to be sexually transmitted (495), which may colonise the female genital tract and induce abortion (miscarriage) and stillbirth (496).

THE SEMEN MICROBIOME

A unique, personalised bacterial community is harboured by individuals and the spectrum of organisms found in semen is both commensal and transient. The individual semen microbiome is comprised of hundreds of bacterial species that have been assessed for relative abundance with respect to different human populations of varying levels of fertility (37, 497). Exploring the biogeography of the semen microbiota of 96 individuals, Ma et al. showed their alpha and beta diversity; however, comparison of the profiles from fertile men (35) with subfertile (28) and infertile (33) men showed no significant relationship. The researchers extrapolated their model to represent the semen microbiome characteristics of the general Chinese male population (497).

In one study that used pyrosequencing of the V1–V2 regions of the 16S rRNA gene, the seminal microbiomes of infertile males and healthy sperm donors were characterised and compared, and, although a diversity of species was found, no significant difference was demonstrated between the microbial profiles of the two groups (37). Of particular significance in this study was the finding of a clearly negative association between the presence of the *Anaerococcus* spp. in the semen and the quality of the sperm, according to multiple statistical analyses. This led the authors to suggest that *Anaerococcus* spp. may be explored as a biomarker for poor sperm quality and therefore male infertility (37). In a survey of men diagnosed with idiopathic infertility, their seminal microbial composition

demonstrated a relative abundance of *Rhodopseudomonas*, *Oligotropha* and *Cutibacterium* genera (225).

Two studies were included in this review although they examined the semen microbiomes of infertile patients, as they reported on the microbial profiles in the semen of healthy controls. Weng et al. profiled probiotic species found in semen using next-generation sequencing technology and bioinformatics and analysed microbiome measures against sperm quality (274). The study recruited 96 male subjects, analysing seven clinical criteria for semen quality (volume, sperm concentration, motility, morphology, antisperm antibodies (IgA), atypical sperm and leukocyte count). *Lactobacillus* was the dominant genus in the samples (19.9%), followed by *Pseudomonas* (9.85%), *Prevotella* (8.51%) and *Gardnerella* (4.21%). Normal semen revealed higher proportions of *Lactobacillus* and *Gardnerella* genera, while *Prevotella* was in much greater abundance in the lower quality semen samples (274). One hypothesis drawn from this research was that probiotic supplementation with *Lactobacillus* spp. might offer a therapeutic solution for correcting poor semen quality, while reducing colonies of *Prevotella* and *Pseudomonas* to correct semen microbiome dysbiosis (274). Mändar et al. used 16SrRNA V6 pyrosequencing to analyse the bacterial profile of semen in their study of healthy men in heterosexual relationships; they discovered no dominant microbes, but a larger proportion of *Flavobacterium* and *Corynebacterium* as well as *Lactobacillus* genera in this cohort, and declared the semen microbiome as having low abundance with high diversity (491).

Similarly, Hou et al. analysed the seminal microbiomes of 19 sperm donors in comparison to those of 58 infertile men and, importantly, was able to suggest that a potential biomarker for low sperm quality may be the presence of *Anaerococcus* genus, which contains common anaerobes of the vaginal microbiome (37). Additional findings from this study were that, although there is a great diversity of personalised semen microbial communities for both fertile and infertile individuals, no diagnostic discrimination could be made based on the taxa of these two cohorts (37).

SEMEN MICROBIOTA AND FERTILITY

How the male genitourinary tract microbiome influences sperm quality and function, and hence fertility, has become a topic of interest to the research community in recent years. Baud et al. analysed the semen of a healthy cohort of 94 men attending a fertility clinic in Lausanne, Switzerland (October 2014 to July 2016), of which 26 demonstrated normal spermiograms and 68 displayed at least one abnormal sperm parameter. The researchers performed 16S rRNA sequencing on the semen of both fertile and subfertile subjects, revealing no overall difference in the microbial composition between the two groups, nor any effect of either alpha or beta diversity of the microbiota on sperm parameters (36). Specific genera were identified as being more prevalent in normospermic males (*Lactobacillus* and *Staphylococcus*) in comparison to the subfertile males, in whom *Prevotella* genus was recorded in higher abundance (36).

DISCUSSION

As human microbiome research progresses globally at an exponential pace, the importance of various organ and tissue microbiomes is recognised in relation to health and disease. Seminal, testicular and genitourinary microbiota have been identified more comprehensively since the advent of next generation genomic sequencing in comparison to basic culturing techniques, which has provided the scope for mapping the microbiomes of these regions. A better understanding has arisen of the crosstalk that exists between microbial and human cells such as spermatozoa, and how these germ cells are influenced by microbial metabolites during their development, which phenomenon has the potential to be harnessed for therapeutic benefit in terms of probiotic therapy to improve sperm quality (40, 319).

