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SYMPOSIUM

Beyond Fermentation: Other Important Services Provided to Endothermic Herbivores by their Gut Microbiota

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Synopsis For decades, comparative biologists have recognized the importance of microbial partners in facilitating herbivory as a successful feeding strategy. Most of this success is attributed to the ability of gut microbes to digest recalcitrant dietary fiber and provides usable nutrients to their hosts. Gut microbes can also provide numerous other functions, such as vitamin synthesis, nitrogen recycling, and the detoxification of plant secondary compounds. Here, we review these microbial functions in herbivorous mammals and birds, highlighting studies that utilize recently developed metagenomic techniques. Several of these studies emphasize that microbial services are the product of interactions and exchanges within a complex microbial community, rather than the product of an individual member. Additionally, a number of these microbial functions are interdependent. For example, levels of dietary nitrogen or plant toxins can influence fiber digestibility. Further studies into the variety of microbial services provided to herbivorous hosts, and how these services might interact will broaden our understanding of host–microbe interactions.

Introduction

Herbivorous vertebrates host a dense and diverse community of microbial organisms in their alimentary tract, or gut. Current estimates suggest that herbivores harbor trillions of microbial cells comprised of hundreds of taxa (Stevens and Hume 2004; Ley et al. 2008; Kohl and Dearing 2012). One of the first functions ascribed to these gut communities was the breakdown of recalcitrant plant fiber into usable forms of energy for the host i.e., volatile fatty acids (VFAs). Given that more than 50% of the energy in plants is locked up in complex carbohydrates for which vertebrate herbivores have no enzymes to digest, the conversion of fiber to VFAs through the process of microbial fermentation represents a significant benefit for the herbivore in terms of energy acquisition from food (Demmet and Van Soest 1985; Mackie 2002). However, in addition to fiber fermentation, gut microbes can deliver myriad services to the host.

Across all animals, gut microbial communities have been documented to provide numerous functions from training the immune system to impacting behavior (Round and Mazmanian 2009; Heijtz et al. 2011). It has been proposed that animals and their microbes may collectively form a "holobiont" upon which natural selection acts (Bordenstein and Theis 2015; Shapira 2016; Theis et al. 2016), though this idea has been debated (Moran and Sloan 2015; Douglas and Werren 2016). Therefore, it is unclear whether the benefits of host-associated microbes are truly "services" and "functions", or merely the byproducts or "accidental" benefits resulting from microbes acting in their own interest (Mushegian and Ebert 2016). Moreover, we recognize that these relationships are nuanced, such that microbes may be beneficial under certain conditions and harmful under a different context. While we use the terms "services", "functions", and "beneficial" in this

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paper, we recognize that more research is needed to understand the nature of these interactions for the microbes and the host.

In this review, we will focus on functions afforded by the gut microflora with respect to the nutritional ecology of endothermic herbivores (mammals and birds). It should be recognized that gut microbes are known to facilitate herbivory in other vertebrate groups [reptiles: Troyer (1982); fishes: Rimmer and Wiebe (1987); and perhaps some larval amphibians: Pryor and Bjorndal (2005)]. Although the specific functions of these communities are understudied in non-mammalian hosts (Colston and Jackson 2016), recent breakthroughs in sequencing technology have led to the discovery of a previously unappreciated diversity of microbes in the gut. We will briefly give an overview and review some new developments with respect to microbes that participate in the fermentation of fiber, and will also review lesser known services of the gut microflora: vitamin synthesis, nitrogen recycling, and detoxification of plant secondary compounds (PSCs). For the latter topic, we provide a case study profiling our recent work on the microbiota of woodrats. While comparative physiologists have long appreciated these other functions of the microbiome, studies integrating recent microbiome techniques with these functions are still lacking (Fig. 1).

