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Exploration du rôle du microbiote intestinal équin dans l'intoxication à l'hypoglycine A : acteur ou témoin ? Éléments de compréhension issus d'approches *in vivo* et *in vitro*.

Exploring the role of the equine intestinal microbiota in hypoglycin A intoxication: player or witness? Insights from *in vivo* and *in vitro* studies.

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Abbreviations

16S rRNA	16S ribosomal RNA
Acyl-	Acyl group
AM	Atypical Myopathy
AMAG	Atypical Myopathy Alert Group
AMOVA	Analysis of Molecular Variance
ANOSIM	Analysis of Similarities
ASVs	Amplicon Sequence Variants
ATD	Acute Toxic Dose
AT pairs	Adenine–Thymine pairs
BCAT	Branched-Chain Amino Acid Aminotransferase
BCAAs	Branched-Chain Amino Acids
BCKA	Branched-Chain Keto Acid
BCKDHc	Branched-Chain α-Keto Acid Dehydrogenase Complex
BW/meal	Body Weight per meal
CFU	Colony-Forming Units
CK	Creatine Kinase
cDNA	Complementary DNA
db-RDA	Distance-based Redundancy Analysis
ddNTPs	Dideoxynucleoside Triphosphates
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EGS	Equine Grass Sickness
EMS	Equine Metabolic Syndrome
FAD	Flavin Adenine Dinucleotide
FISH	Fluorescent In Situ Hybridisation
FOXP3	Forkhead Box P3
GC pairs	Guanine–Cytosine pairs
GIT	Gastrointestinal Tract
HGA	Hypoglycin A (Methylenecyclopropylalanine)
HGB	Hypoglycin B
HMI™	Host-Microbiota Interaction
IL-10	Interleukin-10
IL-17	Interleukin-17A
IVD	Isovaleryl-CoA Dehydrogenase
JVS	Jamaican Vomiting Sickness
km	Michaelis Constant
LCAD	Long-Chain Acyl-CoA Dehydrogenases
MCAD	Medium-Chain Acyl-CoA Dehydrogenases
MCPA-CoA	Methylenecyclopropylacetyl-CoA
MCPA-carnitine	Methylenecyclopropylacetyl-Carnitine
MCPA-glycine	Methylenecyclopropylacetyl-Glycine
MCPrG	Methylenecyclopropylglycine

M-SHIME	Mucosal Simulator of the Human Intestinal Microbial Ecosystem
MTD	Maximum Tolerated Dose
NADH-TR	Nicotinamide Adenine Dinucleotide-Tetrazolium Reductase
NGS	Next Generation Sequencing
NMDS	Non-metric Multidimensional Scaling
OTU	Operational Taxonomic Unit
PCA	Principal Component Analysis
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
Picrust2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
qPCR	Quantitative PCR
RDA	Redundancy Analysis
RESPE	Réseau d'Épidémio-Surveillance en Pathologie Équine
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription PCR
SCAD	Short-Chain Acyl-CoA Dehydrogenases
SCFAs	Short-Chain Fatty Acids
SHIME	Simulator of the Human Intestinal Microbial Ecosystem
T cells	T Lymphocytes
T-RFLP	Terminal Restriction Fragment Length Polymorphism
TGGE	Temperature Gradient Gel Electrophoresis
UniFrac	Unique Fraction
yo	Years Old

Résumé - Abstract

La méthylènecyclopropylalanine, appelée également hypoglycine A, et la méthylènecyclopropylglycine sont des protoxines responsables d'intoxications sévères tant chez l'homme que chez l'animal. Chez les chevaux, l'hypoglycine A a été la première protoxine identifiée comme responsable de la myopathie atypique, une intoxication saisonnière associée à une létalité élevée. Ces protoxines ne sont pas intrinsèquement toxiques mais le deviennent par un processus enzymatique impliquant d'abord l'aminotransférase des acides aminés branchés, puis le complexe déshydrogénase des α-céto-acides branchés. Cette conversion métabolique conduit à la formation du méthylènecyclopropylacétyl-CoA à partir de l'hypoglycine A et du méthylènecyclopropylformyl-CoA à partir de la méthylènecyclopropylglycine. Ces métabolites toxiques perturbent notamment le métabolisme lipidique provoquant une accumulation d'acylcarnitines dans le sérum des chevaux intoxiqués et sont éliminés par le sang et les urines après conjugaison à la carnitine et à la glycine respectivement.

Des travaux menés chez l'homme, les chevaux et d'autres herbivores suggèrent un lien entre le microbiote intestinal et l'intoxication par l'hypoglycine A. Certaines populations bactériennes pourraient intervenir dans la biotransformation des protoxines, soit en facilitant leur activation en métabolites toxiques, soit en contribuant à leur dégradation en composés non-toxiques avant leur absorption par l'hôte. Les perturbations du métabolisme énergétique induites par la toxine chez l'hôte pourraient en retour remodeler l'écosystème intestinal en sélectionnant des communautés microbiennes susceptibles d'aggraver ou d'atténuer la maladie. Des altérations de la composition microbienne pourraient également compromettre l'intégrité de la barrière intestinale et favoriser le passage systémique des protoxines ou de leurs dérivés.

Dans une première étude *in vivo*, des matières fécales ont été collectées chez des chevaux atteints de myopathie atypique (survivants et non-survivants), chez des chevaux pâturant sur la même prairie sans présenter de signe clinique (*i.e.*, les co-pâturants), ainsi que chez des chevaux contrôles (*i.e.*, sans hypoglycine A ou méthylènecyclopropylacétyl-carnitine dans le sang). Les échantillons ont été analysés par séquençage du gène 16S rDNA (régions V1–V3) afin de comparer la composition microbienne entre groupes et de corréler les variations bactériennes avec les différents paramètres sanguins diagnostiques de la myopathie atypique (hypoglycine A, méthylènecyclopropylacétyl-carnitine et acylcarnitines). Le groupe contrôle présentaient une α-diversité statistiquement plus élevée avec une meilleure équitabilité des populations bactériennes. De plus, ce groupe se distinguait des autres sur la base des analyses de β-diversité, avec notamment des différences concernant *Clostridia_ge*, *Bacteria_ge*, *Firmicutes_ge*, *Fibrobacter* et *NK4A214_group*. Les variations de composition des populations bactériennes fécales étaient significativement corrélées aux concentrations sanguines du méthylènecyclopropylacétyl-carnitine et de la C14:1 laissant penser que ces variations étaient davantage représentatives des perturbations énergétiques de l'hôte. Les prédictions fonctionnelles réalisées avec l'outil Picrust2 ont suggéré la présence théorique des enzymes

impliquées dans la transformation de l'hypoglycine A, sans toutefois permettre de conclure sur une éventuelle transformation en méthylènecyclopropylacétyl-carnitine par le microbiote intestinal. La conclusion de cette étude est que le microbiote fécal des chevaux contrôles différait de celui des co-pâturants et des chevaux intoxiqués : cette différence est en partie corrélée à certains paramètres sanguins propres de l'intoxication. Néanmoins, il n'est pas possible de conclure formellement sur l'origine de ces changements ; c'est-à-dire si ces changements sont dus à une exposition directe aux protoxines ou aux altérations métaboliques de l'hôte. Des systèmes de fermentation *in vitro* pourraient contribuer à clarifier ce point.

Dans une seconde étude *in vitro*, des matières fécales provenant de chevaux contrôles ont été regroupées dans un système dérivé du SHIME® représentant six côlons descendants équins réparti en deux groupes : trois fermenteurs « contrôles » et trois fermenteurs « traités avec de l'hypoglycine A ». Deux autres fermenteurs, contenant uniquement le milieu nutritif sans inoculum fécal, ont servi à évaluer la stabilité de l'hypoglycine A. Le système a fonctionné durant 48 heures en anaérobiose avec des paramètres de température et de pH contrôlés par ordinateur. L'hypoglycine A est restée stable dans le milieu nutritif. Cependant, sa concentration a significativement diminué dans les fermenteurs contenant les matières fécales sans augmentation concomitante du méthylènecyclopropylacétyl-carnitine. Le microbiote est donc capable de dégrader l'hypoglycine A en composés non toxiques comme rapporté chez d'autres espèces. Par ailleurs, les fermenteurs contrôles présentent une α-diversité significativement plus élevées que les fermenteurs traités avec l'hypoglycine A, avec une représentation microbienne (*i.e.*, équitabilité) plus équilibrée. Le genre *Paraclostridium* était présent dans le groupe témoin mais absent du groupe traité. Une hypothèse serait que l'hypoglycine A pourrait entrer en compétition avec la leucine ou d'autres acides aminés branchés dans la fermentation de Stickland, induisant un déficit énergétique pour cette population bactérienne.

En conclusion, ces deux études démontrent que le microbiote intestinal équin est influencé par la présence d'hypoglycine A. Les populations bactériennes s'accommodent aux désordres métaboliques énergétiques de l'hôte, suggérant un rôle de témoin dans l'intoxication, et réduisent les niveaux de protoxine en la transformant en dérivés non toxiques, prônant un rôle protecteur vis-à-vis de l'hôte. Toutefois, contrairement aux ruminants, chez qui la fermentation microbienne précède l'intestin grêle et limite l'absorption des protoxines, le microbiote du cheval se situe en aval du principal site d'absorption des acides aminés. Ainsi, l'hypoglycine A peut rejoindre la circulation sanguine avant toute dégradation microbienne, rendant les chevaux plus sujets à cette intoxication. Ces résultats ouvrent des perspectives tant pour la recherche que pour la prévention comme l'usage d'hypoglycine A marquée isotopiquement pour suivre son devenir métabolique dans l'intestin et le développement de stratégies préventives visant à promouvoir certaines populations bactériennes capables de dégrader cette protoxine.

Methylenecyclopropylalanine, also known as hypoglycin A, and methylenecyclopropylglycine are protoxins responsible for severe intoxications in both humans and animals. In equids, hypoglycin A was the first protoxin identified as being involved in atypical myopathy; a seasonal intoxication with a high mortality rate and characterised by clinical signs such as weakness, recumbency, myoglobinuria, stiffness, and depression. These protoxins are not inherently toxic but are activated through a two-step enzymatic process involving first branched-chain amino acid aminotransferase and subsequently the branched-chain α-keto acid dehydrogenase complex. This metabolic conversion results in the formation of methylenecyclopropylacetyl-CoA from hypoglycin A and methylenecyclopropylformyl-CoA from methylenecyclopropylglycine, both of which disrupt lipid metabolism, leading to an accumulation of acylcarnitines in the serum of affected horses. These toxic metabolites are eliminated via the blood and urine after conjugation with carnitine and glycine, respectively.

Studies in humans, equids, and other herbivores suggest a link between gut microbiota and HGA poisoning, with specific populations potentially biotransforming protoxins by either activating them into harmful metabolites or degrading them into detoxified forms. Toxin-induced metabolic disturbances may in turn reshape the gut environment, selecting microbial communities that aggravate or mitigate disease, while alterations in composition could also impair barrier integrity and enhance systemic uptake of protoxins or their derivatives.

In a first *in vivo* study, faeces were collected from horses with atypical myopathy (survivors and non-survivors), from horses grazing on the same pasture without clinical signs, and from control horses free of hypoglycin A or toxic metabolites. Samples were analysed by 16S rDNA sequencing (V1–V3 regions) to compare microbiota composition across groups and to correlate bacterial genera with diagnostic blood parameters including hypoglycin A, methylenecyclopropylacetyl-carnitine, and acylcarnitines. Control horses showed significantly higher α-diversity and were clearly distinct from other groups in β-diversity analyses, with notable differences involving *Clostridia_ge*, *Bacteria_ge*, *Firmicutes*_ge, *Fibrobacter*, and *NK4A214_group*. These variations in faecal bacterial composition were significantly correlated with blood levels of methylenecyclopropylacetyl-carnitine and C14:1, indicating that they mainly reflect the host's energy imbalance. Functional prediction with Picrust2 suggested the potential presence of relevant enzymes, although no conclusion could be drawn regarding microbial transformation of hypoglycin A. Overall, the faecal microbiota of control horses differed from that of cograzers and intoxicated individuals, but it remains

uncertain whether these shifts reflected direct protoxin exposure or secondary host metabolic effects. *In vitro* fermentation systems may help disentangle these mechanisms.

In a second *in vitro* study, faeces from horses considered as control (*i.e.*, free of protoxin or toxic metabolite in their blood test) were pooled in a fermentation system derived from SHIME®. This batch system was adapted to represent six descending colons of horses. Hypoglycin A was added in three of them while the other three were used as a control group. Two additional fermenters were prepared with the nutritive media without any faecal incubation to assess the stability of hypoglycin A. The system ran for 48 hours with parameters as anaerobiosis, temperature and pH maintained constant. Hypoglycin A remained stable in the nutritive media while a significant decrease was recorded in the fermenters due to faecal microbiota. Moreover, no methylenecyclopropylacetyl-carnitine was present allowing the hypothesis that microbiota is able to degrade or to transform hypoglycin A in a non-toxic compound playing a protective role. This is coherent with the results of studies in other species. Furthermore, significant differences were found in the α-diversity and β-diversity between hypoglycine A treated fermenters and control fermenters with a more balanced representation of bacterial populations in the latest group. One genus, *Paraclostridium*, was present in the control group and absent in the other group suggesting that hypoglycin A might compete with leucine or other branched-chained amino acids in the Stickland fermentation and, consequently, leading to an energy deficit for this population.

Taken together, these studies demonstrate that the equine gut microbiota is directly influenced by the presence of hypoglycin A. Bacterial populations adapt to the host's energy-related metabolic disorders, suggesting a role as indicators of host toxicity, and reduce protoxin levels, probably by transforming it into non-toxic derivatives, suggesting a protective role for the host. However, in contrast to ruminants, where microbial fermentation occurs prior to the small intestine and can prevent systemic absorption of protoxins, the equine hindgut microbiota is located downstream of the main site of amino acid absorption. As a result, hypoglycin A can enter the bloodstream before microbial degradation takes place, rendering horses particularly susceptible to atypical myopathy. These findings open several perspectives. Future studies using isotopically labelled hypoglycin A could help clarify its precise metabolic fate within the gut environment. In addition, identifying and promoting bacterial taxa with the capacity to degrade hypoglycin A may represent a novel preventive strategy, either through targeted probiotics or nutritional interventions designed to enhance the detoxifying potential of the equine microbiota.

Foreword

Evidence linking the horse's gut microbiota to certain diseases is expanding. Among these diseases is equine atypical myopathy (AM), a pasture-associated intoxication that occurs during autumn and spring in European regions. Several findings have raised the hypothesis that the intestinal microbiota may influence this poisoning. Research in this field mainly aims to improve prevention and/or treatment strategies to reduce the number of cases. Currently, there is no specific treatment for AM, making prevention a key strategy against this commonly fatal disease, which is characterized by clinical signs such as pigmenturia, generalized weakness, stiffness, tachycardia, and lateral recumbency (Van Galen et al., 2012a). The disease is associated with the ingestion of seeds and seedlings from *Acer pseudoplatanus*, which contain the protoxins hypoglycin A (HGA; methylenecyclopropylalanine) and methylene-cyclopropylglycine (MCPrG) (Fowden and Pratt, 1973; van der Kolk et al., 2010; Votion et al., 2007). Although not toxic *per se*, these compounds are converted through a two-step enzymatic process into toxic metabolites (Melde et al., 1991, 1989) that inhibit fatty acid β-oxidation and branched-chain amino acid metabolism (Billington et al., 1978; Ikeda and Tanaka, 1990; Kean, 1976; Osmundsen and Sherratt, 1975; Tanaka, 1972; Von Holt et al., 1966; von Holt et al., 1964).

In *Acer* spp., HGA occurs at higher concentrations than MCPrG and is therefore considered the main toxic compound involved in AM cases (El-Khatib et al., 2022; Fowden and Pratt, 1973). Although MCPrG has also been detected in affected horses (Bochnia et al., 2019), its investigation began later due to the recent availability of analytical standards and suitable detection methods, which initially limited toxicological and diagnostic studies to HGA. Both HGA and MCPrG exist in plants as γ-glutamyl derivatives (*i.e.*, hypoglycin B (HGB) and γ-glutamyl-MCPrG, respectively) (Fowden and Pratt, 1973), which act as reservoirs for their parent compounds and thus have a bioavailability rather than a toxicodynamic role (Bowen-Forbes and Minott, 2011; Brown et al., 1992). Moreover, HGB is significantly less toxic than HGA (Hassall et al., 1954; Hassall and Reyle, 1955a) and is not believed to contribute directly to clinical intoxication.

Importantly, HGA intoxication has been reported not only in equids but also in other animal species (Brooks and Audretsch, 1971; Bunert et al., 2018; Chen et al., 1957; Feng and Patrick, 1958; Hassall et al., 1954; Hassall and Reyle, 1955a; Hirz et al., 2021; Jordan and Burrows, 1937; Renaud et al., 2022; Scott, 1916) and even in humans (Hassall et al., 1954; Hassall and Reyle, 1955a; Tanaka et al., 1976) . Furthermore, HGA has been detected in animal-derived foodstuffs such as equine muscle (Sander et al., 2023) and milk from both mares (Renaud et al., 2021) and cows (Bochnia et al., 2021; El-Khatib et al., 2023; Engel et al., 2023), raising potential concerns for food safety.

Given the predominant role of HGA in the pathogenesis of AM, its broader toxicological relevance across species, its detection in animal-derived food products, and its long-standing availability for toxicological research, this doctoral research has specifically focused on this protoxin.

Introduction

Hypoglycin A is one of the causative agents of AM in equids (Valberg et al., 2013; Votion et al., 2014). Hypoglycin A intoxication may also affect several species (Brooks and Audretsch, 1971; Bunert et al., 2018; Chen et al., 1957; Feng and Patrick, 1958; Hassall et al., 1954; Hassall and Reyle, 1955a; Hirz et al., 2021; Jordan and Burrows, 1937; Renaud et al., 2022; Scott, 1916) including humans (Hassall and Reyle, 1954; Tanaka et al., 1976). In the latter, HGA has been identified as the likely causative agent of Jamaican vomiting sickness (JVS), following its isolation from the fruit (*i.e.*, ackee) of *Blighia sapida* (*Sapindaceae*) and the detection of its toxic metabolite in the urine of patients (Hassall et al., 1954; Hassall and Reyle, 1955a; Tanaka et al., 1976). The clinical picture of JVS is characterised by a sudden onset of vomiting, primarily during the cooler months of the year (Hassall and Reyle, 1955a; Scott, 1916), and possibly followed by convulsions, coma, and death (Bressler et al., 1969; Jordan and Burrows, 1937). A profound hypoglycaemia, a depletion of liver glycogen stores (Chen et al., 1957; Hassall and Reyle, 1955a; Jelliffe and Stuart, 1954) and encephalopathy are reported (Blake et al., 2006; Melde et al., 1991; Tanaka et al., 1976). This condition is frequently fatal within 3 to 4 days if left untreated (Bressler et al., 1969; Jordan and Burrows, 1937).

Poisoning due to HGA has also been demonstrated in laboratory animals such as mice, rats, guinea pigs, canaries, chicks, pigeons, rabbits, dogs, cats, and monkeys (Brooks and Audretsch, 1971; Chen et al., 1957; Hassall et al., 1954; Hassall and Reyle, 1955a; Jordan and Burrows, 1937; Scott, 1916) as well as in ruminant species such as Père David's deer (Bunert et al., 2018), two-humped camels (Hirz et al., 2021), and gnus (Renaud et al., 2022).

For all these species, HGA is not toxic by itself. After ingestion, HGA is converted into its active CoA derivative: methylenecyclopropylacetyl-CoA (MCPA-CoA). This metabolite disrupts lipid metabolism primarily by inhibiting fatty acid β-oxidation (Billington et al., 1978; Ikeda and Tanaka, 1990; Kean, 1976; Osmundsen and Sherratt, 1975; Tanaka, 1972; Von Holt et al., 1966; von Holt et al., 1964), impairs certain steps in branched-chain amino acids (BCAAs) catabolism (Tanaka, 1972; Tanaka et al., 1971), and decreases mitochondrial respiration (Billington et al., 1978; Kruse et al., 2021; Lemieux et al., 2016; Von Holt et al., 1966). The protoxins conversion into active metabolites is achieved through a two-step enzymatic process involving the branched-chain amino acid aminotransferase (BCAT) and the branched-chain α-keto acid dehydrogenase complex (BCKDHc) (Melde et al., 1991, 1989).

Evidence from studies in humans (Tanaka et al., 1976), equids (Karlíková et al., 2016; Wimmer-Scherr et al., 2021) and herbivorous species (Engel et al., 2025; Renaud et al., 2022) suggests a potential involvement of the gut microbiota in the development of HGA poisoning. In affected individuals, researchers have reported a decrease in urinary hippurate (*i.e.*, a microbial metabolite of benzoic acid conjugated with glycine (Lees et al., 2013)) in both humans (Tanaka et al., 1976) and horses (Karlíková et al., 2016). Additionally, in horses with AM, levels of indole-containing compounds (*i.e.*, derived from microbial metabolism of tryptophan) were also reduced (Karlíková et al., 2016), suggesting a possible disruption in microbial diversity or metabolic activity during intoxication.

Further *in vivo* findings support this hypothesis. In horses suffering from AM, the faecal microbiota composition was significantly altered compared to healthy cograzers (*i.e.*, horses sharing the same pasture as a case of AM but showing no clinical signs of intoxication) (Wimmer-Scherr et al., 2021). However, the interpretation of these findings is limited by the recognition of subclinical cases among cograzers. Some of these animals have been found to carry elevated HGA levels and display early signs of lipid metabolism disruption in the absence of overt clinical symptoms (Renaud et al., 2024).

Complementary insights have emerged from studies in other herbivores. In sheep, *in vitro* fermentation experiments revealed a reduction in protoxin concentrations within the rumen, without concurrent formation of toxic metabolites (Engel et al., 2025). This finding suggests that microbial fermentation chambers located before the site of amino acid absorption might offer protection by degrading the protoxins before they can be absorbed and activated systemically as previously suggested in other ruminant species by (Renaud et al., 2022).

These findings raise the hypothesis that the intestinal microbiota may influence host susceptibility to HGA intoxication. Certain microbial populations could contribute to the bioactivation of protoxins into toxic metabolites, or conversely, promote their degradation or detoxification prior to absorption. Also, disruption of host energy metabolism by the toxins might alter the gut environment, favouring microbial communities that exacerbate or mitigate disease. Additionally, shifts in microbiota composition may compromise gut barrier integrity, potentially increasing absorption of protoxins or metabolites.

This doctoral work combines both *in vivo* and *in vitro* approaches to investigate the role of the intestinal microbiota in HGA intoxication in horses. By characterising the microbiota

composition and its potential functional interactions with the protoxin, this research aims to determine whether the microbiota contributes directly to the pathogenesis of AM as an "actor," or instead serves as a "witness" of the disease, mirroring host physiological alterations through adaptive shifts in its composition.

1. General Concepts about Intestinal Microbiota

The growing interest in microbiota research reinforces the relevance of such investigations. Studying the microbiota is challenging due to its vast complexity. An analysis on veterinary gut health research recorded 1696 documents published between 2000 and 2020, with an annual growth rate of 22.4% and a peak of 417 publications in 2020 (Colombino et al., 2021). In December 2024, a PubMed search for the term "Microbiota" yields over 152,000 records, and "Gut Microbiota" returns nearly 90,000 results, with the earliest entries dating from 1956 and 1977 respectively. Within this expanding field, equine microbiota research has also gained momentum. A search combining "Microbiota AND Horses" retrieves 506 entries, of which 456 specifically concern the gut microbiota and were published between 2015 and 2025. These figures illustrate the rapid growth and increasing scientific interest in understanding the horse's intestinal microbial ecosystem.

In many mammalian herbivores, significant differences in gut microbiota have been observed between wild and captive individuals. However, such differences appear to be less pronounced in horses than in other mammals (Ang et al., 2022; de Jonge et al., 2022; Edwards et al., 2020; Metcalf et al., 2017; Su et al., 2020; Wen et al., 2022; Zhao et al., 2016). This relative stability supports the relevance of studying domestic horses to gain insight into equine gut microbiota under controlled or real-world conditions. Accordingly, this thesis focuses on the digestive microbiota of domesticated horses.

1.1. Definitions and Functions of the Microbiota

The origin of the definition of the word "*microbiota*" itself has sparked debate among scientists. While some authors attribute its definition to Lederberg and McCray in 2001 (Lederberg and McCray, 2001; Marchesi and Ravel, 2015), others argue that the term was

already in common use in microbiology for at least 50 years prior (Prescott, 2017). In the 1960s, when germ-free and specific pathogen-free animal models became standard in laboratory research, studies aimed to characterise the "selected microbiota compatible with sustained health" (Prescott, 2017). Moreover, the term "microbiota" was also employed earlier in the context of rumen microbial ecology, further confirming that its usage predates the definition attributed to Lederberg and McCray (Hungate, 1966).

Beyond this historical note, the terms "microbiota" and "microbiome" must now be clearly distinguished to structure future research (Costa and Weese, 2019, 2018). Microbiota designates the assemblage of microorganisms present in a defined environment, and its use is particularly suited to studies investigating taxonomic composition (*e.g.*, sequencing of the 16S rRNA gene) (Costa and Weese, 2019). The microbiome refers to the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (*i.e.*, genes), and the surrounding environmental conditions (Marchesi and Ravel, 2015): in other words, the microbiome is characterised by its microbial community composition (diversity and structure) and its theatre of activities (metabolic functions and resulting end products) (Fox et al., 2024; Julliand and Grimm, 2016; Tardiolo et al., 2025). Consequently, the term "microbiome" should be reserved for studies investigating the entire genetic content of a microbial community, typically through metagenomic approaches (Costa and Weese, 2019). The "gut microbiota" is defined as the collection of microorganisms colonising the gastrointestinal tract (GIT) (Huttenhower et al., 2012). The "core microbiome" refers to a set of microbial populations present in a stable and coherent manner in the microbiota of a specific environment independently of individual variations. These micro-organisms often play an essential role in the stability and ecological functions of the microbiota (Sharon et al., 2022). All these definitions must also be applied in scientific research concerning horses (Costa and Weese, 2019, 2012; Kauter et al., 2019).

The GIT and its microbial community form a dynamic ecosystem, tightly interconnected with the host. This interaction is bidirectional, as the host environment shapes microbial composition, while microbial activity influences host physiology. Communication occurs both locally, through the enteric nervous system, and systemically via neural pathways linking to the central nervous system (Adekoya et al., 2025; Jin et al., 2024; Merritt and Julliand, 2013).

The intestinal microbiome contributes significantly to host health via both metabolic and protective functions. On the metabolic side, it enables the degradation of complex dietary substrates (*i.e.*, such as non-digestible polysaccharides and plant-derived components) that the human host cannot enzymatically process, thereby enhancing energy harvest. For instance, microbial fermentation in the colon produces short-chain fatty acids (SCFAs: acetate, propionate, butyrate) which not only supply energy to colonocytes but also regulate host glucose and lipid metabolism and influence gut motility (Martin-Gallausiaux et al., 2021). In addition, the gut microbiota acts as a source of essential micronutrients by synthesising B-group vitamins and vitamin K (LeBlanc et al., 2013; Tarracchini et al., 2024; Zhan et al., 2024). A major contribution is the production of short-chain fatty acids (SCFAs), which provide energy to colonocytes, regulate host metabolism, and influence gut motility (Martin-Gallausiaux et al., 2021). Beyond these metabolic activities, the gut microbiota plays a central role in host protection: it strengthens mucosal immunity, promotes antigen tolerance, and acts as a barrier against pathogens by competing for nutrients and adhesion sites on the intestinal epithelium. Moreover, it produces antimicrobial compounds (such as SCFAs and bacteriocins), thereby maintaining intestinal homeostasis (Iacob and Iacob, 2019; Williams et al., 2001). Disruptions in these microbial communities have been linked to a wide range of inflammatory and metabolic disorders (*e.g.*, metabolic syndrome, type 2 diabetes) (Fox et al., 2024; Paul et al., 2025; Rodriguez et al., 2015a).

In equine species, although the literature is less abundant than in humans, comparable metabolic and protective roles of the intestinal microbiome are documented. As hind-gut fermenters, horses rely on microbial communities in their caecum and colon to degrade structural plant carbohydrates and other complex substrates not digestible by the host alone, yielding SCFAs which contribute a substantial portion of the horse's maintenance energy (Wunderlich et al., 2023). Moreover, the equine gut microbiota has been shown to influence immune and barrier functions: shifts in microbial composition are associated with colic or other gastrointestinal disorders (Boucher et al., 2024; de Jonge et al., 2022). Although direct equine data on microbial synthesis of vitamins or bacteriocins are scarce, the presence of similar functional microbial fermentations and barrier-protection mechanisms suggests that the horse's microbiota likewise provides micronutrient support and protection against pathogens albeit with species-specific nuances (Kauter et al., 2019). Overall, the equine

intestinal microbiota performs essential roles in energy metabolism, micronutrient assimilation, epithelial integrity and host defence.

In the equine hindgut, specific bacterial taxa, such as unclassified *Clostridiales*, or members of the *Lachnospiraceae* and *Ruminococcaceae* families, have been associated with the expression of key inflammatory and immunomodulatory genes like FOXP3 (or forkhead box P3, *i.e.*, a transcription factor crucial for the development and function of regulatory T cells which are immune cells playing a central role in maintaining immune tolerance and preventing autoimmune responses.), IL-10 (*i.e.*, the gene encoding for interleukin-10, a major anti-inflammatory cytokine, that plays an essential role in regulating immune responses to prevent excessive inflammation or tissue damage) or IL-17 (*i.e.*, the gene encoding interleukin-17A, a pro-inflammatory cytokine crucial in mediating immune responses against infections, particularly extracellular bacterial and fungal infections). This correlation suggesting immunoregulatory properties has been observed in the ileum (*i.e.*, the terminal small intestine) and caecum (*i.e.*, the first segment of the large intestine) (Lindenberg et al., 2019). Furthermore, the resident microbiota protects the host by competing with pathogenic bacteria for both nutrients and adhesion sites along the gut epithelium (Williams et al., 2001). Moreover, the involvement of the intestinal microbiota has been studied in frequently encountered diseases in the host such as colic, colitis, stomach ulcers and laminitis (Al Jassim and Andrews, 2009; Arnold and Pilla, 2023; Costa et al., 2012; Lara et al., 2022; Milinovich et al., 2006; Moreau et al., 2014; Murray et al., 2009; Steelman et al., 2012).

1.2. Experimental Models for Microbiota Research

In human, regarding the high complexity of GIT environment and the limited accessibility of most gut regions, *in vitro* models have been developed to dynamically monitor microbial processes (Ménard et al., 2014). These models range from simple batch fermentations to complex multi- compartmental continuous systems. They offer standardised and reproducible conditions, and the most advanced models allow dynamic sampling over time in different simulated gut compartments. In addition, these models bypass ethical constraints, making it possible to test pathogenic microorganisms, toxicants, or even radioactive compounds without ethical concerns (Venema and Van Den Abbeele, 2013).

The simplest of these are static batch fermentations, typically conducted in small bioreactors or test tubes. While they are useful for assessing the microbial response to specific substrates, these models suffer from non-physiological conditions, including the accumulation of metabolites, and uncontrolled shifts in pH and redox potential. Such changes inhibit microbial activity over time, thus limiting the utility of batch systems to preliminary screenings rather than physiologically relevant analyses (Moon et al., 2016; Venema and Van Den Abbeele, 2013). However, when performed in parallel, batch experiments allow for high-throughput testing, thereby increasing statistical power and enabling robust comparative analyses. To overcome these limitations, mono- and multi-compartmental dynamic systems have been developed to simulate one or several sections of the GIT, respectively. These advanced models better reflect *in vivo*-like conditions and are more suitable for studying microbial dynamics, metabolic activity, and the impact of diet or drugs on the gut microbiota.

1.2.1. The Dynamic SHIME® Model and the Derived Static Batch model

Among the multi-compartmental models, the SHIME® (Simulator of the Human Intestinal Microbial Ecosystem - Ghent University- Prodigest, Ghent, Belgium) is a highly flexible and standardised system validated in the 90s which evaluates the dynamics of the microbial ecology of the GIT (Molly et al., 1994). The SHIME® consists of five double-jacketed glass reactors that are connected through peristaltic pumps representing both the upper (stomach, small intestine) and the lower (ascending, transverse and descending colon) GIT. The retention time in each part of the simulated GIT is controlled by computer to reflect the *in vivo* digestive process. The system is maintained at 37°C using temperature-controlled water bath pumped to the glass jacket surrounding each reactor. The pH is controlled by computer along the GIT to represent the pH encountered in each compartment of the GIT at each stage of the digestive process. Typically, the pH of the gastric compartment used in the system operates at a pH of 2.0, while the small intestine compartment operates at slightly acidic to neutral conditions. Finally, the pH of the colon compartments is controlled between 5.6 and 5.9 in the ascending, 6.1–6.4 in the transverse and 6.6–6.9 in the descending colon. Magnetic stir bars allowed mixing the contents of each reactor. Finally, anaerobic conditions are maintained by flushing the compartments daily with N_2 gas or a 90/10% N_2/CO_2 gas mixture.

The first reactor (*i.e.*, stomach) receives a nutritional medium (*i.e.*, feed) three times a day and the second reactor (*i.e.*, small intestine) receives a pancreatic and bile juices. As with other multi-stage colon compartment reactors, the system uses faeces, ideally of pooled donors (Aguirre et al., 2014), and requires a stabilisation period to allow the microbial community to adjust to the environmental conditions of each colon region. The stabilisation period is typically 10–20 days for an adult individual: this duration can be adjusted depending on the population targeted. From an engineering perspective, a suitable adaptation time is around 5–10 times the residence time. A subsequent basal period of approximately two weeks was established to allow for the measurement of baseline parameters. Then, a treatment period of two to four weeks is conducted to measure the effect of challenge on microbiota, followed by a two-week wash-out period to determine how long the changes induced by the tested substance persist in the absence of the substance itself (Figure 1.) (Minekus et al., 2014; Molly et al., 1994; van de Wiele et al., 2015).

A feature of the SHIME® model is the possibility of simulating the microbiome from different target groups such as adult *vs.* infant, healthy *vs.* diseased as well as animal microbiome simulation. Another feature is the possibility to use several SHIME®-systems in parallel named Twin-SHIME®, Triple-SHIME® and Quad-SHIME® for direct comparison of several products or to perform placebo-controlled studies (Dupont et al., 2019; Goya-Jorge et al., 2023a; Ménard et al., 2014; Minekus et al., 2014; Molly et al., 1994; Possemiers et al., 2004; van de Wiele et al., 2015; Venema and Van Den Abbeele, 2013). The combination of SHIME® and the Host-Microbiota Interaction (HMI™) model allowed the study of microbiota but also host-microbiota interactions occurring at the level of the intestinal wall. Indeed, the latter is an *in vitro* device that allows a microbial community to encounter "host" cells via a semi-permeable membrane (Dupont et al., 2019; Marzorati et al., 2014).

Recently, a static “batch” model derived from SHIME® has been described in several studies. This batch system allows a close contact between the microbiota and the treatment for 72 hours, under controlled pH, temperature, and anaerobic conditions, with the addition of nutritional medium. This “simplified-SHIME® system” allows the use of a parallel control system for comparison purposes (Gonza et al., 2024; Goya-Jorge et al., 2024, 2023a, 2023b, 2022).

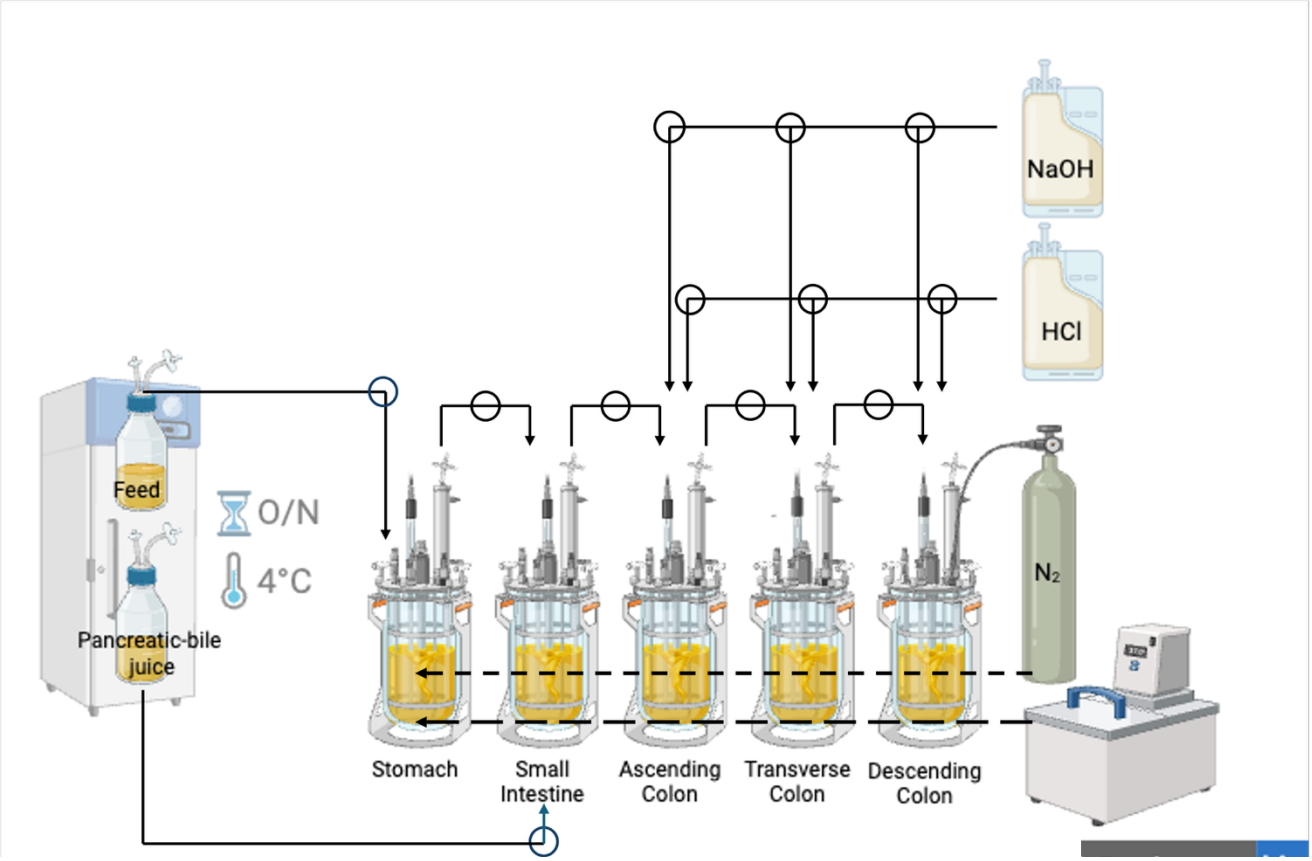

Figure 1. Schematic representation of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) model. Five double-jacketed glass reactors are connected in a dynamic flow representing the stomach, small intestine, and ascending, transverse, and descending colon sections, respectively. Anaerobic conditions (*i.e.*, N_2 flush), specific pH intervals, temperature and volume are computer-controlled automatically by the SHIME® machine. The first reactor (*i.e.*, stomach) is fed with nutritional medium three times a day and the "Small Intestine" reactor receives a pancreatic and bile juices. The black lines represent the tubing of the experimental model, and the black circles represent the peristaltic pumps. Created in BioRender.com

Finally, a mucosal component can be added to *in vitro* models allowing the understanding of the mucosal microbial community. The Mucosal-SHIME® or M-SHIME® model incorporates mucin-covered microcosms, allowing specific bacteria, such as *Lactobacillus mucosae* to adhere to the mucosal layer, mimicking *in vivo* conditions more accurately. This model also replicates the balance of *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* observed in human studies, making it ideal for investigating the mucosal microbiota's role in health and

disease (Dupont et al., 2019; Goya-Jorge et al., 2024; Van den Abbeele et al., 2012, 2011; Venema and Van Den Abbeele, 2013).

1.3. Microbiota Characterisation Methods

Historically, bacterial identification relied on culture-based methods. However, when applied to the gut microbiota, this approach is limited because a large proportion of intestinal microorganisms cannot be cultured under standard laboratory conditions. Consequently, only a restricted subset of fast-growing or aerotolerant species tends to be recovered, leading to a significant underestimation of microbial richness (Eckburg et al., 2005) . The development of diverse culture media and growth conditions has broadened the recovery of previously uncultured species. Nevertheless, this labour-intensive strategy still provides only a partial view of gut microbial diversity (Costa and Weese, 2019; Feng et al., 2018).

To overcome these limitations, a variety of culture-independent methods have been developed. Molecular fingerprinting techniques provided the first comparative insights into microbial communities, followed by sequencing-based approaches (*i.e.,* from early cloning and Sanger sequencing to next-generation sequencing (NGS) technologies). More recently, functional "omics" (*i.e.,* metatranscriptomics, metaproteomic, and metabolomics) have enabled the exploration of microbial activity and host-microbe interactions. Together, these approaches have progressively revolutionised our ability to characterise the microbiota, both in terms of taxonomic composition and functional potential.

1.3.1.Molecular Fingerprinting Methods

The molecular fingerprint methods exploit the separation or hybridisation of nucleic acids to assess microbial community structure and composition. They have provided valuable insights, particularly for comparative profiling and targeted detection, although they are constrained by limited taxonomic resolution.

The denaturing (*i.e.*, with chemical denaturants) and temperature gradient gel electrophoresis (DGGE and TGGE, respectively) as well as the terminal restriction fragment length polymorphism (T-RFLP) methods are based on the separation of deoxyribonucleic acid (DNA) fragments according to their physical or chemical properties into a series of bands.

In DGGE, PCR-amplified fragments of equal length (*i.e.*, amplified by the same primers) but differing in internal sequence are separated according to their differential denaturation behaviour in a chemical gradient containing a denaturing agent (typically urea and formamide). The TGGE method, in contrast, relies on temperature gradient, with melting points determined by sequence stability: GC pairs being more stable than AT pairs due to their three hydrogen bonds *vs.* two hydrogen bonds respectively. Both approaches are relatively fast and semi-quantitative.

T-RFLP, another semi-quantitative method, involves the digestion of PCR-amplified genes labelled with a fluorescent primer using restriction enzymes. The resulting fragments are separated by capillary electrophoresis through automated sequencing, generating reproducible community fingerprints. However, this method is subject to several biases (*e.g.*, DNA extraction, PCR amplification, and terminal restriction sites) and tends to oversimplify microbial diversity, often requiring complementary approaches such as clone libraries (Feng et al., 2018; Fischer and Lerman, 1980; Liu et al., 1997; Strathdee and Free, 2013).

The fluorescent *in situ* hybridisation (FISH) uses labelled DNA oligonucleotide probes to target specific microbial groups based on their nucleic acid sequences. This semi-quantitative method enables the detection of low-abundance organisms and allows quantification of bacterial communities. Depending on the probe, it can target phyla, genera, or species. The fluorescent *in situ* hybridisation provides precise localisation and evaluation of specific taxa, including within host tissues but cannot identify unknown species (Feng et al., 2018).

Microarray-based approaches use oligonucleotide probes immobilised on a solid surface, which hybridise with complementary labelled sequences from the sample. While effective for detecting known taxa, they inherently underestimate community richness since only organisms represented on the array are detectable (Feng et al., 2018).

The quantitative real-time Polymerase Chain Reaction (qPCR) is a molecular technique derived from PCR that allows the specific detection and quantification of DNA sequences in a sample. It relies on primers designed for each target organism or bacterial group, enabling highly specific phylogenetic discrimination. Two main strategies are used to detect amplification products in qPCR (i) non-specific fluorescent dyes that intercalate with any double-stranded DNA and (ii) sequence-specific probes, consisting of labelled

oligonucleotides that hybridise to their complementary target sequences, generating a fluorescent signal. This technique offers several advantages, including rapid analysis, reproducibility, and high specificity, making it a valuable complementary tool for microbial quantification. However, biases can be introduced at several steps, including DNA extraction, primer design, and amplification efficiency, and the method generally cannot detect unknown or uncharacterised species. It is also important to distinguish qPCR from reverse transcription qPCR (RT-qPCR), which first converts RNA into complementary DNA (cDNA) before amplification, enabling quantification of gene expression rather than genomic DNA (Bustin et al., 2009; Feng et al., 2018; Jian et al., 2020). A comprehensive overview of these methods is provided in Table 1.

Molecular fingerprinting methods offered early culture-independent insights into microbial communities, but their limited taxonomic resolution and scope gradually led to their replacement by sequencing-based approaches.

1.3.2. Early Sequencing Methods: Cloning and Sanger Sequencing

Prior to high-throughput sequencing technologies, microbial community analysis relied on cloning-based approaches combined with Sanger sequencing. In the cloning method, DNA fragments from a microbial community were inserted into vectors (*e.g.*, plasmids) and introduced into a bacterial host via transformation. As the host multiplied, the inserted DNA was replicated and sequenced. While this allowed the isolation of individual sequences, the process was labour-intensive, time-consuming, and low throughput.

Sanger sequencing, first described by Frederick Sanger and colleagues in 1977, became the standard method due to its high accuracy. It relies on the incorporation of fluorescently labelled chain-terminating dideoxynucleotides during DNA synthesis. A single-stranded DNA template is replicated in the presence of a primer, DNA polymerase, regular nucleotides, and labelled ddNTPs. Incorporation of a ddNTP terminates elongation, generating fragments of varying lengths that are separated by capillary electrophoresis and read via fluorescence. Despite its precision, Sanger sequencing is low-throughput and suited primarily for short DNA regions, making it less practical for large-scale microbial community studies (Feng et al., 2018; Giani et al., 2020; Sanger et al., 1977; Sanger and Coulson, 1976).

Table 1. Comparison of Molecular Fingerprinting Methods

	Principle	Detection/Signal Type	Taxonomic Resolution	Quantification	Advantages	Limitations
DGGE	Separation of PCR-amplified DNA fragments based on their differential denaturation behaviour in a chemical gradient	Gel electrophoresis with visible banding patterns	Low to medium (partial sequence discrimination)	Semi-quantitative (band intensity)	Fast, simple, allows comparative microbial profiling	Low resolution, PCR bias, limited standardisation, difficult taxonomic identification
TGGE	Separation of DNA fragments according to their melting temperature in a temperature gradient	Gel electrophoresis with visible bands	Low to medium	Semi-quantitative		
T-RFLP	Digestion of fluorescently labelled PCR products with restriction enzymes; separation of fragments by capillary electrophoresis	Fluorescent signal detected by automated sequencer	Medium (fragment size-based)	Semi-quantitative	Reproducible, automatable, suitable for inter-sample comparisons	DNA extraction, PCR, and restriction biases; oversimplifies microbial diversity; cannot detect unknown taxa
FISH	Hybridisation of fluorescently labelled oligonucleotide probes to target microbial cells based on nucleic acid sequences	Fluorescence microscopy or flow cytometry	Variable: from phylum to species depending on probe specificity	Semi-quantitative	Allows spatial localisation, detection of low-abundance taxa, and in situ identification	Cannot detect unknown species; labour-intensive sample preparation
Microarray	Hybridisation of labelled DNA sequences to immobilised oligonucleotide probes on a solid surface	Fluorescent signal on microarray chip	Medium to high (depending on probe design)	Semi-quantitative to quantitative	Rapid detection of multiple known taxa; highly specific	Detects only taxa represented on the array; underestimates community richness
qPCR	Amplification and real-time quantification of DNA using fluorescence (non-specific dyes or sequence-specific probes)	Real-time fluorescence curve during amplification	High (species or gene-level)	Quantitative	High sensitivity and specificity; fast and reproducible	Extraction and amplification biases; cannot detect unknown taxa

1.3.3.Next Generation Sequencing

Next-generation sequencing (NGS) technologies have profoundly transformed microbiome research by enabling the parallel sequencing of millions of DNA fragments, thereby increasing the speed, depth, and cost-efficiency of microbial community profiling. These approaches provide unprecedented access to the complexity of microbial ecosystems, but they remain affected by technical and interpretative limitations that must be carefully considered (Fox et al., 2024; Wensel et al., 2022). Two main NGS strategies dominate microbiome research: shotgun metagenomics and amplicon-based sequencing, particularly of the 16S rRNA gene (Wensel et al., 2022)

Shotgun metagenomics consists of randomly sequencing all DNA fragments extracted from a sample without prior amplification. This strategy minimises PCR-related biases and allows comprehensive profiling of both taxonomic diversity and functional potential by detecting genes associated with metabolic pathways. It also facilitates cross-condition comparisons of microbial functions and provides insights beyond taxonomy (Bars-Cortina et al., 2024; Fox et al., 2024; Wensel et al., 2022). However, its application is limited by the substantial sequencing depth (*i.e.,* average number of times a given nucleotide in a genome or amplicon is read during sequencing, reflecting the coverage and reliability of the data) required, the high costs involved, and the considerable computational resources needed for data processing and annotation (Fox et al., 2024; Wensel et al., 2022). Moreover, because all DNA is sequenced without selective enrichment, contamination from host or environmental DNA may complicate analyses. Finally, in highly diverse samples such as faeces or soil, distinguishing closely related species may remain challenging (Wensel et al., 2022).

The 16S rRNA gene sequencing, in contrast, remains the most widely used method for taxonomic surveys because it is cost-efficient, robust, and adaptable to large sample sets (Jin et al., 2024; Tringe and Hugenholtz, 2008; Wensel et al., 2022). The 16S rRNA gene, ~1500 base pairs in length, is universally present in bacteria and archaea and consists of ten conserved (C1–C10) and nine hypervariable (V1–V9) regions. Primers are designed to bind conserved regions flanking one or more hypervariable regions, enabling selective amplification and sequencing of these segments (Lane et al., 1985). These regions provide sufficient sequence diversity for discrimination at the genus level, though resolution at the species level is often limited (Regueira-Iglesias et al., 2023; Wensel et al., 2022). Because only subsets of variable

regions (*e.g.*, V1–V3, V4–V5) are usually targeted, cost and data complexity are reduced, but taxonomic resolution and cross-study comparability may be compromised (Figure 2.) (Jin et al., 2024; Regueira-Iglesias et al., 2023).

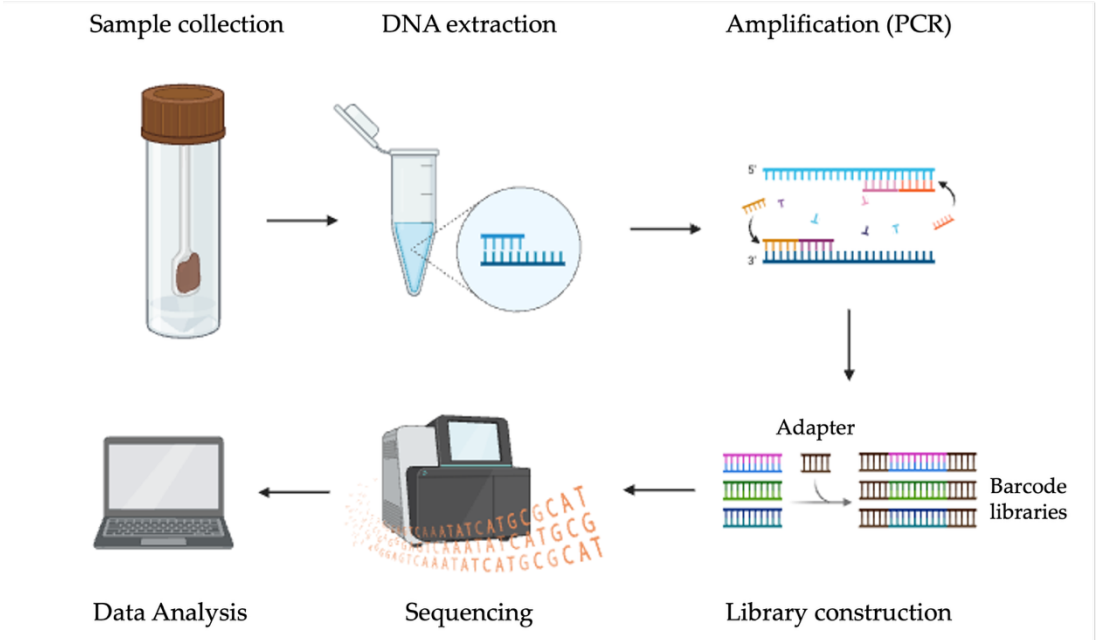

Figure 2. Overview of the 16S rRNA gene sequencing workflow, from sample collection to bioinformatic and statistical analyses. Created in BioRender.com

The 16S rRNA gene has several strengths: its universal presence across bacteria and archaea, the combination of conserved and variable regions enabling primer design and taxonomic discrimination, its relatively long sequence length that supports phylogenetic inference, and the availability of curated, continually updated databases facilitating classification (Fox et al., 2024; Tringe and Hugenholtz, 2008). However, there are also limitations. The choice of targeted variable region critically impacts outcomes, as no single region can resolve all taxa equally, resulting in systematic under- or overrepresentation of certain groups and complicating comparisons across studies (Feng et al., 2018; Rintala et al., 2017; Sirichoat et al., 2021). Furthermore, even so-called conserved primer-binding sites may exhibit variability that biases amplification efficiency among taxa (Regueira-Iglesias et al., 2023).

Another major drawback is that 16S rRNA sequencing generally provides only relative abundance data, not absolute quantification, which complicates interpretation when

community composition changes without an associated shift in bacterial load (Wensel et al., 2022). Technical biases can also arise at multiple stages: DNA extraction, primer design, PCR amplification, sequencing technology, and bioinformatics pipelines (Fox et al., 2024; Wensel et al., 2022). Short-read platforms such as Illumina deliver high accuracy but are constrained by fragment length, limiting resolution at the species level. Conversely, long-read platforms like PacBio and Oxford Nanopore improve resolution but are less broadly applied due to higher costs, error rates, and computational demands (Wensel et al., 2022). Low-abundance taxa are often underrepresented, especially in amplicon-based studies where dominant taxa overshadow minority populations (Jin et al., 2024). Complementary approaches such as qPCR are therefore often required to quantify specific organisms with accuracy. Finally, although NGS-based profiling provides detailed descriptive insights into community structure, it rarely allows causal inference between microbial patterns and host outcomes; such links can only be established through targeted experimental designs (Table 2) (Costa and Weese, 2019; Feng and Patrick, 1958; Kauter et al., 2019).

1.3.4.Functional Omics

Metatranscriptomics, which analyses mRNA, provides insights into the gene expression and the potential activity of microbial communities. It is technically challenging due to the need to deplete abundant rRNA and the inherent instability of RNA, requiring careful sample handling and preparation. Metaproteomics, which examines the proteins produced, more directly reflects microbial functions and metabolic activity. However, this approach requires specialised instrumentation and protein databases remain incomplete for many microbial taxa. Metabolomics, which profiles small molecules and metabolites produced by both the microbiota and host, captures the functional end-products of microbial activity and provides complementary phenotypic readouts. These functional “omics” approaches are valuable for understanding microbial functions and host-microbe interactions (Arıkan and Muth, 2023).

