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The Role of the Gut Microbiome in Cattle Production and Health: Driver or Passenger?

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Abstract

Ruminant production systems face significant challenges currently, driven by heightened awareness of their negative environmental impact and the rapidly rising global population. Recent findings have underscored how the composition and function of the rumen microbiome are associated with economically valuable traits, including feed efficiency and methane emission. Although omics-based technological advances in the last decade have revolutionized our understanding of host-associated microbial communities, there remains incongruence over the correct approach for analysis of large omic data sets. A global approach that examines host/microbiome interactions in both the rumen and the lower digestive tract is required to harness the full potential of the gastrointestinal microbiome for sustainable ruminant production. This review highlights how the ruminant animal production community may identify and exploit the causal relationships between the gut microbiome and host traits of interest for a practical application of omic data to animal health and production.

1. INTRODUCTION

The 3.9 billion ruminants estimated to exist today are important in sustainable agricultural practices, as they can render nonarable land useful via grazing, use industrial by-products (e.g., distillers grains) as a food source (1), and synthesize energy from low-quality forages for milk and meat production. Central to ruminant production and health is the gut microbiome, the complex microbial community that resides in the ruminant gastrointestinal tract (GIT), which is now well-recognized as a crucial contributor to the maintenance of intestinal homeostasis, mucosal and lymphoid structure development, and activation of the host immune cell repertoire (2). Moreover, microbial fermentation of ingested plant biomass in the rumen, a specialized foregut fermentation chamber, allows the animal to harness the nutritional value of host-indigestible plant biomass and so is a critical facet of both beef and dairy systems (3).

Livestock production systems face a myriad of challenges at present. Providing adequate nutrition to the growing global population—estimated to reach 9.15 billion by 2050—will require a 70% increase in food production from 2007 levels in developed countries, and perhaps a doubling of output from developing nations (4). Compounding this, concerns about the environmental footprint of livestock production are also increasing. Recent estimates based on total life cycle assessment indicate that approximately 14.5% of global anthropogenic greenhouse gas (GHG) emissions are derived from agriculture, but less than 5% of the total is attributable to direct emissions from livestock (6). A range of GHG are produced throughout beef and dairy production chains, with the livestock themselves generating methane (CH_4) enterically and nitrous oxide (N_2O) from manure (5). Methane is a particularly prominent GHG associated with ruminant production, synthesized in the rumen and lower gut by methanogenic archaea, and has a global warming potential approximately 28 times greater than that of carbon dioxide (6). In addition to its negative environmental impact, the loss of gross dietary energy to the animal via enteric methanogenesis is estimated at 2–12% and is therefore a major contributor to reduced host feed efficiency (FE) (7).

In light of the intricate relationships between the host animal and its resident gut microbiomes, studies of these microbial communities as a means to improve cattle production efficiency while reducing/removing its environmental impact have been ongoing for many decades (8). The advent of high-throughput sequencing technologies in recent years has generated a large amount of data on the composition and function of the rumen microbiota across a range of hosts and environments (9–11). However, there is increasing evidence that the lower GIT and its resident microbiota also make important contributions to cattle health and production (2), which has not been extensively studied to date (12, 13). Understanding the complex interactions between host and microbe throughout the GIT is key to informing strategies to maximize ruminant production efficiency and tackle the challenges outlined above.

In this review, we highlight recent research concerning the ruminant gut microbiome, discussing the contributions of both the rumen and hindgut microbiota to animal performance. Additionally, we assess recent findings concerning host–microbe relationships in the rumen and their implications for host animal performance. Finally, complementing our recent review on the application of omics technologies to study host-associated microbiomes (14), we discuss the challenges associated with statistical analysis of data generated from such studies and provide guidelines for robust analysis of microbial data sets, to better understand the roles of the gut microbiome in cattle production.