Fermentation

Microbial communities occur along the entire digestive tract, but those responsible for high levels of fermentation are often housed in enlarged digestive organs either proximal to the small intestine as in the case of foregut fermenters (e.g., bovids, kangaroos) or distal to the small intestine [e.g., equids, rodents; see Karasov and Douglas (2013) and Stevens and Hume (2004) for thorough reviews of these differences in gut morphology and the implications for energy acquisition]. These communities are complex and contain bacterial, archaeal, fungal, and protozoan members. Although several factors such as phylogeny and diet can contribute to the bacterial composition, in general, the phyla Bacteroidetes and Firmicutes dominate the gut communities of herbivorous mammals (Ley et al. 2008; Muegge et al. 2011) and birds (Godoy-Vitorino et al. 2008; Matsui et al. 2010). A number of microbial members in the phyla Bacteroidetes, Firmicutes, and Fibrobacteres are able to digest starch, along with more recalcitrant plant carbohydrates: pectin, xylan, cellulose, and hemi-cellulose compounds (Stevenson and Weimer 2007; Flint et al. 2008). We are just beginning to understand the roles of the non-bacterial microbes such as protozoans, archaea, and fungi, in the degradation of plant fiber (Comtet-Marre et al. 2017).

The process of fermentation is dependent on a consortium of microbes that are interdependent with respect to the efficiency of fermentation. A highly simplified description of this complex process reveals three general types of microbes that together result in the conversion of indigestible plant carbohydrates into host energy. These are the primary fermenters, secondary fermenters, and proteolytic microbes (Van Soest 1994). In general terms, the primary fermenting microbes do the work of converting cellulose into VFAs that can be used by the host, and require proteolytic microbes to supply them with an appropriate form of nitrogen, typically ammonia, whereas the secondary fermenters convert the products created by the primary fermenters into acetate, hydrogen, methane, and CO_2 (Van Soest 1994). The primary fermenters responsible for the initial processing of cellulose and other complex carbohydrates are sensitive to low pH and often require B vitamins and other nutrients (e.g., organic acids). Some microbes, such as *Fibrobacter*, require CO_2 as a growth factor (Van Soest 1994). In addition, primary fermenters often require nitrogen in the form of ammonia, typically produced by proteolytic microbes, such as *Clostridium* and *Peptostreptococcus* (Van Soest 1994).

Recent studies employing new approaches such as metagenomics underscore the sheer complexity of the interactions among fibrolytic microbes and illustrate the gaps in our knowledge. Metagenomic inventories of cows (Hess et al. 2011), reindeer (Pope et al. 2012), elephants (Ilmberger et al. 2014), and wallabies (Pope et al. 2010) reveal that each of these herbivores harbors novel microbial enzymes for degrading fiber. Studies on the iconic giant panda have produced conflicting results as to whether or not these herbivores host microbes capable of extensive fermentation (Zhu et al. 2011; Xue et al. 2015). Interestingly, the microbial communities of baleen whales, which are predators of crustaceans and fish, are more like terrestrial herbivorous vertebrate communities with respect to taxonomy and fermentative function (Sanders et al. 2015). Additionally, a recent study using metatranscriptomic techniques identified over 12,000 carbohydrate active enzymes (CAZymes) in the rumen of dairy cattle, which were dominated by enzymes from non-bacterial microbes, such as fungi and protozoans (Comtet-Marre et al. 2017). This work highlights the need for further understanding of the fibrolytic community, given that

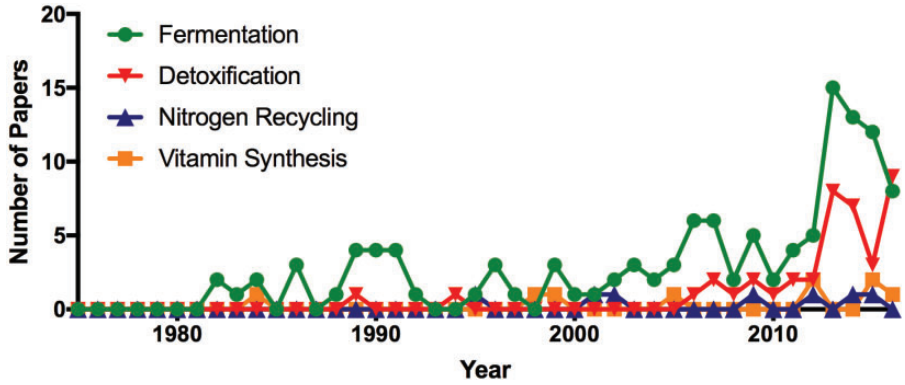

Fig. 1 Number of publications regarding various microbial functions in the guts of herbivores. This search was conducting using the rentrez package in R to search PubMed with the terms (herbivor*) AND (microb* OR bacteria OR microflora) AND (term of interest).

one third of the CAZymes identified came from distantly related strains of microbes (Comtet-Marre et al. 2017). Numerous herbivorous birds digest significant portions of fiber in their diets (Herd and Dawson 1984; Dawson et al. 1989). Given the high amount of enzymatic novelty across mammalian herbivores, it would be interesting to conduct similar metagenomic inventories in avian herbivores.