Table 2. Comparison of Next Generation Sequencing Methods

	16S rRNA Gene Sequencing (Amplicon-based NGS)	**Shotgun Metagenomic Sequencing**
Sequencing target	Specific regions of the 16S rRNA gene	Entire genomic DNA from all micro-organisms present
Taxonomic resolution	Usually genus-level; sometimes species-level with high-quality databases	Species- and strain-level resolution possible
Functional insights	Limited; only inferred from taxonomy (*e.g.*, via PICRUSt)	Direct identification of genes and metabolic pathways
Coverage of microbial groups	Targets bacteria and archaea only	Detects bacteria, archaea, viruses, and eukaryotes
Quantification accuracy	Semi-quantitative (affected by copy number variation of 16S gene)	More quantitative representation of community composition
Data complexity and size	Moderate; manageable computational requirements	High; requires large datasets and advanced bioinformatics tools
Cost per sample	Relatively low	Substantially higher
Sensitivity to contaminants and host DNA	Less affected (due to targeted amplification)	More sensitive; host DNA can dominate sequencing reads
Primer bias	Possible amplification bias depending on primer set	No primer bias (random fragmentation and sequencing)
Typical applications	Microbial diversity surveys, community profiling, comparative studies	Functional metagenomics, metabolic reconstruction, antibiotic resistance or virulence gene detection
Advantages	Cost-effective, simple workflow, suitable for large cohorts	High-resolution, functional, and taxonomically comprehensive
Limitations	Limited functional and taxonomic depth	Expensive, computationally demanding, sensitive to contamination

1.4. Bioinformatics Workflows: from Raw Sequences to Taxonomic Tables

The bioinformatic processing of 16S rRNA gene sequencing data follows a structured workflow designed to convert raw reads obtained from high-throughput sequencing platforms into taxonomic tables. While the specific tools and parameters employed may vary depending on the objectives of the study, the general workflow encompasses four key stages: preprocessing of raw reads, determination of taxonomic units, taxonomic assignment, and the generation of relative abundance tables.

The amount and quality of biological information retrieved from sequencing data depend critically on both sequencing depth (*i.e.*, the number of sequences per sample) and sequencing breadth (*i.e.*, the number of samples analysed). Increased sequencing depth improves the detection of low-abundance or rare microbial taxa, thereby enhancing sensitivity, whereas

broader sampling across individuals, conditions, or time points allows for robust comparative analyses and generalisation across ecological or clinical contexts. These considerations influence not only experimental design but also the reliability of downstream bioinformatic inferences (Bharti and Grimm, 2021; Kang et al., 2021; Regueira-Iglesias et al., 2023; Sanschagrin and Yergeau, 2014).

1.4.1.Preprocessing of Raw Reads and Error Removal

The first step in the bioinformatic analysis of 16S rRNA gene sequencing data involves the preprocessing of raw reads. While the specific procedures may vary depending on the sequencing technology used (*e.g.,* Illumina, Oxford Nanopore), this quality-control phase generally includes:

(i) Merging paired-end reads into contigs by aligning forward and reverse sequences based on specific parameters, such as a minimum required overlap and a maximum number of allowed mismatches. In paired-end sequencing, each DNA fragment is sequenced from both ends, producing two reads per fragment: a forward and a reverse read. These reads usually overlap in the central portion of the fragment, allowing their merger into a full-length amplicon. This reconstruction improves sequence accuracy and often provides full or nearly full coverage of the targeted hypervariable region, although this depends on the amplicon length and the overlap between paired reads.

(ii) Filtering and trimming procedures, including the removal of low-quality sequences, trimming of unreliable bases at the read termini, and elimination of non-biological sequences such as adapters and primers. Adapters are artificial sequences introduced during library preparation to facilitate the binding of DNA fragments to the sequencing flow cell and initiate the sequencing reaction. Primers are short synthetic oligonucleotides used during the PCR amplification step to target specific regions of the 16S rRNA gene. Although adapters and primers are essential for library preparation, they must be removed from the dataset prior to downstream analysis.

Altogether, these preprocessing steps are essential to ensure that the resulting dataset is of high quality and accurately reflects the true composition of the microbial community (Bharti and Grimm, 2021; Kang et al., 2021; Lane et al., 1985; Regueira-Iglesias et al., 2023; Sanschagrin and Yergeau, 2014) .

1.4.2. Dereplication, Sequence Binning and Alignment

Following preprocessing, sequences are grouped into representative sequence units to reduce redundancy and prepare for taxonomic analysis (*i.e.*, dereplication). This dereplication step collapses identical reads into unique sequences, each associated with an abundance count corresponding to the number of times it was observed. This significantly reduces computational load and improves analytical efficiency.

Two main approaches are then employed: clustering sequences (*i.e.*, grouping together based on sequence similarity or phylogenetic relatedness) into Operational Taxonomic Units (OTUs) (*i.e.*, the taxonomic level at which the analysis is carried out and defined by the researcher who can decide to group the sequences into OTUs with a 97% identity threshold similarity (Sokal, 1963) or resolving sequences into Amplicon Sequence Variants (ASVs). The latter represent exact biological sequences inferred from raw reads by applying error-correction and denoising algorithms that distinguish true sequence variants from sequencing errors, enabling discrimination at single-nucleotide resolution without arbitrary clustering thresholds (Callahan et al., 2017). The resulting representative sequences are aligned to a defined region of the 16S rRNA gene to ensure that all ASVs or OTUs span the same targeted hypervariable region. This alignment also helps detect and eliminate artefactual sequences, such as those containing unexpected terminal insertions or deletions caused by amplification or sequencing errors. Retaining only high-quality, consistently aligned sequences ensures the reliability of downstream taxonomic and ecological analyses (Bharti and Grimm, 2021; Regueira-Iglesias et al., 2023; Weinroth et al., 2022).

1.4.3. Clustering or Denoising

In this stage, high-quality dereplicated sequences are grouped into clusters known as OTUs, or alternatively denoised into ASVs. OTUs are typically defined by a 97% sequence similarity threshold, which serves as an approximate proxy for species-level classification. However, this cut-off is imprecise and can vary depending on the sequenced region and taxonomic group. Three main strategies are used for OTU clustering:

(i) *De novo* clustering groups sequences based solely on their pairwise similarity, without reference to external databases. This method is useful for detecting novel or rare taxa but is

computationally intensive and may yield non-reproducible results due to dependence on sequence input order or algorithmic variability.

(ii) Closed-reference clustering matches each sequence against a curated reference database. Sequences that meet the similarity criterion are assigned to predefined OTUs, while unmatched sequences are typically excluded from downstream taxonomic analysis. This method is fast and yields standardised results, facilitating comparisons across studies, but may lead to substantial information loss by omitting unclassified or novel sequences.

(iii) Open-reference clustering combines both approaches: sequences are first compared to a reference database, and unmatched reads are then clustered *de novo*. This hybrid method balances efficiency with inclusiveness, retaining sequences absent from reference databases while maintaining standardisation where possible (Bharti and Grimm, 2021; Kang et al., 2021; Regueira-Iglesias et al., 2023; Sanschagrin and Yergeau, 2014).

1.4.4. Taxonomic Assignment

Each OTU or ASV is compared against a reference database to assign a taxonomic classification, typically from phylum to genus, and occasionally down to species level. The accuracy of taxonomic assignment depends on the comprehensiveness and curation of the reference database, as well as the specific algorithm employed, especially when identifying poorly characterised or rare taxa (Bharti and Grimm, 2021; Kang et al., 2021; Regueira-Iglesias et al., 2023; Sanschagrin and Yergeau, 2014).

1.4.5. Generation of Taxonomic Abundance Tables

The final output of the bioinformatic pipeline is a taxonomic abundance table, where each row represents a taxonomic unit (OTU or ASV), and each column corresponds to a sample. These tables report either raw read counts or normalised values such as relative abundances.

They form the foundation for downstream ecological and statistical analyses, including assessments of α- and β-diversity, differential abundance testing, and multivariate modelling. Ultimately, these analyses support the biological interpretation of microbial community structure, composition, and variation across experimental conditions or ecological gradients (Bharti and Grimm, 2021; Kang et al., 2021; Regueira-Iglesias et al., 2023; Sanschagrin and Yergeau, 2014).

1.5. Ecological Indices and Diversity Metrics

1.5.1. Alpha-diversity Analysis

The α-diversity measures the diversity within a single sample and characterises the structure of a microbial community with respect to its richness and/or evenness (Pielou, 1966; Whittaker, 1960). Commonly used α-diversity metrics, which generate a diversity value for each sample that can be statistically compared using nonparametric tests like the Kruskal–Wallis test, include:

(i) The phylogenetic diversity, defined as the sum of the branch lengths on a phylogenetic tree that spans all members of the community (Faith, 1992),

(ii) The Shannon's index which estimates microbial diversity by integrating both taxa richness and evenness yielding higher values in communities with greater overall diversity (Shannon, 1948)

(iii) The Simpson's index which estimates taxa diversity by accounting for both richness and evenness, represents the probability that two randomly selected individuals belong to the same taxon, with values ranging from 0 to 1 (*i.e.*, 1 indicating minimal diversity when all individuals belong to the same species) (Simpson, 1949), and

(iv) The Gini coefficient, originally developed to quantify inequality in economics, has been adapted to microbial ecology as a measure of unevenness in community structure, where higher values indicate dominance by a few taxa and lower values reflect a more balanced distribution of abundances (Feranchuk et al., 2018; Gini, 1921).

The Shannon Index and Simpson Index are both commonly used to measure biodiversity, but they focus on slightly different aspects of community structure. The Shannon Index places more emphasis on rare species in the community while the Simpson Index places more weight on dominant species. Consequently, Shannon is ideal for highlighting the diversity of communities harbouring rare species, while Simpson offers a better insight into dominance dynamics (Fedor and Zvaríková, 2019; Morris et al., 2014).

Another possibility is to use the formulations of Hill, a family of metrics used to assess diversity (entropy, richness or evenness) in a biological community thanks to a unique mathematical formulation with a variable parameter (*i.e.*, q) whose value 0, 1 or 2 makes it possible to calculate the α-diversity by taking into account all the species, by weighting the

species according to their abundance or by favouring the dominant species respectively (Ricotta and Feoli, 2024).

The richness is the number of taxa, most often defined as an OTUs or as the observed number of amplicon sequence variants (ASVs) (Fedor and Zvaríková, 2019; Kers and Saccenti, 2022). The number of observed ASVs reflects the taxa detected in a given sample, which corresponds to the observed richness and is inherently dependent on sequencing depth and sampling effort. This differs from the true richness of the environment (*i.e.*, the total number of taxa actually present). The latter can be approached through estimators that infer the undetected portion of diversity, thereby providing an estimate of the richness that would have been observed if the entire community had been exhaustively sampled (Callahan et al., 2017; Costa and Weese, 2019). The richness can be estimated by:

(i) The number of OTUs or ASVs (Kers and Saccenti, 2022),

(ii) The Chao1 index is employed to estimate the true number of OTUs present in a sample by inferring the portion of diversity that remains undetected in the observed data. It does so by considering the number of rare taxa, specifically singletons and doubletons, thereby providing a lower-bound estimate of the richness that would be observed with exhaustive sampling (Chao, 1984; Chao et al., 2005; Kers and Saccenti, 2022) and,

(iii) The Abundance-based Coverage Estimator considers the relative abundance of rare species to estimate richness by dividing the species into abundant and rare groups in a sample and by ignoring the abundance information for the abundant species, based on the assumption that the abundant species are observed regardless of their exact abundance (Chao and Lee, 1992).

The evenness is the distribution of abundances of the groups (*i.e.*, the prevalence or relative abundance of each population within a community) or, in other words, the observed diversity over absolute diversity (Pielou, 1966). The evenness can be estimated by:

(i) The Simpson Evenness index whose value, if it is close to 1, indicates an equal distribution of the different populations. Because it gives more weight to abundant species, the Simpson Evenness index is less sensitive to rare taxa and better reflects dominance patterns within the community (Fedor and Zvaríková, 2019),

(ii) The Pielou index which compares the observed diversity to the maximum diversity possible if all species were equally abundant with a result from 0 (*i.e.*, dominance of a species)

to 1 (*i.e.*, equal distribution of individuals between different species). This index is relatively sensitive to the presence of rare species, providing a nuanced measure of how evenly individuals are distributed across taxa within a community (Pielou, 1966) and,

(iii) The Heip index which is better suited for rich communities or when the dominance of a few species is important with a result close to 1 indicating an equitable distribution (Heip and Engels, 1974). A comprehensive synthesis of these indices is provided in (Table 3).

1.5.2. Beta-diversity Analysis

The β-diversity assesses differences in microbial composition between samples and differentiates which samples differ from one another by considering either sequence abundances or presence–absence of sequences (Whittaker, 1960). Common metrics include:

(i) The Bray–Curtis dissimilarity which quantifies compositional dissimilarity between two samples based on taxa relative abundance, ranging from 0 (*i.e.*, identical communities) to 1 (*i.e.*, completely distinct) (Bray and Curtis, 1957),

(ii) The Jaccard index which is the ratio between the number of members that are common between the samples and the number of members that are distinct, ranging from 0 (*i.e.*, different communities) to 1 (*i.e.*, identical communities) (Kers and Saccenti, 2022; Lozupone and Knight, 2008), and

(iii) UniFrac and weighted UniFrac distances assess differences between microbial communities by incorporating phylogenetic relationships, with UniFrac based on the fraction of unshared branch lengths and weighted UniFrac further accounting for the relative abundance of sequences (Lozupone and Knight, 2005).

These distance or dissimilarity indices are used to construct a distance or a dissimilarity matrix, which represents pairwise differences between all samples in a dataset.

To visualise the structure and the variation of microbial communities between multiple samples (*i.e.*, the dissimilarity matrix), non-supervised and non-constrained ordination methods can be used. These methods reduce complex high-dimensional data (*e.g.*, from OTU or ASV abundance tables) into two or three dimensions (*i.e.*, projection in a Cartesian space), allowing patterns of similarity or dissimilarity to be more easily interpreted. Two commonly used ordination methods in microbiome research are:

(i) Principal Coordinates Analysis (PCoA) is a distance-based technique that operates on a dissimilarity matrix, preserving metric distances as faithfully as possible before projecting samples into a lower-dimensional coordinate space. PCoA reports the percentage of variance explained by each axis, thus facilitating interpretation of the relative contribution of underlying components. However, this method is more sensitive to outliers and zero values and may distort patterns when the underlying relationships between samples are non-linear. Such non-linear relationships are frequently observed in microbiome studies, due to the high dimensionality, sparsity, and compositional nature of microbial community data, and
(ii) Non-metric Multidimensional Scaling (NMDS) is a rank-based ordination method that does not assume linear relationships among variables, making it well-suited for analysing complex ecological data such as microbial communities. Unlike metric approaches, NMDS relies on the ranked dissimilarities between samples rather than absolute distances, allowing it to accommodate non-linear structures often present in microbiome datasets. The method is iterative and non-parametric, typically requiring multiple runs with different random starting configurations to ensure a stable solution and avoid convergence on local minima. NMDS outputs a stress value, which quantifies the degree of mismatch between the reduced ordination space and the original distance matrix. A stress value below 0.1 is generally considered a good fit, while values below 0.2 are acceptable for ecological interpretation (Armstrong et al., 2022; Goodrich et al., 2014; Paliy and Shankar, 2016; Ramette, 2007).

To relate variation in microbial community composition to environmental or experimental factors, constrained ordination methods can be applied, allowing the identification of patterns specifically explained by these variables. Redundancy Analysis (RDA) is a constrained ordination method that extends PCoA to relate community composition to explanatory variables, assuming linear relationships and Euclidean distances. By incorporating environmental or experimental factors, RDA identifies the portion of variation in microbial communities that can be explained by these covariates. Distance-based RDA (db-RDA) generalises this approach by allowing the use of any dissimilarity or distance matrix (*e.g.*, Bray-Curtis, UniFrac), making it suitable for non-Euclidean data typical in microbiome studies, including data explored with NMDS or other non-metric ordinations. In db-RDA, the dissimilarity matrix is first transformed into principal coordinates, and then constrained ordination is performed to partition variation according to explanatory variables.

Significance of the constrained axes can be assessed via permutation tests. These approaches provide a complementary perspective to unconstrained ordinations (*e.g.*, PCoA, NMDS) by explicitly linking patterns of community variation to measured environmental or experimental gradients (Paliy and Shankar, 2016; Ramette, 2007).

Once the dissimilarity matrix has been computed, appropriate statistical methods are needed to assess whether the observed patterns of microbial community structure differ significantly between groups. Two widely used non-parametric approaches in microbial ecology are

(i) The Analysis of Similarities (ANOSIM) is a rank-based non-parametric test. It calculates an R statistic, ranging from -1 to 1, where values near 0 indicate no separation between groups, and values closer to 1 indicate strong between-group differences. Significance is assessed by permuting group labels and recalculating the R value. While ANOSIM is robust to non-normal data, it is sensitive to differences in within-group dispersion, which can affect interpretation if dispersion varies among groups, and

(ii) The Permutational Multivariate Analysis of Variance (PERMANOVA) is a robust statistical method for comparing groups of samples when multiple variables are involved. It constructs ANOVA-like test statistics and assesses the significance of group differences through random permutations of the data. The null hypothesis (H_0) tested by PERMANOVA posits that the centroids of all groups, in the space defined by the chosen resemblance measure, are identical. This method assumes that samples are exchangeable under the null hypothesis, which is often interpreted as a form of independence. In more complex experimental designs, such as paired samples, permutations can be restricted or stratified to respect the underlying dependencies. Significance is evaluated by calculating the pseudo-F statistic and comparing it to a distribution generated through repeated permutations of group labels (typically k = 9,999). The p-value is determined as the proportion of permuted pseudo-F values that are equal to or greater than the observed statistic (Kers and Saccenti, 2022; Tang et al., 2016). A comprehensive synthesis of the β-diversity analysis is presented in (Table 4).

Table 3. Summary of α-Diversity Indices

Category	Index / Metric	Definition / Description	Interpretation
Phylogenetic diversity	Faith's Phylogenetic Diversity (PD)	Sum of the branch lengths on a phylogenetic tree that connect all taxa in a community	Higher PD indicates greater evolutionary breadth and phylogenetic richness
Overall diversity	Shannon Index (H')	Integrates both richness and evenness; sensitive to rare taxa	Higher values indicate higher diversity; emphasizes rare species
	Simpson Index (D)	Measures probability that two randomly selected individuals belong to the same taxon; accounts for richness and evenness	Ranges from 0 (high diversity) to 1 (low diversity); emphasizes dominant species
	Gini Coefficient	Adapted from economics to quantify unevenness in taxon distribution	High values indicate dominance by few taxa; low values indicate balanced communities
	Hill Numbers (q = 0, 1, 2)	Family of diversity measures unified by parameter q: 0 (richness), 1 (Shannon), 2 (Simpson)	Allows comparison of richness, evenness, and dominance within a single framework
Richness	Observed OTUs / ASVs	Number of distinct operational taxonomic units or amplicon sequence variants detected in a sample	Reflects observed richness; dependent on sequencing depth
	Chao1 Index	Estimates true richness by accounting for undetected taxa based on singletons and doubletons	Provides lower-bound estimate of total richness
	Abundance-based Coverage Estimator (ACE)	Estimates richness by partitioning taxa into rare and abundant groups; focuses on rare taxa	Captures unseen diversity by emphasizing rare species contribution
Evenness	Simpson Evenness Index	Reflects distribution equality	Values close to 1 = uniform distribution; less sensitive to rare taxa
	Pielou's Evenness (J')	Ratio of observed diversity to maximum possible diversity if all species were equally abundant	0 = dominance by one species; 1 = perfect evenness; sensitive to rare taxa
	Heip's Evenness Index	Modified evenness measure suitable for rich communities with strong dominance patterns	Values close to 1 = equitable distribution

Table 4. Summary of β-Diversity Metrics and Ordination Methods

Category	Index / Method	Definition / Description	Interpretation / Key Features
Dissimilarity Metrics	Bray–Curtis Dissimilarity	Quantifies compositional dissimilarity between two samples based on relative abundances	0 = identical communities; 1 = completely distinct; sensitive to abundance changes
	Jaccard Index	Ratio of shared taxa to total taxa between two samples, based on presence–absence data	0 = completely different; 1 = identical communities; ignores abundance
	UniFrac Distance	Measures community dissimilarity incorporating phylogenetic information based on unique branch lengths	Considers evolutionary relationships; Weighted UniFrac includes relative abundance
Unconstrained Ordination Methods	Principal Coordinates Analysis (PCoA)	Distance-based ordination that projects pairwise dissimilarities into low-dimensional space	Preserves metric distances; variance explained by axes; sensitive to outliers and non-linear relationships
	Non-metric Multidimensional Scaling (NMDS)	Rank-based ordination preserving ranked dissimilarities rather than absolute distances	Captures non-linear structures; robust to sparsity; stress < 01 = good fit, < 02 = acceptable
Constrained Ordination Methods	Redundancy Analysis (RDA)	Linear constrained ordination linking community composition to explanatory variables using Euclidean distances	Identifies variance explained by environmental or experimental factors; assumes linearity
	Distance-based RDA (db-RDA)	Extension of RDA allowing use of any dissimilarity matrix (*e.g.*, Bray–Curtis, UniFrac)	Suitable for non-Euclidean data; partitions variation by covariates; significance via permutation tests
Statistical Tests	Analysis of Similarities (ANOSIM)	Non-parametric rank-based test comparing within- and between-group dissimilarities	R statistic from -1 to 1; 0 = no difference, 1 = complete separation; sensitive to within-group dispersion
	Permutational Multivariate Analysis of Variance (PERMANOVA)	Non-parametric test assessing group differences using permutations of dissimilarity matrices	Tests equality of group centroids; pseudo-F statistic; robust but assumes exchangeability; can be stratified for paired designs

2. Digestive Microbiota of the Horse

2.1. Influence of Digestive Physiology on the Equine Gut Microbiota

Each compartment of the GIT has its own characteristics that can influence the presence of certain bacterial types such as nutrient heterogeneity, topography and fluctuating environmental conditions like pH, oxygen levels and mean retention time which, for example, varies from 5 hours on average through the stomach and the small intestine to 35 hours on average in the caecum and colon (Adekoya et al., 2025; Van Weyenberg et al., 2006). Horses are non-ruminant hindgut fermenters and consequently possess both mammalian enzymatic digestive capacity in the foregut (*i.e.*, stomach and small intestine) and microbial fermentation in the hindgut (*i.e.*, caecum and colon) (Mok and Urschel, 2020).

2.1.1.Influence of Upper Digestive Physiology on the Equine Gut Microbiota

The GIT begins with the oral cavity, where saliva has been reported to contain very low bacterial counts (< 10 colony-forming units (CFU) of total bacteria per millilitre) as reviewed by (Merritt and Julliand, 2013). The functional role of these salivary microbes remains unclear. The mouth is followed by the oesophagus, which measures approximately 1.2 to 1.5 metres in length. The stomach, with a capacity ranging from 8 to 15 litres, harbours an active microbial population. Bacterial activity in this compartment is largely influenced by pH and primarily involves the fermentation of non-structural carbohydrates into lactic acid, and to a lesser extent SCFAs. In horses with continuous access to forage, the pH near the lower oesophageal sphincter typically ranges between 5.0 and 7.0, while the fluid contents at the bottom of the stomach (*i.e.*, glandular stomach) maintain a consistently acidic environment (pH 2.0–3.0). When forage intake is reduced, the pH in the non-glandular stomach can decline sharply, reaching values at or below 4.0. These variations in pH significantly affect microbial fermentation, which is more active at pH levels above 4.0 (Bachmann et al., 2020; Hintz, 1975; Hintz et al., 1978, 1971; Merritt and Julliand, 2013).

The digestive environment of the equine small intestine is characterised by a high density of bacteria, predominantly facultative and strict anaerobes, thriving in a warm (approximately 39 °C), aqueous environment with a high-water content (86.7% – 96.5%) and a relatively

elevated pH, particularly in the ileum. This alkaline pH is largely maintained by bicarbonate secretions from the pancreas (pH ~ 8.0), the duodenum, and the ileum (Argenzio et al., 1977; Bauck et al., 2022). Several features are distinctive to the horse (i) the pancreatic secretions are abundant and appear to be continuous, (ii) the concentrations of amylase and trypsinogen (trypsin) are markedly lower than in other species, and (iii) the lipase, which consequently represents the dominant digestive enzyme in this region (Hintz et al., 1978; Merritt and Julliand, 2013). Only bacteria are present, with no recorded presence of fungi or protozoa. Total anaerobic bacterial counts vary across sections, reaching up to 10^9 CFU/mL in the jejunum and ileum. Although cellulolytic bacteria are present, they occur at low densities. In contrast, starch-degrading and lactate-utilising bacteria are more abundant and tend to dominate the microbial community. Their relative abundances are influenced by the composition of the diet (Wunderlich et al., 2023). However, there is limited data on the specific roles of these bacteria in the digestion of carbohydrates, fats, or proteins within the small intestine and on the absorption of these compounds in the small intestine (Merritt and Julliand, 2013).

2.1.2.Impact of Hindgut Physiology on the Composition and Function of the Microbiota

Equids are hindgut-fermenting species with the caecum and large colon serving as the primary fermentation chambers (Merritt and Julliand, 2013). The hindgut represents approximately 60% of the total GIT volume and comprises the caecum, the ascending colon (anatomically subdivided into the right ventral, left ventral, left dorsal, and right dorsal segments), the small or descending colon, and the rectum. The proximal section (caecum and ventral colon) connects to the distal section (dorsal colon and small colon) at the colonic pelvic flexure (Julliand and Grimm, 2016; Merritt and Julliand, 2013). In addition to its volumetric dominance, the hindgut is more frequently implicated in colic syndromes than other regions of the GIT (Christophersen et al., 2014; Spadari et al., 2023; van der Linden et al., 2003). The microbial community inhabiting these fermentation chambers is diverse and densely populated. Microorganisms are present in increasing order of abundance as follows: fungi (5.3×10^2 and 1.3×10^2 zoospores/mL in the caecum of horses and ponies respectively), protozoa (10^3 to 10^6 cells/mL of caecal or colonic content), archaea (10^4 to 10^6 cells/g wet weight of equine caecal content), bacteriophages (10^{10} to 10^{11} per gram of faeces) and bacteria (total anaerobic

bacteria vary from 10^7 to 10^{11} cells/mL of caecal or colonic content) (Dougal et al., 2013; Julliand and Grimm, 2016). The physicochemical conditions of the hindgut (*i.e.*, strict anaerobiosis, a stable pH near neutrality (~7.0), and a constant temperature of 38°C – 40°C) are favourable to the proliferation of obligate anaerobes (De Fombelle et al., 2003; Julliand and Grimm, 2016). A virtuous cycle exists between the microbiota and the host's differentiated colonocytes. Through oxidative phosphorylation, colonocytes consume oxygen, thereby generating a hypoxic luminal environment that supports the growth of strict anaerobes. These microbes exhibit fibrolytic activity, breaking down dietary fibres to produce SCFAs (*i.e.*, containing 1 to 5 carbon atoms), primarily acetate, propionate, and butyrate. In turn, SCFAs, particularly butyrate, serve as an essential energy source for colonocytes via mitochondrial β-oxidation (Chaucheyras-Durand et al., 2022; Donohoe et al., 2011; Merritt and Julliand, 2013).

The concentration of several functional groups of bacteria has been described in the caecum and colon of horse: glycolytic (10^8 and 10^8 cells/mL, respectively), starch-utilising bacteria (10^8 and 10^8 cells/mL, respectively), lactate-utilising bacteria (10^5 to 10^8 and 10^5 to 10^8 cells/mL, respectively), cellulolytic (10^4 to 10^7 and 10^4 to 10^8 cells/mL, respectively), hemicellulolytic (10^7 to 10^8 and 10^6 to 10^8 cells/mL, respectively), pectinolytic (10^7 to 10^8 and 10^6 to 10^7 cells/mL, respectively), and proteolytic (10^8 and 10^6 to 10^8 cells/mL respectively) (Daly et al., 2006; De Fombelle et al., 2003; Julliand and Grimm, 2016).

In these parts of the GIT, fibrolytic bacteria produce SCFAs that account for about 65% of the horse's energy production (Bergman, 1990; Hintz et al., 1971; Vermorel and Martin-Rosset, 1997). It is also described that at least 50% of the host's energy requirement is furnished by caecum and colon SCFAs (*i.e.*, fatty acids that are volatile at room temperature, including acetate, propionate and butyrate, but also other compounds like lactate and formate) and 30% are provided by the caecum alone (Julliand and Grimm, 2016). The main SCFAs produced are acetate, propionate and butyrate (*i.e.*, SCFAs) in the respective following proportion 74.9%, 18.0% and 6.0% of the total in the caecum and 74.8%, 16.9% and 6.3% in the colon (Argenzio et al., 1974a; Daly et al., 2006).

Passive and active absorption of SCFAs occurs across the luminal mucosal membrane. To be efficiently and passively absorbed across the luminal mucosal membrane, the SCFAs must be in a non-ionic (*i.e.*, protonated with H^+) form but 95% of the SCFAs are in their ionised form

($RCOO^-$) within the caecum and colon where the pH is ~7.0 (pKa = 4.8). The 5% remaining protonated form (RCOOH) is rapidly absorbed by a concentration-dependent passive diffusion process, and the rate of absorption is inversely proportional to molecular weight (acetate > propionate > butyrate > lactate) (Argenzio et al., 1974a; Merritt and Julliand, 2013). The epithelial cells of the caecum and colon establish a distinct microclimate at their surface, where the pH is slightly more acidic than that of the luminal contents. This microenvironment is maintained by several mechanisms, including: the active secretion of H^+ ions via proton pumps or ion exchangers (*e.g.*, the Na^+/H^+ exchanger), and the presence of a mucus layer that slows proton exchange between the epithelial surface and the intestinal lumen. Within this slightly acidic microclimate, ionised volatile fatty acids ($RCOO^-$) can reassociate with protons to form their non-ionised, protonated form (RCOOH), which more readily diffuses passively across the epithelial membrane. Once inside the epithelial cells, where the intracellular pH is closer to neutral, the protonated SCFAs rapidly dissociate, enabling their subsequent metabolism or transport into the bloodstream (Merritt and Julliand, 2013).

Beside this passive transport, carrier-mediated mechanisms allow the transport of SCFA in their anionic form ($RCOO^-$) into colonic epithelium. Two major systems operate in cellular uptake of SCFA: H^+-coupled, and Na^+-coupled. The H^+-coupled mediate electroneutral transport thanks to MCT1 transporter expressed both in the apical membrane and the basolateral membrane and MCT4 transporter specifically in the basolateral membrane. The transporters for the Na^+-coupled systems are both expressed in the apical membrane and are: SMCT1 (electrogenic - Na^+: SCFA stoichiometry; 2:1) and SMCT2 (electroneutral). In the apical membrane, an anion-exchange mechanism also exists allowing the entry of SCFA in anionic form coupled with HCO_3^-. The transporters located in the basolateral membrane allow the transport of SCFA into the bloodstream (Sivaprakasam et al., 2018). These active mechanisms are functionally linked to additional ionic exchanges across the epithelial membrane, including sodium absorption via the Na^+/H^+ exchanger, chloride uptake via the Cl^-/HCO_3^- exchanger, and the secretion of carbonic acid (H_2CO_3) into the intestinal lumen (Merritt and Julliand, 2013; Sivaprakasam et al., 2018).

The large intestine functions as a major water reservoir, with its fluid content modulated by the quantity and composition of ingested feed, as well as by microbial fermentation dynamics. The absorption of SCFAs from the colonic lumen is closely linked to the concurrent

uptake of sodium (Na^+), chloride (Cl^-), and water into the bloodstream. In the large colon, sodium absorption occurs primarily through electroneutral pathways involving Na^+/H^+ exchangers. The H^+ ions exchanged during this process protonate ionised volatile fatty acids ($RCOO^-$), converting them to their non-ionised form (RCOOH), thereby facilitating passive diffusion across the epithelium as mentioned above. By contrast, sodium absorption in the small colon is entirely electrogenic, uncoupled from SCFAs absorption or acid–base regulation, and is specialised for the conservation of sodium and water. Both absorption mechanisms are regulated by aldosterone, with the small colon demonstrating a more pronounced response to its stimulation (Argenzio et al., 1974; Merritt and Julliand, 2013). Feeding strategies (particularly meal feeding *vs. ad libitum* intake) substantially influence colonic hydration and plasma water volume. A single large meal of hay and/or pelleted grain can lead to a reduction in plasma volume by up to 15% within the first three hours post-ingestion, a phenomenon not observed in horses fed *ad libitum*. This effect arises when SCFAs production in the large colon temporarily exceeds their rate of absorption, creating an osmotic gradient that draws water from the bloodstream into the colonic lumen. The resultant hypovolemia activates the renin–angiotensin–aldosterone system, enhancing sodium and water reabsorption, particularly in the small colon. While compensatory, this mechanism may further dehydrate the colonic contents. Notably, this marked reduction in plasma volume is not observed in horses fed grain meals with continuous access to hay. In fact, high-roughage diets may lead to the opposite effect (*i.e.*, an increased water retention) highlighting the critical role of feeding practices not only in maintaining hindgut health but also in preventing disorders such as colonic impaction (Costa and Weese, 2018; Hesta and Costa, 2021; Merritt and Julliand, 2013).

2.2. Experimental Models for Microbiota Research in Equids

2.2.1. Experimental "In Vitro" Study Models in Equine Research

Models simulating the equine GIT have been employed in various studies to effectively represent the horse's gut microbiota. Studies like those by (Lowman et al., 1999; Murray et al., 2014) have demonstrated the suitability of using equine faecal content or inoculum from different gut regions to assess microbial activity. Murray's work, for instance, compared gas

production from different intestinal contents, including faecal and colonic samples, and showed that these *in vitro* techniques can mimic natural microbial processes (Murray et al., 2014). Further, Leng et al. (2020) developed a continuous three-stage fermentation model designed to mimic the equine large intestine. This model used faecal inoculation to establish microbial communities that closely resembled the *in vivo* taxonomic composition. This study shows that the in vitro equine model can reproduce the microbial community at a broad taxonomic level, including phyla and major classes/orders, providing reference proportions (*e.g.*, Firmicutes vs Bacteroidetes) and confirming the relevance of analyses at the class/order level. It also replicated the normal production of SCFAs and branched-chain fatty acids, which are key indicators of microbial metabolic function. Studies using these *in vitro* models highlight their ability to maintain stable microbial communities that represent the equine gut environment, making them valuable tools for investigating how factors like diet, pathogens, and medications influence the equine microbiome. Despite some limitations, these models offer insights into equine gut health and could aid in understanding microbial responses to various conditions (Leng et al., 2020). However, to date, no study has aimed to comprehensively characterise the normal equine microbiota at the genus or specie level using such *in vitro* methodologies.

2.2.2. Alternative Study Methods in Equine Research: Faecal Sampling

In horses, gut microbiota composition is typically studied using digesta collected from different segments of the gastrointestinal tract, either in live animals (*e.g.*, cannulation or endoscopy) (Daly et al., 2006; De Fombelle et al., 2003; Kristoffersen et al., 2016; Steelman et al., 2012) or post-mortem (Costa et al., 2015; Dougal et al., 2013; Ericsson et al., 2016; Raspa et al., 2024). However, these methods could raise ethical concerns when applied to living animals, and post-mortem sampling introduces the question of how accurately the microbiota reflects the *in vivo* state at the time of death.

Faecal sampling has therefore emerged as a practical, non-invasive alternative for characterising the equine gut microbiota. Several studies have shown that the faecal microbiota closely reflects that of the distal colon (Costa et al., 2015; Dougal et al., 2013; Ericsson et al., 2016; Żak-Bochenek et al., 2024). This approach offers several advantages: it allows for repeated sampling without ethical constraints, facilitates fieldwork through rectal

collection or ground sampling of freshly voided faeces, and captures microbial communities under near-physiological conditions.

However, the use of faeces as a proxy for intestinal content has several limitations. Faecal samples mainly represent the microbial communities of the distal colon and rectum and not accurately reflect those of the proximal hindgut or caecum (Costa et al., 2015a; Dougal et al., 2013; Ericsson et al., 2016). They primarily capture the luminal fraction and do not account for mucosa-associated microbes, which may play specific roles in host–microbe interactions (Ericsson et al., 2016). Finally, sample collection and storage conditions can influence microbial community profiles. A recent study reported that freeze–thaw cycles reduced bacterial viability by approximately 47% (Arantes et al., 2025). Nonetheless, several investigations have indicated that storage at – 80 °C does not cause significant alterations in microbial composition compared with fresh faecal samples, even after several months (Carroll et al., 2012; Fouhy et al., 2015). As a result, some studies have used samples stored at – 80 °C as reference controls for their analyses (de Bustamante et al., 2021). Furthermore, other works have shown that storage at – 20 °C also preserves microbial profiles, without inducing notable variations in relative abundance, α -diversity and β-diversity (Bell et al., 2024; Gavriliuc et al., 2021).

2.3. The Normal Horse Gut Microbiota

The composition and function of the equine gut microbiota are known to vary significantly along the different compartments of the gastrointestinal tract, reflecting regional physiological and environmental conditions.

2.3.1. Microbial Composition Along the Equine Gastrointestinal Tract

Dougal et al., (2013) investigated the presence of a core bacterial community in the equine gastrointestinal tract by analysing the V1–V2 regions of the 16S rRNA gene using 454-pyrosequencing. Samples were collected from the terminal ileum and seven distinct regions of the large intestine (*i.e.*, caecum, right ventral colon, left ventral colon, left dorsal colon, right dorsal colon, small colon, and faeces from the rectum) from ten horses. The ileum showed the lowest bacterial richness, α-diversity, and bacterial load. Although not statistically significant,

a progressive increase in richness and DNA quantity was observed from the ileum to the faeces.

At the phylum level, *Firmicutes* and *Proteobacteria* were dominant in the small intestine, while *Firmicutes* and *Bacteroidetes* predominated in the large intestine. In terms of bacterial families, the proximal large intestine was mainly composed of unclassified *Bacteroidales*, *Lachnospiraceae*, *Prevotellaceae*, *Erysipelotrichaceae, Ruminococcaceae*, and *Fibrobacteraceae*. The distal regions were primarily dominated by *Prevotellaceae, Fibrobacteraceae, Lachnospiraceae*, and unclassified *Firmicutes*.

Clustering analyses based on OTUs revealed distinct bacterial community profiles in the ileum, the proximal large intestine (including the caecum, right ventral colon, and left ventral colon), and the distal large intestine (comprising the right dorsal colon, small colon, and rectal faeces). The left dorsal colon appeared to share features with both proximal and distal regions, suggesting a transitional microbial composition.

A core bacterial group was identified in all gut regions, although it differed in composition and abundance. In the ileum, the core represented 32% of sequences and is composed of only seven OTUs of varying abundance with *Lactobacillaceae* being the most abundant (*i.e.*, typical of a more acidic and less fermentative environment). In the large intestine, the core represented a smaller fraction (5 – 15% of sequences) but included a larger number of low-abundance OTUs (Dougal et al., 2013). This change in bacterial community is anatomically situated at the pelvic flexure (Dougal et al., 2013; Reed et al., 2021). This low abundance but highly diverse core in the large intestine suggests that many bacterial taxa contribute to gut function, but each in a subtle, limited way (Dougal et al., 2013). If some of these taxa are lost (*e.g.*, due to dietary changes, stress, or medical treatments), the overall microbial balance can be rapidly disrupted, as no single dominant species is present to compensate (Collinet et al., 2021). This may explain the horse's high sensitivity to digestive disorders (such as colic or diarrhoea) when the colonic microbiota is disturbed (Lara et al., 2022; Park et al., 2021).

Costa et al. (2015) aimed to characterise the bacterial microbiota along different compartments of the equine GIT using DNA extraction and PCR amplification targeting the V4 region of the 16S rRNA gene.

The *Firmicutes* phylum was the most abundant across all compartments, with significantly higher relative abundance in (i) the stomach compared to the caecum, pelvic flexure, small colon, rectum, and faeces, and (ii) the duodenum compared to the pelvic flexure, small colon, and faeces. *Verrucomicrobia* were significantly more abundant in the small colon, rectum, and faeces compared to the stomach. The *Spirochaetes* phylum was more abundant (i) in faeces compared to the stomach, duodenum, and ileum, and (ii) in the small colon and caecum compared to the stomach and duodenum. *Proteobacteria* were significantly more represented in (i) the duodenum relative to the large colon, small colon, and faeces, and (ii) in the ileum and caecum compared to the small colon and faeces. *Fibrobacteres* were more abundant in the large colon, small colon, and faeces than in the stomach and duodenum. *Bacteroidetes* were more abundant in (i) the caecum compared to the stomach, duodenum, and ileum, and (ii) the small colon and faeces compared to the duodenum and ileum.

At the genus level, a notable transition in microbial composition was observed around the caecum, which appeared to represent a microbiological shift between the proximal GIT (stomach, duodenum, ileum) and the distal GIT (pelvic flexure, small colon, rectum, faeces). In the proximal GIT, genera such as *Lactobacillus*, *Sarcina* (exclusively in the stomach), *Streptococcus*, *Pseudomonas*, and *Actinobacillus* were prominent. In contrast, the distal GIT was characterised by genera including *Treponema*, *Fibrobacter*, and a substantial proportion of unclassified taxa, such as unclassified *Bacteria*, *Clostridiales*, *Lachnospiraceae*, and *Ruminococcaceae*.

Analysis of α-diversity using Simpson's diversity index revealed a significantly lower microbial diversity in the proximal GIT (*i.e.*, stomach, duodenum, ileum) compared to the distal GIT (*i.e.*, caecum, large colon, small colon, rectum, faeces). This observed shift in microbial diversity was statistically supported by the comparison between the ileum and caecum, which showed the most distinct contrast. Moreover, the proximal compartments displayed relatively similar microbial compositions within each region, while the distal compartments exhibited stronger intra-individual similarities, suggesting a more stable and consistent microbial community across distal sites within individual horses (Costa et al., 2015a).

Ericsson et al. (2016) aimed to establish a comprehensive map of the equine gastrointestinal microbiota by analysing both luminal and mucosal bacterial communities in various gut compartments. Using 16S rRNA gene sequencing targeting the V4 region, samples were collected from the dorsal and antral stomach, jejunum, ileum, caecum, ventral colon, and dorsal colon.

The analysis of unique sequences (*i.e.*, sequences differing by at least one nucleotide) revealed several significant differences: (i) in luminal contents, the stomach and small intestine harbored microbial profiles distinct from those observed in the ventral and dorsal colon, (ii) in mucosal samples, the dorsal colon exhibited a significantly higher number of unique sequences compared to the ileum. In contrast, when considering the total number of OTUs (*i.e.*, defined as clusters sharing at least 97% sequence identity), it was the mucosa of the dorsal stomach that showed a significantly greater OTU richness than the ileum. This indicates that, although fewer unique sequences were detected in the stomach, they encompassed a broader diversity of closely related taxa; in other words, the gastric microbiota appeared to consist of fewer distinct lineages, but these were represented by a wider array of closely related variants within each taxonomic group.

These findings indicate a greater phylogenetic relatedness and microbial diversity in the lower gastrointestinal tract compared to the upper segments. This was further supported by Shannon and Simpson α-diversity indices, which confirmed an increase in diversity from the proximal to distal GIT, consistent with previous studies. In mucosal samples, significant increases in diversity were observed between the jejunum and the colon (both ventral and dorsal). In luminal samples, the following significant differences were reported: (i) the dorsal colon differed from the stomach, jejunum, and ileum, (ii) the ventral colon differed from the antral stomach, jejunum, and ileum, and (iii) the caecum differed from the antral stomach and jejunum. These comparisons highlighted a clear transition between proximal and distal gut regions, especially around the caecum.

Analysis of relative phylum-level abundance in luminal content revealed high proportions of *Proteobacteria* and *Firmicutes* in the upper GIT, along with lower levels of *Cyanobacteria* and *Bacteroidetes*. A major compositional shift was observed at the junction between the small and large intestine, where the lower GIT harboured increased proportions of *Firmicutes* and

Bacteroidetes, followed by *Verrucomicrobia*, *Tenericutes*, *Spirochaetes*, and *Fibrobacteres*. These trends were less pronounced in mucosal samples.

At the taxonomic level, a similar transition was observed. The upper GIT was dominated by taxa such as *Lactobacillus sp. (*especially in the stomach), *Streptococcus sp.*, Actinobacillus *sp.*, *Sarcina sp.*, and unclassified members of *Enterobacteriaceae* and *Streptophyta* (Ericsson et al., 2016). The caecum and colon harboured unclassified *Bacteroidales*, *Prevotella*, RF16, genus CF231, unclassified *Clostridiales*, *Lachnospiraceae*, *Ruminococcaceae*, *Treponema*, and RFP12 (Costa et al., 2015a; Ericsson et al., 2016).

Principal Component Analysis (PCA) based on OTU composition clearly separated upper and lower GIT samples along the horizontal axis, especially for luminal communities. PERMANOVA analysis confirmed these findings, showing: (i) a significant effect of gut region (upper vs. lower), (ii) significant differences between all regions of the lower GIT lumen, and (iii) a significant difference between upper and lower GIT in mucosal samples as well. When all samples were considered together, PERMANOVA confirmed significant main effects of both anatomical region (*i.e.*, upper *vs.* lower GIT) and sample type (*i.e.*, lumen *vs.* mucosa) (Ericsson et al., 2016).

Altogether, these studies highlight several consistent findings:

(i) Alpha-diversity is generally higher in the hindgut than in the foregut (Costa et al., 2015a; Dougal et al., 2013; Ericsson et al., 2016), consistently indicating a more diverse and complex bacterial community in the hindgut. This overall trend is confirmed by α-diversity indices such as Shannon and Simpson. It is important to note, however, that the observed rise in diversity is not strictly progressive throughout the proximal GIT; for instance, mucosal richness has been shown to decrease gradually along the small intestine before demonstrating a sharp increase in the large intestine. This clear transition is reinforced by a statistically significant difference in overall microbial composition between the upper and lower GIT being consistently demonstrated across multiple studies (Costa and Weese, 2018; Reed et al., 2021; Żak-Bochenek et al., 2024).

(ii) In the foregut, the microbiota is predominantly composed of *Firmicutes* and *Proteobacteria.* Whilst Firmicutes remain the main bacterial phylum overall in all compartments, the relative abundance of *Proteobacteria* becomes highly notable and generally increases substantially in

the small intestine (*e.g.*, in the duodenum and ileum) compared to the hindgut (Costa et al., 2015a; Dougal et al., 2013; Ericsson et al., 2016). For instance, the phylum *Proteobacteria* was found to be significantly more abundant in the duodenum than in the large colon, small colon, and faeces, with the ileum exhibiting a greater relative abundance of this phylum (due largely to *Actinobacillus* spp.). These bacteria are mostly facultative anaerobes, and one hypothesis is that they consume residual oxygen, thereby creating more favourable anaerobic conditions for the microbial populations of the hindgut (Chaucheyras-Durand et al., 2022).

(iii) The proximal GIT microbiota appears more adapted to rapidly ferment simple carbohydrates, such as sugars and starch, and to tolerate fluctuating pH and oxygen levels. For instance, genera like *Lactobacillus* and *Streptococcus*, commonly found in the stomach and small intestine, are efficient sugar fermenters, which may be functionally relevant in early digestive compartments where enzymatic digestion predominates (Chaucheyras-Durand et al., 2022; Perkins et al., 2012).

(iv) In the hindgut, the microbiota is dominated by the phyla *Firmicutes* and *Bacteroidetes*, followed by other important phyla such as *Verrucomicrobia*, *Spirochaetes*, *Fibrobacteres*, and *Actinobacteria* (Costa et al., 2015a; Dougal et al., 2013; Ericsson et al., 2016). Within the *Firmicutes* phylum, families such as *Lachnospiraceae* and *Ruminococcaceae* are crucial components of the core microbiota. Members of the *Ruminococcaceae* family are widely recognised as cellulolytic and fibrolytic bacteria, capable of degrading complex carbohydrates like cellulose. Whilst *Lachnospiraceae* are highly relevant fibrolytic species, they are better known as major producers of SCFAs, particularly butyrate, and do not contain cellulolytic species in the strict. The production of these SCFAs, including acetate, propionate, and butyrate is consistent with the primary function of fibre fermentation expected in this region. These SCFAs play a key role in maintaining gut health by contributing to the epithelial energy supply (as previously mentioned, butyrate being the indispensable fuel for colonocytes), the regulation of intestinal pH, and overall immune modulation; (Chaucheyras-Durand et al., 2022; Den Besten et al., 2013; Venegas et al., 2019).

(v) The faecal microbiota reflects the microbial composition of the distal part of the GIT (Costa et al., 2015a; Dougal et al., 2013; Ericsson et al., 2016). This point is particularly valuable for future research. Indeed, the collection of ingesta from different GIT regions is an invasive procedure, and more ethical alternatives, such as using faecal material, should be promoted in

accordance with the Three R's principle: Replacement, Reduction, and Refinement. *In vitro* models simulating various segments of the equine GIT have also been developed and can serve as useful tools for studying microbial dynamics. However, these systems may not fully replicate the complexity and diversity of *in vivo* communities. In this context, faecal sampling remains a practical, non-invasive, and biologically relevant strategy for characterising the equine hindgut microbiota under both physiological and experimental conditions.

2.3.2. The Equine Faecal Microbiota as a Proxy for Hindgut Communities

This section focuses on the composition of the faecal microbiota in horses as described in studies using 16S rRNA gene sequencing. While faecal samples are widely used as a proxy for hindgut microbiota, limitations must be acknowledged before interpreting the data. First, the targeted region of the 16S rRNA gene varies between studies (*e.g.*, V1–V2, V3–V4, V4), and this choice significantly influences the microbial profile obtained (Costa and Weese, 2019; Hrovat et al., 2024; Kauter et al., 2019). Even when the same region is used, differences in DNA extraction protocols, sequencing depth, and bioinformatic pipelines may affect the comparability of results. Second, sampling methodology is critical. Indeed, the microbial composition differs between the outer surface and the centre of the faecal ball, with the latter more accurately reflecting rectal content (Stewart et al., 2018). Moreover, the time elapsed between sampling and freezing can alter microbial community structure. While some studies recommend immediate processing (Beckers et al., 2017), others have shown minimal changes with up to 6 hours at room temperature (de Bustamante et al., 2021; Stewart et al., 2018). However, changes in α-diversity and phylum-level composition have been observed after 12 hours (Beckers et al., 2017; Theelen et al., 2021). Therefore, the results reported in the following tables from the study by Stewart et al. (2018) correspond to samples taken from the centre of the faecal ball, which were collected directly from the rectum. Third, dietary intake is a major driver of microbiota composition (Garber et al., 2020; Kauter et al., 2019). Whenever possible, only data from horses fed forage-based diets (grass, hay, haylage) were included in this synthesis. For example, Costa et al. (2012) reported values for healthy horses on a controlled fibre-rich diet (Costa et al., 2012). In contrast, Theelen et al. (2021) did not provide dietary information, limiting the interpretation of their findings. Their results were therefore included in the composition of the faecal microbiota at the phyla level but excluded from finer

taxonomic comparisons (Theelen et al., 2021). Finally, to improve clarity and focus on biologically relevant patterns, only taxa with a mean relative abundance of ≥ 1% were retained in the tables. Although this threshold excludes some rare taxa, it reduces noise and aligns with common practices in microbiome research.

At the phylum level, *Firmicutes* and *Bacteroidetes* dominate the faecal microbiota of healthy adult horses (Table 5). In approximately half of the datasets, *Firmicutes* is the most abundant phylum, followed by *Bacteroidetes* (Costa et al., 2012; Fernandes et al., 2021a; Shepherd et al., 2012). The opposite pattern is observed in other studies (Salem et al., 2018; Stewart et al., 2018; Theelen et al., 2021), suggesting that both phyla could be co-dominant in the equine hindgut, though their proportions may vary depending on methodological or physiological factors. Other phyla commonly detected above 1% include *Spirochaetes, Proteobacteria, Actinobacteria, Verrucomicrobia*, and *Fibrobacteres,* though with more variability across studies. Nevertheless, the assessment of phyla is a high-level overview of the microbiota (Costa and Weese, 2018).

Table 5. Composition of the equine faecal microbiota at the phylum level

	16S rRNA Variable Regions Analysed					
Phylum	**V3-V5**	**V4**	**V1-V2**	**V1-V2**	**V3-V4**	**V3-V4**
Firmicutes	55.40%	43.73%	31.64%	32.50%	63.96%	28.40%
Bacteroidetes	10.03%	3.65%	39.04%	56.37%	21.88%	50.10%
Spirochaetes	1.00%	2.06%	9.32%	3.64%	1.37%	7.10%
Proteobacteria	27.53%	3.75%	1.49%	-	0.56%	-
Actinobacteria	5.27%	1.60%	-	-	1.71%	-
Verrucomicrobia	-	4.11%	3.29%	-	3.69%	6.50%
Fibrobacteres	-	0.75%	10.87%	4.16%	0.32%	5.00%
Unclassified/ Other phyla	-	38.14%	-	-	2.89%	-
Reference	(Costa et al., 2012)	(Shepherd et al., 2012)	(Salem et al., 2018)	(Stewart et al., 2018)	(Fernandes et al., 2021a)	(Theelen et al., 2021)

Only major phyla with a mean relative abundance ≥1% are shown, 16S rRNA gene regions targeted are indicated for each study.

At the family level, results from rectal samples analysed by Stewart et al. (2018) show dominance of *Bacteroidales*, *Lachnospiraceae*, and *Ruminococcaceae*, all well-known for their roles in fibre fermentation and SCFAs production (Table 6). Families such as *Prevotellaceae*, *Paraprevotellaceae*, *Fibrobacteraceae*, and *Spirochaetaceae* also appear frequently and contribute to

plant polysaccharide degradation, highlighting the functional adaptation of the equine gut microbiota to an herbivorous diet (Stewart et al., 2018).

Table 6. Main bacterial families identified in horse faeces

Phylum	**Family (or closest taxonomic level)**	**Mean relative abundance ± SE***
Bacteroidetes	*Bacteroidales (Order)*	32.32 ± 1.46 %
Firmicutes	*Lachnospiraceae*	13.43 ± 1.61 %
Firmicutes	*Ruminococcaceae*	8.45 ± 0.47 %
Bacteroidetes	*Prevotellaceae*	7.74 ± 0.93 %
Bacteroidetes	*Paraprevotellaceae*	6.25 ± 0.85 %
Firmicutes	*Clostridiales (Order)*	6.05 ± 0.40 %
Fibrobacteres	*Fibrobacteraceae*	4.16 ± 1.05 %
Bacteroidetes	*Bacteroidaceae*	3.65 ± 1.03 %
Bacteroidetes	*RF16*	3.12 ± 0.66 %
Spirochaetes	*Spirochaetaceae*	3.09 ± 0.42 %

* SE = standard error - Only families with mean relative abundance ≥1% are represented. Only baseline (T0) data from were included

Genus-level data further confirm the dominance of fibre-degrading bacteria such as *Prevotella*, *Ruminococcus*, *Fibrobacter*, and *Treponema* (Tables 7 and 8) (Fernandes et al., 2021a; Stewart et al., 2018). While *Akkermansia* is detected at relatively low abundance (~1%), it may play a significant role in mucin degradation and gut barrier integrity (Chaucheyras-Durand et al., 2022). Notably, many taxa remain unclassified beyond the family or order level, especially in older datasets. For example, Stewart et al. (2018) reported a high proportion of sequences attributed only to higher taxonomic ranks (*e.g., Bacteroidales, Clostridiales*), reflecting the incompleteness of reference databases at the time (Stewart et al., 2018). More recent studies (*e.g.,* Fernandes et al., 2021a) show improved classification, suggesting progress in taxonomic resolution over the past decade (Fernandes et al., 2021a).

