THESIS

GENE EXPRESSION ANALYSIS BEFORE AND AFTER THE PELVIC FLEXURE IN THE EQUINE HINDGUT

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ABSTRACT

GENE EXPRESSION ANALYSIS BEFORE AND AFTER
THE PELVIC FLEXURE IN THE EQUINE HINDGUT

The equine hindgut is the primary site of the horse's nutrient breakdown, absorption, and energy production. More than 60% of the horse's energy comes from hindgut fermentation. In this process, commensal microbes in the hindgut aid in the digestion of plant materials to create volatile fatty acids that can be used by host cells to make energy. Many severe health issues- such as colic, laminitis, or colonic impactions- often occur in the equine hindgut, making it an important site to study to provide better management, treatment, and prevention options for horses suffering from gastrointestinal disease. Although much research exists focusing on the microbiome and overall physiology of the equine hindgut, relatively little addresses the role of gene expression in maintaining a complex yet essential homeostatic balance within the gastrointestinal tract. Previous from our lab found major differences in the microbial content of gastrointestinal compartments of the equine hindgut, separated by the pelvic flexure. The pelvic flexure is a short, narrow, horseshoe-shaped loop in the equine large colon. It defines the ventral and dorsal segments of the colon and is a common site of colonic impaction in horses. Although the pelvic flexure cannot and should not act as a "barrier," something "barrier-like" may be occurring around this region as it pertains to the hindgut microbiome. The mechanism for this action is not defined. As a result, this thesis aims to investigate gene expression in the intestinal epithelial cells of the ventral colon, pelvic flexure, and dorsal colon regions of a healthy hindgut to determine what differences exist. The insight gained from this analysis will provide a baseline for comparison to understand how

gene expression patterns in these tissues adapt to changes in the microbiome and external factors like diet. The results of this thesis are the first steps towards a better understanding of homeostasis in the equine hindgut.

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CHAPTER 1
REVIEW OF LITERATURE

Summary

The research completed for this thesis was an analysis of gene expression in tissues of the equine hindgut by RNA sequencing. The motivation for this work was a previous finding that the microbial communities of the hindgut are distinct and separated by the pelvic flexure. The mechanism by which this is accomplished has yet to be discovered. The interaction between the horse and the various microbial communities is central to maintaining homeostasis in the hindgut, which supports proper digestive physiology. Investigation of gene expression in the hindgut tissues can help to better understand how homeostasis is achieved and maintained. As a background to the experiments and analysis conducted, this literature review covers the topics of homeostasis, the microbiome, the gastrointestinal tract, and gene expression.

Homeostasis

Homeostasis- which combines the words “homeo” meaning similar, and “stasis” meaning steady is the process of maintaining a relatively stable internal environment by responding to potential internal or external variables of change [1]. Some of these variables may include, but are not limited to, changes in temperature, pH, nutrient availability, amount of waste products, cell volume and pressure, solute concentration, CO_2 concentration, and O_2 concentration [1]. Homeostasis is essential for cell survival [1], so understanding how internal and external factors affect homeostatic balance is necessary to understand physiological processes more completely [2]. Intrinsic homeostatic regulators often act automatically [3] during potentially harmful changes by using negative feedback loops to return the body to a healthy, balanced state. Negative feedback

occurs when a controlled variable changes and moves outside of the optimal range and triggers a response that opposes the change, causing the variable to move in the opposite direction of that initial change [1]. For example, when body temperature increases, negative feedback mechanisms, such as sweating and blood vessel dilation, engage in reducing body temperature by increasing the rate of releasing body heat into the environment. The negative feedback loop will end once the body temperature returns to a physiologically appropriate level and balance is restored.

The concepts behind homeostasis were first expressed in 1877 when the German physiologist Edward Pflüger noted that "the cause of every need of a living being is also the cause of the satisfaction of the need" [3]. In 1878, the French physiologist Claude Bernard expanded on Pflüger's observations by defining that "the conditions which must be maintained constant in the fluid matrix of the body to favor freedom from external limitations are water, oxygen, temperature, and nutriment (including salts, fat, and sugar)" [3]. Years later, the term and theory of "homeostasis" was coined by the American physiologist Walter Cannon, expanding upon Bernard's notion of "constancy" of the internal environment by using clear and concrete examples in his work [2]. Cannon's theory of homeostasis focused on maintaining a steady state within an organism regardless of whether the mechanisms involved were passive (such as water movement between capillaries and the interstitium, which reflects a balance between hydrostatic and osmotic forces) or active (such as the process of storing and releasing intracellular glucose) [2]. It was only during the 1960s that homeostatic regulatory mechanisms were more intentionally used to describe discreet processes in physiological systems [2].

The concept of homeostasis can be applied in a “macro-” and “micro-” manner, meaning that disruptions in homeostatic balance in microsystems can affect the overall homeostasis of the macro system. This can be seen when a group of cells in an organ become cancerous, therefore causing disruptions in the overall function of the organ; if a business had 1000 employees and only 500 of them were showing up and working, then the company is less productive and therefore not operating as efficiently. For example, the earlier stages of the growth of cancerous polyps in colon cancer patients can affect the digestive system's overall homeostatic balance by causing bowel obstruction, changes in stool consistency, and the appearance of blood that can cause acute peripheral circulation failure [4-6]. This form of cancer can cause problems for the entire digestive system by affecting the composition of the intestinal lumen (including the microbiome, nutrient content, and presence of immune system elements), the pressure-induced backup from problems releasing harmful toxins from the body, and cancer potentially spreading to other host cells in the intestines and throughout the body. The homeostasis of these living cells is disrupted at the macro or organ-system- level because of the malfunctioning of a group of essential cells at the micro-level.

Homeostasis can be influenced- positively or negatively- by various factors, internal and external to the organism. No matter what the situation is, living organisms often have biochemical systems that actively utilize negative feedback loops- a type of feedback that tends to dampen a process by applying the output against the initial conditions- to return from an altered state back to a normal state since the reaction product leads to a decrease in the initial altered-state response [7]. This type of feedback is often outlined in thermoregulation and blood sugar regulation examples. Every species has a set temperature range that permits normal cellular processes to

function (the range in humans is 97-99 °F, dogs 101–102.5 °F, and horses 99–101 °F); for instance, if a human being has a severe fever of 104.7 °F, that person's proteins may become denatured while the fever potentially damages cell membranes and increases oxidative stress. These changes induced by the fever can lead to problems in cell function and an eventual increase in the presence of white blood cells during an immune response [8]. To maintain temperature homeostasis, a person's body will respond by dilating the blood vessels to release heat into the environment and sweat via the sweat glands [8]. This response causes the body to feel weak and cold, leading to "chills" that people often get during a fever. Still, as heat is lost to the environment via this negative feedback, the body will eventually return to normal body temperature, and the fever will dissipate as the body's natural systems fight off the infection [8]. Negative feedback is a common regulatory mechanism for maintaining homeostasis, with the intrinsic controls of a homeostatic system being built into that system so that balance can be maintained and, hopefully, crises averted [1].

Some intrinsic factors that can alter homeostasis include genetics, fever, ingested food, nutrient deficiencies such as a lack of calcium, and immune response. At the same time, some extrinsic factors that may also cause changes to a homeostatic state include temperature, weather, pressure changes such as traveling to a very high elevation, toxins such as air pollution, external stressors, and lifestyle choices [9]. It is important to remember that homeostasis can be affected by multiple variables. Often, even after significant deviations from a homeostatic state, the body will naturally return to a balanced state and "remember" that previous balance that existed before the dysbiosis occurred [9]. For example, one study involving horses by Te Moller *et al.* in 2017 discussed joint homeostasis and the importance of sustainable athletic training methods. They showed that "biomechanical loading in the form of deliberate exercise has a major influence on

the delicate homeostatic balance within the tissues constituting the diarthrodial joint and their interactions, which is crucial for proper and durable joint functioning" [10]. This means that the amount and intensity of exercise can have a lasting effect on equine joints and their functioning later in life. However, certain joint injuries, such as mild-to-moderate cartilage degeneration within a joint, can be recovered if the horse is allowed three months of stall rest with hand walking before training may resume; resting time may vary depending on the type of injury and how severe the damage is [11]. These examples further display how, naturally, an organism's state of dysbiosis can be recovered. It can often be more easily recovered if the cause of the dysbiosis is known and dealt with safely, methodically, and scientifically soundly.

Additionally, homeostasis as a process is never static; a constant battle exists within a living organism to maintain homeostatic levels that are key to survival, and slight changes in one or many of these homeostatic balances can cause disease-states and even death. In short, the body must maintain relatively stable conditions that allow the organism to survive; homeostasis is an ongoing process rather than a set "state" [17]. For instance, thermoregulation and blood glucose homeostasis are two essential processes that must continuously be monitored and maintained throughout an organism's life, and any deviations from the "average" or "safe" temperature or blood glucose levels can lead to the development of disease states and potentially death [17]. For example, the continuous imbalance of blood glucose levels can lead to various complications, including organ damage, cardiovascular disease, diabetes, etc. [13].

Living organisms would not exist without homeostasis [14]. This is because homeostasis works to maintain the balance of the "self" with the "environment." Disease states will result when

the barriers separating and keeping these distinct elements become blurred. One example discussed how cancers can result when homeostasis is altered in a group of cells, eventually becoming large tumors that can spread throughout the body, becoming more severe and deadly to the host organism. Importantly, homeostasis is influenced by various factors, internal and external, and often, in developing disease states, multiple factors can be involved in developing the health issue (such as a mix of stressors, diet, nutrient deficiencies, and lifestyle). All in all, understanding homeostasis as it works to influence and maintain healthy states in various bodily systems will aid in the scientific community's understanding of how and why certain balanced interactions exist, the development of disease states, potentially how to best recover from disease-states, as well as a better understanding of how to maintain healthier conditions in humans, horses, and other organisms.

GI Homeostasis

GI homeostasis is the balance between the functions of the epithelial cell lining in the gut, the host's immune response to pathogenic organisms, and the observable tolerance that occurs for the present commensal microbes [12,15,16]. In the gastrointestinal tract, homeostasis must be maintained for most processes to happen, and this process involves a complex relationship between a complex network of factors. Some of these factors include, but are not necessarily limited to, the gut structure, host immune system, microbiome, and physiology. On top of this, internal and external changes may influence the homeostatic balance of these systems in the host- for example, a change in diet, environment, or health status could affect healthy GI function and could potentially induce disease-states. In addition, it is important to recognize the importance of the

cellular activity occurring in the mucosal and submucosal layers of the gastrointestinal wall, which actively works to maintain GI homeostasis in various ways.

Maintaining homeostasis in the GI tract requires various factors to work together properly, in a complex yet consistent manner, and in a manner that doesn't end up harming the host. For instance, according to Okumura *et al*., intestinal epithelial cells in the intestinal epithelium greatly contribute to maintaining the symbiotic relationship between gut microbiota and the host by constructing mucosal barriers, secreting various immunological mediators, and delivering bacterial antigens [17]. These cells form mucosal barriers, which include both physical and chemical properties, to segregate the gut microbiome in the lumen spatially from the intestinal epithelium and the host's immune system [17-20], which is vital in protecting the host organism from unnecessary immune responses that can lead to changes in the microbiome, intestinal inflammation, and potential overactive immunity. Therefore, the mucosal lining of the gastrointestinal tract plays an essential role in maintaining GI homeostasis by directly mediating the interactions between the host's epithelial tissues and the microbe-filled lumen of the GI tract.

The mucosal lining of the intestinal epithelium acts as the first line of defense against the infiltration of microorganisms, digestive enzymes, acids, toxins, etc.; this layer coats the internal surface of the GI tract, acting to lubricate luminal contents while also acting as a physical and chemical barrier between self and non-self [21]. Notably, the mucosal layer of the intestinal epithelium can also bind and trap potentially harmful particles during digestion, making it essential in maintaining intestinal homeostasis and the continuous process of protecting host cells from non-self pathogens [21]. The mucosal layer does this by utilizing the beneficial elements present inside

the layer, which contains various protective elements- including but not limited to immune cells, natural antibiotics, and protective lubricants to defend against injury- which may act together or separately to prevent problematic toxins, pathogens, et cetera from entering host cells [22]. It is also structurally important because the stickiness of the mucosal layer can capture foreign particles and effectively remove them while mediating what is allowed or blocked from entering the host cells [22]. Therefore, the mucosal layer plays a key part in maintaining GI homeostasis. It essentially exists as a protective barrier that directly interacts with the intestinal lumen, allowing some aspects through while preventing others from entering host intestinal epithelial cells (IECs).

Another key factor that plays a role in maintaining GI homeostasis is the overall structure of intestinal epithelial cells (IECs) and the communication mechanisms that they constantly employ to promote or inhibit the absorption of certain elements in the intestinal lumen. Besides the protective mucosal layer, IECs possess essential external receptors that can recognize various luminal bacterial signals, with some examples of these receptors including both toll-like receptors- or TLRs- and nod-like receptors (or NLRs) [23]. TLRs are proteins that play a key role in innate immunity by being able to recognize and bind to pathogen-associated molecular patterns or microbes that possess structurally conserved molecules that can be easily read and identified; they are first responders to danger signals that may be present in the gastrointestinal tract [24]. NLRs are found in the cell cytosol and are responsible for detecting invading pathogens and initiating the innate immune response. Still, unlike TLRs, which are membrane-bound proteins, NLRs are not bound to any membrane [25,26]. TLR signaling leads to the expression of inflammatory cytokines and chemokines that respond directly to a signal, which may either activate or inhibit specific immune cells while aiding in the control and development of an immune response [25-

27]. These gastrointestinal immune responses are vital for maintaining homeostasis; for an organism to remain healthy, these IECs must be able to recognize and destroy harmful microbes before- or when- they infect, multiply, and endanger IECs, intestinal tissues, and, therefore, the host. In addition to TLRs, NLRs can act as regulators of a cell's immune response by detecting bacterial components or danger signals that can eventually lead to a host cell's innate immune response; for example, NLRs NOD1 and NOD2 have been shown to induce the activation of MAPK and NF-κB while other NLRs have been shown to induce caspase-1 activation through the assembly of an inflammasome [28-31]. This means that IECs possess receptors that can recognize and identify potentially helpful or harmful microorganisms that are present in the intestinal lumen while also being able to signal to other cells if there is a danger present that needs to be dealt with so that gastrointestinal homeostasis can be reestablished and organismal health will be maintained.

In addition to providing a physical and biochemical barrier that segregates host tissue and lumen microbes, IECs can maintain specialized pathways that deliver luminal antigens and potentially harmful bacterial species to lamina propria-resident antigen-presenting cells, which plays a crucial role in gastrointestinal homeostasis [32]. One especially important example of specialized pathways is the transport pathways for luminal antigens and bacteria, which includes adapting the epithelial barrier for sampling luminal contents to identify and transport certain recognized molecules to efficiently direct appropriate responses to their presence [32]. These transport pathways may occur across the plasma membrane via the transcellular route or tight junctions via the paracellular route; immune system responses are often involved in these specialized transport pathways, and IECs often use active transport methods to absorb certain molecules- such as glucose- from the intestinal lumen into host cells and finally to the animal's

bloodstream [33]. IECs can transport various elements such as antigens, chemical signals, immune system elements, and nutrients, such as glucose, water, and vitamins [34]. In general, active transport is a process that requires energy (often ATP) to move molecules across barriers, while passive transport is defined as a means of transport that does not need energy to move molecules; importantly, active transport is required for the movement of molecules against a concentration gradient while passive transport moves molecules along said gradient [35]. Specific carrier-mediated processes- which are regulated via transcriptional and post-translational mechanisms- also exist in IECs, which tend to be used for absorption of vitamins like biotin, folate, niacin, ascorbate, pantothenic acid, thiamin, pyridoxine, and riboflavin [36]. IECs use active transport to transfer essential nutrients, such as glucose, from the digested food in the intestinal lumen into IECs and then into the organism's bloodstream [37]. In this case, active transport is essential because it ensures that the glucose molecules do not travel back into the intestinal lumen and that glucose absorption via glucose transport continues to occur no matter the glucose concentration. This ensures the body can harvest as much energy as possible from ingested food, which is essential for maintaining gastrointestinal homeostasis. Other important pathways that exist to transfer nutrients include the glucose transport system via sodium-glucose cotransporter SGLT- a form of secondary active transport that utilizes a Na+ concentration gradient- water absorption through the process of osmosis (which is heavily influenced by the absorption of electrolytes), amino acid absorption mechanism which occurs via facilitated diffusion by a co-transport protein utilizing Na+ ions, and many others (vitamins, other sugars, ex cetera) [38-40]. In short, these specialized pathways that exist in the network of IECs that make up the gastrointestinal mucosa play a key role in maintaining homeostasis by controlling digestive and absorptive patterns (i.e., what can and cannot be absorbed), keeping chemical gradients essential for proper functioning,

and actively protecting host cells from potential pathogens that could induce disease-states via physical and chemical means.

Besides utilizing the mucosal layer, creating both physical and chemical barriers between self and non-self, the gastrointestinal mucosa further supports gastrointestinal homeostasis by secreting mucins and antimicrobial peptides [41]. As previously discussed, the mucosal layer of IECs helps maintain GI homeostasis by acting as a physical and chemical barrier between the intestinal lumen and host cells, promoting the digestion and absorption of molecules while actively preventing potential pathogens from invading host cells and directly interacting with other essential systems (i.e., immune system, gene expression, microbiome, etc.) to promote proper responses to specific events [22, 41-45]. Antimicrobial peptides, or AMPs, that are released by IECs are a diverse class of evolutionarily conserved, naturally occurring peptide/protein molecules- which include defensins and cathelicidins- which are produced universally by multicellular organisms as a first line of defense to directly kill various types of potential pathogens such as bacteria, yeasts, fungi, and viruses as well as cancer cells [46]. AMPs are released by specific kinds of IECs release them- including Paneth and immune cells embedded within the intestinal epithelium- which leads to an innate immune response and an inhibitory effect against the present pathogenic threat [47,48]. One example of how this happens can be seen when norovirus is present in the intestinal lumen and binds to cellular binding and recognition sites found on the surface of IECs, which leads to the detection of the viral antigen and an eventual immune response- including cellular inflammation, secretion of antiviral factors, and macrophage activity- which ends up destroying the pathogenic threat, protecting the intestinal tract from infection and also promoting intestinal homeostasis [49-51].

Notably, many different types of IECs exist in the mammalian gastrointestinal tract, some of which perform specific tasks that carry out essential functions. These subtypes include enterocytes, Paneth cells, goblet cells, and neuroendocrine cells. Enterocytes are the primary cell type present throughout the intestinal epithelium [52]. Enterocytes are columnar epithelial cells that play a crucial role in nutrient absorption- such as taking in ions, water, lipids, peptides, and sugar- and in secreting immunoglobins- with immunoglobin A (IgA) being the primary antibody found in the intestinal lumen [52,53]. Enterocytes are found throughout the GI tract, while Paneth cells are only found in the small intestine, particularly in the ileum [52,53]. Paneth cells play a vital role in maintaining GI homeostasis because they synthesize and secrete essential antimicrobial peptides and proteins that work to protect against infection and regulate the composition of the intestinal flora [54]. One study reported that Paneth cells could directly sense enteric bacteria using cell-autonomous MyD88-dependent toll-like receptor (TLR) activation, which triggers the expression of multiple antimicrobial factors [55].

Additionally, unlike other IEC cell types, Paneth cells are long-lived cells that can migrate to base crypts after differentiating from stem cells [52,56,57]. Goblet cells are another type of IEC, and they can secrete mucus to help lubricate the passage of food through the small intestines while also protecting the intestinal wall from digestive enzymes and potential pathogens that exist in the lumen; they have also been found to play a novel “gate-keeping” role for the presentation of oral antigens to the immune system [52,58]. Finally, neuroendocrine cells can release intestinal hormones or peptides into the bloodstream to activate nervous responses while also acting as

chemoreceptors to initiate digestive actions, detect harmful substances such as pathogenic bacteria or toxins, and trigger essential protective responses to help maintain GI homeostasis [52].

In interactions involving IECs and the microbiome or immune system elements, the main goal is almost always to maintain GI homeostasis. For example, if a pathogenic strain of *Salmonella* were to enter a mammalian gastrointestinal tract, IECs would detect and respond to this presence to induce a response that would remove the problem organism from the intestinal lumen and prevent it from entering the host cells. Multiple systems, including the host cell, microbiome, and immune systems, interact effectively to maintain GI homeostasis. All in all, IECs and the intestinal epithelium as a whole possess structural and chemical properties that allow for a robust, well-controlled homeostatic system to exist, which includes balancing the interactions between host cells and external factors; these interactions include ensuring that digestion, immune responses, microbial interactions, and cell-to-cell interactions are functioning correctly to promote gastrointestinal homeostasis and the proper absorption of nutrients to host cells [59]. Importantly, the mammalian gastrointestinal tract must be able to distinguish between self and non-self and react appropriately to protect host cells from pathogens and potentially harmful molecules. Gastrointestinal homeostasis is possible because of this distinction and the essential interactions between IECs, the microbiome, and the host immune system. Problems in GI homeostasis can lead to disease states such as Chron's disease, ulcerative colitis, or other inflammatory diseases [60]. In short, without the careful maintenance and monitoring of digestive activity within and between the host IECs, microbiome, and immune system to distinguish self from non-self and determine what can enter the bloodstream through IECs and what cannot, gastrointestinal homeostasis would not exist.

Equine GI Homeostasis

As hindgut fermenters, horses depend highly on their gut microbial populations to help maintain GI homeostasis [61]. This is because many of these essential microbes present in the equine hindgut perform hindgut fermentation, which aids in the breakdown of plant material like cellulose that cannot be properly broken down without the help of these microbes, leading to proper digestion and energy absorption. During hindgut fermentation, gut bacteria break down plant structural carbohydrates to produce volatile fatty acids, which are short-chain fatty acids with fewer than six carbon atoms that can be absorbed through the cecal and colonic epithelium, and used to build many different organic compounds. They comprise approximately 60-70% of the horse's energy source [62-67]. Without a balanced GI tract, especially in the hindgut, many disease states, including colic, hindgut ulcers, and laminitis can occur and can result in the horse's death [63,68-72]. In short, without homeostasis in the equine GI tract, the animal would most likely get sick and die from whatever condition they have developed.

All aspects of the equine gastrointestinal system have a balance that must be constantly checked, and every example of a homeostatic response will include a receptor, an integrating (or control) center, and an effector [73]. The receptor will receive and transduce a stimulus signal that will be received and processed by the control center (also known as the regulator); then, the effector will be what responds to the initial stimulus and causes the change to regulate the earlier biological stimulation. Additionally, homeostasis is not a static process- a living organism's body systems are constantly working to maintain a set range of conditions required for survival [2,15]. This includes but isn't limited to, sustaining a healthy temperature, blood sugar, calcium, potassium, and osmotic range [2,9]. As stated previously, many of the known regulatory processes

of homeostasis include negative feedback loops- which make up a majority of naturally occurring homeostatic regulatory processes- but there are also examples of positive feedback that exist in nature, including the process of childbirth, blood clotting, and action potential generation [1,2,9,73]. To maintain GI homeostasis, the horse's intestinal cells must be able to distinguish self from non-self, keep internal conditions required for survival, efficiently and effectively fight back against pathogens, and return from disease states.

Much of the research currently published agrees that homeostasis is not only an important factor in ensuring that healthy biological conditions are met, but it is essential for the survival of all living organisms, especially given the fundamental requirement of life to be able to protect and distinguish self from non-self [1,15,74,75]. For GI homeostasis to exist in horses, an important, complex pattern of interactions must occur between the equine IECs, microbiome, and immune system. For example, the study done by Reed *et al*. states that "as hindgut fermenters, horses are especially dependent on the microbiota residing in their cecum and large intestines. Interactions between these microbial populations and the horse are critical for maintaining gut homeostasis, which supports proper digestion" [61]. On a similar note, Steelman *et al*. state in their 2012 study that "bacterial communities are also essential for maintaining gut homeostasis and have been hypothesized to contribute to various diseases including laminitis" [63]. In addition, many "recent studies have identified a critical role for commensal bacteria and their products in regulating the development, homeostasis, and function of innate and adaptive immune cells" [76]. Epithelial cells (or IECs) have been shown to play the role of "segregation" and "mediation" about maintaining gut homeostasis and preventing intestinal inflammation [17]. In particular, "intestinal epithelial cells greatly contribute to the maintenance of the symbiotic relationship between gut

microbiota and the host by constructing mucosal barriers, secreting various immunological mediators and delivering bacterial antigens" [17]. Additionally, when interacting with the microbiome and immune system, IECs can activate and suppress their toll-like receptors (TLRs) using microbial signals, which influence immune responses and help regulate energy homeostasis [42,77,78]. Another interesting study showed that GI homeostasis involves an active migration of IECs up the gut villus during tissue renewal, which could influence gut homeostasis by increasing cell packing towards the villus top, therefore affecting the distribution of younger vs. older cells, which may potentially lead to a more organized, functional digestive system [79]. On top of protecting and maintaining IEC populations and functions- as well as maintaining a microbial balance in the intestinal lumen- the horse's immune system must also be balanced for GI homeostasis to exist overall; in short, disruptions in the horse's immune system can lead to dysbiosis in the gastrointestinal tract overall. For example, "antibiotic-induced dysbiosis compromises immune homeostasis and has been linked to disorders involving inflammation and autoimmunity [80]. It could promote the development of inflammatory gastrointestinal diseases [81]." [65]. The Collinet *et al*. study showed that changes in immune mucosal and peripheral hindgut homeostasis could occur due to TMS administration (a treatment shown to alter hindgut ecosystems), likely due to varying bacterial communities [65]. These studies and various others display the importance of homeostasis and just how intricately intertwined the microbiome, immune system, and host IECs are with one another to maintain GI homeostasis as a whole properly.

Equine GI systems can be susceptible to alterations in microbial homeostasis; when microbiota density is reduced and microbial homeostasis disrupted, this can lead to reduced colonization resistance and potentially a pro-inflammatory host immune response [64,82,83,84]. Having fewer commensal bacteria in the equine hindgut, for example, can lead to problems with energy production and the potential of developing severe illness; for example, one study showed that an increase in the density of *Enterococcus* and *Acinetobacter* populations and a decrease in *Methanobacteriaceae* populations in horses with colic versus the healthy horse control group influenced disruptions in hindgut homeostasis as well as disease-state developments [62]. More pathogenic microbes can also induce disease states; for example, *Fusobacteria*, rarely present in samples taken from healthy horses, can be significantly enriched in cases of diarrhea and colitis [82,85]. Just as these microbiome changes can induce disease-states, it is important to remember that some disease-states can also cause microbiome changes; for example, cancer development, treatments, and medications could alter the gut microbiome, which affects various biological pathways, including drug and antibiotic efficacy, probiotic efficacy, and immune checkpoint inhibitors [86-89]. Finally, equine microbial dysbiosis can also be caused by variables other than a disease, such as age, living space, and antimicrobial treatments such as penicillin, cephalosporins, or fluoroquinolones [62,82,85,90].

Besides the gastrointestinal microbiome itself, the overall structure of the equine hindgut also helps promote GI homeostasis in horses, specifically as it relates to energy creation and conservation. As stated previously, horses rely heavily on hindgut fermentation for energy production, and hindgut digestion occurs in the cecum and large colon, with the process being

most efficient/productive when the horse is allowed to graze and continuously access forage [91]. The physiological structure of the hindgut is longer overall than many other animals and covered in millions of villi and microvilli along the intestinal lumen, which increases intestinal surface area and, therefore, allows the horse to extract more nutrition out of feed [92,93]. The villi in the intestine also move in swaying, contracting motions that likely help increase the flow of blood and lymph to enhance the absorption process [94]. In short, the physiological structure of the equine digestive tract, especially regarding the equine hindgut, plays an essential role in maintaining equine GI homeostasis, which influences equine health.

On top of the overall structure of the equine digestive system playing a role in GI homeostasis, the type of food ingested by the horse- and general feeding patterns- also plays a prominent role in GI homeostasis and, therefore, the continued maintenance of the horse's overall health. For example, one recent study showed that ingesting alfalfa likely improves equine energy efficiency compared to smooth bromegrass because it increases VFA production. Although alfalfa was found to lower the pH in the cecum slightly, it also appeared to increase the abundance of specific microbes in the lumen, including *Streptococcus*, *Lactobacillus*, and *YRC22* [95]. On a different note, GI homeostasis can be disrupted by feeding horses too much starch, and another recent study done by Johnson *et al*. further displayed that modern feeding practices of two to three meals per day that were filled with starch-based concentrates can disrupt normal digestion and potentially lead to disease-states [96]. Finally, GI homeostasis can be promoted and maintained by following the Merck Veterinary Manual's general feeding guidelines: "a horse on anything but a complete feed should eat 1.5-2% of its body weight in good-quality roughage in the form of

pasture, hay, or other types of fiber. This equals 15-20 pounds of hay daily for an average 1,000-pound horse" [97,98].

In general, some of the most important molecules that influence equine hindgut homeostasis are the SCFAs, or short-chain fatty acids, which are produced via hindgut fermentation and can both modulate the intestinal barrier and escape the gut to influence systemic health (Spiliar 2017). As previously stated, short-chain fatty acids are the primary metabolites that are produced by the microbiota in the large intestine through anaerobic fermentation of polysaccharides, which may include dietary fibers and resistant starches. Importantly, SCFAs may influence gut-brain communication and brain function in direct or indirect ways [99]. Three SCFAs that are produced are acetate (which has two carbons), propionate (which has three carbons), and butyrate (which has four carbons). These SCFAs can send signals via G-protein-coupled receptors such as GPR41, GPR43, and GPR109a, which are important in regulating gut homeostasis and participating in epithelial barrier maintenance [42]. Biochemically, these SCFAs can directly produce energy (or ATP) in the equine hindgut via processes like β-oxidation, which is a catabolic process where SCFAs are broken down to generate important molecules like acetyl-CoA, NADH, and $FADH_2$, which can later be used for ATP production in the cell's mitochondria [100]. For example, one butyrate molecule with four carbon atoms in its structure can generate approximately 29 units of ATP [101]. Additionally, SCFAs have been shown to influence activity in nerves, renal arteries, adipose tissue, and the brain, as well as liver and muscle fatty acid oxidation and insulin activity in the pancreas [102]. Thus, SCFAs are essential in maintaining GI

homeostasis because they modulate the intestinal barrier, produce most of the horse's energy, affect various body systems, and influence systemic health.

Another essential factor that plays a direct role in maintaining GI homeostasis in horses is the production of bile acids. Bile acids are "a family of cholesterol-derived amphipathic molecules that solubilize dietary fat in the small intestine to support the digestion and absorption of fat and fat-soluble vitamins," and the liver produces them to serve as detergents that make up to 90% of the organic portion of bile [103,104]. On top of regulating digestion, these important molecules can also act as signaling molecules that regulate metabolic homeostasis and immune cell functioning and homeostasis [103]. Bile acids do this by regulating triglyceride metabolism, cholesterol metabolism, and energy expenditure using a variety of signaling pathways, which can also aid in regulating their homeostasis [105,106]. Additionally, bile acid concentration increases in the horse's serum and liver can be used to detect intestinal disorders such as colic, enteritis, and equine dysautonomia [107]. Generally, a healthy range of bile acids in horse plasma should exist between 5 and 28 □mol/L for normal adult horses [104]. Importantly, serum bile acid concentrations can be increased with liver dysfunction, liver failure, porto-systemic shunts, and bile duct obstruction and can be associated with specific forms of colic [104]. The reason why higher concentrations of bile acids, in particular, can cause these disease states in horses is that this buildup can upset the delicate homeostatic chemical balance, which, in cases of GI bile acid imbalances, can lead to the colon releasing extra water into the lumen and therefore cause diarrhea and other potential gastrointestinal disease-states in the gut [108]. Therefore, maintaining bile acid

concentrations in the horse's liver, GI tract, and bloodstream is essential in maintaining homeostasis and a healthy state of living.