Although most humans are not strict herbivores, it is worth highlighting some recent and elegant work on the fiber degrading strategies of two species of *Bacteroides* found in the human gut. Martens et al. (2011) found that together *B. thetaiotaomicron* and *B. ovatus* are capable of degrading nearly all of the types of fiber in plants. Through a series of gene inactivation experiments, they were able to demonstrate that these two microbial species have diverged in their molecular strategies of fiber degradation such that each microbe is specialized to ferment a subset of the overall fiber in plants (Martens et al. 2011). This work demonstrates the complexity and specificity of interactions among species in the gut microbial community.

Vitamin synthesis

It has long been recognized that mammalian and avian hosts depend on vitamins synthesized by gut microbes (Bentley and Meganathan 1982; Stevens and Hume 2004). Vitamins are organic molecules that perform numerous biological functions, such as acting as coenzymes, and are required through dietary inputs because animals are unable to synthesize them in sufficient amounts (Karasov and Martinez del Rio 2007). The majority of our understanding of vitamin synthesis in herbivores comes from domesticated animals. We lack a thorough understanding of the importance of vitamins to wildlife due to the fact that it is difficult to detect vitamin deficiencies (Robbins 1993). However, given that vitamin requirements are likely met through synthesis by intestinal microbes (Robbins 1993), understanding the nature of these interactions demands further research. Recently developed metagenomic methods may enable such investigations.

Microbes are known to synthesize several types of vitamins (vitamins E, K, B; Bentley and Meganathan 1982; Robbins 1993). Many of these vitamins are present in adequate concentrations in plants, and so herbivores do not necessarily rely on microbial synthesis (Robbins 1993). However, herbivores likely require microorganisms for the synthesis of several B vitamins (thiamin, riboflavin, biotin, folate, cobalamin, etc.), which are necessary coenzymes in metabolic processes and are absent or in low concentrations in plants. Vitamin B12 (cobalamin) is a prime example; plants do not contain it and animals are unable to synthesize this vitamin, so herbivores must acquire it through microbial synthesis (Roth et al. 1996; LeBlanc et al. 2013). Indeed, the gut microbial communities of domesticated ruminants (Bechdel et al. 1928; Wegner et al. 1940) and rabbits (Huang et al. 1954) synthesize several B vitamins. Metagenomic analysis of the cecal contents of the leaf-eating flying squirrel (*Petaurista alborufus lena*) detected many genes associated with vitamin B synthesis, especially folate (Lu et al. 2012). Other recent studies have investigated vitamin synthesis by gut microbes, but not necessarily in the context of herbivores. In humans, members of the genus *Bifidobacterium* can carry out synthesis of folate (LeBlanc et al. 2013), though it has been suggested that the presence of these folate-producing *Bifidobacterium* may be limited to primates (e.g. humans, chimpanzees, orangutans; D'Aimmo et al. 2014). Inoculation of folate-producing microbes into rats can improve their

nutritional status (Pompei et al. 2007). Investigations into additional herbivorous taxa are warranted to understand if production of B vitamins is a standard service provided by gut microbes.

Analyses of microbial genomes suggest that rather than particular microbial taxa performing this function, vitamin synthesis may be the cumulative effect of the microbial community (Magnúsdóttir et al. 2015). B-vitamin precursors may be shared and exchanged between particular microbes, suggesting that changes in microbial community structure could impact vitamin production (Magnúsdóttir et al. 2015). Moreover, the fermenting microbes often require B-vitamins, underscoring the importance of the interactions among the microbial community (Van Soest 1994). Overall, our understanding of the contribution of microbes to vitamin synthesis in wild herbivores lags far behind our understanding in humans and domestic animals. Advances in DNA sequencing and other techniques related to the gut microbiome could be applied to the gut communities of wild herbivores to better understand these relationships.