## 2. IMPORTANCE OF THE GUT MICROBIOME TO CATTLE PRODUCTION AND HEALTH

2.1. The Rumen Microbiome: Composition and Function

The rumen microbiome is a phylogenetically diverse consortium of anaerobic bacteria, fungi, methanogenic archaea, ciliate protozoa, and viruses. The major microbial constituents of this community are detailed in **Figure 1**. This microbial cohort contains cellulolytic, hemicellulolytic, amylolytic, proteolytic, and biohydrogenating (lipolytic) species, exhibiting a high level of functional redundancy, and is capable of effectively degrading host-indigestible plant fiber (15). Volatile fatty acids (VFAs), principally acetate, propionate, and butyrate, are the major products of rumen microbial fermentation and are absorbed and used as energy sources by the host (16). Ruminally derived VFAs can meet up to 70% of the host's energy needs (16), and thus their production is essential to animal performance. Metabolism of nitrogen-containing compounds (including peptides, ammonia, and urea) by the rumen microbiota is also vital in the provision of microbial proteins to the host for muscle and milk synthesis (17). Ingested fiber, carbohydrates, protein, and lipids are first hydrolyzed to shorter chains (or oligomers) and monomers (e.g., glucose, amino acids) by primary members of the microbiota and subsequently used as substrates by various members of the microbial community (18). Investigation of the temporal colonization of ingested feed by the rumen microbiota showed divergent taxonomic and functional profiles among the primary and secondary colonizers, pointing to variation in their role(s) and/or substrate specificity (19, 20). Diet, genetics, age, gender, and geography (9, 21–23) are among the determinants of rumen microbial composition and function; however, influence of diet is the best studied to date. The composition of the rumen microbiota under various production systems and life stages has been reviewed extensively in recent years (1, 24, 25) and is beyond the scope of this review.

The importance of microbial metabolism in the rumen to the well-being of the host has led to interest in the contribution of the rumen microbiome to animal production. Microbial composition of the rumen is associated with variations in FE (26), intensity of CH_4 emission (27), health (28), and milk composition (29). More recently, evidence of the heritability of certain groups of rumen bacteria in beef and dairy cattle has emerged (23, 30), but the extent of the contribution of these microbial species to host traits is not yet clear. If clear relationships between (*a*) the host genome and the rumen microbiome and (*b*) the heritable portion of the microbiome and desirable host traits can be conclusively identified, they could facilitate selective breeding for an optimum rumen microbiome. Finally, extensive efforts have been made to manipulate the rumen microbiome via dietary intervention to improve host performance, particularly in terms of methane abatement (24, 31–34). Below, we discuss in detail the contributions made by the rumen and lower-gut microbiomes to several key aspects of cattle production.