Table 7. Main bacterial genera according to Stewart et al. (2018)

Phylum	**Genus (or closest level)**	**Mean relative abundance ± SE**
Bacteroidetes	*Bacteroidales (Order)*	32.32 ± 1.46 %
Firmicutes	*Lachnospiraceae (Family)*	12.25 ± 1.60 %
Bacteroidetes	*Prevotella (Genus)*	7.90 ± 1.00 %
Firmicutes	*Ruminococcaceae (Family)*	6.15 ± 0.25 %
Firmicutes	*Clostridiales (Order)*	6.05 ± 0.40 %
Fibrobacteres	*Fibrobacter (Genus)*	4.16 ± 1.05 %
Bacteroidetes	*RF16 (Family)*	3.12 ± 0.66 %
Spirochaetes	*Treponema (Genus)*	3.05 ± 0.43 %
Bacteroidetes	*BF311 (Genus)*	2.79 ± 0.88 %
Bacteroidetes	*YRC22 (Genus)*	2.44 ± 0.39 %
Bacteroidetes	*Paraprevotellaceae (Family)*	2.38 ± 0.38 %
Firmicutes	*Ruminococcus (Genus)*	1.59 ± 0.34 %
Firmicutes	*RFN20 (Genus)*	1.32 ± 0.51 %
Firmicutes	*Mogibacteriaceae (Family)*	1.06 ± 0.09 %
Bacteroidetes	*CF231 (Genus)*	1.04 ± 0.22 %

* SE = standard error - Only taxa with a mean relative abundance ≥1% are shown.

Table 8. Main bacterial genera according to Fernandes et al. (2021a)

Phylum	**Genus (or closest level)**	**Mean**
Firmicutes	*Ruminococcaceae* (Family)	22%
Firmicutes	*Lachnospiraceae* (Family)	14%
Firmicutes	*Clostridiales* (Order)	13%
Bacteroidetes	*Bacteroidales* (Order)	10%
	Unassigned genera	3%
Firmicutes	*Mogibacteriaceae* (Family)	2%
Bacteroidetes	*Prevotella*	2%
Firmicutes	*Ruminococcus*	2%
Verrucomicrobia	*RFP12* (Family)	2%
Bacteroidetes	*BS11* (Family)	2%
Bacteroidetes	*Paraprevotellaceae* (Family)	2%
TM7	*F16 (Family)*	2%
Bacteroidetes	*YRC22*	2%
Verrucomicrobia	*Akkermansia*	1%
Firmicutes	*Coprococcus*	1%
Firmicutes	*p-75-a5*	1%
Bacteroidetes	*CF231*	1%
Spirochaetes	*Treponema*	1%
Firmicutes	*Clostridiaceae* (Family)	1%
Firmicutes	*Oscillospira*	1%
Actinobacteria	*Coriobacteriaceae* (Family)	1%
Firmicutes	*Phascolarctobacterium*	1%
Bacteroidetes	*BF311*	1%
Firmicutes	*Clostridium*	1%
Firmicutes	*Christensenellaceae* (Family)	1%
Bacteroidetes	*RF16* (Family)	1%
Firmicutes	*Lactobacillus*	1%
Firmicutes	*Lachnospiraceae* (Family)	1%
Bacteroidetes	*Paludibacter*	1%

* SE = standard error - Only taxa with a mean relative abundance ≥1% are shown.

The data summarised here underline the complexity of comparing faecal microbiota compositions across studies due to methodological heterogeneity, lack of standardisation, and incomplete taxonomic resolution. While certain patterns emerge (*e.g.*, such as consistent dominance of *Firmicutes* and *Bacteroidetes*) and frequent presence of *Lachnospiraceae* and *Prevotellaceae*. Establishing reference faecal microbiota profiles for horses will require studies conducted under tightly controlled and reproducible conditions, with clearly defined diets, animal ages, health status, and standardised sample handling. Such reference datasets would be essential for identifying dysbiotic signatures in pathological contexts or evaluating the impact of nutritional interventions. Lastly, although the focus here is on taxa with relative abundances ≥1%, it is important to acknowledge that low-abundance taxa may have disproportionate functional roles. For example, *Akkermansia* has been linked to anti-inflammatory effects in humans. Some species of *Treponema* contribute to cellulose degradation. *Phascolarctobacterium* produces acetate and propionate from succinate and has been associated with positive health indicators (Chaucheyras-Durand et al., 2022). Therefore, future studies should complement taxonomic profiling with functional metagenomics or metabolomics to fully understand the ecological significance of these microbial populations.

2.4. Factors Influencing the Equine Gut Microbiota

2.4.1. Diet and Supplements

Diet is a fundamental factor influencing the intestinal microbiota. Horses are monogastric herbivores and are "designed" to ingest frequently small amounts of forage and walk during most of the day. However, domestication has introduced readily fermentable carbohydrates (*i.e.*, starch) and lipids into their diet to fulfil energy requirements. Changes in diet can significantly influence the composition and functionality of the equine gut microbiota. The microbial diversity tends to decrease as dietary availability of rapidly fermentable nutrients, especially starch, increases for example when introducing grains rich in starch to fibre-based diet. According to ecological theories, this reduction in diversity often correlates with less stable microbial communities, increasing the likelihood of gastrointestinal dysbiosis and disorders (Boucher et al., 2024; Garber et al., 2020; Morrison et al., 2018).

A high-starch diet is defined as one where starch consumption exceeds the safe upper limit intake of 1g/kg BW per meal (Harris and Dunnett, 2018; Harris and Shepherd, 2021). This type of diet promotes the proliferation in (i) *Lachnospiraceae* family and other propionic acid-producing bacteria, (ii) *Bacteroidetes* family, and (iii) lactic acid-producing bacteria (such as *Streptococci* and *Lactobacilli*). This overproduction of lactic acid can lower large intestine pH below 6.2 for extended periods, creating caecal and/or colonic subclinical acidosis as an unfavourable environment for beneficial bacteria like fibrolytic bacteria (*e.g.*, such as the *Ruminococcaceae* family and the genus *Fibrobacter*) and consequently, can lead to gastrointestinal disorders such as colic or laminitis (Chaucheyras-Durand et al., 2022; Daly et al., 2012; Durham, 2009; Ermers et al., 2023; Garber et al., 2020; Harlow et al., 2016; Morrison et al., 2018; Raspa et al., 2024). A study revealed that four days on feeding on pasture are necessary to horses fed with forage-grain diet to obtain a faecal microbiota like grass diet horses (Fernandes et al., 2014).

In contrast, forage-based diets (*i.e.*, grass, hay, haylage…), which contain less rapidly fermentable carbohydrates and nutrients that degrade at a slower rate, promote higher microbial diversity and help maintain a more diverse and stable microbial community, which is generally associated with better digestive health. Moreover, the fibre fermentation in the hindgut is known to increase acetic acid-producing bacteria: a process associated with a more diverse bacterial population. In this kind of diet, fibrolytic bacteria are more abundant than in high-concentrate diets and these bacteria help break down fibre, producing SCFAs, which are the main source of energy for the horse (Boucher et al., 2024; Durham, 2009; Garber et al., 2020; Raspa et al., 2024). In forage-fed horses, the most abundant bacterial genera were unclassified members within the families of, in descending order, *Ruminococcaceae*, *Lachnospiraceae, Clostridiales,* and *Bacteroidales* which accounted for a range between 54% – 62% of the total abundance of bacterial genera (Fernandes et al., 2021a). The α-diversity indices (Simpson and Shannon index) can be different following the composition of the diet: for example, they are higher in exclusively grass diet *vs.* grass and hay diet (Fernandes et al., 2021b, 2021a). This trend is followed by the evenness (Chao1 index) without being significant following studies. The β-diversity is also significantly different with the creation of clusters explaining 47% of the variation in faecal microbiota between horses fed exclusively with grass and horses fed with a supplement of hay (Fernandes et al., 2021a).

Differences between grass diet and grass/haylage diet are reported in other studies with an (i) increase of the phylum *Fibrobacter* and *Spirochaete* when haylage is introduced and (ii) an apparently associated decrease in the phyla *Firmicutes* (Salem et al., 2018). Differences between hay and haylage are also observed in terms of relative abundance of bacterial families: *Lachnospiraceae* were in the hay-fed ponies and *Rumminococcaceae* were higher in the haylage-fed ponies (Leng et al., 2022). Another type of diet is to use silage in horses' management: the α-diversity (*i.e.*, Shannon and Chao1 indices) is significantly lower in silage-fed horses *vs.* grass-fed horses and some differences in bacterial taxa are also observable (Zhu et al., 2021).

The traditional diets of horses combining forage and concentrate as oat, barley and corn lead to specific shifts in faeces microbial composition with an increase in *Clostridiaceae* and the presence of *Lactobacillus ruminis* compared to forage-based diets. These microbial changes are the result of rapid fermentation that escape digestion in the small intestine, reaching the large intestine and consequently are more marked with barley and corn compared to oat grains (Garber et al., 2020).

Contrary to what is expected, a study revealed no difference in faecal microbiota of horses concerning diet but revealed a difference of β-diversity between horses having access to pasture compared with no access (Theelen et al., 2021).

Interestingly, diet-induced changes in the gut microbiota may not only affect digestion but also behaviour. High-starch diets have been linked to increased stress and behavioural changes; a phenomenon referred to as alimentary stress. Research suggests that changes in gut microbiota could influence neuroendocrine pathways, altering the host's behaviour. This relationship between diet, gut microbiota, and behaviour has also been observed in human studies, where gut microbes are thought to manipulate eating behaviour by producing hormone-like neurochemicals (Boucher et al., 2024; Bulmer et al., 2019; Destrez et al., 2015, 2019; Garber et al., 2020; Hesta and Costa, 2021; Homer et al., 2023; Mach et al., 2020).

As review by (Berreta and Kopper, 2022) and according to the joint expert consultation of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are defined as living microorganisms that, when ingested in appropriate quantities, exert beneficial effects on the health of the host. To comply with this definition, a probiotic formulation must include viable microbial strains capable of (i)

surviving and remaining metabolically active at the target site within the host gastrointestinal tract (*i.e.*, mostly caecum and/or colon), (ii) persisting in adequate concentrations, and (iii) demonstrably contributing to host well-being.

In the equine context, common probiotic candidates include non-pathogenic yeasts (*Saccharomyces cerevisiae* and *boulardii*) and lactic acid bacteria (*Lactobacillus*, *Bifidobacterium*, *Enterococcus*), while prebiotics are typically oligosaccharides (FOS, MOS) intended to increase SCFA production and support fibrolytic taxa.

The effects of *Saccharomyces cerevisiae* supplementation on the equine hindgut microbiota remain inconsistent across studies (Perricone et al., 2022). Several investigations have reported a stimulation of fibrolytic bacterial populations such as *Ruminococcus* and members of the *Lachnospiraceae* family, together with improved fibre degradation and increased production of SCFA. Such responses are thought to mitigate starch-induced lactate accumulation and thereby reduce the risk of hindgut acidosis under high-starch feeding conditions (A. Garber et al., 2020; Jouany et al., 2009). In contrast, other studies have described only minor or negligible effects of *S. cerevisiae* on microbial composition and fibrolytic activity, despite reporting moderate alterations in fermentation parameters (such as pH, lactic acid) especially in the caecum, where yeast abundance is generally higher. These findings suggest that *S. cerevisiae* may help buffer adverse microbial and metabolic shifts when starch digestion in the small intestine is exceeded (Medina et al., 2002). Nevertheless, at least one study observed no measurable effect of *S. cerevisiae* supplementation on faecal microbial profiles or pH, indicating that the response to yeast addition may depend strongly on the specific strain used, dietary context, and individual variability among horses (Taran et al., 2016). Clinical trials report mixed outcomes for diarrhoea reduction and clinical recovery in enterocolitis (Boyle et al., 2013; Desrochers et al., 2005).

In vitro and strain-level evaluations demonstrate that horse-isolated *Lactobacillus spp*. can display desirable probiotic traits (*e.g.*, acid/bile tolerance, adhesion, antimicrobial activity), supporting the potential advantage of equine-adapted strains over generic commercial products (Nogacka et al., 2025; Schoster et al., 2016). Synbiotic formulations produce reproducible increases in SCFAs in fermentation models despite limited compositional shifts, indicating metabolic modulation without necessarily altering community structure (Macnicol et al., 2023).

Most equine probiotic products fail to meet core requirements for viability, accuracy, and proven efficacy (Berreta et al., 2021; Weese and Martin, 2011). Major inconsistencies in product quality persist, with marked discrepancies between labelled and actual microbial contents (Berreta and Kopper, 2022), and the literature is affected by publication bias favouring positive outcomes (Weese, 2025). Overall, evidence supporting probiotic supplementation for improving starch or fibre digestion, preventing gastrointestinal disorders, or managing foal scouring remains weak, and adverse effects have been reported with high doses of poorly characterized strains. Although most species used are of human origin, multistrain formulations show some promise for enhancing performance in exercising horses (Cooke et al., 2021), yet current data remain insufficient to justify routine use in equine health management.

The definition of prebiotics has evolved from substrates that resist host digestion, are selectively fermented, and stimulate beneficial gut bacteria, to the broader concept of "a substrate selectively utilized by host microorganisms conferring a health benefit". Although most established prebiotics are carbohydrate-based oligosaccharides such as fructo-oligosaccharides (FOS), inulin, galacto-oligosaccharides (GOS), mannan-oligosaccharide (MOS), and xylo-oligosaccharide (XOS), other compounds may fit the updated definition if their effects are demonstrated in vivo and mediated through the microbiota. Importantly, prebiotic efficacy and safety must be validated in the target species (Gibson et al., 2017).

In horses, the effects of prebiotics are variable and depend on their site of fermentation. Fructo-oligosaccharides are widely used but can be fermented in the stomach, generating butyrate concentrations capable of damaging both squamous and glandular gastric mucosa (Cehak et al., 2019). Other substrates may offer more favourable profiles. Cellobiose supplementation induced dose-dependent shifts toward saccharolytic taxa such as Clostridium, consistent with potential prebiotic activity (Paßlack et al., 2020). In foals, early-life supplementation with an oligosaccharide-rich diet increased *Akkermansia spp*. and taxa associated with regulatory immunity, and microbiota transfer experiments indicated enhanced anti-inflammatory potential (Lindenberg et al., 2021). These findings highlight both the promise and the need for careful evaluation of prebiotics in the equine species.

2.4.2. Exercise and Metabolic Status

Food digestibility is enhanced by exercise in horses, but its effects on the gut microbiota are only beginning to be elucidated. Gastrointestinal health is crucial for equine athletes, yet current evidence suggests that management and training routines may exert a greater impact on gut microbial composition than exercise alone (Boucher et al., 2024; Garber et al., 2020).

Initial studies found no significant changes in microbial diversity following standardised exercise tests (Szemplinski et al., 2020). However, multi-omics approaches later revealed functional interactions between the gut microbiota and host metabolism, particularly involving energy metabolism, oxidative stress, and inflammation (Mach et al., 2021b). More complex and diverse microbiomes have been associated with improved mitochondrial β-oxidation and glucose metabolism, whereas specific microbiome profiles correlated negatively with cardiovascular capacity (Mach et al., 2022).

Training intensity, stress, and housing conditions also play major roles. Equitation-related stressors were linked to gut microbiota variability and to behavioural indicators of reduced welfare, supporting the existence of a microbiota–gut–brain axis (Mach et al., 2020). Improved welfare through temporary pasture turnout led to more stable microbial profiles and lasting increases in beneficial taxa, suggesting resilience and positive feedback between welfare and microbial balance (Mach et al., 2021a).

Functionally, microbial capacity for fibre degradation and SCFA synthesis appears beneficial for performance. The efficiency of fibre fermentation and predicted pathways related to butyrate and thiamine metabolism were positively associated with maximal running speed, while starch degradation pathways correlated negatively (Vasseur et al., 2024). High-performance horses generally display greater microbial diversity and enrichment in pathways producing polyamines, butyrate, and vitamin K, whereas low-performance horses show metabolic signatures of dysbiosis and fatigue (Park et al., 2024). Moreover, racehorses possess microbiomes enriched in genes for SCFA synthesis, particularly butyrate, potentially supporting higher energy efficiency (Li et al., 2025, 2023).

Altogether, these findings indicate that exercise itself exerts modest effects on microbial diversity, but long-term training, physiological adaptation, and management stress strongly influence gut microbiota composition and function. The enrichment of butyrate-producing taxa (notably *Lachnospiraceae*, *Ruminococcus*, and *Eubacterium*) and metabolic pathways related

to SCFAs, and vitamin synthesis appear to support both metabolic efficiency and resilience to stress in equine athletes. This interplay between gut microbial activity, host energy metabolism, and performance underscores the relevance of the gut–muscle and gut–brain axes in equine sports physiology (Li et al., 2025, 2023; Mach et al., 2022, 2021a, 2020b; Park et al., 2024; Szemplinski et al., 2020; Vasseur et al., 2024).

Obesity and equine metabolic syndrome (EMS) are increasingly recognized as major health concerns in horses, contributing to insulin dysregulation, laminitis, and reduced performance. Diet is a primary driver of both obesity and EMS, and it strongly shapes the gut microbiota. In turn, the gut microbial composition can influence energy extraction and metabolism, potentially exacerbating obesity and metabolic dysregulation (Garber et al., 2020). Horses with EMS or obesity tend to exhibit subtle alterations in hindgut microbial communities, including shifts in fibre-degrading bacteria and taxa involved in carbohydrate and lipid metabolism, rather than large-scale changes in overall diversity (Al-Ansari et al., 2025; Biddle et al., 2018; Coleman et al., 2019; Elzinga et al., 2016). These microbial changes may reflect a reduced functional capacity for fibre fermentation and energy homeostasis, contributing to the metabolic phenotype associated with EMS.

2.4.3. Seasonal & Spatial Interactions

Geographic area, seasonal variations and social interactions appear to play a role in establishing the gut microbiome (Garber et al., 2020; Zhao et al., 2016). Indeed, Salem et al., (2018) studied faecal microbiota composition in normal horses over 12 months. The samples were collected every 14 days in a population of 7 horses managed at pasture with minimal changes in management. Changes in faecal microbiota composition were associated with season, supplementary forage and ambient weather conditions (*i.e.*, ambient temperature and rainfall): these parameters explained 13.2% of variation in the data. The ambient temperature was significantly associated with α-diversity modification and higher ambient temperature, and rainfall were significantly associated with β-diversity (Salem et al., 2018).

The seasonal impact on β-diversity is also underlined by Theelen et al., (2021) with a significant difference between summer samples and winter samples but this difference explained only 2.8% of the total observed variation in faecal microbiota (Theelen et al., 2021).

Another study aimed to characterise seasonal variation in the faecal microbiota of forage-fed horse on pasture over a 12-month period with hay supplemented from June to October. The characterisation of α-diversity showed that (i) Simpson's diversity index was significantly higher in autumn compared to summer, winter, and spring, (ii) the Shannon index (*i.e.,* entropy) was significantly higher in summer and autumn than in winter and spring and (iii) the Chao1 index (*i.e.,* richness) was similar in summer, autumn, and winter, but significantly lower in spring. The β-diversity were also impacted by season with the apparition of clusters: one cluster observed during the drought period, a second cluster during the late autumn and winter months and the third cluster during the remaining months of the year (Fernandes et al., 2021a).

Hypothesis about the influence of season on the horse faecal microbiota could be due to the effect of weather on the composition of environmental bacteria (soil and grass/haylage microbiota) (Chaucheyras-Durand et al., 2022; Salem et al., 2018) or in the nutrient composition of the pasture, which in turn is influenced by climatic conditions (Fernandes et al., 2021a).

2.4.4. Antimicrobial, Nonsteroidal Anti-Inflammatory Drugs and Anaesthetics

Antibiotic use in horses can cause dysbiosis, resulting in conditions like antimicrobial-associated diarrhoea and colitis (Arnold et al., 2021; Barr et al., 2013). There is a consensus that antibiotics are associated with a decreased richness and diversity. Although the incidence is low, antibiotics can pose a risk by allowing the proliferation of pathogens like *Clostridium difficile* (Barr et al., 2013; Harlow et al., 2013; Parker et al., 2024).

Costa et al., (2015) studied the effects on equine faecal microbial populations of intramuscular administration of procaine penicillin and ceftiofur sodium and, also, of oral administration of trimethoprim sulfadiazine. The trimethoprim-sulfadiazine has a more significant impact on microbiota than others reducing bacterial richness and diversity and affecting especially cellulolytic bacteria needed for fibre digestion (Costa et al., 2015). These results are supported by other studies (Di Pietro R et al., 2021; Gomez et al., 2023; Theelen et al., 2023). Moreover, the oral administration of trimethoprim sulfadiazine has a rapid and long-lasting effect on faecal microbiota and resistome (*i.e.*, relative abundance of resistance genes) (Theelen et al., 2023).

Another study compared intravenous administration of ceftiofur, enrofloxacin and oxytetracycline on equine faecal microbiota during 5 days of administration of treatment and over 30 days post treatment. The richness was significantly different at day 3 for ceftiofur and the most significant effect on relative abundance were noted after administration of ceftiofur and enrofloxacin (Liepman et al., 2022). Metronidazole led to microbiota disorders and gastro-intestinal disorders after three days (Arnold et al., 2020).

Additionally, nonsteroidal anti-inflammatory drugs, other medications frequently administered to horses, can also cause transient modification in gut microbiota (Boucher et al., 2024; Garber et al., 2020). The administration of phenylbutazone and firocoxib is characterised by a decrease in *Firmicutes*, *Ruminococcaceae*, *Clostridiaceae* and *Lachnospiraceae* (Whitfield-Cargile et al., 2018).

Anaesthesia and fasting may have an impact on equine gut microbiota and may increase prevalence of diarrhoea, colitis, and colic onset after surgery. However, the study design of studies doesn't allow to separate fasting and anaesthesia (and sometimes transport): consequently, it is not possible to characterise specific alteration of each treatment (Boucher et al., 2024; Garber et al., 2020). Indeed, transportation induces stress that can impact both health and performance, especially in horses that are not regularly exposed to travel. Yet, horses are frequently transported for various purposes, including competition, training, veterinary care, and breeding. Some studies concluded that differences in gut microbiota are effective between transport horses *vs.* control horses, but studies also suggest that stress from transportation or herd separation may confound results, highlighting the need for carefully designed studies to assess the true impact of transportation on the microbiome (Garber et al., 2020).

2.4.5. Helminthes and Anthelmintics

In horses, anthelmintic treatments can disrupt the gut microbiota and are associated with risk of colic. Studies have found that treatments, like fenbendazole and moxidectin, cause a decrease in the *Bacteroidetes* phylum and an increase in *Firmicutes*. The shift in bacterial populations is notable but varies depending on the sample size and treatment used. Parasite infections tend to increase the abundance of specific bacteria linked to inflammation, such as *Clostridium* and *Campylobacter*, while reducing butyrate-producing bacteria that have anti-inflammatory properties. Anthelmintic treatments also tend to reduce microbial diversity,

although the effects may reverse after treatment. Some studies report a temporary decrease in microbial diversity and an increase in inflammatory biomarkers post-treatment. However, overall, the long-term impacts of these treatments on the gut microbiota seem limited, with no major disruptions observed in certain studies (Boucher et al., 2024; Garber et al., 2020).

2.4.6. Age, Gender, and Individual Variation

The colonisation of the GIT commences at birth and progresses through weaning into adulthood. While foals develop in a sterile intrauterine environment, recent research indicates potential prenatal exposure to microbial components originating from the mare's microbiome. Postpartum, the external environment becomes the primary source of microbial colonisation, with microorganisms being introduced through ingestion of bacteria from the mare's skin, vagina, udder, and surrounding environment. As the foal matures, its gut microbiota undergoes continuous adaptation in response to dietary shifts, notably the transition from a milk-based to a forage-based diet around 1 or 2 months of age. The timeline for establishing a stable microbial community varies across studies, with estimates around 60 days post-parturition. The microbiota's change during foal live until weaning are described further (Boucher et al., 2024; Garber et al., 2020).

In mature and elderly horses, some studies reveal a decline in bacterial α- and β-diversity observed with advancing age (Baraille et al., 2024; Theelen et al., 2021). This reduction in diversity may be attributed to age-related physiological changes, including prolonged gastrointestinal transit time, dental deterioration, and modifications in dietary intake and energy requirements (Boucher et al., 2024; Garber et al., 2020).

Recently, Baraille et al., aimed to (i) characterise the faecal microbiome in physically health horses aged from 6 to 30 years old, living in the same environment and consuming the same diet, (ii) assess parameters changing linearly with age and (iii) identify a pivotal age category. For that purpose, four categories were compared: 6 to 10 years old (yo), 11 to 15 yo, 16 to 20 yo and 21 to 30 yo. The richness (*i.e.*, measure of the number of species in the community (Chao et al., 2005) – ASV and Chao 1 index) and the intra-sample diversity (*i.e.*, Inverse Simpson and Shannon indexes which are an estimator of taxa diversity combining richness and evenness (Kers and Saccenti, 2022) with evenness defined as the distribution of abundances of the different species in the community (Chao et al., 2005)) were negatively

correlated with age ($p < 0.05$). According to age category, (i) the number of ASV were higher in 6 to 10 yo and in 11-15 yo compared to 21 to 30 yo ($p < 0.05$), (ii) the inverse Simpson index was higher in 11 to 15 yo compared to 21 to 30 yo ($p < 0.05$) and (iii) the Shannon index was higher in 6 to 10 and 11 to 15 yo compared to 21 to 30 yo. A difference was noted in the β-diversity (*i.e.*, inter-sample diversity) between categories. In terms of families, (i) the *Bacteroidales RF16 group*, *Prevotellaceae* and *Lachnospiraceae* were more represented in the 6 to 10 yo category, (ii) the *Bacteroidales BS11 gut group*, *p-251-o5*, *Prevotellaceae*, *Desulfovibrionaceae*, *Lachnospiraceae* were more represented in 11 to 15 yo category, (iii) the *Muribaculaceae* in the 16 to 20 yo category and (iv) the *Eggerthellaceae* and the *Ruminococcaceae* in the last category. The authors underlined the microbiome rearrangement which appeared to be taking place in the 21 to 30 yo category additionally with an acidification of the faecal environment without any difference in the function of fibre degradation probably due to the rearrangement of bacterial communities (Baraille et al., 2024).

Differences between faecal microbiota of female and male horses have been reported in a study (Hu et al., 2021) but these conclusions are not observed in one other study (Theelen et al., 2021). The faecal microbiota in females during late pregnancy and non-pregnant females have also been reported to be different probably due to the alteration in intestinal capacity, hormonal changes, and stress related to changes happening in the management of the mare before parturition (Garber et al., 2020).

The gut microbiota presents individual variations which are the results of several individual factors. In a study, which aimed to characterise the faecal microbiota of physically healthy horses kept on pasture, the impact of each individual can be significantly visualised on the β-diversity analysis. At the phylum level, the relative abundances of *Verrucomicrobia* and *Tenericutes* were significantly different between the horses without impacting the most dominant phyla (*Firmicutes* and *Bacteroidetes*) and at the genus level, only two genera differed between horses without including the most dominant genera. These two genera are an unclassified genus from *Bacteroidaceae* family and an unclassified genus from *Verrucomicrobia* phyla. From these data, the diet (pasture *vs.* pasture and hay) could be considered as a confounding factor: the data were consequently analysed within each diet period, and the individual impact of horses were also relevant without exclude the possibility of temporal factor influencing the Bray-Curtis dissimilarity index (*i.e.*, β-diversity analysis) (Fernandes et

al., 2021a). These variations during the year without the influenceof the diet is also highlighted in another study (Leng et al., 2022).

2.4.7. Weaning

The development of the intestinal microbiota in foals is a dynamic process that begins at birth and evolves as the animal matures. The gut microbiota plays a crucial role in the health and development of foals, influencing digestion, immunity, and overall growth. Several studies have shed light on how the foal's gut microbiota is established, how it changes over time, and the impact of weaning, a critical life event.

At birth, the equine GIT is sterile. However, colonisation begins immediately as the foal comes into contact with the mother's vaginal and faecal microbiota, as well as the surrounding environment (Husso et al., 2020). The first few hours of life are crucial, as the foal ingests colostrum and is exposed to a variety of bacterial communities that initiate the formation of its gut microbiome. Studies indicate that bacteria, or at least bacterial DNA, can already be detected in the foal's intestine within the first 24 hours, with a significant presence of *Firmicutes* and *Proteobacteria* at this stage (Costa et al., 2016; Husso et al., 2020). The bacterial community in newborn foals is highly susceptible to environmental influences, including the maternal microbiota and dietary intake. Coprophagic behaviour also plays an important role in shaping the microbiota of foals in the first few weeks of life (Costa et al., 2016). The foal's gut microbiota is initially dominated by genera such as *Gemella* and *Corynebacterium* from the mother's vaginal microbiota, with a rapid shift in composition within the first week of life as the foal's gut adapts to its diet and environment (Husso et al., 2020).

Between birth and two months of age, the microbiota of foals undergoes significant changes. In the first month, microbial diversity is lower compared to older animals, with the foal's microbiota being distinct from that of adult horses (Costa et al., 2016). During this period, fibre-fermenting bacteria such as *Akkermansia* begin to proliferate, reflecting the gradual adaptation of the foal's gut to a more fibre-rich diet. Studies show that as foals age, the microbial community becomes more stable, with the diversity of the microbiota increasing as the foal starts to ingest solid feed (Costa et al., 2016). The foal's gut microbiota shows considerable individual variation during this early developmental stage, but some genera remain consistent across studies. For example, *Fibrobacteres* and *Clostridiales* tend to increase

after weaning, which reflects the adaptation of the gut to a diet containing more solid, fibrous feeds. By 60 days of age, the composition of the foal's microbiota begins to resemble that of adult horses, although significant differences in community membership persist up to nine months of age (Costa et al., 2016; Husso et al., 2020).

By two months of age, the foal's gut microbiota resembles that of an adult horse in terms of dominant bacterial genera, although some differences in community composition persist. Bacteria from the *Firmicutes* phylum, particularly members of the *Lachnospiraceae* and *Ruminococcaceae* families, become more abundant as the foal matures (Costa et al., 2016). The microbial community continues to evolve as the foal's diet becomes more fibre-based, and by the time the foal reaches one year of age, the microbiota is largely similar to that of adult horses (Husso et al., 2020). However, subtle changes in microbial diversity and community structure can continue into the yearling stage. For example, foals that experience significant stress events, such as abrupt weaning or dietary changes, may exhibit temporary shifts in their microbiota that gradually stabilise over time (Mach et al., 2017).

Weaning is a stressful event in a foal's life, and it has a profound impact on the gut microbiota. Weaning typically occurs between six and eight months of age, when the foal transitions from a milk-based diet to solid food. This dietary shift, combined with the social and physical separation from the mare, introduces significant changes to the microbiota. Several studies have investigated the effects of weaning on the composition of the gut microbiota in foals. Mach et al. (2017) compared progressive and abrupt weaning and reported marked microbial shifts in both groups. One month before weaning, coinciding with the introduction of a cereal-based diet, foals subjected to abrupt weaning showed higher abundances of the genera *Prevotella*, *Paraprevotella*, and *Ruminococcus* compared to progressively weaned foals. These genera, known for their saccharolytic and fibrolytic activities, suggest that the gut microbiota of abruptly weaned foals was better adapted to the upcoming dietary transition. The authors proposed that repeated separations in progressively weaned foals may have induced sustained stress responses, thereby inhibiting the proliferation of these beneficial taxa. After weaning, Streptococcus displayed the opposite trend, showing higher abundance in abruptly weaned foals, possibly reflecting stress-related alterations in the gut environment (Mach et al., 2017). Further research by Lindenberg et al., (2019) showed that the microbial community became increasingly dominated by fibre-

fermenting species such as *Akkermansia* after weaning, and by day 50, the microbiota had reached a relative level of stability. Interestingly, this study found that weaning did not significantly impact the microbial composition, highlighting the need for further research to clarify the effects of weaning on the gut microbiota (Lindenberg et al., 2019).

2.5. Equine Diseases associated with Microbiota Imbalance

When an alteration of the intestinal microbiota is associated with a disease, the question arises whether this alteration reflects the host's health status or actively influences it, especially with intestinal disorders. Indeed, a study compared the faecal microbiota composition of horses with large intestinal disease, small intestinal disease and control horses. Horses with intestinal disease showed a significant decrease in the Shannon index (*i.e.*, α-diversity) and horses with large intestinal disease showed a significant diminution of richness (*i.e.*, measure of the number of species in the community) compared with healthy horses. The β-diversity was also significantly different among the three groups (Park et al., 2021).

2.5.1. Colitis and Diarrhoea

The primary infectious bacterial agents linked to colitis include *Clostridium difficile*, *Clostridium perfringens*, *Salmonella* spp., and *Neorickettsia risticii* (Costa and Weese, 2018). Current research is exploring the contribution of facultative pathogens to diarrhoea in horses, though it remains unclear whether certain bacteria are overrepresented in horses with colitis due to their role in disease development or because they proliferate more rapidly in affected gut environments.

In a study by Costa et al. (2012), healthy horses had a predominance of *Firmicutes* (68%), followed by *Bacteroidetes* (14%). However, in horses with colitis, *Bacteroidetes* (40%) became the dominant phylum, surpassing *Firmicutes* (30%). Healthy horses also exhibited higher levels of *Actinobacteria* and *Spirochaetes*, while horses with colitis showed an increase in *Fusobacteria*. Additionally, *Lachnospiraceae*, a family within the *Clostridia* class, were more abundant in healthy horses, suggesting their potential role in maintaining a stable gut microbiome (Costa et al., 2012). Alterations in the gut microbiota during colitis are expected, as enteropathogenic bacteria have been associated with the disease. However, the exact cause

is often unclear. Moreover, intestinal barrier dysfunction may contribute to microbial imbalances (Garber et al., 2020).

Diarrhoea is a common issue in foals, *Rotavirus* being the most frequent cause, followed by *C. perfringens*, *Salmonella*, and *C. difficile* (Frederick et al., 2009). Foals with diarrhoea show decreased bacterial richness, particularly in *Lachnospiraceae* and *Ruminococcaceae* families (Schoster et al., 2017). A potential equine enteropathogen that requires further investigation is *Fusobacteria* because it is found in horses with colitis and diarrhoea but absent in healthy individuals (Costa et al., 2012; Rodriguez et al., 2015). The α-diversity analysis of faecal microbiota in diarrheic horses decreased compared to healthy control (Li et al., 2022) in term of richness and evenness (Rodriguez et al., 2015).

2.5.2. Colic

Colic, or abdominal pain, is common in horses and its impact on the gut microbiota has been explored in only a few studies. Research has shown that during colic episodes, specific bacterial populations tend to increase, while post-colic periods see a return to baseline levels. Recent longitudinal data confirmed that microbial communities fluctuate during hospitalization and that the dynamics of these changes differ depending on the type of colic (Loublier et al., 2025). Additionally, there appears to be a relationship between the ratio of *Firmicutes* and *Proteobacteria* and the likelihood of developing colic; a higher ratio would be associated with a lower risk of colic. It is not fully understood whether these changes are causes or effects (Garber et al., 2020; Lara et al., 2022; Loublier et al., 2025).

Further studies have demonstrated that horses suffering from colic exhibit reduced microbial diversity and richness compared to those undergoing elective surgeries. The relative abundance of commensal bacteria, such as *Prevotella* and *Lachnospiraceae*, is notably decreased in colic-affected horses, while opportunistic pathogens like *Streptococcus* and *Sphaerochaeta* are more prevalent. In colic caused by inflammatory obstruction, the increase of the relative abundances of *Bacilliculturomica* and *Saccharofermentans* were associated with survival (Loublier et al., 2025).

Identifying early microbial shifts in the faeces, which may occur before clinical symptoms arise, could help in the early detection of horses at increased risk of colic. There is also evidence to suggest that the onset of colic might be linked to the inability of the gut microbiota

to adapt to environmental changes, such as variations in forage type, season, or weather conditions, which could differ between individual horses. This variability may explain why some horses are more susceptible to colic, while others remain unaffected (Garber et al., 2020).

2.5.3. Inflammatory Bowel Disease

The article by Hashimoto-Hill and Alenghat, (2021) reviews the composition of inflammation-associated microbiota across various domestic animals, focusing on how gut dysbiosis correlates with intestinal inflammation. In horses, the microbiota is primarily composed of Firmicutes and Bacteroidetes, essential for fibre fermentation and nutrition. Dysbiosis in horses, particularly increases in *Fusobacteria* and decreases in *Clostridia*, has been linked to conditions like idiopathic colitis and antibiotic-induced colitis, though specific studies on equine inflammatory bowel disease (IBD) are limited. This pattern aligns with other species like dogs, which are often studied due to their close similarity to human IBD. In dogs, decreases in *Firmicutes* and *Fusobacteria* are observed, alongside increases in *Gammaproteobacteria,* indicating microbial shifts associated with chronic gastrointestinal issues. The review also highlights how microbiota imbalance does not necessarily correlate with pathogenic bacteria but suggests functional disturbances within microbial communities. For example, some functional pathways, like benzoate degradation, are enriched in canine and feline microbiota during inflammation, similar to findings in human IBD. The authors suggest that a multi-species comparison and further functional analysis of dysbiosis microbiota may offer insights into shared mechanisms of intestinal inflammation (Hashimoto-Hill and Alenghat, 2021).

Recently, profiling the equine gut microbiota has emerged as a promising diagnostic tool for detecting IBD in horses. Studies show that horses with IBD exhibit distinct microbial signatures that correlate with clinical markers of intestinal inflammation. Importantly, these microbial patterns could potentially allow for non-invasive monitoring of disease progression and responses to therapy, highlighting the translational value of microbiota profiling in veterinary medicine (Sävilammi et al., 2024).

2.5.4. Laminitis

Laminitis can be triggered by multiple factors, including dietary starch overload and fructan consumption from lush pastures. Other causes include non-dietary factors like repeated hoof trauma, retained placenta, colitis-associated laminitis, and colic. Changes in the equine gut microbiome associated with carbohydrate-induced laminitis – or oral administration of oligofructose mimicking pasture-induced laminitis - are mainly attributed to alterations in *Streptococci* and *Lactobacilli* (Garber et al., 2020; Milinovich et al., 2006; Moreau et al., 2014).

2.5.5. Equine Grass Sickness

A study revealed that both α- and β-diversity are significantly different in horses with Equine Grass Sickness (EGS) *vs.* control horses. In fact, EGS is associated with a significant reduction in the α- diversity of colonic microbiota (*i.e.*, difference in the number of observed species and Chao1 index). The bacterial diversity within the faeces of horses with EGS has greater variation than the control groups. At phylum level, a higher relative abundance of *Bacteroidetes* and *Proteobacteria* was observed in EGS horses compared with the control horses, while the relative abundance of *Firmicutes* and *Verrucomicrobia* was lower (Leng et al., 2018). This increase in the relative abundance of *Bacteroidetes* and the concomitant decrease in *Firmicutes* have previously been reported in horses with colitis (Costa et al., 2012; Leng et al., 2018). Five bacterial classes were found to be significantly more abundant in the faecal microbiota of EGS horses compared to controls. These classes are, in order of importance of percentage of different OTU between groups, Clostridia, *Gammaproteobacteria*, *Fusobacteria*, *Bacteroidia,* and *Deltaproteobacteria* (Leng et al., 2018). These bacteria are also more represented in horses with colitis and diarrhoea compared to control horses (Costa et al., 2012; Leng et al., 2018; Rodriguez et al., 2015).

2.5.6. Equine Atypical Myopathy

Equine AM is a severe intoxication in equids due to the ingestion of protoxins called HGA (Valberg et al., 2013; Votion et al., 2014) and MCPrG (Bochnia et al., 2019). Studies have explored potential microbiota-related mechanisms involved in this intoxication syndrome in equids (Karlíková et al., 2016; Wimmer-Scherr et al., 2021) and in other species (Chen et al.,

1957; Engel et al., 2025; Renaud et al., 2022). In ovine species, a study challenged the hypothesis of a potential involvement of the microbiota in HGA poisoning (González-Medina et al., 2021). However, this study did not consider the specific retention times in the ovine digestive tract (*i.e.*, 7–35 h for solutes and 10–50 h for dry matter (Clauss et al., 2006)), nor the potential conversion of HGB into HGA during the first two hours following incubation with sycamore maple seeds that contain the protoxins. These aspects, along with a comprehensive overview of AM pathophysiology, will be discussed in the next chapter.

3. Equine Atypical Myopathy

Atypical myopathy is a seasonal intoxication which affects pastured equids and is linked to the ingestion of seeds and seedlings mainly from sycamore maple (*Acer pseudoplatanus*), a species widespread in temperate regions of Europe (Valberg et al., 2013; Votion et al., 2014). Field maple (*Acer campestre*) and Norway maple (*Acer platanoides*), two species also present in the European temperate regions are non-toxic (Fowden and Pratt, 1973; Westermann et al., 2016).

The sycamore maple tree contains the protoxins (*i.e.*, HGA and MCPrG) responsible for the disease (Bochnia et al., 2019; Valberg et al., 2013; Votion et al., 2014) as well as their γ-glutamyl peptide (El-Khatib et al., 2022; Fowden and Pratt, 1973). The main protoxin, HGA, is contained in seeds (Baise et al., 2016; Doležal et al., 2020; El-Khatib et al., 2022; Fowden and Pratt, 1973; Unger et al., 2014; Westermann et al., 2016), seedlings (Baise et al., 2016; El-Khatib et al., 2022; Westermann et al., 2016), and to a lesser extent, in leaves(Doležal et al., 2020; El-Khatib et al., 2022; Westermann et al., 2016). As a result, AM outbreaks predominantly occur in autumn (*i.e.*, October–December) and spring (*i.e.,* March–May) when horses consume seeds and seedlings, respectively (Votion et al., 2020).

3.1. History and Epidemiology

A first case of enzootic myoglobinuria in a pony was reported in Australia in 1951 with clinical signs compatible with AM as an inability to rise, presence of dark urine, normothermia, and a preserved appetite (Irwin and Pulsford, 1951). After that, an outbreak of myoglobinuria

under unusual circumstances in four horses was reported in Canada in 1960 (Popef and Heslopt, 1960). Finally, a report of an outbreak of acute myopathy in animals kept in the meadow during the cold season was described in cattle with signs and biochemical blood analyses (*i.e.*, recumbency, ataxia and serum creatinine kinase values) compatible with a diagnosis of AM (Linklater et al., 1977).

During the 1980s, the entity of AM was finally recognised, and long before the aetiology was known, several papers reported outbreak of acute myopathy affecting horses at pasture during high-risk seasons in Europe: in autumn 1984 and in spring 1985 in east and south-west of Scotland (Hosie et al., 1986), in spring 1985 in England (Whitwell et al., 1988), in autumn 1995, and spring 1996 in Germany (Brandt et al., 1997).

In 2004, an alert group named "Atypical Myopathy Alert Group" (AMAG) was created in Belgium to alert practitioners and horse owners, and in 2006 this network expanded across Europe to ensure epidemiological surveillance and the dissemination of information. In France, a similar network for equine diseases, composed of French sentinel practitioners, already existed *i.e.*, the "Réseau d'Épidémio-Surveillance en Pathologie Équine (RESPE)" with which AMAG collaborates closely. Thanks to these networks, additional cases considered highly probable cases or confirmed cases of AM were reported in Europe: in Belgium (D. Votion et al., 2007), in Spain (Rivero and Palencia, 2007), in the Netherlands (van der Kolk et al., 2010; Westermann et al., 2016), in Sweden (Gröndahl et al., 2015), in New Zealand (McKenzie et al., 2016), in Denmark (Høffer et al., 2016), in England (Dunkel et al., 2020; François et al., 2024), and in the Czech Republic (Jahn et al., 2024).

In the United States, an acute severe non-exertional rhabdomyolysis called "Seasonal pasture myopathy" was gradually correlated with multiple acyl-CoA dehydrogenation deficiency (MADD), similar to AM (Finno et al., 2006; Sponseller et al., 2012; Valberg et al., 2013). The first epidemiological analyses of European cases then emerged, making it possible to highlight characteristics of the disease such as its clinical picture, environmental characteristics, possible risk and protective factors (Votion et al., 2007; Votion et al., 2009; van Galen et al., 2010; Van Galen et al., 2012b, 2012a), and especially the diagnostic algorithm used to categorise cases reported to the AMAG (Van Galen et al., 2012a).

The penultimate European counting of AM cases registered by AMAG covered a period of 13 years (2006–2019) and includes 2,371 horses from, in order of prevalence, France,

Belgium, the United Kingdom, Germany, the Netherlands, Switzerland, Spain, the Czech Republic, Ireland, Austria, Denmark, Sweden, Luxembourg, and Portugal. This paper answers to the frequently asked questions regarding horse feeding and management practices to reduce the risk of AM thanks to additional data collected by AMAG. A relevant clinical finding for European veterinarians from this study is that 94% of "autumnal" cases occurred between the 1st of October up to the 31st of December, and 94% of "spring" cases occurred between the 1st of March and the 31st of May (Votion et al., 2020). The most recent European survey, covering 2006 to 2023 in the AMAG database, records 3,199 horses and adds Hungary to the list of countries where AM cases have been reported (François et al., 2024).

3.2. Hypoglycin A: Structure and Toxic Doses

The structure of HGA was discovered as the methylenecyclopropylalanine (*i.e.*, $C_7H_{11}NO_2$) during the 1960s (Black and Landor, 1968, 1963). The amino acid structure of HGA typically consists of one carboxyl group (-COOH) and one amine group ($-NH_2$) linked to the same alpha carbon (C_α) and by a radical group composed of a methylene group ($=CH_2$) adjacent to a cyclopropyl cycle composed of three carbon atoms (Figure 3). The presence of this three-carbon cyclopropyl ring in the HGA structure is particularly noteworthy due to the unique binding properties of cyclopropane. Indeed, this cycle is characterised by a marked angular deformation which creates tension in the cycle, and a high reactivity due to the C=C double bond (Nelson et al., 2021).

Hypoglycin A has a molecular weight of 141 daltons (Da) (Bressler et al., 1969) which is similar to the molecular weight of BCAAs. This characteristic allows HGA to use BCAA metabolic pathways (Billington and Sherratt, 1981). The structure of HGA is similar to that of leucine, which explains the difficulty to separate these two compounds chromatographically (Billington and Sherratt, 1981).

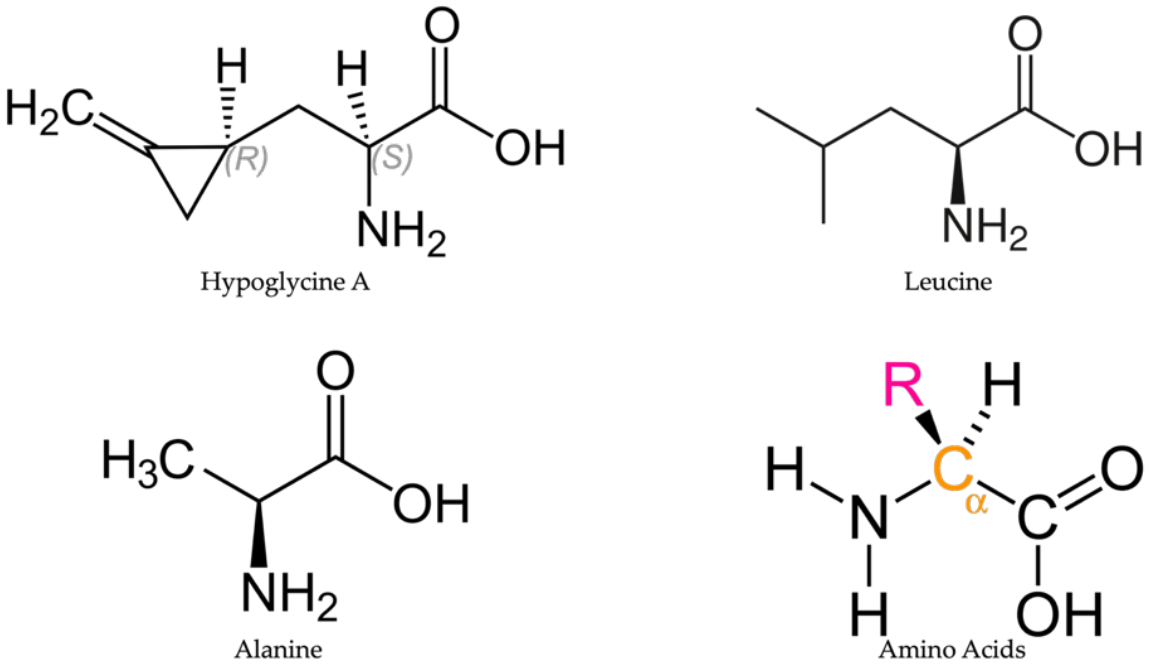

Figure 3. Chemical formulation of hypoglycin A, leucine, alanine and amino acids

Finally, the carboxyl group (-COOH) and the amino group (-NH_2), which make HGA a hydrophilic molecule, also influence its acid-base behaviour. The carboxyl group can release a proton (H^+), making HGA a weak acid. The pKa of the carboxyl group is generally between 2 and 3, as for most amino acids, indicating that it is dissociated (*i.e.*, COO^- and therefore deprotonated) at physiological pH (~7.4). The amine group (-NH_2) has a higher pKa, usually around 9-10, meaning it remains mostly protonated (NH_3^+) at physiological pH. Accordingly, at physiological pH, HGA exists primarily as a zwitterion, with the carboxyl group deprotonated (COO^-) and the amine group protonated (NH_3^+), giving it overall neutrality but with polar sites(Bressler et al., 1969; De Meijere, 1979; Nelson et al., 2021). This polarity strongly limits its passive diffusion through lipid membranes. The fact that it uses the metabolic pathways of BCAAs (Billington & Sherratt, 1981) suggests that it also uses their transport systems to enter the cell.

In the first clinical studies on HGA toxicity, the varying toxic doses of HGA observed among animal species were attributed to variations in metabolic rates characteristic of each species (Bressler et al., 1969). For instance, rabbits and monkeys are particularly sensitive to HGA (Chen et al., 1957). Toxicity was also found to vary according to the metabolic state of

the animals (*e.g.*, fasted *vs.* fed, female *vs.* male), the route of administration, and the formulation of HGA (*i.e.*, part of the fruit and whole fruit *vs.* extract of HGA) (Bressler et al., 1969).

The lethal dose 50 (LD_{50}) of HGA isolated from ackee seed in rats was estimated at 90 mg/kg (Hassall, Reyle, et al., 1954), and at 98 and 97 mg/kg for oral and intraperitoneal administration of graded doses, respectively (Feng and Patrick,1958). Toxicity was greater in fasted rats without the possibility to characterise precisely the amount of HGA (Feng and Patrick, 1958; Hassall, Reyle, et al., 1954).

A single oral dose of 100 mg/kg of a crude aqueous HGA extract in rats causes hypoglycaemia followed by recovery: this is consequently considered as the acute toxic dose (ATD)(Feng and Patrick, 1958). Another study, using ackee directly as source of HGA, reported an ATD of 231.19 ± 62.55 mg HGA/kg BW for males and 215.99 ± 63.33 mg HGA/kg BW for females. The difference between these studies probably results from the form of HGA administered, as the fruit matrix could play a role (Blake et al., 2006).

The maximum tolerated dose (MTD) of HGA in rats was determined with control diets over a 30-day period with a result of 1.50 ± 0.07 mg HGA/kg BW/day (Blake et al., 2006). An attempt to extrapolate this MTD to horses can be found in the literature, using body surface area and the following equation 1.5 mg/kg BW x $500^{0,75}/6$ which leads to a MTD of 26.5 mg/horse (Valberg et al., 2013).. However, the explanations about the choice of equation as well as k_m value was not provided, and this calculation does not consider the horses specificity as metabolic rates characteristic.

3.3. Mechanism of Toxicity and Toxic Metabolites

The general toxicity of HGA observed *in vivo* shows multiple blockages in metabolism causing a restriction in the availability of energy sources as demonstrated in laboratory animals (Chen et al., 1957; Feng and Patrick, 1958; Jordan and Burrows, 1937). The observable hypoglycaemia in human patients led to the hypothesis that the gluconeogenetic pathway was impacted by HGA (Jelliffe and Stuart, 1954) probably due to interference with one of the coenzymes derived from the vitamins B group because thiamine cured patients with JVS as reviewed by (Bressler et al., 1969).

Afterwards, two major discoveries about the mechanism of HGA poisoning were highlighted. First, an interaction with fatty acid oxidation has been suggested through several observations: (i) the serum concentration of free fatty acids and fatty liver infiltration increased after HGA administration, (ii) HGA increases glucose oxidation, (iii) hepatic glycogen was depleted following HGA administration without interfering with its synthesis or degradation (Von Holt et al., 1966), and (iv) riboflavin (*i.e.*, a precursor of flavin adenine dinucleotide (FAD)) administration prevented the effects of HGA (Gregersen, 1985; Von Holt et al., 1966). Secondly, the latency period between HGA administration and the symptom onset, as well as the inactivity of HGA under certain conditions *in vitro* suggested that the hypoglycaemic agent was a metabolite of HGA (Von Holt et al., 1966). Furthermore, HGA is rapidly metabolised by rats *in vivo* (Von Holt et al., 1966) in a compound, extracted from the liver of HGA-treated rats, that inhibits the oxidation of long chain fatty acids (LCFA) (von Holt et al., 1964). To explore this last hypothesis of the presence of a toxic metabolite of HGA, [I-^{14}C] hypoglycin was injected into rats and, thereafter, the two-step metabolism of HGA was characterised (von Holt et al., 1964) with the final product, *i.e.*, methylencyclopropylacetyl-CoA (MCPA-CoA).

From a biochemical point of view, MCPA-CoA impacts mainly the initial step of fatty acid β-oxidation, gluconeogenesis, and the degradation of BCAAs in rats. The inhibition of fatty acid β-oxidation is due (i) to the rapid, severe and irreversible inhibition of short-chain-acyl-CoA dehydrogenases (SCAD), and (ii) to the slower inhibition of medium-chain-acyl-CoA dehydrogenases (MCAD). The impact on gluconeogenesis is indirect via the depletion of acetyl-CoA (*i.e.*, a product of normal β-oxidation) for use in the Krebs cycle, and potentially directly via the inhibition of glucose-6-phosphatase. Finally, the inhibition of isovaleryl-CoA dehydrogenase (IVD) impacts the degradation of BCAAs, and more precisely, leucine (Figure 4) (Billington et al., 1978; Broadway and Engel, 1998; Feng, 1957; Feng and Patrick, 1958; Hue et al., 1986; Ikeda and Tanaka, 1990; Kean, 1976; Kean and Rainford, 1973; Osmundsen and Sherratt, 1975; Tanaka, 1972; Tanaka et al., 1971; Von Holt, 1966; Von Holt et al., 1966).