Nitrogen recycling and synthesis of essential amino acids

Plant material is often low in protein content (roughly 9%), including deficiencies in essential amino acids that animals cannot synthesize themselves (Karasov and Martinez del Rio 2007). Therefore, many herbivores rely on microbes for nitrogen recycling and the synthesis of essential amino acids. These processes greatly enhance the nitrogen balance of herbivores and allow them to subsist on challenging diets.

Nitrogen recycling is the process by which microbes can convert nitrogenous waste products into forms that animals are able to utilize again. Mammals and birds primarily produce urea and uric acid, respectively, as metabolic waste products (Karasov and Martinez del Rio 2007). Many animals are able to transport these waste products into the gut where they serve as substrates for gut microbes (Stevens and Hume 2004). Gut microbial communities produce urease or uricase enzymes that convert these nitrogenous wastes into ammonia (Suzuki et al. 1979; Campbell and Braun 1986; Vecherskii et al. 2015), which can then be reabsorbed by the host and synthesized into nonessential amino acids in the liver (Stevens and Hume 2004). Urea recycling has been demonstrated in the wallaby (Kinnear and Main 1975), brushtail possum (Foley and Hume 1987), rock hyrax (Hume et al. 1980), rabbit (Regoeczi et al. 1965), and several ruminant species (Mousa et al. 1983). In birds, uric acid recycling was shown in the Willow Ptarmigan (Mortensen and Tindall 1981), Gambel's Quail (Campbell and Braun 1986), and has been suggested in emus (Dawson and Herd 1983). Applications of recently developed technologies should be applied to understand the diversity of microbes that perform nitrogen recycling in herbivorous animals.

Gut microbes can also utilize nitrogenous wastes or resulting ammonia to synthesize amino acids and microbial proteins, which hosts can then digest and absorb (Stevens and Hume 2004). These microbial proteins can act as substantial nutrient sources for animals (Bergen 2015). Some of the amino acids synthesized by microbes are essential amino acids that animals cannot produce themselves (e.g., valine, leucine, methionine, and others; Stevens and Hume 2004). Metagenomic analyses have revealed that the gut microbial communities of mammalian herbivores are enriched in enzymatic machinery associated with the synthesis of these essential amino acids compared with the gut microbiota of carnivorous mammals (Muegge et al. 2011). Specifically, the biosynthetic pathways for the essential amino acids histidine, lysine, methionine, phenylalanine, and tryptophan were enriched in herbivorous mammals (Muegge et al. 2011). Conversely, the gut microbiota of carnivorous mammals had higher abundances of genes associated with the degradation of amino acids (Muegge et al. 2011). In birds, the gut microbiota of the herbivorous Greater Sage Grouse is also enriched in genes associated with the synthesis of these essential amino acids when compared with the gut microbiota of domestic chickens or even mammalian herbivores (Kohl et al. 2016a). Additional metagenomic studies could investigate which microbes are performing such functions, and whether it is the function of particular members, or a community function, as is the case for other microbial functions (e.g., vitamin synthesis; Magnúsdóttir et al. 2015). Further, physiological studies could investigate the functions of these gut communities. For example, to understand the role of microbial symbionts in supplying amino acids to insect hosts, researchers have used antibiotics to disrupt the symbionts and then test the effects of diets lacking particular amino acids (Douglas and Prosser 1992; Douglas 1996). Similar studies in vertebrate herbivores would help to illuminate the contribution that microbes make to the essential amino acid pool of herbivores.

Detoxification

Herbivores often confront the dilemma of being poisoned by their food. Nearly all plants defend

themselves against herbivory with a wide array of PSCs, which can be toxic and/or inhibit digestion (Dearing et al. 2005). The notion that gut microbes could degrade a significant fraction of toxic plant compounds is a long-standing hypothesis independently suggested by Freeland and Janzen (1974) and Janis (1976). Freeland and Janzen (1974) proposed it as one of the many strategies required for herbivory whereas Janis (1976) went a step further to propose that this capacity of microbes drove the evolution of foregut fermentation. In the more than 40 years since this hypothesis was proposed, there have been few documented examples or experiments to test this idea.

