

Role of the gut microbiota in equine health and disease

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Implications

- The health of the equine gastrointestinal microbiota is irreversibly linked to the overall health of the animal.
- More work is needed to improve diagnostic and management treatment protocols for horses suffering from health issues associated with nutrition or feeding.
- Current research has only begun to interpret the wealth of information contained in the equine gastrointestinal microbiota.

Key words: colic, equine, gut health, microbiota

Introduction

The equine gastrointestinal physiology is unique and complex (Bentz, 2014). Horses are hindgut fermenters, utilizing the colon and cecum for fermentative activity via the production of volatile fatty acids, which are utilized by the horse for energy (Costa et al., 2012). These acids produced during fermentation include acetate, a source of energy for tissue repair and regeneration; butyrate, a preferential energy source for colonocytes and gut epithelia; and propionate, a precursor for gluconeogenesis (Hoffman, 2001; Milinovich et al., 2010; Costa et al., 2012). Gut microbes also help to synthesize vitamins B and K, which are required nutrients for equines (NRC, 2007).

While the hindgut itself serves as a fermentation vat, the microbiota contained within contribute to stimulation of the immune response, help protect against pathogens, neutralize toxins, and regulate gene expression in the host's epithelial tissues (Milinovich et al., 2010). Clearly, maintaining a healthy and stable gastrointestinal ecosystem is vital to the overall health of the horse. Unfortunately, this ecosystem is extremely sensitive and can be affected by factors such as disease and dietary changes, resulting in systemic consequences and even death (Costa et al., 2012).

Gastrointestinal Location

Some very early work alluded to the effect of gastrointestinal location on microbial content (Mackie & Wilkins, 1988). Authors reported the total culturable bacteria obtained from the lumen of the duodenum, jejunum, and ileum of euthanized horses were $2.9 \times 10^6/g$, $29.0 \times 10^6/g$, and $38.4 \times 10^6/g$, respectively. This is substantially less than the numbers reported for the cecum (25.9×10^8) and colon (6.1×10^8) and is easily explained when one considers the difference in function across those gastrointestinal locations.

More recently, studies have utilized culture-independent techniques (Table 1) to examine the effect of location on the microbial profile (Costa et al., 2012; Dougal et al., 2012; Schoster et al., 2013; Costa et al., 2015a). Dougal et al. (2012) used both horses and ponies in a terminal study using terminal restriction fragment length polymorphism and demonstrated a significant elevation in the bacterial DNA per gram of digesta in the cecum that was not representative of the right dorsal colon or feces. This is in direct contradiction to the results of Schoster et al. (2013) who also used the same microbial profiling method and reported that fecal samples were most closely related to cecal samples collected from 10 euthanized horses. Although both studies offered new insights into gastrointestinal location and its effect on microbial community similarity, neither study provided any information regarding specific taxa. Instead, they were simply a comparison of the similarity or diversity of the populations and locations. In addition, although both studies utilized a similar technology, the lack of control diet and other confounding factors limit the comparisons that may be made.

Most recently, next-generation sequencing technology has begun to provide specific and detailed information regarding the taxa present in each segment of the equine gastrointestinal tract. Researchers reported an increase in diversity at the class level from the cecum through the feces when 11 horses of varying age, breed, gender, and health status were examined (Costa et al., 2015 a). Although Firmicutes was the predominant phylum across all compartments, microbial diversity increased as sample location became more distal. As a result of this increase in diversity, authors concluded that fecal samples might be appropriate as a model for changes occurring in the distal region of the gastrointestinal tract. Dominance by Firmicutes at the phylum level has been confirmed repeatedly across several studies as shown in Figure 1.

It is important to note that each of the above referenced studies utilized a combination of breeds and ponies, across a wide range of age groups and lacked a control diet, and each study analyzed gastrointestinal and fecal samples with different amplifying techniques. All of these factors may have contributed to the contradictory findings, suggesting that sampling protocols may need to be revised and standardized. Furthermore, the horses utilized were managed under different systems and often had incomplete medical histories. Also, the sequencing technique utilized for some of the early studies, terminal restriction fragment length polymorphism, is unable to culture a large percentage of the microbiota in highly diverse samples (Orcutt et al., 2009), which suggests the possibility of underrepresentation or underestimation of the microbial profile and its relative abundance. In addition, although important in their novelty, several of the early studies did not report taxa below the taxonomic level of phylum (Costa et al., 2012; Daly et al., 2012), and others reported no individual taxonomic data whatsoever (Dougal et al., 2012; Schoster et al., 2013). Again, this seems to highlight the need for a more standardized approach in study design and reporting.