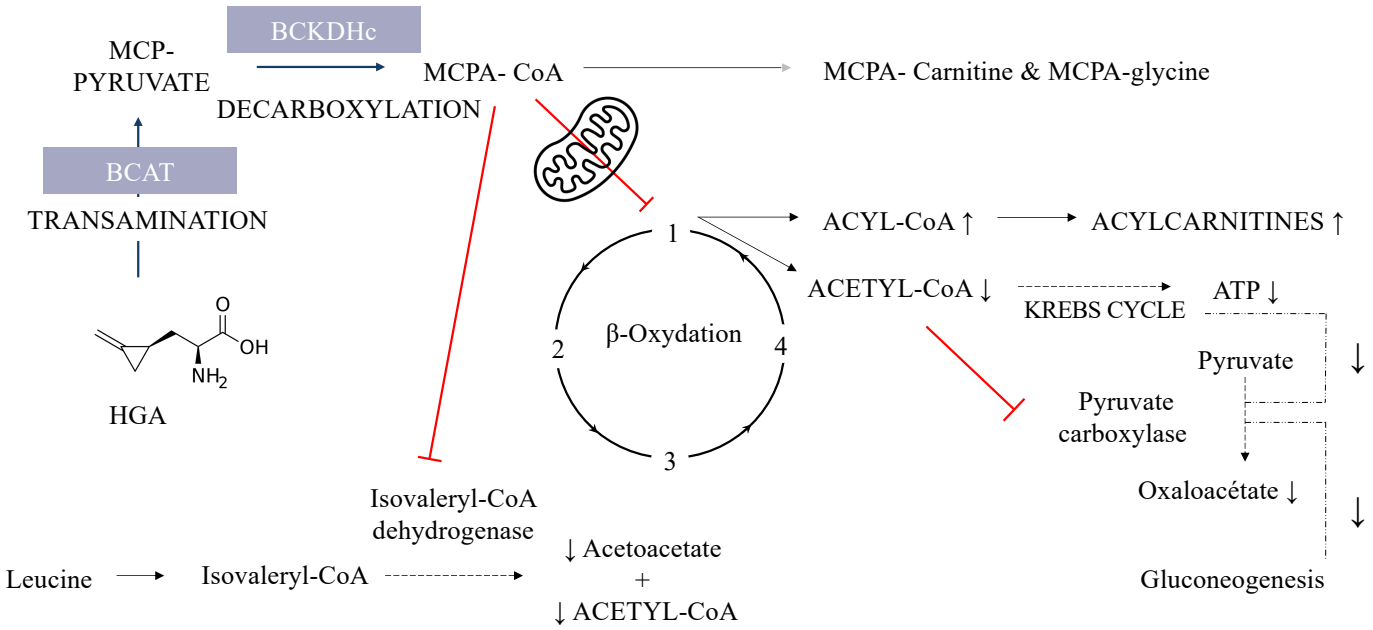

Figure 4. Main biochemical pathways affected by Methylenecyclopropylacetyl-CoA.

The inactivation of the two flavoproteins, acyl-CoA-dehydrogenases (*i.e.*, SCAD and MCAD), is due to a covalent modification of the flavin coenzyme of the acyl-CoA dehydrogenases by a "suicide inhibition". Briefly, this type of inhibition creates a reactive intermediate which chemically reacts with the enzyme, and the enzyme is covalently and permanently modified. In the case of the inhibition of acyl-CoA-dehydrogenases by MCPA-CoA, the removal of the α-proton from the inhibitor allows the formation of a reactive carbanion intermediate. The exact mechanism of this reaction is still unclear (Ghisla et al., 1980; Wenz et al., 1981). The SCAD and MCAD are essential for the first step of the fatty acid oxidation cycle (Kean, 1976; Kunau et al., 1995; Osmundsen and Sherratt, 1975). However, a study reported that MCPA only inhibits SCAD in rats (Qiu et al., 2018).

In equids, the inhibition of SCAD and MCAD seems to be effective as demonstrated by the measurement of enzymatic activity in AM horses (Westermann et al., 2008, 2007) and by the expected accumulation of acylcarnitines in the serum and urine of horses with AM (Bochnia et al., 2019; Boemer et al., 2017; Karlíková et al., 2016; Lemieux et al., 2016; Mathis et al., 2021; Renaud et al., 2024; Sponseller et al., 2012; Valberg et al., 2013; van der Kolk et al., 2010; Westermann et al., 2008, 2007). However, some studies revealed increases of long chain

acylcarnitines in the serum of AM horses compared to cograzers or healthy horses, without the confirmation of elevated enzymatic activity of long-chain acyl-CoA dehydrogenases (LCAD) (Boemer et al., 2017; Karlíková et al., 2016; Lemieux et al., 2016; Renaud et al., 2024; Valberg et al., 2013). Some of these studies corroborate the major increase in short- and medium-chain acylcarnitines (Lemieux et al., 2016; Renaud et al., 2024; Valberg et al., 2013). The possible reasons for differences between studies are the absence of measurement of long-chain acylcarnitines, the lack of measurement of enzyme activity of LCAD and the alternative pathway of β-oxidation in peroxisomes (Kunau et al., 1995; Poirier et al., 2006), which can reduce the serum concentration of acylcarnitines in the beginning or in mild case of AM (Karlíková et al., 2016; Sponseller et al., 2012). Peroxisomes contribute to the initial chain-shortening of very-long-chain fatty acids through β-oxidation, producing shorter-chain acylcarnitines that can subsequently be metabolised in mitochondria; this alternative route can therefore mask early accumulation of long-chain acylcarnitines in serum. Long-chain acylcarnitines could have an impact on the myocardium (Ferro et al., 2012) and AM horses have increased plasma cardiac troponin I, a specific biomarker of myocardial injury. The IVD inhibition is also reported in AM horses (Karlíková et al., 2016; Westermann et al., 2008, 2007) with a decrease in enzymatic activity (Westermann et al., 2008, 2007) and an increase in leucine concentration in the serum and urine of horses with AM (Karlíková et al., 2016). There is no evidence of any glycolytic deficiency at the mitochondrial level (Cassart et al., 2007) or at the clinical level, with more frequent hyperglycaemia in horses and no reported hypoglycaemia (Votion et al., 2007).

From a cellular point of view, in equids, the primary impact of AM is located inside the mitochondria with a sharp decrease of mitochondrial NADH reductase activity and its altered cytoplasmic distribution. This means that the first and most direct target of the toxin is the mitochondrial machinery responsible for energy production, rather than other cellular compartments. This is confirmed by the lack of ultrastructural changes at the sarcoplasmic and endoplasmic reticulum membranes and the absence of calcium precipitates (*i.e.*, both are typical signs of secondary mitochondrial alterations that occur when the initial lesion is located elsewhere, such as at the plasma membrane or the nucleus; their absence here clearly indicates that mitochondria are the primary site of injury). In other words, the muscle cell membranes remain structurally intact, ruling out a primary sarcolemma or nuclear pathology and

supporting a direct mitochondrial toxic effect (Cassart et al., 2007). A general decrease in mitochondrial respiratory capacity, accompanied by severe impairment of oxidative phosphorylation and the electron transport system, has also been reported (Kruse et al., 2021; Lemieux et al., 2016). This global mitochondrial failure leads to a major energy deficit within muscle fibres, as ATP synthesis becomes insufficient to sustain normal cellular metabolism. This mitochondrial dysfunction is further evidenced by the accumulation of lipids in the cytoplasm and the lack of calcium salts accumulation: under normal conditions, mitochondria oxidize fatty acids to generate energy and buffer cytosolic calcium. When this function is impaired, fatty acid oxidation slows down, leading to the accumulation of acyl-CoA esters within lipid droplets, while the inability to sequester calcium prevents its normal uptake from the cytosol. Indeed, the impact of AM on mitochondria results in a slowing of mitochondrial oxidation of fatty acids leading to the accumulation of acyl-CoA esters within lipid droplets and preventing normal calcium uptake from the cytosol (Cassart et al., 2007). Together, these features constitute clear morphological and biochemical evidence of a primary mitochondrial myopathy rather than a secondary degenerative process.

From a macroscopic and microscopic point of view, this mitochondrial impairment leads to an acute degenerative process affecting muscular fibres; indeed, the inability to provide sufficient energy to muscle fibres lead to an inability of fibre repair and maintenance mostly the slow contracting type I fibres which rely heavily on fatty acids as an energy substrate (Cassart et al., 2007; Votion et al., 2024).

Recently, a proteomic study highlighted new pathophysiological facts in AM with an impact on the glycolysis/gluconeogenesis pathway, the coagulation/complement cascade, and on the biosynthesis of amino acids. Consequently, Kruse et al., (2024) pointed out that AM is a combination of inflammation, oxidative stress, and impaired lipid metabolism, which is trying to be counteracted by enhanced glycolysis (Kruse et al., 2024).

3.4. Metabolism of Hypoglycin A

The metabolism of HGA is a two-step procedure similar to that of BCAAs (*i.e.*, valine, leucine and isoleucine) which seems to be of great importance in the context of HGA toxicity. The first reversible reaction (*i.e.*, transamination) leads to the formation of

methylenecyclopropylpyruvate (MCP-pyruvate) via the action of the Branched-Chain Aminotransferase (BCAT). The second irreversible step is an oxidative decarboxylation and a reversible conjugation to coenzyme A (CoA) and leads to the final formation of unstable MCPA-CoA (von Holt et al., 1964) with the Branched-Chain α-Keto Acid Dehydrogenase Complex BCKDHc (Melde et al., 1991; Neinast et al., 2018).

It is interesting to note that the fact that HGA is metabolised in the liver (von Holt et al., 1964) is, in fact, probably partially true. Indeed, in rats, the main BCAT activity is located in the skeletal muscle unlike the BCKDHc activity which is mainly located in the liver (Harper et al., 1984; Hutson, 2001). However, no specific information about activity or expression of BCAT or BCKDHc in horses was found. Nevertheless, recently, a study confirmed that activation of HGA to MCPA-CoA occurred mainly in the skeletal muscles (Sander et al., 2023).

In BCAAs, BCAT catalyses bidirectional amino-transfer between BCAAs and their Branched-Chain keto-Acid (BCKA) (*i.e.*, 2-ketoisocaproate (KIC), 2-keto-3-methylvalerate and, 2-ketoisovalerate for leucine, isoleucine and, valine respectively) in cells with glutamate/2-ketoglutarate as partner to receive or transfer the amino group (Adeva-Andany et al., 2017a; Brosnan and Brosnan, 2006; Chen et al., 2023; Harper et al., 1984; Krebs and Lund, 1976; Suryawan et al., 1998) and BCKDHc catalyses the irreversible oxidative decarboxylation of BCKA leading to the acyl-CoA derivative with one less carbon (Adeva-Andany et al., 2017). Thereafter, the pathways resemble those for fatty acid oxidation and lead to end products that can enter the Krebs cycle (Harper et al., 1984; Krebs and Lund, 1976; Suryawan et al., 1998).

It is interesting to note that BCAAs (*i.e.*, valine, leucine and isoleucine) biosynthesis and catabolism take place in bacteria, archaea, yeasts, and lower eukaryotes. Higher eukaryotes can catabolize but not synthesize BCAAs, which are essential amino acids and must therefore be obtained from the diet. As a result, the BCAA biosynthetic pathway represents an attractive target for antimicrobial, herbicidal, and antifungal agents (Bezsudnova et al., 2017a; Chen et al., 2023; Dimou et al., 2022; Neinast et al., 2018).

The physiological significance of BCAAs extends beyond their catabolic pathways, playing a crucial role in metabolic regulation and cellular signalling. Indeed, BCAAs are involved in activating the mechanistic target of rapamycin (mTOR) pathway, which controls essential cellular processes such as protein synthesis, autophagy, and energy metabolism. Among the BCAAs, leucine stands out as the most potent stimulator of mTOR, linking its availability to

muscle protein synthesis and anabolic responses. Furthermore, BCAAs contribute to glucose homeostasis by modulating insulin secretion and sensitivity, highlighting their broader metabolic roles (Dimou et al., 2022).

3.4.1.Branched-Chain-Amino-Acid Aminotransferase

The BCAT (EC 2.6.1.42) occurs in both the cytosol and the mitochondria (Harper et al., 1984; Ichihara and Koyama, 1966). The preferred BCAA substrate is isoleucine or leucine followed by valine (Hutson, 2001). In fact, two BCAT isoenzymes exist in mammals: a mitochondrial (BCATm) and a cytosolic (BCATc) isoenzyme (Brosnan and Brosnan, 2006; Hall et al., 1993; Hutson et al., 1992, 1988; Hutson and Hall, 1993; Kadowaki and Knox, 1982; Neinast et al., 2018; Suryawan et al., 1998). The activity and mRNA expression levels of BCATm and BCATc should be distinguished to understand the activity of general BCAT.

The BCAT activity in rats was highest – in descending order- in pancreas, stomach, heart, kidney, brain, and skeletal muscle; the lowest levels of activity were detected in the colon, small intestine, and adipose tissue. In monkeys, BCAT activity was also highest in the pancreas followed by kidney, stomach, and brain and the lowest activity was found in adipose tissue. In humans, pancreas and kidney had the highest activity followed by brain and stomach and finally by tissues of the gastrointestinal tract (Goto et al., 1977; Suryawan et al., 1998). The activity of BCATm have also been found in the submaxillary gland in rat, rabbit, guinea pig, and macaque monkey (Hutson, 2001). Interestingly, in sheep, the BCATm contributes for 57% to 71% of the BCAT activity in sheep skeletal muscles. The remaining part of the activity is due to the presence of mRNA of BCATc in muscles including heart contrarily to the previous species (*i.e.*, rats, humans and monkeys) where the BCATm is the sole muscular isoenzymes (Bonfils et al., 2000).

Moreover, the inhibition of activity of purified BCATc and BCATm in rats was established by measuring the transformation of leucine in its respective keto-acid with α-ketoglutarate as cofactor and the presence of selected inhibitors. For the BCATc, the bromoleucine allows an inhibition greater than 95% of the isoenzyme followed by gabapentin (*i.e.*, a leucine analogue) (> 80%) followed by HGA, MCPrG and valproate, MCPA and, oxfenicine. For BCATm, bromoleucine, HGA and MCPrG allow an inhibition followed by valproate and MCPA but not gabapentin (Hutson, 2001). The structure of HGA is similar to leucine which explains the

difficulty to separate these two compounds chromatographically (Billington and Sherratt, 1981) and consequently, the inhibition of BCAT by HGA is probably more a competitive action (*i.e.*, better affinity for the enzyme).

The expression of enzymes thanks to immunologic analysis in rats show that BCATm is found in most tissues whereas BCATc is restricted to the neurologic tissue (mainly brain, retina and spinal cord), ovary, and placenta (Hall et al., 1993). Moreover, a marked induction of the BCATm in mammary epithelial cells has been found during late pregnancy and lactation in the rat (Brosnan and Brosnan, 2006). In human, BCATm mRNA expression was detected in all tissues with the most abundant in skeletal muscle, brain, kidney, and intestine whereas liver had low concentrations of BCATm message. BCATc mRNA was clearly found in human brain but only in trace amounts of transcript in other tissues (expression in ovary and placenta were not determined) (Suryawan et al., 1998).

A proposed hypothesis for the brain function of the BCATc isoenzyme is the existence of a specific nitrogen shuttle based on BCAAs, particularly in regulating levels of the major excitatory neurotransmitter glutamate in glutamatergic neurons. Glutamate undergoes a recycling process between neurons and astroglial cells, involving its conversion to glutamine and vice versa. However, this cycle presents inefficiencies, requiring additional glutamate synthesis via pyruvate carboxylase and transamination processes. In this context, BCATm in neurons plays a crucial role by converting α-ketoglutarate into glutamate, while BCATc in astroglia facilitates BCAA recycling to maintain this balance: this operation in series aims to provide nitrogen for optimal rates of *de novo* glutamate synthesis. Further studies have revealed that BCATm co-localises with BCKDHc in secretory cells, suggesting a direct interaction in BCAA oxidation. In contrast, BCATc does not show this co-localisation, indicating a distinct physiological role, potentially focused on regulating the availability of BCAAs as metabolic substrates. These findings support the idea of a finely regulated mechanism where BCAAs serve not only as energy substrates but also as essential intermediates in maintaining neuronal homeostasis and glutamatergic signalling (Brosnan and Brosnan, 2006; Chen et al., 2023; Hutson, 2001). Moreover, gabapentin inhibits only BCATc and not BCATm: the "shuttle" can therefore only be inhibited in the direction of glutamate synthesis (Hutson, 2001).

3.4.2. Branched-Chain α-ketoacid Dehydrogenase Complex

The BCKDHc (EC 1.2.4.4) is a multienzyme complex located on the inner surface of the inner mitochondrial membrane (Harper et al., 1984; Neinast et al., 2018). The BCKDHc catalyses the oxidative decarboxylation of α-ketoisocaproate (ketoleucine), α-keto-β methylvalerate (ketoisoleucine), and α-ketoisovalerate (ketovaline), to form isovaleryl-CoA, 3-methylbutyryl-CoA, and isobutyryl-CoA, respectively (Harper et al., 1984). In both structure and function, BCKDHc resembles the pyruvate dehydrogenase complex, which catalyses the conversion of pyruvate to acetyl-CoA (Patel et al., 2014; Wieland, 1983).

The BCKDHc is composed of three separate subunits: (i) a thiamin-dependent branched-chain a-ketoacid decarboxylase (E_1 - EC 1.2.4.4) arranged in an $\alpha_2\beta_2$ substructure encoded by the *BCKDHA* and *BCKDHB* genes respectively, (ii) dihydrolipoyl transacylase (E_2 - EC 2.3.1.168.) with lipoate as a prosthetic group encoded by the *DBT* gene, and (iii) dihydrolipoyl dehydrogenase (E_3 - EC 1.8.1.4.) with flavin adenine dinucleotide (FAD) as a prosthetic group that transfers the released electrons to nicotinamide adenine dinucleotide (NAD^+) and is encoded by the *DLD* gene (Adeva-Andany et al., 2017b; Berg and de Kok, 1997; Brosnan and Brosnan, 2006; Chuang, 1989; Danner et al., 1979; Harper et al., 1984; Johnson and Connelly, 1972; Paxton and Harris, 1982; Pettit et al., 1978; Suryawan et al., 1998; Yeaman, 1989). In addition to the covalently bound prosthetic groups listed above, BCKDHc requires CoA and NAD^+ as cofactors for oxidation of BCKA. The complex also requires Ca^{2+} and Mg^{2+} for optimal activity (Harper et al., 1984). The activity of the complex is regulated by phosphorylation and dephosphorylation catalysed by a specific kinase and phosphatase (Brosnan and Brosnan, 2006; Paxton and Harris, 1984; Suryawan et al., 1998).

Like BCAT, BCKDHc is distributed ubiquitously and nonuniformly throughout the body (Harper et al., 1984). In humans and monkeys, the distribution of BCKDHc activity is similar, with the highest levels found in the kidney, followed by the liver, brain, and heart. However, human liver exhibits lower total BCKDHc activity compared to monkey liver. Both humans and monkeys display limited BCKDHc activity in the gastrointestinal tract, indicating minimal loss of indispensable BCAA carbon during metabolism in these tissues. In contrast, rats exhibit much higher BCKDHc activities than humans and monkeys with a liver BCKDHc activity which is approximately ten times greater. Surprisingly, high activity is also observed in the stomach, surpassing levels in skeletal muscle and brain. The activity state of the BCKDHc

complex (*i.e.*, the ratio of activity before activation to total activity obtained after activation by phosphatase treatment) is also higher in rats than in humans or monkeys (Harper et al., 1984; Khatra et al., 1977; Suryawan et al., 1998).

Expression patterns of the BCKDHc complex and its regulatory kinase further underscore interspecies differences. In humans, the kinase's mRNA is ubiquitously expressed, with the highest levels in skeletal muscle, followed by the brain and kidney. In contrast, the liver and intestine exhibit the lowest expression. Consequently, skeletal muscle is the predominant site of BCKDHc activity in humans, with significant contributions from the kidney and brain. Monkeys share a similar pattern, with higher BCKDHc activity states than humans in several tissues, particularly the liver. Rats exhibit both higher BCKDHc activity and greater expression of the complex and its regulatory kinase across tissues compared to primates. This contributes to their greater overall oxidative capacity for BCAAs. Despite these quantitative differences, the ratio of BCAT to BCKDHc activity is relatively conserved across species, suggesting shared regulatory mechanisms. These findings highlight the unique metabolic adaptations in BCAA oxidation among species (Suryawan et al., 1998).

3.5. Clinical Signs and Diagnosis

The clinical picture of AM can be confused with other pathologies frequently encountered in horses, such as colic or acute laminitis. Nevertheless, the identification of HGA intoxication remains very important to establish the appropriate supportive treatment, and to isolate and protect cograzers from the different potential sources of HGA (Votion et al., 2019). The diagnosis is based on (i) different elements of the anamnesis, the environment and the clinical examination, (ii) the results of routine laboratory analyses, and (iii) the performance of specific analyses including necropsy. The identification of risk or protective factors (in Belgium (Votion et al., 2009) and at a European level (Renaud et al., 2024; Van Galen et al., 2012a)) can also help in this diagnostic approach.

Access to pasture is a major element of the anamnesis and obviously, has been identified as a risk factor (Van Galen et al., 2012a; Votion et al., 2009) especially if previous cases of acute death in horses had been reported (Votion et al., 2009). Indeed, 99.8% of horses affected by AM were pastured for more than 6 hours a day when clinical signs appeared (Van Galen et

al., 2012a; Votion et al., 2020). The remaining 0.2% had been indoors (box, stabling, etc.) for less than a week and consequently these horses had the possibility of being in contact with the protoxins: this information was verified by the presence of HGA and/or MCPA-carnitine in the blood analyses of these horses (Votion et al., 2020). The weather-dependent pasturing time in spring and in autumn is considered as protective factor (Votion et al., 2009). The second major element of the anamnesis is the season. The autumn is the riskiest season with 76% of the cases and the spring represents the remaining 24% but it is important to note that "autumnal" cases are continuing in early winter as for "spring" cases in the early summer (Votion et al., 2020). Finally, two protective factors could remain of interest in the anamnesis: the horses over 10 years of age and geldings are less susceptible to develop AM compared to younger equids and to males or females respectively. These two observations take into account certain facts that could be considered as confusing: for example, there are more geldings after a certain age (males are regularly castrated before getting to work), or that females can be more in the meadow in certain physiological periods as during gestation and before weaning the foal (Renaud et al., 2024).

The observation of the environment can reinforce the suspicion of AM. Firstly, the presence of trees at pasture (Van Galen et al., 2012a; Votion et al., 2020) or in the vicinity (Votion et al., 2020). In fact, *Acer* species are classified as Anemochorous (*i.e.*, seeds are dispersed by wind, often with the help of specific organs as the "wings" around the seed for sycamore maple tree) and, more precisely, as pterometeorochory (or pterochory) which means that seeds dispersal is improved through wings: consequently, seeds can be dispersed up to 80 to 314 meters from the original trees (Vittoz and Engler, 2007) thanks to climatic condition such as moderate to intense wind. In our temperate European regions, as previously stated, of the three *Acer* species frequently encountered, only the sycamore has been associated with AM. These trees can be differentiated by their leaves, seeds, and inflorescence, and to a lesser extent by identification of seedlings (Figure 5). Recently, the box elder (*Acer negundo*) has been associated with AM cases in the Czech Republic (Jahn et al., 2024). This variety of *Acer* species has been described in North America cases of AM, known as seasonal pasture myopathy in the USA (Finno et al., 2006; Valberg et al., 2013). Secondly, pastures surrounded by or containing a stream, or a river and humid pastures have been identified as riskier (Votion et al., 2009): indeed, HGA is a water-soluble protoxin and can be "extracted" from plants by

water (Votion et al., 2019). Thirdly, sloping or steep pasture have been also identified as risk factors and finally, the access to dead leaves piled in autumn (Van Galen et al., 2012a; Votion et al., 2009) or dead wood (Van Galen et al., 2012a). These last two points could be linked: the slope creating areas of accumulation of leaves (*i.e.*, leaves of *Acer pseudoplatanus* contain HGA (Westermann et al., 2016)) and other debris such as dead wood. Moreover, the slope can also create areas of accumulation of seeds in autumn or seedlings in spring.

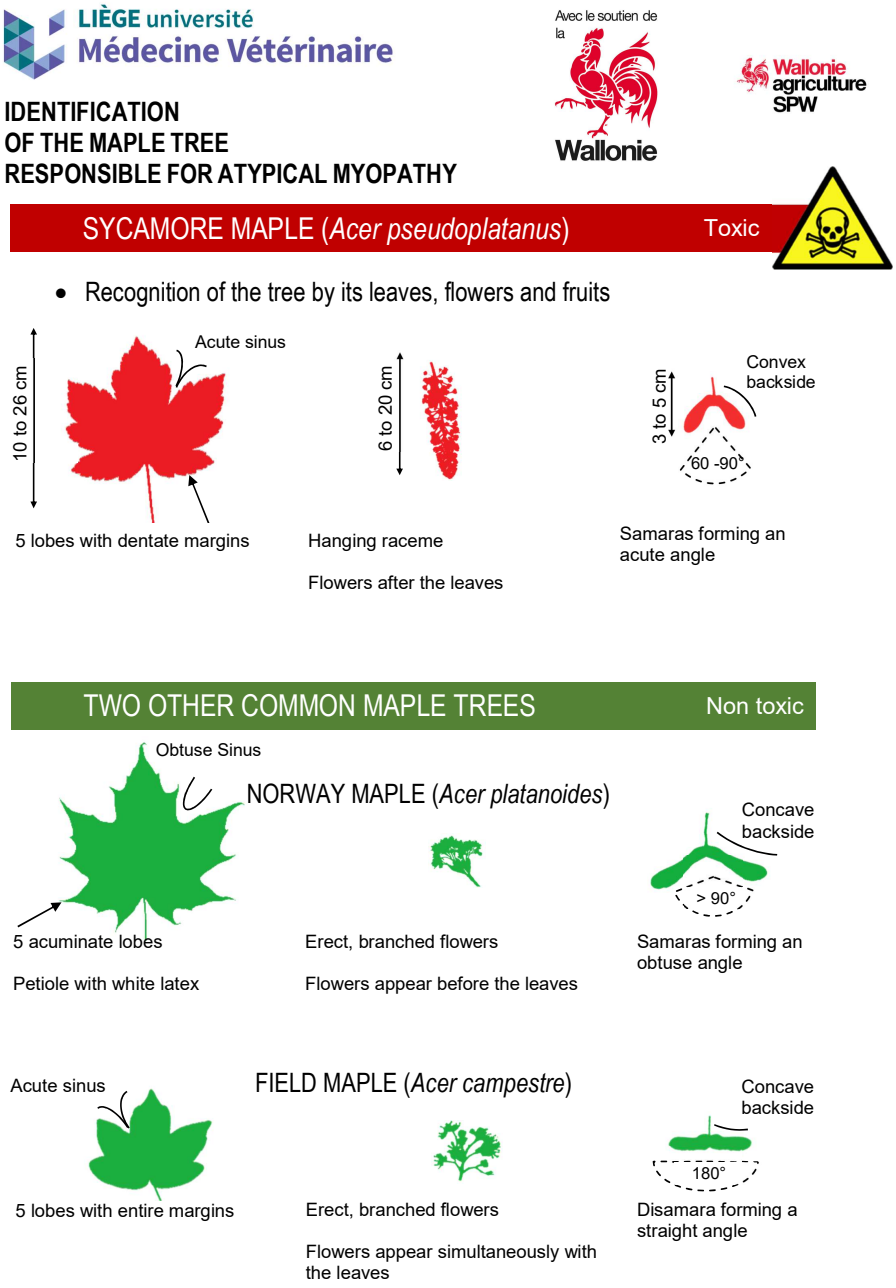

Figure 5. Identification of *Acer pseudoplatanus* - © (Renaud, 2019)

The most frequently encountered clinical signs are pigmenturia (93%), generalised weakness (85%), stiffness (83%), depression (80%), tachycardia (79%), lateral recumbency (78%), maintained appetite (72%), tremors (68%) and sweating (64%), normothermia (60%) and congested mucous membranes (53%). Pigmenturia is very specific of myopathy and AM but exceptionally, normal urine can be observed at the very beginning of the intoxication probably when the myoglobin of the muscles is not yet released in sufficient quantity to colour urine. The clinical examination reveals tachycardia > 45 bpm (79%), normothermia (37°C – 38,5°C; 60%), bladder distension (58%), congestive mucous membranes (53%) (in % of cases according to (Van Galen et al., 2012a)). The distension of the bladder can cause abdominal pain, which itself causes signs of colic reported by owners. Dyspnoea is observed in 49% of the cases (Van Galen et al., 2012a) and would be the result of the degeneration of respiratory muscles including intercostal muscles and the diaphragm (Cassart et al., 2007). Some clinical signs are more frequently observed in some outbreaks as oesophageal obstruction, and dysphagia (Votion et al., 2007) without explanation proposed to date. Cardiac damages can be observed clinically with tachycardia in 79% of cases (Van Galen et al., 2012a) and histologically (*i.e.*, more than 50% large whitish areas on the ventricular myocardium) (Cassart et al., 2007) but also via the alterations on electrocardiogram recordings and cardiac ultrasound examination during the time of clinical sign (Verheyen et al., 2012). In fact, the heart QT_{cf} interval was increased in all AM horses included in the paper of Verheyen et al., (2021) without knowing if these parameters were normal before the intoxication and cardiac abnormality can be found in survivors after 10 weeks follow-up. The QT interval represents the time between the depolarisation and the end of the repolarisation of the ventricles. This interval required a correction (QT_{cf}) in case of increase heart rate and the resulting physiological shortening QT (Verheyen et al., 2012).

The serum activity of creatine kinase (CK) is increased (Votion et al., 2007) and generally well beyond the 10,000 IU/L needed to consider that muscle breakdown (myolysis) is significant (Volfinger et al., 1994). However, in the beginning of the intoxication, the CK concentration seems to be in the high standards: indeed, as for the coloration of urine, a delay is necessary between the beginning of the destruction of muscle cells and the apparition of disturbance in CK measure.

The protoxin, HGA, and its toxic metabolite linked to carnitine (*i.e.*, MCPA-carnitine), or glycine (*i.e.*, MCPA-glycine) can be detected in blood (Baise et al., 2016; Bochnia et al., 2016, 2015; Boemer et al., 2015; Renaud et al., 2024; Sander et al., 2023; Valberg et al., 2013; Votion et al., 2014) and urine (Bochnia et al., 2016, 2015; Valberg et al., 2013) respectively as well as in tissue samples (*i.e.*, diaphragm, myocardium, liver, kidney, pancreas, cerebral fluid, muscle *semitendinosus*, muscle *triceps brachii* and muscle *gluteus medius* (Sander et al., 2023)). The presence of HGA is considered as an exposure marker to the protoxin and not as a disease marker as some cograzers (*i.e.*, physically healthy horses) can also present this protoxin in their blood, urine or tissue. Moreover, the presence of MCPA-carnitine or MCPA-glycine in cograzers blood, urine or tissue does not allow this parameter to be used as the only diagnostic marker (Baise et al., 2016; Renaud et al., 2024, 2022).

Up to now, the association of significant quantities of both HGA and MCPA-carnitine or MCPA-glycine and the increase of blood acylcarnitines outside the reference ranges (*i.e.*, C2, C3, C3-DC, C4, C5, C5:1, C5-OH, C5-DC, C6, C8, C8:1, C10, C10:1, C10:2, C12, C12:1, C14, C14:1, C16, C16:1, C18, and C18:1) allow to confirm the intoxication (Bochnia et al., 2016, 2015; Boemer et al., 2017, 2015; Karlíková et al., 2016; Mathis et al., 2021; Sander et al., 2023; Votion, 2018; Votion et al., 2014). Free carnitine and the following acylcarnitine (C3, C6, C8, C10, C10:1, C12, C12:1, C14, C14:1, C16 and C16:1) were used to confirm diagnosis without the possibility to distinct horses surviving from AM and non-survivors horses. In contrast, acylcarnitine (C2, C3DC, C4, C5, C5-OH, C5DC, C8:1, C10:2, C18 and C18:1) were used to confirm diagnosis and are significantly different between survivors and non-survivors horses which can be considered as a prognosis tool. From all these acylcarnitine, a partial least square revealed that C2, C10:2 and C18–carnitine were the best parameters to predict survival and were integrated in a prognostic logarithm. The concentration of toal calcium is also considered as a prognosis marker: horses with a total calcium lower than 2.69 mmol/L have a 70% chance of dying (specificity 92% and sensitivity 64%) (Boemer et al., 2017).

But recently, a large-scale study confirmed, invalidated or highlighted several important facts. Firstly, the HGA and the MCPA-carnitine blood concentrations were not sufficient to make a diagnosis or a prognosis. Indeed, although the mean blood HGA concentration was significantly higher in diseased horses compared to cograzers ($p < 0.0001$), the dispersion of these parameters did not allow to differentiate them. Moreover, the blood HGA values cannot

be used to differentiate non-survivors horses from surviving horses. The mean serum MCPA-carnitine concentration was significantly higher in diseased horses compared to cograzers ($p < 0.0001$) and in non-survivors horses *vs.* survivors ($p < 0.0001$), but the dispersion of values around this mean did not allow the distinction of groups based on a simple value. Consequently, this study confirmed the exposure-related nature of HGA but invalidated the possibility of using high concentrations of MCPA-carnitine or both HGA and MCPA-carnitine as diagnosis tools. Secondly, a partial least square (PLS) analysis revealed a selection of six acylcarnitines (*i.e.*, C2, C3, C3DC, C4, C5, and C10:1), which had a variable importance in projection (VIP) score greater than 1, indicating the importance of each explanatory variable in the model. A subsequent analysis of sensitivity and specificity (ROC curves) of these six markers in differentiating diseased horses *vs.* cograzers and survivors *vs.* non-survivors horses was performed. The C5-carnitine has been highlighted to be the best candidate for both diagnosis and prognosis. A first cutoff of 3.04 µmol/L C5-carnitine allowed to differentiate diseased horses from cograzers (*i.e.*, diagnostic tool): indeed, 92% of horses with a C5-carnitine serum concentration below this cutoff were effectively cograzers, and 97% of horses with concentration above the cutoff were effectively diseased horses. A second cutoff of 12.21 µmol/L C5-carnitine can be used as prognostic tool: 76% of horses presenting with serum C5-carnitine above this cutoff were likely to die, and 81% of horses with serum C5-carnitine below this cutoff were effectively survivors. The clinical implication of these cutoffs is that a quantitative analysis of blood C5-carnitine in a horse presented to a veterinarian for AM would allow the diagnosis to be invalidated/confirmed and the animal's outcome to be predicted more reliably and more rapidly (Renaud et al., 2024). Thirdly, this study confirmed the existence of subclinical cases among cograzers as proved in other mammalian herbivore species as explained by their significant increase in acylcarnitine profiling (Renaud et al., 2024, 2022). Cograzers are exposed to the same environment without displaying signs of intoxication (Votion et al., 2007; Bochnia et al., 2015; Baise et al., 2016) and were used as a control group in some scientific papers: this point of view can be challenged now. Moreover, this information implies that preventive or protective measures must be applied to all horses at pasture with an animal diagnosed with AM.

The post-mortem examination of AM cases revealed discolouration of certain muscles (*i.e.*, intercostal, shoulder, neck and masseter) and bilateral congestion of the lungs with abundant

foamy fluid (Cassart et al., 2007). In fact, an acute and severe myonecrosis of respiratory and proximal postural muscles and, sometimes, in the myocardium is noticed (Cassart et al., 2007; Finno et al., 2006; Westermann et al., 2007; Żuraw et al., 2016). A multifocal and monophasic process compatible with Zenker's necrosis/degeneration (*i.e.*, disappearance of striation and myofibrils, and presence of hyaline substance) is noticed especially in type I fibres (*i.e.*, slow-twitch fibres (Votion et al., 2007)) which is consistent with the macroscopic observation (*i.e.*, involvement of respiratory and postural muscles). The principle of the histochemical technique for nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) is to employ a colourless, soluble tetrazolium salt as an electron acceptor which is reduced to a deeply coloured, insoluble formazan product at the site of enzyme activity. Thus, the intensity of the formazan reaction product reflects the number of mitochondria within a fibre and reveals the characteristic checkerboard pattern of fibre types (Dubowitz et al., 2020). In AM, this histochemical technique revealed a weak and disorganised pattern which underlines the mitochondrial disorder process during AM (Cassart et al., 2007; Westermann et al., 2007; Żuraw et al., 2016). A dramatic lipid accumulation was demonstrated with the Oil Red O stain in the neck, proximal forelimb and hindlimb, intercostal, myocardial, and diaphragm muscles (Cassart et al., 2007; Finno et al., 2006). The following observations suggest a primary mitochondrial disorder during the AM process: (i) the sharp decrease in mitochondrial NADH reductase activity, (ii) the altered cytoplasmic distribution of mitochondria, and (iii) the concomitant intact nuclei (Cassart et al., 2007). As previously explained, the alteration of mitochondrial oxidation of fatty acids results in the accumulation of long-chain fatty acid acyl-CoA esters leading to accumulation in lipid globules which is exacerbated by the inhibition of β-oxidation of fatty acids.

The survival prognosis remains poor with the European lethality rate at around 74%, which varies between years, countries or some variability factors, and most horses are euthanised within 72 hours. In a study of hospitalised AM cases, a lethality rate of 56% was reported (Dunkel et al., 2020). The authors hypothesised that the medical support provided to these horses contributed to this lower lethality rate as the initial status of horses referred to the clinic (*i.e.*, mildly affected horses can be transported to the clinic and severely affected horses cannot be transported without risking of worsening the myopathic process).

3.6. Treatment and Prevention

The treatment remains supportive and symptomatic with the aims of (i) resolving dehydration and balancing electrolyte and acid-base levels, (ii) providing energy for muscles, (iii) eliminating the protoxins, (iv) supporting mitochondrial function, (v) eliminating pain, and (vi) preventing injuries (Fabius and Westermann, 2018; Van Galen and Votion, 2013a, 2013b).

Firstly, the correction of hydration *(i.e.*, an increase of packed cell volume (Votion et al., 2007)), acid-base imbalance (*i.e.*, (i) an increase of blood lactate due to cellular destruction or shift from aerobic to anaerobic metabolism and consequent metabolic acidosis, (ii) the presence of respiratory acidosis due to dyspnoea and hypoventilation resulting from respiratory muscle damage, (iii) the presence of respiratory alkalosis due to hyperventilation in case of stress or pain (Van Galen et al., 2013)) and electrolyte disturbances (*i.e.*, hypocalcaemia, mild hyponatraemia, and hypochloraemia (Van Galen et al., 2013)) should be performed to optimise cardiovascular, respiratory, muscular, renal and digestive functions in cases of AM and to promote myoglobin and protoxins excretion (Van Galen and Votion, 2013a). If needed, oxygen should be administered to maintain PaO_2 (Votion and Serteyn, 2008) or to regulate acid-base status of the intoxicated horse.

Next, the severe metabolic disturbances cause an inability to use the horse's most efficient energy source (*i.e.*, lipids) and therefore, the carbohydrate metabolism must be supported (Westermann et al., 2008) through (i) intravenous glucose and insulin administration, (ii) a diet rich in structural fibres such as grass, hay or alfalfa, (iii) grains or concentrates to a lesser extent (Van Galen and Votion, 2013a), and (iv) other kind of food such as carrots or apples, for example.

Then, the elimination of the protoxins can be achieved by the administration of fluids and adsorbents such as activated charcoal (Krägeloh et al., 2018). The use of carnitine (Fabius and Westermann, 2018) or glycine as agents promoting the excretion of the protoxin (as MCPA is excreted as MCPA-carnitine or MCPA-glycine in urine) has not been proven useful in AM. The administration of carnitine would contribute to elevating plasma leptin concentrations in healthy horses (Kranenburg et al., 2014), thereby stimulating glucose metabolism, as leptin might stimulate cellular glucose uptake (Dardeno et al., 2010). However, the delay between

the oral administration of carnitine and the increase of leptin remains unclear. The oral availability of carnitine is low but results in an increase in carnitine and conjugated carnitine in plasma and urine (Kranenburg et al., 2014) : consequently, intravenous administration which presents a more rapid action is therefore to be preferred (Harris et al., 1995).

After that, the activity of mitochondrial respiratory chain complexes is decreased in horses with AM (Cassart et al., 2007; Kruse et al., 2021; Lemieux et al., 2016; Westermann et al., 2011); because FAD is a cofactor of the deficient mitochondrial dehydrogenases in MADD as in AM horses and because riboflavin (*i.e.*, vitamin B2) is a precursor of FAD, riboflavin administration should be considered or at least, vitamins B group administration (Westermann et al., 2008).

Finally, the mild to severe pain experienced by AM horses must be controlled. The use of non-steroidal anti-inflammatory drugs should be considered with regard to renal and gastrointestinal side effects (Van Galen and Votion, 2013a). Emptying the bladder may relieve pain for some cases (Votion et al., 2007).

Moreover, to prevent injuries and as a first aid measure, owners may be advised while awaiting the arrival of a veterinarian to move the horse to a comfortable stall, provided the animal can move without exacerbating its condition (*i.e.*, physical effort and stress should be avoided so as not to aggravate the myopathy and the energetic imbalance (Westermann et al., 2008)), which facilitates the warming (*i.e.*, blanket or heat lamps) of hypothermic animals and further therapeutic intervention (Van Galen et al., 2012a; Votion and Serteyn, 2008).

As there is no specific treatment against AM, prevention by avoiding contact with toxic plant material, by using or creating low-risk pastures and, by management of grazing time remains the best way to fight this intoxication.

As a prerequisite, the identification of *Acer pseudoplatanus* and its seeds and seedlings is very important. Some climatic conditions (*i.e.*, wind) can disperse seeds of *Acer* species to hundreds of meters (Vittoz and Engler, 2007); therefore, pasture contamination is not necessarily linked to the presence of a tree within the pasture. Herbicidal spraying or mowing do not change the HGA concentration in seedlings (González-Medina et al., 2019) and do not eliminate all seedlings (Ghislain et al., 2022) consequently, the manual removal of seeds or seedlings must be considered (Votion, 2016). However, when seeds or seedlings are too abundant and/or too widely dispersed, the grazing area must be prohibited, for example by parcelling the pasture (Votion et al., 2019). It is noteworthy that a proportion of seedlings

naturally declines over time, while mowing contributes to maintaining the nutritional quality of the pasture. Therefore, mowing should be considered a beneficial management practice, as it reduces seedling density without compromising the overall nutritive value or botanical composition of the pasture (Ghislain et al., 2022). Supplementary feeding (such as hay) all year round has been identified as a protective factor (Van Galen et al., 2012b): horses are probably less tempted to ingest samaras and/or seedlings if they receive enough food (Votion et al., 2020). However, hay can be both a risk factor (Votion et al., 2009) and a protective factor(Van Galen et al., 2012b); in fact, hay can contain HGA (González-Medina et al., 2019) (*i.e.*, presence of seedlings) and if the hay is distributed directly on the ground, the horses could ingest seeds or seedlings at the same time (Votion et al., 2020).

At the pasture level, humid pastures have been identified as risk factors (Votion et al., 2009) probably because of the water-soluble nature of HGA (Votion et al., 2019) which can lead to its transfer from plant material to drinking water, such as rivers and/or freestanding water. This last point helps to explain why drinking water provided via the distribution network was identified as protective factor (Votion et al., 2009).

Finally, weather-dependent pasturing during autumn or spring, or limiting pasturing time to less than 6 hours per day allows a reduction in the likelihood of HGA ingestion (Gonzalez-Medina et al., 2016; Van Galen et al., 2012b; Votion et al., 2009), as does the practice of pasture rotation from risky to less risky pastures (Van Galen et al., 2012a).

4. Hypothesis of a Role of the Intestinal Microbiota in Atypical Myopathy

Several studies investigating HGA poisoning have proposed potential links between intoxication and alterations in the intestinal microbiota. In equids, Karlikova et al., (2016) notably observed that hippurate levels were significantly decreased in the serum and urine of horses (*i.e.*, first time sample) suffering from AM compared to healthy controls, while indole-containing metabolites were also found to be reduced in their urine. Hippurate, also known as hippuric acid, is the glycine conjugate of benzoic acid normally found in urine. As certain bacterial species are involved in the metabolism of phenolic compounds, the production of hippurate is linked to the gut microbiota (Lees et al., 2013). Moreover, germ-free animals do not excrete hippurate and are able to secrete hippurate after two to three weeks of being

exposed to a normal environment, and antibiotic-treated rats showed low levels of hippurate compared to three weeks post-administration of antibiotics: these facts reinforce the link between this carboxylic acid and the gut microbiota(Lees et al., 2013). Additionally, hippurate has previously been detected in horse urine (Escalona et al., 2015). The variation in hippurate concentration has been previously correlated with possible changes in gut microbiota in diseases such as EGS (Leng et al., 2018) or under different diets, where hay-fed horses secreted more hippurate than horses fed haylage (Leng et al., 2022). Moreover, hippurate was the only compound detected in urine of normal children, unlike children with JVS (Tanaka et al., 1976). The indole-containing metabolites are directly derived from tryptophan by intestinal microorganisms and are ligands for the Aryl hydrocarbon Receptor (AhR) (Alexeev et al., 2018; Galligan, 2018; Smith and Macfarlane, 1996; Su et al., 2022). The AhR signaling is a key component of the immune response and is important for intestinal homeostasis, affecting epithelial turnover, barrier integrity, and many immune cell types such as intraepithelial lymphocytes, innate lymphoid cells, macrophages, dendritic cells, and neutrophils (Agus et al., 2018). Consequently, these indole-containing metabolites play a role in the homeostasis of the gut and in systemic immunity, and they could probably also affect the occurrence and development of diseases such as inflammatory bowel diseases, obesity, and metabolic syndrome, as well as diseases of the nervous system (Agus et al., 2018; Alexeev et al., 2018; Galligan, 2018; Su et al., 2022). Furthermore, several bacterial species have been significantly correlated with the production of these indole-containing metabolites (Agus et al., 2018; Galligan, 2018; Nie et al., 2023; Smith and Macfarlane, 1996). Accordingly, the decreases in hippurate and indole- containing metabolites in the urine of AM horses compared to control horses seem to indicate a dysbiosis of the gut microbiota in AM horses (Karlíková et al., 2016).

Wimmer-Scherr et al. (2021), analysed the faecal microbiota of AM horses (survivors and non-survivors) compared to cograzers. At the genus level, the α-diversity and the evenness were significantly lower in cograzers compared to AM horses, without differences between survivors and non-survivors, while richness was not significantly different between groups. These results for richness mean that the number of bacterial genera present in the faeces was not significantly different between groups. Moreover, the result for evenness indicates that the distribution of the relative abundance of bacterial genera in the faeces of cograzers present the predominance of certain bacterial genera, indicating a more structured microbiota. The β-

diversity AMOVA analysis revealed significant differences between cograzers and AM horses. Furthermore, certain bacterial families were highlighted because of their significantly different relative abundance between cograzers and AM horses and between survivors and non-survivor horses: *Ruminococcaceae*, *Christensenellaceae* and *Akkermansiaceae* were increased in AM horses *vs.* cograzers but also in non-survivors *vs.* survivors whereas *Lachnospiraceae*, *Bacteroidales* and *Clostridiales* were decreased for both comparisons. Several bacterial genera were also identified as significantly different between cograzers and AM horses as well as between survivors and non-survivors: *Lachnospiraceae_ge*, *Bacteroidales_ge*, *Christensenellaceae_R-7_group, Ruminococcaceae_NK4A214_group, Ruminococcus_1*, *Akkermansia* and *Ruminococcaceae_UCG-002*. As expected regarding the results at family level, the relative abundance of *Christensenellaceae_R-7_group, Ruminococcaceae_NK4A214_group, Akkermansia* and *Ruminococcaceae_UCG-002* increased and *Lachnospiraceae_ge* and *Bacteroidales_ge* decreased gradually from cograzers to non-surviving AM horses (Wimmer-Scherr et al., 2021). It is important to note that since the discovery of subclinical cases among cograzers (Renaud et al., 2024), the choice of cograzers as control group is no longer relevant for AM studies. Consequently, the observations of this study must be considered as modifications progressing from subclinical cases to clinical cases, and not a comparison with a control group which should ideally be free of toxins in the body or at least in the serum. However, this study corroborates the hypothesis of a link between the gut microbiota and the development of AM intoxication as some of the modifications encountered are gradually modified from subclinical cases to clinical cases and from survivors to non-survivors horses.

In other species, as early as the 1950s, the hypothesis of a possible role of the digestive tract in dogs was proposed; indeed, the oral administration of HGA led to less severe hypoglycaemia compared to parenteral administration of the same dose of HGA (Chen et al., 1957).

More recently, Renaud et al. (2022) observed that herbivorous species in which the fermentation part of the digestive tract (*i.e.*, the major site of intestinal microbiota in herbivorous species) is located proximal to the small intestine (*i.e.*, the major site of amino acid absorption in mammals), and particularly those with a longer retention time were less likely to develop AM poisoning. Therefore, a ruminal transformation of HGA is hypothesised (Renaud et al., 2022).

Finally, the transformation of HGA, HGB, and MCPrG in ovine ruminal fluid batch cultures was investigated with a control made of autoclaved ovine ruminal fluid batches thanks to the addition of (i) pure HGA and MCPrG mixture and (ii) sycamore maple seeds containing HGA, HGB, MCPrG, and the glutamyl-peptide of MCPrG. In the ovine ruminal fluid with pure mixture of HGA and MCPrG, the concentrations of both protoxins rapidly decreased with a significant decrease after 8 hours and no protoxins detected after 24 hours. The same mixture in an inoculum made from autoclaved ruminal fluid led to an incomplete decrease of both protoxins. Similar conclusions were drawn from the incubation of seeds with two major differences: indeed, in seeds incubation, (i) the concentration of HGA increased in the first 24 hours with a concomitant decrease in HGB concentration before decreasing, and (ii) measurable HGA and MCPrG concentrations remained after 48 hours. The increase in HGA is explained by the release of HGA from HGB in seeds in both normal and autoclave ruminal fluid: this phenomenon seemed, consequently, to be independent of ruminal microbial assistance. The decrease in HGA in ovine ruminal fluid and the less marked decrease in autoclaved medium highlighted the possible transformation of protoxins by the rumen microorganisms but also by abiotic processes (Engel et al., 2025).

Whether the gut microbiota plays an active or passive role in AM remains an open question. On the one hand, the observed microbial changes could simply reflect the profound metabolic disturbances occurring in the host as a result of HGA poisoning, with the microbiota acting as a biomarker of systemic dysfunction. On the other hand, several lines of evidence support the possibility that the microbiota may actively contribute to the course of intoxication. Certain microbial populations might be involved in the bioactivation of protoxins such as HGA into their toxic metabolites, thereby enhancing toxicity. Conversely, other members of the microbiota may participate in the degradation, detoxification, or elimination of HGA before systemic absorption, potentially offering a protective effect as shown by (Engel et al., 2025). Moreover, shifts in microbial composition could influence gut barrier function, altering permeability to protoxins and their metabolites. Disruption of host energy metabolism by the toxins may also reshape the gut environment in ways that either favour or hinder disease progression. These contrasting hypotheses warrant further investigation using complementary *in vivo* and *in vitr*o models to clarify the functional role of the intestinal microbiota in the pathophysiology of AM.

4.1. The microbiota as a Witness

In AM, the protoxin HGA is metabolised into MCPA-CoA, which inhibits fatty acids β-oxidation, as evidenced by the accumulation of acylcarnitines in the serum and urine of affected horses (Bochnia et al., 2019; Boemer et al., 2017; Karlíková et al., 2016; Lemieux et al., 2016; Mathis et al., 2021; Renaud et al., 2024; Sponseller et al., 2012; Valberg et al., 2013; van der Kolk et al., 2010; Westermann et al., 2008, 2007). In addition, inhibition of IVD (Westermann et al., 2007, 2008; Karlíková et al., 2016) impairs the degradation of BCAAs (*i.e.*, especially leucine) and increased leucine concentrations in serum and urine (Karlíková et al., 2016). In contrast to findings in rats, no evidence of glycolytic impairment has been reported in AM horses (Cassart et al., 2007; Votion et al., 2007). Consequently, the formation of acetyl-CoA from triglycerides is severely compromised, since β-oxidation of free fatty acids cannot occur, and the main source of cellular energy shifts toward pyruvate derived from glycolysis. While both carbohydrates and lipids are major energy substrates for skeletal muscle, lipids represent the horse's most efficient source of energy (Votion et al., 2007). The gut microbiota is known to be altered in several metabolic disorders in humans (Fan and Pedersen, 2021), and could therefore be secondarily affected by the profound metabolic disturbances observed in AM. The detection of subclinical cases among cograzers (Renaud et al., 2024) suggests that these metabolic alterations may precede the onset of clinical signs, meaning that changes in gut microbiota composition could begin early in the disease course, potentially during the subclinical phase. Taken together, these considerations could support the hypothesis that observed *in vivo* microbiota alterations in AM could reflect an active adaptive response to host-level metabolic dysfunctions. This interpretation is consistent with the findings of Karlíková et al. (2016) and Wimmer-Scherr et al. (2021) (Karlíková et al., 2016; Wimmer-Scherr et al., 2021), but contrasts with the hypotheses of Renaud et al. (2022), which propose a protective role for the microbiota (Renaud et al., 2022), and Engel et al. (2025), who reported a reduction in HGA concentrations mediated by the ruminal microbiota of ovine *in vitro* models (Engel et al., 2025).

4.2. The microbiota as an Active Player

To play an active role in AM, HGA must encounter the intestinal microbiota after ingestion. Consequently, the following section examines the digestion and absorption of HGA and amino acids along the equine GIT.

4.2.1. Digestion and Absorption of Hypoglycin A along the Gastro-Intestinal Tract

The only study that partially addresses this point in horses is that of Krägeloh et al., (2017) which investigated the transport of HGA across intestinal mucosae from a resection of the terminal jejunum obtained from clinically healthy horses using the Ussing chamber technique. After one hour of incubation (a time frame chosen to ensure mucosal viability), HGA was detected in serosal compartment. Both the serosal release of HGA and its accumulation within mucosal tissues increased proportionally to its concentration on the mucosal side (Krägeloh et al., 2018). These results confirmed that HGA can be absorbed, at least in part, by equine jejunal cells. The experiment was based on the assumption that HGA, as an amino acid analogue, would follow similar digestive and absorptive pathways to those of natural dietary amino acids.

As previously mentionned, HGA is a non-proteinogenic amino acid, meaning that its structure contains both an amino group ($–NH_2$) and a carboxyl group (–COOH) bound to the same α-carbon, but it is not incorporated into proteins during anabolism. Its side chain consists of a methylene group ($–CH_2$) and a cyclopropane ring with three carbon atoms, containing no aromatic ring. This chemical structure classifies HGA as an aliphatic amino acid, similar to alanine, from which it is derived (Nelson et al., 2021).

The low molecular weight of HGA (*i.e.*, 141 Dalton) (Bressler et al., 1969) is comparable to that of BCAAs, which can make chromatographic separation challenging (Billington and Sherratt, 1981). It can also enter BCAA metabolic pathways, involving enzymes such as BCATs and BCKDHc (Melde et al., 1991). In AM, the protoxin HGA is ingested by horses and therefore undergoes the digestion process within the digestive tract, probably in the same way as amino acids or BCAAs. These BCAAs are hydrophobic neutral amino acids such as alanine, methionine, and phenylalanine. Other amino acids are positively charged at physiological pH (*i.e.*, lysine, arginine, histidine), and other are negatively charged (*i.e.*, aspartate, glutamate).