2.2. The Rumen Microbiome and Feed Efficiency

With global food demands projected to rise significantly in the coming decades, the efficiency of food production, both animal and crop derived, must be improved (35). The term feed efficiency (FE) describes the efficacy at which the conversion of feed to useable product occurs, and it is a moderately heritable trait in cattle (35). Given that feed inputs account for up to 75% of variable costs in beef operations, and 40–60% of those in dairy systems (36, 37), improving FE is a means of increasing output while minimizing costs. Several measurements of FE have been used in cattle [e.g., feed conversion ratio (38) and partial efficiency of growth (39)], but residual feed intake (RFI) has emerged as the most common measure. First proposed in 1963, RFI is defined as the difference between actual and predicted feed intake of an animal for maintenance of body
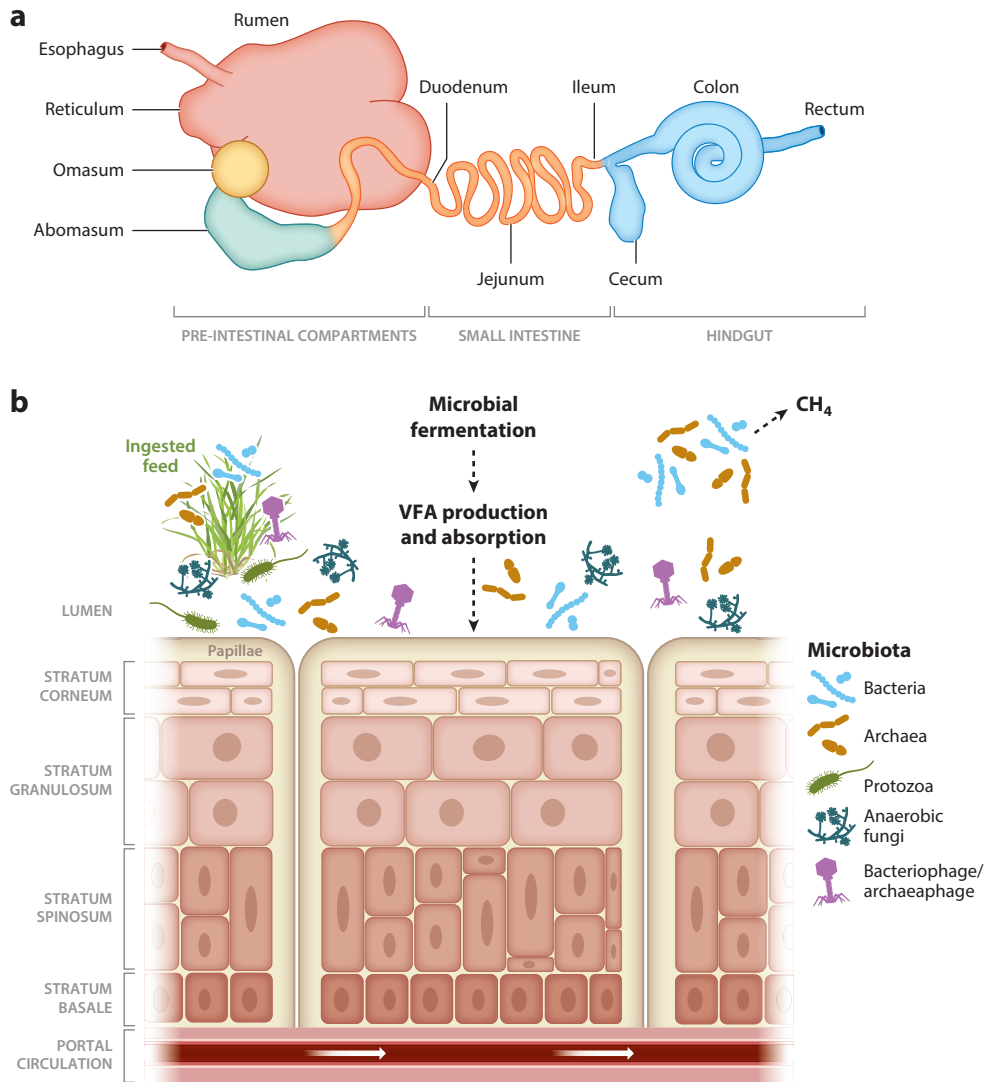
Figure 1

(*a*) Schematic of the bovine gastrointestinal tract and (*b*) depiction of rumen wall structure and microbial diversity and function. Bacteria: The most numerous microbial group in the rumen, bacteria are present at a density of 10^{10}–10^{11} cells/ml rumen fluid. The rumen bacteriome is dominated by members of the Firmicutes, Bacteroidetes, and Proteobacteria phyla, containing numerous genera like *Prevotella*, *Fibrobacter*, and *Butyrivibrio*, capable of metabolizing a range of dietary polysaccharides and peptides. Archaea: The rumen methanogens (10^6–10^8 cells/ml rumen fluid) belong exclusively to the Euryarchaeota phylum and are dominated by members of the *Methanobrevibacter ruminantium* and *Methanobrevibacter gottschalkii* clades. Protozoa: The ciliates are found in the range of 10^4–10^6 cells/ml in the rumen fluid, and the most abundant genera are *Entodinium*, *Polyplastron*, *Epidinium*, and *Eudiplodinium*. Anaerobic fungi: Discovered only in the 1970s and present at rates of 10^3–10^6 zoospores/ml, the cellulolytic anaerobic fungi in the rumen belong to the phylum Neocallimastigomycota and are currently grouped into eight genera (*Neocallimastix*, *Piromyces*, *Ontomyces*, *Buwchfawromyces*, *Caecomyces*, *Orpinomyces*, *Anaeromyces*, and *Cyllamyces*). Bacteriophage/archaeaphage: The rumen virome is dominated by *Caudovirales*, and the phage are key regulators of microbial populations and facilitators of horizontal gene transfer. Abbreviation: VFA, volatile fatty acid.

weight and for weight gain (40). Genetically independent of growth, animals may be classified as Low-RFI (efficient) or High-RFI (inefficient), with a view to selecting animals that will have the same or greater output value (e.g., meat yield/quality) with lower input costs (i.e., feed).