Table 1. Dominant phyla and study parameters for equine microbial data sets currently published.

Citation	N	Gender ^a	Diet ^b	Breed ^c	Age ^d (years)	Dominant phyla	Treatment ^e	16 s rRNA Region	Method	Samples/ horse	Sample type
Costa et al., 2012	16	Mixed	NC	Multiple	4-23	Firmicutes	C 68% CL 30%	V3 – V5	454	1	Fecal
						Bacteroidetes	C 14% CL 40%				
Costa et al., 2015	11	Mixed	NC	Multiple	2-30	Firmicutes	> 40% across locations	V4	MiSeq	8	Intestinal
						Proteobacteria	> 30% duodenum				
						Verrucomicrobia	> 15% feces				
Fernandes et al., 2014	12	Fillies	C	TB	1	Firmicutes	FG 80% P 74%	V1 – V3	454	6	Fecal
						Bacteroidetes	FG A 18% P 22%				
Dougal et al., 2012	8	Mixed	NC	H & P	6-20	NR		NR	TRFLP	3	Fecal and intestinal
Dougal et al., 2014	17	Mares	C	Multiple	5-28	Firmicutes	45% across treatments	V1 – V2	454	9	Fecal
						Bacteroidetes	37% across treatments				
O' Donnell et al., 2013	6	Mixed	NC	ITB	3-8	Firmicutes	47-74%	V4	454	1	Fecal
						Bacteroidetes	13-31%				
						Proteobacteria	1-11%				
						Verrucomicrobia	2-11%				
Schooster et al., 2013	10	Mixed	NC	H & P	2-14	NR		V6 – V8	TRFLP	3	Fecal and intestinal
Schooster et al., 2015	8	Mixed	C	Multiple	5-19	Firmicutes	63%	V4	MiSeq	6	Fecal
						Verrucomicrobia	12%				
Shepherd et al., 2012	2	Gelding	C	Arabian	NR	Firmicutes	43.7%	V4	Genome Sequencer FLX	2	Fecal
						Verrucomicrobia	4.1%				
Steelman et al., 2012	18	Mixed	NC	Multiple	UNK	Firmicutes	C 69.2% L 56.7%	V4 – V5	454	1	Fecal
						Verrucomicrobia	C 18.1% L 27.6%				

^aMixed group of genders.^bNC denotes diet was not controlled; C denotes control diet.^cMultiple denotes multiple breeds; H & P denotes horses and ponies; TB denotes Thoroughbreds; ITB denotes Irish Thoroughbreds.^dNR denotes Not Reported in referenced citation; UNK original citation reports as Unknown.^eC denotes control; CL denotes colitis; FG denotes forage-grain diet; P denotes pasture diet; L denotes laminitis.

Diet

Few studies have been published regarding the effects of diet on the equine gastrointestinal microbiota. Controlled diets of orchard grass have been utilized to characterize the fecal bacterial community of forage-fed horses (Shepherd et al., 2012), which provided key insights into the taxonomic structure of the resident microbiota. Predominant phyla (Firmicutes, 43.77%, Verrucomicrobia, 4.1%, Proteobacteria, 3.8%, and Bacteroidetes, 3.7%) were identified, and bacterial richness was confirmed by pyrosequencing of 16S rRNA gene (V4 region). Unfortunately, more than 38% of the total bacterial sequences were unclassified. This fact emphasizes the underrepresentation of the equine microbiota in current microbial databases.

Fernandes et al. (2014) examined the microbial community of yearling thoroughbred fillies when transitioned from an ensiled forage/grain ration to *ad libitum* ryegrass-clover pasture with no acclimation period. This resulted in a significant effect on microbial diversity with a decrease in Firmicutes and an increase in Bacteroidetes from 79.7–68.4% and 18.1–27.6%, respectively, in as little as four days. Decreasing Firmicutes has also been associated with a compromised gastrointestinal system (Costa et al., 2012; Rodriguez et al., 2015; Weese et al., 2015) and provides further microbiological evidence necessitating a slow transition period when changing a horse's diet.