However, given the structural analogy, the next section will focus primarily on the digestion and absorption of neutral amino acids, with only brief reference to positively and negatively charged amino acids.

4.2.2. Digestion and Absorption of Amino Acids along the Gastro-Intestinal Tract

In mammals' stomach, hydrochloric acid secreted by parietal cells activates the proteolytic enzyme (*i.e.*, pepsinogen) into pepsin. In horses, the importance of pepsin in protein catabolism remains unknown (Merritt and Julliand, 2013; Mok and Urschel, 2020). Nevertheless, proteolytic activity is noted in the pyloric region, but this activity is negligible in the fundic region and ten-fold less compared to that of the small intestine (Merritt and Julliand, 2013).

In small intestine, peptides undergo enzymatic breakdown by trypsin (*i.e.*, an endoprotease produced in the pancreas) whose role is to hydrolyse the C-terminal region of peptides. The oligopeptides produced are degraded into di or tripeptides (*i.e.*, ~ 25%) or free amino acids (*i.e.*, ~ 75%) by both pancreatic carboxypeptidases that remove one amino acid at a time from the carboxyl end of the chain, and oligopeptidases which are located in the brush border of mucosal cells (Merritt and Julliand, 2013; Bröer and Fairweather, 2019; Mok and Urschel, 2020; Bröer, 2024). In horses, the ileum possesses the highest proteolytic activity (23.85 μg of hydrolysed protein/mg of ileal content/min) compared to the rest of the GIT. However, the intestinal digestion and absorption of protein in the horse remains poorly documented (Merritt and Julliand, 2013). Nevertheless, studies on the kinetics of nitrogen in the equine GIT indicated that 11 to 30% of apparent N fermentation is attributed to the small intestine *vs.* 40% to 70% to the hindgut (Gibbs et al., 1988; Glade, 1983; Reitnour and Salsbury, 1972)

Despite this highest proportion of hindgut N fermentation, absorption appears to occur primarily in the jejunum and ileum, as observed by recording the net disappearance of N from the equine small intestine, which varied from 16% up to 58% pre-caecally, and as supported by an amino acid transporter mRNA study. The absorption form of N from the equine small intestine has not been specified, but it is probably mainly in the form of amino acids (*i.e.*, the form identified in humans and other mammals) (Merritt and Julliand, 2013; Mok and Urschel, 2020). In humans, the absorption process in the form of di- and tripeptides and free amino

acids takes place at the level of the apical membrane of the enterocytes. Di- and tri- peptides are transported via an H^+-driven transporter into the cytoplasm after which a hydrolysis by cytoplasmic oligopeptidases allows cleavage into amino acids which then passively (*i.e.*, via a concentration gradient) diffuse into the portal blood vein (Merritt and Julliand, 2013).

The transporter for free neutral amino acids at the apical membrane level belongs to the system B^0 and is called B^0AT1 (broad neutral amino acid transporter 1, gene designation *SLC6A19*). This system is primarily expressed in the apical membranes of small intestinal enterocytes, with increasing expression from the duodenum to the ileum. This transporter requires ancillary proteins (*i.e.*, non-essential accessory proteins that support or stabilise the function of a primary enzyme or protein complex) and a cotransporter which are respectively angiotensin converting enzyme II (ACE2) and sodium (Na^+). The cotransporter works according to Na^+ electrochemical gradient. The ACE2 is also a general carboxypeptidase located in the intestinal brush border. The ACE2 and B^0AT1 co-expression generates a metabolon (*i.e.*, a functional and transient assembly of enzymes involved in the same metabolic pathway, organised to promote the efficiency of metabolic flow) for the instantaneous transport of all neutral amino acids, but preferentially BCAAs (especially those with hydrophobic side chains) and methionine. Finally, B^0AT1 and ACE2 mRNA, as well as their protein expression, increases along the crypt-to-villus axis: when enterocytes move from the crypts (*i.e.*, the differentiation point) to the villus tip, the genes are increasingly expressed (Bröer et al., 2004; Bröer, 2023; Broër, 2008; Bröer and Fairweather, 2019).

Transporters for free neutral amino acids at the basolateral membrane level are LAT2 (large neutral amino acid transporter 2, *SLC7A8*), LAT4 (large neutral amino acid transporter 4, *SLC43A2*) and TAT1 (system T amino acid transporter 1, *SLC16A10*). The LAT2 transports all neutral amino acids except proline and is mostly expressed in the jejunum (K_m range 50-200 μM for extracellular binding and 200-fold higher for intracellular binding). The LAT4 has a very low affinity for its substrates (*i.e.*, leucine, isoleucine, valine, phenylalanine, and methionine). The TAT1 is selective for aromatic amino acids with a high K_m (Bröer, 2023; Broër, 2008; Bröer and Fairweather, 2019).

Antiporters in both membranes (*i.e.*, apical and basolateral membranes) are used for cationic free amino acid transport. Expressed in the distal jejunum and colon, these antiporters are composed of two subunits: the trafficking subunit rBAT (related to $b^{0,+}$ amino acid

transporter, *SLC3A1*) and the catalytic subunit $b^{0,+}$AT (*SLC7A9*). The antiport activity involves an exchange of cationic with neutral amino acids which are then recaptured by B^0AT1 located in cells downstream. The transporter accepts most neutral and all cationic amino acids, with preferred substrates being arginine, lysine, alanine, cysteine, cystine, leucine, methionine, phenylalanine, and tyrosine. Another transporter of cationic amino acid called $ATB^{0,+}$ (*SLC6A14*) is expressed in the distal jejunum and colon (Bröer, 2023; Bröer and Fairweather, 2019).

The dominant transporter for free anionic amino acid is EAAT3 (excitatory amino acid transporter 3, *SLC1A1*) involving the cotransport of aspartate or glutamate with 3 Na^+ and 1 H^+ followed by return of the carrier with K^+ bound to it (Bröer, 2023). In the rabbit ileum, another transporter ASCT2 (*SLC1A5*) contributes to the absorption of anionic amino acids (Bröer, 2023; Bröer and Fairweather, 2019).

Finally, the transport of glycine, proline, and β-amino acids (*e.g.*, taurine) relies on specific transports due to their short side chains and/or special conformation, and/or distance between the α-amino and the α- carboxyl group. These transporters include the neutral amino acid transporter B^0AT1, the proline and glycine transporter PAT1 (proton amino acid transporter 1, *SLC36A1*) and the proline transporter SIT1 (*SLC6A20*). The PAT1 is a low-affinity, high-capacity transporter for glycine, betaine, proline, alanine, the β-amino acid taurine, and the γ-amino acid GABA. This transporter has the capacity to accept D-amino acids with similar affinity and is proton dependent with the highest activity at low pH levels, as in the intestinal brush border. The SIT1 transport proline, betaine, and hydroxyproline with Na^+ and Cl^- and is mainly located in brush-border membrane vesicles with ACE2 as an ancillary protein (Bröer, 2023; Bröer and Fairweather, 2019).

It is important to note that both circulating and intraluminal amino acids can also be used by the intestinal epithelial cells as a source of metabolic energy (Merritt and Julliand, 2013).

The large intestine absorbs amino acids mainly from endogenous secretions and microbial protein, whereas the small intestine is responsible for the absorption of amino acids resulting from the digestion of dietary proteins. These amino acids are derived from endogenous secretions such as mucus, epithelial cells, and microbial amino acids. In human, it has been estimated that 2–7% of the daily protein uptake arrive to the large intestine. The mRNA expression of the major transporters described in the previous paragraph and involved in

vectorial (*i.e.*, process by which a substance passes through a cell in a specific direction) transport, such as B^0AT1 (*i.e.*, transport of neutral amino acid through the apical membrane), $b^{0,+}AT$ (*i.e.*, transport of cationic amino acid through the apical membrane), and LAT2, decreases from the jejunum to the colon. The PAT1, by contrast, shows a constant expression along the intestine. In fact, the main transporters in the large intestine are $ATB^{0,+}$ (*SLC6A14*) and ASCT2 (*SLC1A5*) both located in the apical membrane and initially detected in the distal ileum of rabbits. The $ATB^{0,+}$ is mainly expressed in the distal ileum and colon and accepts all neutral and cationic free amino acids. However, it accumulates mainly glycine and BCAAs. The ASCT2 is an obligatory antiporter which seems to be less effective in vectorial transport, but it could mediate the removal of glutamate and aspartate in the acidic microclimate of the brush border in exchange for nonessential neutral amino acids (Bröer, 2023).

In pigs, absorption of amino acids from colon was confirmed by using the appearance of ^{15}N-labeled amino acids in the portal venous blood after endoluminal injection of ^{15}N-labeled bacteria into the caecum as reviewed by (Davila et al., 2013).

In the hindgut of horses, apparent nitrogen digestion occurs from 40% to 70%; in fact, microbes ferment dietary nitrogenous components such as gelatin, casein, peptones, amino acids, or ammonia but hardly urea. Indeed, bacterial isolates obtained from caecal contents of horses were cultured on medium containing urea, ammonia, peptones, or amino acids as the sole nitrogen source and only 17.9% and 20.5% of bacterial isolates were able to use urea and ammonia for growth, respectively while 100% and 35.9% of bacteria grew in the peptone and amino acid media, respectively. This suggests that caecal bacteria can contribute to the amino acid metabolism of the horse, but the exact mechanism remains unknown (Merritt and Julliand, 2013; Mok and Urschel, 2020).

In the caecal compartment, an injection of nitrogen isotope (*i.e.*, ^{15}N) led to the appearance of labelled essential and non-essential amino acids, urea, ammonia, and lysine in the caecal veins. This supports evidence of the horse's ability to digest and absorb microbial protein from the large intestine (Slade et al., 1971). However, active transport of amino acids through the caecal or colonic mucosa has not been directly demonstrated in horses. Nevertheless, both cationic and neutral amino acid transporter genes are expressed in the equine large intestine, suggesting their potential role in microbial and dietary amino acids absorption (Woodward et al., 2010). Another hypothesis is the possibility of reflux from the caecal to the ileal

compartment in horses and consequently, the absorption of amino acids from the caecum could take place in the ileum via this reflux.

Nevertheless, the large quantity of nitrogen disappearing from the large intestine is a direct result of (i) the deamination of dietary or endogenous amino acids or (ii) the catabolism of other "nitrogen-containing" compounds (such as urea to ammoniac) by the microbiota. The resulting ammoniac diffuses across the intestinal wall and is excreted by the urine (Hendriks et al., 2012). Therefore, it appears reasonable to hypothesise that the amino acids ingested, such as HGA, by the horse may encounter the caecal and colonic microbiota.

4.2.3. The Microbiota as a Negative Player in the Facilitation of Toxicity

As described, the metabolism of HGA is a two-step process similar to that of BCAAs, catalysed by two enzymes: BCAT and BCKDHc (Melde et al., 1991). Moreover, the BCAAs are also catabolised in bacteria, mainly by the same two enzymes involved in HGA metabolism (Massey et al., 1976).

In bacteria, BCAT catalyses the final step in the biosynthesis of BCAAs and also initiates their degradation; it therefore fulfils both anabolic and catabolic functions (Bezsudnova et al., 2017b; Hutson, 2001). Bacterial BCAT is distinguished from its eukaryotic counterparts by a broader substrate specificity (Chen et al., 2023; Hutson, 2001) and variability in size and subunit composition (Hutson, 2001). For example, BCAT from *Pseudomonas* (PsBCAT) exhibits significant activity against many aliphatic L-amino acids (Chen et al., 2023) such as HGA.

Described originally in crude extracts of *Escherichia coli*, BCAT activity was referred to three transaminases A, B, and C. Transaminase A was composed of aspartate aminotransferase and aromatic amino acid aminotransferase. Transaminase C is a unique alanine/valine transaminase. Transaminase B (*i.e.*, the product of the *ilvE* gene) is the bacterial version of the BCAT enzymes found in eukaryotes and catalyses transamination of all three BCAAs and glutamate. The "ilv" designation was later applied to the genes encoding BCAT in bacteria (Bezsudnova et al., 2017b; Hutson, 2001).

Unlike higher eukaryotes, which possess both mitochondrial and cytosolic BCAT isoforms (*i.e.*, including those in yeast, fungi, plants, nematodes, and mammals), bacteria generally contain a single BCAT. The apparently ubiquitous expression of BCAT in the bacterial kingdom has been confirmed by the bacterial genome sequences now available in the

databases and is consistent with the enzyme's role in the biosynthesis of BCAAs (Hutson, 2001).

The BCKDHc is also found in several bacteria (Martin et al., 1973; Perham and Lowe, 1988; Singh et al., 2018; Sokatch et al., 1981; Sykes et al., 1987; Wang et al., 1993) and appears to be composed mainly of the same three subunits described in mammals (Chuang, 1989; Massey et al., 1976), although this has not been systematically demonstrated for all bacterial species. Structural studies of bacterial BCKDHc have, in fact, provided the basis for the currently accepted model of the mammalian complex (Berg and de Kok, 1997; Yeaman, 1989), although structural variations may occur in prokaryotes (Berg and de Kok, 1997).

Additionally, BCKDHc also shares similarities with the pyruvate dehydrogenase complex (Massey et al., 1976; Patel et al., 2014; Wieland, 1983). Some bacterial species possess multiple genes encoding the same subunit (Berg and de Kok, 1997) or exhibit fusion between enzyme subunits (de Kok and Westphal, 1985; Powles and Rawlings, 1997), which preserves enzymatic functionality while simplifying the overall structure of the complex.

Consequently, it could be hypothesised that gut microbiota may also transform HGA into its toxic metabolite, thereby playing an active detrimental role in AM. This hypothesis finds some supports in the study of Wimmer-Scherr et al., (2021) in which certain bacterial populations appeared to be progressively affected from cograzers (*i.e.*, subclinical cases) to clinically affected AM horses, and especially in non-survivors (Wimmer-Scherr et al., 2021). However, it is not supported by the protective hypothesis of Renaud et al., (2022) (Renaud et al., 2022), and no toxic metabolites (*i.e.*, MCPA MCPA-glycine, MCPA-carnitine, and MCPF-glycine were detected in batch cultures with ovine ruminal fluid, thereby undermining this assumption (Engel et al., 2025).

4.2.4. The Microbiota as a Positive Player in Detoxification and Resistance

Since amino acids can serve as an important source of carbon and nitrogen for bacteria in the GIT, it is conceivable that the intestinal microbiota could metabolise HGA either into a non-toxic compound or directly utilise it for growth, thereby removing HGA from the digestive ingesta.

Amino acids and ammonia are generally considered as the preferred nitrogen sources for gut bacteria. Among amino acids, glutamine/glutamate, asparagine/aspartate, lysine, serine,

threonine, arginine, glycine, histidine, and BCAAs are more efficiently degraded and preferentially used as metabolic substrates by the gut microbiota compared to other amino acids (Dai et al., 2011). Moreover, gut bacteria contain a higher proportion of BCAAs relative to other amino acids (Neis et al., 2015), which further supports the hypothesis that the intestinal microbiota could degrade HGA.

Deamination and decarboxylation are the two major pathways of microbial amino acid catabolism, which occur mainly in the large intestine. The resulting products include ammonia, SCFAs and branched-chain fatty acids, phenolic and indolic compounds, organic acids, gaseous compounds, and amines (Dai et al., 2011; Neis et al., 2015). Along the GIT of both humans and animals, strains belonging to the *Clostridium* clusters have been identified as predominant amino acid-fermenting bacteria (Dai et al., 2011). In the human large intestine, bacteria of the classes *Clostridia* and *Peptostreptococci* appear to be the most actively involved in amino acid fermentation (Neis et al., 2015). Interestingly, the large intestine is the fermentative compartment in animals such as the horse, which is a hindgut fermenter.

In horses, several bacterial species are already known to utilise amino acids as substrates as (i) *Escherichia coli* has arginine, asparagine, aspartate, glutamine, glutamate, lysine , serine, and threonine, as preferred substrates, (ii) *Clostridium difficile* prefers isoleucine, leucine, and threonine as substrates but this species also has the possibility to generate a transfer of electron via the Stickland reaction (*i.e.*, a metabolic pathway used by certain anaerobic bacteria to produce energy from amino acids, which involves a coupled fermentation of two amino acids, where one acts as an electron acceptor (reduced) and the other as an electron donor (oxidised) (Nisman, 1954)), between alanine and valine as an H^+ donor and leucine as an H^+ acceptor, and (iii) *Veillonella spp.* preferentially uses lysine and ornithine (Dai et al., 2011).

The involvement of gut microbiota in the degradation or detoxification of phytotoxins is well documented, as illustrated by mimosine, a toxic heterocyclic non-protein amino acid found in the seeds and leaves of tropical *Leucaena* genus (*Mimosaceae* family) (Adams and Jones, 1947; Bickel, 1947). This leguminous is used as forage for ruminants in a range of sub-tropical and tropical environments for its high fibre and protein content (National Research Council, 1984). This protoxin is known to cause depressed serum thyroxine levels, enlarged thyroid glands, ulceration of the oesophagus, depressed appetite, alopecia, weight loss, and death, mostly in ruminants. As reviewed by (Hegarty et al., 1964), the adverse effects of

mimosine have also been described in pigs, small laboratories animals and in horses (Hegarty et al., 1964; Machado et al., 2024). The goitrogen effect of mimosine is due to its ruminal degradation product namely 3,4-Dihydroxypyridine (DHP), which is partly excreted in urine (Hegarty et al., 1979, 1964; Kudo et al., 1984).

Interestingly, the consumption of *Leucaena* is toxic to ruminants in Australia, New Guinea, and Africa but is not reported in ruminants from Asia, Hawaii, or Indonesia (Jones, 1981; Jones and Lowry, 1984). At first, the hypothesis was that the difference lay in the plants from these different parts of the world, but these plants contain the same range of mimosine concentration, as demonstrated between Australian and Hawaiian plants (Jones, 1981). Another hypothesis concerned the quantity of Leucaena administered to sheep, but the rumen fluid of Hawaiian sheep fed 100% *Leucaena* degraded 70% of mimosine after 22 hours, which explains the very low levels of DHP in their blood and consequently the absence of clinical signs (Jones, 1981). Finally, an infusion of ruminal fluid from Indonesian goats fed with *Leucaena*, known to be resistant to the toxic effect of mimosine, was transfauned to Australian goats to detoxify the goitrogen effect of DHP. Following infusion, the increased feed intake and the strong decline in DHP excretion in Australian goats seem to corroborate the implication of ruminal microbiota in tolerance and detoxification (Jones, 1981). Moreover, an *in vitro* propagation of rumen population able to degrade DHP was used to inoculate the rumens of sensible cattle: once colonised by these DHP-degrading bacteria, the animals were protected against mimosine toxicity (Jones and Megarrity, 1986). Consequently, the difference in DHP detoxification among ruminants is due to the differences in ruminal microbiota, which can degrade mimosine and protect animals (Jones and Lowry, 1984; Jones and Megarrity, 1983). Four strains of strictly anaerobic, gram-negative, rod-shaped bacteria able to degrade DHP were isolated from rumen contents from a goat in Hawaii. These bacteria do not ferment carbohydrates, as usually observed, but use DHP for growth, and consequently use amino acids as the main source of energy. A new genus and species designation was proposed: *Synergistes jonesii* (Allison et al., 1992, 1990)

Lastly, the hypothesis that HGA may be degraded into a non-toxic compound supports the hypothesis of Renaud et al., (2022) and Engel et al., (2025), while also aligning with the findings of other studies suggesting a possible link between the gut microbiota and AM (Engel et al., 2025; Karlíková et al., 2016; Renaud et al., 2022; Wimmer-Scherr et al., 2021).

Objectives

This thesis aimed to elucidate the role of the equine gut microbiota in HGA intoxication and the development of AM. Given the limited understanding of how HGA interacts with and affects intestinal microbial communities, the objectives were divided into three complementary and interconnected sub-objectives:

1. Identifying *in vivo* alterations in gut microbiota composition

The first objective was to investigate statistically significant differences in gut microbiota composition and diversity between horses affected by AM and healthy controls. As subclinical cases were confirmed among cograzing horses, they could no longer be considered a valid control group (Renaud et al., 2024). Establishing robust *in vivo* differences between affected and control animals provided the foundation for subsequent mechanistic investigations.

2. Characterising microbiota responses to HGA in the absence of host influence

The second objective was to assess the direct impact of HGA on equine gut microbial communities under controlled *in vitro* conditions free from host influence. A static fermentation model (derived from SHIME®) was used used to isolate the microbial response. Faecal inocula from healthy horses were exposed to HGA and compared with unexposed controls. This approach will identify bacterial populations directly impacted by the protoxin, independently of host-mediated influences.

3. Elucidating the microbiota's role in the pathophysiology of atypical myopathy

The third objective was to investigate how the equine gut microbiota contributed to the metabolism and fate of HGA and to the overall pathophysiological processes of AM. This includes determining whether specific bacterial taxa could degrade, transform, or utilise HGA as a carbon or nitrogen source. Insights obtained from the *in vitro* experiments guided an in-depth characterisation of bacterial taxa most likely to influence protoxin fate and disease outcome, helping to clarify whether the microbiota acts as a protective or detrimental factor in AM.

The three objectives of this thesis were addressed through two complementary studies forming its core. The first, published in *Animals* (François et al., 2025: "Unravelling Faecal Microbiota Variations in Equine Atypical Myopathy: Correlation with Blood Markers and Contribution of Microbiome"), investigates *in vivo* alterations in the gut microbiota of horses affected by AM compared with toxin-free controls. The second, an *in vitro* fermentation study (François et al., 2025; "*In Vitro* Investigation of Equine Gut Microbiota Alterations under Hypoglycin A Exposure"), explored the direct effects of HGA on equine faecal microbial communities and the potential microbial transformation or degradation of the protoxin, thereby linking microbial composition to functional capacity.

Experimental section

STUDY 1: Unravelling Faecal Microbiota Variations in Equine Atypical Myopathy: Correlation with Blood Markers and Contribution of Microbiome

Anne-Christine François, Carla Cesarini, Bernard Taminiau, Benoît Renaud, Caroline-Julia Kruse, François Boemer, Gunther van Loon, Katrien Palmers, Georges Daube, Clovis P. Wouters, Laureline Lecoq, Pascal Gustin and Dominique-Marie Votion

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INTRODUCTION TO THE FIRST STUDY

The first study aimed to characterise the composition and diversity of the faecal microbiota in horses affected by AM compared with toxin-free controls. Particular attention was given to exploring potential associations between microbial community shifts and biochemical blood markers linked to HGA intoxication. To achieve this, faecal and blood samples were collected from affected and healthy horses across multiple outbreaks. Microbial DNA was extracted and analysed using 16S rRNA gene sequencing to determine taxonomic composition and diversity indices. Statistical analyses were then applied to identify significant differences in microbial structure between groups and to explore correlations with metabolic indicators of AM.

animals

MDPI

Article

Unravelling Faecal Microbiota Variations in Equine Atypical Myopathy: Correlation with Blood Markers and Contribution of Microbiome

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Simple Summary: Equine atypical myopathy is a severe intoxication caused by protoxins synthesised by certain maple trees, notably sycamore maple (*Acer pseudoplatanus*). These protoxins are activated into harmful toxins that disrupt lipid metabolism by inhibiting specific steps of fatty acid β-oxidation, leading to the accumulation of acylcarnitines in the blood. This activation process is catalysed mainly by specific mitochondrial enzymes, which are also present in some bacteria. Horses grazing in close proximity to affected animals have shown differences in their faecal microbiota composition, suggesting that the role of gut microbiota in atypical myopathy could be more substantial than previously understood. Recently, blood analyses have demonstrated the existence of subclinical cases among these cograzers. The present study compares the faecal bacteria of horses affected by atypical myopathy, their cograzers, and a group of toxin-free horses serving as a control group. Results show significant differences in faecal bacterial diversity and composition between groups, particularly for certain bacterial genera. Additionally, blood levels of specific compounds appear to be associated with these bacterial changes. The theoretical presence of the enzymes involved in the protoxin activation process was also studied. These results highlight the importance of comprehensively studying intestinal microbiota to better understand its role in this generally fatal poisoning.

Abstract: Hypoglycin A and methylenecyclopropylglycine are protoxins responsible for atypical myopathy in equids. These protoxins are converted into toxins that inhibit fatty acid β-oxidation, leading to blood accumulation of acylcarnitines and toxin conjugates, such as methylenecyclopropylacetyl-carnitine. The enzymes involved in this activation are also present in some prokaryotic cells, raising questions about the potential role of intestinal

microbiota in the development of intoxication. Differences have been noted between the faecal microbiota of cograzers and atypical myopathy-affected horses. However, recent blood acylcarnitines profiling revealed subclinical cases among cograzers, challenging their status as a control group. This study investigates the faecal microbiota of horses clinically affected by atypical myopathy, their cograzers, and a control group of toxin-free horses while analysing correlations between microbiota composition and blood parameters. Faecal samples were analysed using 16S amplicon sequencing, revealing significant differences in α-diversity, evenness, and β-diversity. Notable differences were found between several genera, especially *Clostridia_ge*, *Bacteria_ge*, *Firmicutes_ge*, *Fibrobacter*, and *NK4A214_group*. Blood levels of methylenecyclopropylacetyl-carnitine and C14:1 correlated with variations in faecal microbial composition. The theoretical presence of enzymes in bacterial populations was also investigated. These results underscore the critical need to investigate the potential role of intestinal microbiota in this poisoning and may provide insights for developing prevention and treatment strategies.

Keywords: equine atypical myopathy; microbiota; gut microbiota; faecal microbiota; microbiome; faecal microbiome; horses; equine; hypoglycin A; methylenecyclopropylacetyl-carnitine; MCPA-CoA; acylcarnitines; 16S rRNA gene sequencing; next generation sequencing; NGS; blood metabolites; toxin; poisoning

1. Introduction

Equine atypical myopathy (AM) is a seasonal and highly fatal [1] intoxication caused by the ingestion and metabolism of two protoxins contained in some *Acer* spp., including *Acer pseudoplatanus* and *Acer negundo* [2,3]: methylenecyclopropylalanine, also known as hypoglycin A (HGA), [2] and methylenecyclopropylglycine (MCPrG) [4].

Both HGA and MCPrG are non-proteinogenic amino acids [5] that are not toxic *per se*. Their metabolism into toxic metabolites requires the action of enzymes, mainly located in the mitochondria, involved in branched-chain amino acids (BCAAs) catabolic pathway [6]. The first step is a transamination, catalysed by the branched-chain amino acid aminotransferase (BCAT) [6,7]. The second and irreversible step, catalysed by the branched-chain α-keto acid dehydrogenase complex (BCKDHc), is an oxidative decarboxylation with coenzyme A (CoA) added to oxidised products [6,8]. This results in the formation of methylenecyclopropylacetyl-CoA (MCPA-CoA) for HGA and methylenecyclopropylformyl-CoA (MCPF-CoA) for MCPrG [6]. These toxic metabolites impair the β-oxidation of fatty acids, leading to increased levels of acylcarnitine in tissues [9], urine [2,10,11] and blood [5,12–16]. The diagnosis of AM relies on a combination of clinical signs, acylcarnitine profiling, and the detection of toxic metabolites conjugated with carnitine or glycine, such as MCPA-carnitine [3,16–19].

Both prokaryotic and eukaryotic cells can metabolise amino acids [20]. Indeed, some bacteria have the above-mentioned enzymes: for example, *Lactococcus lactis* and *Escherichia coli* express BCAT [21,22], and *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas putida* express BCKDHc [23–31]. This raises the hypothesis that the intestinal microbiota may play a role in the metabolism of HGA and/or MCPrG, potentially affecting horses that have ingested these protoxins. Analysis of the faecal microbiota is of twofold interest in exploring this hypothesis: on top of being a non-invasive technique, the faecal microbiota is reflective of the colonic microbial population in equids [32–34].

Wimmer-Scherr et al. (2021) conducted a study exploring the differences in faecal microbiota between horses with AM and a control group consisting of their cograzers (CoG)

(i.e., horses grazing in the same pasture as a poisoned horse but having a normal physical examination). They described that (1) the relative abundance of families *Ruminococcaceae*, *Christensenellaceae* and *Akkermansiaceae* was higher in horses suffering from AM compared to their CoG, and (2) the relative abundance of families *Lachnospiraceae*, *Bacteroidales* and *Clostridiales* was lower in horses with AM compared to their CoG, especially in non-surviving AM animals [35].

Clinically healthy CoG of HGA-poisoned horses are exposed to similar toxic pressure as horses affected by AM, yet they do not exhibit any clinical signs of poisoning [36–38]. Due to their shared environment and, therefore, exposure to the same protoxins, their blood often contains detectable levels of HGA and, in some cases, MCPA-carnitine [10,17,37,39] at levels that sometimes overlap with those observed in clinically affected AM horses [16,38]. Despite the absence of overt clinical signs, acylcarnitines profiling—which is the diagnostic and prognostic gold standard of AM [15,16]—revealed that many CoG has increased levels of acylcarnitines compared to control horses (i.e., horses free of toxin) [16]. This observation indicates the existence of subclinical cases among CoG, challenging their status as a healthy control group. Furthermore, another study on *Acer pseudoplatanus* poisoning in herbivorous species highlighted the existence of subclinical poisoning in species other than equids and hypothesised a potential role of gut morphology and intestinal microbiota in the risk of HGA intoxication [40]. Therefore, to study the potential role of microbiota in AM, it is necessary to include a control group composed of clinically healthy toxin-free horses and compare this group to both AM horses and their CoG.

As previously mentioned, some bacteria possess the enzymatic machinery necessary to metabolise HGA into MCPA. This raises the hypothesis that there could be a correlation between the host's microbiota and blood parameters related to intoxication (such as HGA, MCPA-carnitine and the acylcarnitines profile). This hypothesis was not addressed in the study of Wimmer-Scherr et al. (2021), highlighting a second point for improvement in understanding the possible role of intestinal microbiota in AM [35].

Therefore, the aims of this study were (1) to compare the faecal microbiota of AM horses (both survivors and non-survivors) and their CoG with that of control horses (i.e., grazing horses without protoxins or toxic metabolites in their blood) and (2) to analyse the correlation between faecal microbiota and blood parameters associated with AM intoxication in horses (i.e., HGA, MCPA-carnitine and the acylcarnitines profile).

2. Materials and Methods

All procedures in this study adhered to both national and international guidelines on animal welfare. The Animal Ethics Committee of the University of Liege was consulted, and it was confirmed that the sampling process was part of routine veterinary practice for diagnosing or preventing AM. As a result, formal ethical approval was not required. Informed consent was obtained from horse owners prior to their inclusion in the study.

2.1. Horses: Study Design, Inclusion Criteria and Group Definition

Four separate groups were defined for the purpose of this study:

- Control horses (CONTROL): clinically healthy horses free of HGA and MCPA-carnitine in the blood (i.e., HGA and MCPA-carnitine levels below the limit of detection), living in a place where AM cases had previously been observed and spending at least 6 h a day at pasture;
- Cograzers (CoG): clinically healthy horses grazing in a pasture where a case of AM had been diagnosed in the previous 24 h, which had a normal physical exam and a normal dynamic examination at walk (no signs of AM or other obvious disease) at the time of sampling;

- AM survivors (AM–S): horses diagnosed with AM that were discharged from the clinic once free of clinical signs after a variable hospitalisation period;
- AM non-survivors (AM–NS): horses diagnosed with AM that died from the intoxication during hospitalisation or had to be euthanised due to significant clinical deterioration or continuous or unmanageable pain leading to a poor prognosis [41].

These last two groups (i.e., AM-S and AM-NS) were merged into a single group, referred to as 'diseased horses', for certain statistical analyses to facilitate comparisons with previously published data [16].

The groups AM-NS, AM-S and CoG include published data from a prospective clinical study conducted from autumn 2016 until spring 2019 [35]. The horses from the CONTROL group were prospectively sampled in autumn 2020.

The diagnosis of AM was based on (1) the algorithm proposed by van Galen et al. (2012) [1] (i.e., a compatible history and clinical signs highly suggestive of AM during spring/autumn) and on (2) the presence of HGA and MCPA-carnitine in serum, a modified acylcarnitines profile compatible with the diagnosis of AM, and elevated serum activities of creatine kinase (CK) when available. For inclusion in the present study, only horses with available data on blood levels of HGA, MCPA-carnitine, and acylcarnitines were considered [14–16].

2.2. Comparison of Faecal Microbiota Between Groups

A fresh faecal sample was collected at the time of clinical admission for horses suspected of AM. Faecal samples of CoG of these diseased horses were taken within 24 h of the first horse in the shared pasture displaying clinical signs of AM.

The centre of a faecal ball was sampled after direct collection from the rectum or from a pile of recently passed faeces (<30 min) as described by Stewart et al. (2018) [42] and was directly placed in a conservation medium (Stool DNA stabiliser, PSP® Spin Stool DNA Plus Kit 00310, Invitek, Berlin, Germany) and stored at −20 °C until total bacterial DNA extraction.

2.2.1. Bacterial DNA Extraction and High-Throughput Sequencing

The PSP Spin Stool DNA Plus Kit 00310 (Invitek, Berlin, Germany) was used to extract total bacterial DNA from stool samples as recommended by the manufacturer. The following primers (with Illumina overhand adapters), forward (5′-GAGAGTTTGATYMTGGCTCAG-3′), and reverse (5′-ACCOGCOGGCTGCTGGCAC-3′) were used to perform PCR amplification of the 16S rDNA V1–V3 hypervariable region and library preparation. Each PCR product was purified with the Agencourt AMPure XP bead kit (Beckman Coulter, Pasadena, CA, USA) and subjected to a second PCR round for indexing using Nextera XT index primers 1 and 2. After purification, PCR products were quantified using the Quant-IT PicoGreen (ThermoFisher Scientific; Waltham, MA, USA) and diluted to 10 ng/µL. A final quantification of each library was performed using the KAPA SYBR® FAST qPCR Kit (KapaBiosystems; Wilmington, MA, USA) before normalisation, pooling and sequencing on a MiSeq sequencer using V3 reagents (Illumina; San Diego, CA, USA). Commercial Mock community positive controls using DNA from 10 defined bacterial species (ATCC MSA-1000, ATCC, Manassas, VI, USA) and negative controls (from extraction and PCR steps) were included in the sequencing run [43].

Raw amplicon sequencing libraries were submitted to the NCBI database under bioproject number PRJNA1170059.

2.2.2. Sequence Analysis and 16S rDNA Profiling

Sequence read processing was performed as previously described [43] using the MOTHUR software package v1.48 [44] and the VSEARCH algorithm for chimera detection [45]. For operational taxonomic unit (OTU) generation, a clustering distance of 0.03 was used. 16S reference alignment and taxonomical assignment, from phylum to genus, were performed with MOTHUR and were based upon the SILVA database (v1.38.1) of full-length 16S rDNA sequences [46].

2.2.3. Data Analysis

Subsampled datasets with 10,000 cleaned reads per sample were obtained and used to evaluate α-diversity and β-diversity using a vegan package (v 2.6-6.1) [47].

The analysis of α-diversity (i.e., measuring diversity within the community) and β-diversity (i.e., measuring diversity between communities or within the same community at different time points by considering sequence abundances or by considering only the presence–absence of sequences) are used to assess the ecology of a microbial community [48,49]. Indicators of α-diversity include the Chao richness index, reciprocal Simpson microbial diversity, and Simpson-derived evenness. Richness quantifies the number of species present within a community, while evenness describes how uniformly individuals are distributed among the species, highlighting the presence or dominance of certain species [50].

Differences in α-diversity between groups (AM-NS, AM-S, CoG, CONTROL) were evaluated with an ANOVA test followed by paired post hoc tests corrected with a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli using PRISM 10 (GraphPad Software; San Diego, CA, USA). Differences were considered significant for a p and q-value of 0.05 or less.

The β-diversity was analysed using vegan and vegan3d packages (v 1.3-0) [51] in R. Sample β-diversity was visualised with a Bray–Curtis dissimilarity matrix-based non-parametric dimensional scaling (NMDS) model. Differences between groups for the sample clustering and β-dispersion were assessed with analysis of variance (Adonis2), using Bray–Curtis dissimilarity matrices (Adonis2) tests, and post hoc paired tests (pairwise adonis package v 0.4.1) [52] with p threshold of 0.05, using R studio.

The differential abundance analysis of the genus populations of the whole system was carried out using the Aldex function (unpaired test in ALDEX2 package v1.36.0) [53]. The Monte Carlo method used in Aldex involves generating random samples from the dataset and calculating the statistics of interest for each sample. By repeating this process, an empirical estimate of the null statistical distribution is obtained—that is, the distribution assuming no significant difference between the randomly generated samples. The p-values can then be calculated by comparing the observed statistics with this empirical null distribution.

A differential abundance analysis was performed with the Deseq2 package in R (v1.44.0) to highlight statistical differences in population abundance between pairs of groups.

Finally, the theoretical presence of BCAT (EC 2.6.1.42) and BCKDHc (E_1: EC 1.2.4.4–E_2: EC 2.3.1.168.–E_3: EC 1.8.1.4. for the three different subunits respectively) in bacterial profiles were investigated with Picrust2 tool [54]. This tool allows for theoretical metagenome function prediction based on 16S sequences. Orthologous sequences corresponding to these enzymes were identified using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (ver. 2024-11-20, http://www.kegg.jp/kegg/, accessed on 23 November 2024). The presence of these orthologous sequences in the genus populations from our dataset was identified from known genome content present in the database. The statistical analyses and

graphical representations of the number of pseudo-counts of each ortholog identified by groups of horses were performed using GraphPad Prism 10 (GraphPad Software; San Diego, CA, USA). The Kolmogorov-Smirnov test was used to assess the normality or lognormality of the data distribution, followed by a Kruskal-Wallis multiple comparisons test.

2.3. Correlation Between Faecal Microbiota and Blood Parameters

A blood sample was collected from each diseased horse upon admission to the clinic. Cograzers of these diseased horses were sampled in the field within 24 h after the first affected horse in the pasture showed clinical signs of AM. Blood samples were obtained via jugular venipuncture, aliquoted within one hour of collection, and stored at −80 °C until analysis. Both blood samples and faecal samples were collected simultaneously from each horse.

2.3.1. Quantification of Hypoglycin A, Methylenecyclopropylacetyl-Carnitine and Acylcarnitines

The quantification of HGA was performed on serum according to a previously described methodology using aTRAQ® kit (Sciex, Framingham, MA, USA) for amino acid analysis of physiological fluids [17]. Briefly, HGA was derivatised using an isotopic tag (mass m/z 121), while a second labelling reagent (mass m/z 113) allowed absolute quantification. The samples were derivatised and introduced into a TQ5500 tandem mass spectrometer (Sciex, Framingham, MA, USA) using a Prominence AR HPLC system (Shimadzu, Kyoto, Japan). The lower limit of quantification associated with this method is 0.090 µmol/L and a coefficient of variation below 8% [17].

An ultra-performance liquid chromatography combined with subsequent tandem mass spectrometry (UPLC-MS/MS) was used for MCPA-carnitine quantification with a limit of detection of approximately 0.001 nmol/L, as previously described [2].

Free carnitine and acylcarnitine profiling were quantified in serum by tandem mass spectrometry. Serum proteins were precipitated with a methanol solution with labelled internal standards. After evaporation with a nitrogen stream and derivatisation with butanolic-HCl, the samples were analysed with a TQ5500 mass spectrometer (Sciex, Framingham, MA, USA) [15,55].

2.3.2. Statistical Analysis of Group Parameters and Blood Markers Associated with Intoxication

The statistical analyses and graphical representations were performed using GraphPad Prism 10 (GraphPad Software; San Diego, CA, USA). The Kolmogorov-Smirnov test was employed to assess the assumption of normal or lognormal distribution: the data were normalised through Log10 transformation if needed.

A comparison of the means of the parameters "Age" and "Sex" and the blood concentration of HGA and MCPA-carnitine was conducted by comparing groups as previously described in the literature [16].

The acylcarnitines profiling was studied by comparing the averages between groups of horses and by focusing on the recently published cutoffs isovaleryl-/2-methylbutyrylcarnitine (i.e., C5 acylcarnitine) [13,15,16].

2.3.3. Statistical Analysis of the Correlation Between Faecal Microbiota and Blood Parameters

A Mantel test was performed to assess the correlation between faecal microbiota and blood parameters related to intoxication: HGA, MCPA-carnitine and a selection of acylcarnitines (i.e., C2, C4, C5, C10, C12:1, C14, C14:1, and C18:1) in serum, based on previous publications [15,16].

A multivariate analysis of the influence of selected acylcarnitines, HGA and MCPA-carnitine on microbiota distribution was performed using a distance-based redundancy analysis (dbRDA) with the vegan package in R software. This dbRDA was performed (1) to explore the interactions between the chemical variables (i.e., HGA, MCPA-carnitine and selected acylcarnitines) and the microbial composition of samples, (2) to identify the most important variables, (3) to visualise results, and (4) to statistically validate relationships between variables and sample composition. The significance of parameter influence on the dimensional model was assessed with ANOVA (vegan package). A p-value of 0.05 or less was considered statistically significant. The dbRDA model was illustrated with the ggord package in R (v1.1.8) [56].

3. Results

3.1. Horses

Changes in the classification of bacteria since 2021 justified a re-analysis of faecal samples from AM horses and CoG coming from Wimmer-Scherr and collaborators' study [35]: during this process, one animal was discarded due to lack of bacterial DNA in the faecal samples to allow further analysis.

A total of 36 horses were included: 13 horses in the AM-NS, 12 horses in the AM-S, 5 horses in the CoG, and 6 horses in the CONTROL group.

The final population included a mix of different breeds: Andalusians, Belgian Warmblood Horses, Belgian Draft Horses, Friesians, Haflingers, Hanoverians, Irish Cobs, Merens, Ponies, Quarter Horses, French saddlebreds, Trotters and Zangersheides. The age and sex distribution of each group are presented in Table 1.

Table 1. Demographic data of horses classified by group.

	CONTROL	CoG	AM-S	AM-NS	Diseased Horses
Number of horses	6	5	12	13	25
Age mean ± SD (Years)	13.3 ± 8.5	7.1 ± 7.5	6.5 ± 7.7	5.8 ± 4.7	6.1 ± 6.2
Age minimum (Years)	4.0	1.5	0.4	0.5	0.4
Age maximum (Years)	23	17	25	16	25
Age CI [LLCI-ULCI]	[6.5–20.1]	[0.5–13.7]	[2.1–10.9]	[3.3–8.3]	[3.7–8.5]
Ratio of					
entire male	33%	40%	33%	15%	24%
gelding	33%	20%	33%	23%	48%
female	33%	40%	33%	62%	28%

SD = Standard deviation, CI = Confidence interval, LLCI = Lower Limit of Confidence Interval, ULCI = Upper Limit of Confidence Interval. The groups represented are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy, as well as all diseased horses (AM-S + AM-NS).

3.2. Comparison of Faecal Microbiota Between Groups

3.2.1. Composition of Faecal Microbiota

Starting with 6,099,718 raw reads, 3,649,756 reads were kept after read cleaning and chimera removal. We further proceeded with 10,000 reads per sample to the taxonomic identification, leading to a table of 184,346 OTUs.

Among the 21 phyla identified in the faeces, the 4 most abundant defined phyla were Firmicutes, Bacteroidota, Verrucomicrobiota and Fibrobacterota. A total of 206 families and 440 genera were identified. The dominant bacterial populations for each group and individual are detailed in Appendix A. Figures show results by group and for each individual in terms of phyla (Figure A1), family (Figure A2) and genera (Figure A3). The determination of α- and β-diversities of the faecal bacterial populations were assessed at the genus level.

3.2.2. α-Diversity Analysis

A test of normality was performed, and a normal distribution of data was indicated. Consequently, an ANOVA and a paired test were performed ($p = 0.05$). The outcomes of Benjamini, Krieger and Yekutieli with a false discovery rate (FDR) ($q = 0.05$) indicated significant disparities among the groups concerning bacterial α-diversity (reciprocal Simpson Index) and evenness (i.e., distribution of abundances of the groups—Simpson Evenness index), though not for richness (i.e., number of taxonomic groups—chao1 richness index) (Figure 1).

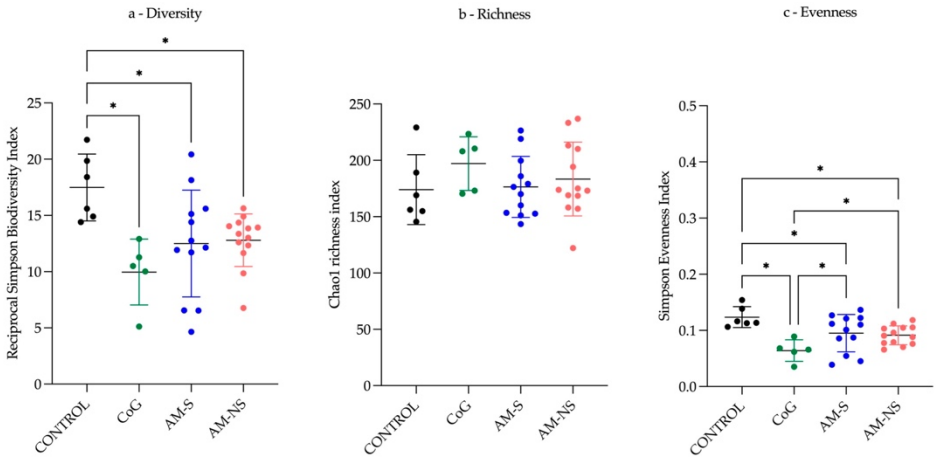

Figure 1. Representation of bacterial intrinsic diversity deduced from inverse Simpson index ((**a**)—Diversity), bacterial genus richness deduced from Chao1 index ((**b**)—Richness) and bacterial genus evenness deduced from Simpson index ((**c**)—Evenness). Data are scatter dot plots at the genus level for individual horses in the defined groups (control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) among atypical myopathy cases), with the mean and the standard deviation. The two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli reveals significant differences with a q-value of 0.05 or less: * < 0.05.

Pairwise comparisons showed that α-diversity was significantly higher in CONTROL horses than in CoG ($q < 0.01$), in AM-S ($q < 0.01$), and in AM-NS ($q < 0.05$). Similarly, genus evenness was found to be (1) significantly higher in the CONTROL group vs. CoG ($q < 0.001$), AM-S ($q < 0.05$), and AM-NS ($q < 0.05$) and (2) significantly lower in the CoG group vs. AM-S ($q < 0.05$) and AM-NS ($q < 0.05$) (Table 2).

Table 2. α-diversity: p-value for pairwise comparisons and q-value for the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli.

DIVERSITY			***p*-Value**	***q*-Value**
CONTROL	**vs.**	**CoG**	**	**
CONTROL	**vs.**	**AM-S**	**	**
CONTROL	**vs.**	**AM-NS**	*	*
CoG	vs.	AM-S	nsig	nsig
CoG	vs.	AM-NS	nsig	nsig
AM-S	vs.	AM-NS	nsig	nsig

Table 2. *Cont.*

EVENNESS			*p*-Value	*q*-Value
CONTROL	**vs.**	**CoG**	***	***
CONTROL	**vs.**	**AM-S**	*	*
CONTROL	**vs.**	**AM-NS**	*	*
CoG	**vs.**	**AM-S**	*	*
CoG	**vs.**	**AM-NS**	*	*
AM-S	vs.	AM-NS	nsig	nsig

Significantly different with a *p*- or *q*-value of 0.05 or less: * < 0.05; ** < 0.01; *** < 0.001, nsig: Not significant. The defined groups are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy.

3.2.3. β-Diversity Analysis

β-diversity of the faecal microbial profile was visualised using a Bray-Curtix matrix-based NMDS model (k = 3, stress = 0.082) (Figure 2). Sample clustering showed that bacterial profiles were not homogenous between groups (p = 0.001). Results from paired tests (p = 0.05) showed that the microbial profile of the CONTROL group was different from the other groups, and CoG was different from AM-NS, as shown in Table 3.

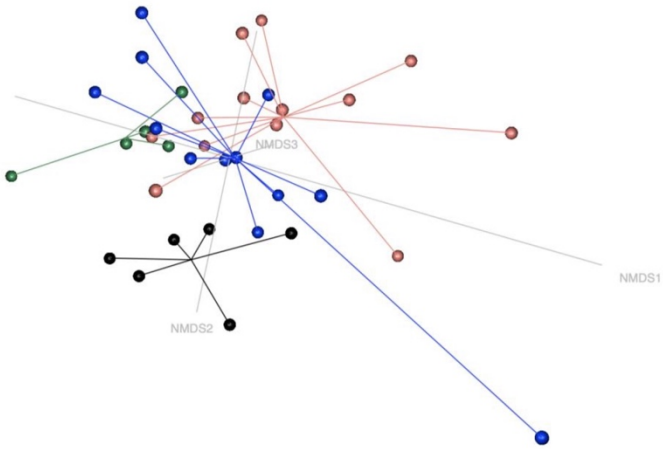

Figure 2. Nonmetric multidimensional scaling model (NMDS) plots in three dimensions of the horse's faecal microbiota. Black symbols represent control horses (CONTROL), green symbols represent cograzers (CoG), blue symbols represent survivors of atypical myopathy (AM-S), and red symbols represent non-survivors (AM-NS). The model stress is 0.082.

Table 3. β-diversity: *p*-value adjusted for pairwise comparisons.

Pairs			*p*-Value Adjusted
CONTROL	**vs.**	**CoG**	*
CONTROL	**vs.**	**AM-S**	*
CONTROL	**vs.**	**AM-NS**	**
CoG	vs.	AM-S	nsig
CoG	**vs.**	**AM-NS**	*
AM-S	vs.	AM-NS	nsig

Significantly different with a *p*-value of 0.05 or less: * < 0.05; ** < 0.01; nsig: Not significant. The defined groups are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy.

3.2.4. Differences in Global Faecal Microbiota Composition

The Aldex function revealed six significantly different genera in the samples studied (without the notion of group), compared with the null statistical distribution empirically determined by this aldex function: *Clostridia_ge*, *Bacteria_ge*, *Firmicutes_ge*, *Phascolarctobacterium*, *Fibrobacter* and *NK4A214_group* (Table 4).

Table 4. Aldex function: adjusted *p*-value of selected genera.

Genera	*p*-Value Adjusted
Clostridia_ge	**
Bacteria_ge	**
Firmicutes_ge	**
Phascolarctobacterium	*
Fibrobacter	*
NK4A214_group	*

Significantly different with a *p*-value of 0.05 or less: * < 0.05; ** < 0.01.

3.2.5. Differences in Faecal Microbiota Composition Between Groups

At a genus level, the statistically significant differences in population abundance between the CONTROL group and the three other groups (CoG, AM-S and AM-NS, respectively) are represented in Table 5. From a total of 34 genera, 4 genera were statistically different between CONTROL and CoG, 12 genera were statistically different between CONTROL and AM-S, and 32 genera were statistically different between CONTROL and AM-NS. These genera are graphically represented in Figure 3.

The 4 genera statistically different between CONTROL and CoG were also different between CONTROL and AM-S and AM-NS. Interestingly, the relative abundance of these four genera was significantly lower in CONTROL vs. CoG, AM-S and AM-NS. These genera are *Firmicutes_ge*, *Clostridia_ge*, *Bacteria_ge* and *Oligosphaeraceae_ge*. Three of them (i.e., *Firmicutes_ge*, *Clostridia_ge* and *Bacteria_ge*) were also highlighted by the Aldex function. The genera *NK4A214_group* and *Fibrobacter*—also revealed by the Aldex function—presented a significantly lower and higher relative abundance between CONTROL and AM-NS, respectively.

Table 5. Adjusted *p*-value corresponding to comparisons between groups.

Adjusted *p*-Value	CONTROL vs. CoG	CONTROL vs. AM-S	CONTROL vs. AM-NS
Firmicutes_ge	****	****	****
Clostridia_ge	****	****	****
Bacteria_ge	**	****	***
Oligosphaeraceae_ge	*	***	**
Lachnospirales_ge		**	*
Bacilli_ge		**	**
Oscillospirales_ge		**	**
RF39_ge		*	
Catenibacillus		*	*
Treponema		*	*
Anaerovoracaceae_ge		*	
Anaeroplasma		*	**
Streptococcus			***
Bradymonadales_ge			***
Candidatus_Soleaferrea			***

Table 5. *Cont.*

Adjusted *p*-Value	CONTROL vs. CoG	CONTROL vs. AM-S	CONTROL vs. AM-NS
Oscillospiraceae_ge			**
Candidatus_Saccharimonas			**
Prevotellaceae_UCG.001			**
Akkermansia			**
Clostridiaceae_ge			**
NK4A214_group			**
Christensenellaceae_R.7_group			*
Prevotellaceae_UCG.003			*
Gastranaerophilales_ge			*
Campylobacter			*
Selenomonadaceae_ge			*
Prevotella			*
Bacteroidales_ge			*
Endomicrobium			*
COB_P4.1_termite_group_ge			*
Verrucomicrobiota_ge			*
Fibrobacter			*
Muribaculaceae_ge			*
Bacteroides			*

Significantly different with a *p*-value of 0.05 or less: * < 0.05; ** < 0.01; *** < 0.001, **** < 0.0001. The defined groups are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy. The genera that were also highlighted by the Aldex function are underlined.