The maintenance of GI homeostasis is also influenced directly by the horse's immune system, which is deeply interconnected with other essential systems such as the microbiome and host IECs. The epithelial tissue of the equine intestinal tract is lined with layers of IECs but also contains important differentiated epithelial cells- which include but aren't limited to Paneth cells, enterocytes, goblet cells, tuft cells, ex cetera- and gut resident-immune cells- which include but aren't limited to T cells, B cells, dendritic cells, innate lymphoid cells, et cetera [109]. Of these cell types, enterocytes are the major cell type in the intestinal epithelium. One of the main ways that the immune system acts to help maintain GI homeostasis is in response to pathogenic threats; in short, the immune system does this by using the innate and adaptive immune response to a) detect pathogens at the cell surface or intracellularly, b) use signaling pathways to direct a specific chemical response, and finally c) induce an immune response which could lead to the infected cell's death [110,111]. One horse-related study by Collinet *et al*. in 2021 emphasizes the importance of the immune system by displaying that the microbial and immune functions of 9 healthy horses were influenced only two days after receiving the orally administered antibiotic oral trimethoprim sulfadiazine (or TMS), which demonstrates that decreasing microbial diversity, lowering the concentration of helpful cellulolytic bacteria, and altering the integrity of the hindgut's mucosal layer can impact the horse's immune system in various ways [65]. Importantly, these nine horses fully recovered from microbial dysbiosis after 2-9 days post-TMS administration, displaying the general tendency of living systems to naturally trend back towards homeostasis even

after enduring periods of dysbiosis [65]. Additionally, IECs can signal to macrophages, goblet cells, and other immune system elements when a pathogen is present to induce a response that removes the threat and therefore protects GI homeostasis; for example, if a horse were to get cut on a sharp object that was accidentally ingested, then epithelial cells would signal to immune cells to recruit phagocytes, platelets, and other elements to help repair the area and induce inflammation (notably, the injury may take time to heal depending on where the injury was located, how deep the cut was, and what kinds of cells were damaged) [112,113]. Finally, the horse's immune system can influence GI homeostasis by directly interacting with the microbiome, influencing lumen contents, bacterial population sizes, and locations throughout the GI tract [114].

Finally, one key aspect of equine GI homeostasis is the capacity for recovery from illness; horses are susceptible to colic, and in some cases, a strangulating obstruction will occur that induces ischemia, or reduced blood flow, that can be life-threatening depending on location and severity [115]. The mechanism of repair when the mucosal epithelium- an essential aspect of homeostasis in the GI tract- is damaged can be affected by inflammation, which is a natural response that occurs when the host cells interact with the immune system to fight off an infection or heal an injury [115]. The potentially available repair mechanisms from this kind of disease state include villus contraction, epithelial restitution, and tight junction closure, all of which may aid directly in reforming the mucosal barrier to a healthier, pre-colic state [115]. Another factor that can help return to GI homeostasis is using nonsteroidal anti-inflammatory drugs that may promote IEC repair by influencing tight junction activity and other essential repair mechanisms [115]. The specific, dynamic communications- which directly include the immune system, microbiome, and

IECs themselves- that are involved in maintaining gastrointestinal homeostasis lie directly with the horse's ability to distinguish self from non-self, maintain internal conditions required for survival, efficiently and effectively fight back against pathogens, and return from disease-states. In essence, GI homeostasis would not occur in the equine gastrointestinal tract if IECs were unable to effectively recover from disease states such as colic, which may affect the mucosal layer of IECs and influence immune responses that can lead to inflammation, cell communication issues, et cetera.

As displayed previously, many essential, interconnected factors play a role in maintaining GI homeostasis in horses. Communication between the microbiome, immune system, and host gastrointestinal cells is critical in maintaining GI homeostasis in horses and other mammals. Without this active communication between these different systems, the organism would lose the ability to actively distinguish between self and non-self and protect itself against potential pathogens that could threaten organismal health. Additionally, many specific biochemical structures in the equine digestive system, such as the SCFAs and bile acids, directly promote and maintain GI homeostasis. Finally, GI homeostasis is an active, dynamic system of balance that requires constant monitoring to promote proper digestive functioning, energy creation (especially during hindgut fermentation), and overall horse health.

Gastrointestinal Anatomy and Physiology

The mammalian gastrointestinal system begins at the mouth and ends at the anus, with the primary goal of the system being to digest food material and convert the nutrients from food into

energy, which therefore keeps the organism alive [116]. The main types of digestive systems in animals include monogastric, ruminant, and pseudo-ruminant [117,118]. Each type of digestive tract depends highly on the food the organism eats. Smaller animals – like cats – that eat meats have less complex large intestines, while larger animals – like horses – that eat grasses and fibrous plant materials have more developed and voluminous large intestines [119]. Notably, despite the differences between species, the general structures within the digestive system- including the alimentary canal, oral structures, and accessory digestive glands- remain consistent across systems [119]. As an example, the dog and the horse both have a pancreas, which is essential for food digestion and managing the animal's use of sugar for energy after digestion; the pancreas is required for producing hormones such as insulin and enzymes such as lipase and protease [120,121]. Despite general similarities, systems differ by animal based on size, diet, overall health, stressors, et cetera [18].

As stated previously, every mammalian digestive system has macroscopic compartments that are separated from each other by sphincters- or rings of muscle that work to guard or protect an opening or tube [122]- and these components consist of the buccal cavity, esophagus, stomach, small intestine, and large intestine including the colon, and rectum, along with accessory organs like the liver and pancreas [18,122]. The buccal cavity, or the mouth of the organism, is the initial place that food enters the digestive system, and it functions to begin the process of breaking food material down via physical, such as chewing and grinding from the teeth, and chemical, such as digestive enzymes in saliva, means. Carbohydrate digestion is performed by several enzymes that break down larger, more complex molecules into smaller, more easily digestible molecules, including amylase, maltase, sucrase, and lactase [123]. In mammalian systems, the mouth is where

carbohydrates begin to get broken down into sugars so the body can more easily absorb them; additionally, the buccal cavity includes structures like the lips, the oral mucosa, the front two-thirds of the tongue, the upper and lower gums, the floor of the mouth under the tongue, the hard palate, and the small area behind the last molars [124,125]. The buccal cavity will lead the food material to the pharynx and then the esophagus, which is a muscular, mucous-covered viscus that directly connects the throat to the stomach in the organism [18,126]. The main function of the esophagus is to carry ingested food material and liquids from the mouth to the stomach and, when needed/possible, allow for reverse flow in the case of regurgitation, belching, or vomiting [126].

In most digestive systems, the stomach is located immediately following the esophagus, and it works to create enzymes and acidic digestive juices to break down food, holding the food until it is completely ready to enter the small intestine [186]. In general, the stomach actively digests fats and proteins via digestive enzymes such as pepsin and gastric lipase, which the body makes to break down certain foods and aid digestion [18,123,128]. Pepsin, an enzyme that aids in protein digestion, is secreted from gastric chief cells in the inactive form called pepsinogen, which is converted to pepsin through the low pH environment of the stomach maintained by hydrochloric acid [123]. This highly acidic environment also aids in killing bacteria in the stomach chyme before it continues through the rest of the gastrointestinal tract [480]. Lipase, another essential enzyme, breaks down fats into fatty acids and glycerol so that these nutrients can be absorbed in the intestinal tract; lipase is produced in the mouth, pancreas, and stomach [130]. Carbohydrates, in particular, are not chemically broken down in the mammalian stomach but are further digested in the small intestine because the carbohydrate digestive enzyme amylase does not function in the stomach's acidic conditions [131]. However, the stomach can aid in the further digestion of

carbohydrates by physically mixing the chyme into a more uniform mixture via strong peristaltic contractions before proceeding through the rest of the digestive system [131].

The chyme enters the small intestine from the stomach. The small intestine is a long, narrow muscular structure that extends from the stomach to the large intestine. In humans, it is where most digestion and absorption of food takes place and is often categorized into three parts: beginning with the duodenum, followed by the jejunum, and finally ending with the ileum; it also utilizes digestive enzymes like those discussed in previous sections [132]. In the small intestine, the duodenum- the first portion that the stomach chyme enters- will be aided by the pancreas- a large gland behind the stomach that secretes digestive enzymes and hormones- to help break down fats, proteins, and carbohydrates [133]. In species possessing a gall bladder- another important structure that is small and sac-like located close to the liver where bile is stored after the liver secretes it and before it is released into the intestine- the gall bladder also releases the bile that is produced by the liver- a larger organ which cleanses the blood by removing toxins and aids in digestion by secreting bile- to help further break down fats so that they can be properly absorbed by the intestinal epithelial cells [18,133]. Notably, the small and large intestinal epithelial cells are lined with finger-like projections called villi, which create a large surface area to help facilitate the absorption of nutrients into the organism's bloodstream (microvilli themselves can increase cell surface area by up to 25 times) [18,133-135]. This happens because more surface area leads to more nutrient absorption, energy production, and healthier organisms. Finally, the small intestine is essential in regulating blood glucose levels because it contains many receptor cells that can detect specific macronutrients and signal the pancreas to secrete the necessary hormones to maintain glycolic homeostasis [136,137]. For example, suppose an organism's body is low on

glucose. In that case, it may release glucagon to indicate to the liver that glucose needs to be released into the blood, or if it has too much glucose present, it may release insulin, which helps move glucose out of the bloodstream and into cells [138]. In general, the main tasks that the small intestine accomplishes are as follows: finishing the process of digestion (carbohydrates, fats, and proteins) via microbial and chemical breakdown, absorption of nutrients into the bloodstream and body cells, and eventually passing the leftover residue on to the large intestine [139].

Once the small intestine reaches the large intestine, chemical digestion will be completed, and the body will absorb around 90% of nutrients and water [140]. The main goal of the large intestine is to absorb water, electrolytes, and salts from the indigestible food residue left over from the small intestine [140]. In general, the large intestine is a large, tube-like organ that absorbs water and salts, stores remaining waste material before it can be removed by defecation, and contains a wide variety of gut microbes that perform many essential functions (such as host vitamin formation); structurally, the large intestine consists of the appendix, cecum, colon, and rectum [141]. Some examples of vitamins that colonic bacteria can produce are thiamine, riboflavin, and vitamin K [142]. Additionally, the large intestine is where bacteria break down digestive fibers for their nourishment, creating acetate, propionate, and butyrate as waste products that can be used by host cells as an energy source [142]. Finally, any remaining nutrients can be absorbed around the colon before fecal matter can pass through the rectum and out the anus. In general, once the fecal matter leaves the body, it will be composed of water, undigestible food matter (such as fats, proteins, or cellulose in some animals), some microorganisms and dead bacteria, organic and inorganic substances such as calcium phosphate and iron phosphate, and other undigested materials- like specific vitamins- that are not needed by the host organism [143-145].

Although there are many similarities in the digestive patterns of mammals, the external environment, including factors like diet and lifestyle, has directly influenced the process of evolution and, therefore, played a role in the development of apparent physiological differences that exist between herbivores, carnivores, and omnivores [146]. Herbivores, which consume plant matter as their main source of nutrition, have digestive systems that can break down the cell walls of plant vegetation; this happens by microbial fermentation and rumen digestive activity, specifically with the help of commensal bacteria in the gut that can produce digestive enzymes like cellulase to help break down and digest plant cellulose [147]. Additionally, their digestive systems are much longer than those of carnivores or omnivores, and many herbivores also have multiple stomach chambers available to aid in specific digestive processes (such as microbial fermentation) [148]. By having a longer digestive tract, herbivores can process plant and grass-based foods that are high in cellulose - an insoluble polysaccharide that exists as a linear chain of hundreds to thousands of β-linked D-glucose units - [149], which is tougher to digest and is often not digestible by the digestive enzymes present in animal systems alone [149,150]. Herbivore digestive systems support microorganisms that can produce cellulase enzymes- which include β-1,4-endoglucanase, cellobiohydrolase, and β-glucosidase- and they can help directly with the breakdown of plant material in the digestive tract; many herbivores also utilize physical means of further breaking down plant material, such as chewing cud [151,152].

Another important distinction in mammalian gastrointestinal systems lies when comparing ruminant animals with non-ruminant animals. Ruminants – herbivorous animals that acquire nutrients via microbial fermentation – are characterized by a rumen, the first and largest compartment of their intestinal tract where cellulose begins to be broken down by the action of

microbial fermentation [153,154]. Since the rumen is such a large structure, it can act as a storage or holding vat for feed as well as a fermentation vat that favors microbial growth and the formation of volatile fatty acids, which are short-chain aliphatic monocarboxylic acids that are produced in anaerobic digestive processes and exist as the primary energy source for ruminants [155-157].

Additionally, many animals go through different forms of microbial fermentation at different locations along the digestive tract, which includes foregut fermenters- which include animals like deer, cattle, and some monkeys- and hindgut fermenters- which include animals like horses, rabbits, rhinos, and some rodents [158]. During fermentation, wherever it occurs, symbiotic bacteria aid in the digestion of plant materials like cellulose via microbial fermentation, which helps break down large organic plant-based molecules into simpler ones for easier digestion. Foregut fermenters have a digestive system that contains a pre-gastric fermentation chamber, while hindgut fermenters have enlarged fermentation compartments in the cecum or colon, and these physiological systems distinguish these animals from others that do not heavily utilize these particular fermentation processes [158,159]. The location where the fermentation process occurs along the digestive tract is important because it begins in the areas of the gut where active digestion and absorption of nutrients, water, electrolytes, and vitamins can occur, making it an ideal place to begin breaking down larger, more complex molecules into a form that can be more easily digested. Additionally, hindgut fermenters typically can process food more rapidly than foregut fermenters, which gives hindgut fermenting animals with a considerable body size an advantage since they can accommodate significantly larger food intakes and process that nutrition more efficiently [160,161]. Finally, the location of fermentation processes along an animal's gastrointestinal tract dictates how different types of host IECs present in specific areas interact

with the microbiome and intestinal lumen contents, as well as directly influencing how certain tissues function; in short, the microbial presence of certain beneficial microbes in particular areas of the gastrointestinal tract will directly impact the activity of digestive tissues, and therefore affect overall physiological structure and function [162,163].

Another essential factor to recognize in the overall physiological function of an organism's gastrointestinal system is average retention time, which varies depending on species, organism age, and health, what the animal is eating, and other factors such as external stressors, exercise levels, or living environment [164,165-167]. A much longer overall digestive retention time exists for herbivorous animals, while carnivorous animals (and sometimes omnivorous animals) often have a faster retention time due to their diets and how their bodies process ingested nutrients; differences also exist between ruminant retention time based on browsing and grazing feeding types [165,167,168]. The average retention time varies between different areas of the equine digestive tract; for example, passage through the stomach and small intestine is relatively rapid- approximately 5 hours on average- while a much longer retention time has been recorded in the cecum and colon- which takes about 35 hours to pass through on average [169]. This difference in average retention time between the earlier and later portions of the equine digestive tract exists because a) the large intestine is where much of the critical yet slower digestive steps occur, specifically including microbial digestion, water absorption, and vitamin production, b) the size and structure of the cecum itself naturally slows the passage of digesta and provides microbes with more time to digest plant fibers, and c) microbial fermentation itself can take a long time because plant cell walls are more difficult to digest and therefore more difficult to efficiently break down into smaller nutrient-rich molecules that can be utilized by host cells [170,171]. Importantly,

individual horses, however, can have altered retention times because of various internal and environmental factors (such as health condition, body weight, pregnancy, exercise, et cetera), with one study showing that smaller particles and feed with a higher water-holding capacity move slower through the gut. At the same time, reduced fiber length, increased feeding level, and increased forage/concentrate ratio accelerated passage rate [169], meaning that mean retention time in the equine GI can be changed with diet, feed type, and feeding frequency [172]. Another excellent example of how environmental factors can affect the average retention time of a meal in the equine digestive tract was shown in a Thoroughbred horse study from Pagan *et al*. in 1998, which displayed that exercise reduced mean retention time of digestion and likely reduced blood flow to the digestive tract [173]. Various other studies have also proven that internal and external factors can affect mean retention time in horse digestive systems [174-179]. Therefore, the average retention time of digesta in mammalian digestive systems will vary between individuals- due to factors such as stress, exercise levels, organism age, ex cetera- and between different species- due to factors such as diet, microbiome differences, evolutionary history, and whether the animal is ruminant or non-ruminant [180].

Compared to other mammals, the equine digestive system is unique in many ways; as hindgut fermenters, horses produce most of their energy from the nutrients they ingest in the hindgut, where commensal bacteria aid in the breakdown of plant material into a more usable chemical form. The equine digestive system is also unique because it digests portions of food enzymatically first in the foregut before fermenting it in the hindgut. In the foregut, food often takes a couple of hours to pass through, while in the hindgut, digesta can take 1-3 days to pass through before being excreted in feces [169,181]. The equine digestive system is also

physiologically capable of breaking down cellulose and other plant-based materials that many other animals- specifically those that are not herbivorous or do not have GI systems that participate in heavy microbial fermentation processes- cannot break down. The mere length of the horse's digestive tract is also notable- on average about 100 feet in length- especially given the large size of the animal and the fact that a horse typically consumes 1.5-2.5% of his body weight in food per day [182].

Interestingly, compared to other mammals, the equine gastrointestinal tract has a smaller stomach than the rest of the digestive system, with the stomach only being able to hold about two gallons of material at a time and only for around 15 minutes [183]. Another uniquely equine feature of the digestive system is that the esophagus only works in one direction, so unlike humans, dogs, cats, and other mammals, horses cannot vomit [183]. Finally, some other equine-specific digestive tract features include the fact that they do not have a gall bladder, they can only chew on one side of their mouths at a time, they produce many gallons of saliva a day as a result of an almost constant chewing and grazing behavior, they require a minimum of 1.5-2% of their body weight daily of plant material for normal digestive activities to occur, the overall process of digestion can take place between 36 and 72 hours total, and they cannot digest the dietary fiber lignin commonly found in hay. Hence, it passes through the digestive tract into the feces [97,183].

The main goal of all mammalian digestive system structures physiologically is to break down and digest all food nutrients that enter the system properly. Every organism has a specific digestive system that has evolved to physically and chemically break down particular foods that the organism eats based on evolutionary history and whether or not the animal is a herbivore,

carnivore, or omnivore. For example, the herbivore digestive system is often longer than that of carnivores or omnivores because plant cell walls contain cellulose- an insoluble polymer categorized as a complex carbohydrate or polysaccharide- and this substance takes longer to digest. Additionally, to maintain a homeostatic balance with host cells, the tissue structures of each organ in the digestive system must distinguish self from non-self while interacting directly with the lumen contents, including any essential microbes that may aid in the digestive process. Finally, the physiological structures in the digestive system must properly interact with the host immune system, be able to identify beneficial versus harmful bacteria that populate the digestive microbiome (and work with the commensal microbes to aid in digestive processes), and actively maintain gastrointestinal homeostasis by properly distinguishing self from non-self and constantly communicating with other internal and external variables that often do not remain the same throughout an animal's life (such as diet, exercise, stressors, health condition, et cetera).

Equine Gastrointestinal System

As hindgut fermenters, most of the energy obtained from food in the horse is made after microbial fermentation in the hindgut [63-65]. As stated previously, hindgut fermentation is a process where microbes aid in the breaking down of plant material, allowing the horse to digest certain foods properly while also creating essential vitamins (including vitamins B and K) [184]. GI anatomy and physiology play a prominent role in when this process of breaking down plant material takes place, how it occurs, how nutrients flow into the intestinal epithelial cells and get circulated to other locations in the body, why the differences of specific intestinal structures dictate function, and why certain areas of the hindgut look relatively different from one another. This section will dive into what makes the equine GI tract unique compared to other mammals, how

different anatomical structures of the GI tract can influence the essential digestive process, and why understanding what is currently known about equine GI physiology is critical for comprehending system interactions, why the different segments of the digestive system perform the tasks that they do, and for what purpose (i.e., how can it help organismal health or promote GI homeostasis?). This section will generally go over what is currently known, what is possible, and what is presently unknown regarding the anatomy and physiology of the equine GI system.

"The equine gastrointestinal tract (GIT) is a remarkable organ system with a potential length and volume in the adult horse of over 30 meters and 150 liters, respectively… each compartment must function correctly and in concert with the other regions to support the health of the animal" [185]. The mere length and size of the equine GI tract play a role in digestive processes compared to other smaller animals because it allows for more time and space to break down food, absorb nutrients, and absorb water and electrolytes, making the longer GI tract length helpful in extracting maximum nutrition from digesta. Regarding intestinal pH changes throughout the equine GI tract, the pH levels increase along the length of the small intestine and then decrease slightly in the hindgut, specifically because this is the site of VFA production in the horse [186]. The foregut is mainly involved in the digestion and absorption of sugars from starch, amino acids from proteins, fatty acids, and fat-soluble vitamins (A, D, and E). At the same time, the hindgut mainly digests, processes, and absorbs plant fibers that cannot be processed in the foregut, water, electrolytes, and some vitamins and minerals [187]. Like other mammals, horses have a gut microbiome that directly affects digestive health and overall health. Notably, the microbes in the hindgut- such as the fibrolytic and amylolytic species of bacteria- help with digestion by

hydrolyzing carbohydrates into simple sugars that can be fermented further, resulting in products like SCFAs, lactate, and gases like CO_2 and CH_4 [66].

In general, the equine GI system can be categorized into two main digestive sections- the foregut and hindgut- which include the mouth, esophagus, stomach, small intestine, and a highly developed large intestine, which consists of the caecum, large colon, small colon, and rectum [188]. Digestion begins when plant matter enters the mouth, and the horse begins to chew it, using physical means to break it down into a bolus. The horse's mouth includes digestive elements like the teeth, tongue, and salivary glands, which all contribute to the breakdown of food matter [188]. When food enters the mouth, chewing reduces feed particle size by physically breaking it down into smaller parts, while saliva can break down certain food elements in a chemical matter [188]. Saliva- a critical component of early-stage equine digestion- is essential because it can act as a lubricant to provide easier passage through the esophagus. This lubrication buffers acid in the stomach [188]. Additionally, after chewing, horse saliva was found to have higher amounts of potassium, calcium, and bicarbonate and less phosphate than human saliva [189]. Finally, unlike in other species, the saliva produced by horses is not important in an enzymatically digestive way, mainly because horse saliva only contains a small amount of amylase, little actual digestion occurs in the stomach of most horses, and horse saliva does not have the same prevalence of digestive enzymes compared to other animals; interestingly enough, one study displayed that changes in saliva analytes correspond to altered stress response reactions and potential disease-states in horses [190-192].

The esophagus is a muscular structure around 4 to 5 feet in length and carries food from the horse's mouth into the stomach via the cardiac sphincter [193]. Importantly, no digestion occurs in the esophagus of the horse [193]. When mashed food particles pass through the horse's esophagus, they can only go in one direction; this is because they possess a valve at the entrance of the stomach called cardias or "Swiss tie," and this valve has muscles that are so strong that they prevent food from returning into the horse's mouth [194]. This means that, unlike many other mammals, the horse cannot vomit, mainly because they have a physiological structure present near the entrance of the stomach called a cardias or "Swiss tie" that acts as a one-way valve to prevent food from coming back up [194]. In this way, horse anatomy and physiology- including the presence of a one-way valve and the lower angle that the esophagus joins with the stomach- prevents food matter from moving in any other direction besides the mouth to the esophagus to the stomach.

Once the ingested food enters the horse's stomach- which is the smallest unit in the equine digestive tract and can hold around 2 to 4 gallons of matter, making up approximately 10% of the horse's total digestive system volume- further physical and chemical digestive activities will occur [188]. For example, the stomach will begin mixing, storing, and periodically releasing food matter into the horse's small intestine, with one crucial function being to control when and how much feed is being released into the small intestine for further digestion [188]. Additionally, pepsin is secreted in the stomach to begin protein digestion by cleaving peptide bonds in the amino-terminal side of the cyclic amino acid residues (such as tyrosine, phenylalanine, and tryptophan) and then breaking the polypeptide chains down into smaller peptides; pepsin is also responsible for initial and partial protein hydrolysis processes with the aid of hydrochloric acid [18,188,195]. Finally,

although hardly any nutrient absorption occurs in the stomach of the horse, a healthy horse will constantly have food coming into the stomach, and the stomach's smaller size corresponds well to a diet consisting of continuous, small meals [170]. Food matter remains in the stomach for about 30 to 45 minutes on average, and the stomach will consistently stay less than two-thirds full. Still, it should consistently replenish feed and water throughout the day [196,197]. Feeding the horse little and often- and with consistent, healthy meals- is essential for overall health because a) it helps their digestive system operate efficiently since they are herbivores that have GI tracts that are always designed to digest small amounts of forage and since they graze consistently throughout the day and b) it ensures optimum hindgut fermentation processes are occurring, therefore allowing for proper energy formation and nutrient absorption [198].

When digesta is released from the horse's stomach, it enters the small intestine, which is physically around 70 feet long and makes up about 30% of the horse's total digestive system volume [188]. The small intestine is comprised of the duodenum, jejunum, and ileum structures [199]. In general, food digestion in the equine small intestine is rapid- with food digesta passing through within 1-3 hours [200]- but when the horse digests a large volume of feed or if the feed exhibits an increased rate of passage through the small intestine, then there will be an overall decrease in digestion and absorption of nutrients [188]. The small intestine is the site where most non-structural carbohydrates- such as starches- as well as proteins and fats are digested and absorbed by host cells; the enzymes that work to digest these starches are amylase enzymes while lipase enzymes and proteins digest fats are digested by protease enzymes [188,195]. The pancreas- a large gland present near the horse's stomach that secretes digestive enzymes into the duodenum of the small intestine- is the enzyme "powerhouse" of digestion, meaning it is the main producer

of the most important digestive enzymes: amylase, lipase, and protease [128,195]. Other digestive enzymes are also produced by the mammalian small intestine, which include lactase- which breaks down lactose- and sucrase- which breaks down sucrose [128,195]. Regarding the three principal digestive enzymes mentioned, starches are broken down by amylase into glucose; fats- or oils- are broken down into glycerol and fatty acids by lipase; proteins are broken down by protease into amino acids [188]. In equine systems, the small intestine is where extensive digestion occurs, specifically for fats and proteins; the digestion of a majority of the non-structural carbohydrates (starches) will happen in the small intestine, but the overall digestion of plant matter will be incomplete at this stage due to the presence of cellulose and other tougher-to-digest materials in the lumen [188]. This undigested plant matter will then be delivered to the hindgut, where essential hindgut fermenting bacteria will aid in the digestive process of structural carbohydrates, such as the dietary fibers from forages [188].

The equine hindgut- the site where 60-70% of the horse's energy is made from microbial fermentation and the production of volatile fatty acids (or VFAs)- includes structures like the cecum, large colon, small colon, and rectum [64,188,201-203]. The horse's large intestine makes up more than half of the total volume of the animal's overall digestive tract, and it plays an essential role in digestion by utilizing commensal bacteria to perform hindgut fermentation while also acting as a major reservoir for water [204]. The main function of the hindgut is to perform microbial fermentation on the dietary fibers found in the horse's diet, which includes structural carbohydrates that are found in grass, hay, and other plant sources [188]. Hindgut fermentation produces essential end products called volatile fatty acids (VFAs), which include carbon-rich structures like acetic, propionic, and butyric acids that can be used as an energy source for horses by converting acetate

to acetyl CoA, propionate to glucose, and butyrate to B-OH butyrate or 1 mol of butyrate to 2 mols of acetate and hydrogen [188, 205-207]. Additionally, this process produces methane, B vitamins, vitamin K, carbon dioxide, water, and some essential amino acids [188,205-207]. The hindgut is also a valuable part of the digestive system because of its role in electrolyte absorption and water reabsorption; it is a site where water can be re-absorbed back into host cells via osmosis [140,208]. The epithelial cells in the horse's intestines can move food matter by peristalsis, which happens by involuntary muscle constrictions and relaxations in a wave-like pattern that forces the contents of the intestinal canal forward, and it requires constant cell-to-cell interaction along the intestinal tract [209]. Intestinal epithelial cells can interact with one another by using different kinds of chemical signals- such as histamines, hormones such as epinephrine and norepinephrine, mRNA and proteins- and various cell junctions- such as tight junctions, gap junctions, adherens junctions, and desmosomes- which can directly influence immune system reactions, the rate of digestion, digestion itself and other processes [19,210-212]. Therefore, cell physiology and intracellular interactions between cells in both the small and large intestine contribute to the movement and digestion of food matter.

As discussed in the microbiome section, the microbial population in the equine hindgut is affected by various factors, including diet composition [188]. The beneficial microbes that exist in the hindgut- mostly consisting of the amylolytic bacteria found in the *Firmicutes* and *Bacteroidetes* phyla- ferment starches that are delivered from the small intestine into the equine hindgut [64,65,213-217]. This rapid fermentation produces large quantities of lactic acid and volatile fatty acids (or VFAs), which both provide energy to herbivorous animal species [65,216,188,206,220]. Additionally, since lactic acid and volatile fatty acids are acidic, their production will cause the pH

in the equine hindgut to fall [68,218]. For the horse to remain healthy and not develop hindgut acidosis, the hindgut pH should stay between 6.5 and 7 for healthy VFA production and efficient energy production from a balanced diet high in fiber and lower in starch and certain sugars [219]. Importantly, suppose the pH in the hindgut drops too low (below 6, especially if the change is rapid). In that case, pathogenic bacteria (like *Salmonella*) may proliferate and contribute to the development of disease states in the horse [219]. Some examples of disease states that can result from such pH changes in the hindgut include laminitis, colic, metabolic acidosis, and other gastrointestinal diseases [219].

In the equine digestive system, passage through the stomach and small intestine is rapid (approximately 5 hours on average). In comparison, a longer retention time is present in the cecum and colon (about 35 hours on average) [169]. The average mean retention time (or MRT) of food matter in the equine hindgut is different in each section of the hindgut, with the slower rates of passage existing in the cecum and ventral colon and the faster rates of passage being in the stomach and rectum [169,175,221]. As stated by a study done by Miyaji *et al*., the average MRT of hay and silage in the cecum, right ventral colon, left ventral colon, left dorsal colon, right dorsal colon, and small colon was 2.9, 3.1, 5.9, 1.0, 4.0, and 4.0 h, respectively (Miyaji 2008). Another informative study performed later by Miyaji *et al*. showed that high feed intake decreased both the total tract fiber digestibility and the MRT, displaying a clear relationship between MRT and fiber digestion along the total GI tract of the horse [222]. These apparent differences in MRT between different hindgut segments and in-total across the equine hindgut may be attributed to many potential influencing factors [164], which may include horse breed (i.e., light vs. draft breeds), physiological state such as pregnancy, age, feed type/frequency, ex cetera. Although the intestinal epithelial cells

that exist across the equine hindgut all share many of the same structures and functions- such as absorbing VFAs, the ability to distinguish self from non-self, interactions with the microbiome and potential toxins or pathogens, ex cetera- some differences may also exist between the earlier and later portions of the hindgut which may also contribute directly to these MRT observations.

To fully understand fiber digestion in the equine hindgut, it is essential to look at the structure and function of intestinal epithelial cells, how these cells interact with each other, and other factors that exist in the lumen. Individual intestinal epithelial cells have organelles, including a nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, and ribosomes like many other cell types; however, their overall shape, cell sheets, and cellularity, connective cell junctions to other epithelial cells, polarity, basement membrane, high regeneration, nerve innervation and lack of blood vessels makes epithelial tissues unique [223]. The intestinal epithelium itself is very diverse and consists of many different cell types- such as enterocytes, goblet cells, neuroendocrine cells, tuft cells, Paneth cells, and M cells- and each of these cell types can develop further into specialized subsets [44]. As a result, these diverse functions arising from different epithelial cell types can work together to maintain GI homeostasis while promoting host defense [44]. Intestinal epithelial tissues are composed of four main layers: mucosa- which faces the internal intestinal lumen and can be further divided into a) the surface epithelium, b) the lamina propria, and c) the muscularis mucosa - submucosa, muscularis, and the serosa [224]. Because of how these intestinal epithelial cells are oriented, the mucosal layer is the layer that directly interacts with digesta, microbes in the microbiome, chemical signals such as mRNA, immune system elements, and potential pathogens or toxins. This layer is key in providing a barrier against any foreign particles that may interact directly with host cells, making it essential in the animal's

determination of self versus non-self while acting as the first line of defense against any problematic microorganisms, digestive enzymes, acids, food particles, microbial by-products, and food-associated toxins [21].