The effects of high fiber, high oil and high starch on the equine gastrointestinal microbiota have been characterized using pyrosequencing (Dougal et al., 2014), and the relative abundance of Clostridiales appears to be most susceptible to dietary change. This dietary effect has been re-

ported in humans as well (Claesson et al., 2011). Authors noted a very small “core” (microbiota present across all treatments) associated with all three diets that was predominantly populated by members of the family Lachnospiraceae. Interestingly, the high-fiber treatment had the largest core, followed by the high-oil treatment, with the smallest core associated with the high-starch diet, which suggests a relationship between fiber content and core microbiota. This is consistent with our current understanding of the importance of fiber in the equine diet and its critical role in maintaining equine gastrointestinal health. Given the apparent effect of diet on the core microbial profile, this may explain part of the connection between high-starch diets and their link with microbial dysbiosis. In addition, this suggests that future studies should include a measure of microbial stability such as the Jaccard Index (Faith et al., 2013).

Laminitis

The microbiota of healthy horses has been compared with those suffering from laminitis with significant taxonomic differences. The relative abundance of Firmicutes decreased (69.21%, healthy horses and 56.72%, laminitic horses), and the relative abundance of Verrucomicrobia increased (18.13%, healthy horses and 27.63%, laminitic horses) (Steelman et al., 2012). Horses with laminitis also had higher levels of the cellulolytic bacteria, Ruminococcaceae and Clostridiaceae, as compared with the control (Steelman et al., 2012). It's interesting to note that horses with laminitis were reported as having greater microbial diversity compared with the control

horses. The authors offer the possibility that species-level shifts within individual genera may contribute to different disease states. One other interesting point is that this study identified *Clostridium* as the most highly diverse genus, which contains both pathogenic and purportedly beneficial species.

Other results from a starch-induced laminitis study, where *Escherichia coli* decreased and the lactic acid bacteria, *Streptococcus bovis* and *Streptococcus lutetiensis*, increased are somewhat consistent with the aforementioned study (Milinovich et al., 2010; Goer, 2010). The proliferation of lactic acid bacteria decreased the intestinal pH to 4 as compared with a normal range of 6.5–7.1 (Milinovich et al., 2010; Goer, 2010). In addition, the acid-intolerant obligate fibrolytic bacteria died off in large numbers, possibly triggering a release of endotoxins and exotoxins, which have been associated with hindgut epithelial cell damage and increased intestinal permeability (Goer, 2010). Along with clinical laminitis symptoms, the dysbiosis linked with sudden bacterial die-off can lead to other serious conditions such as colic (Reed et al., 2004), further supporting the concept of microbial stability as a factor for overall health.

Gastrointestinal Disease

Colic is a major cause of morbidity and mortality in horses (USDA, 2001) and has long been a critical problem in the equine industry. One study reported that mares post-partum had increased concentrations for members of the Firmicutes phylum (specifically Streptococcaceae) and a corresponding decrease in fibrolytic taxa Lachnospiraceae and Ruminococcaceae (both in the Firmicutes phylum) as well as a decrease in the relative abundance of the phylum Proteobacteria compared with pre-partum fecal samples (Weese et al., 2015). This reduction in the typically dominant Firmicutes phylum has also been observed in horses with colitis (Costa et al., 2012) and in hospitalized horses with diarrhea (Rodriguez et al., 2015). Again, this further supports the theory that microbial dysbiosis is intrinsically connected with overall horse health and the need for studies aimed at designing protocols associated with measuring microbial stability.

Similar to the dysbiosis that may accompany laminitis, it has been theorized that the hindgut microbial environment may become too acidic due to the proliferation of lactic acid bacteria. Researchers have demonstrated that overloading a horse with starch can result in the proliferation of *Streptococcus*, lactobacilli, and *Clostridium*, which results in the production of lactate. The resulting accumulation may lead to a decrease in hindgut pH and associated fiber digestion and may negatively affect volatile fatty acid production (Hussein et al., 2004; Milinovich et al., 2010). Other work has shown that dietary change and intestinal disease can significantly increase the relative abundance of members from the Lachnospiraceae, Bacteroidetes, and the lactic acid-producing *Bacillus*-*Lactobacillus*-*Streptococcus* group (Daly et al., 2012). In this study, authors also reported a decrease in *Fibrobacter* and Ruminococcaceae (fibrolytic, acid-intolerant bacteria). Interestingly, the concentration of the family of lactate-utilizing bacteria, Veillonellaceae, remained constant. The authors concluded that the inability of the lactate-utilizing bacteria to adjust in concert with the increasing lactic acid concentration may be a causal factor related to the accumulation of lactic acid that is typical for horses on concentrate diets suffering from simple colonic obstruction and intestinal disease.