Although a range of physiological processes contribute to divergence in FE within a population (41), the fact that the conversion of ingested feedstuff to energy substrate (e.g., VFA) is dependent on the rumen microorganisms suggests that the rumen microbiome may play an important role in determining an animal's FE status. In a landmark study, Guan and colleagues (42) demonstrated that the rumen microbial ecology of efficient (Low-RFI) cattle differed from that of their inefficient (High-RFI) counterparts, and there was also a greater similarity in microbial profiles among the efficient animals. More recently, the use of high-throughput sequencing demonstrated that efficient cattle (both dairy and beef) had lower rumen microbial diversity and richness, in terms of both microbial species and gene content (26, 43) and metabolic profile (44). This suggests that the rumen of efficient animals contains fewer non-essential microbes, though it is unclear if this is a cause or a consequence of the efficiency phenotype. Variation in VFA concentration according to RFI classification has also been reported, but these differences appear to be diet dependent (42, 43, 45).

A range of microbial groups, from phylum to species level, have been associated with FE in the literature, including associations between improved FE and the abundances of the *Lachnospiraceae* and *Veillonellaceae* families (26, 46), and several archaeal taxa, such as *Methanomassiliicoccale*, *Methanobrevibacter.* sp. *AbM4*, and *Methanosphaera stadtmanae* (26, 47, 48). However, there are some inconsistencies in these reports; for instance, while the ruminal abundance of *Dialister* was associated with improved FE in steers (46), species belonging to this genus were associated with reduced efficiency in lambs (49). Because the rumen microbiome is influenced by dietary composition (9), and FE classification is not always consistent in individuals across diets (50), associations between the rumen microbiota and FE may be driven, at least partially, by diet. However, several studies have demonstrated diet-independent effects of FE on the rumen microbiota (45, 49, 51), indicating that a core group of microbes associated with variation in FE could be used to identify efficient animals irrespective of diet (1). Furthermore, selection for improved FE may also contribute to reduction in ruminal methanogenesis (43, 52), as discussed in a later section.

2.3. The Lower-Gut Microbiome: Unexplored Potential to Improve Animal Health and Performance

In contrast to that of the rumen, the fundamental role(s) of the lower-gut microbiota and its contribution to ruminant health and production are poorly understood. For the purposes of this review, the lower gut is defined as the post-gastric intestinal tract and thus consists of both the small intestine and the hindgut regions.

2.3.1. Feed efficiency and the lower gut.
Bacteria are present at levels of 10^{12}–10^{14} cells/ml in the hindgut digesta (cecum, colon, rectum; **Figure 1a**) of cattle (53, 54). Microbial fermentation in the hindgut may be responsible for up to 30% of cellulose and hemicellulose degradation in ruminants (55), though smaller figures have also been proposed (56). Lower dietary energy production in the hindgut compartments is likely due to a combination of factors, including reduced retention time of digesta in the hindgut compartments versus in the rumen, as well as the fact that substrates entering the cecum and colon already have been partially digested by enzymes in the rumen (microbial) and small intestine (host and microbial). However, dietary energy derived from the hindgut is likely an important contributor to energy availability in cattle throughout all stages of production, and hindgut fermentation could be of elevated importance to the calf during the first days and weeks of life, before the rumen becomes fully developed (57).