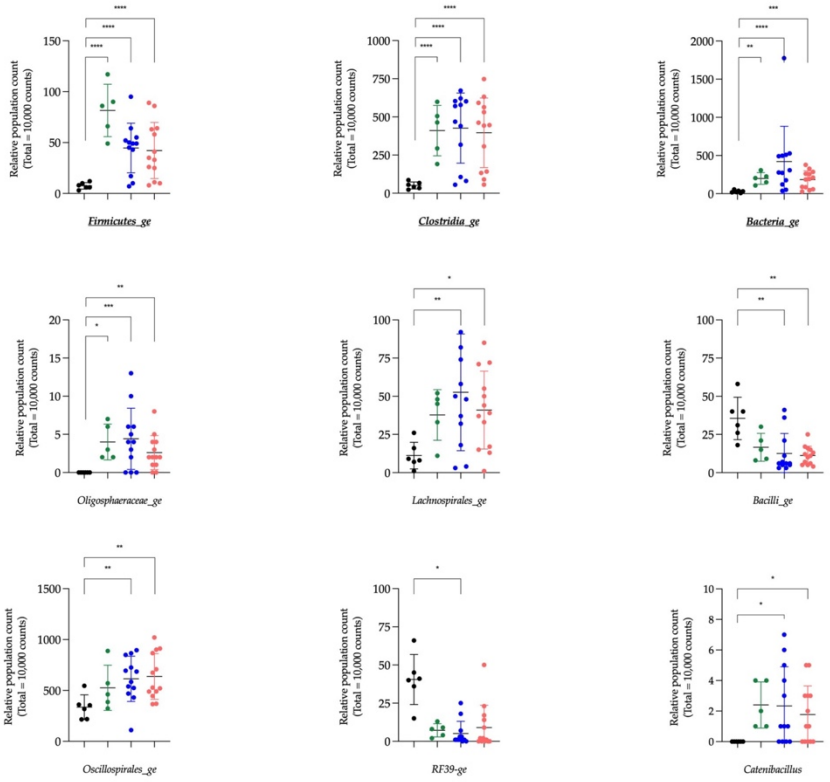

Figure 3. *Cont.*

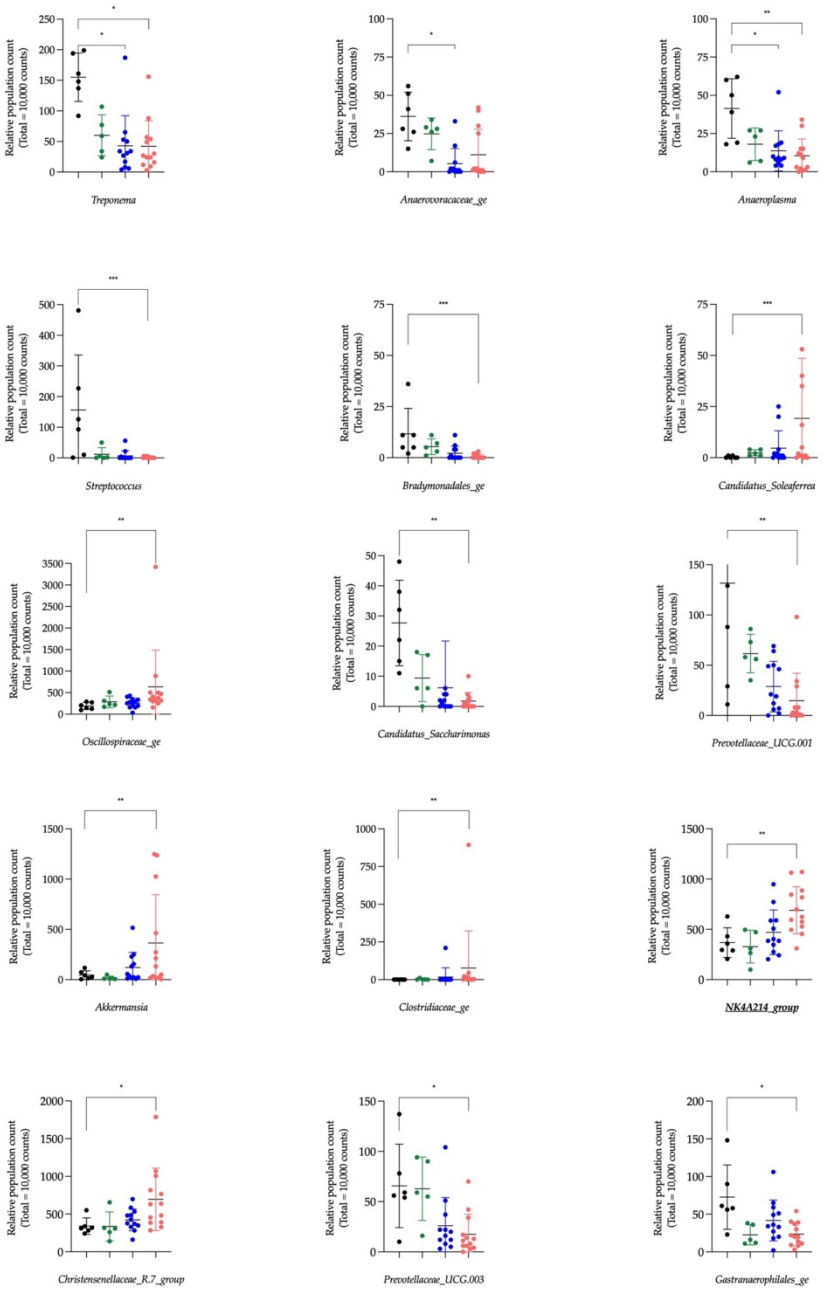

Figure 3. *Cont.*

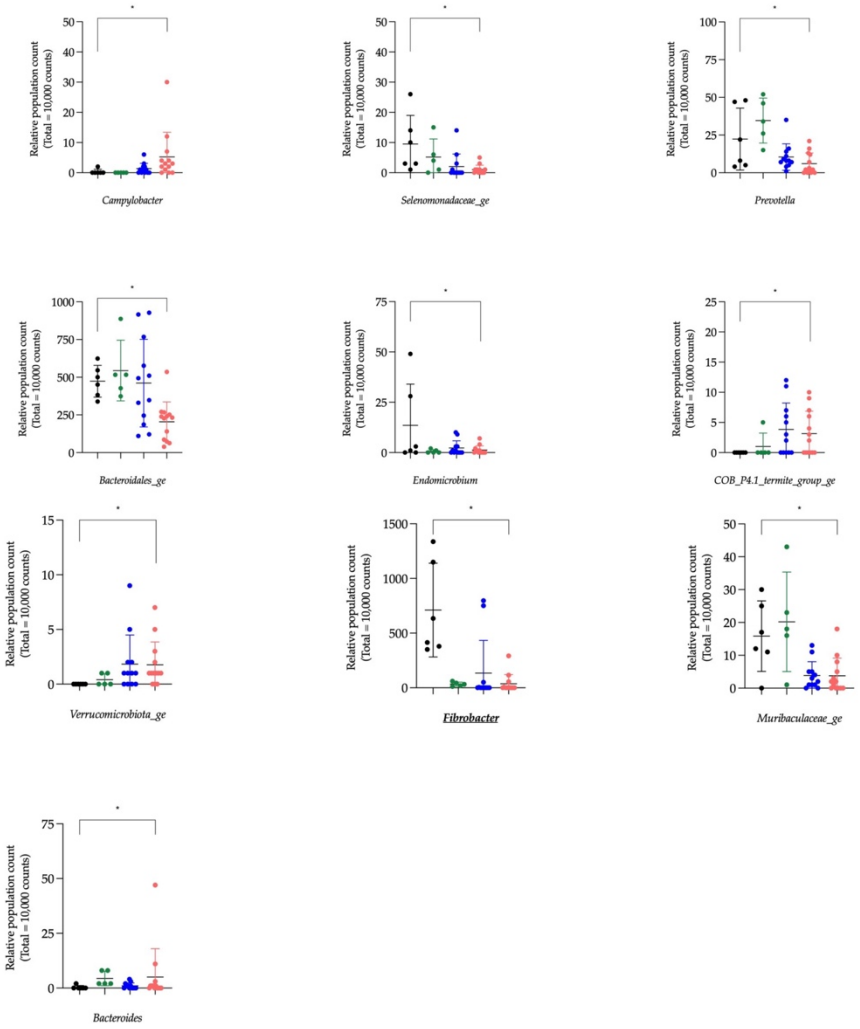

Figure 3. Graphical representation of relative population count (mean and standard deviation, total of 10,000 counts). Data are scatter dot plots at the genus level for individual horses in the defined groups, with black dots for control horses (CONTROL), green dots for cograzers (CoG), blue dots for survivors (AM-S) and red dots for non-survivors (AM-NS) of atypical myopathy. Genus names in bold and underlined were also highlighted by the Aldex function. Significantly different with a *p*-value of 0.05 or less: * < 0.05; ** < 0.01; *** < 0.001, **** < 0.0001.

3.2.6. Enzymes of the Kyoto Encyclopaedia of Genes and Genomes Orthologous Pseudo-Counts Analysis

The KEGG orthologous for BCAT (EC 2.6.1.42) is K00826. For the BCKDHc, the subunit E1 presents three KEGG orthologous: K00166 for 2-oxoisovalerate dehydrogenase E1 component subunit alpha, K00167 for 2-oxoisovalerate dehydrogenase E1 component subunit beta and, K11381 for 2-oxoisovalerate dehydrogenase E1 component. The KEGG orthologous for subunits E2 and E3 are K09699 and K00382, respectively.

Using the Picrust2 tool, the repartition of pseudo-counts of each KEGG Orthology number (KO) by groups of horses is represented in Figure 4. As observed and expected, the Kruskal-Wallis analysis of the different pseudo-counts of each K0 did not reveal any statistical differences between groups of horses (i.e., CONTROL, CoG, AM-S and AM-NS).

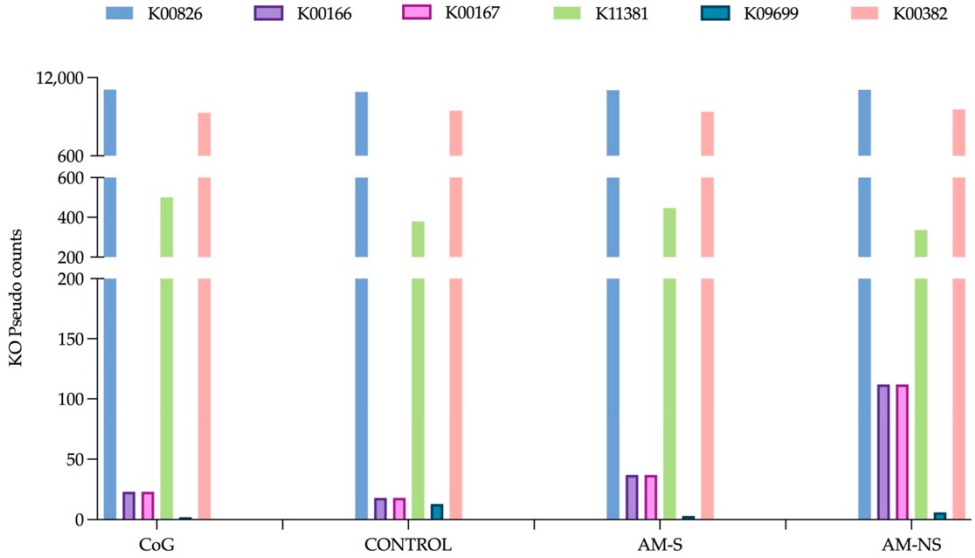

Figure 4. Graphical representation of each KO pseudo-counts in the defined groups: control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy.

The general analysis of the presence of KO in each OTU indicated that (1) K00826 (i.e., BCAT) was mainly represented in 99.18% of total OTUs, (2) K00382 (i.e., BCKDHc—E3) was represented in 70.13% of total OTUs and, (3) BCKDHc E1 and E2 subunits were the main limiting subunits. The latter were poorly represented within the entire 16S rDNA (0.35%, 0.35%, 3.28% and 0.08% for K00166, K00167, K11381 and K09699, respectively), with only four OTUs possessing all the enzymes, and these same four OTUs were the only ones to possess the entire E1 and E2 subunits. Moreover, the relative abundance of these four OTUs was low. The identification of the genus of these four OTUs was *Alphaproteobacteria_ge*, *Rhizobiaceae_ge*, *Sphingomonas*, and *Sphingomonadaceae_ge*.

Among the bacterial genera highlighted by Aldex and/or Deseq2 functions, the mostly represented are K00826 (i.e., BCAT) and K00382 (i.e., BCKDHc—E3), which agrees with the general analysis (Table 6). Moreover, many of the genera analysed only presented these two orthologs. *Phascolarctobacterium* did not present any pseudo-count for any KO number like some OTUs from some genera (i.e., *Anaerovoracaceae_ge*, *Bacilli_ge*, *Bacteria_ge*, *Bacteroidales_ge*, *Christensenellaceae_R.7_group*, *Clostridia_ge*, *Firmicutes_ge*, *Lachnospirales_ge*, *Oscillospirales_ge*). None of the genera identified possessed all the orthologs. Nevertheless, some OTUs from the genera *Bacilli_ge* and *Bacteria_ge* presented 5/6 orthologous sequences with the same missing K11381 (i.e., 2-oxoisovalerate dehydrogenase E1 component). Some OTUs from the genera *Akkermansia*, *Bacteria_ge* and *Verrucomicrobiota_ge* owned 4/6 orthologous sequences, with K11381 and K09699 missing (i.e., BCKDHc E2 subunit). Finally, one OTU from *Verrucomicrobiota_ge* and *Muribaculaceae_ge*, three OTUs from *Prevotella*, and several OTUs from *Bacteria_ge*, *Bacteroidales_ge*, *Bacteroides*, *Christensenellaceae_R.7_group*, *Clostridia_ge*, *COB_P4.1_termite_group_ge*, *Firmicutes_ge*, *NK4A214_group*, *Oscillospiraceae_ge*,

Oscillospirales_ge, *Prevotellaceae_UCG-001*, *Prevotellaceae_UCG-003* presented the same 3/6 orthologous sequences (i.e., K00826, K11381 and, K00382).

Table 6. The presence of orthologous sequences in bacterial genera is of interest.

Orthologs Number KEGG	K00826	K00166	K00167	K11381	K09699	K00382
Akkermansia	✔ ✔	✔	✔			✔ ✔
Anaeroplasma	✔					✔ ✔
Anaerovoracaceae_ge	✔					✔
Bacilli_ge	✔ ✔	✔	✔		✔	✔ ✔
Bacteria_ge	✔ ✔ ✔ ✔ ✔	✔ ✔	✔ ✔	✔	✔	✔ ✔ ✔ ✔
Bacteroidales_ge	✔ ✔ ✔			✔		✔ ✔
Bacteroides	✔ ✔			✔		✔ ✔
Bradymonadales_ge	✔					✔
Campylobacter	✔					
Candidatus_Saccharimonas	✔					✔
Candidatus_Soleaferrea	✔ ✔					✔
Catenibacillus	✔ ✔					✔
Christensenellaceae_R.7_group	✔ ✔ ✔			✔		✔ ✔
Clostridia_ge	✔ ✔ ✔			✔		✔ ✔ ✔
Clostridiaceae_ge	✔ ✔					✔
COB_P4.1_termite_group_ge	✔ ✔			✔		✔ ✔
Endomicrobium	✔					✔
Fibrobacter	✔ ✔					✔

Table 6. *Cont.*

Orthologs Number KEGG	K00826	K00166	K00167	K11381	K09699	K00382
Firmicutes_ge	✔ ✔ ✔			✔		✔ ✔
Gastranaerophilales_ge	✔					✔
Lachnospirales_ge	✔ ✔					✔
Muribaculaceae_ge	✔ ✔			✔		✔ ✔
NK4A214_group	✔ ✔ ✔			✔		✔ ✔
Oligosphaeraceae_ge	✔					✔
Oscillospiraceae_ge	✔ ✔ ✔			✔		✔ ✔
Oscillospirales_ge	✔ ✔ ✔			✔		✔ ✔
Phascolarctobacterium						
Prevotella	✔ ✔ ✔			✔		✔ ✔
Prevotellaceae_UCG-001	✔ ✔ ✔			✔		✔ ✔
Prevotellaceae_UCG-003	✔ ✔ ✔			✔		✔ ✔
RF39_ge	✔					✔
Selenomonadaceae_ge	✔ ✔					✔
Streptococcus	✔					✔
Treponema	✔ ✔					✔
Verrucomicrobiota_ge	✔ ✔ ✔	✔	✔	✔		✔ ✔ ✔

Unfilled rows within a bacterial genus represent one or more OTUs of this genus that do not possess any of the orthologues.

3.3. Correlation Between Faecal Microbiota and Blood Parameters

3.3.1. Group Parameters and Blood Markers Associated with Intoxication

The average blood concentrations of HGA, MCPA-carnitine and selected acylcarnitines, as well as their standard deviation for each group, are referenced in Tables A1 and A2.

Statistical comparison of the "Age" parameter between (1) CoG vs. diseased horses, (2) AM-S vs. AM-NS, (3) CONTROL vs. CoG and (4) CONTROL vs. diseased horses did not reveal any significant difference in mean age (Mann-Whitney test, $p \geq 0.05$) or in the age distribution (Kolmogorov-Smirnov Z test, $p \geq 0.05$).

Fisher's exact test used to study contingency about parameter "Sex" and parameter "Health Status" or "Final Outcome of the disease horses" did not reveal any significant difference ($p \geq 0.05$) for both comparisons.

The mean serum concentration of HGA was significantly lower in CoG vs. diseased horses (unpaired *t*-test, $p < 0.001$), but the difference was not significant for the comparison AM-S vs. AM-NS (unpaired *t*-test, $p \geq 0.05$) (Figure A4).

When comparing the mean serum concentration of MCPA-carnitine, diseased horses presented values significantly higher than CoG (unpaired *t*-test, $p < 0.0001$). Similarly, the mean serum concentration of MCPA-carnitine was significantly higher in AM-NS vs. AM-S (unpaired *t*-test, $p < 0.05$) (Figure A4).

The mean serum concentration of selected acylcarnitines in diseased horses was found to exceed the 99th percentile of the CONTROL group values. Additionally, the C5-carnitine concentration of each horse was analysed, according to what's been proposed by Renaud et al. (2024), to classify horses into the appropriate groups. A cutoff value of 3.04 µmol/L of C5 was described: above this cutoff, 92% of horses are diseased horses, and below this cutoff, 97% of horses are CoG. A second C5 cutoff is described with a value of 12.21 µmol/L: above this cutoff, 76% of diseased horses are likely to die, and below this cutoff, 81% of diseased horses are likely to survive [16]. In this study, only one AM-S horse (i.e., AM-S 04) presented a suspect profiling with a concentration of C5 below the cutoff of 3.04 µmol/L of clinically affected AM cases. Three AM-affected horses survived despite a poor prognosis based on C5 (Figure 5), and four diseased horses died while having a C5 level, suggestive of a positive outcome.

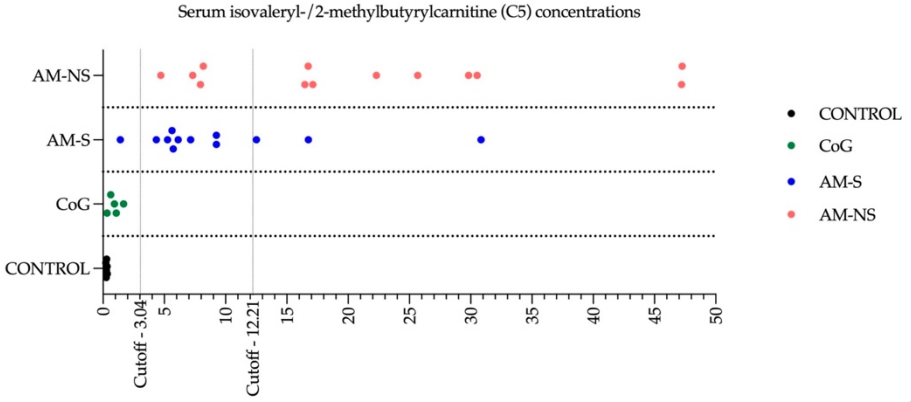

Figure 5. Serum isovaleryl-/2-methylbutyrylcarnitine (C5) concentrations. The groups are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy.

3.3.2. Correlation Between Faecal Microbiota and Serum Concentration of Hypoglycin A, Methylenecyclopropylacetyl-Carnitine and Acylcarnitines

The Mantel test revealed a significant correlation (r = 0.2351, $p = 0.031$) between the matrix of microbiota and the matrix of blood parameters, suggesting that this correlation is unlikely due to chance.

A first dbRDA model was performed: the most important variables identified were MCPA-carnitine ($p = 0.004$), C2 ($p = 0.089$), C10 ($p = 0.099$) and C14:1 ($p = 0.079$).

A series of dbRDA models were further built iteratively by sequentially removing the significant chemical variables identified previously. This allowed us to select the most important variables to explain the variation in microbial composition between groups:

these variables were MCPA-carnitine ($p < 0.01$) and C14:1 ($p < 0.01$), resulting in a final dbRDA model with two constrained dimensions (Figure 6).

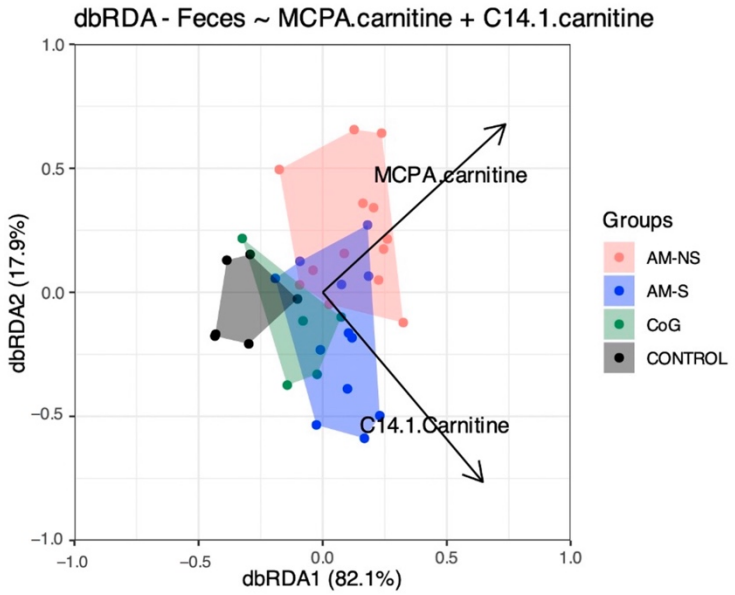

Figure 6. Illustration of the dbRDA model. The horizontal dbRDA1 axis explains 82.1%, and the vertical dbRDA2 axis explains 17.9% of the repartition of the different groups: control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy.

4. Discussion

The present study reveals that (1) faecal microbiota differs between CONTROL horses (i.e., toxin-free horses) and horses suffering from both subclinical (CoG) and clinical (AM-S and AM-NS) *Acer pseudoplatanus* intoxication and (2) the blood concentrations of MCPA-carnitine and C14:1 (i.e., tetradecenoylcarnitine, a long-chain acylcarnitine) significantly correlate with the variation in faecal microbial composition observed between the different groups of horses.

Microbiota statistical analyses were performed at the genus level to detect differences at the highest possible taxonomic resolution. This approach allows for a more accurate identification of the microbial populations influencing the system under investigation using the most up-to-date bacterial taxonomy. Unfortunately, recent updates in bacterial taxonomy since the publication of Wimmer-Scherr et al. (2021) have complicated a direct comparison of the results between the two studies [35]. Nevertheless, the findings of this new study will facilitate future comparisons with other research.

Regarding α-diversity, the CONTROL group presents the highest α-diversity and exhibits a more uniform distribution of populations compared to the three other groups, with an evenness index closer to one. The CoG group presents a lower α-diversity and an evenness index close to zero, indicating the emergence of certain populations more abundant than others. "Dysbiosis" is defined as the loss of central mutualistic relationship among microbiota members, metabolic products, and the host immune system [57]. Consequently, it is not possible to characterise the observed changes as dysbiosis only based on α-diversity indicators.

The Aldex function identified six genera having a significant impact in the global system (i.e., *Clostridia_ge*, *Bacteria_ge*, *Firmicutes_ge*, *Phascolarctobacterium*, *Fibrobacter* and *NK4A214_group*) compared to the null statistical distribution empirically determined by this function. The paired tests (Deseq2) compared bacterial genera between the CONTROL group and the three other groups and revealed 34 significantly different genera. Among these bacterial genera, five were also highlighted by the Aldex function. Indeed, *Clostridia_ge*, *Bacteria_ge*, and *Firmicutes_ge* presented a significantly lower relative abundance in the CONTROL group compared to CoG, AM-S and AM-NS groups, and *NK4A214_group* and *Fibrobacter* in the CONTROL group exhibit a significantly lower and higher relative abundance, respectively, compared to AM-NS horses.

The genera *Clostridia_ge*, *Bacteria_ge*, and *Firmicutes_ge* are composed of bacteria with features belonging to the *Clostridia* class, Bacteria kingdom or Firmicutes phyla, respectively, without the possibility of further characterisation. The genus *Fibrobacter* is a major and highly specialised cellulolytic bacterial genus and is detected in the intestinal tract of several herbivorous animals, including horses [58,59]. Cellulose is not digested and not absorbed in the mammal's gut, so cellulolytic bacteria play a vital role by providing energy to the host via the metabolism of cellulose into short-chain fatty acids (SCFAs). The SCFAs have beneficial effects on colonocytes, intestinal membrane integrity and local intestinal immunity [60,61]. The toxic metabolites of protoxins (i.e., MCPA-CoA and MCPF-CoA) inhibit the β-oxidation of fatty acids with subsequent decreased mitochondrial respiration and uncoupled phosphorylation [5,12,13]. Therefore, lipids can no longer be used as an energy substrate, but the glycolytic pathway is preserved [14,62,63]. Consequently, it is generally recommended to give a complete mix of grains or glucose in another form to horses affected by AM [64]. Although the appetite of diseased horses is preserved, the clinical picture (weakness, recumbency, depression, stiffness, etc. [1,36,65]) can make an inadequate ingestion of food. It is hypothesised that the inhibition of β-oxidation by toxic metabolites alters the overall energy metabolism of the host and the intestinal environment, potentially leading to conditions that are less favourable for the growth and activity of cellulolytic bacteria, thus explaining the observed decrease in the relative abundance of this genus from CONTROL horses to intoxicated horses (from CoG to AM-NS).

The genus *NK4A214_group* is associated with the degradation of structural carbohydrates, such as cellulose and hemicellulose, other less complex polysaccharide substrates and the production of butyrate (i.e., SCFAs) [66,67]. The relative increase in this genus from CONTROL horses to intoxicated horses, as previously reported [35], could be linked to the preservation of the glycolytic pathway and its ability to deal with less complex polysaccharides, as mentioned above. This preservation may provide a more favourable environment for the *NK4A214_group* by maintaining substrate availability for carbohydrate fermentation despite the metabolic disruptions caused by toxic exposure.

Other clearly identified genera, such as *Akkermansia*, *Bacteroides*, *Prevotella*, and *Treponema*, might be considered interesting in the context of AM. The genus *Akkermansia* attracts the attention of scientists for its anti-inflammatory and anti-obesity effects in mice and men by reducing insulin resistance, glucose intolerance, and gut permeability. One of the distinguishing features of *Akkermansia* is its ability to (1) promote the renewal and thickening of the mucin layer, which reduces intestinal permeability, and (2) degrade intestinal mucin glycoproteins and to use them as a source of carbon and nitrogen when dietary fibres are defective; this process leads to an increase of the intestinal permeability and to the production of SCFAs [68–72]. This ability to use mucin as a source of carbon and nitrogen may give *Akkermansia* an advantage when facing disruptions in host energy metabolism in the context of AM, despite the potential repercussions on intestinal microbiota. On the other hand, the resulting increase in intestinal permeability represents a disadvantage,

as this could increase the absorption of HGA and other toxins present in the digestive tract and into the bloodstream. Lastly, the resulting production of SCFAs—which account for 65% of the horse's energy production [73,74]—cannot be used as an energy source by the horse. Indeed, in the case of AM, the muscle cannot use them effectively as energy substrates following the inhibition of acyl-CoA dehydrogenases [5,12,75–77].

The genus *Bacteroides*, which increases in AM-NS compared to CONTROL horses, is known for its ability to metabolise polysaccharides and oligosaccharides such as starch [78,79], which could make sense in the framework of the modification of the metabolic pathways available in horses affected by AM. This genus has interesting features to thrive in the gut as (1) complex systems to sense and adapt to available nutrients, (2) systems to get rid of toxic substances, and (3) the ability to control the environment by interacting with the host's immune system, which is an opportunity to control of other pathogens considered as competitors by *Bacteroides* [78]. Moreover, the relative abundance of *Prevotella* is inversely correlated with that of the *Bacteroides* in human gut microbiota [80], and this is what is observed in the faecal microbiota analysis between CONTROL and AM-NS horses.

The genus *Treponema* is associated with the degradation of structural carbohydrates and correlates positively with different dietary fibres in pigs and horses [66,81]. In this study, *Treponema* was present in faecal microbiota in CONTROL horses and significantly decreased in AM-NS, which could also be explained by the modification of energetic metabolism in AM horses.

The explanations provided remain hypothetical. At this stage, it is not possible to draw definitive conclusions about the role of these bacterial populations in the context of AM based solely on relative abundance. By definition, the analysis of relative abundance reveals populations that are increasing and others that are decreasing relative to each other. This phenomenon is illustrated by the inverse correlation observed between *Prevotella* and *Bacteroides* in humans [80]. This illustration of the ecological niche can also explain variations in populations of genera like *Campylobacter*, *Endomicrobium*, and *Streptococcus*. It is important to remain cautious when interpreting increases or decreases in bacterial populations expressed in relative abundance values: only an absolute quantification of these populations would make it possible to truly quantify the increases and decreases (for example, via RT-PCR). Furthermore, the bacteria present in the gut microbiota are also influenced by the host's metabolism. As a result, observations made in vivo, at the level of the faeces, may not necessarily reflect changes in the microbiota itself following exposure to the protoxins studied. Finally, it is possible that bacteria identified as significantly different between groups are involved in host-related metabolic functions rather than in the direct metabolism of HGA. The discovery of the subclinical character of metabolic processes in horses that have ingested toxins involved in AM [16] suggests that the gut microbiota also has time to modify before the appearance of the clinical phase, which remains acute.

The enzymes responsible for HGA metabolism (i.e., BCAT and BCKDHc) are involved in the catabolism of BCAAs in bacteria [82], leading to the hypothesis that bacteria can transform HGA into toxic metabolic compounds. In mammals, the BCAT exists in two isoenzymes: a mitochondrial (BCATm) ubiquitously present (mainly in skeletal muscle, brain, kidney, and intestine in humans), and a cytosolic (BCATc) isoenzyme, mainly presents in the brain in humans and rats, and in ovary and placenta in rats [83–90]. In contrast to the mitochondrial and cytosolic isoenzymes found in higher eukaryotes, a single form of BCAT is ubiquitously expressed in bacteria. Moreover, this BCAT is involved in the final step of anabolism and in the first step of catabolism of BCAAs, which explains that many bacterial species possess this BCAT. Finally, bacterial BCAT is also distinguished from eukaryotes by broad substrate specificity [91–93]. The mammals BCKDHc is a mul-

tienzyme complex of three separate subunits located on the inner surface of the inner mitochondrial membrane [89,94]. The bacterial BCKDHc seems to mainly consist of three subunits, as described for mammalian species [82,95] and is also found in several bacterial species [25,27,28,31,96,97]. Among the bacterial genera highlighted by Aldex function and/or Deseq2 analysis, some of them have in their known genome content one or more copies of the orthologous sequences encoding for the BCAT and/or subunits of BCKDHc (i.e., enzymes involved in the metabolisation of HGA). However, no bacterial genus appears to possess all the expected enzymes, and consequently, the question of the ability of these genera to completely transform HGA in MCPA-linked to carnitine or glycine arises. Interestingly, (1) a pattern of distribution can be observed with the same ortholog missing in groups of genera owning 5/6, 4/6 or 3/6 orthologous sequences and (2) if we compare the pattern of distribution of genera identified in the scientific literature as having the BCKDHc (i.e., *Bacillus*, *Pseudomonas*, *Staphylococcus* [24–26,28–31,96], this pattern is exactly the same as the pattern encountered for the genera having 5/6 orthologous sequences with only the K11381 missing (i.e., 2-oxoisovalerate dehydrogenase E1 component). Unfortunately, these OTUs presented this interesting pattern belonging to the non-clearly identified genera *Bacilli_ge* and *Bacteria_ge*. Indeed, the identification of these OTUs is stopped at the *Bacilli* class and Bacteria kingdom, respectively, preventing further possible characterisation and explanations. Nevertheless, *Bacteria_ge* was also highlighted by Aldex function (i.e., having a significant impact on the global system) and Deseq2 function (i.e., being significantly higher in CoG, AM-S and AM-NS horses vs. CONTROL horses). It will be valuable to investigate these specific OTUs from *Bacteria_ge* and compare them across groups to determine whether these specific OTUs are significantly different. The *Bacilli_ge* was highlighted by the Deseq2 function and was significantly different between CONTROL and AM-S and AM-NS but in a decreasing way from CONTROL to AM-NS: the opposite of the *Bacteria_ge*. This last fact seems to be contradictory (i.e., having the machinery to metabolise HGA but presenting an opposite evolution between the groups). However, some bacteria might possess analogous (non-orthologous) enzymes capable of performing similar functions.

The "Age" parameter did not seem significant in the horse population of the present study, contrary to recent findings in the scientific literature, which reported that diseased horses were either younger than 2 years or older than 10 years, while 90% of CoG were between 2 and 10 years of age [16]. In our study, there was an age overlap between groups. Regarding the "Sex" parameter, Renaud and collaborators found that, among horses exposed to the protoxins, geldings were less susceptible to developing AM compared to intact males and females [16]. However, in our study, neither "Sex" nor the parameters "Health Status" or "Final Outcome" were significantly associated. This may be explained by the lower number of individuals in our study as compared to the above-mentioned one.

As found previously [16], the mean serum concentrations of HGA and MCPA-carnitine were significantly different when comparing CoG and diseased horses. Among the latter, only MCPA-carnitine could differentiate AM-S from AM-NS. Interestingly, MCPA-carnitine was identified as one of the most important blood variables to explain the variation in microbial composition between groups and seemed to correlate better with AM-NS vs. AM-S.

Acylcarnitines are esters formed by the conjugation of acyl groups (notably fatty acids) with carnitine. They are typically divided into four groups: short-chain (C2–C5), medium-chain (C6–C12), long-chain (C13–C20) and very-long-chain (>C21) acylcarnitines. The primary biological function of acylcarnitines is to facilitate the transport of acyl groups from the cytosol into the mitochondrial matrix for β-oxidation, which in turn contributes to cellular energy production. Atypical myopathy is characterised by a severe alteration of the serum acylcarnitines profile with an increase in nearly all acylcarnitines, regardless of their chain length [4,11,13–16,63,98,99]. In the present study, acylcarnitines profiling revealed

significant changes comparable to those previously described in the literature. Moreover, C5-carnitine has been highlighted as the best candidate for helping in both the diagnosis and prognosis of the disease. According to the model described by Renaud et al. (2024), a serum concentration of C5-carnitine lower than 3.04 µmol/L would make it possible to identify a diseased horse vs. a cograzer in more than 90% of cases. A second threshold at 12.21 µmol/L of C5-carnitine would identify an animal likely to die (i.e., negative predictive value) in 76% of cases and a surviving animal (i.e., positive predictive value) in 81% of cases [16]. In this study, the analysis of the C5 concentration identified one horse in the AM-S group (i.e., AM-S 04) with a profile similar to that of a CoG, as well as four other cases with prognostic survival estimates that differed from the real outcome: nevertheless, these results are consistent with the percentages announced in the literature. Of the four diseased horses that ultimately died despite having C5 levels indicative of a positive outcome, it should be noted that 3 of them were euthanised following the worsening of clinical signs despite the intensive treatment put in place, and the last was also euthanised, but the reason (i.e., medical or financial constraints) was not clearly identified. Moreover, to the authors' knowledge, none of the CoG developed symptoms of AM during the sampling season, supporting the results obtained from the analysis of these horses' blood concentration of C5 carnitine. Rapid access to C5 assay results remains a significant challenge, preventing clinicians from promptly updating prognoses in cases characterised by severe clinical signs.

Lastly, the C14:1 was identified as one important blood variable to explain the variation in faecal microbiota between groups. The elevated C14:1 level in neonates may indicate a very-long-chain acyl-coenzyme A dehydrogenase deficiency known under the following abbreviation VLCADD, an autosomal recessive disease [100]. In horses, AM is recognised as an acquired multiple acyl-CoA dehydrogenase deficiency [14,63,98]. The long-chain carnitine C14:1 concentration was above the reference range in AM horses [3,13], though serum concentrations showed no significant difference between surviving and deceased horses [15]. In a recent study involving a larger group of AM-affected horses, C14:1 was also identified as one of the variables with the most significant impact on distinguishing groups (comparable to those used to compare faecal microbiota in this paper), further corroborating the results presented here. However, it did not prove to be a reliable diagnostic or prognostic indicator [16]. In humans, tetradecenoylcarnitine (i.e., C14:1), as other long-chain acylcarnitines, plays a role in insulin resistance and in the development of cardiovascular diseases [101]. Most horses affected by AM show elevated plasma concentrations of cardiac troponin I, a specific biomarker of myocardial injury [36,102], and some horses exhibit specific alterations in electrocardiogram (ECG) recordings and cardiac ultrasound examination similar to those observed in mice and humans with VLCADD [103]. Its role in muscle insulin resistance [104] might contribute to explaining the hyperglycaemia observed in AM horses.

In equids, individual variations in faecal microbiota are influenced by factors such as nutrition, management practices, seasonal variation, medications, animal-related factors, pathological conditions, and stress-related factors [105]. To reduce these individual variations, horses included in the present study were all pasturing for a minimum of 6 h per day during a high-risk season for AM (i.e., autumn and spring), including the CONTROL horses that were sampled in November 2020, as 94% of "autumnal" cases occurred between October and December [106]. Seasonal variation and associated weather conditions are known to influence gut microbiota composition in horses [107–109]. This seasonal effect could be attributed to changes in the composition of environmental bacteria (e.g., soil and grass/haylage microbiota) or in the nutrient composition of pasture which are in turn influenced by climatic conditions [107,109,110]. In this study, AM-S and AM-NS horses were sampled in autumn and spring, while CoG and CONTROL horses were sampled in

autumn. However, seasonal variations between winter and summer are reported to have only a minor impact on faecal microbiota, accounting for 2.8% of the variation, according to Theelen et al. (2021) [108]. Similarly, the variations between autumn and spring in the present study could also be considered minor. It is important to note that the year of sampling differed between groups (2016, 2017, and 2018 for clinical cases of AM, 2018 for selected CoG, and 2020 for CONTROL horses), which may introduce potential biases. To minimise the impact of this temporal variation, samples were collected under a strict protocol, stored immediately in a conservative medium, and frozen at −20 °C until analysis [42,111,112]. Moreover, analyses were conducted on a regular basis over the years to ensure that samples did not remain frozen for extended periods. Finally, sampling was also standardised using a protocol described by Stewart et al. (2018) [42], further minimising potential sampling bias.

There are some limitations in this study. Some of them are already described by Wimmer-Scherr et al. (2021): the potential bias introduced by euthanasia for ethical reasons based on the severity of the clinical signs in the AM-NS group as well as the possible medications received by some horses prior to referral to the clinic [35]. The method used for microbiota assessment also presents inherent limitations, potentially favouring or underestimating certain bacterial taxa due to the lack of absolute quantification and selection bias during the process (e.g., DNA extraction, primer selection, PCR amplification, and bioinformatics parameters) [113].

When a modification of the intestinal microbiota is associated with a pathology, the question arises whether this modification reflects the state of health of the host or influences the host's health. Recently, Renaud et al. (2022) suggested that protoxins may be transformed by rumen microbiota, particularly in species with a long retention time, which would protect these species from developing AM-clinical signs. Indeed, the hypothesis proposed is that having a proximal fermentation compartment located before the absorption site of amino acids might be protective while having a distal fermentation compartment located after the absorption site of protoxins—as is the case in horses—might make the species more sensitive to poisoning [40]. This hypothesis can be partly explained by the fact that (1) certain bacteria can metabolise peptides and amino acids, and the protoxins are non-proteinogenic amino acids [114,115], and (2) some bacteria possess the enzymes involved in the metabolism of BCAAs [6,114], HGA, and MCPrG [6].

The identification of bacteria within the equine microbiota that can metabolise HGA and MCPrG into their corresponding toxic metabolites or potentially degrade these protoxins could be valuable in understanding species sensitivity. Moreover, this could contribute to a broader comprehension of intoxication and potentially aid in the discovery of molecules and/or bacteria capable of preventing this intoxication. To study the intestinal microbiota in this context, the use of alternative models, such as in vitro dynamic (i.e., SHIME® or static fermentation models (i.e., batch), makes sense [116–118]. In addition to aligning with the three R's—replacement, reduction, and refinement—this type of model would eliminate the direct influence of host metabolism on the microbiota by using faeces of healthy donors (i.e., CONTROL horses) and by adding the studied challenge (for example, the addition of HGA). As such, the changes observed in the digestive microbiota would be directly linked to the challenge applied to the system: the addition of protoxins in the AM model. The identification of bacteria that could play a role in HGA and/or MCPrG poisoning would then be easier. Another benefit of this kind of in vitro dynamic or static fermentation model is that it can offer opportunities for studying treatments by also adding targeted molecules. Finally, another possibility would be to use metagenomic shotgun analysis. This analysis makes it possible to describe the taxonomic composition of a community of organisms and its diversity, as well as its genes and, therefore, its functional capacities.

5. Conclusions

For the first time, a correlation has been observed between blood parameters and the intestinal microbiota of horses suffering from AM. At this stage, it remains to be determined whether these changes result directly from the protoxins' effect on the microbiota, the metabolism of protoxins by bacteria, and/or the host's pathological state. Further investigation is necessary to elucidate the underlying mechanisms and determine the specific contributions of each factor. Understanding these relationships could deepen our knowledge of the role of the intestinal microbiota in AM and open the path to potential therapeutic strategies.

Author Contributions: Conceptualisation, A.-C.F. and C.C.; methodology, A.-C.F. and B.T.; validation, A.-C.F. and B.T.; formal analysis, A.-C.F., B.T. and F.B.; investigation, A.-C.F., C.C., B.R., C.-J.K., G.v.L. and K.P.; resources, D.-M.V., F.B., P.G. and B.T.; data curation, B.T.; writing—original draft preparation, A.-C.F.; writing—review and editing, A.-C.F., C.C., D.-M.V., B.T., B.R., G.v.L., L.L., C.P.W., G.D. and C.-J.K.; visualisation, A.-C.F. and B.T.; supervision, D.-M.V. and P.G.; project administration, A.-C.F.; funding acquisition, D.-M.V. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval were waived for this study because all procedures in this study adhered to both national and international guidelines on animal welfare. The Animal Ethics Committee of the University of Liege was consulted, and it was confirmed that the sampling process was part of routine veterinary practice for diagnosing or preventing atypical myopathy.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Raw amplicon sequencing libraries were submitted to the NCBI database under bioproject number PRJNA1170059.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

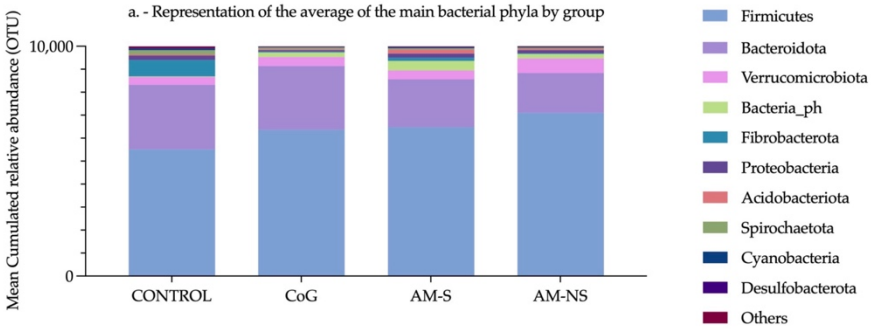

Figure A1. *Cont.*

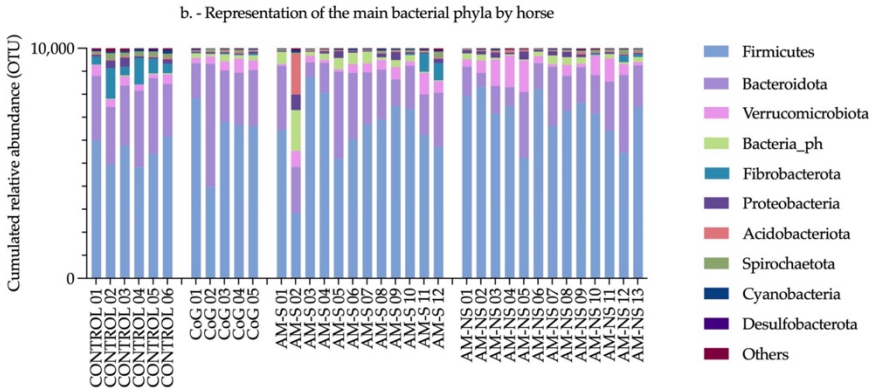

Figure A1. Main dominant bacterial phyla: (**a**) by group and (**b**) by horse. The horses represented are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy.

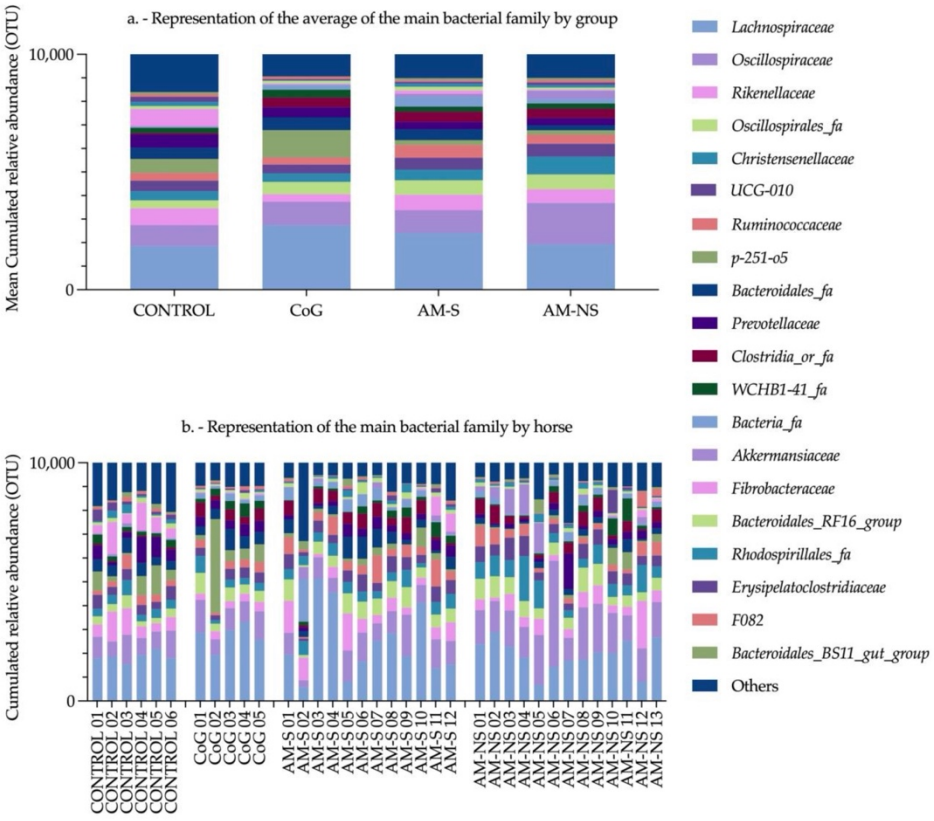

Figure A2. Main dominant bacterial family: (**a**) by group and (**b**) by horse. The horses represented are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy.

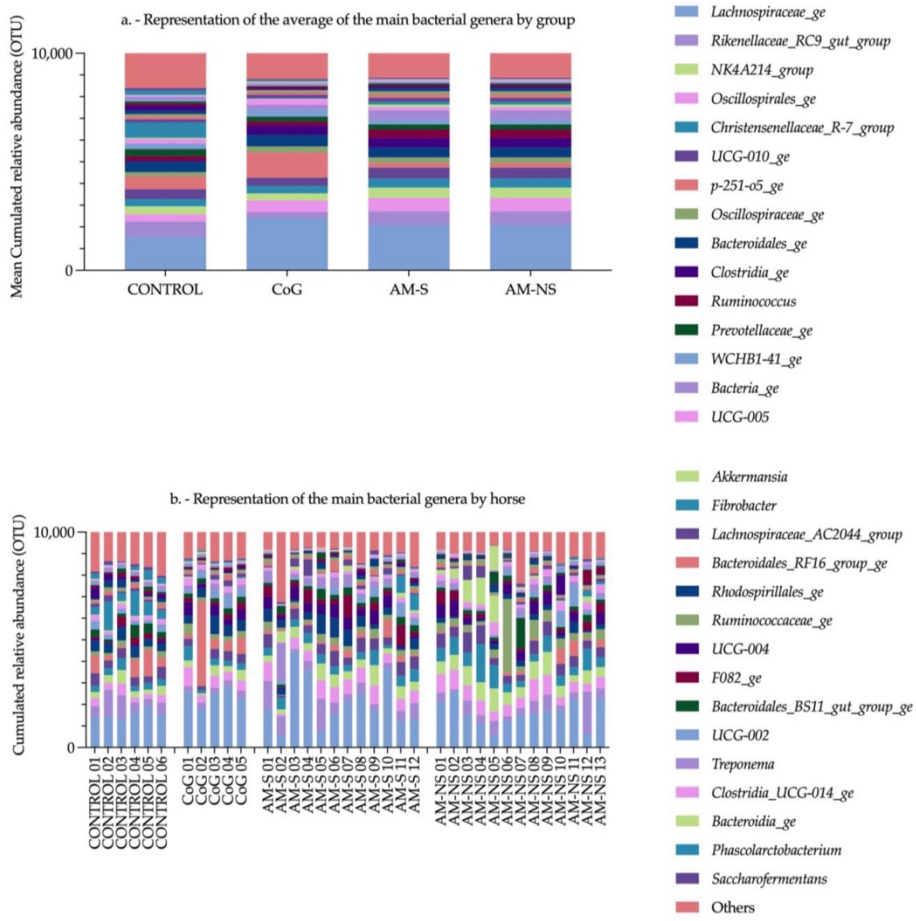

Figure A3. Main dominant bacterial genera: (**a**) by group and (**b**) by horse. The horses represented are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy.

Table A1. Serum concentrations of hypoglycin A (µmol/L) and MCPA-carnitine (nmol/L).

		CONTROL	CoG	AM-S	AM-NS	Diseased Horses
HGA	Mean ± SD CI [LLCI-ULCI]	<LD	1.46 ± 1.81 [a] [0.00–3.05]	4.59 ± 3.67 [2.52–6.67]	7.32 ± 5.61 [4.28–10.37]	6.01 ± 4.88 [a] [3.88–7.72]
MCPA-carnitine	Mean ± SD CI [LLCI-ULCI]	<LD	9.63 ± 14.03 [b] [0.00–21.92]	369.62 ± 972.82 [c] [0.00–920.04]	507.73 ± 485.45 [c] [243.84–771.62]	441.44 ± 746.02 [b] [149–733.88]

HGA: Hypoglycin A/MCPA-carnitine: methylenecyclopropylacetyl-carnitine/< LD = below the limit of detection (0.09 µmol/L for HGA and 0.01 nmol/L for MCPA-carnitine), SD = standard deviation, CI = Confidence interval, LLCI = Lower Limit of Confidence Interval, ULCI = Upper Limit of Confidence Interval. The groups represented are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy and diseased horses. a—Significant difference in mean serum concentration of HGA, $p < 0.01$. b—Significant difference in mean serum concentration of MCPA-carnitine, $p < 0.0001$. c—Significant difference in mean serum concentration of MCPA-carnitine, $p < 0.05$.

Table A2. Serum concentrations of selected acylcarnitines (µmol/L).

		CONTROL	CoG	AM-S	AM-NS	Diseased Horses
C2	Mean ± SD CI [LLCI-ULCI]	7.43 ± 0.96 [6.67–8.39]	6.59 ± 2.15 [4.71–8.73]	23.87 ± 14.64 * [15.58–38.51]	43.05 ± 25.42 * [29.23–68.47]	33.84 ± 22.74 * [24.93–56.58]
C4	Mean ± SD CI [LLCI-ULCI]	0.65 ± 0.28 [0.43–0.93]	0.85 ± 0.43 [0.48–1.28]	10.76 ± 7.02 * [6.79–17.78]	21.06 ± 15.16 * [12.81–36.22]	16.12 ± 12.85 * [11.08–28.97]
C5	Mean ± SD CI [LLCI-ULCI]	0.29 ± 0.05 [0.25–0.33]	0.93 ± 0.51 * [0.48–1.44]	9.52 ± 7.83 * [5.10–17.35]	21.64 ± 14.15 * [13.94–35.79]	15.82 ± 12.90 * [10.77–28.72]
C10	Mean ± SD CI [LLCI-ULCI]	0.01 ± 0.00 [0.01–0.01]	0.04 ± 0.03 * [0.01–0.07]	0.55 ± 0.54 * [0.25–1.10]	0.96 ± 1.39 * [0.21–2.35]	0.77 ± 1.07 * [0.35–1.84]
C12:1	Mean ± SD CI [LLCI-ULCI]	0.01 ± 0.00 [0.00–0.01]	0.01 ± 0.01 [0.00–0.02]	0.20 ± 0.13 * [0.13–0.33]	0.28 ± 0.26 * [0.14–0.55]	0.24 ± 0.21 * [0.16–0.45]
C14	Mean ± SD CI [LLCI-ULCI]	0.01 ± 0.00 [0.01–0.02]	0.05 ± 0.07 [0.00–0.12]	0.22 ± 0.13 * [0.14–0.35]	0.34 ± 0.28 * [0.19–0.62]	0.28 ± 0.23 * [0.19–0.51]
C14:1	Mean ± SD CI [LLCI-ULCI]	0.01 ± 0.00 [0.01–0.01]	0.03 ± 0.01 [0.01–0.04]	0.33 ± 0.22 * [0.21–0.55]	0.54 ± 0.56 * [0.24–1.10]	0.44 ± 0.43 * [0.27–0.87]
C18:1	Mean ± SD CI [LLCI-ULCI]	0.04 ± 0.02 [0.03–0.06]	0.08 ± 0.06 [0.02–0.14]	0.61 ± 0.48 * [0.34–1.09]	0.92 ± 0.86 * [0.46–1.78]	0.77 ± 0.71 * [0.49–1.48]

* Mean over the percentile 99 of reference range obtained with control horses, SD = standard deviation, CI = Confidence interval, LLCI = Lower Limit of Confidence Interval, ULCI = Upper Limit of Confidence Interval. The groups represented are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy and diseased horses.

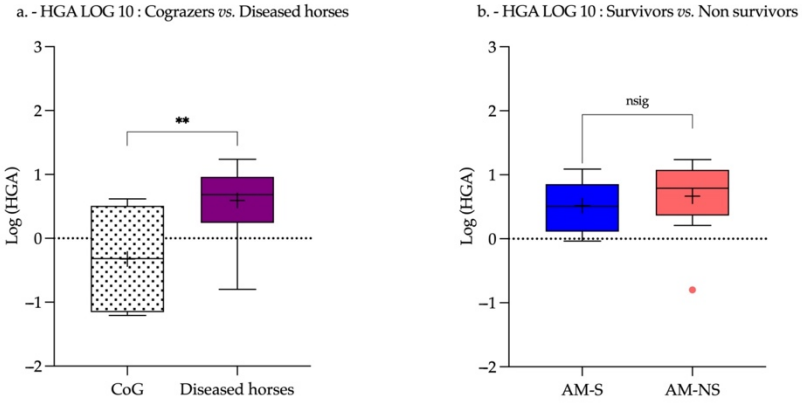

Figure A4. *Cont.*

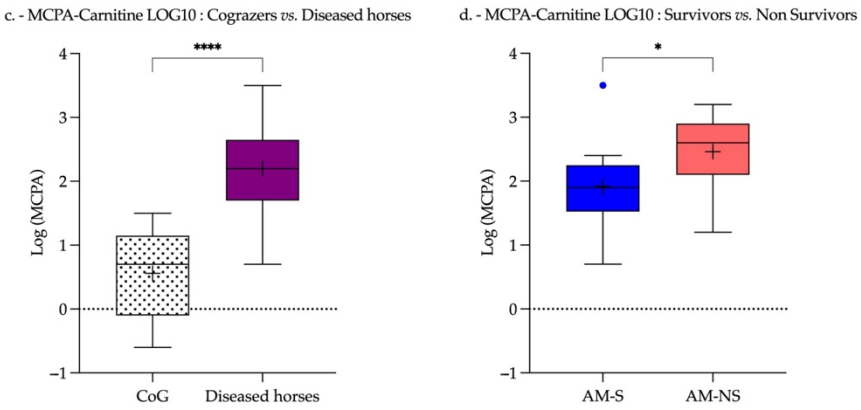

Figure A4. Serum hypoglycin A (HGA) (**a**,**b**) and methylenecyclopropylacetic-carnitine (MCPA-carnitine) (**c**,**d**) concentrations. Boxes range from the 25th to 75th percentiles. Means are represented by horizontal lines and medians by a cross. Box plot whiskers were established using the Tukey method. The groups are control horses (CONTROL), cograzers (CoG), survivors (AM-S) and non-survivors (AM-NS) of atypical myopathy and diseased horses. Significantly different with a *p*-value of 0.05 or less: * < 0.05; ** < 0.01; **** < 0.0001, Not significant "nsig".