With an understanding of the holographic interconnectedness of human anatomical and physiological systems and their functions), it is unlikely that any one organ or tissue exists or operates in isolation from others in the same organism (498). The functional continuum

of the gut/immune/nervous/endocrine systems, mediated by metabolic and inflammatory processes that affect both local and distant tissues, implicates an influence on human fertility (319). The immune system learns to tolerate its growing intestinal microbial biomass throughout development from foetal to adult life, leading to a symbiotic state of regulation with the internal and external environments, especially in the gastrointestinal tract where the microbiome dominates by sheer numbers (499). It is recognised that the blood-testis barrier is maintained by the same adhesion proteins, such as zonulin, that modulate intestinal mucosal permeability (500) and that toxins that increase intestinal permeability may also do the same for the testis, exposing developing gametes to molecular injury (235).

Inflammatory bowel disease is often associated with male infertility; however, until now the link has been attributed to complications of medical (specifically sulphasalazine treatment) and surgical management of these cases (501). Genitourinary infections are common predisposing causes of poor sperm quality and function; however, intestinal infections or infestations are yet to be explored for the possibility of playing a role in the causation of male infertility, considering their contribution to producing systemic inflammation (502).

IMMUNITY AND THE TESTIS

To respond to microbial attack, the testis recruits innate immune responses to microbial infections. It is necessary for the testis to co-ordinate both processes of immune privilege and local innate immunity to enable maintenance of immune homeostasis. Disruption of this homeostasis can result in testicular inflammation and production of abnormal sperm, impacting fertility (503).

THE GASTROINTESTINAL MICROBIOME

The bacteria, viruses, fungi, parasites and archaea that inhabit the gastrointestinal tract play essential roles for the host organism, in terms of nutrient digestion for energy production, neurotransmitter and vitamin production, immune regulation, and maintenance of the mucosal barrier. The interface between the gastrointestinal tract and other organs, such as liver, lung, skin, and in particular, the brain, has been the subject of

extensive research over the past decade, identifying molecular crosstalk between separate systems. Only recently has such a connection between explored between the gut and the testis via the blood-testis barrier.

THE BLOOD-TESTIS BARRIER (BTB)

Similar to the blood-brain barrier, which acts as a gatekeeper membrane, protecting delicate cerebral tissue from infection and a variety of exogenous and endogenous toxins, the blood-testis barrier (BTB) also exists; the BTB relies on the integrity of tight junctions between the Sertoli cells, the peritubular myoid cells and interstitial epithelial cells of the testis to protect spermatogonia from immune attack, rendering the testicular tissue an 'immune-privileged site' (235). The permeability of the BTB, under the influence of testosterone and cytokines (236), allows the passage of spermatocytes into the seminiferous tubules where meiosis occurs. Xenobiotics, toxins from tissue infections, endotoxins from intestinal putrefaction, toxic environmental chemical substances, as well as physical injury and heat stress, may damage DNA during spermatogenesis, resulting in poor sperm quality and impaired fertility (15).

The selectivity of the BTB can be weakened by bacterial toxins, but conversely protected by beneficial bacterial flora in the semen, which can manipulate intercellular adhesion molecules, occludin, zonulin-2 and E-cadherin, to alter the permeability of the BTB (131). The non-selectivity of a porous BTB membrane can be recovered by exposure to butyrate, restoring levels of intercellular adhesion molecules, as also occurs with the action of butyrate on intestinal mucosal cells (504). Furthermore, murine studies demonstrate that levels of the hormones FSH, LH and testosterone are lower in germ-free male mice, hence it is posited that the intestinal microbiome influences the production and regulation of these sex hormones in men, necessary for normal sexual functioning (131) and optimal male fertility.

INTESTINAL DYSBIOSIS

An altered microbial composition of the intestinal environment that disrupts metabolic activity and harms the host has been termed 'intestinal dysbiosis' (505). This condition is often a sequela of antibiotic therapy and is now purported to contribute to the

development of both functional and inflammatory bowel disorders in adults and children (506, 507, 508, 509, 510). A dysbiotic intestinal microbiome can produce endotoxins, such as pro-inflammatory lipopolysaccharides secreted by gram-negative bacteria, which can injure mucosal membranes and damage their tight intercellular junctions, rendering these membranes more permeable (511). Hence it is posited that this process may also impact the BTB and that, for example, antibiotic therapy may potentially adversely affect male fertility (275).