The first demonstration of this concept was in an agricultural herbivore, the goat. Jones and colleagues initially documented differences in tolerance to a tropical shrub, leucaena (*Leucaena leucocephala*), among goats in different geographical locations (Jones 1981). In a series of follow-up studies, they found that these differences were caused by differences in the community composition of rumen bacteria (Allison et al. 1990) and ultimately identified a bacterium responsible for the degradation of mimosine, one of the more bioactive compounds in this shrub (Allison and Mayberry 1992). By transferring ruminal fluid from goats with a high tolerance to leucaena, they were able to improve the ability of less tolerant goats to feed on this toxic shrub (Jones and Megarrity 1986).

Recent experimental work on reindeer, *Rangifer tarandus*, provides additional support for this hypothesis (Sundset et al. 2010). During the winter, reindeer rely heavily on various species of lichens, including species containing high levels of phenolics. In a study with captive reindeer, the researchers monitored levels of usnic acid, a common phenolic in a diet of lichen, along with the concentration of usnic acid in the digestive tract, feces, and urine of reindeer. Usnic acid present in the diet was not detected in any tissue samples, which is consistent with the hypothesis that the microbial community in the rumen degrades this compound (Sundset et al. 2010). Moreover, bacteria resistant to usnic are documented to occur in the rumen (Sundset et al. 2008; Glad et al. 2009).

In addition to these studies, recent work on the degradation of pharmaceuticals by a microbe in the human gut offers direction for future exploration into the microbes of herbivores that ingest PSCs. Haiser et al. (2013) demonstrated that *Eggerthella lenta* (Actinobacteria), a microbe found in human gut communities, is capable of directly degrading digoxin, a common PSC used in the treatment of heart disease. The presence of particular strains of *E. lenta* with a cardiac glycoside reductase operon significantly reduced digoxin levels in mice (Haiser et al. 2013). Curiously, they found an interaction with protein levels in the diet such that high protein levels dampened the ability of the microbe to degrade digoxin (Haiser et al. 2013). Cardiac glycosides like digoxin are ingested by herbivores (e.g., monarch butterflies and African crested rats; Brower and Moffitt 1974; Kingdon et al. 2012), thus it is possible that consumption of cardiac glycosides by herbivores is enabled by the direct activity of *E. lenta* or other microbes that degrade these compounds.

A case study for microbial detoxification: the woodrat and its microbiome

We have been developing a tractable system to investigate the role of the gut microbiota with respect to facilitating the ingestion of dietary toxins. Our explorations focus on numerous woodrat species within the genus *Neotoma* (Kohl and Dearing 2016). This genus is ideal because of its diversity of species and dietary strategies (including multiple specialists) coupled with a well-documented evolutionary and dietary history (Edwards et al. 2001; Edwards and Bradley 2002; Matocq 2002; Patton et al. 2007). Moreover, these animals thrive in captivity. In the subsequent paragraphs, we review the evidence from this system testing the hypothesis that gut microbes facilitate the ingestion of dietary toxins.

We have been investigating whether the woodrat gut supports a community of microbes that could play a role in degrading plant toxins. To that end, we have conducted studies to evaluate the presence and abundance of microbes living in the foregut chamber of the woodrat gut (Kohl et al. 2014a). Our work revealed that the foregut of woodrats houses a vast diversity of microbial taxa. In the foreguts of *Neotoma lepida* and *N. bryanti*, we identified over 4500 operational taxonomic units from seven phyla, including the Actinobacteria, with the predominant genus being *Lactobacillus* (Kohl and Dearing 2012). Members of both the phylum Actinobacteria and genus *Lactobacillus* are known for their capabilities in degrading hydrocarbons. For example, Actinobacteria are thought to be important in the degradation of plant phenolics in the guts of termites (Le Roes-Hill et al. 2011). In addition to the vast diversity, the foregut is home to a high density of microbes, at 10^{10} cells per gram of contents. This microbial density is comparable to that of the cecum, an organ known for extensive microbial fermentation (Kohl et al. 2014a). Finally,

the microbial community of the foregut is metabolically active as evidenced by the high level of VFAs produced (Kohl et al. 2014a).

To determine whether the unique microbial communities present in the woodrat gut facilitate the ingestion of dietary toxins, we conducted several performance-based assays. These studies were primarily conducted on *N. lepida*, which has natural populations of woodrats with ecological and evolutionary experience to creosote bush and its phenolic-rich resin (experienced) and populations without such experience (naïve). In laboratory feeding trials, experienced and naïve woodrats exhibit differential tolerance to creosote PSCs, such that the experienced population can consume 25% more creosote resin (Mangione et al. 2000). We conducted a study where we disrupted the gut microbiota of experienced woodrats through the administration of oral antibiotics. This treatment significantly impaired their ability to consume creosote resin (Kohl et al. 2014b). We extended this work by transplanting fecal microbial communities from the experienced population into individuals collected from the naïve population. The microbial transplant from the experienced woodrats significantly increased the ability of the naïve woodrats to maintain body mass and persist on the toxic diet compared with control animals (Kohl et al. 2014b).