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CONCLUSION OF THE FIRST STUDY

The study revealed that AM in horses is associated with significant alterations in faecal microbiota composition and diversity compared with toxin-free controls.

Horses affected by AM showed a reduction in microbial diversity and evenness, indicating a "dysbiosis-like" process that may reflect or contribute to metabolic disturbances induced by HGA intoxication. Specific bacterial genera (*i.e.*, *Firmicutes_ge*, *Clostridia_ge*, *Bacteria_ge*, *NK4A214_group* and *Fibrobacter*) were differentially abundant between affected and healthy animals. Some of these taxa are known to play key roles in fibre degradation, SCFAs production, and energy metabolism, suggesting a potential link between microbial imbalance and impaired energy pathways observed in AM.

Furthermore, correlations between microbial profiles and biochemical blood markers, including acylcarnitines and other metabolites indicative of disrupted β-oxidation, strengthened the association between gut microbiota composition and host metabolic state.

These findings support the hypothesis that the intestinal microbiota may be both affected by and potentially involved in the pathophysiology of AM.

Overall, the study provides the first comprehensive *in vivo* evidence of microbiota dysregulation in horses suffering from AM. It highlights the importance of considering gut microbial dynamics in understanding the disease process and lays the groundwork for further mechanistic investigations into whether the microbiota acts as a passive biomarker or an active participant in HGA intoxication.

STUDY 2: *In vitro* Investigation of Equine Gut Microbiota Alterations during Hypoglycin A Exposure

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INTRODUCTION TO THE SECOND STUDY

The second study aimed to investigate the direct interaction between HGA and the equine gut microbiota under controlled *in vitro* conditions. Using a static fermentation system derived from the SHIME® model, faecal inocula from healthy horses were incubated with or without HGA to isolate the microbial response from host-related factors. The study focused on identifying bacterial taxa whose abundance or activity was directly affected by HGA exposure and on evaluating whether microbial communities could transform or degrade the protoxin. Microbial composition was assessed through 16S rRNA gene sequencing, and HGA concentrations were monitored by targeted LC-MS/MS analysis over time. This combined approach provided a controlled framework to explore both the ecological and metabolic responses of the equine microbiota to HGA.

animals

MDPI

Article

In Vitro Investigation of Equine Gut Microbiota Alterations During Hypoglycin A Exposure

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Simple Summary

Atypical myopathy is a severe and often fatal poisoning of equids caused by the ingestion of sycamore maple tree seeds or seedlings that contain hypoglycin A. Once ingested, the protoxin is converted into harmful compounds that block energy production in muscles, leading to muscle breakdown. Scientists have questioned whether the microbes living in the gut could influence how this toxin behaves. Previous studies have shown differences in gut microbial communities between horses affected by atypical myopathy, their clinically healthy co-grazers, and a group of toxin-free horses serving as a control. This suggests that the microbiota may influence the outcome of intoxication. In this in vitro study, we recreated part of the horse's large intestine in the laboratory and exposed it to hypoglycin A. Our results show that the toxin's concentration decreased significantly when microbes were present, while no toxic breakdown products were detected. This study suggests that the equine gut microbiota may contribute to protection against hypoglycin A, providing new insights into the understanding and potential prevention of atypical myopathy.

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Abstract

Hypoglycin A is a plant-derived protoxin that causes atypical myopathy in equids. In atypical myopathy-affected horses, metabolomic and microbiome studies have reported alterations in metabolic markers and faecal microbiota composition, pointing to a potential disruption of microbial homeostasis. However, in vivo observations are strongly confounded by host-related factors, underscoring the need for controlled in vitro approaches. To address this, we used an in vitro static batch fermentation model simulating the equine colon to investigate the direct effects of hypoglycin A on microbiota composition and

activity. Faecal inocula from healthy horses were incubated in control and hypoglycin A-treated fermenters for 48 h, with serial analyses of hypoglycin A concentration, short-chain fatty acids, and 16S rRNA gene profiles. Hypoglycin A remained stable in the nutritive medium in the absence of microbiota, confirming that its degradation in inoculated fermenters was microbiota-dependent. The results showed significant microbial-associated hypoglycin A degradation without evidence of toxic metabolite formation. The analysis of α- and β-diversity revealed both an effect of incubation time, reflecting the natural temporal dynamics of microbial communities under batch fermentation, and a specific impact of hypoglycin A exposure, with certain taxa such as *Paraclostridium* being affected. This study provides the first in vitro evidence that the equine microbiota contributes to hypoglycin A degradation.

Keywords: equine atypical myopathy; microbiota; intestinal microbiota; horses; hypoglycin A; methylenecyclopropylacetyl-carnitine; 16S rRNA gene sequencing; next generation sequencing; toxin; poisoning; in vitro batch fermentation; short chain fatty acids

1. Introduction

Atypical myopathy (AM) is a commonly fatal pasture-associated intoxication of equids that occurs predominantly during the autumn and spring in temperate European regions. The disease is strongly linked to the ingestion of seeds or seedlings of the sycamore maple (*Acer pseudoplatanus*), which contain plant-derived protoxins [1]. The analysis of plant materials from the sycamore maple tree showed that seedlings [2] and seeds [3] contain hypoglycin A (HGA) as well as methylenecyclopropylglycine (MCPrG) [4,5]. Both are non-proteinogenic amino acids structurally related to branched-chain amino acids (BCAAs), which likely allows them to enter the same metabolic pathways [6,7].

Although both HGA and MCPrG are present, HGA is considerably more abundant in seeds [4,8]. Moreover, HGA intoxication has been reported in humans and other animal species and is therefore better characterised in the literature [9–17]. Hypoglycin A is not toxic per se [18] but is metabolised into the toxic compound methylenecyclopropylacetyl-coenzyme A (MCPA-CoA) [19], which is responsible for the pathogenesis. The diagnosis of AM relies on the detection of its conjugated metabolites (i.e., methylenecyclopropylacetic acid-carnitine (MCPA-carnitine) and -glycine (MCPA-glycine)) and marked alterations of the acylcarnitines profile in serum and urine of AM horses [17,20–23], resulting from the inhibition of *β*-oxidation of fatty acids [24,25] and the inhibition of isovaleryl-CoA-dehydrogenase [21,24,25].

The gut microbiota constitutes a complex microbial ecosystem capable of metabolising a wide range of substrates, including plant secondary metabolites and toxins [26–28]. Depending on the enzymatic pathways involved, microbial activity can lead either to detoxification or to bioactivation of xenobiotics. Given its structure, HGA could be susceptible to microbial transformation or degradation, potentially influencing its toxicity.

The potential involvement of the microbiota in HGA poisoning has been suggested on several occasions in animals. One hypothesis proposes that ruminal transformation of HGA may occur, based on the observation that herbivorous species possessing a proximal fermentation chamber seem less susceptible to sycamore poisoning [11]. Supporting this hypothesis, an in vitro study using sheep ruminal fluid reported a decrease in HGA concentrations after incubation with either pure HGA or sycamore maple seeds. However, this effect was also observed, though to a lesser extent, in autoclaved ruminal fluid, suggesting that abiotic processes could contribute to the effect. Although the microbial

markers used to evaluate community activity did not reveal significant differences [29], these markers may not provide sufficient resolution to accurately characterise microbiota dynamics [30].

In horses, which are hindgut fermenters, differences in faecal microbiota composition have been reported between AM-affected animals and healthy co-grazers [31,32]. In particular, an in vivo study comparing the faecal microbiota of AM-affected horses, co-grazers, and protoxin-free control horses revealed significant differences in α-diversity, evenness, and β-diversity, as well as in the relative abundance of several bacterial genera [32]. Metabolomic analyses further suggest the disruption of microbial homeostasis in the diseased animals [21]. Together, these findings indicate a possible interaction between HGA toxicity and gut microbial composition. Nonetheless, these in vivo studies are observational and subject to significant confounding by host factors such as diet, clinical condition, and environment [33,34].

Therefore, controlled in vitro approaches are essential to dissociate the host and microbial contributions. Batch fermentation models using equine faecal inocula are a well-established method to investigate microbial activity under defined conditions [35,36]. Nevertheless, no previous study has directly evaluated whether the equine hindgut microbiota can metabolise HGA or how exposure affects the microbial community structure. This represents the main gap addressed by the present work.

Based on existing evidence, we hypothesised that HGA toxicity may be influenced by the equine gut microbiota, which could transform or degrade the compound and, thereby, alter its bioavailability and effects. Specifically, we postulated that faecal microbial communities from healthy horses would be capable of transforming HGA in vitro, resulting in measurable decreases in protoxin concentration without the production of known toxic metabolites, and that HGA exposure would be associated with specific changes in microbial diversity, potentially reflecting some of the trends observed in vivo in horses exposed to HGA.

Using a static batch fermentation system, the objectives of this study were (i) to investigate, under in vitro conditions free from host influence, the modifications in colonic bacterial populations induced by HGA exposure compared with unexposed controls, and (ii) to characterise the temporal dynamics of HGA concentration within the batch fermentation system.

2. Materials and Methods

2.1. Selection of Donor Horses and Sampling

The inclusion criteria were as follows: (i) clinically healthy horses; (ii) normal general examination parameters; (iii) access to pasture for at least 6 h a day; and (iv) absence of detectable levels of HGA (aTRAQ® kit (AB Sciex Germany GmbH, Darmstadt, Germany —LOQ 0.090 µmol/L [37]) and MCPA-carnitine (UPLC-MS/MS—LOD 0.001 nmol/L [38]) in blood serum. Sampling occurred in spring 2022.

All procedures in this study adhered to both national and international guidelines on animal welfare. The Animal Ethics Committee of the University of Liège had confirmed that the sampling process was part of routine veterinary practice to establish a diagnosis. As a result, formal ethical approval was not required. Informed consent was obtained from horse owners prior to the inclusion of the horses in the study.

The faecal samples were collected directly from the rectum in anaerobic jars and anaerobiosis was ensured using AnaeroGen™ bags (Oxoid, Basingstoke, UK). The samples were transported under cooled conditions and stored at −80 °C until batch analysis. The pool of faeces was prepared by mixing equal proportions of samples from the two donors [39]. A phosphate-buffered solution containing per litre: 8.8 g of K_2HPO_4, 6.8 g of KH_2PO_4, and 0.1 g of sodium thioglycolate was used to prepare a 20% *w*/*v* faecal homogenate.

Mechanical homogenisation was achieved in a Stomacher VWR® Star-Blender LB400 (VWR International GmbH, Darmstadt, Germany). Double-coated sterile stomacher bags (300 × 190 mm) were used to collect and filter the faecal suspensions.

2.2. Static In Vitro Batch Model Derived from SHIME® System

2.2.1. Chemical Reagents and Consumable Materials

The materials and nutritional media were obtained from ProDigest (Ghent, Belgium). The nutritional medium, referred to as "feed", used for the simulation of the gastrointestinal environment contained per litre of distilled water: arabinogalactan (1.2 g), pectin (2.0 g), xylan (0.5 g), glucose (0.4 g), yeast extract (3.0 g), special peptone (1.0 g), mucin (3.0 g), L-cystein-HCl (0.5 g), and starch (4.0 g). The prepared feed was autoclaved at 121 °C for 30 min.

2.2.2. Determination of HGA Concentration in Batch System

An estimated maximum tolerated dose (MTD) of HGA in horses was determined thanks to a human equivalent dose (HED) of the MTD values for HGA in rats. The MTD of HGA in rats was determined with controlled diets over a 30-day period, and the result was 1.50 ± 0.07 mg HGA/kg BW/day [40].

The human equivalent dose (HED) was determined as previously described with the following mathematical formula [41] using K_m as a correction factor:

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \times (\text{Animal } K_m/\text{Human } K_m)$$

Then, the HED obtained was used to calculate the equivalent dose for horses using the same formula.

The K_m of the horse was calculated based on its average height and weight (1.61 ± 0.073 m and 565.08 ± 69.81 kg, respectively) as previously described [42].

The Km was calculated by dividing the average body weight (BW, kg) of species by its body surface area (BSA, m^2) as follows [41]:

$$K_m = \text{BW (kg)}/\text{BSA } (m^2)$$

The BSA calculation is based on height and weight with the following mathematical formula [43]:

$$\text{BSA } (m^2) = \sqrt{} (\text{Height (cm)} \times \text{Weight (kg)}/3600)$$

Consequently, the estimated MTD of HGA in horses was 0.08 mg HGA/kg BW/day or 45.4 mg HGA/day with the same average weight used (i.e., 565.08 ± 69.81 kg) [42]. The digestive volume of the gastrointestinal tract of an adult horse is around 200 L [44]: the concentration of HGA representing the MTD of HGA in the horse's digestive system is 227 ng HGA/mL. Considering the acute exposure context of our in vitro fermentation system (i.e., 48 h), a 2-fold increase in HGA concentration (i.e., 454 ng HGA/mL) was tested. This concentration of HGA (purity 85%, Toronto Research Chemicals, Canada) was used in the three HTFs of the batch system.

2.2.3. Static Batch Model and Sampling Procedure

The batch model is a short-term in vitro fermentation that uses the SHIME® model to mimic the descending colon of the human intestinal tract [45,46]. For this study, the system was adapted to represent the descending colon in horses, maintaining a constant temperature of 38 °C and a pH range of 6.6–6.9 [44,47]. Indeed, the colon is the part of the digestive tract where most fermentation occurs in horses [47] and humans [48]. It is therefore the most appropriate environment in which to study the changes in the microbiota linked with the compounds ingested during feeding. Specifically, six double-jacketed

fermenters were filled with 300 mL of feed and inoculated with the faecal homogenate (5% *v*/*v*). The fermenters were connected to a water bath that kept the system at a constant temperature. The pH was automatically controlled by adding either acid (0.5 M HCl) or base (0.5 M NaOH) as required to maintain values between 6.6 and 6.9. The anaerobic conditions were guaranteed by a N_2 flush. Three fermenters served as "control fermenters" (CFs) containing only the nutritional media and faecal inoculate, while the other three additionally received a single addition of HGA (454 µg/L) and acted as "HGA-treated fermenters" (HTFs). This configuration allowed for obtaining triplicate results for each studied group.

The samples were obtained from each fermenter every 2 h for the first 24 h of the experiment, and every 6 h thereafter, until reaching 48 h. Consequently, the sampling time points were T0, T2, T4, T6, T8, T10, T12, T14, T16, T18, T20, T22, T24, T30, T36, T42, and T48. The system was started, and 10 min later, HGA was added to the HTF. At this moment, an additional sampling time point (T0*) was included to monitor the system immediately after HGA addition.

Several samples were taken for different analyses, as shown in Figure A1, Appendix A: HGA and MCPA-carnitine quantification, short-chain fatty acids (SCFAs) quantification, and microbiota analysis. In practice, only a subset of samples was analysed to balance analytical depth and sample coverage.

2.3. Microbiota Assessment

2.3.1. Bacterial DNA Extraction and High-Throughput Sequencing

The total bacterial DNA was extracted from faecal samples using the PSP Spin Stool DNA Plus Kit 00310 (Invitek, Berlin, Germany), following the manufacturer's instructions. The PCR amplification of the 16S rDNA V1–V3 hypervariable region and the library preparation were performed using the following primers with Illumina overhang adapters: forward (5′-GAGAGTTTGATYMTGGCTCAG-3′) and reverse (5′-ACCOGCOGGCTGCTGGCAC-3′) [49].

The PCR products were purified using the Agencourt AMPure XP bead kit (Beckman Coulter, Pasadena, CA, USA), followed by a second PCR round for indexing with Nextera XT index primers 1 and 2. After purification, amplicons were quantified with Quant-IT PicoGreen (ThermoFisher Scientific, Waltham, MA, USA) and diluted to 10 ng/µL.

Final quantification was conducted using the KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) before normalisation, pooling, and sequencing on a MiSeq platform with V3 reagents (Illumina, San Diego, CA, USA). The sequencing run included commercial mock community positive controls containing DNA from 10 defined bacterial species (ATCC MSA-1000, ATCC, Manassas, VA, USA) as well as negative controls from the extraction and PCR steps [50].

Raw amplicon sequencing libraries were submitted to the NCBI database under bioproject number PRJNA1335877.

2.3.2. Sequence Analysis and 16S rDNA Profiling

Sequence read processing was performed as previously described [50] using the Mothur software package v1.48 [51] and the VSEARCH algorithm for chimaera detection [52]. For operational taxonomic unit (OTU) generation, a clustering distance of 0.03 was used. Briefly, 16S reference alignment and taxonomical assignment, from phylum to genus, were performed with Mothur and were based upon the SILVA database (v138.1) of full-length 16S rDNA sequences [53]. These OTUs were further clustered into a final relative abundance table at the genus level.

2.3.3. Microbiota Analysis

Subsampled datasets with 10,000 cleaned reads per sample were obtained and used to evaluate α-diversity (i.e., measuring diversity within the community) and β-diversity (i.e., measuring diversity between communities or within the same community at different time points by considering sequence abundances or by considering only the presence–absence of sequences) using the vegan R package (v 2.6-6.1) [54–56].

The indicators of α-diversity computed are the reciprocal Simpson microbial diversity index, Chao richness index (i.e., richness which quantifies the number of species present within a community), and Simpson-derived evenness index (i.e., evenness which describes how uniformly individuals are distributed among the species, highlighting the presence or dominance of certain species) [55–57]. Differences in α-diversity between groups (CF vs. HTF) were evaluated with an ANOVA test (with a Geisser–Greenhouse correction) followed by paired post hoc tests corrected for multiple comparisons by controlling the False Discovery Rate (FDR) with a two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli using PRISM 10 (GraphPad Software; San Diego, CA, USA), and were considered significant for a p and q-value of 0.05 or less. As T0 represents the moment when the system was started following pooling of donor faeces, the microbial populations inevitably have to adapt to this new environment (i.e., descending colon). Consequently, T0 is expected to be statistically different from subsequent time points. Moreover, no treatment (i.e., HGA addition) was applied at T0, and the two groups were therefore not different. As a result, the figure presented in the main text excludes T0 from the α-diversity statistical analysis, while a second informative figure including this time point is presented in Figure A2, Appendix B.

The β-diversity was analysed using vegan and vegan3d packages (v 1.3-0) in R [58]. A principal coordinate ordination analysis (PCoA) based on the Bray–Curtis dissimilarity matrix was used to visualise samples in β-diversity analysis. Differences between groups were assessed with a PERMANOVA (Adonis2) and post hoc pairwise comparisons (pairwise adonis package v 0.4.1) [59] with a significance threshold p of 0.05. These analyses were conducted on both the complete dataset and after T0 exclusion. A distance-based redundancy analysis (dbRDA) was further applied as a constrained ordination model to assess the effect of the variables *Time* and *Treatment* on the β-diversity. This step of the statistical analysis was also realised without the T0. The significance of the model and axes was assessed with ANOVA.

Finally, a differential abundance analysis was performed using the DESeq2 package in R (v1.44.0) to examine statistically significant changes in microbial abundance between treatments and across time points. This step was also carried out after excluding T0.

2.4. Quantification of Short Chain Fatty Acids and Statistical Analysis

A previously validated method of solid-phase microextraction (SPME), followed by gas chromatography coupled to mass spectrometry (GC–MS) [60], was used to simultaneously quantify the SCFAs produced in each fermenter at T0, T12, T24, T36, and T48. The measurement of SCFAs was essential, as they represent major metabolic end products of microbial fermentation and serve as indicators of intestinal microbiota activity and functionality within the batch model.

The analytical technique has the following lower and upper limits of quantification (LLOQ and ULOQ, respectively): acetic acid (C2): 2.00–99.90 mmol/L, propionic acid (C3): 0.97–48.60 mmol/L, butyric acid (C4): 0.57–28.37 mmol/L, isobutyric acid (iC4): 0.16–7.94 mmol/L, isovaleric acid (iC5): 0.10–4.90 mmol/L, valeric acid (C5): 0.29–14.69 mmol/L, caproic acid (C6): 0.0086–0.43 mmol/L, heptanoic acid (C7): 0.008–0.04 mmol/L, and octanoic acid (C8): 0.0007–0.03 mmol/L [60].

The proportion of each SCFA as a percentage of the total of SCFAs was calculated. The statistical analyses of SCFA concentrations were performed using GraphPad Prism 10 (GraphPad Software; San Diego, CA, USA) with a p threshold value of 0.05. The values which were under the LLOQ of the methods were replaced by the value of the LLOQ for the statistical analysis. The D'Agostino–Pearson, Anderson–Darling, Shapiro–Wilk, and Kolmogorov–Smirnov tests were used to assess the normality or lognormality of the data distribution. A two-way ANOVA was performed for each SCFA with a Greenhouse–Geisser correction, followed by post hoc multiple comparisons adjusted using Tukey's method.

2.5. Quantification of Hypoglycin A and Statistical Analysis

Hypoglycin A concentrations were quantified using liquid chromatography coupled with mass spectrometry, without derivatisation, as previously described by González-Medina et al. (2021) [61]. The LOD of this method is 0.055 ng/mL.

Statistical analyses of HGA concentrations were conducted using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA), with a significance threshold set at $p < 0.05$.

2.5.1. Preliminary Assessment of Hypoglycin A Stability in the Nutritional Medium

To evaluate possible degradation of HGA, two additional fermenters containing 300 mL of autoclaved nutritional medium were prepared, and HGA was added (i.e., 150 ng HGA/mL). The temperature (i.e., 38 °C) was maintained by a water bath. The pH was controlled before each sampling and adjusted manually if needed by the addition of 0.5 M NaOH or 0.5 M HCl. For this parallel experience, sampling points were: T0*, T6, T12, T20, and T24. These fermenters are designed as "nutritional medium fermenters" or NMFs.

Normality and lognormality tests (D'Agostino–Pearson, Anderson–Darling, Shapiro–Wilk, and Kolmogorov–Smirnov tests) were performed. An ANOVA with a Greenhouse–Geisser correction was performed, followed by a multiple comparisons test, which compared the mean concentration of HGA of each time point to the T0* considered as "the control time point" with a correction of False Discovery Rate (FDR) of Benjamini, Krieger, and Yekutieli ($q = 0.05$).

2.5.2. Hypoglycin A Concentration in the Batch Fermenters: Data Analysis

Time points were selected to provide a representative overview of HGA degradation over the incubation period while maintaining a balance between analytical depth and the number of samples processed.

Changes in HGA concentration over time (T0, T12, T24, T36) were analysed within the CF. Normality and lognormality were assessed using the D'Agostino–Pearson, Anderson–Darling, Shapiro–Wilk, and Kolmogorov–Smirnov tests. Data were log-transformed when required to meet distribution assumptions. A repeated measures one-way ANOVA was then performed, applying the Greenhouse–Geisser correction. Multiple comparisons were first carried out using Dunnett's test to compare each time point to the baseline (T0). When statistically significant differences were detected, additional pairwise comparisons were performed using Tukey's correction to assess differences between all the time points.

The same statistical approach was subsequently applied to the HTF, analysing HGA concentrations at T0, T0*, T2, T4, T6, T12, T16, T20, T24, T36, and T48. However, because one of the three replicates at T6 was missing, the repeated measures one-way ANOVA for the HTF was replaced by a mixed-effects analysis.

Finally, a two-way ANOVA was performed to compare both groups (CF vs. HTF) at the time points common to both groups (T0, T0*, T12, T24, and T36), and a multiple

comparison test with Tukey's correction was applied. The values at T0* for the CF were supposed to be the same as the values at T0, which made analysis between the two groups possible (CF vs. HTF).

2.5.3. Kinetic Data Analysis of Hypoglycin A Concentration

In addition to the ANOVA tests performed to compare HGA concentrations between time points, linear regression analyses were conducted to compare the overall degradation trend within CFs, HTFs, and NMFs. The slope of the regression line was used as an indicator of the apparent degradation rate of HGA. The significance of the regression was assessed using the F-test for the null hypothesis that the slope equals zero.

2.6. Quantification of Methylenecyclopropylacetyl-Carnitine

An ultra-performance liquid chromatography combined with subsequent tandem mass spectrometry (UPLC-MS/MS) was used for MCPA-carnitine quantification, as previously described [38]. The LOD is 0.01 nmol/L. The MCPA-carnitine was quantified only in HTF.

3. Results

3.1. Donor Horses

Two horses were involved in the study.

Horse 1: The horse was an 8-year-old Zangersheide female with a 5/9 body-score. The horse lived at pasture for more than 6 h a day and received hay (12 kg/1 time a day). The horse had access to a salt block. The horse is a leisure horse and was ridden 3 times/week for 1 h session each time.

Horse 2: The horse was a 3-year-old Haflinger and Spanish cross-breed female with a 5/9 body-score. The horse lived at pasture for more than 6 h a day and received ad libitum hay. The horse had access to a salt block. The horse is a leisure horse.

3.2. Microbiota Analysis

Starting with 2,760,444 raw reads, 2,050,624 reads were kept after read cleaning and chimaera removal. We proceeded with 10,000 reads per sample to taxonomic identification, leading to a table of 3489 OTUs. The determination of α- and β-diversities of the bacterial populations were assessed at the genus level.

The relative abundance of genera identified is represented in Figure 1. The genera at T0 seemed to be different from those at the other time points. The most represented genus at T0 was *Lachnospiraceae_ge*. The five most represented genera at T12, T24, and T48 were *Streptococcus*, *Clostridium_sensu_stricto_1*, *Escherichia-Shigella*, *Veillonella*, and *Bacteroides*. The decrease in the proportion of the "OTHERS" genera over time reflects the general dynamic of the batch system.

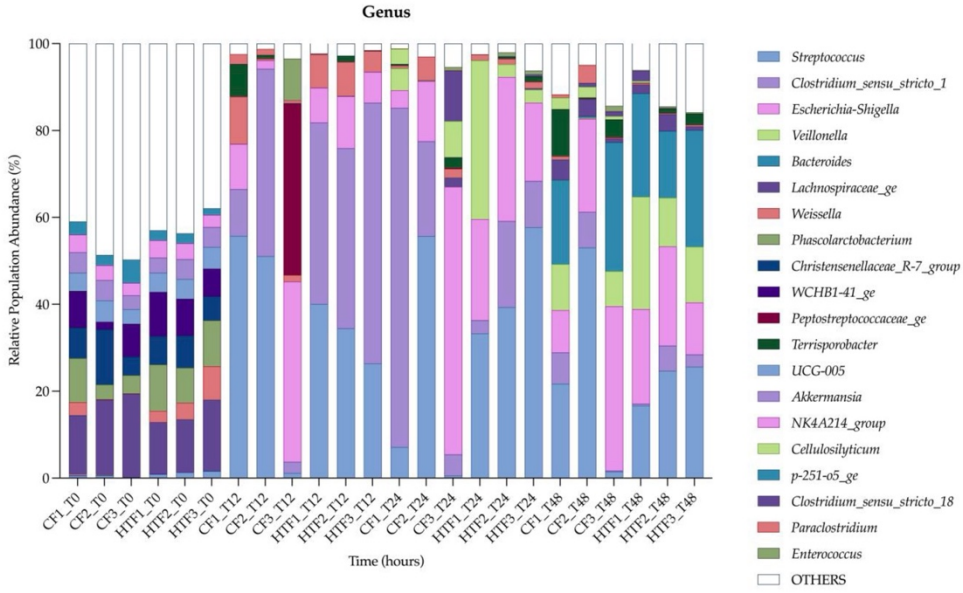


Figure 1. Main dominant genera at each time point for each control fermenters (CF) and hypoglycin A-treated fermenters (HTF) in relative population abundance by percentage.

3.2.1. α-Diversity Analysis

The α-diversity indexes (i.e., richness, diversity, and evenness) were compared between treatment groups (*Treatment* effect) and within groups (*Time* effect). Concerning the Reciprocal Simpson Index (i.e., diversity), a significant effect of *Time* was observed (p = 0.0320, *). The evenness (i.e., Simpson Evenness Index) was significantly different between CF vs. HTF at T12 (q = 0.0329, *) and between T12 vs. T48 (q = 0.0319, *) in the HTF (Figure 2).

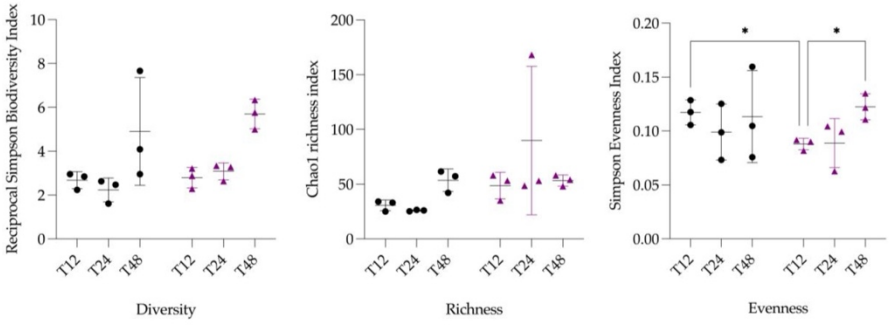


Figure 2. Representation of bacterial α-diversity derived from Reciprocal Simpson Biodiversity index, bacterial genus richness derived from Chao1 index, and bacterial genus evenness derived from Simpson Evenness index. Data are scatter dot plots at the genus level for each fermenter in both groups (i.e., control fermenters and HGA-treated fermenters). Horizontal bars indicate the mean, and vertical bars indicate the standard deviation for each group. Black circles represent control fermenters, and purple triangles represent HGA-treated fermenters. Groups were compared with

ANOVA followed by post hoc pairwise tests, using FDR multiple-test correction (q-value threshold: * < 0.05).

3.2.2. β-Diversity Analysis

The first PERMANOVA β-diversity analysis, including the interaction between *Time* and *Treatment*, revealed a significant effect of both factors and their interaction ($p = 0.001$, ***). However, inclusion of T0 samples influenced the ordination pattern (Figure 3a).

To better capture the effects of the experimental factors independently from baseline, a second PERMANOVA was performed after excluding T0 samples. This analysis confirmed a significant global effect ($p = 0.014$, *) explaining 33.6% of the total variance in microbial community structure. The factor *Time* alone accounted for 26.2% of the variance and had a significant impact on β-diversity ($p = 0.001$, ***), whereas *Treatment* explained only 6.6% of the variance and was not significant. A permutation design stratified by *Time* confirmed the absence of a *Treatment* effect ($p = 0.264$). Pairwise comparisons for the *Time* factor are summarised in Table 1. The influence of both *Time* and *Treatment* was further illustrated by a db-RDA constrained ordination model (Figure 3b, 3c), for which only the first axis (db-RDA1) and the *Time* factor were significant ($p = 0.002$, **).

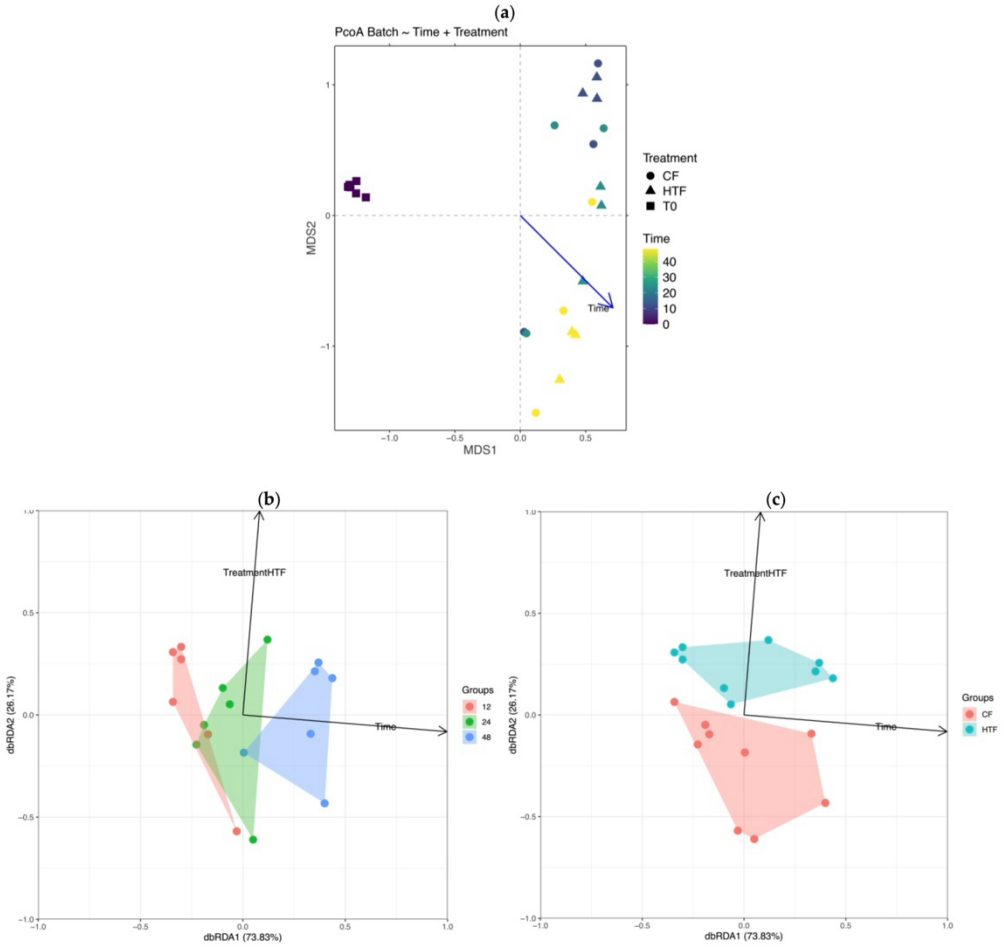


Figure 3. Representation of bacterial β-diversity analysis with (**a**) Principal Coordinates Analysis—Rounds symbols represent control fermenters (CF) and triangular symbols represent hypoglycin A-treated fermenters (HTF) after adding hypoglycin A, while square symbols represent fermenters at T0 before adding HGA, (**b**) Bray–Curtis-based Redundancy Analysis model of microbiota samples for T12, T24, and T48 coloured by *Time* value and (**c**) Bray–Curtis-based Redundancy Analysis model of microbiota samples coloured by *Treatment* group. Variable fitting to model is represented by a vector for each variable for both Bray–Curtis-based Redundancy Analyses.

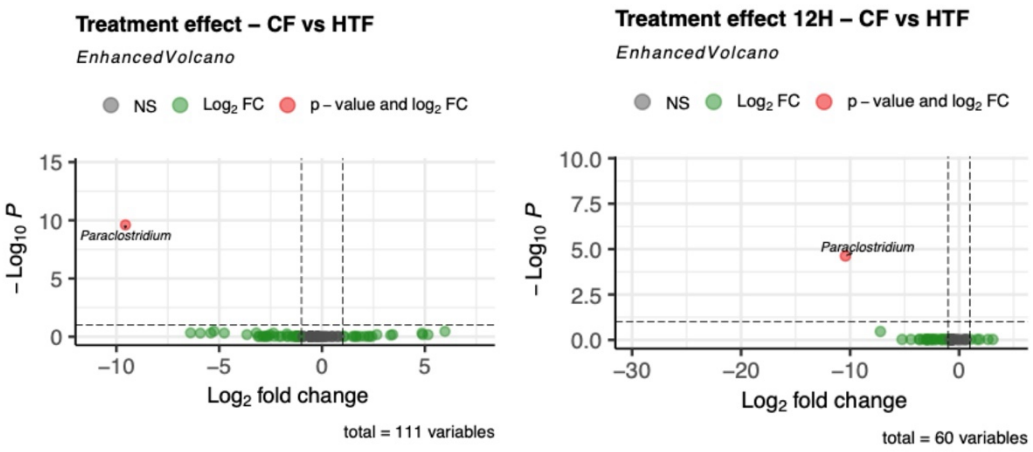
Table 1. β-diversity pairwise comparisons without T0.

Pairs	*p*-Value Adjusted	
T12 vs. T24	0.5416	nsig
T12 vs. T48	0.0189	*
T24 vs. T48	0.0265	*

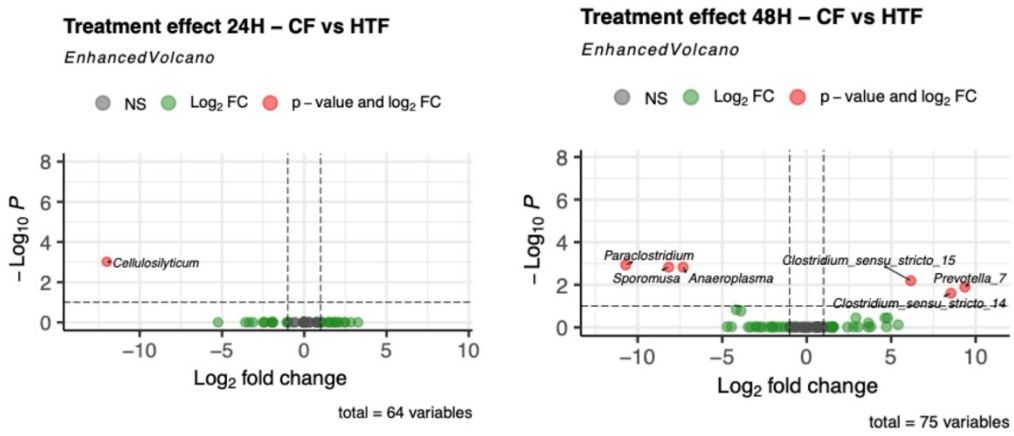
Significantly different with a *p*-value of 0.05 or less: * < 0.05; nsig: not significant; CF: control fermenters; HTF: hypoglycin A-treated fermenters.

3.2.3. Differences in Microbiota Composition Between Groups

Differences in microbial population abundance between CF vs. HTF were assessed with Deseq2. The analysis revealed a significantly higher abundance of the *Paraclostridium* genus in the CF group compared to HTF ($p = 2.5136 \times 10^{-10}$, ***) (Figure 4). Pairwise comparisons between *Treatment* groups at each sampling time highlighted several genera, as shown in the volcano plots (Figure 5). At T12, the only significant genus between CF and HTF was *Paraclostidium* ($p = 2.4113 \times 10^{-05}$, ***). At T24, the significant genus between groups was *Cellulosilyticum* ($p = 9.6744 \times 10^{-04}$, ***). At T48, significantly different genera between groups included *Paraclostridium* ($p = 1.1703 \times 10^{-03}$, **), *Sporomusa* ($p = 1.4903 \times 10^{-03}$, **), *Anaeroplasma* ($p = 1.4903 \times 10^{-03}$, **), *Clostridium sensu stricto 15* ($p = 6.4399 \times 10^{-03}$, **), *Prevotella 7* ($p = 1.2989 \times 10^{-02}$, *), and *Clostridium sensu stricto 14* ($p = 2.5008 \times 10^{-02}$, *). The genera *Paraclostridium*, *Cellulosilyticum*, *Sporomusa*, and *Anaeroplasma* were mainly present in CF (i.e., in the left part of the volcano plots) while *Clostridium sensu stricto 15*, *Prevotella 7*, and *Clostridium sensu stricto 14* were mainly present in HTF (i.e., in the right part of the volcano plots). These differences are illustrated in Figure 5, which displays the relative abundance (population count) of each genus across time points.



(**a**) (**b**)



(**c**) (**d**)

Figure 4. Volcano plot for *Treatment* effect with differences in microbial population abundance between control fermenters (CF) and hypoglycin A-treated fermenters (HTF). (**a**) all *Time* combined; (**b**) T12; (**c**) T24; (**d**) T48. X-axis represents log2 fold change, indicating relative change between two conditions: CF on left and HTF on right. Y-axis represents -log10 *p*-value. Horizontal dotted line indicates significant threshold ($p = 0.05$). Red dots represent populations that are significantly different between CF and HTF.

3.3. Quantification of Short Chain Fatty Acids and Statistical Analysis

Calculated across all sampling times, the proportion of acetate, propionate, and butyrate was 85 ± 13.7%, 16 ± 5.8%, and 8 ± 2.1%, respectively, in CF and 79 ± 14.4%, 19 ± 9.4%, and 10 ± 4.1%, respectively, in HTF.

Two-way ANOVA analysis performed on SCFAs proportions pooled across all sampling times revealed a significant effect of "SCFAs" ($p \leq 0.0001$, ****) and *Treatment* ($p = 0.0207$, *).

Two-way ANOVA analysis of acetate concentrations revealed a significant effect of *Time* ($p = 0.0002$, ***). Post hoc comparisons showed significant differences: (1) in the CF, between T0 and T36 ($p = 0.0279$, *) and between T0 and T48 ($p = 0.0178$, *); (2) in the HTF, between T0 and T12 ($p = 0.0294$, *), T0 and T24 ($p = 0.0099$, **), and T0 and T36 ($p = 0.0241$, *).

Two-way ANOVA analysis of propionate concentrations indicated a significant effect of *Time* ($p = 0.0016$, **). Post hoc comparisons revealed that significant pairwise differences occurred only in the HTF group, specifically between T0 and T36 ($p = 0.0090$, **), T0 and T48 ($p = 0.0050$, **), T12 and T36 ($p = 0.0090$, **), and between T12 and T48 ($p = 0.0050$, **).

Two-way ANOVA analysis of butyrate concentrations presented a significant effect of *Time* ($p = 0.0038$, **) and *Fermenter* ($p = 0.0241$, *). Post hoc comparisons showed a significant difference in the CF between T0 and T48 ($p = 0.0300$, *). No difference was revealed between CF and HTF for any of the SCFAs analysed (Figure 6).

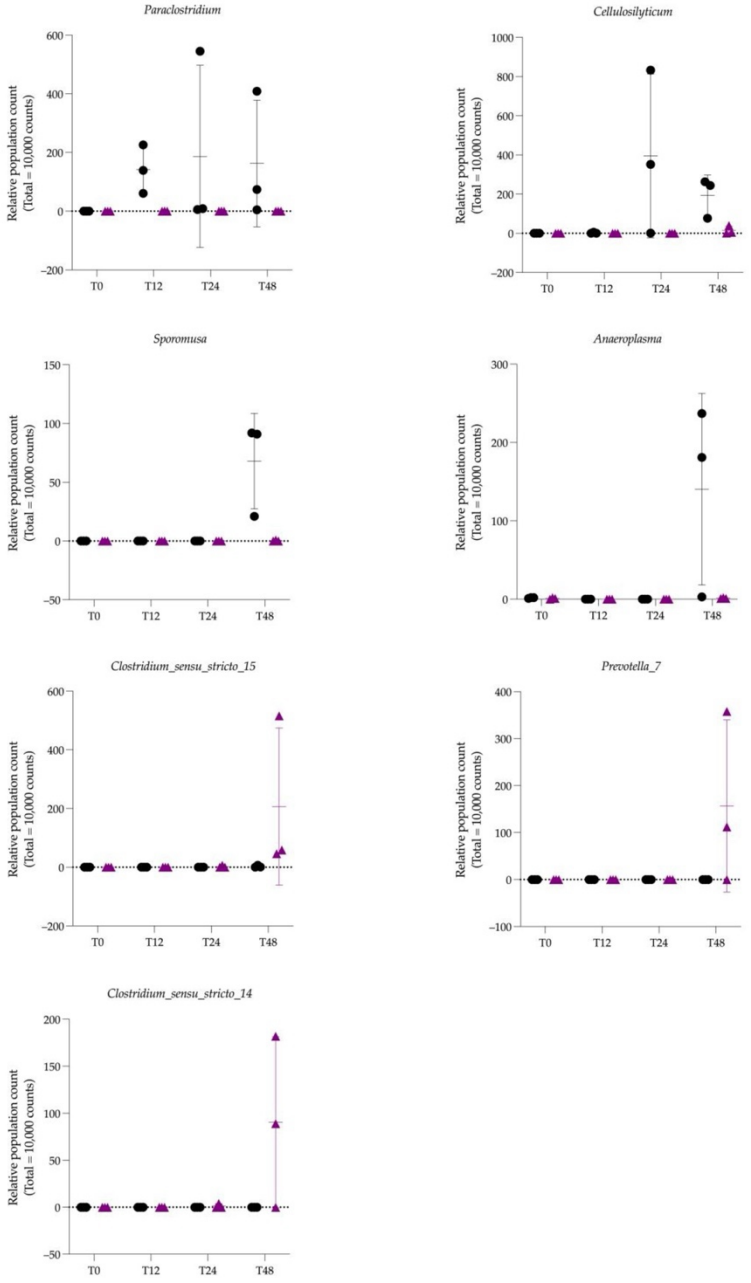


Figure 5. Graphical representation of relative population count (total 10,000 counts). Each symbol represents one fermenter: black circles correspond to control fermenters and purple diamonds correspond to hypoglycin A-treated fermenters. Horizontal bars indicate the mean, and vertical bars indicate the standard deviation for each group.

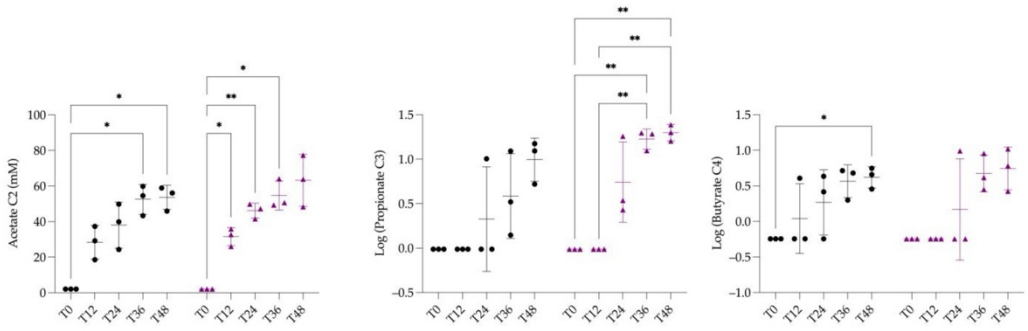


Figure 6. Graphical representation of acetate concentration (mM) and log-transformed concentration of propionate and butyrate in batch experiment. Black circles represent individual measurements from control fermenters, and purple diamonds represent individual measurements from hypoglycin A–treated fermenters. Horizontal bars indicate the mean, and vertical bars indicate the standard deviation for each group. Significantly different with a *p*-value of 0.05 or less: * < 0.05; ** < 0.01.

3.4. Quantification of Hypoglycin A and Statistical Analysis

3.4.1. Stability of Hypoglycin A in the Nutritional Medium

No statistical difference was found at the sampling time points, indicating that HGA remains stable in the autoclaved nutritional medium when the pH and temperature are kept constant.

3.4.2. Hypoglycin A Concentration in the Control Fermenters

A repeated measures one-way ANOVA was performed with either the Dunnett or Tukey correction. The multiple comparison test with the Dunnett correction was applied to compare each time point to the control reference (i.e., T0) and revealed a significant difference between T0 and T12 ($p = 0.0128$, *) and between T0 and T36 ($p = 0.0001$, ***). The multiple comparison test with the Tukey correction was applied to identify specific time points that differed significantly from others. This second test revealed significant differences between T0 and T12 ($p = 0.0167$, *) and T36 ($p \leq 0.0001$, ****) but also between T12 and T36 ($p = 0.0095$, **) (Figure 7).

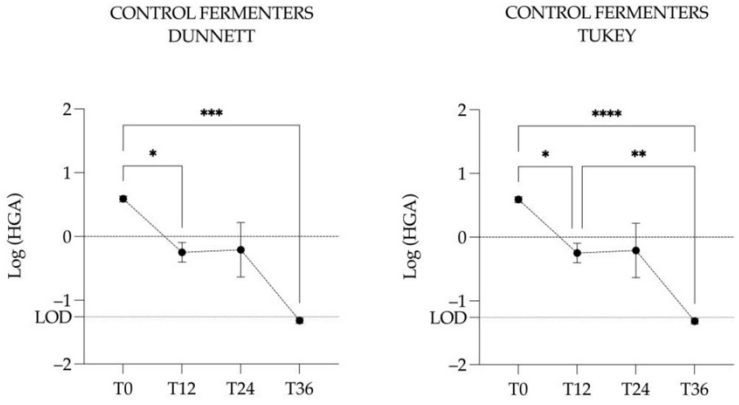


Figure 7. Graphical representation of the log-transformed concentration of hypoglycin A in the control fermenters. Circles represent the mean concentration across the three control fermenters, and vertical bars indicate the corresponding standard deviation. The LOD is the limit of detection and is expressed in Log (LOD). Significantly different with a *p*-value of 0.05 or less: * < 0.05; ** < 0.01; *** < 0.001; **** <0.0001.

3.4.3. Hypoglycin A Concentration in the Hypoglycin A-Treated Fermenters

Considering T0 as the reference time, the multiple comparison test with the Dunnett's correction revealed that T0 was significantly different from T0* (p = 0.0110, *), T2 (p = 0.0207, *), T4 (p = 0.0418, *), T12 (p = 0.0085, **), T16 (p = 0.0143, *), and T20 (p = 0.0196, *).

Considering T0* (i.e., after adding HGA) as the reference time, the multiple comparison test with the Dunnett's correction revealed that T0* was significantly different from T0 (p = 0.0110, *), T2 (p = 0.0087, **), T4 (p = 0.0002, ***), T6 (p = 0.0189, **), T16 (p = 0.0078, **), and T20 (p = 0.0043, **) (Figure 8).

The Tukey multiple comparison test identified significant differences between all the time points without using a reference time: T0 vs. T0* (p = 0.0176, *), T0 vs. T2 (p = 0.0341, *), T0 vs. T12 (p = 0.0142, *), T0 vs. T16 (p = 0.0232, *), T0 vs. T20 (p = 0.0322, *), T0* vs. T2 (p = 0.0145, *), T0* vs. T4 ($p \leq 0.0001$, ****), T0* vs. T6 (p = 0.0036, **), T0* vs. T16 (p = 0.0134, *), T0* vs. T20 (p = 0.0089, **), and T4 vs. T20 (p = 0.0424, *). Tukey test results are presented to emphasise significant differences between time points that were not identified by the Dunnett test (Figure 8).

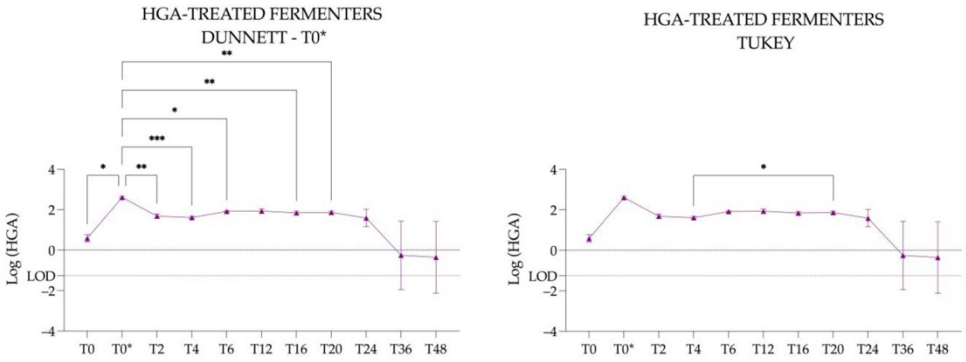


Figure 8. Graphical representation of the log-transformed concentration of hypoglycin A in the hypoglycin A-treated fermenters. Triangles represent the mean concentration across the three hypoglycin A-treated fermenters, and vertical bars indicate the corresponding standard deviation. The LOD is the limit of detection and is expressed in Log (LOD). Significantly different with a *p*-value of 0.05 or less: * < 0.05; ** < 0.01; *** < 0.001.

3.4.4. Hypoglycin A Concentration Comparison Between Control Fermenters and Hypoglycin A-Treated Fermenters

The two-way ANOVA on log-transformed data revealed a significant effect of *Time* (p = <0.0091, **) and *Treatment* (p = 0.0056, **). Moreover, the source of variation is explained by *Time* for 40.38%, by *Treatment* for 33.85%, by the interaction of "*Time* × *Treatment*" for 10.92%, and, finally, by the factor *Fermenter* for 4.62%.

Moreover, Tukey's multiple comparison test revealed that (1) the HGA concentration was significatively different between CF and HTF at T0* ($p \leq 0.0001$, ****), T12 ($p \leq 0.0001$, ****), and T24 (p = 0.0067, **); (2) within the CF, the significant differences were T0 vs. T12

(p = 0.0204, *), T0 vs. T36 ($p \leq 0.0001$, ****), and T12 vs. T36 (p = 0.0117,*); (3) within the HTF, the significant differences were T0 vs. T0* (p = 0.0110, *), T0 vs. T12 (p = 0.0087, **), and T0* vs. T12 (p = 0.0499, *) (Figure 9).

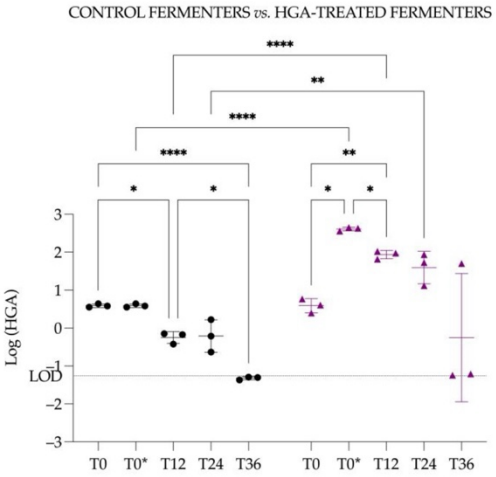


Figure 9. Graphical representation of the log-transformed concentration of hypoglycin A in the control fermenters and in the hypoglycin A-treated fermenters. Black circles represent individual measurements from control fermenters, and purple diamonds represent individual measurements from hypoglycin A–treated fermenters. Horizontal bars indicate the mean, and vertical bars indicate the standard deviation for each group. The LOD is the limit of detection and is expressed in Log (LOD). Significantly different with a p-value of 0.05 or less: * < 0.05; ** < 0.01; ****<0.0001.

3.4.5. Kinetic Data Analysis of Hypoglycin A Concentration

The slope of the regression line was significantly steeper in the HTF (−3.80, p = 0.0156) compared with the CF (−0.09, p = 0.0011). No degradation occurred in the NMF (0.69, p = 0.6428). The linear regression analysis of HGA concentrations over time showed a significant decrease in both CF and HTF containing faecal microbiota, but not in the nutrient medium without microbiota (Figure A3, Appendix C).

3.5. Quantification of Methylenecyclopropylacetyl-Carnitine and Statistical Analysis

The results of MCPA-carnitine quantification in HTF were <LOD (i.e., 0.001 nmol/L) at T0, T2, T6, and T10; consequently, no further analyses were performed.