CONCLUSIONS

Very little research has been directed to date towards exploring the various male genitourinary microbiomes and their significance in influencing healthy spermatogenesis and male fertility. Although the semen microbiomes of fertile and subfertile men are not clearly distinguishable, one bacterial species, *Anaerococcus*, has been purported as a possible biomarker of male infertility. Normal semen contained higher proportions of *Lactobacillus* and *Gardnerella* genera, while *Prevotella* predominated in the lower quality semen samples. Genitourinary microbiota are transient occupants of the lower urethra in men, influenced by sexual intercourse and their female partner's vaginal microbiome; hence, defining a healthy semen microbiome continues to defy researchers in this field.

APPENDIX D

Summary table of semen quality parameters and semen microbiome characteristics for miniature pony stallions (Chapter 2)

TABLE 2.1 SIX SEMEN QUALITY BIOMARKERS AND THREE SEMEN MICROBIOME MEASURES FOR THE FOUR MINIATURE PONY STALLIONS

PONY	Total Count $x10^6$	% Motile	% Progressive Motility	% Normal Morphology	% DNA Intact	% Total 4HNE Positive	% Live MSR Positive	Faith_pd/ Richness	Evenness	Shannon Diversity Index
B	1.84	3.88	17.9	53	82.7	48.4	10.3	13.50	0.66	3.89
C	5.05	69.05	22.6	34.5	93.85	66.7	12.3	15.87	0.72	4.39
P	4.675	70.05	37.15	59.5	79.25	62.7	17.2	31.79	0.73	3.92
Z	17.3	74.8	26.95	62	88.75	35.8	15.5	79.92	0.70	4.40

APPENDIX E

CONFERENCE ABSTRACTS

International Conference on Probiotics and Prebiotics (PROBIOTICS 2022)

Rome, Italy, June 15-16, 2022

Presentation: Equine pro/pre/symbiotic supplementation for fertility enhancement

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ABSTRACT:

How does synbiotic supplementation of the standard equine diet affect both the intestinal and semen microbiomes? To date, no research has been conducted in this domain nor explored this particular question.

Commercial pre- and probiotics are used widely as nutritional supplements and treatment interventions in the management of livestock and companion animals, although evidence to support their use in equine health and disease is currently lacking. Beyond gastrointestinal health, there is a little evidence to support the use of probiotics in the

health maintenance and disease management of horses and none in relation to stallion fertility. Studies have largely focussed on the prevention and treatment of gastrointestinal disease using human probiotic species and strains.

Probiotics are defined by the World Health Organisation (WHO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host'. To date, an extensive number of species and sub-species from the lactobacillus and bifidobacterium genera have been evaluated, while Streptococcus thermophilus and Escherichia coli strains have also received research attention in human health. Many of these probiotic species are regularly administered to horses with little evidence for therapeutic benefit. Prebiotics are non-digestible food ingredients that promote the growth of such beneficial microorganisms in the intestines and are marketed for use in supporting equine digestion, metabolism, growth and immunity. Synbiotics are supplements that contain combinations of prebiotics and probiotic bacteria and/or yeasts. Both are commercially available and promoted for use in enhancing equine athletic performance and reducing morbidity associated with intestinal disease.

Our research team conducted two reviews into the use of pro- and pre-/synbiotics respectively in equine management; we discovered that an opportunity exists for further research to characterise the microbiome of various equine tissue sites to assist in the development of safe and effective therapeutic formulations. It is also evident from our research that male infertility in both horses and humans has been neglected in the rapidly growing research sphere of the microbiome.

In a crossover intervention trial, we enlisted a small cohort of miniature stallions for standard feed, prebiotic beet flakes, a commercial probiotic formulation or a combination of beet flakes and probiotics. Faecal and semen samples were collected prior to and following each treatment period for PacBio 16S-RNA genomic processing to identify bacterial communities, as well as semen analysis for various health and fertility parameters. Our research has identified key microbiome species correlating to sperm quality, for which research there is not current precedent. We also identified colonies of key nascent semen and faecal microbiome species improved by synbiotic

supplementation. Lead by our research, potential exists to develop equine-specific synbiotic formulations to improve stallion sperm quality and hence fertility, which could prove very valuable in the management of equine athletes

KEY LEARNINGS

Insights into the characterisation of the semen and intestinal microbiomes of stallions and gaining an understanding of how that might impact their fertility

Discussion of the current use of prebiotics and probiotics in equine management, for application by veterinarians in clinical practice

Potential for this equine probiotic intervention study concept to be applied to human fertility research, with the possible development of pro/synbiotics to improve sperm quality and male fertility outcomes

Presentation Category: Veterinary research using probiotics and prebiotics

Session Name: Benefits of Probiotics and Prebiotics OR Beneficial microbes from humans and animal intestines

INTERVIEW FOR ARTICLE

"What Research is Saying About Supplements for Horses**", *The Horse*** online article posted by Stacey Oke, DVM, MSc, December 5, 2022. Interviewed regarding probiotic use in horses following publication in JEVS