We also investigated whether microbial transplants were effective across host species lines and other PSCs. In one study, we transplanted microbes from *N. lepida* with experience feeding on creosote resin, which is high in phenolics, into laboratory rats (*Rattus norvegicus*) that typically do not feed on PSCs. The microbial transplant significantly increased the ability of the laboratory rat to consume tannic acid, a PSC representative of the phenolics in the diets of experienced woodrats (Kohl et al. 2016b). In other studies, we transplanted gut microbes from *N. albigula* into the laboratory rat. The woodrats used in this study feed on high levels of oxalate, a PSC common in the woodrat's diet of cactus. The woodrat transplant significantly improved the short and long-term degradation of oxalate in the laboratory rat (Miller et al. 2016b). Because oxalate is only degraded by microbes and not mammals, we are confident that this effect was due to microbial and not host degradation. Collectively, these studies demonstrate the critical role that gut microbes play in facilitating the ingestion of PSCs by mammalian herbivores.

Gut microbes likely facilitate the ingestion of dietary toxins through direct metabolism of these compounds in the gut, prior to absorption into the blood stream. This mechanism is well established for the degradation of oxalate in the gut of *N. albigula*, as mammals do not produce enzymes capable of degrading this compound (Justice 1985; Miller et al. 2016a). In this example, a gene for a key enzyme in the metabolism of oxalate, oxalyl-CoA decarboxylase, was identified in several bacteria isolated from the woodrat gut (Miller et al. 2014). There is also evidence for direct degradation of phenolics. In our studies with the experienced *N. lepida* fed creosote resin, the abundances of genes associated with the metabolism of aromatic compounds, like phenolics, were higher in the metagenomes of woodrats feeding on resin-amended diets compared with animals fed the same diets lacking resin. One microbial gene in particular, aryl-alcohol dehydrogenase, was highly enriched in the woodrats feeding on creosote resin, indicating its importance in the process of degrading phenolics (Kohl et al. 2014b).

Interactions between microbial functions

Given that the gut microbiota performs multiple services for the host, as described above, there may be limitations, tradeoffs, and interactions between these functions. For example, supplementing diets of cattle with urea can enhance microbial fermentation as revealed by improved fiber digestibility (Souza et al. 2010). Additionally, some animals suffer decreases in the digestibility of fiber and nitrogen when diets contain PSCs (Adams et al. 1992; Dearing 1997; Dawson et al. 1999). These effects could be due to the antimicrobial and inhibitory properties of many PSCs (Encarnación and Garcia 1991). Alternatively, PSCs may alter the acid–base balance of herbivores, reducing urea production and provisioning to the microbiota, which could result in decreased microbial fermentation (Dearing 1997). In contrast, some herbivores are able to maintain high digestive performance even on diets containing PSCs (Meyer and Karasov 1989; Skopec et al. 2008). It could be that the gut microbes of experienced herbivores are adapted to PSCs in their host's typical diet, and therefore are able to continue providing digestive benefits even when exposed to high doses of toxins. Understanding the interactions and tradeoffs between microbial functions, and the mechanisms underlying them, could be studied further.

Future directions

In closing, the interactions between gut microbes and their hosts are complex ones that we are only beginning to comprehend. More in-depth knowledge of the exchanges between hosts and their gut

symbionts will advance our knowledge of ecological interactions in general. For example, are these services the product of individual microbes, or interactions within a complex community, and if the latter, how are these communities maintained? How does an increased demand on one function affect other microbial functions? Are these microbial functions evolved, or simply the effect of herbivores feeding on high-fiber diets? Are the services provided to the host merely an extension of those provided to the microbial community itself (i.e., byproducts/"accidental" benefits; Mushegian and Ebert 2016)? Answering these and other questions will expand our understanding of host–microbe interactions and may be applicable to issues related to society, including human health and agriculture practices.

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