Intestinal epithelial cells are structured with many of the same organelles that other cells possess but also have many unique qualities. For example, these cells are tightly linked to one another and may constantly sense the intestinal lumen to determine whether to absorb specific molecules. They are designed to tell whether a food particle is worth absorbing or if a particular antigen is potentially harmful. In many cases, an epithelial cell can signal to other cells that a potentially harmful antigen is present and, therefore, mount an immune response when it is present in the gut [225-227]. Additionally, suppose an intestinal epithelial cell detects a nutritious particle and signals for it to be absorbed. In that case, that particle can take many different routes through the epithelial cell layers to reach other parts of the body. For example, it may travel via the transcellular route, paracellular route, or across tight junctions present between epithelial cells. In general, the intestinal epithelium is constructed so that the apical membrane faces the lumen, tight junctions exist between the lateral membranes, and the basal membrane is located away from the intestinal lumen [92]. Cells in the epithelium can form different layered structures- including transitional, simple cuboidal, stratified cuboidal, simple squamous, stratified squamous, simple columnar, stratified columnar, and pseudostratified columnar- and the intestinal epithelium forms tube-like structures called villi and cave-like structures called crypts, which both increase intestinal surface area and promote effective nutrient absorption into the body [92,93,228]. Finally, these epithelial cells often live for around 3-5 days [229], meaning the intestinal epithelium constantly

sheds and replenishes cells with proliferative stem cells found explicitly within the crypts. These proliferative cells can then migrate out of the crypts as they age and develop [229].

Intestinal epithelial cells (IECs) have unique microvilli- which are short, narrow finger-like projections that contain bundles of parallel actin filaments held together by cross-linking proteins called villin and fimbrin [230]- on the surface facing the lumen, which increase the surface area of the cell exposed to the lumen to enhance absorption and secretion [231]. These structures are also unique in that as many as 1,000 microvilli can exist on each epithelial cell, and they can increase the absorbing surface by approximately 25 times, improving nutrient intake and digestive efficiency [232]. Importantly, intestinal epithelial cells also possess many essential organelles shared by other living cells, including a Golgi complex, peroxisomes, mitochondrion, a nucleus, rough and smooth endoplasmic reticulum, et cetera [233]. Many of these essential organelles are required for cell life; for example, the mitochondrion is an essential double-membrane organelle that uses aerobic respiration to generate cellular energy in the form of ATP, which can be used in various biochemically important processes [18,234,235]. Finally, the unique properties of intestinal epithelial cells- such as their ability to transport specific nutrient-rich molecules across barriers, their overall cellular shape, and their specific communication/orientation to one another- are present because these cells are performing cell and tissue-specific tasks, including digestion, epithelial cell-to-cell interactions, host and non-host interactions, and the recognition and possible elimination of potentially harmful pathogens found in the intestinal lumen.

The cells lining the small and large intestines must constantly interact with each other and other structures, including the microbiome and immune system, in order for digestive processes to

happen efficiently and in a balanced, homeostatic way. They can communicate information via chemical signaling, such as mRNA or hormone signaling, leading to a direct response that may influence digestive activities [236]. One example of critical regulators of epithelial homeostasis, barrier function, and cell-to-cell interactions is the RAS superfamily of small GTPases, which cycle between an inactive state- where they bind to GDP- and an active state- where they instead bind to GTP in human studies, the transition between these two states was found to be regulated by GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), which can convert GTP to GDP and vice-versa [237]. Some RAS oncoprotein members play a fundamental role in cell signaling by interacting with multiple effectors, such as those that can control critical cellular processes like polarization, adhesion, and proliferation [237]. Some examples of these RAS oncoprotein members are HRAS, NRAS, and KRAS, and some effectors that can be influenced to induce a response include RalGDS and phosphoinositide 3-kinase (or Raf kinase) [237]. Previous studies have found that mutations in RAS oncogenes can lead to harmful mutations, which can cause GDP-GTP insensitivities or locked positioning that may lead to developing disease states such as those found in human pancreas, lung, and colorectal cancer [237]. Finally, the RAS superfamily of small GTPases can help coordinate and control cell-fate decisions- more specifically in the KRAS-mediated signaling pathways- while environmental factors and growth factors directly influence the cell; all in all, these molecules directly contribute to promoting cell homeostasis, cell-to-cell-interactions-,-maintenance, influencing cell fate decisions, and protection against intestinal and colorectal diseases [237].

Additionally, IECs can bind and recognize specific pathogens in the lumen and signal for an immune response. This can happen in various ways but often includes a biochemical signal or

binding that leads to a specific response [238]. PRRs, or pattern recognition receptors, are innate immune receptors in mammalian intestinal cells and can be divided into multiple subtypes, all of which influence specific signaling pathways to cause different responses [45]. Some examples of PRRs found in humans include TLR, NLR, RLR, CLR, and Cytosolic DNA sensors [45]. The signals induced by PRRs often cause direct functional responses that aim to protect cell, tissue, and overall organismal health, such as increasing an antiviral response, increasing inflammatory mediators, signaling for B-cell and dendritic cell recruitment, recruiting innate immune cells, increasing natural killer cell activity, or promoting CD8+ T cell proliferation [45]. These responses often lead to a whole-tissue reaction rather than being excluded to a single cell; for example, if a horse were to eat something toxic- such as chocolate, avocados, or certain meat/dairy products- then the intestinal system may swell in a larger area or cause a "warning" chain reaction from the earliest intestinal tissues to the later [239]. Additionally, suppose one cell is secreting a chemical signal. In that case, other cells will pick up on that signal and potentially secrete even more of that signal, causing a chain reaction so that the need for an immune response is met in the most timely, efficient way possible [17]. However, this signaling chain must stop once the response task has been fulfilled and the pathogen dealt with to maintain intestinal homeostasis. Otherwise, disease states may result, and an organism's health could be compromised [224].

Intestinal epithelial cells (IECs) also regulate microbiome activity, and vice versa; this can be done via what is known as "crosstalk" between gut microbiota and IECs via processes such as cell signaling, chemical binding of pathogenic bacterial markers to external cell receptors, secretion of mucus and chemical signals into the mucosal layer and lumen space, et cetera [17,240]. One example of how IECs regulate the composition and activity of the intestinal microbiome is by

providing signaling molecules- such as mediators like cytokines and chemokines- which can regulate certain pathways and, therefore, specific biological responses or by utilizing alternative energy resources, such as AMPs, hormones, and mucins [17,241]. One way that pathogenic bacteria can compromise or destroy intestinal barrier integrity is by utilizing specific biological pathways- such as mTOR, AhR, FXR, and TLR- to influence the physiological structure of IECs and interactions between IECs; for example, some gut microbiota can produce secondary bile acids which could inhibit the repair of the gut barrier, which may be detrimental during an infection [17,241]. The production and activation of SCFAs- or short-chain fatty acids- in an organism's intestinal tract can promote the production of RegIII γ and defensins from IECs via the activation of mTOR; mTOR is a key catalytic subunit of a protein complex that acts as a central regulator of growth in animals and also controls most of the anabolic and catabolic processes that may occur in response to nutrients and nutrient-induced signals [17,241-244]. SCFAs and indole activation processes have also been shown to improve the function of tight junctions in IECs, therefore allowing for better communication between cells [240]. IECs can also produce antimicrobial molecules in response to specific components of the gut microbiota, such as lipopolysaccharides (or LPS molecules); LPS molecules are key components of the outer membrane of gram-negative bacteria that can act as endotoxin and work as a toll-like receptor 4 (or TLR4) agonist with potential immunostimulatory activity, making it a strong immunostimulant that can be positive in small doses and potentially lethal in large quantities, inducing known side-effects like inflammation, fever, septic shock, and even death [245,246]. Additionally, LPS is known as the major bacterial molecule recognized by the human innate immune system, and this statement may also apply to other mammalian systems [247]. IECs can regulate LPS activity at the gene expression level (such as via *lpxC* gene regulation), at the protein-production level (such as

producing the enzyme acyloxyacyl hydrolase (or AOAH) to detoxify LPS), and even though cytokine activity (such as by utilizing the immune cell Th1 cytokine IFN-γ, which is critical to innate and adaptive immune responses and functions as the primary activator of macrophages while also stimulating the activity of both natural killer cells and neutrophils) [247-249]. As a result, these intestinal epithelial cells are key mediators of hindgut homeostasis because they directly interact, establish, and control the intestinal environment so that communication with the body's immune system is firmly established. The gut lumen is stable and nutrient-rich enough to allow for colonizing commensal bacteria, which are key players in various biological processes, including hindgut fermentation.

Within and between IECs, messenger RNAs (or mRNAs) also play a major role in signaling and regulation; for example, mRNA molecules "interact with RNA-binding proteins throughout their lifespan to carry genetic information and provide precise spatiotemporal regulation within cells" [250]. mRNAs are single-stranded molecules of RNA that carry the necessary genetic information needed to make proteins; they are transported from the cell's nucleus to the cytoplasm, where ribosomes can use them to translate the gene's original "message" into a corresponding protein. Importantly, the small size of these mRNA molecules makes them easier to transport, even when associated with various proteins during RNA processing and transportation [251]. There are many potential regulation methods that mRNAs can participate in, including translation regulation, which can affect what kind of protein is being produced and at what levels [252]. In general, translation regulation- or a form of control over protein synthesis- can be altered by mRNA via single or combinations of mRNA modifications, which could be co-

or post-transcriptionally incorporated into mRNAs, altering their length, composition, cap, and poly(A) tail, and even binding affinity to ribosomes in the cytoplasm [253,254].

Additionally, the amount of mRNA produced can signal how much protein can be made; in short, mRNA levels in the cytoplasm primarily determine protein amounts at a steady state [252]. This means that transcription can be directly controlled by limiting the amount of mRNA transcribed by a specific gene. Finally, mRNA molecules can also a) undergo alternative splicing, which ends up making different mRNAs and proteins from the same RNA transcript, b) become targets of microRNAs that can regulate mRNA expression by chopping them up or blocking their translation, and c) go through RNA processing that can change the sequence and adds elements like a poly-A tail, which can therefore affect how an mRNA molecule is used and what protein product will result from translation [255]. In IECs, in particular, mRNAs participate in both internal and external cell activities but are processed in different locations along the GI tract; one study displayed that out of all the differentially expressed genes discovered in the intestines of mice, significant enrichment existed for genes involved in cell cycle progression, translation, and importantly, RNA processing [256]. This means that along the GI tract of these mice, the genes involved in RNA processing were expressed at different levels in different locations, likely corresponding to each segment's microbiome, overall structure, and digestive activity (i.e., one area of the gut may need a specific RNA molecule present to code for a needed protein while another area of the gut may not need that particular RNA). MRNAs are key intermediate players in gene expression and essential components in gut cell signaling and homeostasis.

Returning to the broader equine model, heavily digested material in the intestinal lumen will eventually reach the horse's colon. Finally, once as much water, ions, and other reusable materials are reabsorbed back into the horse's body, fecal matter will pass through the hindgut into the rectum and then out of the system as feces. Fecal matter can tell horse owners a lot about their horse's health, such as if the horse is dehydrated or suffering from worms or parasites, et cetera [257]. However, fecal samples do not necessarily tell us everything about the horse or his health; although feces are often used in microbiome studies, many recent studies have shown that these samples do not reflect the microbial composition of the entire gastrointestinal tract [61]. Many other studies have also demonstrated that fecal samples- taken from horses and other mammals- deviate compositionally from samples from other areas of the gut, meaning that these samples cannot tell researchers anything accurate about earlier portions of an animal's gastrointestinal system [258-261]. Reed *et al*. showed that fecal samples may tell us about later parts of the GI tract (such as the distal section of the large intestine). Still, they cannot be used to analyze any earlier or mid-section areas of the GI tract. As a pilot study to this current project, this is important because it emphasizes that compositional differences exist between every section of the gut- with larger differences existing between earlier and later portions of the GI tract- meaning that only certain comparisons are viable [61]. Finally, horse fecal samples may have a general composition of water, microbes (living or dead), undigested grass and grain fibers, minerals and nutrients (like nitrogen, phosphorous, and potassium), shed cells, sand, and grit [262,263].

All in all, the horse's digestive physiology is unique in the sense that it relies heavily on microbial fermentation in the hindgut, requires a constant flow of plant material from grazing and "little and often" feeding patterns, and a single meal can take days to fully digest since horses are

large, herbivorous animals. Their intestinal epithelial cells, in particular, are variable and perform many vital functions, which makes them essential players in maintaining the horse's GI homeostasis and overall health. Importantly, the overall structure of the equine hindgut directly influences the microbial fermentation process, with its longer length directly aiding in plant digestion, vitamin production, water absorption, and efficient energy production. Finally, equine gastrointestinal physiology influences and is influenced by the host cells' gene expression (including mRNA production), the intestinal microbiome, and the immune system.

Gene Expression

Gene expression is a process where genetic information from the host's DNA is effectively transcribed into RNA, which can then be translated into a protein product (or non-coding RNA) used in biological processes to influence cell function directly and/or induce a biological change. Gene expression is also used to maintain homeostasis since it directly controls what products can be made through various mechanisms involving the host's genes and regulatory elements, which will be discussed in depth later. By directly regulating what biological products are being produced, as well as being used as a homeostatic mechanism which is required for normal functioning in every cell throughout the body, gene expression plays a major role in the overall functioning of cells, cellular structures, tissues, organs, and therefore the whole body of an organism. Problematic gene expression events can cause severe illness, such as benign tumors and malignant forms of cancer [264].

To begin understanding gene expression, it is essential to recognize and understand how The Central Dogma of molecular biology works, coined in 1957 by Francis Crick. In general, The

Central Dogma explains how genetic information flows within a biological system, almost like how the post office works in our everyday lives; it addresses where the product and package are made, where the package is transported, the route it travels along the way, and what ends up getting delivered. The Central Dogma is often stated as “DNA makes RNA, and RNA then makes Protein-” a simple way of explaining a complex process, but the idea still stands today in many biology classes worldwide. To summarize, DNA is replicated using genetic machinery like DNA polymerase, which is then transcribed into RNA using RNA polymerase, which can be further translated into protein via the cell's ribosomes [265]. Although it is taught in this manner, the transcribed RNA is not always converted into proteins; sometimes, the RNA can exist on its own and carry out functions without needing a protein product, and some examples of RNA that can operate on their own and aren’t translated into proteins are tRNA, rRNA, and other ncRNAs [266]. Importantly, although they are different molecules, mRNA, tRNA, and rRNA must work together during the process of translation to build a protein, with mRNA delivering the “blueprint” manual instructions to the ribosome, tRNA carrying the amino acids to the ribosome, and rRNA acting as the “factory” that makes up the actual ribosome [266,267]. Gene expression plays a direct role in The Central Dogma because it dictates what can be transcribed into RNA, with certain genetic factors aiding in expression and others inhibiting expression.

DNA, or deoxyribonucleic acid, is the site at which gene expression occurs. It is a polymer composed of two polynucleotide chains that coil around each other to form a double helix that carries essential genetic instructions for a cell to exist [268]. In general, the instructions carried by DNA can influence the development, functioning, growth, and reproduction of all organismal cells and the systems they inhabit [269]. The two polynucleotide chains in DNA are commonly referred

to as a “double helix,” a structure that was discovered by scientists Watson and Crick in 1953 as well as with images developed by the English scientist Rosalind Franklin’s previous X-ray diffraction work that clearly showed a visual double-helical structure (notably, Watson and Crick won a Nobel prize in 1962 for their work but Franklin’s contribution was not correctly recognized until recent years) [270]. It was also discovered around this time that DNA consisted of complementary base pairs, which include adenine, thymine, guanine, and cytosine. Adenine and guanine are purines, which means they have double-ring chemical structures, while cytosine and thymine are pyrimidines or single-ring structures [271]. In short, the adenine pairs with thymine, and the guanine pairs with cytosine, but a distinction exists in the way that these pairs bind; three hydrogen bonds hold together guanine and cytosine while adenine and thymine are only held together by two hydrogen bonds [271]. The covalent bonds that form between the phosphate group of one nucleotide and the sugar molecule of the next nucleotide form a long polymer of nucleotide monomers, which end up making a “backbone” of sugar-phosphate groups that line up in each single strand of DNA; in this formation, the nucleotides stick out from the backbone [271]. In both strands of DNA, the sugar-phosphate groups face outwards while the nitrogenous bases interacting with one another face inwards; this formation exists because the nitrogenous bases are significantly hydrophobic and, therefore, prefer avoiding water. Importantly, the hydrogen bonds that hold these bases together play a key role in maintaining the overall structure and shape of the DNA molecule. However, in general, hydrogen bonds are weak on their own, but in DNA, because there are so many of them present, they are collectively quite strong; in short, hydrogen bonding is a weak molecular force, but it does have an additive effect that stabilizes the DNA molecule and makes it stronger in numbers [18,272,273].

A couple of essential processes play a role in maintaining DNA structure and function in living cells. Firstly, as discussed previously, proper bonding between nitrogenous bases and the sugar-phosphate backbone is vital to maintaining the overall structure of a DNA molecule; factors that could affect this include helicase activity or environmental exposure to radiation or ultraviolet (UV) [274,275]. Secondly, gene regulation processes such as the use of methylation and histone activity can affect DNA structure and function by packing nucleosomes tightly together and preventing transcription factors from binding to the DNA, therefore halting gene expression in certain disease states induced by improper gene expression- which includes a wide range of diseases ranging from cancer to cardiovascular to autoimmune to neurological- sometimes disruptions in gene regulation processes can be a key cause to the onset and development of the disease-state [276,277]. Finally, on top of internal regulation and gene regulatory processes, many external structures exist to protect the DNA in a cell from biochemical components that could be damaging; for example, the nucleus has an envelope that separates the cell's genetic material from the rest of the cell, acting as a protective mechanism in eukaryotic cells [276,278]. Other examples of critical external structures that protect DNA structure and function include many proteins, such as RPA, NAPs, SASPs, and SSBs, and cell cycle regulatory elements (such as tumor suppressor proteins). Transcription regulators (such as the presence/absence of transcription factors) and many other structures also exist to monitor DNA activity constantly, especially if structural damage could compromise the function of transcription products [279-281]. In general, without these elements in place, the overall biochemical balance of the DNA molecule wouldn't exist in a natural, functioning manner, affecting the homeostasis of cells and potentially leading to cell death and the development of disease states.

When DNA is transcribed into RNA, a lot of structural changes occur. RNA, or ribonucleic acid, is a nucleic acid present in all living cells, but unlike DNA, it is single-stranded. RNA also has a backbone of alternating phosphate groups and ribose sugar rather than deoxyribose, found in DNA [282]. Another major difference between DNA and RNA is that RNA uses the base-pair uracil instead of thymine, and uracil has a pyrimidine structure similar to thymine [18,271,273]. RNA is transcribed from DNA via an enzyme called RNA polymerase, which creates an RNA sequence that is complementary to the DNA template strand being read; this means that if a sequence of "ATGCCG" is being transcribed by RNA polymerase, the corresponding RNA structure will be "UACGGC." First, RNA polymerase binds to the DNA sequence at the promoter during initiation, and it separates the DNA strands to provide the single-stranded templet needed for transcription; then, elongation occurs where the template strand acts as a template for RNA polymerase, which builds an RNA molecule in the 5' to 3' direction one base at a time (notably, this RNA transcript has the same genetic information as the template- or coding- strand of DNA, but with uracil instead of thymine); finally, termination occurs when termination sequence signals that the RNA transcript is complete, allowing the transcript to be released from RNA polymerase (there are many ways to terminate a transcription process, and the formation of a "hairpin" is one such mechanism) [18,283]. The process of transcription will always take place in the nucleus of eukaryotic cells since that is where the DNA is readily available; additionally, because of the size and presence of DNA in the nucleus of eukaryotes, transcribing the smaller, easy-to-read, more transport-friendly RNA structures makes it easier to move genetic information from the nucleus to other parts of the cell through nuclear pore complexes via mobile export receptors, and potentially between cells via a transport method like exocytosis [283,284]. In a way, it is like going to a public library and making a partial copy of a short story needed for a class of 30 students- the book (i.e.,

the DNA) stays in the library. Still, the copies (i.e., the RNA) are readily distributed outside the library.

There are three main types of RNA: mRNA, tRNA, and rRNA [283,285]. mRNA- or messenger RNA- is a type of single-stranded RNA that carries protein-coding information from the DNA in the nucleus to the cell's cytoplasm, where protein translation can occur [286]. In this study, we are interested in analyzing mRNA activity in the equine hindgut because it contains the genetic blueprint to make proteins and because of its overall mobile structure (the 5' cap protects it from degradation and the 3' poly(A) tail adds stability and aids in the ease of transport) [286]. tRNA- or transfer RNA- plays a key role in protein synthesis by matching an mRNA codon with the amino acid it codes for, making it act as an adaptor molecule that carries an amino acid directly to the site on the ribosome necessary for protein translation to occur [286]. rRNA- or ribosomal RNA- are non-coding structures forming part of the ribosome to directly aid in translating mRNAs into proteins via ribosomal protein synthesis, accounting for around 80% of the total RNA in cells [287]. Another type of RNA that exists is called SnRNA, or "small nuclear" RNA, which exists as part of spliceosomes that can catalyze the splicing and editing of mRNAs; they may be only around 150 nucleotides in length, but they often form larger RNA-protein complexes (or snRNPs) in the cell nucleus [288]. ncRNAs- or non-coding RNAs- are a type of RNA that does not code for a protein product, and functional ncRNAs may be categorized as infrastructural or regulatory; some examples of ncRNAs include microRNAs (miRNAs), small interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), and importantly, tRNAs and rRNAs [289-292]. Some RNA may be further synthesized into proteins via translation. At the same time, other ncRNAs may circulate freely in the cell and perform other tasks, such as long ncRNAs like thymus-specific non-coding

RNA1 (Thy-ncR1) or HOX antisense intergenic RNA myeloid 1 (HOTAIRM1) [293]. Additionally, if an RNA needs to get degraded for whatever reason, the cell's ribonucleases (or RNases) act to degrade the RNA by performing enzymatic reactions that cleave the phosphodiester bonds and eventually yield mononucleotides, making them non-functional and therefore marking them for cellular detection and elimination; importantly, RNases operate at both the levels of transcription and translation [294]. These RNases are hardy and very difficult to remove in the cell. Still, they are essential in maintaining a balanced system of RNAs in the cell, ensuring no massive buildup of RNA molecules could lead to problems maintaining cellular homeostasis and overall health [294].

Before discussing translation- or the RNA-to-protein-making process- it is essential to understand how genetic information is read and synthesized into tangible products. Each of the four bases in an RNA molecule can be combined to mean different things, similar to how humans read, process, and digest written information on paper to create thought, action, and response. Base triples, or recurrent clusters of three bases put together, can be read and transcribed into actual amino acids, of which 20 exist. Some of these triplets can code for the same amino acid- for example, "CUU" and "CUC" both code for leucine, a branched-chain amino acid that can help build and repair muscle tissue in mammals [295]. Additionally, there exists important "start" and "stop" signals that indicate when the "story" begins and when it ends; the "AUG" codon will always code for methionine, which will code for the "start" of the process, while "UAA," "UAG," and "UGA" will all code for the process to "stop." Without these four essential codons, the process of transcribing proteins could not begin and would not end, which could, therefore, cause problems with protein formation and disturbances in gene expression.

During translation, the RNA molecule is transported outside the eukaryotic cell's nucleus and into the cytoplasm, where it interacts directly with ribosomes. These ribosomes are essentially "factories" for the synthesis of specific protein products and the site at which translation occurs; however, this process could not happen effectively without readily available amino acids circulating nearby, along with the proper tRNAs that have complementary anticodons to the mRNA codons being translated [296]. Translation begins with the small and large subunits of the ribosome coming together and binding to the mRNA during the initiation stage, with the first tRNA entering the large ribosomal subunit and the tRNA-ribosome complex, then attaching to the 5' cap of the mRNA molecule to begin the process [297]. Initiation is then followed by the elongation stage, where the amino acids are brought to the ribosome via tRNAs and linked together by the ribosome via peptide bonds to form a polypeptide chain; importantly, protein formation requires energy, including ATP (for attaching an amino acid to a tRNA molecule) and GTP (used throughout elongation) [297]. Once the polypeptide chain is complete, the process ends with a termination stage where the finished structure is released after a stop codon- or release factor-enters the P site of the ribosome, allowing the polypeptide chain to either start performing its job in the cell or potentially enter an editing/processing stage (which can help with folding into its proper 3D structure, direction to certain areas of the cell, or forming essential interactions with other polypeptides) [297]. As the polypeptide chain is formed via peptide bonds, tRNAs will be released once their amino acid has been successfully bound to the growing polypeptide chain. The process ends with a stop codon, which acts as a termination signal for releasing the polypeptide chain into the cytosol of the cell and the later release of the ribosome subunits. After translation, the ribosome can be reused to synthesize other proteins, a process termed "ribosome recycling" [298].

Proteins, which are the products of translation, are nitrogenous organic compounds that contain one or more amino acid chains linked together to form a large biomolecule because 20 potential amino acids exist, and 20^n represents the number of different combinations possible (with n being the number of amino acids in the polypeptide chain, which may vary from ten to a hundred to even around 1-2 thousand), this means that there can be millions- and potentially more- of possible proteins that can be made as a result of translation [299]. Proteins perform diverse functions, including organization, transportation, defense, and structural functioning [300]. Proteins are also directly involved in organismal metabolism because they provide overall structural support and often act as enzymes, carriers, or hormones [300], all necessary for normal functioning and, therefore, GI homeostasis. One example of how integral proteins are in mammalian systems includes the process of oxygen transportation via the body's red blood cells, which contain hemoglobin- a protein compound that carries oxygen throughout the body by readily binding oxygen to an iron-containing heme group- and without that compound oxygen would not be able to circulate to needed areas successfully. The organism would not survive [301].

As a brief introduction, various regulatory elements can act as a "go" signal or a "stop" signal for gene expression. A promoter region, for example, is a region of DNA located close to the gene's transcription site and around where transcription machinery can assemble to begin transcribing RNA from DNA [302]; the promoter can be controlled by various regulatory sequences that can either enhance or repress transcription at that site on the DNA. Additionally, the promoter's orientation, positioning, and distance from the gene's transcription site can affect the speed and efficiency of that gene's transcription [303-305]. Without the promoter region, which directly controls the process of gene expression, transcription could not be properly

initiated, and the production of RNA and a later protein product would not happen, meaning the gene would not be transcribed/expressed [303-305]. However, promoter deletion analysis is a common, widely used technique used to identify critical regulatory regions involved in transcriptional control of gene expression, making it a successful method for determining if any cis-acting elements or specific transcription factor binding sites within a promoter could potentially be responsible for the transcriptional regulation of a gene [306].

Regulatory elements can be used at the transcriptional or translational levels of gene expression. During transcription, some important regulatory features may include promoters, enhancers, silencers, insulators, activators, repressors, transcription factors, and other structural components like histones. During translation, regulation occurs predominantly at the initiation step. Still, the process may also be regulated using signal-dependent covalent modifications of general translation (initiation) factors and trans-acting RNA-binding factors (RBPs and miRNAs) [283,307]. Translation rates can also be affected by a) the ability of the miRNA to travel to the ribosome, b) the ribosome's efficiency at "coming together," c) the length, structural accessibility, presence of open reading frames, and content of the RNA being translated (and where the "STOP" codon is located), d) the availability of tRNAs bound to their correctly paired amino acids, and e) the cellular environment (i.e. heat, cell size, pH, et cetera), along with other factors [283,308-310]. Cellular environment, or the environment in the cell's nucleus such as the pH, cell size, or temperature, as well as the availability of essential structural elements- such as RNA polymerase, transcription factors, available promoter and enhancer regions on the DNA template, ex cetera- involved in the process, can also affect transcription [283,308-310].

Besides internal regulation, various environmental factors, including diet, temperature, oxygen levels, humidity, light cycles, and the presence of mutagens, can affect how an animal's genes are expressed [311]. Chronic exposure to the stress hormones called glucocorticoids has been found to cause epigenetic changes that could influence gene expression and affect an individual's health [312]. There are many ways to test how environmental factors can shape gene expression; for example, studies using twins have been performed to see how organisms born with the same genes may exhibit differences- phenotypically and potentially regarding gene expression- due to environmental differences [313]. These studies can be important because they allow researchers to estimate the proportion of variance in a specific trait of interest that is attributable to genetic variation versus the proportion that is due to the shared or unshared environment; in short, using twins in a study can reduce the amount of genetic or environmental variability, which can therefore improve a study's statistical power [314].

Regarding the history of gene expression, Barbara McClintock displayed at the Cold Spring Harbor Symposium in 1951 that there existed an interaction between two genetic loci- activator and dissociator- in color formation that occurs in maize seeds; she later received the Nobel Prize in Physiology or Medicine in 1983 for her experiments detailing the evidence for transposons [315-318]. Her findings were important because they highlighted the instability of genetic material and the existence of transposable elements in the genome [315-318]. However, gene expression itself was not officially discussed until the summary of the central dogma was formulated by James Watson and Francis Crick in the early 1950s and published by Francis Crick in 1958, with further development on these ideas appearing in a 1970 article that expanded upon the concepts of reverse transcription and RNA replication [319-320]. In addition, the idea of a gene "regulation" system

was not relatively understood until the discovery and identification of the lac operon in 1961 by Francois Jacob and Jacques Monod, whose study showed that bacteria could control the production of an enzyme called beta-galactosidase and became the first model available for the control of protein production [321]. Finally, modern technological advances in genetic profiling, analysis, and resources in general (i.e., having access to the internet, virtual resources, et cetera) have led to faster, more efficient work pipelines. More recent research on gene expression has been driven by many major technological advances- such as the utilization of microarray analyses and qRT-PCR- that have made it easier to analyze and study various aspects of the process in vitro, in vivo, and in real time [322].

There is a range of approaches that exist to study gene expression, which includes at the RNA level (such as qPCR, RNA-seq, microarray, or Northern blotting) or the protein level (such as mass spectrometry, Western blotting, or ELISA) [323-326]. Some commonly used methods of studying gene expression include microarray analysis or real-time PCR, with our study utilizing RT-PCR to study RNA activity to analyze gene expression in the equine hindgut at the transcription level. In general, DNA microarray analysis consists of two complementary strands of DNA joining together via hydrogen bonds to form a double-stranded molecule to compare and analyze the sequences; microarray analysis happens via the following steps: sample isolation/preparation, hybridization, washing, and image analysis [327,328]. Some of the positives of the microarray analysis method are that it is a practical, reproducible, and reliable form of analysis that can be applied to various experimental procedures, and it can also examine the expression of thousands of genes simultaneously [329]. Some drawbacks to microarray analysis include the high cost of a single experiment, the large number of probe designs based on sequences

of low specificity, and the potential need for more control over the pool of analyzed transcripts [329]. Quantitative reverse-transcriptase PCR (qRT-PCR or qPCR), another commonly used method, is a truly quantitative method that combines PCR amplification and detection into a single step, which eliminates the need to use processes like gel electrophoresis to detect specific products [330]. Some of the most prominent advantages of qRT-PCR compared to other PCR-based quantification approaches are that it eliminates post-amplification handling, has more straightforward automation, and processes a larger number of samples while having a very large dynamic range of template determination [331-334]. However, some potential drawbacks to this method include that it requires expensive equipment and reagents, it can be relatively complex, there may be problems associated with sensitivity, reproducibility, and specificity based on assay design, human error, or another factor, and it also requires separate priming reactions for each target, meaning that it isn't possible to go back to the same preparation and amplify other targets potentially in a later stage of the research [335-338].

All in all, the complex, multistep process of gene expression is essential to all eukaryotic life. Without gene expression and regulation, cell development and differentiation would not occur, and the cell would be unable to produce the essential RNA and proteins critical to many cellular processes. Since proteins dictate cell function, without gene expression creating proteins, there would be no direction or functioning, and life as we know it would not exist; in short, nothing would get done or work properly without gene expression. Importantly, any problems with the gene expression pathway could directly affect homeostasis and cellular functioning, leading to severe illnesses, including but not limited to autoimmune diseases, neurological disorders, or cancers [339]. This means that gene expression is the process that controls and directs cell

structure and function, communications between cells and external, non-self-entities like the microbiome, and is an essential component in maintaining internal homeostatic processes and, therefore, organismal health.