The lower-gut microbiota diverge in composition according to intestinal segment (58, 59), likely reflecting differences in physical, chemical, and biological conditions in each compartment. The jejunum is a major site of post-ruminal protein and carbohydrate digestion and absorption, with *Firmicutes* (up to 90%) being the predominant phyla detected here (60). The hindgut regions, the cecum and colon, have similar functions, with *Firmicutes* and *Bacteroidetes* dominating their microbial communities. Augmenting the hypothesized importance of the lower-gut microbes to animal performance, several taxa in both the small and large intestine have been related to feed efficiency status, with abundances of *Butyrivibrio*, *Pseudobutyrivibrio*, *Prevotella*, *Anaeroplasma*, *Paludibacter*, *Faecalibacterium*, and *Succinivibrio* in the hindgut, and that of *Butyrivibrio* in the jejunum, reported as being divergent across FE phenotypes (12, 61). These findings indicate that the microbial communities of the small intestine and hindgut may indeed be closely related to cattle production efficiency. Future studies examining such relationships should be reticent of this and include analysis of the lower gut microbiomes in their work.

2.3.2. Contribution of the lower-gut microbiome to host gut health.

Unlike in the rumen, where there remains incongruence over the presence of any robust host immune mechanisms that propagate gut health, the lower-gut regions are highly active in terms of immune function, with the mucosal immune system comprising physical (mucosal/epithelial layers) and chemical (antimicrobial peptides, secretory IgA) barriers, as well as pattern-recognition receptors (for example toll-like receptors, TLRs) and containing a wide array of immune cells that contribute to host defense (2, 62). As such, with the lower-gut regions known to be vital to immune system development in monogastric animals (63), there is also increasing evidence that the microbial communities of the lower gut contribute to immune system establishment and homeostasis in beef cattle (2) that directly impact animal gut health in addition to their role(s) in feed digestion and energy production. In this regard, starter feeding as part of normal early-life calf management influenced both bacterial diversity and the expression of genes (*TLR10* and *TLR2*) related to the effectiveness of the host mucosal immune response in the lower gut (64). In a follow-up study, total counts of mucosa-associated and luminal bacteria in the small intestine of pre-weaned dairy calves were closely correlated with the expression of genes encoding host immune response (65), while the same authors also showed that interaction between the commensal gut microbes and expression of specific host microRNAs may contribute to immune system development in the neonatal calf gut (66). A recent study of functional metagenomic profiles derived from the ileal tissue of *Lactobacillus*-dominant calves showed elevated expression of genes involved in "leukocyte and lymphocyte chemotaxis" and the "cytokine/chemokine-mediated signaling pathway" (67). Taken together, these observations suggest the importance of lower-gut microbiota in immune system development in dairy calves, which may lay the foundation for improving the health of neonatal calves through nutritional manipulation strategies. This is supported by the close relationship between microbial perturbation or dysbiosis in the gut and ruminant health. One example is the onset of hindgut acidosis, which occurs when rapidly digestible carbohydrates overflow to the hindgut for fermentation. The accumulation of acidic fermentation products, such as short-chain fatty acids, is suspected to decrease the luminal pH, leading to changes in microbial composition and damage to the gut epithelium, with detrimental effects on animal productivity and health. While clear relationships between the ruminal microorganisms and acidosis have been demonstrated (68, 69), relationships between hindgut acidosis and the changes of lower-gut microbiota in the ruminant remain poorly understood. Evaluating this relationship in future studies may pave the way for manipulation of lower-gut communities as an avenue to improve intestinal health in cattle.

Overall, research on the lower gut and its role in adult cattle (especially beef cattle) remains scarce. Maintenance of host immune function and gut health requires energy expenditure (70),

and therefore stress and disease can reduce the growth and production efficiency of the animal. Further research is needed to fully understand the lower-gut microbiome and its contribution to animal health and production.

3. THE ENVIRONMENTAL IMPACT OF THE GUT MICROBIOME IN CATTLE PRODUCTION

3.1. Rumen Methanogenesis

Livestock industries are a significant source of environmentally harmful GHG, with carbon dioxide (CO_2), CH_4, and N_2O being the major greenhouse gases emitted from food and agricultural production chains. The potent global warming potential of CH_4 means it is the most extensively studied GHG in terms of ruminant emissions, and reducing rates of enteric methanogenesis is desirable in terms of both improved animal productivity and environmental stability. As stated previously, CH_4 is produced in the rumen by methanogenic archaea, which are estimated to account for 0.3–3.3% of the rumen microbial population, based on 16S ribosomal RNA (rRNA) gene analysis (71).