Extensive research has not been conducted observing lactic acid-utilizing bacterial changes over time. It has been suggested (Weese et al., 2015)

that the profile of the fecal microbiota preceding a colic event could provide new diagnostics for early detection and prevention of colic. Even though colic is the number one killer of horses, little is known about the factors and mechanism of the condition. Venable et al. (2013) examined the equine fecal microbial profile both during and 30 days post-recovery and reported an increase in the presence of taxa from the phylum Bacteroidetes in the colic state as compared with the healthy state.

This effect of microbial dysbiosis has also been reported for horses with colitis (Costa et al., 2012). Fecal samples from healthy horses and horses with colitis were compared.

Once again,



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Firmicutes was the predominant phyla with 68% of the relative abundance in healthy horses, followed by Bacteroidetes with 14%. However, researchers observed a shift in the predominant phyla for the horses with colitis (Firmicutes at 30%, Bacteroidetes at 40%), again supporting the theory that gastrointestinal microbial balance influences overall health.

Some changes have been reported in equine microbiota as a result of fasting, travel stress, and anesthesia (Schoster et al., 2013). These data are particularly intriguing because it is common veterinary practice to withhold food and water from horses that present with colic symptoms and because travel is a common factor for many performance horses. Schoster et al.'s (2013) work identified significant changes at each taxonomic level (phylum, class, order, family). These data support earlier work (Faubladier et al., 2013) that identified an increased risk of microbial dysbiosis as a result of transportation. When one considers the large number of horses

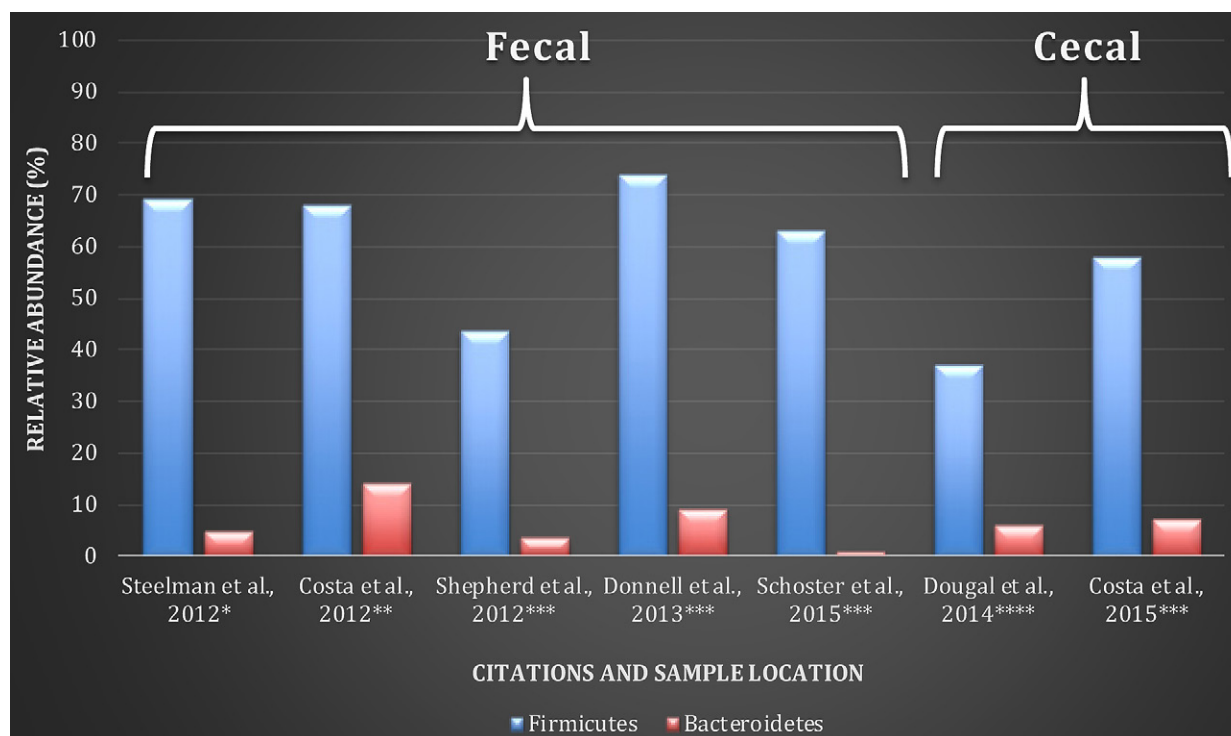


Figure 1. Relative abundance of dominant phyla in equine studies using next generation sequencing techniques. *V4-V5 Region; **V3-V5 Region; ***V4 Region; ****V1-V2 Region; *****V1-V2 Region.

that are transported regularly for recreation and other activities, it seems clear that this is an area requiring further investigation.