Gene Expression in Mammalian GI Systems

Gene expression plays a role in the biochemical activities of the mammalian digestive system in many ways. For cells to function properly, proteins must be produced in appropriate quantities and with the best quality possible; this means that gene expression as a system must be actively expressing or inhibiting specific protein production processes to ensure proper protein folding, post-translational modifications, and product assembly [340]. In essence, gene expression in mammalian cells is a necessary process that dictates cell function and overall biological activity, and problematic alterations in expression patterns can lead to dysbiosis and, therefore, disease states in an organism. Problematic mutations in mammalian cells that result from issues relating directly to gene expression and its regulation include but are not limited to cancers, autoimmune disorders, neurological problems, developmental syndromes, diabetes, cardiovascular disease, and obesity [339]. As a result, the scientific community needs to understand what "normal" gene expression events look like in mammals so that it will be easier to detect and potentially treat disease states when abnormal events happen.

Mammalian gastrointestinal systems, in particular, have patterns of gene expression that are often shared between different species, with the primary goal being to aid in digestion, for epithelial cells to remain in communication with the contents of the intestinal lumen, for a proper response to occur when potential toxins, allergens, ex cetera have entered the body using the

immune system, and to work to maintain GI homeostasis constantly. Of course, the pattern of digestion and when certain events occur in the organism's body will heavily depend on physiology, what that animal has eaten, that animal's way of eating, and if that animal has any history of illness. In general, gene expression plays a direct role in digestive activity by actively controlling what can be digested, interacting with the GI microbiome and immune systems, directing which elements can enter or leave epithelial cells, and directing which parts must remain in the digestive system for further digestion or so that they may later be excreted [341]. The process of gene expression, laid out in the previous section, will be essentially the same for every mammal. Still, the level of expression, where it happens in the digestive tract, and how effective the expression will be in performing a certain task will be different for every animal. As an example, some people who consume the same amount of alcohol will have symptoms of nausea, dizziness, or overall drunkenness right away. In contrast, others may develop those symptoms later, showing that mammalian gastrointestinal systems share the same general qualities but may still react to certain factors differently or at different rates.

Every mammalian cell must have shared fundamental gene expression activity; for example, gene expression events that promote the proper use of energy and moderate protein production are essential components of every cell, and without them, cells may not function properly. One example of necessary gene expression events in every cell is housekeeping genes, which are required to maintain essential cellular function; some examples of housekeeping genes are actin, GAPDH, and ubiquitin [342]. Constitutive genes, which are continuously transcribed in cells regardless of environmental conditions, are key components in maintaining cell structure and function, with one example being the enzymes present in the citric acid cycle [343]. Importantly,

all mammalian species share a significant amount of their genetic code, with most of the major differences being at the expression level; this is because we all share a common ancestor, and over time, many changes- dietary, environmental, social, ex cetera- have shaped how evolution takes place [344]. For example, humans share ~98% of their genetic code with chimpanzees, 80% with cows, and 87% with horses [345-348]. Two radically different species- the human and zebrafish- which digest different foods and have different sizes, lifespans, and lifestyles- share 73% of their DNA, meaning they are more similar genetically despite their different phenotypes [349]. This means that many of the basic segments of DNA that code for essential processes for life are likely the same between different mammalian species, and these segments are actively expressed at the genetic level in every cell of every individual animal.

Some gene expression events are consistent throughout every living cell in a mammal's body, while others are more specific to a particular cell type or group of cells. Often called tissue-restricted expression, this gene expression is only exclusive to one or a group of specific cell/tissue types in a living system. This tissue-restricted expression leads to tissue differentiation, which creates cells that function in a particular way or have specific tasks that other cells in the body may not perform. One example of tissue-restricted expression in mammals can be seen when stem cells differentiate into other cell types- such as fat, muscle, bone, blood, nerves, epithelial, immune, or sex cells- which is an important process because this specific expression leads to the creation of different tissue types which perform separate, yet essential tasks, which in effect allows for organ differentiation and therefore the development and maintenance of a living organism [350]. On the other hand, gene expression events that are consistently present throughout most of the cells in an organism's body are often essential processes for cell survival and functioning. For example, the

16S rRNA gene codes for the eventual production of ribosomal RNA that makes up the ribosome, an essential structure during translation [351]. In essence, gene expression events must occur in all cells no matter what type, but some gene expression events are exclusive to a certain tissue area in an organism, and this differential expression is key in differentiating organ systems and their functions.

Without tissue-restricted expression, we would not witness the formation of different organs in living things, meaning that larger, more complex lifeforms would likely not exist. The heart, a critical organ in mammalian systems that actively pumps oxygenated blood throughout the body to tissues that need it, is an excellent example of an organ with tissue-restricted expression. Cardiac tissues contain "pacemaker" cells that contract and expand in response to electrical impulses from the nervous system and can generate action potentials that direct cardiac muscles to contract and relax. These cells have specific transcription factors (such as Gata 4, Gata 6, Mef2C, and Tbx5) and gene expression events that are absent in other tissue types [352,353]. As a result of this type of expression, heart tissue can pump blood throughout an organism in an organized, evenly paced, pressure-controlled way and, therefore, circulate oxygenated blood to needed tissues in a constant, efficient manner. This kind of tissue expression is present within every specific organ in the body, including other essential systems that include bone, muscle, and sex organs. A perfect example of how important tissue-restricted expression can be seen in the body's hox genes; hox genes, or a group of related genes that specify where each organ system along the head-tail axis of an embryo will develop, code for body segments to exist in certain places of the body during embryonic development. William Bateson first identified and studied them in 1894 [354-356]. In 1915, Thomas Hunt Morgan's lab analyzed homeotic transformations

and created the first homeotic mutant, with the result being a bithorax *drosophila* fly that had a duplicated thorax, displaying that these genes dictate where body segments develop in the early stages of life [357,358]. It was further confirmed by later experiments- such as those done by Ed Lewis, Christiane Nüsslein-Volhard, Eric F. Wieschaus, and others- that mutations in hox genes can cause abnormal events, such as legs growing on the head of flies where antennae should be, which can therefore alter proper functioning and thus the organism's overall survivability rate [359,360]. As a result, previous studies on hox genes showed that gene expression could directly influence an organism's phenotype, further emphasizing the importance of proper tissue-restricted expression, particularly during an organism's early development.

Other examples of genes present in every mammal that are consistently expressed for a major function required for cells to survive are genes that operate by activating and regulating cell growth, proliferation, and survival via energy-making and delegating mechanisms [361]. Proto-oncogenes, for example, are normal cell genes that regulate cell growth and differentiation; they operate by coding for products such as growth factors, cell cycle regulators, DNA-binding proteins, transcription factors, and others, which can play key roles in the processes of transcription, translation, cell signaling, ex cetera [362,363]. The other genes listed above also go through transcription and translation to code for essential proteins and mRNA molecules, all of which operate to perform a specific task intended to aid in essential cellular functioning. Interestingly, important genes are not always coded at the same rate during an organism's development; a recent study showed that neuronal essential genes (or NEGs) are expressed highly before birth during the early development of the human brain and maintain a relatively high expression after birth. In contrast, ACEG expression tended to be expressed highly during

development but dropped quickly after the organism's birth [364]. This finding is interesting because it displays that changes in NEG or ACEG trends of expression can lead to neurodevelopmental disorders or neuropsychiatric diseases, emphasizing the importance of not only understanding what gene expression looks like in healthy individuals but also how healthy individuals' gene expression tends to look overtime [364]. In short, gene expression can be influenced by other factors- such as time, disease states, environment, et cetera- to protect and maintain the organism as a whole.

All in all, gene expression plays a key role in forming important mRNAs and proteins essential for cell functioning. Some gene expression events are tissue-specific- with some segments of the organism's genetic code being expressed in one area of the body and not others- while other gene expression events are consistently present in every living cell. Some of these genes, like the hox genes, are key in the early development of an organism's body layout. In contrast, others operate to regulate the cell cycle, catalytic activity, binding, et cetera. In short, gene expression is required for mammalian cells, tissues, organs, and the entire body system to operate. As a process, it interacts directly with other bodily systems- such as the microbiome and immune system- to best maintain homeostasis and promote survival.

Gene Expression in the Equine GI

Gene expression is essential in directing biochemical activities in the equine digestive tract. Specifically, IECs in the equine gastrointestinal tract code essential mRNAs and proteins that can carry out important tasks, such as the AMP or Reg3 protein families that play a critical role in segregating intestinal bacteria and IECs [17]. The release of these molecules into the lumen from

host cells can directly influence other systems that play a role in digestion, including the GI microbiome and immune system [17]. As a result, gene expression in the horse's gastrointestinal epithelial cells is directly involved in equine digestion because it actively directs how host cells interact with intestinal lumen contents (such as whether or not to absorb specific molecules, such as VFAs or salts). In short, alterations in gene expression activity can directly or indirectly lead to changes in digestive activity and potentially develop disease states, such as laminitis or colic [83].

Adipose tissue location in horses, for example, is associated with differences in mRNA expression of inflammation-related genes and, therefore, in certain facets of gene expression [365]. The study, which used GeNorm analysis and was done by Bruynsteen *et al*. in 2013, focused on inflammation-related genes leptin, chemokine ligand 5, interleukin 1β, interleukin 6, interleukin 10, adiponectin, matrix metalloproteinase 2, and superoxide dismutase 2, and took samples from 8 different adipose tissue sites [365]. They found that HPRT1, RPL32, and GAPDH mRNA expression were the most stable genes in every adipose tissue site. In contrast, the expression of leptin, chemokine ligand 5, interleukin 10, interleukin 1β, adiponectin, and matrix metalloproteinase 2 significantly differed across the eight tissue collection sites [365]. There were also differences between adipocyte area and the number of antigen-presenting cells per adipocyte, which makes sense given that certain body tissues might have more immediate interactions with potential pathogens and will, therefore, have cells that are more likely to present antigens [365]. In the end, this research is important because it displays that an inflammatory response in horses could be partially determined by the relative proportion of different adipose tissue depots, meaning that the area where the adipose tissue is located will influence expression and not all adipose tissue will share the same gene expression processes.

The findings of Bruynsteen *et al*. may apply to other body regions besides those containing adipose tissue, such as epithelial cells in the equine digestive tract. Similar to their study, taking samples from multiple regions of the equine hindgut that are a) found in different places along the gastrointestinal tract, b) potentially digesting different nutrients at different rates, and c) may have differences in gene expression based on location, function, and other factors such as microbial distribution, and comparing them to the same physiological regions in other horses could lead to similar findings. Similarities will likely be shared between the different gastrointestinal samples taken from the horses, given that they are all the same type of cell from the same species with similar digestive tasks. Still, the differences could lead to specific, essential functions only targeted at certain portions of the hindgut. These differences, which are currently not well understood, are the key aspects of our current study that could further characterize a) what gene expression activity is shared throughout the hindgut and why, b) what gene expression activity is targeted only at tissue-specific regions of the hindgut and why, and c) how is this process efficient enough in aiding the horse to maintain the proper digestion of nutrients and gastrointestinal homeostasis [16].

Notably, gene expression also directly influences the microbiome in the gastrointestinal tract. One specific example is when the gene *foxp3* is expressed; there is a positive correlation with the abundance of *Verrucomicrobia* in the equine hindgut [114]. Additionally, if gene expression directly alters the intestinal lumen, this can greatly affect the microbiome and potentially disturb microbial homeostasis if the change is great enough; for example, any change in lumen pH due to problems with gene expression could lead to changes in the microbial population and the death of vast microbial populations [114]. In mammalian systems, genetics play a role in the overall composition of the gut microbiome, meaning that microbial species and their compositions are

heritable from one generation to the next; this translates to the development of certain GI diseases- such as IBD- if relatives were previously diagnosed [366,367-370]. The microbiome can also influence gene expression that can lead to downstream effects on immunity, metabolism, and other biological systems in the host [371]; for example, the general presence of certain microbes can induce IECs to release miRNAs, which can enter bacteria and regulate their expression and growth and therefore affect processes like nutrient digestion and absorption [372]. Another example of how the microbiome can influence gene expression is when the ratio of Firmicutes to Bacteroidetes is altered, leading to gut dysbiosis and changes in gene expression (such as via immune response genes). As a result, the microbiome and gene expression influence each other, and therefore, any alterations in this relationship can directly affect the organism's overall health.

Although not directly related to the GI tract, one recent study by Lepage *et al*. found that enzymatic digestion of cartilage significantly impacts the gene expression profile in equine articular cartilage [373]. In-tact regions had consistent gene expression of SOX9, COL1A2, COL2A1, ACAN, and COLX, while the digested cartilage showed a significant decrease in expression, particularly in COL1A2, COL2A1, and ACAN [373]. Additionally, they found an increase in COLX expression in only the non-weight-bearing cartilage, showing that the type of cartilage influences the response to degradation since non-weight-bearing regions may have fewer extracellular matrix molecules (or ECM) [373]. This study clearly shows that enzymatic digestion- whether in the equine fetlock joint or other areas of the body- can affect gene expression. Therefore, gene expression analysis should only be conducted on intact cartilage so that only reliable *in vivo* results are used in future research experiments [373]. This study also displays that gene expression can be influenced by external forces, such as enzymatic digestion or the type of

cartilage being analyzed via RNA isolation, which can also apply to other areas of the body besides the equine fetlock joint.

Importantly, the expression of many immune regulatory genes can directly influence the microbiome and overall equine health. One study done by Lindenberg *et al*. in 2019 showed that the expression of immune regulatory genes- specifically with *foxp3* or *il10*- was directly correlated with certain microbial abundances, including specific *Clostridiales* and *Verrucomicrobia* species that were found to be present in the ileum and cecum [114]. Additionally, they found that increasing the expression of the anti-inflammatory gene *tgfb* was found to be correlated with increasing abundances of *Bacteroidetes*, and the expression of the pro-inflammatory *il17* gene was found to be negatively correlated with unclassified *Ruminococcaceae* expression [114]. These results suggest that the abundance of intestinal microbiota in horses is directly influenced by immune regulatory gene expression and the activity of specific transcription factors, meaning that these bacteria may play various digestive, immune, and regulatory roles, which can also be directly influenced by gene expression [114]. Finally, the bacterial species discussed in Lindenberg *et al*. may also be useful targets for increasing regulatory immunity in the horse's gastrointestinal tract [114].

Another equine-specific study that shows the importance of gene expression in the equine gastrointestinal tract is one done by Cappelli *et al*. in 2019, which displays that the expression of the *GUCA2a*, *GUCA2b*, and *GUCY2* genes- all of which encode for guanylate cyclase-c (or GC-C) multifunctional receptors- were highly expressed in the horse jejunum, ileum, descending colon and rectum [374]. GC-C is a type I transmembrane receptor expressed on the apical surface of

intestinal epithelial cells; they are important because they help control the flow of ions and water from the lumen to the IECs and vice-versa and also play a role in mucosal barrier function, inflammation, intestinal cell proliferation, and pain sensation [374]. This study further confirms that this particular set of genes is not only highly expressed in the GI tract of horses but is present to serve a wide variety of important functions and may be a potential target for future therapeutic approaches- such as using a selective antagonist of GC-C to prevent uncontrolled electrolyte and water released into the intestinal lumen and therefore the development of secretory diarrhea- to help horses that have GI disease [374].

Gene expression is a dynamic, necessary process for cellular functioning, and it is especially important in essential processes like digestion. Since horses are hindgut fermenters, a majority of the energy they receive from ingested food is formed in the hindgut, which relies heavily on microbial fermentation to function [64,65,375]. Gene expression can direct and shape microbial systems, immune responses, and overall cellular structure and function. In the equine gastrointestinal tract, gene expression can determine whether the entire GI system functions in a healthy, homeostatically balanced manner, or if unregulated, gene expression can cause various health concerns, including gastrointestinal disease and cancer. Importantly, gene expression- along with the GI microbiome, host cells, and immune system- all influence and interact with one another to ensure that GI homeostasis can exist and, when working correctly, ensure that the horse remains healthy. Given that very little is still known about gene expression in the equine hindgut, it makes sense that current research follows the study of gene expression to ensure that every key system is explored and understood in the most straightforward, most scientifically credible way possible.

This is especially true as it relates to the intestinal regions surrounding the pelvic flexure, which was shown previously as an area of interest.

The Microbiome

A microbiome can be loosely defined as a community of different microorganisms, or microbiota, that are found in a particular part of an organism's body. Most living organisms with a digestive system have a gut microbiome, which plays a vital role in that organism's overall health [62]. For example, intestinal microbiome diversity has been proven to be significantly lower in individuals suffering from obesity, and these findings have been proven true across multiple species [213]. Many variables may influence the microbiome, including but not limited to age, genetic background, environment, diet, vaccinations, stress, and disease states [62,63,372]. Other key systems in the body- which we will discuss further- may also harbor an important relationship with the microbiome, including the body's physiology, gut structures, and immune system.

The microbiome of an organism contains bacteria, viruses, and eukaryotes that have been shown to interact with one another to maintain systematic balance and overall health [376,42]. For example, endosymbiosis occurs when eukaryotes and bacteria become intimately associated with one another- often for a purpose that can enhance or suppress certain biochemical processes- further displaying the complex relationships within the microbiome [42]. Our knowledge about bacterial communities far outpaces that of viral and eukaryotic communities [376], and this knowledge gap exists across various animal species. However, recent time series data has shown that the overall microbial composition is relatively stable within healthy adult individuals over time for bacteria, viruses, and eukaryotes, but this is assuming that numerous external variables

are held constant (such as diet, disease, stress, et cetera) [376]. In short, visual changes in the microbial composition may happen over time as an organism ages. Still, the overall presence of the most essential, influential, and often dominant bacterial, viral, or eukaryotic species will remain relatively the same (otherwise, the organism may experience severe illness and death).

Specific microbial phyla are commonly present across various species, but the percent composition and variation between species can be different. For instance, the gram-negative *Bacteroidetes* and gram-positive *Firmicutes* are the most abundant phyla in the human gut [42]. Similarly, in horses, *Firmicutes* represents the largest phylum in the equine gastrointestinal bacterial community [82,85]. However, variations can occur between digestive segments or even between individuals [8]; for example, a recent study displayed a large variability in the microbial composition and community in healthy individuals, with twins in that study sharing less than 50% of their species-level bacterial taxa and even fewer viral sequences [376]. Understanding these variations can help the scientific community determine a) what constitutes a "normal" range for healthy individuals, b) when deviations occur from the "normal" range, what disease states can result, and c) what can be done to treat the disease-state and bring the "abnormal" microbiota levels back to "normal."

Various studies have shown a shared core of functionalities in the microbiome [376], and it has been suggested that intestinal microbiome stability during adulthood may be a feature of mammals [213]. For example, the microbiome plays an essential part in development, immunity, and nutrition, and commensal bacteria, in particular, are key participants in the digestion of food and are responsible for the extraction and synthesis of nutrients and other metabolites that are

essential for the maintenance of mammalian health [76]. Every microbiome has commensal, symbiotic, and pathogenic microbiota that influence the host's digestion, immunity, and overall health. Commensal microbiota are microorganisms that exist in a space without harming the host organism's health, and in many cases, their presence can be beneficial; for example, commensal bacteria that reside within the large intestine of the equine gastrointestinal tract are vital for the horse to be able to utilize a forage-based diet because they produce short-chain fatty acids (SCFAs) that can be absorbed through the gut wall and contribute to the energy requirements of the horse [377]. Symbiotic microbiota are microorganisms living in symbiosis with the host (and potentially other microorganisms). As a result, both organisms benefit; for example, the horse has a diverse symbiotic microbial population that contributes heavily to fiber digestion and proper nutrient absorption [378]. Finally, pathogenic microbiota are microorganisms that can cause disease, especially when prevalent in higher numbers; changes in the gut environment, which can be easily altered by diet, drugs, stress, infection, et cetera, can cause an increase in the presence of pathogenic microbiota, which can therefore lead to disease-states [17]. For instance, one study showed that *Bacteroidetes* was the most abundant phylum among horses that had colitis, which supports the fact that *Firmicutes* play an important role in gut function and an increase in the potentially problematic, pathogenic bacteria in the hindgut can aid in the development of disease-states [63].

Interestingly enough, some microbes can fall into multiple categories; for example, some of the once commensal or symbiotic portions of the microbiome may become pathogenic when certain conditions arise. For instance, in the horse, there are forms of the diverse genera *Clostridium* that can be both beneficial and pathogenic [63]. In addition, horses that have too much

Lactobacilli spp. present in their microbiomes were shown to have an increased risk of developing conditions like osmotic diarrhea, colic, laminitis, and founder [379]; this is because *Lactobacilli* facilitates rapid fermentation and the production and buildup of lactate, which can be poorly absorbed in the gut and causes a decrease in pH, the death and lysis of other bacterial species, and mucosal damage that can result in disease states such as laminitis [62,63,68,380]. However, at lower levels, the amylolytic bacteria *Lactobacilli* can be good for the horse and may help protect the animal from disease and stop pathogens from colonizing in the gut by releasing antimicrobial substances in response to invaders [380] while also aiding in the process of fermenting starch to lactic acid before it is enzymatically degraded into glucose in the small intestine [68]. Another example of this can be seen with another well-known probiotic, *Bifidobacterium*, which was shown to increase in horses with large intestinal colic and small intestinal colic but did not induce such health risks at lower levels [62].

Studies involving the microbiomes of various species have become popular in recent years [381], likely because of the broad implications that microbiome data may have to further our understanding of what a healthy state looks like in various organisms and what certain microbiome changes could mean for current and future health conditions. Metagenomics, which is the process of identifying species present in body fluid, is often used in microbiome research, and it has two methods of sequencing: amplicon and shotgun sequencing [64]. Amplicon sequencing is a next-generation sequencing (NGS) technique that enables researchers to analyze genetic variation in specific genome regions. Integrated DNA technologies stated that this method "uses PCR to create sequences of DNA called amplicons. Multiplexing – barcoding samples so that they can be mixed into pools – allows multiple samples to be sequenced on a single sequencing run. Before

multiplexing, individual samples used for amplicon sequencing must be transformed into libraries by adding adapters and enriching target regions by PCR amplification. The adapters allow the formation of indexed amplicons and enable them to adhere to the sequencing flow cell [382]. Many projects utilize Illumina-based 16S rRNA gene amplicon sequencing to reveal similarities and differences between organs and their microbial compositions [185]. This form of amplicon sequencing utilizes ribosomal RNA (or rRNA), which is present in all living organisms and around 80% of all bacterial RNA, making it compatible with specific targeting, PCR amplification, and sequencing, and often at a lower cost [375]. Shotgun sequencing, on the other hand, can be used to determine the DNA sequence of an organism's genome by first randomly breaking up the genome into smaller DNA fragments that can be sequenced individually and then using computer programs to a) search for overlaps in these individual DNA sequences and then b) use them to reorganize and reassemble the smaller DNA fragments in the correct order as a way to "reconstitute" the genome [383]. One example of a whole-genome shotgun sequencing was done using DNA from a thoroughbred mare named Twilight to generate a complete, comprehensive genome sequencing of a horse for the first time [384]; once completed, the first genome sequence of the domestic horse was then subsequently published in November 2009 [384].

To summarize, the microbiome contains various microorganisms- including bacteria, viruses, and eukaryotes- that survive in specific niches of the host's body and work together to perform tasks- such as aiding in digestive processes- while also maintaining the host's overall health. Since many mammals share similar phyla in their microbiomes, it can be easy to see why there is a shared core of functionality between different species and also at the individual level. There is also a degree of stability in the microbiome [213], meaning that once specific changes

arise, the microbiome is relatively consistent at maintaining most of the bacteria that were present before that change while eventually recovering to "normal" levels. In general, the microbiome has commensal, symbiotic, and pathogenic microbiota present, and some of these microorganisms can fall into multiple categories depending on certain conditions (such as pH, levels present, and other environmental factors). Metagenomics processes that are commonly used in the study of an organism's microbiome include, but are not necessarily limited to, amplicon and shotgun sequencing, with many options being available for generating statistical analyses. This introduction outlines the microbiome and what can be used to study it, with the next section diving into the gastrointestinal microbiome.

GI Microbiome

The gastrointestinal (GI) tract is the digestive system passageway from the mouth to the anus of the animal. It contains many essential organs that play a role in the processing and digestion of nutrients. The GI microbiome consists of the microorganisms that live in all mammals' digestive tracts, including bacteria, archaea, and eukaryotes. Viruses are also present in the GI microbiome. It is well established that all vertebrate animals have intestinal microbiota that is associated with a) the community of viruses infecting or produced by microorganisms within the microbiome and b) the viruses of the macro-host and eukaryotic symbionts and parasites that also may exist in the host's digestive system, which may include protozoa or helminths [385]. Different sections of the GI tract will share some overall similarities in microbial composition but also often have some important differences due to various factors such as physiological structure, microbial differences, and tissue-specific digestive tasks at that point in the GI tract [61,386,387]. These microbial differences that exist between different sections of the GI tract can be seen in recent horse research

that displays separation differences between the foregut and hindgut, as well as between different body segments of the hindgut, which will be discussed later [27,61,185,378,387].

The GI microbiome is often analyzed using specific variables that can be key to discovering more about the structure and function of certain microbes within and between individual organisms and each other. Firstly, microbiome testing measures the different levels of microorganisms present in the GI tract as well as determining which phyla are the most "dominant" in certain areas [388]. Percent abundance, in particular, refers to the relative amount of a specific microbe in a particular place in the host's system. For example, in the equine GI tract, *Firmicutes* has been proven through microbiome testing to be the dominant phylum, existing at 50% or higher of the total relative abundance in every single area of the GI tract tested, with *Bacteroides* following closely behind [185,215,65,380]. This shapes the overall community structure of the GI tract by determining how much actual "space" and resources are taken up by certain phyla groups in the gut to promote the survival of specific microbes directly and maintaining this structure is essential because these microbes directly influence organismal health in various ways, including digestive, immune, metabolic, and even neurological health, and any major alterations in microbial percent abundance can lead to the development of disease-states [389]. Some of the most common ways that the microbiome levels are collected include taking fecal samples or performing a biopsy to take tissue samples; these fecal or tissue samples can then be further studied and analyzed using methods such as 16S/18S RNA gene sequencing, deep sequencing, whole genome sequencing, phylogenetics techniques or microbial diagnostics [82,390]. Notably, the results gathered from these different research methods can be used to compare/contrast the different sections of an individual organism's GI tract and similar physiological structures between other individuals. This

is essential when developing a picture of what healthy, i.e., "normal," levels look like in the GI tract, as well as a better understanding of what harmful changes to the microbiome levels can cause disease states in a host organism.

Microbiome diversity is also an essential component of the GI microbiome, directly influencing an individual organism's health status and displaying how "strong" the microbial community is [391]. It can be defined as the measurement of "the amount of individual bacteria from each of the bacterial species present in an organism's gut microbiome," and these measurements can vary based on an individual's species, age, culture, diet, antibiotic intake, stress levels, and current health status [392-397]. Often, microbial diversity can also indicate an organism's health status; for example, 75% of the world's food accessible to humans today originates from only 12 plants and five animals, and a lack of food diversity as well as the use of antibiotics in food industries- such as in meat and fish production- has been shown to actively reduce microbial diversity and therefore affect human health in various ways (such as weakening the immune system and increasing the chance of developing diseases such as asthma or IBD) [397-399]. There are many ways to study microbial diversity, with some examples being culture-based methods, 16S/18S RNA gene sequencing, and non-sequence-based molecular techniques such as PCR, FISH-flow, or bacterial DNA microarrays, which can lead to further alpha-diversity and beta-diversity calculations that can give researchers a clear sense of what types of diversity might exist in their sample set [400]. Generally, "alpha diversity is used to describe the compositional complexity of a single sample while beta diversity is used to describe any taxonomical differences between samples" [401]. However, because of the many ways to study microbial diversity, these different types of studies can also be a source of variation and, therefore, confusion regarding the

interpretation of results (for example, using only one sample type or using culturing vs. molecular approaches) [402].

Very importantly, there are different ways of analyzing microbiome diversity that involve what is known as alpha vs. beta diversity. Alpha diversity was previously defined as a method used to describe the compositional complexity of a *single* sample; we can claim that a sample has high alpha diversity when it contains a high number of equally abundant [401]. Beta diversity, on the other hand, describes the taxonomical differences *between* samples; we can claim that beta diversity is high between two samples if they share few species and that the comparison has low beta diversity if most of their species are in common [401]. Many times, alpha diversity is calculated in a research dataset by either a) calculating a weighted generalized mean of the within-subunit species proportional abundances and then taking the inverse of this mean or b) calculating the species diversity for each subunit separately and then taking a weighted generalized mean [403,404]. The most commonly used index of beta diversity is Whittaker's species turnover equation, $\beta_w = S/\alpha - 1$, where S (sometimes represented as "γ" instead of "S") is the total number of species and alpha is the average number of species per site [405,406]. In many cases, one of these diversity types may be more significant than the other due to various factors, such as sample type, sampling methodology, and sampling error, as well as if there are confounding variables present in the dataset that could be influencing the microbiomes of the study's set of organisms. As mentioned, alpha and beta diversity in the GI microbiome can be affected by diet, age, vaccination, external stressors, disease states (known or unknown), etc. This is another reason why it is so important to have control variables in microbiome studies, such as having many subjects

who are all the same species/breed, were around the same age, were on the same diet, and were raised under similar conditions.

The term “normal levels” has been consistently used so far, but what does “normal” even mean, and how can it be defined scientifically? Generally, “normal levels” of a specific microbial community can be defined as a % abundance value within a certain range widely considered “healthy” and contributes directly to organismal health in various ways. For example, there is a “healthy range” that exists for maintaining a healthy gut microbiome in humans; a Firmicutes/Bacteroidetes ratio (or an F/B ratio) is often used to analyze a range of pathological conditions in humans, with the optimal result being a ratio between 12 and 620 [407]. In addition to this ratio, the average % abundance level of *Firmicutes* in the human GI microbiome tends to fall between 11% and 95%. In comparison, *Bacteroidetes* tend to fall between 0.6% and 86.6%, respectively, and this variability between individual humans likely exists based on many factors, including their health status and the country in which they live [51]. When these “normal levels” are altered by internal or external forces, this can result in various health problems, including but not limited to obesity, diabetes, and non-alcoholic fatty liver disease [407,408]. Finally, it is important to remember that “normal” microbial levels in an organism’s GI tract will naturally vary with age, given that as an organism ages from birth to adulthood, the overall Firmicutes/Bacteroidetes ratio has been shown to increase, especially from ages 0-9 and 60-69 in humans [409].

The GI microbiome is intricately involved in various digestive processes, and diet profoundly affects the microbiome of individual animals [376,8]. In humans, a high-fat diet can

alter the *Firmicutes* to *Bacteroidetes* ratio in the GI microbiome and lead to changes in metabolism that can develop into health conditions like diabetes, IBD, and obesity [410]. Microbial dysbiosis- or a significant, potentially harmful disruption in what is considered a "normal" percent composition of microbes that exist in the GI tract- can also lead to inflammatory bowel disease (IBD), autoimmune diseases, and various allergic diseases [63,376]. A meal's nutritional value, how often the host ingests food, how much food is ingested at that point, and what food is being eaten can also influence the host's GI microbiome [410-413]. One study by Zhu *et al*. involving horses investigated how different diets- a diet heavy in hay, pasture grass, or silage- influenced the intestinal microbial community, and the researchers' results displayed that the intestinal microbiome- especially the fecal microbiome- is directly shaped by diet type, meaning that not only is the type of food important in one's diet, but overall nutritional value can also play a role in the productive maintenance or harmful alteration of the GI microbiome [414]. In short, regarding Zhu *et al*.'s specific equine diets, the source of food, how heavily processed that food is, how easily it is digested, and how nutritious it is can all directly influence the horse's intestinal microbiome.

For instance, let's say two six-foot-tall male humans of the same age with nearly identical microbiome compositions and no prior health issues eat two different diets over a period of 6 to 8 weeks. Person #1 ingests three meals daily, but they are all fast-food, high-fat meals, and he rarely exercises. Person #2 consumes three meals daily but eats all the food groups and primarily natural foods like apples and salads, and he exercises sometimes. Over 6 to 8 weeks, Person #1 may experience weakness, mood changes, and changes in weight due to not getting enough nutrition, thus altering his GI microbiome. On the other hand, Person #2 will have a much more diverse,

healthy microbiome with no serious changes in the percent composition of essential microbial phyla like *Firmicutes* or *Bacteroidetes*. However, recent studies have shown that Person #1 could still potentially restore their microbiota to "normal" levels by consistently eating healthier meals with less sugar and more nutritional value, exercising more often, sleeping well, and reducing stress levels [415].