4. Discussion

Given the robust biochemical evidence linking HGA exposure to equine AM, the present study focuses specifically on HGA as the primary protoxin of interest, while recognising that co-exposure to MCPrG may occur in the natural setting

This study represents the first in vitro investigation of the equine colonic microbiota conducted under conditions that exclude any host–microbiota interactions, providing a controlled system to explore microbial mechanisms potentially relevant to equine AM. First, this model reveals differences in colonic bacterial populations between HGA-exposed and non-exposed conditions. Second, the study demonstrates that the protoxin HGA is specifically degraded by the descending colon microbiota of horses, as HGA

remains stable in the sterile nutritive medium, without any detectable production of the toxic metabolite MCPA-carnitine.

The α-diversity analysis revealed significant differences between groups (i.e., CF vs. HTF) and within the HTF group. The reciprocal Simpson microbial diversity index was significantly impacted by the factor *Time* of the ANOVA analysis, which is consistent with the intrinsic functioning of the in vitro batch technique. Among the diversity indices, evenness exhibited significant differences between groups (i.e., CF vs. HTF), suggesting that HGA exposure mainly altered the relative distribution of taxa rather than community membership. At T12, the distribution of abundance (i.e., evenness) was significantly more uniform in the CF compared to the HTF, reflecting a more balanced representation of populations in the CF and suggesting that exposure to HGA may have selectively promoted or inhibited particular taxa early in the incubation. This pattern is consistent with in vivo observations, where horses classified as controls exhibited a more uniform distribution of microbial populations compared to co-grazers or intoxicated horses [32]. Moreover, the significant difference in evenness within HTF between T12 and T48 likely reflected the progressive disappearance of HGA, which alleviated selective pressures and allowed a more balanced distribution of taxa. Regarding the Figure A2, including T0 in the alpha diversity analysis, and as expected, T0 is similar between the groups. The decrease in diversity was also expected with the batch system, as well as the fact that this decrease is primarily due to a decrease in richness.

The β-diversity (i.e., between community) analysis, including T0, revealed that 66.8% of the microbial variation was significantly explained by the factor *Time*, the factor *Treatment* applied (i.e., adding HGA), and the interaction of both factors. Among these factors, *Time* exerted the strongest influence, consistent with the batch fermentation model where faecal inocula evolve in a closed environment mimicking part of the gastrointestinal tract. Moreover, the experimental design and the inclusion of T0 samples (i.e., representing a single baseline condition shared by all fermenters prior to any treatment) artificially inflated the explained variance and limited the ability of the permutation design to detect treatment-specific differences. When T0 samples were excluded, temporal variation remained the dominant factor structuring microbial communities. The PERMANOVA confirmed that *Time* alone significantly accounted for the largest share of variance (i.e., 26.2%), while the contribution of *Treatment* was comparatively small (i.e., 6.6%) and was not significant. This finding indicates that community changes over time were largely driven by intrinsic microbial succession rather than by direct HGA effects at specific sampling points. Consistent with these results, the Bray–Curtis distance-based RDA confirmed a strong and significant effect of Time on the first canonical axis, reinforcing the predominant role of temporal evolution in shaping the community structure.

To minimise the confounding influence of temporal dynamics and to better disentangle treatment-specific effects from the natural evolution of the microbial communities, DESeq2 analyses were performed by contrasting CF and HTF samples within each individual time point. The DESeq 2 analysis exploring CF vs. HTF at each time point (i.e., T12, T24, and T48) and for all times combined revealed that some bacterial populations are significantly different. Notably, the genus *Paraclostridium* was consistently more abundant in CF than in HTF samples at T12, T48, and when all times were combined. This genus is known to perform Stickland fermentation using leucine and BCAAs. The Stickland reaction is an anaerobic fermentation pathway in which one amino acid is oxidised as an electron donor, while another is reduced as an electron acceptor, thereby maintaining redox balance and generating ATP through substrate-level phosphorylation [62–65]. Hypoglycin A has a molecular weight close to that of BCAAs and can interfere with BCAA metabolic pathways [7]. Moreover, its structural similarity to leucine accounts for the chromatographic challenges in separating the two compounds [7]. Based on these

properties, we hypothesise that *Paraclostridium* may have incorporated HGA instead of leucine or other BCAAs in Stickland fermentation, leading to an energy deficit and population collapse in the fermenters exposed to the toxin. To further validate these findings, the use of absolute quantification approaches, such as qPCR, would be valuable for determining whether the observed relative changes in bacterial populations genuinely reflect true increases or decreases in abundance. It is important to note that *Paraclostridium* was detected at a low relative abundance, suggesting that its direct impact on the overall community dynamics is likely limited. However, the Stickland reaction, a key metabolic pathway potentially involved in HGA utilisation, is not exclusive to *Paraclostridium* and can be carried out by other bacterial taxa as well. This raises the possibility that additional, more abundant populations could contribute substantially to the observed metabolic shifts in the fermenters. Consequently, a targeted investigation focusing on bacteria capable of performing Stickland fermentation could help identify the key taxa driving these functional responses and better elucidate the microbial mechanisms underlying HGA metabolism in this system. Such an approach would provide a more comprehensive understanding of both the compositional and functional impacts of the toxin and could guide future studies aimed at mitigating its effects in vivo. Interestingly, *Anaeroplasma* is present in CF but not in HTF, which is consistent with in vivo observation. Indeed, in a previous study, *Anaeroplasma* was significantly higher in horses considered as control horses vs. horses with AM [32]. Reductions in *Anaeroplasma* abundance have been reported in hypercholesterolemic subjects, a condition also characterised by altered lipid homeostasis [66]. Therefore, the observed depletion of *Anaeroplasma* in the HGA-treated batch may reflect the sensitivity of this genus to disruptions in fatty acid metabolism caused by the toxic metabolite MCPA-CoA.

The proportions of acetate, propionate, and butyrate are similar to those described in horses in the literature: 70–80%, 10–20%, and 5–10%, respectively, in the caecum and the colon [67]. Moreover, these concentrations of SCFAs indicate that the batch system worked as expected and without a significant difference between CF and HTF. The significant effect of *Time* observed for all three SCFAs reflects the natural progression of microbial fermentation in the batch model. The donor's faeces were introduced into the system under conditions mimicking the colonic environment, and the microbiota were required to adapt to these conditions. Acetate appeared as early as T12 in all fermenters, whereas propionate and butyrate became detectable at T36 and T48, respectively—a pattern consistent with previous findings [35]. Propionate formation from pyruvate involves two major microbial pathways: the succinate pathway, which proceeds via oxaloacetate and succinate, and the acrylate pathway. These multi-step conversions likely contribute to the delayed accumulation of propionate in the system [67]. Butyrate is synthesised by strictly anaerobic bacteria and many of these anaerobic bacteria rely on acetate as a co-substrate or precursor via the butyryl-CoA/acetate CoA-transferase pathway. The delayed detection of butyrate and its higher fermenter-to-fermenter variability may reflect the complex microbial interactions and slower establishment of cross-feeding relationships required for its production [68–70]. Moreover, the multiple comparisons for each SCFA also revealed some interesting facts despite the presence of significant differences between CF and HTF. The acetate post hoc comparisons showed early significant differences in HTF compared to CF, which can be explained by the fact that HGA modifies the microbial composition associated with acetate production. The post hoc comparisons for propionate revealed a significant change only in HTF, suggesting the potential influence of HGA on metabolic pathways involved in propionate synthesis. Finally, the post hoc comparisons test for butyrate reflects the necessary time to produce this SCFA, but only in CF, suggesting a possible impact of HGA on butyrate-producing anaerobic bacteria. However, no significant difference was observed between groups (i.e., CF vs. HTF)

Hypoglycin A remained stable in the autoclaved nutritional medium at constant pH and temperature. In contrast, one study reported no change in HGA concentration after incubation with equine gastric or ovine rumen fluid for two hours [71]. However, this study did not consider the retention time in the sheep rumen, nor the fact that in horses, the majority of the gut microbiota is located in the colon rather than in the stomach. These methodological differences may explain why the results of that study do not align with our observations. Conversely, another study documented a decrease in HGA concentration in autoclaved ruminal fluid. Since sterilisation renders microorganisms non-viable, the authors attributed this reduction to abiotic processes, most likely related to changes in pH and/or temperature. This interpretation is consistent with the known stability of HGA in pure water [29] and supports our conclusion that, under certain conditions, HGA degradation can occur independently of active microbial metabolism. The chemical structure of HGA is composed of—as amino acids—one carboxyl group (-COOH) and one amine group (-NH_2) linked to the same alpha carbon (Cα) and by a radical group composed of a methylene group (=CH_2) adjacent to a cyclopropyl cycle composed of three carbon atoms. The carboxyl group and the amine group, which make HGA a hydrophilic molecule, can also influence its acid-base character. Indeed, the carboxyl group can release a proton (H^+), making HGA a weak acid molecule. The pKa of the carboxyl group is generally between 2 and 3, as for most amino acids, indicating that it is dissociated (and therefore deprotonated) at physiological pH (~7.4). The amine group (-NH_2) has a higher pKa, usually around 9–10, meaning it remains mostly protonated (NH_3^+) at physiological pH. Accordingly, at physiological pH, HGA exists primarily as a zwitterion, with the carboxyl group deprotonated (-COO^-) and the amine group protonated (NH_3^+), giving it overall neutrality but with polar sites [72–74]. These polar sites could be potential sites for attracting other molecules. Consequently, the hypothesis of abiotic processes influencing the detection of HGA is a possibility. Especially since the concentration of HGA in our model of stability in an autoclaved medium with constant pH and temperature was stable during the experiment.

Within the CFs, the HGA concentrations drop to values close to or below LOD from T12 to T36. This significant decrease in HGA, in parallel with the stability in the nutritional medium, confirms that HGA is decreasing due to the activity of the microbiota in the batch system. In the HTFs, post hoc comparisons (Dunnett and Tukey) identified numerous significant differences between T0 (or T0*, immediately following HGA addition) and subsequent time points up to T20, indicating a progressive decline in HGA concentration as confirmed by the kinetic analysis. This trend, in parallel with the stability of HGA in the NMF, also suggests a microbial involvement.

In the HTFs, the kinetic profile of HGA degradation shows, at first, a decrease, followed by a plateau phase, and then a second decline. This biphasic pattern may reflect a mechanism of catabolite repression. Catabolite repression is a regulatory process in which microorganisms preferentially metabolise easily available carbon or nitrogen sources, repressing the expression of genes involved in the degradation of alternative or less favourable substrates [75]. In our system, the initial decrease in HGA may result from its direct utilisation by microorganisms, but the subsequent plateau suggests that once other nutrients in the medium are still readily available, microbial metabolism may shift away from HGA. The second decline observed after T20 could then indicate that, as preferred substrates become depleted, the microbial community resumes the degradation of HGA. This phenomenon is well described in microbial ecology and supports the idea that HGA degradation is not only possible but also subject to regulation depending on the nutritional context [75].

The two-way ANOVA comparing the CF and the HTF revealed significant effects of *Time* and *Treatment*. These findings collectively demonstrate a temporal evolution of HGA

concentrations and a difference between the CF and HTF. As expected, HGA levels differed significantly between groups at T0*, T12, and T24, consistent with the timing of HGA exposure and subsequent microbial transformation. Moreover, HGA levels were not significantly different at T0 between CF and HTF (i.e., as no HGA had yet been added). Interestingly, results from a previous study on ruminal batch cultures provide a useful point comparison. In that system, the incubation of HGA in ruminal fluid batch cultures revealed a decrease in HGA over time, with a significant decrease after 8 h and undetectable levels after 24 h [29]. In the present study, within the HTF, the significant difference was already noted after 2 h and values <LOD after 48 h. Despite an initial concentration of HGA being relatively similar (i.e., 564 ± 133 ng/mL HGA in the sheep ruminal fluid and 411 ± 46 ng/mL HGA in the equine colonic fluid), the difference between the first time point of significant decrease in HGA (i.e., 8 h in sheep ruminal fluid and 2 h in equine colonic fluid) underlines the "*Time* × *Treatment*" effect observed. Moreover, this difference may be due to the difference in microbial composition between sheep ruminal fluid and horse colonic fluid. Indeed, the authors explained that the decrease in HGA in the active ruminal fluid was due to the activity of microbial enzymes in this medium via the integration of HGA (i.e., an amino acid) into microbial protein [29].

The kinetic analysis confirmed the results obtained by ANOVA, showing a clear decrease in HGA concentration over time only in the fermenters containing faecal microbiota. Moreover, the difference in the slope of regression in CF vs. HTF indicated a dose-dependent microbial degradation of HGA. The absence of a significant slope in NMF indicated that no degradation of HGA occurred in an autoclaved medium. As HGA concentrations remained unchanged in the NMF without microbiota during the initial incubation period, additional measurements at later time points were not performed.

The concordance between both approaches strengthens the evidence that HGA degradation occurs through microbial activity rather than abiotic processes.

The absence of MCPA-carnitine was expected as previously described in the literature on sheep ruminal fluid batch cultures (as well as MCPA-glycine) [29]. The absence of toxic metabolites in the sheep ruminal fluid (i.e., MCPA-carnitine, MCPA-glycine, MCPF-carnitine, and MCPF-glycine) [29] and in the present horse colonic batch system (i.e., MCPA-carnitine) demonstrates the absence of toxic metabolism (in the sense that conjugation with glycine and carnitine does not take place) of HGA (or MCPrG) in the digestive tract of herbivores. In the present study, HGA concentrations decreased markedly during the incubation in the faecal batch cultures, while remaining stable in the corresponding sterile nutritive medium, thereby confirming that its degradation was microbially mediated. Therefore, it can be assumed that if the digestive microbiota played a role in HGA poisoning, this role might be more "protective", supporting the hypothesis that microbial activity promotes detoxification rather than activation into toxic metabolites. However, this detoxification occurs in the distal part of the digestive tract, suggesting that such microbial degradation may occur too late to prevent the host's systemic uptake of the protoxin, which primarily happens in the small intestine. This interpretation is consistent with previous conclusions regarding proximal fermenter species, which stated that: "Their gut morphophysiology may act as a protection as toxins may be transformed in the rumen. Indeed, the degree of protection against A. pseudoplatanus may be directly linked to the rumen retention time of soluble molecules" [11]. These findings reinforce the hypothesis that the site and timing of microbial fermentation are critical determinants of susceptibility to HGA poisoning. Species in which fermentation occurs before the small intestine (e.g., ruminants) may inactivate HGA before absorption, whereas in hindgut fermenters like horses, the protoxin is likely absorbed upstream of microbial degradation. Moreover, this "protection" conferred by the proximal position of the fermentation tank could explain the greater number of HGA intoxication in horses compared to other

herbivores, even considering differences in species management (i.e., the fact that horses are more often at pasture in autumn or spring compared to other herbivores as cattle or sheep)". The implication of intestinal microbiota as a "protective actor" is already known for other phyto-protoxins as illustrated by mimosine metabolism in ruminants [76,77]. This example of mimosine metabolism in ruminants highlights that the ability of certain microbial communities to metabolise plant protoxins can evolve as an adaptive mechanism, conferring host tolerance to otherwise harmful compounds. Such examples illustrate the potential ecological and evolutionary relevance of microbial detoxification in herbivorous species. Despite its toxic effects, ruminants in certain regions tolerate mimosine ingestion due to microbial degradation of its goitrogenic and toxic metabolite [78,79]. Transfaunation experiments confirmed that ruminal microbiota confer resistance to mimosine toxicity [78,80]. Notably, *Synergistes jonesii*, an anaerobic bacterium isolated from Hawaiian goats, utilises the toxic metabolite for growth, demonstrating microbial adaptation to detoxification [81,82].

Gut microbiota is known to vary from one individual to another due to age or physiological status, but also due to several factors, such as diet, nutritional supplements, exercise, seasons, and medications [33]. To limit the individual impact on the faecal microbiota used in this study, two horses were selected, and faecal samples were pooled. Nevertheless, the number of horses in the present study could have been greater (n = 4) as suggested for in vitro fermentation studies [39]. Considering "age", the two horses are considered adult horses and not mature or elderly horses; consequently, they did not exhibit a microbiome rearrangement observed in horses over 20 years of age [83] or a decline in bacterial α and β-diversity observed with advancing age [83,84]. The impact of sex is not clearly established in the literature, with contradictory results in horses [84,85]. Moreover, the horses involved in this study were not pregnant or lactating, cancelling out the possible impact of this specific physiological status on their intestinal microbiota [33]. Horses developing AM were at pasture for at least 6 h a day, and some of them were supplemented with hay [86,87]. Consequently, selected horses encountered this condition and received no grain or supplement: thus, their diet was a forage-based diet (i.e., grass and hay). Although differences exist in α-diversity of faecal microbiota between grass diet or grass/hay diet [88,89], these differences are less important compared to a diet partially composed of grains [90]. Fibrolytic bacteria are therefore expected, as these bacteria help break down fibre with the production of SCFAs [67,90]. The bacterial community in the forage-based diet is dominated by two phyla, Firmicutes and Bacteroidetes, which both account for more than 80% of the overall abundance of bacterial phyla [89]. Seasonal variation also influences faecal microbiota [84,89,91] either via the composition of the bacterial community in the environment (soil, grass, …) or via the nutrient composition of the pasture [89,92]. The main seasons at risk for AM are autumn and spring; horses involved in this study were sampled during spring 2022. Finally, the conservative procedure of storing faeces at −80 °C prior to the experimental procedure can be debated. Indeed, a recent study reported that freeze–thawing decreased the bacterial viability by 47% [93]. However, other studies have shown that storage at −80 °C does not lead to significant differences compared to fresh faecal samples, even over several months [94,95]. Consequently, some studies have used a temperature of − 80 °C for the storage of their control samples for their analyses [96]. Moreover, other studies have demonstrated that storage at −20 °C does not induce significant changes in terms of relative abundance, α-diversity, and β-diversity [97,98].

The use of in vitro systems to study equine microbiota has already been scientifically approved and is considered reflective of in vivo conditions [35,36]. However, the absorption of amino acids, such as HGA (i.e., a small non-proteogenic amino acid [7,74]), mainly takes place in the jejunum and ileum, with a net disappearance of nitrogen (N) from 16%

to 58% pre-caecally [47,99,100]. Consequently, the possibility that HGA reaches the colon in horses could be challenged by the distal location of this fermentation tank. However, studies about nitrogen dynamics in the equine gastrointestinal tract indicate that 11 to 30% of the apparent N digestion occurs in the small intestine vs. 40% to 70% in the hindgut [101–103]. Both cationic and neutral amino acid transporter genes are expressed in the equine large intestine, suggesting their potential role in microbial and dietary amino acid absorption [104]. In the caecal compartment, an injection of the 15N isotope of nitrogen led to the appearance of labelled essential and non-essential amino acids, urea, ammonia, and lysine in the caecal veins. These observations indicate that horses are able to digest and absorb microbial protein from the large intestine [105]. In humans, the microbiota is greater in the more distal part of the intestine compared to the proximal part of the intestine, where the concentrations of proteins, peptides, and amino acids are relatively high. Amino acids are not significantly absorbed by the colonic mucosa but are intensively metabolised by the large intestinal microbiota. The preferred amino acid substrates of human colonic bacteria include lysine, arginine, glycine, and the branched chain amino acids (i.e., leucine, valine, and isoleucine), resulting in the generation of a complex mixture of metabolic end products, including, among others, ammonia, SCFAs (acetate, propionate, and butyrate), and branched-chain fatty acids (valerate, isobutyrate, and isovalerate) [106].

Moreover, the unexpected presence of HGA in the faeces of the horses used in the present study (i.e., considered as control and toxin-free horses, since neither protoxin nor toxic metabolites were detected in their blood analysis) indicates that HGA does reach the distal part of the digestive tract and comes into contact with the colonic microbiota in vivo. This finding could be explained by the difference in sensitivity between the LOQ (i.e., 0.090 µmol/L) of the method used to quantify HGA in the blood of horses and the LOD (i.e., 0.055 ng/mL) of the method used to quantify HGA in the batched faecal samples and/or by the fact that part of the ingested amino acid is not absorbed in the small intestine and thus reaches the colon. Therefore, it can be hypothesised that the amino acids ingested by the horse such as HGA, may reach the caecal and colonic microbiota. Nevertheless, the presence of HGA in the faeces of horses selected as "toxin-free" may have already influenced the composition of their colonic microbiota, potentially introducing a bias in the present experimental design.

Future research should aim to fully unravel the fate of HGA within the gut ecosystem and its interactions with microbial communities. Radiolabelled carbon tracing could provide decisive insights into the metabolic pathways involved and clarify whether HGA is transformed, assimilated, or detoxified by specific microbial taxa. In parallel, metabolomic approaches could comprehensively profile the chemical transformations of HGA and related metabolites, allowing a deeper understanding of how microbial activity shapes the local metabolic landscape. Beyond mechanistic understanding, the identification of bacterial groups with protective potential would pave the way for innovative interventions, ranging from microbial transfaunation strategies to the rational design of next-generation probiotics. Such approaches hold the promise not only of mitigating the risks associated with HGA exposure in equids but also of advancing our broader comprehension of host–microbiota interactions in the context of xenobiotic metabolism.

5. Conclusions

For the first time, an in vitro experiment confirmed that the equine intestinal microbiota is modified by the presence of HGA. Moreover, the decrease in the protoxin concentration during the experiment supports the protective role of the microbiota, given that HGA remains stable in autoclaved nutritive media without faecal inocula. Similar observations have also been reported previously in sheep ruminal content. In particular, one genus, *Paraclostridium*, was identified as having a significant impact on the system. Further

investigations are needed to confirm which genera or species are involved in HGA degradation. Understanding which microorganisms participate in this process could pave the way for potential therapeutic strategies.

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Institutional Review Board Statement: Ethical review and approval were waived for this study because all procedures complied with both national and international guidelines for animal welfare. The Animal Ethics Committee of the University of Liège was consulted and confirmed that the sampling procedure was part of routine veterinary practice for diagnostic purposes or annual health check-ups.

Informed Consent Statement: Informed consent was obtained from the owner of the horses included in the study.

Data Availability Statement: Raw amplicon sequencing libraries were submitted to the NCBI database under bioproject number PRJNA1335877.

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Abbreviations

The following abbreviations are used in this manuscript:

AM	Atypical myopathy
HGA	Hypoglycin A
MCPrG	Methylenecyclopropylglycine
BCAA	Branched-chain amino acids
MCPA-CoA	Methylenecyclopropylacetyl-coenzyme A
MCPA-carnitine	Methylenecyclopropylacetic acid-carnitine
MCPA-glycine	Methylenecyclopropylacetic acid-glycine
UPLC-MS/MS	Ultra-performance liquid chromatography with mass spectrometry
LOQ	Limit of quantification
LOD	Limit of detection
MTD	Maximum tolerated dose
HED	Human equivalent dose
BSA	Body surface area
CFs	Control fermenters
HTFs	HGA-treated fermenters
SCFAs	Short-chain fatty acids
PCR	Polymerase chain reaction
ANOVA	Analysis of variance
PERMANOVA	Permutational multivariate analysis of variance
dbRDA	Distance-based redundancy analysis
SPME	Solid phase microextraction
GC-MS	Gas chromatography coupled to mass spectrometry
LLOQ	Lower limits of quantification
ULOQ	Upper limits of quantification

NMF Nutritional medium fermenters
FDR False discovery rate

Appendix A

Sampling Design and Analysis

Time (hours)	CONTROL FERMENTERS (CF) HGA = 0 µg/L												HGA-TREATED FERMENTERS (HTF) HGA= 454 µg/L												NUTRITIONAL MEDIUM FERMENTERS (NMF)	
	Fermenter 1				Fermenter 2				Fermenter 3				Fermenter 4				Fermenter 5				Fermenter 6				Fermenter 7	Fermenter 8
	HGA	MCPA-carnitine	MICROBIOTA	SCFAs	HGA	MCPA-carnitine	MICROBIOTA	SCFAs	HGA	MCPA-carnitine	MICROBIOTA	SCFAs	HGA	MCPA-carnitine	MICROBIOTA	SCFAs	HGA	MCPA-carnitine	MICROBIOTA	SCFAs	HGA	MCPA-carnitine	MICROBIOTA	SCFAs	HGA	HGA
0	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
0*	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
2	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
4	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
6	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
8	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
10	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
12	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
14	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
16	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
18	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
20	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
22	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
24	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
30	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
36	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
42	✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓			✓	✓
48	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Figure A1. The first column indicates the sampling time. T0 corresponds to the baseline time point, while T0* refers to 10 min after adding HGA in HGA-treated fermenters. ✓ indicates the samples taken; white cells represent the samples that were analysed, whereas grey, textured cells represent the samples that were not analysed. HGA: hypoglycin A; MCPA-carnitine: methylenecyclopropylacetyl-carnitine; SCFAs: short-chain fatty acids.

Appendix B

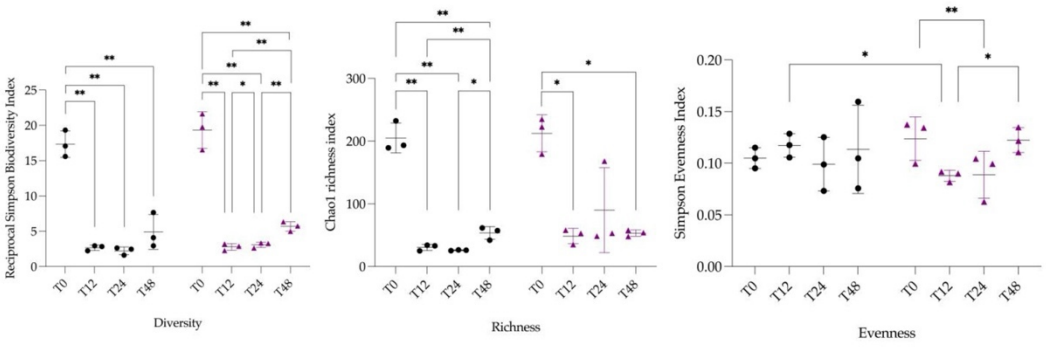

Figure A2. Representation of bacterial α-diversity derived from Reciprocal Simpson Biodiversity index, bacterial genus richness derived from Chao1 index, and bacterial genus evenness derived from Simpson Evenness index. Data are scatter dot plots at genus level for each fermenter in both groups (i.e., control fermenters and HGA-treated fermenters), with mean and standard deviation.

Horizontal bars indicate the mean, and vertical bars indicate the standard deviation for each group. Black circles represent control fermenters, and purple triangles represent hypoglycin A-treated fermenters. Groups were compared with ANOVA followed by post hoc pairwise tests, using FDR multi-tests correction (q-value threshold: * < 0.05; ** < 0.01).

Appendix C

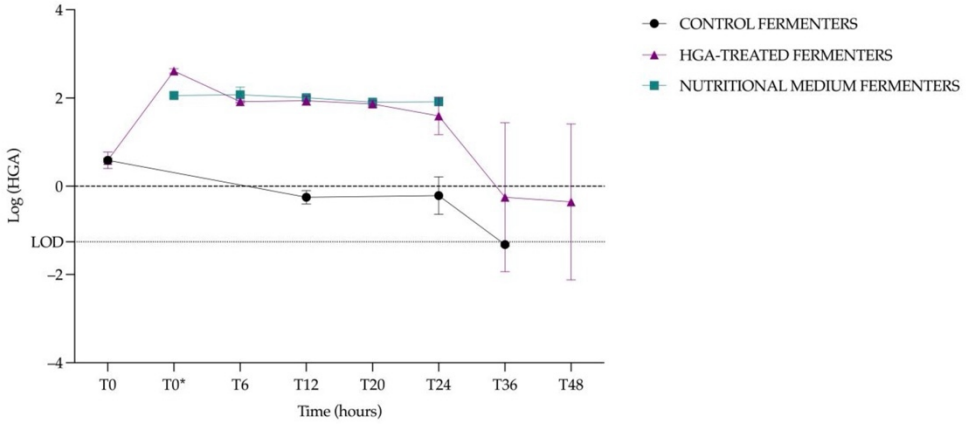

Figure A3. Graphical representation of log-transformed hypoglycin A (HGA) concentration in Nutritional Medium Fermenters, hypoglycin A-treated fermenters, and control fermenters.

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CONCLUSION OF THE SECOND STUDY

This *in vitro* study constitutes the first investigation of the equine colonic microbiota conducted under conditions entirely excluding host–microbiota interactions, thereby offering a controlled framework to elucidate microbial mechanisms potentially relevant to AM.

The results revealed distinct microbial community profiles between HGA-exposed and control fermenters. Analyses of α-diversity indicated significant differences between treatments, primarily affecting evenness rather than richness, suggesting that HGA exposure selectively modified the relative distribution of taxa at early incubation stages. Several taxa were significantly affected by HGA exposure, notably *Paraclostridium*, which was consistently less abundant in HGA-treated fermenters. This genus is known to perform Stickland fermentation, and it is plausible that HGA interferes with this metabolic pathway, thereby disrupting energy metabolism and leading to the collapse of sensitive microbial populations. Moreover, the depletion of *Anaeroplasma* in HGA-treated fermenters mirrors previous *in vivo* observations in horses affected by AM and may reflect sensitivity to disturbances in fatty acid metabolism linked to MCPA-CoA.

The protoxin is degraded in the fermenters containing microbiota while remaining stable in sterile medium, and this degradation occurs without the production of MCPA-carnitine. The degradation of HGA revealed a biphasic pattern consistent with a catabolite repression mechanism in which microorganisms preferentially metabolise more accessible substrates before resuming HGA utilisation as nutrients become depleted.

The results complement the *in vivo* observations from the first study and reinforce the hypothesis that the intestinal microbiota may play a protective role rather than a deleterious one in AM.

Discussion

This work aimed to elucidate the potential role of the equine gut microbiota in HGA intoxication and the development of AM. Given the scarcity of knowledge of how this protoxin may interact or influence intestinal microbial communities, the study was structured around three interconnected objectives: (i) to identify *in vivo* alterations in gut microbiota composition and diversity between AM-affected horses and toxin-free controls horses; (ii) to characterise microbiota responses *in vitro* under controlled fermentation conditions, thereby isolating the direct of HGA from host-mediated influences; and (iii) to explore the potential contribution of intestinal bacteria to the pathophysiology of AM, particularly their ability to metabolise or transform HGA. By integrating *in vivo* and *in vitro* approaches, this thesis represents the first comprehensive investigation into the possible involvement of the equine gut microbiota in HGA intoxication.

The primary objective of this thesis was to establish statistically supported differences in gut microbiota composition and diversity between horses suffering from AM and control horses. The latter were defined as toxin-free animals with unremarkable blood profiles, indicating the absence of any detectable metabolic disturbances, thereby providing a robust framework for comparison. This goal was achieved through a comparative analysis of faecal samples, which demonstrated that microbial community structure was altered not only in clinically affected horses but also in subclinical cograzers.

The results revealed two major findings: first, faecal microbiota composition differed consistently between control horses, cograzers and intoxicated horses (both survivors and non-survivors), and second, blood concentrations of MCPA-carnitine and C14:1 were significantly correlated with these microbial shifts, suggesting a link between systemic toxin exposure and intestinal ecosystem changes.

Regarding α-diversity, control horses displayed the highest richness and evenness, whereas cograzers and AM horses exhibited markedly reduced diversity and an uneven distribution of taxa. Although such patterns may reflect perturbations in the microbial community, they cannot be classified as dysbiosis *per se*, since dysbiosis requires evidence of disrupted host–microbe mutualism beyond diversity indices (Gagliardi et al., 2018). Furthermore, the identification of subclinical cases may change our understanding of HGA poisoning (Renaud et al., 2024). While its clinical manifestation remains acute, low-level

metabolic alterations occurring over an extended period may give the microbiota sufficient time to actively adapt. Consequently, the shifts in microbial populations may reflect an active adaptive response rather than an unbalanced change indicative of true dysbiosis. Nonetheless, the reduced evenness observed in intoxicated groups suggests the selective enrichment of certain taxa under toxic stress.

At the taxonomic level, both multivariate and pairwise analyses identified key genera driving community differentiation. Six genera exerted a global impact across all groups, while 34 showed differential abundance between control and intoxicated horses. Notably, genera such as *Clostridia*_ge, *Bacteria*_ge, *Firmicutes*_ge, *Fibrobacter*, and *NK4A214_group* emerged as central to the observed compositional changes. Several taxa (*e.g.*, *Clostridia_ge*, *Bacteria_ge*, *Firmicutes_ge*) cannot be resolved at the genus level and consequently, hypotheses regarding changes in their relative abundance are more difficult to formulate. However, significant shifts observed in *Fibrobacter* and *NK4A214_group* provide plausible hypotheses regarding how the host's metabolic disturbances (*i.e.*, inhibition of β-oxidation by toxic metabolites, altered energy metabolism, and preserved glycolytic pathway) may differentially affect their ability to adapt. Specifically, the decrease in *Fibrobacter* likely reflects the reduced availability or utilisation of complex structural carbohydrates in an intestinal environment affected by impaired lipid metabolism, whereas the increase in *NK4A214_group* may result from its greater ability to exploit simpler carbohydrates that remain accessible when the host shifts toward glycolysis as its primary energy source.

Collectively, these findings demonstrate that AM intoxication is associated with measurable shifts in faecal microbiota composition, characterised by reduced diversity, a decline in cellulolytic taxa, and the proliferation of genera adapted to alternative substrates utilisation.

The correlation between microbial variation and blood markers relevant to atypical myopathy (*i.e.*, MCPA-carnitine and C14:1) further supports a systemic link between the host's metabolic state and gut ecology.

By establishing these *in vivo* differences, the present study provides a strong foundation for subsequent mechanistic investigations designed at disentangling the bidirectional interactions between host energy metabolism, toxic exposure, and microbial community dynamics.

The second objective of this thesis was to characterise microbiota modifications under controlled *in vitro* conditions, thereby eliminating host-related metabolic and immunological influences. This approach provided a unique opportunity to distinguish microbial dynamics directly attributable to toxin exposure from those shaped by systemic *in vivo* responses. The *in vitro* study revealed two main findings: first, the gut microbiota of the equine descending colon is capable of degrading HGA without producing toxic metabolites (*i.e.,* MCPA-carnitine) and second, bacterial community composition differed between fermenters exposed to HGA and unexposed controls, although temporal progression remained the dominant factor shaping microbial trajectories.

Focusing on the first finding, further analysis confirmed that HGA degradation is performed by the microbiota rather than abiotic processes. Indeed, HGA remained stable in autoclaved nutritional medium under constant pH and temperature, confirming that its significant decrease in the batch system is microbiota-dependent. The kinetic profile of HGA degradation is consistent with catabolite repression (Magasanik, 1961), whereby microorganisms preferentially metabolize more readily available nutrients, temporarily delaying HGA utilization until other substrates become depleted. These results demonstrate that HGA can be actively degraded by the equine gut microbiota and that its catabolism is regulated according to nutrient availability, reflecting typical ecological strategies of microbial communities. These findings are consistent with observations from a sheep model, which also reported a decrease in HGA concentration when the protoxin was exposed to the digestive microbiota, without evidence of toxic metabolite formation (Engel et al., 2025). In the same study, the fate of MCPrG and HGB was also examined. Incubations of *Acer pseudoplatanus* seeds with active ruminal fluid revealed that HGA concentrations initially increased, coinciding with a decrease in HGB, suggesting a transformation of HGB into HGA through γ-glutamylation. This process is catalysed by γ-glutamyl transpeptidase (GGT), an enzyme also described in ackee fruit (Blighia sapida, *Sapindaceae*) (Bowen-Forbes and Minott, 2011). A similar mechanism is likely applicable to MCPrG, which can also exist as a γ-glutamyl peptide. γ-Glutamylation is a reversible reaction in which the γ-glutamyl residue of glutathione (GSH) is transferred to an acceptor amino acid, producing a γ-glutamyl-amino acid and cysteinyl-glycine, catalysed by GGT (Meister, 1981; Orlowski and Meister, 1970; Osuji, 1980). Beyond its metabolic role, this modification is known to facilitate the absorption and transport of

amino acids across cell membranes, particularly in the kidneys, liver, and intestine (Hahn et al., 1978; Meister, 1981; Orlowski and Meister, 1970). Such a property may therefore enable HGA and MCPrG, in their γ-glutamylated forms, to cross biological membranes and potentially influence their bioavailability and toxicity.

Analysis of α-diversity indicated that community richness and evenness increased over time in both conditions, reflecting the intrinsic functioning of the batch fermentation model. The rise in diversity was more pronounced in control fermenters, partly driven by a significant increase in richness. In contrast, HGA-treated fermenters exhibited a delayed and less marked increase, suggesting that toxin exposure selectively influenced the establishment or persistence of certain taxa. Notably, at early incubation stage (*i.e.*, shortly after HGA spiking, when the toxin is still largely present), evenness was lower in HGA-treated fermenters compared to controls, consistent with a selective promotion or inhibition of taxa shortly after toxin addition. This pattern closely mirrors the *in vivo* findings, where intoxicated horses displayed reduced evenness compared to toxin-free controls.

The β-diversity analysis confirmed that "*Time*" factor significantly shaped community variation. These results highlight the strong structuring role of temporal succession in batch systems. Differential abundance analyses reinforced the predominance of temporal dynamics, with twenty-five genera differing significantly across time points, compared to only one genus consistently associated with treatment. Among the temporally affected genera were several unclassified groups (*e.g.*, *Lachnospiraceae*_ge, *Anaerovoracaceae*_ge, *Ruminococcaceae*_ge), complicating functional interpretation. In contrast, the genus *Paraclostridium* was identified as significantly impacted by HGA treatment. This taxon is notable for performing Stickland fermentation, an anaerobic pathway where amino acids, including BCAAs, serve as electron donors and acceptors to sustain redox balance and ATP production (Britz and Wilkinson, 1981; Nisman, 1954; Pavao et al., 2022). Given the structural similarity of HGA to leucine (Billington and Sherratt, 1981) , it is plausible that *Paraclostridium* mistakenly incorporated HGA into Stickland fermentation, disrupting its energy metabolism and contributing to its decline under toxin exposure. This observation provides the first experimental evidence of a potential microbial interaction with HGA metabolism independent of the host in an equine model. Interestingly, *Anaeroplasma* was detected in control fermenters but absent in HGA-treated fermenters, reflecting *in vivo* observations where this genus is higher in control horses than in

horses with AM, and suggesting that its depletion may result from sensitivity to disruptions in fatty acid metabolism induced by MCPA-CoA, similar to patterns reported in hypercholesterolemic subjects with altered lipid homeostasis (Granado-Serrano et al., 2019).

These findings demonstrate that *in vitro* models can capture key features of microbial community responses to HGA exposure while ruling out host-dependent confounders. Although temporal variation dominates microbial dynamics in batch fermentation systems, specific taxa such as *Paraclostridium* may play a role in protoxin degradation. However, the observed decline of taxa such as *Paraclostridium* in HGA-treated fermenters may reflect competitive replacement or niche reorganisation rather than direct protoxin degradation or active suppression of other populations. Microbial community dynamics in response to environmental perturbations are shaped not only by the direct effects of toxic compounds but also by the intricate network of interactions among taxa. These interactions encompass a wide range of ecological strategies including cooperation, cross-feeding, competition, nutrient sequestration, and habitat modification (Adekoya et al., 2025). Cooperation can occur when microbial species jointly contribute to a process that benefits the community as a whole, while cross-feeding refers to the metabolic exchange in which by-products from one population serve as substrates for another. Closely related, division of labour describes the partitioning of metabolic functions among distinct taxa, enhancing community efficiency. Detoxification processes also illustrate cooperative behaviour, where one population reduces the toxicity of the environment for others. At the same time, coexistence mechanisms allow multiple populations to persist stably in the same niche, often through resource partitioning, whereas competition emerges when taxa contend for overlapping resources. Nutrient sequestration represents another competitive strategy, whereby microbes monopolise essential substrates to exclude others. In more direct antagonistic interactions, some bacteria induce the lysis of competitors through the production of bacteriocins or other antimicrobial compounds. Finally, habitat modification occurs when microbial activity alters the local environment, for instance through pH shifts or metabolite accumulation, thereby reshaping ecological niches (Adekoya et al., 2025). In this context, the observed decrease of *Paraclostridium* in relative abundance following HGA exposure may not reflect its true disappearance, but rather the outcome of competitive replacement, niche restructuring, or indirect effects mediated by these diverse ecological interactions. This highlights the limitations of relative abundance data for

disentangling absolute population changes and underscores the need for complementary approaches (*e.g.*, quantitative PCR or metagenomic) to more accurately capture microbial population dynamics. Importantly, the demonstration that HGA can be degraded without the accumulation of toxic metabolites highlights a detoxification capacity of the equine hindgut microbiota. These *in vitro* results support the hypothesis that the gut microbiota can directly interact with HGA and potentially mitigate its toxicity, providing a solid foundation for future studies aimed at identifying the specific taxa and metabolic pathways involved in protoxin degradation.

The third and final objective of this thesis was to explore whether the gut microbiota participates in the metabolism of HGA and thereby contributes to the pathophysiological mechanisms underlying AM. By combining *in vivo* and *in vitro* findings, new insights emerge into the potential capacity of microbial communities to interact with the protoxin, and whether their role is deleterious, neutral, or protective.

First, *in vivo* data indicated that changes in faecal microbiota relative abundance were correlated with alterations in host metabolism (*i.e.*, MCPA-carnitine and C14:1), suggesting that the microbiota may act either as a witness or as an active participant in adapting to host metabolic changes.

Furthermore, *in vivo* analyses identified several bacterial genera that were significantly altered between control horses and AM horses were found to contain orthologous genes encoding enzymes involved in BCAAs metabolism (*i.e.*, BCAT and BCKDHc). These enzymes are theoretically capable of participating in the metabolism of HGA given its structural similarity to leucine. However, no genus appeared to possess the complete enzymatic repertoire required for the full conversion of HGA into toxic metabolites such as MCPA-conjugates, which is consistent with the absence of MCPA-carnitine in the *in vitro* fermenters and suggests that HGA is not metabolised into toxic derivatives due to the lack of a fully effective enzymatic pathway.

The *in vitro* study further clarified this issue by directly testing HGA degradation under controlled conditions. Kinetic analyses revealed that HGA remained stable in the absence of microbial activity but underwent rapid and consistent degradation in fermenters containing colonic microbiota, with half-lives decreasing from over 130 hours in sterile medium to

approximately 21 hours in microbial systems. The biphasic degradation observed in toxin-exposed fermenters suggests a regulatory process resembling catabolite repression. Importantly, despite clear evidence of microbial degradation, no toxic metabolites such as MCPA-carnitine were detected, mirroring observations in ruminal fluid incubations (Engel et al., 2025). Collectively, these results suggest that microbial communities can catabolise HGA without producing toxic derivatives (*i.e.*, MCPA-carnitine and MCPA-glycine), pointing toward a potential protective rather than pathogenic role. Nevertheless, it is possible that HGA is degraded into another metabolite or into an MCPA-conjugate with an as yet unidentified molecule. The absence of toxic metabolites in both equine colonic and ruminant batch cultures provides a strong argument that the digestive microbiota of herbivores may contribute to detoxification rather than toxin activation (Engel et al., 2025). This interpretation aligns with earlier hypotheses that the gut microbiota of proximal fermenters such as ruminants may protect their hosts from intoxication by metabolising HGA before systemic absorption, in contrast to hindgut fermenters such as horses (Renaud et al., 2022). Comparable examples exist in ruminant adaptation to other phytotoxins, exemplified by the microbial degradation of mimosine, where specific bacteria such as *Synergistes jonesii* confer protection against toxicity (Allison et al., 1990; Hegarty et al., 1964; Jones, 1981; Kudo et al., 1984). Taken together, these findings suggest that interspecies differences in fermentation site, microbial composition, and retention time explain the higher susceptibility of horses to AM compared to ruminants.

To summarize the common findings between the *in vivo* and *in vitro* studies, both approaches reveal consistent alterations of the gut microbial ecosystem in response to HGA exposure. Firstly, α-diversity, and particularly evenness, was reduced in HGA-intoxicated horses, indicating a disruption of the balance among microbial populations; this effect was similarly observed in the *in vitro* model, suggesting a direct influence of HGA on microbial community structure. Secondly, the genus *Anaeroplasma* was significantly affected in both experimental settings, with its relative abundance decreasing in the presence of HGA. *In vivo*, this may reflect the known sensitivity of *Anaeroplasma* to host lipid metabolism alterations (Granado-Serrano et al., 2019), which are likely perturbed during HGA intoxication. For the

in vitro study, the limited literature on this genus precludes clear mechanistic hypotheses, although the observed decrease suggests a direct or indirect susceptibility to HGA.

Despite its widespread use, 16S rRNA gene sequencing has several inherent limitations that must be considered when interpreting microbiome data. First, taxonomic resolution is often restricted, with many sequences assignable only to higher taxonomic levels such as family or order, rather than to the genus or species level. This limitation can obscure biologically relevant differences between closely related taxa and impede the identification of specific microbial players involved in metabolic processes or toxin degradation. Second, 16S rRNA gene sequencing analyses typically report relative abundances, which reflect the proportion of sequences rather than absolute microbial counts. As a result, observed increases or decreases in relative abundance do not necessarily correspond to true increases or decreases in population size, as shifts may be driven by changes in other community members rather than by expansion or loss of the taxon itself (Regueira-Iglesias et al., 2023). To overcome these limitations, complementary approaches such as quantitative real-time PCR (qRT-PCR) can be employed. This technique uses taxon-specific primers and fluorescent probes to amplify and detect target DNA sequences in real time, allowing precise quantification of absolute abundance (Arya et al., 2005; Jian et al., 2020). By combining 16S rRNA profiling with qRT-PCR, it becomes possible to validate whether changes in relative abundance reflect actual shifts in microbial populations. Additional approaches, such as metagenomics or metatranscriptomics, can provide higher taxonomic resolution and functional insights, enabling the identification of specific genes and metabolic pathways potentially involved in processes such as HGA degradation.

The *in vitro* approach employed offer a rapid, convenient, and cost-effective alternative for investigating microbial responses than the SHIME® model. However, one limitation of this method is the pronounced effect of the factor '*Time*' on statistical analyses, which can potentially obscure treatment-related effects. In this setup, fresh or frozen faecal material from multiple donors is combined and incubated for 48 hours, meaning that microbial populations are subject to continuous temporal shifts throughout the experiment. By contrast, the original SHIME® methodology incorporates an initial stabilization period, followed by the introduction

of a challenge, and a subsequent stabilization phase after challenge removal. Although this approach is more time-consuming, it reduces temporal variability by allowing microbial communities to stabilize before exposure to the experimental treatment, such as HGA (van de Wiele et al., 2015). Consequently, the continuous SHIME® system provides a clearer distinction between effects due to the passage of time and those directly attributable to the treatment, which can be particularly important when assessing subtle microbial responses.

At last, in the *in vitro* study, faecal donors were selected as control horses, showing no signs of atypical myopathy and no detectable HGA or its metabolites in blood analyses. However, HGA was unexpectedly detected in their faecal samples, indicating that the toxin can reach the distal digestive tract and come into contact with the colonic microbiota. This discrepancy may be due to differences in analytical sensitivity between blood (LOQ = 0.090 µmol/L) and faeces (LOD = 0.055 ng/mL), or to incomplete absorption of ingested HGA in the small intestine. Importantly, this finding suggests that even horses considered HGA-free in blood analyses may have been exposed to the toxin in the gut, implying that the number of exposed animals in natural conditions could be underestimated. Consequently, the presence of HGA in "control" faecal material may have influenced the colonic microbiota prior to *in vitro* incubation, introducing a potential bias in the experimental design.

Overall, the results of this thesis support the view that the equine colonic microbiota is a witness of the host's metabolism and, importantly, actively degrades HGA but does not appear to generate toxic intermediates known to drive AM pathogenesis, highlighting its role as a positive player. Unfortunately for horses and other hindgut fermenters, the distal location of their fermentation chamber (and therefore of their microbiota) relative to the primary site of amino acid absorption in mammals (*i.e.*, the small intestine) results in protoxin absorption into the host bloodstream, in contrast to foregut fermenters. In the latter, the fermentation chamber is positioned proximally to the small intestine, allowing a form of "detoxification" of ruminal contents prior to their passage into the small intestine, and thus before amino acid absorption occurs. Nevertheless, the identification of genera with incomplete but relevant enzymatic repertoires leaves open the possibility that certain taxa may contribute indirectly to host susceptibility by interacting with HGA or its analogues. Combining metagenomics,

metatranscriptomics, and metabolomics would provide a comprehensive view of the microbial contribution to HGA bioavailability, transformation, and clearance.

By providing both genomic and functional evidence, the present thesis establishes that the gut microbiota (i) participates in shaping the metabolic fate of HGA, potentially acting as a protective, rather than a pathogenic, player in the pathophysiology of AM and (ii) is a witness of the metabolic disturbances of the host.

Conclusions & Perspectives

Taken together, the present findings provide novel insights into the question posed by this thesis: ***is the equine intestinal microbiota a player or a witness in hypoglycin A (HGA) intoxication?*** Evidence from both *in vivo* and *in vitro* approaches converges towards a limited but active role of the gut microbiota as well as a witness of the host's metabolism disturbances. *In vitro* degradation assays demonstrated that HGA is consistently metabolised by equine microbial communities, with rapid disappearance in faecal and colonic fermenters compared with its stability in sterile controls. Crucially, this microbial activity was not associated with the formation of toxic metabolites such as MCPA-carnitine, supporting the view that equine gut microbiota does not contribute directly to the pathophysiological conversion of HGA. In parallel, the *in vivo* functional analysis identified bacterial taxa harbouring orthologues of enzymes theoretically involved in HGA metabolism (*e.g.*, BCAT and BCKDHc subunits), but no genus presented a complete enzymatic repertoire to achieve the full toxic transformation pathway. The detection of nearly complete orthologue sets in poorly resolved taxa, combined with the possibility of analogous enzymatic functions, leaves open the hypothesis of partial microbial processing of HGA. Moreover, the bacterial populations highlighted in the *in vitro* investigation are not fully consistent with those identified in the *in vivo* study, further underlining that the microbiota may also act as a witness of the pathological process (as also demonstrated by the correlation of the *in vivo* modifications of bacterial populations and the modifications of blood parameters of the host). Nevertheless, the absence of toxic intermediates in both the equine and ruminant digestive environments (Engel et al., 2025), together with kinetic profiles suggesting HGA utilisation as a nutrient source, indicate that the microbiota may act more as a protective barrier reducing toxin persistence rather than as a driver of its toxic fate. Therefore, the intestinal microbiota of horses should be considered an active participant in HGA degradation, but ultimately as a mitigating player in intoxication rather than a direct contributor to disease pathogenesis.

Future research should aim to refine our understanding of whether the equine intestinal microbiota functions predominantly as a player or a witness in HGA intoxication. A priority will be the identification and characterisation of poorly resolved taxa, such as *Bacteria*_ge and *Bacilli*_ge, which exhibited genomic patterns compatible with partial HGA metabolism. While batch fermentation provided valuable insights, its strong susceptibility to

temporal effects limits the resolution of treatment-specific responses. An alternative approach would be to employ a full SHIME® system, which allows the microbiota to stabilise prior to experimental intervention. By introducing HGA only after such stabilisation, the confounding influence of time on statistical analyses would be reduced. This design would better highlight microbial populations whose dynamics are driven predominantly by the treatment rather than by temporal succession, thereby refining the identification of taxa genuinely involved in protoxin metabolism.

Metagenomic and metatranscriptomic approaches, coupled with targeted cultivation, could clarify whether these communities harbour alternative or analogous enzymatic pathways enabling HGA transformation. In addition, isotopic labelling and metabolomic tracking in controlled *in vitro* systems would help to map the metabolic fate of HGA and its potential assimilation into microbial biomass. Complementary untargeted metabolomic analyses could further identify novel metabolites produced during HGA degradation and reveal broader shifts in microbial metabolic activity, providing insights into functional consequences of intoxication. Comparative studies across herbivorous species could also reveal whether differences in microbial composition contribute to the observed variation in susceptibility to AM. Ultimately, such perspectives may open avenues for microbiota-targeted interventions, either through modulation of gut communities or through the exploitation of protective microbial taxa, to mitigate the risk of intoxication in horses.

An additional strategy that deserves investigation is transfaunation (also termed faecal microbiota transplantation or microbiota transfer (Tuniyazi et al., 2023)) directed specifically to the proximal gastrointestinal tract, particularly the stomach and the small intestine, upstream of the major site of amino acid absorption. The rationale would be to increase the abundance of microbial populations capable of utilising HGA as source of carbon and nitrogen before the toxin can be absorbed or converted systemically. Transfaunation is already applied in equine medicine for several gastrointestinal pathologies as chronic diarrhoea, colitis, and post-antibiotic dysbiosis. Nevertheless, outcomes remain variable, relapse is not uncommon, and treatment protocols lack standardisation (Tuniyazi et al., 2023). In the context of HGA intoxication, the application of transfaunation would require selecting donor horses whose microbiota are enriched in candidate HGA-degrading taxa. Administering the transplant in a

way that targets the stomach and/or the small intestine, rather than exclusively the colon, would be crucial to intercept the toxin prior to absorption. If successful, this approach could enhance the role of the equine intestinal microbiota in removing HGA, thereby reducing the risk and severity of intoxication.

Pre- and probiotics represent another potential strategy to modulate the equine intestinal microbiota in the context of HGA intoxication. However, current evidence does not provide a clear consensus regarding their efficacy. The findings of several studies are constrained by experimental design, uncertainties regarding the accuracy of strain identification, and the presence of publication bias, which may lead to overestimation of positive effects. Moreover, it remains unclear which specific microbial populations should be targeted to enhance HGA degradation, and there is currently no formal proof that the strains commonly used as probiotics induce lasting and functionally relevant shifts in the gut microbiota. Thus, while pre- and probiotics may hold theoretical promise, their practical utility in preventing or mitigating HGA intoxication requires rigorous evaluation, including identification of candidate taxa and demonstration of durable functional effects (Berreta et al., 2021; Berreta and Kopper, 2022; Cooke et al., 2021; Weese, 2025; Weese and Martin, 2011).

From a One Health standpoint, the detection of HGA in horses considered as controls (*i.e.*, free of HGA and MCPA-carnitine in blood) raises important questions about food safety and cross-species risk. In equines, HGA metabolism largely occurs in muscle tissue (Sander et al., 2023), suggesting that contaminated horse meat could carry implications beyond veterinary health. This begs the question of whether systematic screening for HGA in animal-derived foodstuffs should be considered, especially in light of the JVS (Hassall et al., 1954; Hassall and Reyle, 1955). Moreover, AM has been documented in other herbivores, including cervids (Bunert et al., 2018), species that may enter the food chain. While one might assume that ruminants (*e.g.*, cattle, sheep, goats) are protected by their rumen's detoxifying capacity (Engel et al., 2025; Renaud et al., 2022), some studies have detected HGA in cow's milk (Bochnia et al., 2021; Engel et al., 2023), indicating that systemic transfer is indeed possible. The presence of HGA was also described in mare milk (Renaud et al., 2021). Among ruminants, differences in digesta retention time could influence risk of developing AM (Renaud et al., 2022).

Consequently, mammalian herbivores with shorter retention times could pose a non-negligible risk to humans or other animals if HGA is not sufficiently degraded before systemic absorption.

Thus, from a One Health perspective, it would be prudent to further investigate: (1) the prevalence of HGA in meat, milk, and other products from equines and ruminants; (2) which species or production systems are most at risk; and (3) whether regulatory monitoring of HGA in food of animal origin may be warranted.

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