Probiotics and Medications

The use of probiotic supplements has skyrocketed in recent years. Despite their popularity among horse owners, there are no probiotic supplements currently approved for use in equines by the FDA (Schoster et al., 2014). Unfortunately, this lack of oversight often results in serious inconsistencies and discrepancies (Weese and Martin, 2011) associated with their production, labeling, and use. Yuyama and colleagues (2004) examined a mixture of five *Lactobacillus* strains from healthy adult horses for their potential to reduce diarrhea in neonatal foals. The authors reported higher body weight in 1-month-old foals along with a lower incidence of diarrhea at 3 wk of age (Yuyama et al., 2004). Unfortunately, contradictory work (Weese and Rousseau, 2005) demonstrated that not only did probiotic administration fail to provide any benefit, it was associated with an increased incidence of diarrhea and other clinical symptoms such as depression, anorexia, weakness, and colic. Due to the inaccuracies and inconsistencies associated with production of probiotics (Weese and Martin, 2011), it is difficult to deduce whether or not the lack of consistent field data is associated with poor selection of species, strain, incorrect dosage, or some other heretofore unknown factor.

Many probiotic studies focus on mixed products instead of individual, cultured strains. Although lactobacilli are commonly found in the gastrointestinal tracts of mammals and have demonstrated beneficial health results (Tanabe et al., 2014), researchers have been unsuccessful at demonstrating consistent benefits associated with the use of probiotics from this genus (Weese et al., 2003). Strains within the genera *Lactobacillus*, *Bifidobacteria*, and *Enterococcus* are most commonly utilized in probiotics despite the fact that these are not the predominant genera within the large colon. This suggests that perhaps

researchers should focus their efforts on taxa within the Clostridiales order whose members have been identified as dominant throughout the equine hindgut (Costa et al., 2012). This is supported by the findings of Schoster et al. (2015), who reported minimal effects associated with the use of a multi-strain probiotic in foals and suggested that a probiotic developed from strains of lactobacilli and bifidobacteria are perhaps limited in their therapeutic potential. Although it is commonly assumed that one of the advantages associated with probiotics is improved microbial stability necessary to mitigate gastrointestinal upset, there are very little data to support this concept. Studies should be developed that examine the effects of probiotics at ameliorating or preventing the onset of various gastrointestinal upsets in conjunction with their use.

In addition to neonatal health, other work associated with microbial effect of probiotic use has concentrated on the incidence of *Salmonella* outbreaks via fecal shedding in hospital environments. Parraga et al. (1997) reported no change in *Salmonella* shedding with the administration of two different probiotics in horses following abdominal surgery. Ward et al., (2004) found that oral probiotic administration reduced the shedding of *Salmonella* in fecal samples from hospitalized horses by 65%. Although both studies had similar objectives, authors noted different results. Since all three probiotics that were used across both studies were comprised of different bacterial strains, it is possible that the microbial strains selected for use in the latter study were more effective.

Antimicrobial agents are one of the more frequently prescribed drugs in veterinary practice. Their benefits are obvious, as they disable pathogenic bacteria within the host; however, there is a possibility of negative side effects, including antimicrobial resistance and alterations in the host's microbiome (Widenhouse, 2004; Jernberg et al., 2010). Antibiotic-associated diarrhea (AAD) is a common side effect of antibiotic administration. According to Gustafsson (2004), colitis in the horse consists of inflammation primarily

in the large colon and cecum but can also be seen in the small intestine and small colon (Gustafsson, 2004). Colitis is often associated with a microbial shift from beneficial anaerobic fermentative bacteria to opportunistic bacteria, including *Clostridium difficile*, *Salmonella*, and *Clostridium perfringens* and may occur when horses are treated with antibacterial drugs, such as erythromycin, rifampicin, doxycycline, enrofloxacin, and penicillin or penicillin/gentamycin combinations (Corpet, 1987; Gustafsson, 2004; Barr et al., 2013; Widenhouse, 2004). Gustafsson (2004) reported that in 40% of the AAD cases examined, *C. difficile* was present. Authors propose a link between AAD and the presence of *C. difficile* as it was not present in the colitis cases that were not treated with antibiotics.