Other variables besides diet that can influence the microbiome include the evolutionary history of the host, age, drugs, probiotics, stress, and infection [17,62,63]. Antibiotics, in particular, have a profound effect on the GI microbiome, and their overuse has been shown to cause an increase in antibiotic-resistant pathogens [68,376]. Mobile genetic elements such as plasmids, transposons, and integrons enable the spread of antibiotic resistance genes among bacteria, and the overuse of antibiotics drives this process by providing selection pressure for resistance genes to establish and persist in microbial populations in the GI tract [416]. In short, antibiotics affect the GI microbiome by damaging the gut microbiota and immune system by reducing microbial diversity, altering metabolic activity, changing functional attributes of the microbiota, formation, and selection of antibiotic-resistant strains to make hosts more susceptible to infection (i.e., the development of antibiotic resistance in certain pathogens), and by disrupting the balance of GI microbiota by allowing the proliferation of pathogenic bacteria [65,90,415,417]. In horses, antibiotics are known to disrupt the hindgut microbiota by affecting the fibrolytic activity of important microorganisms, which can be detrimental since the hindgut microbes are essential for hindgut fermentation and the source of most of the horse's energy, and this can lead to the development of harmful disease-states [65].

Many recent studies have focused on the GI microbiome's specific interactions with other essential host systems, including gut physiology, the immune system, and genetic expression. The physiology of different mammalian gut structures also directly influences microbial concentration in specific areas, mainly as a means of aiding in specific digestive processes. Microbial communities also directly affect host cells, which can benefit host physiology. This was proven in various studies, including Martin *et al*., Jones *et al*., Sharpton *et al*., Grover *et al*., Krishnan *et al*., and Contijoch *et al*. [418-425]. The earlier portions of the gut will have a different digestive composition than the later portions, emphasizing the need for different absorption tactics at different parts of the digestive system. Digestion and nutrient absorption will occur along the pathway of the small intestine. In contrast, active stool formation, water absorption, vitamin formation, and toxin protection will appear later in the large intestine [426]. This means that microbes aiding in digesting fibers in the equine model, for example, will be located in the areas closest to where exposure to said fibers occurs, while less of these microbes may be present in other areas of the digestive tract. In short, microbes will live in portions of the gut where nutrients are available and where a stable, relatively balanced coexistence is prevalent between different microbes living in that space (i.e., active competition for space and resources will still exist, but at a less prevalent/destructive level) [427].

The type of digestion needed in an animal system will also depend on what it eats- therefore, herbivores, carnivores, and omnivores have digestive tracts evolutionarily designed to fit their dietary needs [428-430]. For example, herbivores (like the horse) tend to have the longest mean retention time of digesta due to their more extended intestinal systems and the fact that their diets are composed of plant matter, which must be properly broken down via specific microbial

fermentation processes which are not present in the shorter, often less complex digestive systems of meat-eating carnivores (like the dog, coyote, or wolf) [428-430]. Another common observation of the physiological differences in digestive tracts between herbivores and carnivores is that carnivores tend to have a majority of their digestive processes occur in the small intestine, which is a direct result of the generally high digestibility of the food they eat. In contrast, herbivores may have their main digestive processes occur in various stages in the foregut or hindgut to maximize fiber digestibility and energy production [428-430]. Finally, although there are a lot of structural differences that exist between herbivores, carnivores, and omnivores, many of the same essential functional tasks that are necessary for survival exist in all of these systems, such as using chemical means to break down whatever food molecules are present for digestion into smaller, more digestible parts that can be used to make energy [431].

On top of dietary needs, an animal's digestive system is also shaped by whether or not it is categorized as a "ruminant" or "non-ruminant." Ruminants are herbivorous mammals that acquire most of their nutritional needs from plants via fermentation. This process requires microbes to aid digestion and typically requires a specialized, multi-compartment chamber, or rumen, to break down the food matter properly [432]. Some examples of ruminant animals include cattle, sheep, goats, antelopes, deer, and giraffes [433]. Because of this specialized process of digesting plant matter, ruminants have microbiomes shaped to specifically help in fermentation to produce volatile fatty acids and, therefore, energy; for example, one study showed that ruminants have evolved to possess diverse symbiotic microbiota in their rumen, with more bacterial diversity overall compared to non-ruminants [434]. Additionally, this study also found that some rumen microbial features in cattle are heritable and could be influenced by host genetics (such as breed, genetic

predisposition, and sex), management practices (such as genetic selection and breeding), and environment (such as feed type and feed efficiency) [434]. Non-ruminants, on the other hand, are animals with single-compartment stomachs and, therefore, cannot digest fibers found in forage-based diets. Some examples of non-ruminants include humans, dogs, cats, swine, poultry, and horses. Besides the noticeable structural differences, non-ruminants also have different microbiomes compared to ruminants; for example, non-ruminants must utilize digestive enzymes- such as amylase, lipase, and protease- that aid in the breakdown of different food types while ruminant animals do not use these digestive enzymes and instead rely on the chemical activity of microbes, such as *Ruminococcus* and *Selenomonas* in cows [435].

The immune system influences the microbiome by maintaining a symbiosis with diverse groups of microbes in the GI tract, essentially learning over time what microbes and microbial levels are okay and which microbes may become problematic [436]. It does this by utilizing innate and adaptive immune system responses to constantly learn and adapt to changing environmental conditions and react self-preserving to the presence of new and recognized non-self entities, which directly affects the microbiome by shaping it based on immune responses [437]. As an example, if the immune system detects a growing number of a certain species of harmful pathogenic bacteria in the gut of a mammal, it can target it for destruction and create memory B and T cells to provide a rapid future response to the same pathogen, therefore altering the gut's microbiome by decreasing the amount of the pathogenic bacteria and allowing for new space to be taken up by another potentially symbiotic microbial species. The immune system can also target harmful microbes in the GI tract before they enter and potentially harm host cells, which is essential in maintaining microbial balance because a) there is only so much available space in the digestive tract, and

having harmful bacteria present can threaten commensal and symbiotic bacteria that need said space, b) on a similar note, there are only so many nutritional resources available for these microbes to share, and c) this immune response benefits not only the host cells but also the microbes sharing that environment by protecting key resources and therefore keeping their ecological niches- or "homes-" safe and healthy [438]. During an infection, innate immune cells will act to immediately prevent the spread and movement of foreign pathogens into and throughout host tissues by using physical, chemical, and cellular defenses against potentially harmful pathogens [439]. This quick response will cause activated macrophages to release pro-inflammatory cytokines, chemokines, and other specialized pro-resolving mediators (or SPMs) to the infected region, causing the damaged, inflamed cells to release chemicals that can cause swelling and tissue inflammation [440]. This inflammation can directly influence the balance of the gut microbiome by changing its composition and potentially disrupting essential microbial digestive activities [441]. As an example, humans suffering from inflammatory bowel disease (or IBD) with *NOD2* mutations have been shown to have a decreased abundance of *Faecalibacterium* species and an increased abundance of *Escherichia* species, as well as changes in commensal bacterial activity that leads to altered mucosal barrier function and inflammatory gene expression [442]. Since these bacteria are known to influence the immune system, inflammation, and the intestinal cell mucosal layers, it can be claimed that the changes in specific microbial species abundances and diversity can directly lead to host intestinal dysbiosis and symptoms of disease, people suffering from IBD often have symptoms of diarrhea, abdominal pain, and fever as a result of these altered levels [443]. Many studies have linked problems with the gut microbiota to inflammatory diseases, specifically because inflammation disables the gut microbiome so that it can't metabolically run properly, directly affecting digestive health [441,444]. Additionally, the

gut's mucosal barriers contain immune elements that help protect the host cells from infection, with the primary goal being to form a protective barrier between the intestinal epithelium and the luminal contents [43]. The microbiome also plays a fundamental role in the induction, training, and functioning of the host immune system by teaching immune system cells- specifically T cells- to distinguish foreign entities from self-tissues and, therefore, help educate the immune cells on how to recognize and respond to the presence of specific molecules properly [445,446]. As an example, a recent study has shown that the composition, gene expression, and epigenetic profile of innate lymphoid cells (ILCs) are shaped by the microbiome, specifically because microbial colonization in some regions of the gut alters overall microbial compositions and, therefore affects cell-to-cell communications, potential immune system responses, gene regulation, and gene expression, [447]. Therefore, the immune system can affect the microbiome by directly interacting with the ILCs of the innate immune system and by responding to problematic alterations in the gut microbiota to remove potential pathogens. Microbial composition can influence the immune system by using microbial-associated molecular patterns (or MAMPs) to directly influence the number, function, and maturation of hepatic Kupffer cells (or KCs) and other elements of the immune system (the microbiome also "trains" and "develops" the host's innate and adaptive immune system by maintaining key features of the existing host-microbiome symbiosis) [447-449].

Gene expression also affects the microbiome in various ways, specifically by influencing microbial composition and acting as a potential mediator between microbial communities and host function [366,450]. Gene regulation, in particular, dictates what RNAs and proteins can be produced, which can influence the microbiome by promoting or inhibiting specific microbial gene

expression, microbial levels, activity, and growth patterns [451]. For example, in a study done by Santos *et al*. in 2021, the composition of gut microbiota in miR-21 knockout mice was found to be previously categorized by an increase in *Lactobacillus,* and the incubation of synthetic miR-21 with *Lactobacillus reuteri* led to reduced growth [451,452]. This study is important because it further establishes that specific mRNA molecules can directly influence the microbiome, which therefore helps in maintaining gut health by increasing, maintaining, or decreasing specific microbial levels; however, pathogenic organisms in an organism's gut- such as *Listeria monocytogenes, Salmonella Typhimurium* and *Helicobacter pylori*- can also modulate host gene expression specifically through the activation of miRNAs or changes in miRNA activity [453-457]. As another example, riboswitches are mRNA elements that have been found to interact with metabolites to regulate the expression of the coding region, which can, therefore, affect gene expression, cell activity, the microbiome, and host health [458]. In addition, the microbiome can also influence the process of gene expression via crosstalk, which may involve the use of transcription factors, epigenetic modifications, chromatin remodeling, ex cetera [459,460]. As an example, a study involving zebrafish displayed that the microbiome suppresses the transcription factor hepatocyte nuclear factor 4A (or HNF4a), which directly affects gene expression by preventing the regulation of host inflammatory pathways and potentially leads to an inflammatory state [461]. Therefore, gene expression and the microbiome also influence each other via various crosstalk mechanisms to properly maintain gut homeostasis by directing digestive processes, influencing what RNAs and proteins are produced and therefore circulating in the GI tract, and actively distinguishing between the host cells and the microbes living in the intestinal lumen.

The mammalian microbiome is a complex, dynamic system that plays a key role in GI homeostasis and overall health. The microbiome is essential because it aids in digestion, immune responses, gene expression pathways, and host cell activities, and any internal disruptions of the microbiome can lead to disease states (such as colic and laminitis in horses). By interacting with other essential host systems, including the immune system, gut physiology, and genetic expression, the microbiome can help maintain internal balance by responding to any potentially harmful changes that may occur inside and outside of the system. However, it is important to note that the microbiome can be influenced and changed by many different variables, including evolutionary history, age, drugs, probiotics, stress, and infection. This means that when analyzing microbiome data, it is important to have robust and well-planned control groups (such as a study using organisms that are all the same age, have no drugs/probiotics, have no infections, and the only difference between individuals is stressors) and recognize all the variables that could be influencing the study's results.

Equine GI Microbiome

Horses are nonruminant herbivores whose digestive systems have evolved to utilize the fibers in the roughages in their hindgut [62]. Although horses are categorized as non-ruminants, they rely heavily on the hindgut fermentation in their large intestines. Since horses are hindgut fermenters, the equine intestinal microbiome plays an essential role in the animals' nutrition, allowing them to digest cellulose while aiding in essential vitamin formation [385]. Horses gain approximately 60% of their energy during hindgut fermentation, meaning they have become highly dependent on their microbial symbionts for proper fiber digestion, energy formation, and survival [216,462]. Overall, the equine GI microbiome is influenced by various factors, including but not

limited to diet, age, evolutionary history, lifestyle, vaccinations, stressors, and health status, meaning that many variables can contribute to microbiome health and, therefore, the development of disease-states, in horses. Given the important role that the GI microbiome plays in horse health- especially as it relates to digestion, energy, and disease prevention- it is essential for horse owners, professors, researchers, scientists, and veterinarians to understand the critical role that the microbiome plays in keeping horses healthy, happy, and alive. To better care for these animals, we must further our knowledge of the microbiome itself, what constitutes a "healthy" range or microbial composition, what constitutes a potentially "harmful" range or microbial composition, and how the microbiome influences and is influenced by other variables (such as diet and stress) and systems (such as the horse's immune system, gene expression, and gut physiology).

Regarding microbial composition, the two most abundant bacterial phyla found in horse feces and the GI tract are gram-negative *Bacteroidetes* and gram-positive *Firmicutes* [64, 65,185,213,215,380]. Both phyla are also highly prominent in other mammalian GI tracts, including the human gut, displaying similar digestive patterns in the co-evolutionary processes between closely related mammalian species [463]. In the equine system, *Bacteroidetes* and *Firmicutes* are essential for proper fiber digestion in the equine hindgut, aiding in microbial fermentation via the anaerobic breakdown of carbohydrates and plant matter via chemical means [82,215]. Therefore, active digestion of ingested fiber material is possible only with essential microbes such as those found in the *Bacteroidetes* and *Firmicutes* phyla. Together, these phyla make up ~82% of the total microbes present in the equine gastrointestinal system [462,464], meaning that they inhabit the most space and are, therefore, very actively involved in the important, mutual (and often symbiotic) relationship that exists between the host and the GI microbiome.

Some more specific examples of active gut gram-negative *Bacteroidetes* are *Prevotella spp., B. thetaiotaomicron,* and *B. fragilis*, and gram-positive *Firmicutes* include *Clostridia spp., Erysipelotrichaceae spp.* and *Bacilli spp.* [85]. *Lactobacillus*, which is an example of a *Firmicutes* bacterial genus, actively inhabits the equine hindgut and produces lactic acid by breaking down carbohydrates, and as a commensal organism, helps to break down food, absorb nutrients, and fight off potentially harmful organisms that may cause disease [213,465,466]. Studies have shown that altered *Lactobacillus* levels can induce or indicate the presence of disease states in horses because of dietary alterations, microbial composition alterations, and lactic acid production changes; specifically, one study showed that *Lactobacillus* and *Streptococcus* levels both increased in laminitis cases that were induced by oligofructose or cornstarch [217,466]. An example of an important *Bacteroidetes* genus that lives in the equine GI tract is *B. fragilis*, an aerotolerant, anaerobic chemoorganotroph that actively the mammalian gut and works to ferment and degrade the glycans available in the gut and equine digestion. These *Bacteroidetes* organisms also actively aid cellulose and xylan metabolism [85,467,468]. The presence of this particular microbe is important to digestive health because when their levels are altered, leading to changes in gut microbiome composition and distribution, it can end up harming the GI tract and causing health issues such as diarrhea and anaerobic infection in the horse [467-469].

The equine GI tract has other bacteria outside the gut gram-negative *Bacteroidetes* and gram-positive *Firmicutes* categories. For example, *Spirochaetes*, *Verrucomicrobia*, and *Euryarchaeota* are also microbial phyla relatively prevalent in the equine hindgut but at different levels depending on the tissue site [61]. One important example is *Verrucomicrobia.*, which includes various species that are categorized as mucin-degrading bacteria that reside in intestinal

mucosa and have been found to contribute to intestinal health and glucose homeostasis by helping maintain the mucus layer between the gut lumen and the enterocytes [215,470]. The equine hindgut is sensitive to significant changes in this particular microbe's presence, meaning that even a slight alteration above or below average can induce gastrointestinal disease states [215]. One study showed that *Verrucomicrobia spp.* and *Clostridiales spp.* are associated with increased expression levels of regulatory cytokines *il10*, *tgfb*, and the Treg transcription factor *foxp3* in the horse, indicating the importance of maintaining their expression levels and further proving that any compositional deviations from the "norm-" even slight ones- can lead to health issues, such as problems regarding regulatory immunity and immune-related communications in the equine GI [114].

After *Firmicutes* (~50%) and *Bacteroidetes* (~41%), the next most prominent phyla found in the equine hindgut are *Proteobacteria* (~4%) and *Fibrobacteres* (~2.5%) [65]. Of the 25 phyla identified by Weese *et al*. in 2015, only *Firmicutes, Verrucomicrobia, Actinobacteria,* and *Proteobacteria* were present at a relative abundance of 1% or greater [471]. Only some of these microbes are VFA-producing bacteria that directly help out the horse by digesting fibrous material. In a study done by de Fombelle in 2003, they found that the equine hindgut had higher concentrations of cellulolytic bacteria compared to the ante-caecal segments, and the caecal concentration of total anaerobic bacteria was lowest while the concentration was highest in the stomach [472]. As expected, given the higher presence of cellulolytic bacteria and requirements of fiber digestion in the hindgut, de Fombrelle *et al*. found that VFA concentrations were greater in the large intestine than in other intestinal segments [472]. Some examples of these cellulolytic bacteria that directly affect fiber breakdown are cellulolytic species from the *Prevotella*,

Ruminococcus, *Firmicutes,* and *Bacteroidetes* genera, among others [65,82,85,123]. Additionally, the stomach and small intestine tended to have the highest number of *lactobacilli, streptococci*, and lactate-using bacteria, suggesting a high interference of these microbes in digesting fermentable carbohydrates [472]. Importantly, the family, genus, and species level of *Firmicutes* will vary in horses based on digestive tract location [185].

The commensal bacteria that make up the equine digestive tract are required for the production of bile acids and short-chain fatty acids (or SCFAs), which are both essential because a) they carry anti-inflammatory properties in multiple immune cell populations, b) they are important sources of vitamins and amino acids, c) they regulate systemic lipid homeostasis and d) any alterations of these essential metabolites can influence immune function [472]. Bile acids are cholesterol-derived amphipathic molecules that solubilize dietary fat in the small intestine to support the digestion and absorption of fat and fat-soluble molecules, and they also act as signaling molecules to regulate metabolic homeostasis and immune cell homeostasis and function [472]. Bile acid synthesis is aided by commensal bacteria when they help in converting primary bile acids- such as cholic and chenodeoxycholic acids- into secondary bile acids- such as deoxycholic, lithocholic, and muricholic acids- via dehydration reactions [472]. These bile acids- as a direct product of the interaction between commensal bacteria and the host- are then used as detergents to help emulsify fats, aid in their digestion and absorption, and regulate cholesterol homeostasis [474,475].

Regarding SCFA production, acetate, butyrate, and propionate are all produced by bacterial anaerobic metabolism of ingestible dietary components, which includes fibers [476-479]. The

main difference between these three SCFAs is the size of the molecules, how many of each are produced, and their overall systemic effects on the host. Acetate has two carbons, propionate has three, and butyrate has 4; however, despite being relatively similar in structure, these SCFAs have substantially different effects on brain cells, digestive activity, and overall health [480]. For example, butyrate is an essential energy source for colonocytes, with the colonic epithelium receiving around 70% of its energy from SCFAs in humans; butyrate also enhances insulin sensitivity and energy expenditure [480]. Propionate, on the other hand, is a precursor of protein synthesis, gluconeogenesis, and lipogenesis in the liver, and it can impair insulin action; finally, acetate is a substrate for cholesterol synthesis, suppressor of appetite through a central hypothalamic mechanism, and it also works to improve glucose homeostasis [480]. SCFAs are produced via commensal bacteria by the saccharolytic fermentation of complex resistant carbohydrates (such as fructooligosaccharides, sugar alcohols, resistant starches, plant wall material, et cetera), which essentially involves biochemically breaking down carbohydrates into butyrate, acetate, and propionate metabolites that can be eventually used to create energy [481]. Notably, the SCFAs produced with the help of these commensal bacteria can modulate the intestinal barrier and escape the gut to influence systemic health [42], making them essential molecules in contributing to the energy requirements of the horse while remaining motile enough to be able to circulate throughout the body properly. In general, SCFAs are one of the resulting end products of fermentation in herbivores [66]. In short, carbohydrate digestion will occur with the hydrolysis of complex polysaccharides and then fermentation into simple sugars.

Complex polysaccharide degradation in the equine digestive tract begins in the small intestine, and the first step involves the attachment of these microorganisms to the plant cell walls

[66]. This adhesive interaction directs concentrated enzymes to the substrates to ensure that hydrolysis will occur [66]. The main amylolytic bacteria identified in the equine hindgut are *Streptococcus* and *Lactobacillus*; both fibrolytic and amylolytic species of bacteria can hydrolyze carbohydrates into simple sugars, which are further fermented [66]. The resulting products will include SCFAs, lactate, and gases like CO_2 and CH_4 [66].

In the equine hindgut, the phylum relative abundance of Bacteroidetes will generally decrease from the cecum to the feces. At the same time, *Verrucomicrobia* will increase, and *Firmicutes* and *Spirochaetes* will stay relatively the same, with the main phyla groups persistently present throughout the equine digestive tract [61]. The biggest changes in composition that were observed in previous studies were based on the specific digestive activity required in a particular portion of the hindgut (i.e., microbial fermentation is more prevalent in the hindgut rather than the foregut, meaning microbiomes of these areas are going to have comparative differences) as well as if a disease-state was already present in particular horses [82,85,466,482]

As discussed previously, many variables contribute to microbiome composition in the equine digestive tract. These include age, diet, vaccination, stressors, health status, evolutionary history, and environment. Dietary changes have indicated that high-starch diets can harm microbial populations in the hindgut [66]. Reduced fecal diversity was observed in the feces of horses that were fed this high-starch diet when compared to horses that ate a high-fiber diet, and the *Ruminococcaceae* and *Lachnospiraceae* bacterial families (which act as important fibrolytic bacteria) were shown to be decreased in the hindguts of horses on the high-starch diet [66]. Importantly, all excess nonstructural carbohydrates (including starches, fructans, and simple

sugars) that aren't digested in the foregut will enter the hindgut, where bacterial fermentation will occur and produce byproducts like lactic acid and gas; the buildup of these byproducts can cause imbalances in the gastrointestinal microbiome, leading to colic, laminitis, and potentially other diseases in horses [63,68].

Changes to the horse's gastrointestinal microbiome, no matter how slight, can directly affect horse health. For example, if the lumen pH drops below 6.0, the growth of many fiber-fermenting bacteria will be suppressed. At the same time, the number of acidophiles will increase, leading to more lactic acid production in the hindgut and even more of a pH drop [68]. This pH decrease, specifically when observed in horse feces, can be attributed to changes in the predominantly gram-negative to gram-positive microbial population since gram-negative bacteria are susceptible to acidic environments [62,68,70]. This can lead to hindgut acidosis and the eventual development of colic, colitis, laminitis, systemic inflammatory response syndrome, or anorexia in the horse [62,68]. Additionally, horses with laminitis had apparent compositional differences in their gastrointestinal microbiomes, with a proliferation of the *Streptococcus* species often associated with laminitis [70,201,378,483-485]. In general, horses with chronic laminitis tend to have higher levels of bacteria diversity with differences in abundance that can contribute to the development of laminitis. These horses often develop the disease by carbohydrate overload, which causes mucosal damage, increases the release of endotoxins, and increases the bacterial population of *Lactobacillus* spp. by a factor of 10^5, and following the detrimental pH decrease, these changes can lead to the death and lysis to other bacterial species such as *Enterobacteriaceae* spp. and *Bacilli* spp. [62,63]. The Steelman *et al*. study also found two *Clostridiales* genera in horses with laminitis (37.99%) that differed in abundance from control

horses (41.63%), which was important because it was found to be one of the most diverse genera. This means that even a slight change in composition can be detrimental to horse health, mainly since *Clostridiales* contains a wide variety of functional microbes that can be beneficial and pathogenic [63]. In short, if these horses with laminitis have less of the functionally beneficial *Clostridiales* microbes in their guts, this- and the alteration of the hindgut microbiome as a whole due to causes like carbohydrate overload [63] or an interruption of the natural grazing cycle [83]- can directly contribute to the development of laminitis.

Colic, which was found to be the number one killer of horses from ages 1 through 20 [486-488], has a crude incidence density rate of 10.6 colic cases per 100 horses a year, with 13% of horses having more than one episode of colic per year and the proportional mortality rate of colic existing at 28% [489]. Some common colic symptoms include distension or spasms in the gut, which causes the horse to respond to abdominal pain with actions such as looking, biting, or kicking at their flank or belly [186]. Diets rich in starch, similar to what was discussed about laminitis, are one prominent example of what can cause microbiome changes and gastrointestinal disease that leads to the incidence of colic in horses [68,186]. A higher presence of *Salmonella* spp., which causes salmonellosis, is often responsible for developing fatal colic [68,490,491]. Recent studies have also found that the overgrowth of two lactic acid bacterial families, *Lachnospiraceae* and *Lactobacillaceae*, decreased hindgut pH and interfered with normal fermentation processes, playing a potential role in the development of large intestinal colic [62]. As mentioned previously, *Streptococcus* overgrowth can also lead to a decrease in hindgut pH, altering the microbiome and reducing methanogenesis in horses with small intestinal colic [62].

Regarding large intestinal colic, there are a couple of important observational differences in the microbiomes of these horses compared to healthy horses:

A) *Lactobacillaceae* and *Coriobacteriaceae* were significantly more abundant in horses with large intestinal colic [62].
B) The density of *Enterococcus* and *Acinetobacter* increased significantly, while *Methanobrevibacter* was reduced significantly [62].
C) *Lactobacillus* and *Bifidobacterium*, commonly known probiotics in horses, were more prevalent [62].
D) Their microbiomes had lower species evenness and richness than healthy horses, with some species no longer detectable and generating greater evenness [62].

This shows that when certain changes occur in the equine gastrointestinal microbiome, dysbiosis can affect fermentation patterns and harm horse health, leading to disease states like colic. In general, colic, laminitis, and other equine disease states are often brought on by the decrease or alteration of bacterial richness and diversity, reducing resilience and, therefore, higher susceptibility to dysbiosis [62,63,66,217].

Importantly, some similarities and differences exist between different regions of the hindgut. Principle component analyses (or PCAs) performed by Ericsson *et al*. have shown that the composition of microbial communities within the stomach, jejunum, and ileum are distinct from those in the cecum and colon; variability between individual horses was also found when comparing similar gut regions to one another [185]. The biggest compositional change observed in this study was shown to exist at the junction between the small and large intestines, which makes sense given that horses are hindgut fermenters and segments found later in the GI tract are

performing different digestive tasks compared to the earlier gut segments [185]. Bacterial community structure tends to be more similar between areas closer together on the digestive tract; for example, Sadet-Bourgeteau *et al*. found that the right ventral colon and caecum had very similar community structures, but they were different from that of the horse feces [402]. In this case, their fecal samples had higher lactate-utilizing and lactate-producing bacteria concentrations than the samples they collected from the right ventral colon [402,493]. Previous studies have also shown differences in microbial composition between regions such as the cecum, dorsal colon, pelvic flexure, and ventral colon [61,494]. These observed differences between different anatomical locations along the equine digestive tract are likely due to selective pressures acting on microbes based on various factors [492], including the composition of the lumen (nutrients, pH, et cetera), presence of other microbes that may compete for space, the digestive functions of specific regions (highly-fermenting or not), and interactions with host cells (gene expression, immune system, et cetera).

A previous lab member, Kailee Reed, performed a key research study that further reinforces the potential anatomical differences in microbial presence along the equine hindgut [61]. The goal was to study and compare different digesta/fecal samples taken from the cecum, ventral colon, dorsal colon, and fecal communities of 6 mixed-breed miniature horse hindguts. Importantly, these miniature horses were euthanized for reasons unrelated to GI disease, were all yearlings managed in the same location at the University of Kentucky, ate the same diet of mixed grass-hay, and had no deworming treatment before sample collection. The results of this study indicated that there is a clear distribution of microbial constituents across each of the four hindgut sections analyzed, with the most evident separation existing between the cecum and ventral colon

and the dorsal colon and feces. The taxonomic distribution showed the microbial differences between different hindgut regions by visually comparing each of the dominant phyla and family categories to the tissues and fecal samples collected; in general, the relative abundance at both the phylum and family levels displayed that all four are unique, but there are obvious differences between the taxonomic abundances of the cecum and ventral colon versus the dorsal colon and feces. Beta diversity analysis also clearly showed that microbial composition differences existed between the proximal and distal segments of the large colon in these animals. A principal coordinate analysis (or PCoA) graph was created, visually displaying a clear compositional separation between these groups. Reed *et al*. also performed an indicator species analysis in this study, which combines information about the abundance of a feature (i.e., species, genus, family, etc.) and the faithfulness of occurrence of a feature in a particular group. The indicator species analysis revealed that several families were highly exclusive/faithful to the cecum and ventral colon, but no families were faithful or exclusive to the dorsal colon and feces. Together, these results indicate that there is a clear distinction in microbial content of the cecum and ventral colon compared to the dorsal colon and feces, which points specifically to the digestive structure that lies between the ventral and dorsal colon [61]. This structure, the pelvic flexure, seems to be playing a role in this apparent separation, specifically in this case as it relates to exclusiveness/faithfulness.

As a result of their study, Reed *et al*. concluded that something is going on at the pelvic flexure in the equine hindgut. However, it is important to note that the pelvic flexure is not meant to act as a barrier; in fact, when barrier-like properties happen at this section of the hindgut, colic and other equine diseases often result. Therefore, this difference in microbial composition that was

observed between the cecum and ventral colon versus dorsal colon and feces may not be because of any a physical "barrier," but maybe from a physiologically/structurally influenced chemical or microbial "barrier". To summarize, the main findings of Reed *et al*.'s study included a) the various compartments of the equine hindgut support a microbial population specific to that region, with the largest differences resulting from a "separation" at the pelvic flexure, b) fecal samples are not informative of the proximal hindgut, and c) the pelvic flexure is playing a role in the visual differences observed between these four regions, which should be investigated further [61]. As a result of these major findings, Reed *et al*. suggest that future research further investigates the "role of the pelvic flexure and potentially the physiological aspect from the host as an important component of the curation of microbial communities in different parts of the GI…" [61].

As a result of the findings by Reed *et al*., the current study – presented in chapter 2 of this thesis) – will focus on gene expression and investigate further how the pelvic flexure may play an important physiological role in hindgut activity and may explain the observed microbial differences between earlier and later portions of the hindgut. It is crucial to investigate every element that contributes to digestion in the equine hindgut because there are many present variables- internal (such as gut microbiome, gene expression, immune system, et cetera) and external (such as stressors, diet, exercise, et cetera) to the system- that can directly influence digestive activity, and researchers may not fully understand all of these variables and their effects, as well as how prevalent/active certain variables are. As a result, we may better understand how the digestive system works, how it is influenced by various factors, including the microbiome and gene expression, and how the dysbiosis of normal activity can end up leading to disease states.

Host/Microbiome Interactions

The host and host microbiome are in constant interaction with one another, directly influencing each other in various ways. Microbiomes directly influence the physiology and fitness of the host [496], specifically by responding to the host's diet, external stressors, and potential disease states. For example, people who drink caffeine regularly were found to have increased richness and evenness of the mucosa-associated gut microbiota and higher relative abundance of anti-inflammatory bacteria such as *Faecalibacterium* and *Roseburia*, as well as lower levels of the potentially harmful bacteria *Erysipelatoclostridium* [497]. In short, caffeine intake can actually reduce the growth of "harmful" bacteria while promoting the growth of "good" bacteria [498]. Caffeine consumption has also been shown to lead to a decreased transit time throughout the gut, leading to a direct change in microbiome composition by affecting water and nutrient availability [499-501]. This positive impact on the host's microbiome can aid in improving the host's bowel movements, stimulating the digestive system, decreasing inflammation, and also reducing the risk of developing chronic diseases such as metabolic syndrome, obesity, diabetes, cardiovascular disease, and cancer [502]. The health benefits associated with caffeine, specifically with coffee consumption, are often tied to the fact that these drinks are derived from plant sources, which are rich in fibers that can resist absorption in the small intestine while acting as a direct nutrient source for gut microorganisms [502]. This example clearly outlines how alterations in the gut microbiome- in this case, an increase in caffeine consumption- can cause changes that directly influence host health.

However, although caffeine may be good for people in moderation, having too much of it can cause damage to the gut. Because of the acidity associated with coffee, for example, it can

adversely affect a person's stomach and intestinal lining, which could potentially lead to health issues like dyspepsia, esophageal burns, gastritis, ulcers, GERD, ex cetera [503,504]. Additionally, coffee consumption can lead to leaky gut symptoms, such as diarrhea and stomach pain, which could be easily triggered in individuals who have been diagnosed with leaky gut or an associated autoimmune condition [505]. The type of caffeine a person decides to consume is also important- for example, energy drinks have high caffeine and sugar contents and have actually been proven to decrease the activity, diversity, and gene expression of bacteria in the gut [502,506,507]; they have also been linked in recent studies to the development of metabolic syndrome diseases, an elevated heart rate, blood pressure issues, obesity, and type 2 diabetes [502,508]. By choosing to have 100-400 mg of caffeine from tea instead of from an energy drink, a person could have all the benefits of caffeine while not feeling as tired or sluggish later in the day. That same person could also split up the caffeine consumption, drinking 100 mg in the morning and 100 mg in the afternoon and maybe sleeping in and taking the next day off from caffeine altogether. However, if that same person were to drink more than the recommended daily caffeine intake (which is 400 mg or less a day), let's say 600 mg, for every single day over the course of a week or two, that person could suffer from caffeine intoxication, causing a direct disruption in the microbiome and causing symptoms such as anxiety, insomnia, stomach issues, muscle twitching, restlessness, inexhaustibility, ex cetera [502,509]. This goes to show how important it is to have a form of "balance" in the host diet- just like how parental figures tell us that eating too much ice cream is bad for us, but eating some ice cream on occasion is okay for us, it is essential to host health and the health of the microbiome to be careful with what we decide to consume, how much we decide to consume, and how often we consume it. This is because what we choose to eat directly affects our microbiomes, which can, therefore, affect our overall health.

Just as the microbiome can influence host health, the host's GI cells can also influence the microbiome. The physiology of the host's mucosal layer of the GI epithelial cells directs what can enter and leave the host's intestinal tissues- allowing useful substances to be absorbed into the body while restricting the entry of harmful substances [510,511]. Therefore, this layer of the intestinal epithelium is what controls much of the transit of essential nutrients, electrolytes, water, and chemical signals between the intestinal lumen and the host. The mucosal layer does this by trapping, interacting, and either absorbing, targeting for destruction via the immune system, or releasing the elements in the intestinal lumen; importantly, the inner mucosal layer is impervious to bacteria and is renewed every hour or so by surface goblet cells, making it essential for protecting and distinguishing IECs from non-self molecules [512]. If this communication between epithelial cells and the intestinal lumen- which includes the microbiome- is disrupted, for example, from the host cell's mucosal layer being damaged (potentially by harmful microbes or environmental antigens), then inflammation may occur due to an abnormal immune response, which influences the balance of the gut microbiome and results in the development of intestinal diseases [513]. The gut mucosal immune system- which includes lymph nodes, lamina propria, and epithelial cells- directly maintains intestinal homeostasis by influencing the gut microbiota specifically by acting as a protective "barrier" for the integrity of the intestinal tract; the mucosal immune system does this via the release of mucins- or large, highly glycosylated proteins- which maintains homeostasis of the GI epithelial barrier, providing physical protection to IECs while also regulating the concentration and passage of water, ions, AMPs, ex cetera [512-514]. These mucins also interact with microbes in the GI tract, and an immune response can be mounted if any pathogenic microbe reaches the mucosal layer of IECs. In summary, the host's GI cells can influence the microbiome by interacting directly with the intestinal lumen contents, controlling

what can and cannot enter host GI cells, and using biochemical elements like mucins to protect host cells from potentially harmful microbes.

All in all, homeostasis is involved in this balance between the microbiome and the host organism, playing an essential role by actively distinguishing self from non-self while protecting the delicate balance of individual host IECs and the digestive tract as a whole; this "protection" could include the use of biochemical elements- such as mucins, cellular communication, or immune signals- to address the presence of harmful pathogenic microbes, viruses, toxins, or other digestive elements that may disrupt GI homeostasis. This means that the IECs in the GI tract must constantly work to maintain homeostasis by consistently sensing and responding to the external, nonself factors existing inside the intestinal lumen, which can be seen as an active maintenance process that is necessary for maintaining intestinal balance and survival. Without homeostasis, the relationship that exists between the host and the microbiome would be disrupted, leading to dysbiosis and the development of disease states in the organism. In the gastrointestinal tract, some examples of disease states that could develop because of this dysbiosis- or disruption of homeostatic conditions- include but aren't limited to obesity, metabolic syndrome, inflammatory bowel disease, cardiovascular disease, and other autoimmune diseases [515]. To prevent these diseases from occurring or developing into severe cases, the scientific community must continue to study and understand the important relationship that exists between the host's cells and the microbiome and develop methods of treatment that can benefit both.

The Microbiome Influences Mammalian GI Systems

The microbiome influences mammalian GI systems by interacting directly with the host epithelial cells, which results in the microbes directly helping maintain the integrity of the mucosal barrier, providing nutrients such as vitamins to host cells, protecting against pathogens, and interacting with the host immune system [516]. They interact with mammalian intestinal epithelial cells via specific molecules called pattern recognition receptors, which work by recognizing molecular patterns of bacteria and other microorganisms that can effectively bind and interact with the receptor [517]. Additionally, commensal microbes consume nutrients in the gastrointestinal tract and can then generate metabolites, which can influence mammalian GI systems by mediating communication between the microbiome and the immune system that can directly affect the balance between pro- and anti-inflammatory mechanisms and responses in the intestinal tract [518,519]. In short, these metabolites produced by commensal bacteria are important in various ways, including the development, homeostasis, and function of the immune system, and therefore, they play a key role in the overall protective mechanisms of IECs throughout the gut [76]. Importantly, these metabolites that are produced by microbiota act as energy substrates and biological mediators, which can be both beneficial- such as the proper production and distribution of SCFAs, which can used as a source of energy- and harmful- such as high TMAO plasma levels which could indicate a higher chance of patients developing cardiovascular problems [520,521]. As a result, the microbiome can actively influence the host in various ways, including via pattern recognition receptor activity and the generation of specific metabolites, which can affect nutritional availability and digestion, overall development, immunity, and behavioral responses in the host organism [522].

The Microbiome Influences the Equine GI

As stated previously, the equine microbiome interacts directly with host health and influences various processes essential for survival, including the immune system, nutrient digestion, and gene expression. In the equine gastrointestinal tract, in particular, the microbiome influences it directly by playing an important role in hindgut fermentation, which is where most of the animal's energy is made [523]. The microbiome also influences the IECs in the equine gastrointestinal tract by interacting directly with the host immune system using metabolites and cell signaling. The equine digestive tract is sensitive in the sense that any changes in the composition of major microbial groups can lead to dysbiosis events, meaning that only slight alterations in the microbiome of a horse can lead to the development of disease states [524]. As a result, alterations in microbial communities in the equine GI tract have been implicated in host susceptibility to nutritional and metabolic diseases like colic, laminitis, or other gastrointestinal diseases [525]. Alterations at the community level of the essential microbes found in the dominant phyla, *Firmicutes* and *Bacteroidetes*, have led to disease states such as obesity, colic, and inflammatory bowel disease [42,471]. Additionally, the prevalence of problematic bacteria such as *Salmonella spp.* will cause the horse to develop salmonellosis, which can result in severe diarrhea, colic, and potentially death [526,527]. As another example, Equine Metabolic Syndrome (or EMS) has been associated with decreased overall microbial diversity in the digestive tract, displaying an increased presence of the phylum *Clostridium*, *Verrucomicrobia*, *Lactobacillus*, *Cellulosilyticum*, and *Elusimicrobium* and decreased levels from the families *Lachnospiraceae*, *Flavobacteriaceae*, *Rhodospirillaceae*, *Anaerovorax*, *Fibrobacter*, and *Saccharofermentans* [528,529]. Finally, another example of how altering a microbial category in the microbiome can cause dysbiosis and therefore lead to a disease state is when the Firmicutes/Bacteroidetes ratio

increases in an obese horse's gut compared to the guts of healthy horses; this is especially a problem with domesticated horses that may lead a more sedentary lifestyle, get consistently overfed, or may be predisposed to obesity due to genetic factors [408,529-531). All in all, the equine microbiome influences the digestive tract by aiding in hindgut fermentation and energy production, influencing the host cell's immune system processes and responses, and directly aiding in the maintenance of GI homeostasis; any alterations in this microbial balanced state can often lead to serious health conditions.

Moving Forward

Gastrointestinal (GI) homeostasis in horses results from dynamic interactions between a horse's gut physiology and the microbiota that reside in the various intestinal compartments. Gene expression directly influences the physiological expression of epithelial cells in the hindgut and, therefore, influences the microbiota and overall digestive process in the horse. Although not heavily discussed, it is also important to note the importance of the horse's immune system, which also plays a heavy role in maintaining GI homeostasis and horse health. This essential balance that exists between gene expression, hindgut physiology, the microbiome, and the horse's immune system can easily be altered by factors such as age, diet, vaccinations, external stressors, and present disease states. Many disease states have been discussed in previous research, displaying the critical nature of studying and maintaining GI homeostasis in horses, including colic, laminitis, colitis, ulcers, and other GI diseases. Little has been studied on the topic of gene expression in the equine hindgut, making it a crucial element to analyze when considering equine health and potential treatment methods for common disease states. Additionally, previous findings on the nature of the pelvic flexure in Reed *et al*. 2021 have indicated that future studies should further

dive into the strange observation that the pelvic flexure separates the biological activity and contents of the ventral and dorsal colons, even though it cannot act as a true “barrier” in the equine hindgut. As a result, performing a gene expression study in the same region of the equine hindgut may further the research community’s knowledge of what is happening around the pelvic flexure and potentially why these visual differences may exist.

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CHAPTER 2
GENE EXPRESSION ANALYSIS BEFORE AND AFTER THE PELVIC FLEXURE IN THE EQUINE HINDGUT

Summary

Previous research demonstrated that the pelvic flexure helps regulate the distribution of microbial communities in the equine hindgut. Since the horse's gastrointestinal microbiota directly influences tissue function, digestion, and the immune system, it is crucial to understand these regulatory processes better. One possible mechanism is the physiological differences in the various segments of the equine hindgut contributing to niche environments that support distinct microbiota. The objective of this study was to evaluate gene expression surrounding the pelvic flexure to characterize the active physiological processes. RNA was isolated from the epithelium of the right and left ventral colon (RVC and LVC), right and left dorsal colon (RDC and LDC), and pelvic flexure (PF) from three 4-year-old American Quarter Horses. Single-end libraries generating an average of 23 million reads per sample were prepared using the NEBNext® Ultra II Directional RNA library kit and sequenced with an Illumina® NextSeq 500 platform. An average of 18,330 +/- 191 genes were expressed across the five tissue locations (RVC= 18,445, LVC= 18,258, PF= 18,146, LDC= 18,195, RDC= 18,606). A majority (16,750 of 31,215) of annotated equine genes were identified in all five sampled tissues. All five tissue locations also demonstrated tissue-restricted expression, with 1,203 genes expressed exclusively in 1 of the five tissues. The tissue-restricted genes identified in the LVC and LDC showed trends in performing essential communicative, signaling, and regulatory functions which correlate with their known tissue physiology. In contrast, genes expressed exclusively in the PF had diverse functions. DESeq2 assessed differential expression between the various segments. The number of differentially expressed genes varied between 32 and 280, with the largest group of differentially expressed

genes identified between the RVC vs PF and the smallest between LVC vs LDC (32). Gene ontology analysis did not identify any significantly enriched functional categories in the lists of differentially expressed genes but showed trends associated with immune functions and signaling processes. These trends were reinforced by the functions of genes which showed statistically significant differential expression between the regions of the equine hindgut. Many of these differences provided potential explanations for the distinct microbiota between the hindgut segments observed and reported in previous research. The results reported here regarding gene expression patterns across regions of the equine hindgut provide insight into the physiological mechanisms that influence the microbiota and its distribution in the equine hindgut.

Introduction

The equine gastrointestinal (GI) tract is an essential and complex organ system divided into the foregut- which includes the glandular and non-glandular regions of the stomach and small intestine- and the hindgut, which consists of the cecum, large colon, and small colon [1-4]. The foregut is where enzymatic digestion and nutrient absorption occur, and the hindgut is where fiber is digested, and water is reabsorbed. Compared to the foregut, the hindgut accounts for more length and overall volume in the GI – 62% versus 30% of the total volume [1-4]. Importantly, as horses are hindgut fermenters, most of the energy derived from the diet – more than 60% [5] – results from microbial fermentation in the cecum and ventral colon. In these hindgut compartments, commensal microorganisms actively break down plant-based fibers such as cellulose to produce the volatile fatty acids (or VFAs) used as the horse's primary energy source. This means that hindgut microbes play an essential role in digestive physiology and that disruptions of this microbiome can contribute to performance deficits, health issues, and potential mortality [6-11].

The equine foregut and hindgut are hosts to distinct microbial populations, mainly because these areas of the digestive tract perform different digestive functions [12]. These differences correlate with the different roles of both segments in the digestive process. Differences appear in the microbiota of the various hindgut compartments as well. Previous work by our group demonstrated that the pelvic flexure separates distinct microbial communities (cecum and ventral colon from dorsal colon and feces) of the equine hindgut [12]. The pelvic flexure is a horseshoe-shaped bend (180 degrees) connecting the ventral colon's distal end and the dorsal colon's proximal end in the equine hindgut. The role of this and other flexures is to define hindgut compartments and prevent the backflow of digesta [13]. The pelvic flexure is neither a physical barrier nor supposed to block material transit through the GI. However, it can often be the site of obstruction resulting in colic [14]. In the present study, we sought to investigate gene expression in the equine hindgut surrounding the pelvic flexure to understand better the factors influencing the digestive physiology and microbiota composition of this.

Homeostasis is a self-regulating process to maintain stability in biological systems while possessing the ability to respond to changing external conditions [15]. Maintaining homeostasis is essential for any living system to operate efficiently and effectively [16,17]. In the GI tract, homeostasis results from constant interactions between the host tissues and physiology and the microbes, nutrients, and other contents of the intestinal lumen [18]. In the hindgut of horses, the microbiome plays a crucial role in digestion, energy production, vitamin synthesis, and maintaining gastrointestinal homeostasis [19]. To better understand GI homeostasis and its influence on equine health, we must improve our understanding of the various hindgut factors – microbiome, immune system, physiology – and their interactions. Such knowledge can help

researchers, veterinarians, and horse owners understand how to better diagnose and treat horses with apparent variations from a healthy state while potentially limiting GI illness progression and severity.

The equine hindgut is complex, and its overall structure and function are critical to GI homeostasis and digestive health. The physiology of digestion in the equine hindgut [20-24] and the impact of anatomy [25,26] have been well studied and reviewed. There have been relatively few studies of gene expression or the differential patterns which exist in the tissues of the equine hindgut to complement our understanding of digestive physiology; in general, sequencing of the equine genome has only begun to emerge since Twilight was sequenced in 2006 by the Horse Genome Project and was later updated in 2014 [27-29]. A gap, therefore, exists in our understanding of the underlying biological processes in hindgut physiology and how disruptions and dysregulation can result in disease pathology and dysfunction. A crucial first step in developing this understanding is to investigate gene expression in the tissues of the GI from healthy animals to characterize a pattern of expression across the various compartments.

Here, we collected intestinal epithelium, isolated total RNA, and used RNA sequencing to characterize gene expression patterns in the intestinal epithelium of the pelvic flexure and surrounding ventral and dorsal colons. These regions were targeted in our analyses as they correlate with the shifting patterns in the microbial communities of the equine hindgut. The objective was to determine baseline expression profiles in the equine GI to understand better intestinal epithelial cell function related to hindgut digestive physiology and to identify gene

expression differences between the hindgut compartments, which may contribute to the shifting microbial patterns and distinct functional roles in each.

Materials and Methods

Animal Subjects and Sample Collection

Samples were collected from three four-year-old quarter horses (two males and one female). All three horses had body condition scores (BCS) between 5 and 6 [30], did not receive any prior antibiotics, and were fed mixed grass hay with ad libitum access to food and water. Animal care, handling, and euthanasia were approved by the Colorado State University Institutional Animal Care and Use Committee (protocol 16-6405A). The horses were euthanized as part of an unrelated project for reasons unrelated to gastrointestinal disease.

Intestinal epithelial samples were collected from the right ventral colon (RVC), left ventral colon (LVC), pelvic flexure (PF), left dorsal colon (LDC), and right dorsal colon (RDC) between 40- and 45-minutes post-mortem. The GI tract was removed, and each compartment was identified, beginning with the pelvic flexure. The left ventral and left dorsal colon were marked 10-15 cm caudal or rostral from the PF. The right ventral and right dorsal colon samples were collected from the opposite end of each compartment relative to the PF. A 2 to 3 cm^3 full-thickness section was cut from each site with a sterile scalpel and transferred to a clean sample cup. The tissue was rinsed with sterile PBS before dissecting the mucosal and submucosal layers from the serosal muscle. The mucosal/submucosal epithelium was divided into ~0.5 cm^3 pieces and placed in 5 mL of RNALater [31]. The tissues were incubated at 4^{o}C for 24 hours, removed from the RNALater, and transferred to -80^{o}C for storage according to the manufacturer's protocol.

RNA Isolation and Quality Control

Total RNA Isolation was performed using a modified TRIzol™ (Thermo Fisher) protocol [32,33]. The procedure was as follows: samples were removed from -80ºC and placed on ice to thaw. Fifty milligrams of tissue were weighed into a sterile tube filled with garnet shards and a zirconium bead (D1033-30G, Benchmark Scientific), 1 ML of TRIzol was added, and samples homogenized using a BeadBug 3 homogenizer (Benchmark). Following homogenization, the samples were incubated at room temperature for 5 minutes, combined with 200 uL of chloroform, vortexed, and centrifuged to separate the aqueous and organic phases. The aqueous phase was removed to a clean tube, and the total RNA precipitated with isopropanol. All samples were treated with DNase (TurboDNase, Ambion) to remove genomic DNA contamination. The quality of the isolated samples was verified by checking concentration and purity on a NanoDrop 1C (Thermo Fisher) and integrity using a QuBit 4 (Invitrogen) and the RNA IQ assay (Thermo Fisher). All samples had concentrations above 200 ng/□L, 260/280, and 260/230 ratios above 1.7, and RNA IQ scores of at least 7.

Library Prep and Sequencing

mRNA sequencing libraries were prepared for each tissue sample. There was a "no-RNA" (water) negative control using the NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs Inc., Catalog # 7760S). The overall goal of this step is to enable the capture of the messenger RNA on the sequencing flow cell. Messenger RNA was isolated from total RNA using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs Inc., Catalog # E7490) and fragmented with a target size of 200 nucleotides. First- and second-strand synthesis proceeded according to the manufacturer's protocol. Each of the 15

samples and the negative control were indexed using a unique oligo sequence from the NEBNext® Multiplex Oligos for Illumina Sets 1 and 2 (New England Biolabs Inc., Catalog # E7335S and E7500S). Following PCR enrichment, library size, quality, and abundance were assessed using an Agilent 2200 Tapestation (Agilent Technologies Inc., Santa Clara, CA) and High-Sensitivity D1000 Screen Tape (Agilent Technologies Inc., Catalog # 5067-5584). Equimolar quantities of each library were combined, and the size, quality, and abundance of the combined libraries were re-assessed. Sequencing was performed on an Illumina NextSeq 500 (Illumina Inc., San Diego, CA) using a NextSeq 500/550 75-cycle v2.5 High-Output kit (Illumina Inc., Catalog # 20024906).

Bioinformatic Analysis

Data from the sequencer was uploaded to BaseSpace™ (Illumina Inc., San Diego, CA) and demultiplexed to generate the individual FASTQ files from each sample for secondary analysis. The Galaxy platform [34] was used to perform quality control via FastQC [35] and MultiQC [36], read trimming via Trimmomatic [37] and read alignment to EquCab3.0 [38] using HISAT2 [39]. Post-alignment QC was also performed using FastQC. Gene quantification was performed on the CU/CSU Summit high-performance computing system using featurecounts [40]. This analysis used an equine gene annotation from Ensembl 106 [41]. The differential gene expression analysis was performed in R-Studio [42] using DESeq2 [43]. A diagram of the analysis workflow is shown below (Figure 2-1).

Tissue specificity was assessed using the gene list, and normalized expression values generated DESeq2. Genes were labeled as “expressed” if they had detectable expression in at least two of the three horses. Genes were labeled as “tissue-restricted” if labeled as “expressed”

exclusively in 1 of the five tissues. The manually curated tissue-restricted expression patterns were validated by calculating a Tau Index value for each gene across all tissues and samples. The Tau Index analysis was performed in R-Studio using the Tau Algorithm Protocol [44-46] and required the tispec [47], remotes [48], and knitr [49-51] packages. This analysis took the original dataset and ranked individual genes from 0 to 1, with a Tau value of 0 indicating consistent expression across all five tissue sites and 1 indicating tissue-restricted expression.

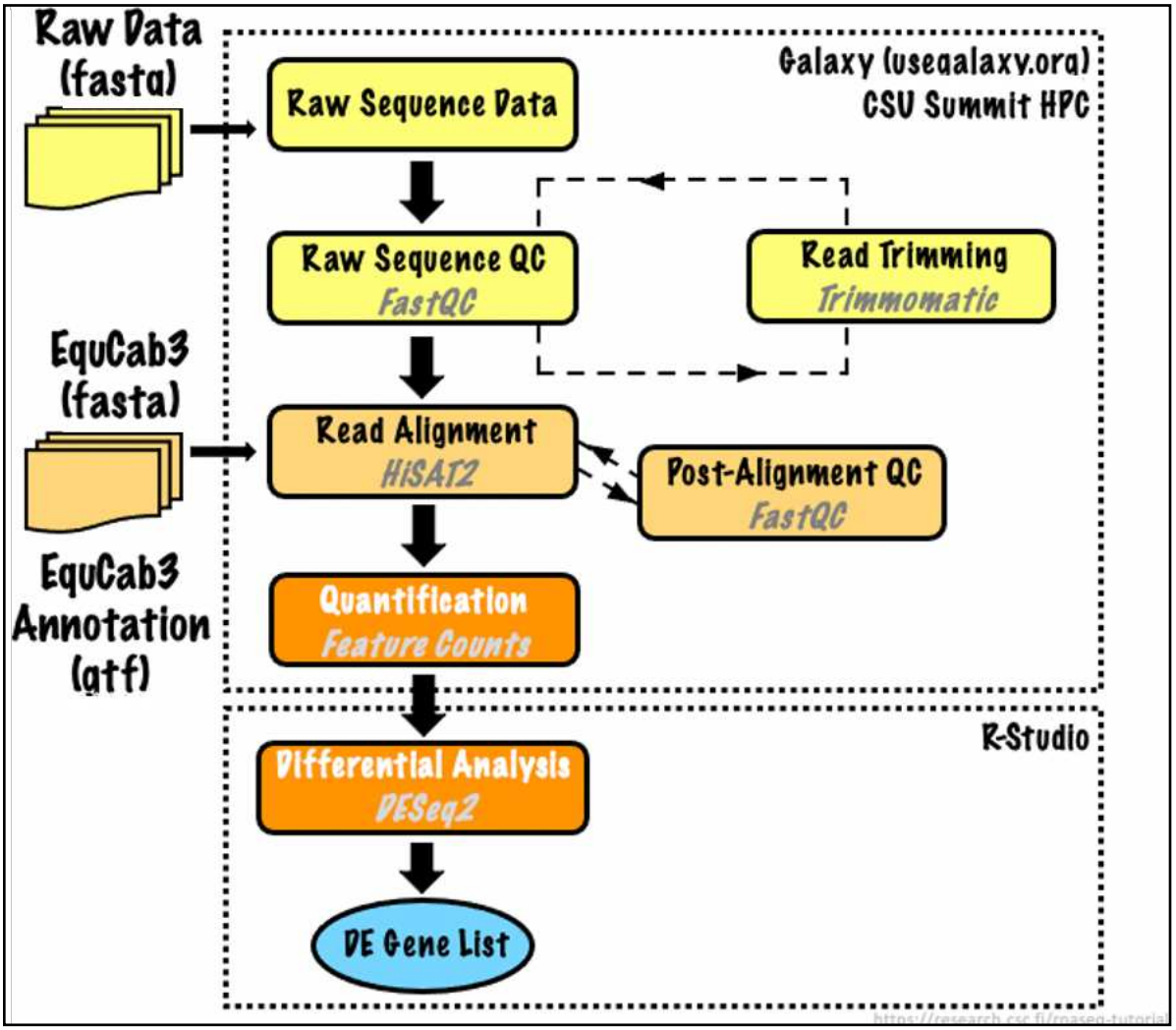

Figure 2-1. Gene Expression Analysis Workflow.

Differential expression between the tissues was determined by DESeq2 [43]. Briefly, the raw read counts values determined by featurecounts were normalized to account for sequencing depth and composition variation. This resulted in the normalized expression values used to analyze tissue-restricted gene expression. Dispersion for each gene was estimated across all samples, and a negative binomial distribution model was used to determine differential expression. A Wald test of the likelihood ratio was used to assess the significance, and a Benjamini-Hochberg correction was applied to account for multiple testing. The comparisons used for this analysis were (1) PF versus LVC, (2) PF versus LDC, (3) LVC versus LDC, (4) PF versus RVC, and (5) PF versus RDC.

We used the subpopulation analysis algorithm from the Kharchenko lab single-cell analysis toolkit [56] to further analyze the gene expression differences present in the dataset. A smooth scatterplot was generated in R-Studio. This was done by mapping all the expressed genes based on the log of the coefficient of variation versus the log of gene means, and the smooth scatterplot generated visually displays how many genes are highly variable versus how many genes are not highly variable. This analysis aimed to visualize just how many highly variable genes exist in the equine hindgut. In the end, this graphical analysis displays that there are some genes present in the equine hindgut that are highly variable.

Functional analysis of the differential gene expression identified between the five tissue sites was performed using the functional annotation tool from DAVID [55]. Biological processes, molecular function, and cellular components were all included in the analysis. The input lists used for the analysis were those of differentially expressed genes identified and described above using

DESeq2 against a background of all annotated genes from the Ensembl 106 annotation. Enrichment analysis was performed to determine if gene ontology terms were overrepresented in the differentially expressed gene lists. The functional annotation tool calculates enrichment p-values to determine if the enrichment observed significantly differs from what would be expected by chance.

Results

Sequencing Results

Sequencing of 16 samples (15 tissue samples and the negative control) generated a total of 360,021,067 seventy-six basepair sequence reads. Sequence data are available in the Sequence Read Archive under Bioproject PRJNA631014. A total of 14,468,231 reads were removed from the analysis during the demultiplexing step as they could not be confidently assigned to a sample group based on the index sequence. The average sequence generated for the fifteen tissue samples was 23,033,024 reads with a range of 18,178,616 (pelvic flexure – horse 2) to 30,913,609 (right dorsal colon – horse 2) reads. By contrast, the negative control produced only 57,475 sequence reads. While assessing the sequence quality, it was observed that the first 8-10 basepairs of the reads in each sample had lower per-base quality and an unexpected distribution of the per-base sequence content compared to the other 66 bases of the read. To avoid any potential ambiguity resulting from including these bases in downstream analyses, they were removed using the HEADCROP function of Trimmomatic [37]. The average GC content of all samples was 47.1%, with a range of 44% to 49%. Figure 2.2 displays the total sequence generated and %GC content for the fifteen tissue samples compared with the negative control. Table 2.1 shows the sequencing results for all samples.

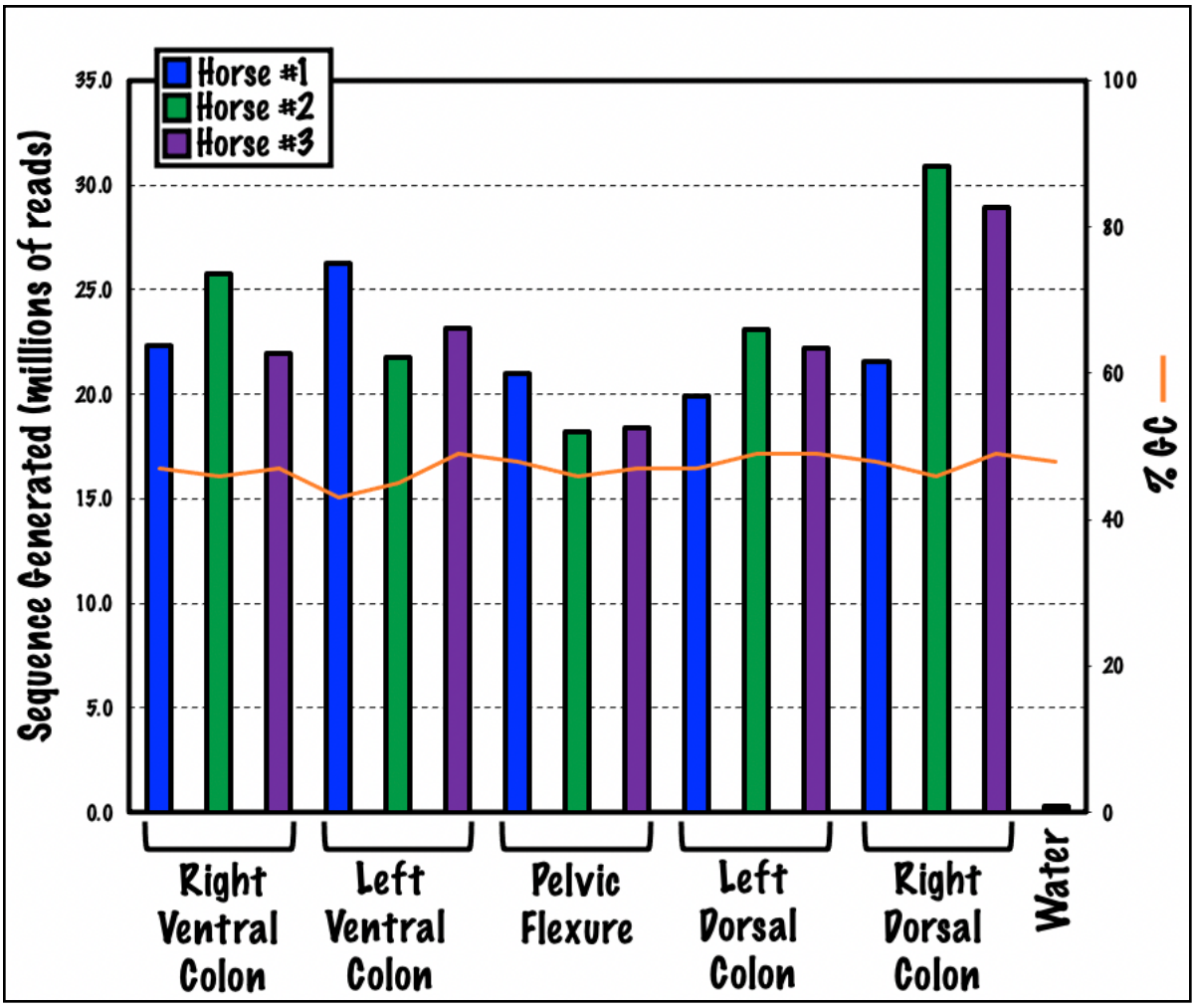

Figure 2.2. Summary of sequence data generated by sample.

Mapping Results.

Sequence reads assigned to the individual tissue samples were aligned to the equine reference genome (EquCab3) using HISAT2 [39]. The average overall alignment rate was 93.44% (unique and multiple mapping reads), ranging from 89.16% to 95.89%. A complete mapping summary is presented in Table 2.2.