Though microbial population shifts can occur after only a few days of medication with reported effects occurring by Day 5 post-antibiotic administration, it may take several weeks or more to restore the diversity of the gut microbiota (Jakobsson et al., 2010; Liepman, 2015). Costa et al. (2015 b) reported that the largest impact on fecal richness and diversity occurred Day 5 post-administration and that the microbial population was similar to the baseline by Day 30 but not yet fully restored. These data seem to indicate that oral antimicrobial agents have long-lasting effects on the gut microbiota and caution should be exercised accordingly.

Age

Age also affects the microbial communities residing within the equine gastrointestinal tract (Earing et al., 2012). One study paired mare and foal fecal samples and monitored species richness over six sample collection time points. Researchers describe microbial richness as a function of the bands present for the PCR-denaturing gradient gel electrophoresis method that was used. Other more robust data collected on mare/foal pairs until 9 mo of age (Costa et al., 2015c) offer specific taxonomic information rather than just shifts in population richness. The authors reported that, contrary to prior work, foals were born with a surprisingly complex intestinal microbial ecosystem, comprised of the predominant phylum Firmicutes, which continued to develop until approximately 60 d of age. The techniques associated with the individual studies cited offer insight regarding potential reasons behind this contradiction. The denaturing electrophoresis methodology utilized provides a superficial (at best) assessment of population membership. The work done by Costa et al. (2015c) utilized next-generation sequencing techniques and targeted the V4 region of the 16s RNA gene prior to sequencing. This approach provides a much more robust evaluation of the complex ecosystem contained within the equine gastrointestinal tract.

Older age has also been associated with a reduction in microbial diversity. As previously mentioned, Dougal et al (2014) were investigating the effects of three different



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dietary treatments and utilized two groups of aged horses. Horses designated as “adult” (5–12 yr) displayed higher microbial diversity as compared with their “elderly” (19–28 yr) counterparts. This is consistent with other work conducted in humans that has shown a similar reduction in diversity associated with age. The reasons for this reduction in microbial diversity are as yet unclear (Zwieblehner et al., 2009).

Conclusion

While it is true that we have made significant headway in an initial characterization of the equine gastrointestinal microbiota, we clearly have a great deal of work left to do. The importance of the health of the microbiota in the gastrointestinal tract in relation to the well-being of the horse is one that cannot be overstated. More work is needed to fully elucidate the impacts of age, gender, diet, breed, and other health conditions on the hindgut microbiota. Future research should evaluate stability as well as diversity as we investigate mechanisms for maintaining homeostasis as a preventative measure to microbial dysbiosis. Greater investigation into dietary supplements and probiotics is warranted as horse owners seem more determined than ever to provide supplementation to their horse’s diet. Veterinarians and nutritionists must have better information regarding the effects various supplements may have on the critically important equine gastrointestinal microbiota.

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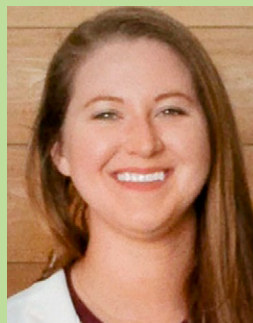
About the Authors



Stephanie Bland is a Ph.D. candidate at Southern Illinois University with more than 10 years of experience working with a variety of species. She is a licensed veterinary technologist through the state of Illinois and has more than 5 yr of experience in emergency animal hospitals. Bland's research is in the area of animal nutrition with emphasis on profiling the microbiome of equines and working canines and examining the therapeutic efficacy of nutraceuticals in osteoarthritic equines and canines. She would like to continue her work in companion animal nutrition after graduation.



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Jennifer McPherson attained her bachelor's degree in animal and dairy science at Mississippi State University. Currently, she is a master's student at Southern Illinois University Carbondale majoring in animal science, food, and nutrition with an emphasis in equine nutrition. Her graduate research focuses on the impact of probiotics on opportunistic bacteria found in the equine cecal microbiome. She aids and leads class and laboratory periods for equine courses.



Dr. Erin Venable is an Assistant Professor at Southern Illinois University with more than 15 years of experience working with equines. She has worked with many species, including cattle, horses, and canines. She specializes in both horses and working canines and hopes to develop better diagnostic tools for identifying causes of microbial dysbiosis. Recently, she conducted several research trials utilizing cecally-cannulated horses in order to better understand the shifts that occur within the gastrointestinal tract. She continues to focus her research on the microbial stability of horses and canines with particular emphasis given to effects associated with stress. **Correspondence:** evenable@siu.edu