Table 2.1. Sequencing Summary

Sample		Read Length	Sequence Reads	% GC	% Duplication
Horse	**Tissue**				
One	*Right Ventral Colon*	66 bp	22,311,131	47%	52%
	Left Ventral Colon	66 bp	26,261,555	44%	65%
	Pelvic Flexure	66 bp	20,973,831	48%	50%
	Left Dorsal Colon	66 bp	19,928,926	47%	57%
	Right Dorsal Colon	66 bp	21,598,494	48%	50%
Two	*Right Ventral Colon*	66 bp	25,793,114	46%	59%
	Left Ventral Colon	66 bp	21,763,631	45%	53%
	Pelvic Flexure	66 bp	18,178,616	46%	52%
	Left Dorsal Colon	66 bp	23,124,398	49%	59%
	Right Dorsal Colon	66 bp	30,913,609	46%	62%
Three	*Right Ventral Colon*	66 bp	21,950,810	47%	53%
	Left Ventral Colon	66 bp	23,166,097	49%	54%
	Pelvic Flexure	66 bp	18,403,414	47%	51%
	Left Dorsal Colon	66 bp	22,219,139	48%	57%
	Right Dorsal Colon	66 bp	28,908,596	49%	57%
H2O Negative Control		66 bp	57,475	48%	33%
Undetermined Reads		66 bp	14468231	37%	69%
Total			360,021,067	////////	////////
Mean			23,033,024	47%	55%
SD			3,581,617	1%	5%

Table 2.2 – Mapping Summary

Sample		Unique Alignment		Multiple Alignment		No Alignment		Overall
Horse	**Tissue**	*Reads*	*%*	*Reads*	*%*	*Reads*	*%*	**Alignment**
One	*Right Ventral Colon*	19,478,652	87.30%	1,614,274	7.24%	1,218,205	5.46%	94.54%
	Left Ventral Colon	21,573,527	82.15%	1,841,687	7.01%	2,846,341	10.84%	89.16%
	Pelvic Flexure	18,364,036	87.56%	1,442,260	6.88%	1,167,535	5.57%	94.43%
	Left Dorsal Colon	15,311,253	76.83%	3,283,848	16.48%	1,333,825	6.69%	93.31%
	Right Dorsal Colon	18,806,179	87.07%	1,632,993	7.56%	1,159,322	5.37%	94.63%
Two	*Right Ventral Colon*	21,616,000	83.81%	1,823,801	7.07%	2,353,313	9.12%	90.88%
	Left Ventral Colon	18,380,890	84.46%	1,489,666	6.84%	1,893,075	8.70%	91.30%
	Pelvic Flexure	15,859,532	87.24%	1,193,734	6.57%	1,125,350	6.19%	93.81%
	Left Dorsal Colon	18,235,066	78.86%	3,524,129	15.24%	1,365,203	5.90%	94.10%
	Right Dorsal Colon	25,707,539	83.16%	2,309,218	7.47%	2,896,852	9.37%	90.63%
Three	*Right Ventral Colon*	18,883,039	86.02%	1,776,660	8.09%	1,291,111	5.88%	94.12%
	Left Ventral Colon	20,530,630	88.62%	1,684,450	7.27%	951,017	4.11%	95.89%
	Pelvic Flexure	16,094,008	87.45%	1,288,641	7.00%	1,020,765	5.55%	94.45%
	Left Dorsal Colon	19,256,044	86.66%	1,965,147	8.84%	997,948	4.49%	95.51%
	Right Dorsal Colon	25,334,402	87.64%	2,092,143	7.24%	1,482,051	5.13%	94.87%
Mean			84.99%		8.45%		6.56%	93.44%
SD			3.47%		3.07%		1.99%	1.99%

Gene Expression

Raw read count data were summarized by sample using the featureCounts program [40] and the Ensembl 106 annotation of the equine reference genome. Normalized count values were produced using DESeq2 [43]. The number of genes expressed in each tissue (reads mapped to annotated gene model in at least 2 of 3 horse samples for that tissue) was 18,445 in the RVC, 18,258 in the LVC, 18,146 in the PF, 18,195 in the LDC, and 18,606 in the RDC, respectively. The average number of genes expressed per tissue was 18,330 +/- 191. The number of expressed genes by sample is displayed in Figure 2.3, and the summation of genes expressed by tissue is presented in Table 2.3.

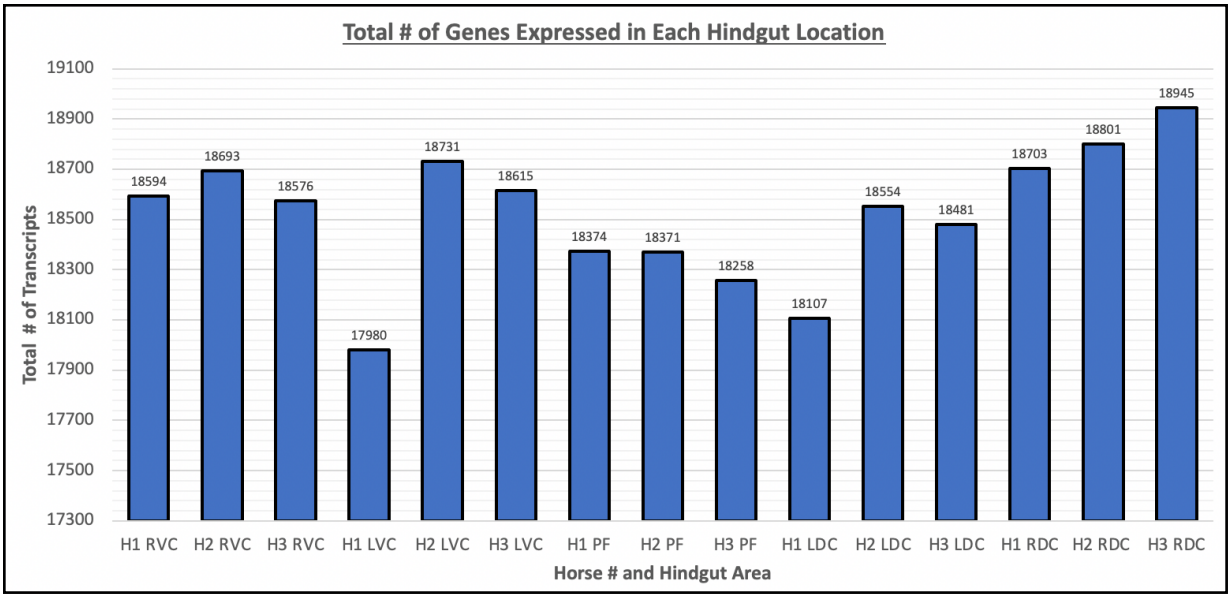

Figure 2.3. The number of genes expressed in sections of the equine hindgut by sample. The average across all tissues was 18,330 +/- 191 genes (red dashed line).

Table 2.3. The average number of genes expressed in each hindgut location.

Hindgut Area	Horse 1	Horse 2	Horse 3	Number of Genes
RVC	18594	18693	18576	18445
LVC	17980	18731	18615	18258
PF	18374	18371	18258	18146
LDC	18107	18554	18481	18195
RDC	18703	18801	18945	18606

Patterns of expression were compared across the five sampling locations. Of the 31,215 gene features included in the Ensembl 106 annotation, expression was detected for a majority, 16,750 (53.7%), at all five intestinal sites. A second set of 1,203 genes was determined to have detectable expression in only 1 of the 5 locations. The comparison of expression patterns also identified genes expressed in four, three, and two out of five sample locations. The distribution of detected gene expression by tissue is presented in Figure 2.4.

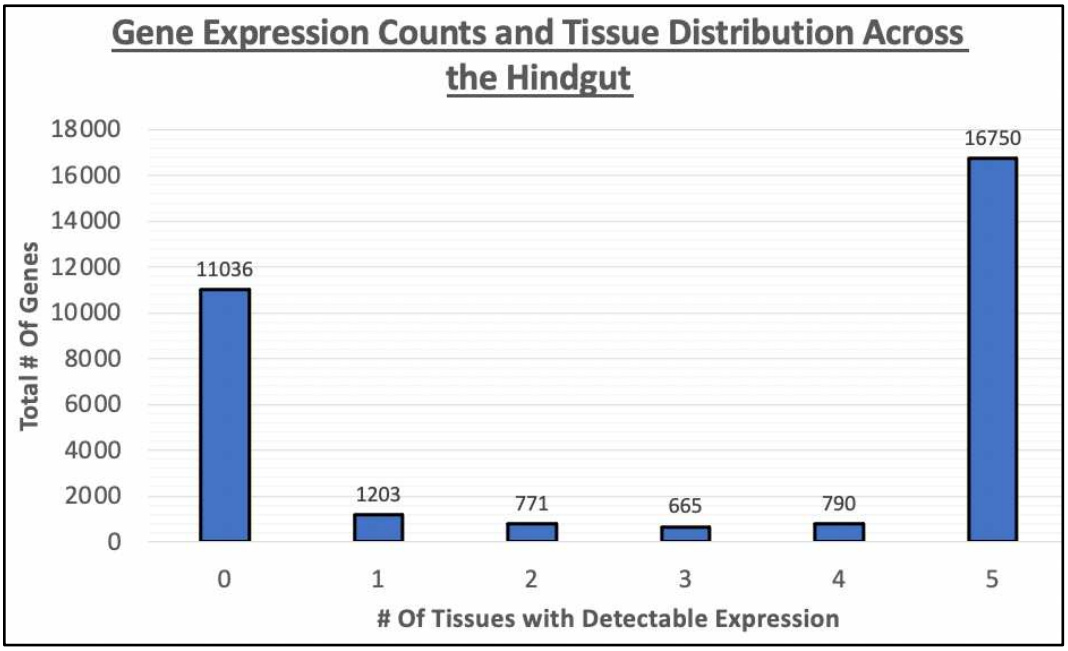

Figure 2.4. Distribution of gene expression by tissue. The number of tissues with detectable expression represents the number of tissues where sequence reads were generated and mapped to a gene model in at least 2 out of 3 horse samples for that tissue. The 11,036 genes without tissue representation had no sequence data meeting this threshold.

Tissue-specificity of expression patterns was confirmed by calculating the tau index. The results of this analysis were comparable to the analog analysis described above. The tau index value frequency distribution across the five tissues is presented in Figure 2.5. All unique, tissue-restricted genes were filtered based on whether they were expressed in 2 or more horses and if that expression was exclusive to 1 of the five tissue areas. The top 20 unique genes per tissue site are shown in Table 2.4, along with the total number of tissue-restricted genes found per tissue site. Tissue-restricted gene expression was found in all five tissue sites, with the pelvic flexure having the least (179 genes) and the right dorsal colon having the most (311 genes) tissue-restricted gene expression. A complete list of genes with restricted expression is included in supplemental table S2.1.

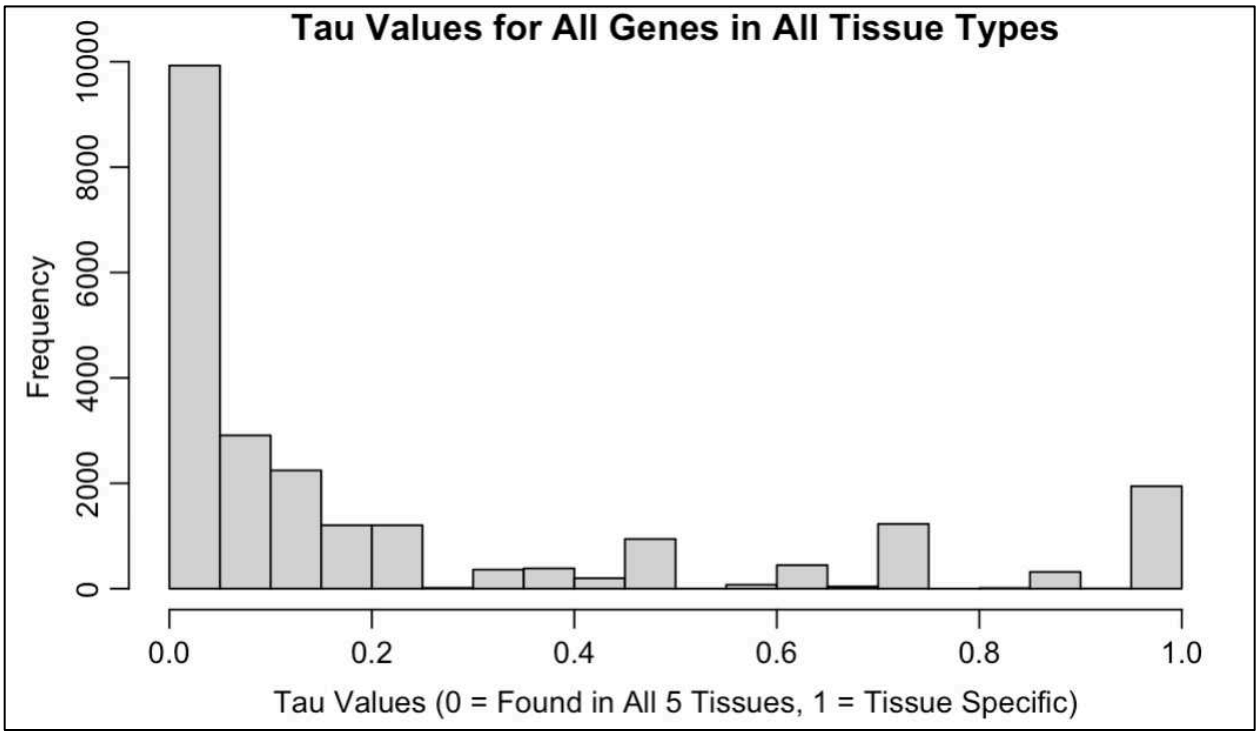

Figure 2.5. Tau index value frequency distribution across hindgut locations. Tau index values ranged from 0 to 1, with 0 indicating consistent expression across hindgut tissues and 1 indicating differential expression (i.e., tissue-restricted expression in 1 of the five hindgut areas). The frequency displays how many genes were categorized at a particular Tau index value. The pattern shown is similar to Figure 2.4, with most genes being expressed across all five tissue sites (at Tau=0) and some displaying tissue-restricted expression (at Tau=1).

Differential Gene Expression.

Differential gene expression between the GI regions was determined using DESeq2 [43]. The specific comparisons used were (1) PF versus LVC, (2) PF versus LDC, (3) LVC versus LDC, (4) PF versus RVC, and (5) PF versus RDC. The number of differentially expressed genes in each comparison is presented in Table 2.5.

Table 2.4. Top 20 and Total # of Unique Genes per Tissue Site.

	Right Ventral Colon	Left Ventral Colon	Pelvic Flexure	Left Dorsal Colon	Right Dorsal Colon
Top 20 Unique Genes (Tissue-Specific)	ADGRA1	FOXB1	SNORA19	LRRTM3	OR6B13
	CFAP46	eca-mir-545	NYX	SNORD108	PNLIP
	CUZD1	IRS4	GPR119	CYP1A2	ANKRD1
	CDHR1	eca-mir-138-1	TKTL1	SLC24A1	SLC35F3
	TMEM266	CDH8	TLE7	TGM5	SNORA74
	DUT	eca-mir-9074	DTHD1	DHRS2	GJD2
	ACTC1	CSN1S1	eca-mir-9077	ARSF	KLHL34
	OR4F13	GABRG1	KLHDC7A	RS1	IL1RAPL1
	MYH7	GJB4	SRARP	NCBP2L	PLAC1
	TAF7L	HCRTR1	LRRD1	SNORA69	ENAM
	RAB9B	ELOA	TAS2R3	TERB1	TMPRSS11A
	C3H16orf86	PRSS55	OR2A75	MIR140	ADRA2C
	AGBL4	eca-mir-703	ASB10	C3H4orf17	CDCP2
	ZMYND12	PON1	OAZ1	AMBN	eca-mir-9061
	POU3F1	DLX6	TRIM29	DMBX1	CITED4
	OPRD1	CPA4	NTM	SCARNA1	TFAP2E
	RPL11	PLK5	eca-mir-1271b	XKR4	NR0B2
	NPM2	GRIA4	PTPN5	SLC26A5	SLC30A2
	GNRH1	OR7D20	SRRM4	CPA1	FAM131C
	NPY2R	CCKBR	SNORA49	STRA8	CFAP74
Total # of Unique Genes	244	237	179	232	311

The top 20 unique genes- or genes with tissue-restricted expression- found at each tissue site were chosen based on significance (with $p<0.05$), expression presence in 2 or more horses, and gene expression present in only 1 of the five tissues. The total # of tissue-restricted genes per tissue site was also included, and all five tissue sites had differential expression present.

Table 2.5. Differentially expressed genes between regions of the equine GI.

Comparison	Total # DEG	Upregulated DEG	Downregulated DEG
LVC vs PF	57	7	50
LDC vs PF	185	73	112
LVC vs LDC	32	16	16
RVC vs PF	280	136	144
RDC vs PF	107	64	43

DEG stands for differentially expressed genes; all five major comparisons are included.

Lists of the differentially expressed genes (Benjamini Hochberg adjusted p-value < 0.05) from each comparison are presented in supplemental tables S2.2-S2.6. The top ten differentially expressed genes from each comparison are shown in Table 2.6-2.10. Notably, the LVC vs. PF only had seven annotated genes expressed with a positive fold change. A principal component analysis (or PCA) was generated using the differential expression data generated by DESeq2. The graph resulting from the first two principal components (PC1 and PC2) is presented in Figure 2.6. PC1 accounts for 34% of the variance in differential gene expression and appears to correlate with anatomical location along the hindgut. The LVC and LDC are closer anatomically to the pelvic flexure and closer on the graph than the RVC and RDC, which are more distal anatomically. PC2 accounts for 20% of the variance and appears to differentiate the ventral and dorsal colons.

Table 2.6. Top 10 differentially expressed genes in RVC vs. PF in both directions.

Top 10 Upregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
LOC111767452 (ABO)	Histo-blood group ABO system transferase 1-like	ENSECAG00000020130	10.7918689	6.51E-04
AKR1C1	aldo-keto reductase family 1, member C1	ENSECAG00000037198	6.930792828	5.04E-03
RSPH4A	radial spoke head component 4A	ENSECAG00000012533	6.403097093	1.71E-02
TLR5	Toll-like receptor 5	ENSECAG00000006701	4.170467646	5.60E-03
ACER1	alkaline ceramidase 1	ENSECAG00000022733	3.885728913	4.67E-02
LRRN3	leucine rich repeat neuronal 3	ENSECAG00000015351	3.680315223	1.84E-04
NXPE1	Neurexophilin And PC-Esterase Domain Family Member 1	ENSECAG00000011963	3.429843186	8.10E-03
GRIA2	glutamate ionotropic receptor AMPA type subunit 2	ENSECAG00000017620	3.391936323	1.81E-02
HIF3A	hypoxia inducible factor 3 subunit alpha	ENSECAG00000020693	3.37174113	1.03E-07
TMEM108	transmembrane protein 108	ENSECAG00000012889	3.359641271	4.36E-03
Top 10 Downregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
ABO	ABO, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase	ENSECAG00000014463	-8.801280999	2.91E-03
ACP7	acid phosphatase 7, tartrate resistant (putative)	ENSECAG00000014105	-5.423258499	4.30E-02
ABCC11	ATP binding cassette subfamily C member 11	ENSECAG00000021168	-4.901956444	4.98E-02
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif 4	ENSECAG00000024172	-4.889426401	1.60E-03
CRP	C-reactive protein	ENSECAG00000024874	-4.617477905	2.04E-03
FOSL1	FOS like 1, AP-1 transcription factor subunit	ENSECAG00000023092	-4.398746637	4.67E-02
SHARPIN	SHANK Associated RH Domain Interactor	ENSECAG00000002550	-4.312673418	1.30E-02
DUOX1	dual oxidase 1	ENSECAG00000023918	-4.311399894	1.42E-04
HMGB1	High-mobility group box 1	ENSECAG00000003655	-4.123804904	2.24E-02
FOSB	FosB proto-onco, AP-1 transcription factor subunit	ENSECAG00000018002	-4.01599	1.31E-03

[A] The top annotated genes from Ensembl 106. [B] The official gene symbols (VGNC). [C] Log2 transformed fold change. [D] The adjusted p-value is based on a Benjamini-Hochberg correction for multiple testing.

Table 2.7. Top differentially expressed genes in LVC vs. PF.

Top 7 Upregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
TLR5	Toll-like receptor 11	ENSECAG00000006701	3.092047395	3.10E-02
LRRN3	leucine rich repeat neuronal 3	ENSECAG00000015351	3.025578512	2.59E-03
HIF3A	hypoxia inducible factor 3 subunit alpha	ENSECAG00000020693	2.118792064	5.12E-03
LY9	solute carrier organic anion transporter family member 2B1	ENSECAG00000035548	1.317290392	2.13E-04
CD163	CD163 Molecule	ENSECAG00000019111	1.314810892	5.80E-03
F2RL2	coagulation factor II thrombin receptor like 2	ENSECAG00000010531	1.053307274	2.19E-02
SLCO2B1	Solute carrier organic anion transporter family member 2B1	ENSECAG00000024423	0.843843252	4.16E-02
Top 10 Downregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
CRP	C-reactive protein	ENSECAG00000024874	-4.524837704	8.90E-03
CXCL6	C-X-C motif chemokine ligand 6	ENSECAG00000012742	-3.311993252	1.02E-02
DPEP1	dipeptidase 1	ENSECAG00000022966	-3.272777732	4.60E-02
KLHL14	kelch like family member 14	ENSECAG00000012922	-2.719377738	8.91E-03
MGST1	microsomal glutathione S-transferase 1	ENSECAG00000020763	-2.716176372	3.75E-06
RASAL1	RAS protein activator like 1	ENSECAG00000009544	-2.500457024	2.87E-02
PRX	periaxin	ENSECAG00000023423	-2.437410327	5.96E-03
DRB	MHC class II DR-beta chain	ENSECAG00000022072	-2.325751152	2.13E-04
APOA1	apolipoprotein A1	ENSECAG00000009752	-2.230444782	4.60E-02
TERT	telomerase reverse transcriptase	ENSECAG00000001347	-2.101503102	1.39E-02

[A] The top annotated genes from Ensembl 106. [B] The official gene symbols (VGNC). [C] Log2 transformed fold change. [D] The adjusted p-value is based on a Benjamini-Hochberg correction for multiple testing.

Table 2.8. Top 10 differentially expressed genes in LVC vs. LDC in both directions.

Top 10 Upregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
CRP	C-reactive protein	ENSECAG00000024874	4.101368799	2.94E-02
IGKV6D-41	Immunoglobulin Kappa Variable 6D-41	ENSECAG00000001747	3.464993582	8.53E-03
HSPH1	heat shock protein family H (Hsp110) member 1	ENSECAG00000018428	2.383140312	1.31E-03
PDK4	pyruvate dehydrogenase kinase 4	ENSECAG00000008335	2.121550823	2.46E-02
DRB	MHC class II DR-beta chain	ENSECAG00000022072	1.994120722	8.53E-03
DNAJA1	DnaJ Heat Shock Protein Family (Hsp40) Member A1	ENSECAG00000010324	1.675528133	4.25E-02
CCDC117	coiled-coil domain containing 117	ENSECAG00000020497	1.652460464	7.08E-03
HLA-DQB1	Major Histocompatibility Complex, Class II, DQ Beta 1	ENSECAG00000006492	1.361772319	2.94E-02
CD5L	CD5 molecule like	ENSECAG00000023772	1.197277418	3.19E-02
DNAJB4	DnaJ heat shock protein family (Hsp40) member B4	ENSECAG00000010808	1.082730766	4.79E-02
Top 10 Downregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
LRRN3	leucine rich repeat neuronal 3	ENSECAG00000015351	-2.541033001	4.25E-02
ZNF420	Zinc Finger Protein 420	ENSECAG00000002797	-2.486144389	3.77E-02
LPO	lactoperoxidase	ENSECAG00000023550	-2.313252949	4.38E-02
LAMC3	laminin subunit gamma 3	ENSECAG00000000296	-1.991460251	4.25E-02
NSMF	NMDA receptor synaptonuclear signaling and neuronal migration factor	ENSECAG00000012467	-0.907115321	8.53E-03
AP1G2	adaptor related protein complex 1 subunit gamma 2	ENSECAG00000015018	-0.897861789	4.25E-02
PLCH2	phospholipase C eta 2	ENSECAG00000031378	-0.863717126	2.46E-02
PNPLA7	patatin like phospholipase domain containing 7	ENSECAG00000017449	-0.811453437	2.68E-02
SLC35A4	solute carrier family 35 member A4	ENSECAG00000004900	-0.735022319	2.46E-02
ACO2	aconitase 2	ENSECAG00000023762	-0.660535767	4.25E-02

[A] The top annotated genes from Ensembl 106. [B] The official gene symbols (VGNC). [C] Log2 transformed fold change. [D] The adjusted p-value is based on a Benjamini-Hochberg correction for multiple testing.

Table 2.9. Top differentially expressed genes in LDC vs. PF.

Top 10 Upregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
AKR1C1	aldo-keto reductase family 1, member C1	ENSECAG00000037198	6.06354858	2.68E-02
IGKV6D-41	Ig-like domain-containing protein	ENSECAG00000001747	2.898486379	2.03E-02
PDK4	pyruvate dehydrogenase kinase 4	ENSECAG00000008335	2.690992218	1.99E-04
TMTC2	transmembrane O-mannosyltransferase targeting cadherins 2	ENSECAG00000014638	2.330117534	8.94E-03
ZNF365	zinc finger protein 365	ENSECAG00000010245	2.280354416	1.18E-03
ADGRG2	adhesion G protein-coupled receptor G2	ENSECAG00000023252	2.272317065	3.02E-02
OMD	osteomodulin	ENSECAG00000014287	2.055684563	1.18E-03
PLPPR4	phospholipid phosphatase related 4	ENSECAG00000004854	2.036549395	3.68E-02
ABCA10	ATP binding cassette subfamily A member 10	ENSECAG00000000323	2.014144378	5.60E-03
GRAMD1C	GRAM domain containing 1C	ENSECAG00000018023	1.916139626	2.93E-02
Top 10 Downregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
ABO	ABO, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase	ENSECAG00000014463	-9.752463371	1.36E-03
DEFB4B	Defensin Beta 4B	ENSECAG00000007755	-6.050128442	1.36E-02
PRG3	Proteoglycan 3, Pro Eosinophil Major Basic Protein 2	ENSECAG00000004473	-5.684841153	2.34E-02
SHARPIN	SHANK Associated RH Domain Interactor	ENSECAG00000002550	-4.53235244	1.25E-02
MHCX1	Major histocompatibility complex class I protein	ENSECAG00000034339	-4.403132226	1.60E-03
SYT13	synaptotagmin 13	ENSECAG00000023889	-3.519219929	4.64E-02
MGST1	microsomal glutathione S-transferase 1	ENSECAG00000020763	-3.493391885	4.05E-12
MPTX1	mucosal pentraxin 1	ENSECAG00000009260	-3.160755817	2.40E-02
PRKCG	protein kinase C gamma	ENSECAG00000019249	-3.116156464	2.68E-02
LOC100071942	Galectin	ENSECAG00000029414	-3.105030159	1.01E-04

[A] The top annotated genes from Ensembl 106. [B] The official gene symbols (VGNC). [C] Log2 transformed fold change. [D] The adjusted p-value is based on a Benjamini-Hochberg correction for multiple testing.

Table 2.10. Top 10 differentially expressed genes in RDC vs. PF in both directions.

Top 10 Upregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
ADIPOQ	adiponectin, C1Q and collagen domain containing	ENSECAG00000002962	5.995718744	5.76E-03
PLIN1	perilipin 1	ENSECAG00000013202	5.962423963	3.67E-02
NGEF	neuronal guanine nucleotide exchange factor	ENSECAG00000011222	5.79292885	1.15E-02
PPP1R17	protein phosphatase 1 regulatory subunit 17	ENSECAG00000021847	3.537438723	4.95E-02
PI16	peptidase inhibitor 16	ENSECAG00000024845	3.328511702	1.23E-02
LOC100066745	Proteoglycan 3	ENSECAG00000029882	3.23049618	2.43E-03
ASPG	asparaginase	ENSECAG00000013195	3.226517882	6.74E-05
CCDC172	coiled-coil domain containing 172	ENSECAG00000024829	3.109763041	4.83E-02
LILRA6	Leukocyte Immunoglobulin Like Receptor A6	ENSECAG00000012768	3.084430732	1.39E-04
CFAP43	cilia and flagella associated protein 43	ENSECAG00000021278	3.029422891	3.14E-02
Top 10 Downregulated Genes [A]				
Gene Symbol [B]	Gene Name	Ensembl ID	Fold Change [C]	P-value [D]
ABO	ABO, alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase	ENSECAG00000014463	-10.37065221	3.43E-04
PRG3	Proteoglycan 3	ENSECAG00000004473	-5.656424674	3.67E-02
MHCX1	MHC class I heavy chain	ENSECAG00000034339	-4.6703655	4.97E-04
SHARPIN	SHANK Associated RH Domain Interactor	ENSECAG00000002550	-4.628004847	1.16E-02
SYT13	synaptotagmin 13	ENSECAG00000023889	-4.055177448	1.91E-02
MUC6	mucin 6, oligomeric mucus/gel-forming	ENSECAG00000015992	-3.900361858	4.55E-02
LOC100071942	Galectin	ENSECAG00000029414	-3.130294445	6.74E-05
KIF12	kinesin family member 12	ENSECAG00000019503	-3.044600027	3.57E-02
MGST1	microsomal glutathione S-transferase 1	ENSECAG00000020763	-2.870408207	3.17E-07
PLEKHD1	pleckstrin homology and coiled-coil domain containing D1	ENSECAG00000008982	-2.840670634	1.65E-02

[A] The top annotated genes from Ensembl 106. [B] The official gene symbols (VGNC). [C] Log2 transformed fold change. [D] The adjusted p-value is based on a Benjamini-Hochberg correction for multiple testing.

To identify the genes with expression differences underlying the patterns revealed by PCA, the differential expression results were further analyzed using the subpopulation analysis algorithm from the Kharchenko lab single-cell analysis toolkit [56], which identifies genes with highly variable expression profiles across a mixed dataset. The resulting graph based on the log transformation of the coefficient of variation is presented in Figure 2.7.

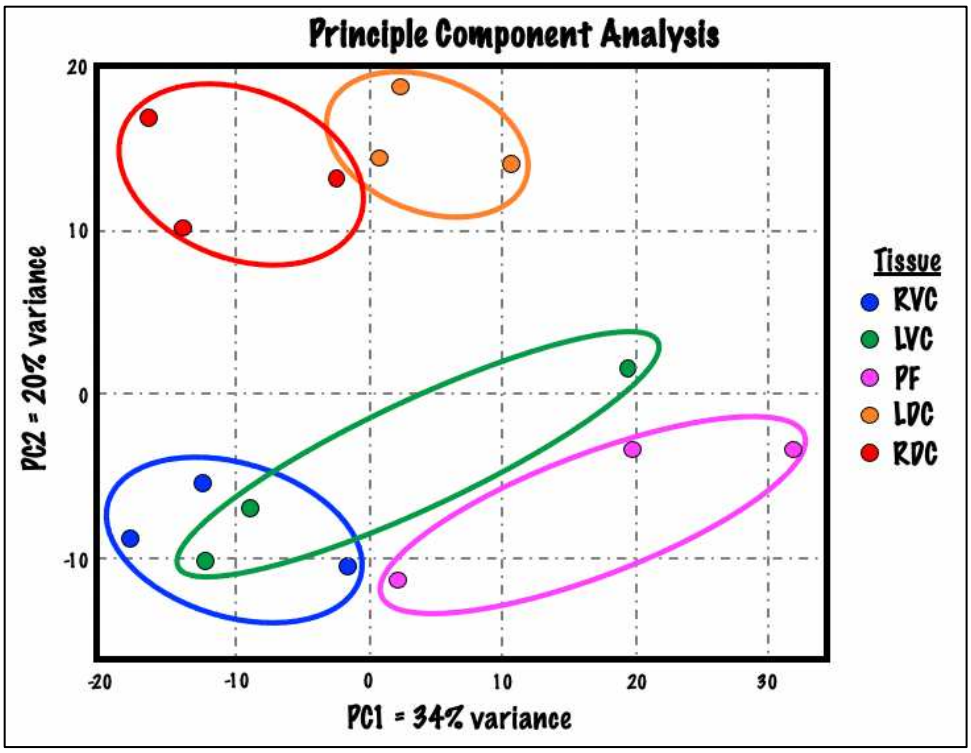

Figure 2.6. Principle component analysis showing expression differences between the right and left ventral colon (RVC and LVC), the pelvic flexure (PF), and the left and right dorsal colon (LDC and RDC).

Functional Analysis.

Enriched gene ontology terms in the lists of differentially expressed genes were determined using DAVID [55]. The top 10 biological processes enriched in each tissue comparison for differential expression are displayed in Table 2.11, with the entire list of results included as supplemental tables S2.7-S2.11.

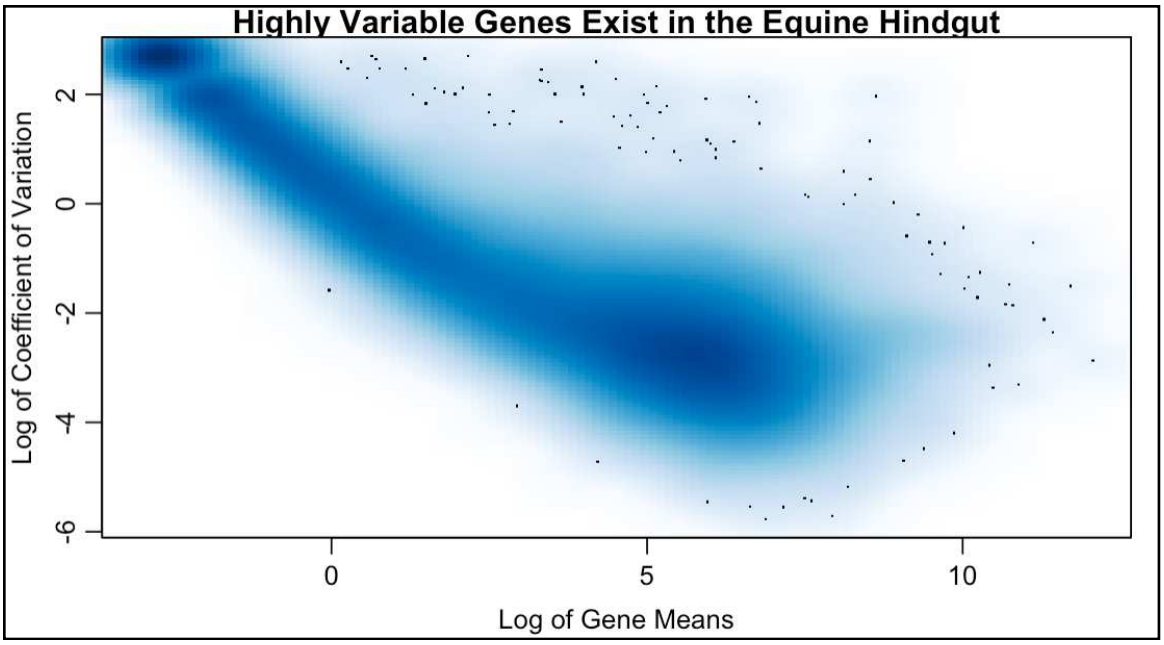

Figure 2.7. Highly variable gene expression patterns drive tissue expression profile differences in the hindgut. This scatterplot compares the log of the coefficient of variation to the log of means. The larger the log(means), the larger the observed difference in means between tissue sites; the larger the log(CoV), the more dispersion exists around the mean (i.e., a larger standard deviation about the mean). The highly variable genes are scattered above the densely packed dark blue area representing the bulk of the dataset.

Table 2.11 - top 10 enriched biological process GO categories by comparison.

	Accession	Biological Process	Fold Enrichment	PValue
PF vs LVC	GO:0006958	complement activation, classical pathway	16.0	0.0144
	GO:0002455	humoral immune response mediated by circulating immunoglobulin	14.2	0.0179
	GO:0006956	complement activation	12.5	0.0228
	GO:0072376	protein activation cascade	11.2	0.0281
	GO:0016064	immunoglobulin mediated immune response	8.4	0.0472
	GO:0019724	B cell mediated immunity	8.4	0.0478
	GO:0007369	gastrulation	8.2	0.0491
	GO:0006959	humoral immune response	6.7	0.0206
	GO:0002250	adaptive immune response	6.0	0.0084
	GO:0009968	negative regulation of signal transduction	3.4	0.0074
PF vs LDC	GO:0055096	low-density lipoprotein particle mediated signaling	71.0	0.0277
	GO:0071447	cellular response to hydroperoxide	35.5	0.0547
	GO:0033539	fatty acid beta-oxidation using acyl-CoA dehydrogenase	31.9	0.0037
	GO:0010886	positive regulation of cholesterol storage	30.4	0.0635
	GO:0032688	negative regulation of interferon-beta production	26.6	0.0722
	GO:0046007	negative regulation of activated T cell proliferation	23.7	0.0809
	GO:0055098	response to low-density lipoprotein particle	23.7	0.0809
	GO:0071404	cellular response to low-density lipoprotein particle stimulus	23.7	0.0809
	GO:0044793	negative regulation by host of viral process	21.3	0.0895
	GO:0034372	very-low-density lipoprotein particle remodeling	21.3	0.0895
LVC vs LDC	GO:0002504	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	51.5	0.0014
	GO:0006955	immune response	3.7	0.0038
	GO:0019882	antigen processing and presentation	17.2	0.0122
	GO:0002250	adaptive immune response	7.3	0.0150
	GO:0006457	protein folding	11.6	0.0255
	GO:0051085	chaperone mediated protein folding requiring cofactor	44.9	0.0421
	GO:0051084	'de novo' posttranslational protein folding	41.7	0.0452
	GO:0006458	'de novo' protein folding	40.3	0.0468
	GO:0002376	immune system process	2.2	0.0470
	GO:0061077	chaperone-mediated protein folding	24.8	0.0748
PF vs RVC	GO:0033561	regulation of water loss via skin	13.5	0.0030
	GO:0045742	positive regulation of epidermal growth factor receptor signaling pathway	11.5	0.0048
	GO:0046323	glucose import	7.8	0.0038
	GO:0046889	positive regulation of lipid biosynthetic process	7.5	0.0044
	GO:0032436	positive regulation of proteasomal ubiquitin-dependent protein catabolic process	6.7	0.0020
	GO:1901800	positive regulation of proteasomal protein catabolic process	6.0	0.0033
	GO:0008643	carbohydrate transport	5.5	0.0047
	GO:1903052	positive regulation of proteolysis involved in cellular protein catabolic process	5.4	0.0050
	GO:0019882	antigen processing and presentation	5.3	0.0020
	GO:1903364	positive regulation of cellular protein catabolic process	5.3	0.0021
PF vs RDC	GO:0045019	negative regulation of nitric oxide biosynthetic process	69.1	0.0282
	GO:0075509	endocytosis involved in viral entry into host cell	59.2	0.0328
	GO:0010739	positive regulation of protein kinase A signaling	51.8	0.0375
	GO:0031953	negative regulation of protein autophosphorylation	46.1	0.0420
	GO:0016264	gap junction assembly	46.1	0.0420
	GO:0002504	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	18.3	0.0114
	GO:0042149	cellular response to glucose starvation	15.6	0.0155
	GO:0055117	regulation of cardiac muscle contraction	13.5	0.0202
	GO:1903426	regulation of reactive oxygen species biosynthetic process	13.2	0.0210
	GO:0019882	antigen processing and presentation	12.2	0.0001

Discussion

The equine hindgut hosts diverse microbial communities critical for digesting a horse's forage-based diet. The pelvic flexure – a common site of impactions, colic, and other digestive disorders [14] – helps regulate transit in the hindgut and, it would seem, segregates distinct microbiota [12]. The roles of the various hindgut segments (cecum, ventral colon, dorsal colon, small colon) in digestive and absorptive processes have been well studied. Physiological differences between the GI regions at the gene expression level have not been determined. The present study used RNA sequencing to investigate epithelial differential gene expression in the equine hindgut, specifically in the regions surrounding the pelvic flexure. Establishing and maintaining homeostasis in the hindgut is critical for normal physiology and function. The results of the current study provide new insight into the gene expression patterns underlying intestinal epithelial cell physiology in different sections of the equine hindgut.

The intestinal epithelium of the equine hindgut comprises multiple cell layers assembled into villi and microvilli structures which project into the intestinal lumen, increasing surface area to support nutrient absorption [62]. The cells of the epithelium (intestinal epithelial cells or IECs) are renewed every 3-5 days [63,64], and their gene expression profiles are shaped by many factors, including cell type, phase in the cell cycle, and responses to varied environmental stimuli [60,61]. The tissues for this study were all sampled mucosal and submucosal layers of the intestine and each anatomic site. Given the broad similarities observed in structure and function based on the shared type and distribution of cells in these intestinal epithelia, we anticipated that gene expression patterns at all five tissue sites sampled would be broadly similar [59]. Still, given that the functional roles of the ventral and dorsal colons differ in equine digestive physiology, we also

expected to find both tissue-restricted and different gene expression profiles across the five locations.

A total of 360,021,067 basepair sequence reads were generated from the 15 tissue samples and one water negative control sample, with the average amount of sequences generated per tissue sample existing around 23,033,024. The negative control produced only 57,475 sequence reads, indicating that contamination and sequencing issues were absent. Read mapping rates were consistent across the subjects and samples, with an average rate of 93.44% +/- 1.99%, including unique and multiple alignments. The number of annotated genes with expression was also similar across subjects and sampling sites, with an average of 18,330 +/- 191, with a range of 18,146-18,606. These results indicate that the data is consistent, comparable, and free of issues that might impair the subsequent analyses.

Most genes expressed in this dataset – (16,750/31,215 or 53.7%) had detectable expression at all 5 sample locations (i.e., at all 5 GI sites). It is important to note that just because a gene was identified in all five tissue locations does not indicate that the expression level detected at all five sites was equivalent. Broadly, the functional roles associated with this gene category were related to cellular metabolism, catalytic activity, cellular responses to stimuli or stress, and other essential components of physiology [58].

In addition to the genes with detectable expression at all five sample sites, 1,203 genes were identified in only 1/5 of tissues – indicating tissue-restricted expression patterns. These tissue-restricted may be informative about the unique physiology of the different tissue sites. They

may also help explain the diverse microbiota hosted in the different hindgut segments. However, that connection will need to be addressed in future work.

The ventral colon of the equine hindgut extends from the cecum to the pelvic flexure. Its primary roles in digestion include support of microbial fermentation of dietary fiber and some absorption of the products resulting from that fermentation. Genes with expression restricted to the LVC include forkhead box B1 (FOXB1) and insulin receptor substrate 4 (IRS4). FOXB1 is a member of the forkhead box family of transcription factors. These transcription factors are involved in various biological processes, including cell proliferation, cell differentiation, immune responses, and signaling. Several related forkhead box family members (FOXA1, FOXA2, and FOXO1) have been implicated in epithelial cell development and commensalism [Tuteja and Kaestner 2007, van de Sluis *et al*. 2008, Chen *et al*. 2021], indicating the potential for FOXB1 to be involved in similar processes. IRS4 expression has been associated with cell proliferation, and its overexpression has been correlated with the development and staging of colorectal cancers [sanmartin-salinas *et al*. 2018]. IRS4 has been poorly studied but may be involved in regulating cell proliferation in the ventral colon. Another gene with expression restricted to the LVC was cadherin 8 (CDH8), an integral membrane protein associated with calcium-dependent cell-to-cell adhesion [65]. The expression of CDH8 in the LVC could help ensure proper cell-to-cell interactions to help maintain the integrity of the mucosa in that hindgut region [reference]. A final example of a gene with expression restricted to the LVC in our dataset was the cholecystokinin B receptor (CCKBR) gene. CCKBR encodes a G-protein coupled receptor for gastrin and CCK. It has demonstrated expression in the brain and gastrointestinal tract, primarily in the stomach [66], enhancing mucosal growth [reference]. CCKBR has also been associated with biological processes

involved in pH regulation, processes which may be necessary as digesta transition from the ventral to dorsal colons.

The dorsal colon extends from the pelvic flexure to the transverse colon and runs along the dorsal aspect of the horse's intestinal cavity. The role of the dorsal colon is the absorption of water, electrolytes, and volatile fatty acids (VFAs) which result from microbial fermentation in the cecum and ventral colon. As with the LVC, several genes appeared to have expression restricted to the LDC. Solute carrier family 24 member 1 (SLC24A1) encodes a potassium-dependent sodium/calcium exchanger protein family member. Notably, this gene has relatively broad expression across multiple tissue types but was only detected in the LDC from our samples. SLC24A1 has been implicated in maintaining intestinal homeostasis by promoting ion balance/transport, absorption/secretion of molecules in the intestinal lumen, proper stool formation, or late-stage nutrient absorption [67]. As the dorsal colon is a primary site of absorption, the expression of SLC24A1 in the LDC is likely associated with that task. Finally, SLC24A1's functional role in ion transport and membrane potential is important in maintaining GI homeostasis in this region of the hindgut, in particular as it relates to the proper absorption of nutrients at the proximal dorsal colon region that may directly influence absorption processes in the RDC. Another gene with LDC-restricted expression in our data was cytochrome P450 subfamily A member 2 (CYP1A2), which codes for a member of the cytochrome P450 superfamily. This family of enzymes is involved in drug metabolism and cholesterol, steroid, and lipid synthesis [68]. There is not much information regarding the expression of this enzyme superfamily in the hindgut. Still, the expression of CYP1A2 in the LDC may be associated with biological processes involved in metabolizing microbial fermentation's absorbed products. Finally, our data shows that

transglutaminase 5 (TGM5) expression appears restricted to the LDC. Transglutaminases can stabilize protein structures by catalyzing glutamine-lysine crosslinking. This stabilization can improve barrier function in the epidermal layers of the skin. In the LDC, it could be essential for helping to maintain mucosal integrity. Alternatively, stabilizing protein structures can facilitate antigen presentation and subsequent immune responses.

There were several genes of interest with expression restricted to the pelvic flexure region of the hindgut in our dataset. First was the steroid receptor-associated and regulated protein (SRARP) gene. SRARP is currently described as enabling estrogen receptor binding and positive regulation of the estrogen receptor signaling pathways [70]. The presence of estrogen receptors in IECs has been previously reported [116], and estrogen signaling has been implicated in the modulation of epithelial cell secretion and epithelial barrier functions [117,118]. There is also evidence that glucocorticoid hormones in the intestinal epithelium help regulate T cell activation [119]. Together, these functions may provide insight into how distinct microbial communities are separated at the pelvic flexure. However, additional investigation is necessary. Similarly, expression of the transketolase-like 1 (TKTL1) gene was also found to be restricted to the pelvic flexure. The associated protein forms a homodimer complex to convert intermediate metabolites and links the pentose phosphate and glycolytic pathways. [71]. The expression of transketolase supports ATP production, which would be required in metabolically active tissues [120]. In IECs, TKT expression has also been demonstrated to help maintain epithelial barrier function and inhibit apoptosis-induced colitis [120]. A final gene with expression restricted to the pelvic flexure in our data was neurotrimin (or NTM), a protein-coding gene that promotes neurite outgrowth and adhesion, stabilizes synapses, and is closely linked to a related opioid-binding protein/cell

adhesion molecule-like (OPCML) family member [72]. As a possible explanation for its expression in the pelvic flexure, this could indicate the importance of proper enteric innervation, structural sensing, stabilization, and community interaction with other cells and contents of the intestinal lumen in this region specifically. Such interactions may be possible at the pelvic flexure because of its narrower luminal space. This is especially true compared to the broader structures of the equine ventral and dorsal colons. Additional research is required to determine whether this explanation is possible.

In addition to examining tissue-restricted expression patterns, we investigated differential gene expression between the five hindgut regions collected for this study. These comparisons focused on identifying differences between the pelvic flexure and the other four surrounding regions. Our focus was on the pelvic flexure as the anatomical feature identified previously [12] as separating distinct communities of microbes in the equine hindgut. This investigation aimed to provide insight into the gene expression patterns underlying the physiology of these five locations in the hindgut to understand why the microbial populations are so distinct in the different regions.

As discussed, all five sites in the equine hindgut region sampled for this study had a broad distribution of gene expression. On average, 18,330 +/- 191 genes had detectable expression in each tissue, representing 58.7% of the features in the Ensembl 106 equine annotation set. Further, 16,750 annotated genes had detectable expression in all five tissue locations. Overall, the differences in the expression patterns of the tissues were relatively narrow, with the number of differentially expressed genes ranging from just 32 (left ventral versus left dorsal colons) to 280 (pelvic flexure versus right ventral colon). It is important to remember that all samples used in this

analysis comprised intestinal epithelial cells collected from the mucosal layer of the equine large colon. They are, therefore, compositionally comparable, and so logically would be expected to share broadly similar expression patterns.

There are also important structural and physiological differences between the ventral colon, dorsal colon, and pelvic flexure to appreciate. The ventral colon has a uniform diameter over its length, and the luminal space is surrounded by four bands of smooth muscle on either side. In contrast, where the dorsal colon connects with the pelvic flexure, the intestinal structure is relatively narrow, lacks sacculation, and has only a single band of muscle. The diameter expands towards the distal end of the dorsal colon and incorporates additional bands of muscle and subtle sacculations [76]. Physiologically, the ventral colon supports many essential digestive and absorptive functions – not the least of which is microbial fermentation - while the dorsal colon is mainly responsible for the absorption and transportation of ingesta, including the last-minute absorption of electrolytes, solutes, water, ex cetera [69]. The principal component analysis completed using the DESeq2 analysis highlights the gene expression differences associated with these structural and functional distinctions. The expression patterns of the five tissue sites appear to separate based on location within each intestinal compartment (principal component 1) and by the intestinal compartments themselves (principal component 2). The ventral and dorsal colons, including the left and right aspects of each one, are well distinguished based on observed differences in gene expression. The pelvic flexure is distinct from the ventral and dorsal colons but appears to cluster with the ventral colon. The basis of this separation is further supported by identifying highly variable genes within the dataset. As has been presented as the motivation for the current study, previous work by our group [12] highlighted and supported the existence of

distinct microbial communities in the equine hindgut separated by the pelvic flexure. The fact that the ventral and dorsal colons are distinguished by the microbial communities they support, and their gene expression profiles add credence to the possibility that gastrointestinal and digestive physiology aspects play a role in managing that microbiota. Correlation is not causation, however, and additional work beyond the scope and capabilities of the current study (we do not possess microbiota samples from the horses used for the expression analysis) will be required to determine if and how exactly host physiology could be regulating the composition of the gastrointestinal microbial communities. The expression differences identified by our study should provide avenues for future investigations.

Gene ontology terms associated with the lists of differentially expressed genes identified by DESeq2 were evaluated for over-representation of functional categories or descriptors. The relatively small size of the data set (3 biological replicates) and narrow lists of differentially expressed genes identified between sampling sites limited the effectiveness of this analysis. Multiple testing corrections eliminated all statistical significance. Still, there were some potentially valuable trends in the results, which may help and direct future investigation. All the differential expressed gene lists represented biological processes associated with adaptive immune responses (both humoral and cell-based) (Table 2.12). This result is undoubtedly expected, given the well-described role of the immune system in the gastrointestinal tract [121].

Based on our analyses, there appears to be an apparent shift from humoral to cell-based immune responses moving from the proximal (right ventral colon) to the distal (right dorsal colon) ends of the large colon. This transition is highlighted by comparing the categories represented

before and after the pelvic flexure. In the ventral colon, the top categories reflect humoral and immunoglobulin-mediated immune responses (GO:0006959, GO:0002455, GO:0016064), B-cell mediated immunity (GO:0019724), and complement activation (GO:0006956, GO:0006958). In the dorsal colon, several of the top categories relate to the regulation of T-cell proliferation (GO:0046007), antigen processing and presentation via MHC class II (GO:0002504), and the regulation of interferon-beta production (GO:0032688). Additionally, multiple ontologies are associated with lipoprotein-particle signaling and responses (GO:0055096, GO:0010886, GO:0055098, GO:0071404, GO:0034372). The appearance of these biological processes following the pelvic flexure likely reflects the dorsal colon's role in the absorption of nutrients resulting from the microbial fermentation and digestion occurring in the ventral colon, but are also linked with inflammation and immune responses, which may also contribute to the immune system's role in influencing the distribution of various microbial constituents in the different compartments of the GI. Further examination is necessary to analyze the specific genes expressed in this particular case.

Our previous work focused on the pelvic flexure as an important anatomical marker that separated distinct microbial communities in the equine hindgut [12]. Our goal in this work was to investigate the gene expression underlying physiological differences in the hindgut regions surrounding the pelvic flexure. As such, we highlighted the patterns of differential expression for genes with distinct expression in the pelvic flexure and demonstrated a difference relative to the other regions (ventral and dorsal colon), the upstream region (ventral colon), or the downstream region (dorsal colon).

Several genes with notable annotated functions were found to be more abundant in the pelvic flexure compared to the ventral and dorsal colons. The ABO alpha 1-3-N-acetylgalactosaminyltransferase and alpha 1-3-galactosyltransferase (ABO) and SHANK-associated RH domain interactor (SHARPIN) genes were both detected at higher levels in the PF compared to the RVC, LDC, and RDC. The ABO gene indirectly encodes the ABO blood group antigens, with the A and B alleles each encoding a glycosyltransferase that actively catalyzes the final step in synthesizing the A and B antigens [83]. The ABO blood group antigens are expressed on red blood cells, in tissues of the salivary glands, and, notably, on epithelial cells [122]. It has also been demonstrated that individuals with different ABO antigens (A, B, AB, or O) present with distinct populations and diversity metrics for the bacterial populations in their intestinal microbiota [123]. Therefore, the increased abundance of ABO in the pelvic flexure compared to the ventral and dorsal colons could be associated with the separation of distinct microbial communities reported by Reed *et al*. [12]. SHARPIN is a highly conserved autosomal gene that is a part of the linear ubiquitin chain assembly complex (LUBAC) and plays a key role in regulating immune and inflammatory responses by enabling polyubiquitin modification-dependent protein binding activity and being actively involved in protein linear polyubiquitination and signal transduction regulation [89-93]. Linear poly-ubiquitin chains are widely involved in innate and adaptive immune signaling pathways [93]. SHARPIN has been found to help initiate systemic inflammatory responses and regulate cell survival and apoptosis. It operates as an essential regulator of immune and inflammatory responses [107-108]. It is, therefore, plausible that SHARPIN's higher expression exclusively in the PF directs specific immune-related responses that could contribute to the microbial differences observed before and after the PF.

Another notable gene, microsomal glutathione S-transferase 1 (MGST1), was detected at higher levels in the PF versus the LVC, LDC, and RDC. MGST1 codes for a protein that is an important mediator of inflammation and plays an essential role in pathways associated with the innate immune system. Expression of MGST1 helps protect cells from toxic, carcinogenic, and other potentially harmful compounds, which result in increased oxidative stress [94]. Upregulation of MGST1 has been implicated in initiating changes associated with oxidative stress resulting from inflammatory bowel disease (IBD) pathogenesis in rat models. In these experiments, IBD altered the epithelial expression of MGST1, resulting in differing metabolite profiles and changes to the colonic microbiome [109]. In humans with Crohn's Disease or IBD, thickening of the intestinal wall is associated with immune responses that can lead to inflammation and abdominal pain [95-97], and similar reactions have been reported in other species, including horses [98-101]. Given the potential pressure differences and closer interaction between host and non-host elements, the increased abundance of MGST1 in the pelvic flexure relative to the surrounding regions could indicate a need for more host cell protections.

Several genes demonstrated differential expression between the PF and segments of the ventral colon. C-Reactive Protein (CRP) was more abundant in the PF than in the preceding ventral colon. CRP is a biomarker of inflammation, involved in the activation of the complement system, and an important part of host defense against pathogens [102]. Microbiome differences reflected by altered gut microbial diversity metrics have been associated with higher white blood cell counts and increased high-sensitivity C-reactive protein (hsCRP) levels [110]. The increased abundance of CRP observed at the PF could therefore help explain the observed microbiome composition differences between the VC and DC reported previously. A second gene, C-X-C motif chemokine

ligand 6 (CXCL6), showed increased mRNA abundance in the PF versus the LVC. CXCL6 encodes a member of the CXC chemokine protein family. This protein is chemotactic for neutrophil granulocytes and antibacterial against certain gram-negative and gram-positive bacteria [84]. Interestingly, chemokines - including CXCL6 - have been demonstrated to influence the abundance of gut microbe species and strains [85,86]. Interleukin 17 (IL-17) - implicated in inflammatory responses, neutrophil recruitment, and protection against extracellular bacterial pathogens – upregulates the expression of CXCL6 [87,88]. Higher levels of CXCL6 expression in the pelvic flexure could indicate targeting of specific microbes, which prevents their movement past the PF resulting in the differentiation of the microbiota composition between the ventral and dorsal colons.

Our analysis also identified genes with differential expression between the PF and dorsal colon. Proteoglycan 3 (PRG3) was more highly expressed in the narrower and angled PF versus the wider and straighter dorsal colon. PRG3 is involved in granulocyte activation, histamine biosynthesis, regulation of gene expression, and, importantly, compression resistance [103]. A recent study in mice examined differential gene expression associated with feeding a diet containing resistant potato starch (RPS). There was an observed increase in the abundance of *Citrobacter rodentium* – a pathogenic bacteria found in the mouse colon and shares 66.7% of encoded genes with E. coli [124] – in the distal colon of mice receiving RPS that correlated with, amongst other changes, a decrease in the expression of PRG3 (111). The observed reduction in PRG3 expression in the dorsal colon relative to the PF could help explain differences in microbial content of the VC and DC by supporting environments that are more permissive to certain bacteria at specific locations. The PF must also resist compression to avoid collapse of the intestinal lumen,

a function that expression of PRG3 supports. A second gene with more abundant expression in the PF compared to the dorsal colon was MHC Class 1 heavy chain (MHCX1). MHCX1 plays important roles in the signaling, binding, and immune responses associated with extracellular and intracellular pathogens [125-127]. Horses have high levels of variation in their MHC haplotypes, which could result in variations in the responses to bacteria and other microbes in the gut [104]. Recent research has demonstrated that MHC heterozygosity promotes functional diversification of the microbiome, enhancing microbial network connectivity and enriching a variety of microbial functions that positively affect host fitness [105, 112]. A final gene, Synaptotagmin 13 (or SYT13), was more abundant in the PF compared to the dorsal colon. SYT13 codes for a membrane trafficking protein actively involved in intracellular vesicle trafficking and exocytosis and, importantly, plays a role in modulating insulin secretion [106,113]. Insulin signaling has been demonstrated to shape gut community composition [114,115]. As a result, differential expression of SYT13 in the PF versus the dorsal colon could help determine microbiota composition before and after the pelvic flexure by influencing the regulation of insulin and nutrient transport.

Conclusion

The differences in gene expression that exist between the ventral and dorsal colons in the equine hindgut relate to tissue function and could impact microbial composition. Previous results from Reed *et al*. demonstrated that differences exist between the microbial composition of the proximal and distal hindgut, pointing to the potential role of the pelvic flexure region in influencing these observed differences. Similarly, there are differences in epithelial gene expression patterns observed relative to the pelvic flexure. Many of the differentially expressed genes play key roles in immune function and the digestion and absorption of specific compounds, directly correlating

to tissue-specific functions. One theory is that the pelvic flexure region is acting not as a barrier to these microbes, but as a "toll road" – the genes highly expressed in the pelvic flexure are directing who may pass through the pelvic flexure into the dorsal colon and who may not. Future research could further analyze these differences by a) expanding sample size from 3 horses to 30 or more horses and by sampling more locations in each hindgut region, b) more narrowly focusing on the gene expression of specific genes that were found in this study which could be closely directing these genetic, microbial and functional differences, and c) introducing healthy versus unhealthy equine subjects to further the research community's understanding of how health status may also affect microbial composition and gene expression in the equine hindgut.

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CHAPTER 3: LOOKING FORWARD

This study on gene expression in the equine hindgut is a stepping stone for future research that may further help in our understanding of GI homeostasis in the hindgut and our translation of this knowledge into useful medical treatment options for horses suffering from GI disease. In this case, we took intestinal samples from 3 four-year-old quarter horses that were raised under similar conditions (i.e., same age, diet, breed, living facility, health status, ex cetera) and had no history of GI disease. This is an important starting point for gene-related hindgut research because most of the potentially influencing factors were controlled for (*i.e., makes for good "baseline" data*). Therefore, this dataset may offer a solid beginning analysis of gene expression in the equine hindgut that can later be taken and expanded upon to a) confirm some of these initial results, b) see how other variables may influence gene expression, c) compare expression in healthy vs GI-diseased horses, and d) further analyze specific genes and their proteins, what they do, and how they may act as targets in drug-related developments. For example, a future study may take the same intestinal samples from 36 additional horses, but those horses now have varying breeds which may be a variable that could influence gene expression.

It is also important to note that each of the 5 tissue sites in this study had over 4,000 previously unidentified genes present, some of which were shown to be significantly expressed. During the analysis, some of the unidentified genes were also found to be differentially expressed, meaning that they may perform a specific function exclusively in one of the five tissues; this function may be coding for an easily identifiable extracellular protein

on that region's intestinal epithelial cells, which may become a target for the development of a future equine hindgut medication. As a result, future research may also benefit from a deeper analysis of these thousands of unidentified genes, which may provide key information on GI homeostasis and how to better treat problematic equine hindgut health conditions such as IBD, colic, impactions, ex cetera. As an example, a future researcher may take the top 5 significantly expressed genes in each tissue site (including some differentially expressed genes) and use modern technological identification programs to construct a) a structure of each gene in question, including length, location, ex cetera, b) what exons code for what RNA/protein structures, and how many potential products may result from transcription and translation, and c) what other factors may interact with this gene to enhance, repress, or influence transcription processes.

Since this current study focused on hindgut tissues that were not separated into distinct cell types, one future research possibility may focus on identifying specific cell types and analyzing their individual gene expression patterns. Intestinal epithelial tissue samples have various cell types, including but not limited to enterocytes, goblet cells, Paneth cells and stem cells. Samples could be taken at various places in the equine hindgut, but various separation techniques may have to be considered. For example, some cell separation techniques that could be considered are Fluorescence activated cell sorting (FACS), Buoyancy activated cell sorting (BACS), magnetic based cell sorting, or centrifugation techniques. Finally, after each cell type is isolated and gene expression analysis completed, comparisons can be made between different cell types within each tissue and between different tissue sites. This potential future study could further develop key biological insights and further analyze the similarities and

differences in gene expression that may exist between different cell types that exist within the intestinal epithelium of the equine hindgut.

Another future research possibility may involve taking a control group of say 6 healthy horses and a separate group of 6 horses with GI-disease and comparing/contrasting the results, utilizing the same methods in this study. However, in this experimental design it may be wise to make sure that all 12 horses are as closely related as possible, meaning that they have similar diets, living/health conditions, breed, age, ex cetera. Having less externally influencing variables in any future research design would benefit early stages of hindgut gene expression research, especially given that the effects of breed, age, previous health conditions, ex cetera may not fully be understood. In short, it would make sense that if a variable affects, say, the equine microbiome, that the same variable would likely affect other interacting systems, such as gene expression in intestinal epithelial cells. Therefore, it is key for future studies to focus the best they can on preventing external factors from influencing results, such as changes in diet, stress, medications, ex cetera, especially if such changes were previously found to affect the overall macro-system of GI homeostasis (such as changing immune system expression or GI microbiome distribution).

It is important to note that the previous experimental outline (a handful of control horses vs an experimental group of horses) can be used to test other conditions. As an example, instead of comparing “normal” vs “GI-diseased” horses, we could take the same design and test “normal” vs “stressed,” or “normal” vs “medicated.” The main goal, however, should be to answer a question based on gene expression in the hindgut, such as “does gene expression in

the equine hindgut vary between healthy and overly-stressed horses?" That question may even lead to deeper questions and theories, such as "may this stress contribute to say, changes in gene expression that can directly affect immune system activity, and potentially make a horse more susceptible to illness?" What makes this research compelling is that there are so many interesting unknowns, and various unanswered questions that can definitely be solved utilizing the right resources, tools, focus, and previous experience. These future research projects can be focused on gene expression in the equine hindgut, or they can even dive into its connection to other systems actively maintaining GI homeostasis, such as "how does gene expression in the hindgut influence the immune system and vice versa?"

There is still much to understand about gene expression in the equine hindgut, as well as how these various biological systems work together to promote GI homeostasis. We know that the immune system, microbiome, IECs, and genes within IECs interact constantly with one another to promote internal balance, self-vs-nonself differentiation, and overall host survival, but we still do not fully understand the full scope of "how" this complicated system of interactions works. This is especially true regarding equine science, where most studies that exist on the hindgut are either purely physiological or microbial and do not necessarily fully connect all these systems together to for a bigger, more accurate picture of what's going on. All in all, future research is needed to continue piecing these puzzle pieces together, and as mentioned previously, there are many avenues that can be taken to help further our understanding of the equine hindgut, gene expression, and equine health.