There are three major pathways of ruminal methanogenesis: (*a*) hydrogenotrophic, in which H_2 is used as an electron donor to reduce CO_2 to CH_4 [formate can also be used as an electron donor and may contribute to the production of up to 18% of ruminal CH_4 (72)]; (*b*) methylotrophic, involving the use of methylamines or methanol; and (*c*) acetoclastic, involving the use of acetate and H_2 to produce CH_4 (73). Hydrogenotrophic methanogenesis is the predominant pathway in the rumen and is carried out mainly by *Methanobrevibacter* species (**Figure 1**), which typically account for more than 90% of archaeal 16S rRNA gene reads (74), though several other less abundant methanogen species are also found in the rumen (75) (**Figure 1**). The rumen archaea have been closely studied for their role in methanogenesis, and interestingly, it does not appear that their total abundance is directly related to the intensity of CH_4 emission (76, 77). Rather, it seems that the expression of certain archaeal genes may be a more measurable predictor of rumen methanogenesis (44), as the transcription of methanogenesis pathway genes within the rumen microbiome is greater in high-CH_4-emitting sheep compared with their low-emitting counterparts (78).

There are many factors underlying the rate and intensity of rumen methanogenesis. Dietary composition can have a major effect on the volume of measurable ruminal CH_4; high-forage diets favor microbial acetate synthesis in the rumen, leading to increased H_2 and consequentially more CH_4 production than under concentrate-rich diets, where starch is mainly metabolized to propionate (79). Although it may seem profitable to simply move away from feeding forages to cattle, reduced rumen pH under high-starch diets may contribute to imbalance of the microbial community and fermentation and lead to subacute ruminal acidosis (80). Furthermore, given that the majority of global livestock rely on forage sources for growth, different strategies for reducing CH_4 formation across a range of diets are needed. A variety of methods for reducing ruminal CH_4 emissions have been investigated and work by either directly targeting the methanogen community or attempting to reduce/redirect H_2 flow in the rumen, thus providing less substrate for methane production. These mitigation strategies have been comprehensively described elsewhere and include dietary manipulation (for example, using seaweed extract), plant lipid feeding, synthetic methanogen inhibitor supplementation, and genetic selection for low-emitting animals (4, 25, 81, 82). Methanogens may also acquire H_2 via interspecies hydrogen transfer, particularly from protozoan populations, as some methanogens are symbiotically associated with protozoan cells (71). Consequentially, some studies have examined the significance of defaunation on CH_4 production (83), finding that defaunation reduces enteric methanogenesis by 11% on average (84). However, the absence of a reliable farm-level method of defaunation has precluded

its widespread adoption to date. Arguably the most effective mitigation strategy demonstrated to date is basal dietary supplementation with 3-nitroproxypropanol (3-NOP). Developed in 2012, 3-NOP acts by inhibiting the methyl coenzyme-M reductase (MCR) enzyme in the terminal step of methanogenesis (85). Supplementation of 3-NOP has been shown to dramatically reduce ruminal CH_4 production in lactating dairy cows and crucially does not have any adverse effect on milk yield (32), though an increase in milk fat has been reported (86). It has also proven to be an effective CH_4 inhibitor in sheep (33) and beef cattle (34). Furthermore, there is no current evidence of microbial adaptation to this additive, as has been observed when other MCR inhibitors, such as bromoethanesulfonate, were added to the basal diet (87). Yet, with a large proportion of the world's domesticated ruminants existing in open pasture, the practicalities and economics of continued supplementation with 3-NOP (or any dietary additive) are unclear. Furthermore, the compound is yet to be approved for commercial use, and critically the effect of 3-NOP on the composition and function of the rumen microbiome has not been studied in depth.

A critical aspect of an effective CH_4 abatement strategy or indeed any intervention that aims to elicit a change in rumen microbial composition or function (for example, to improve animal FE or reduce CH_4 production) is the persistence of such changes in the long term. However, in mature animals it has proven difficult to permanently modify the established microbiota, which generally reverts to the original composition following the cessation of treatment/supplementation (88). This phenomenon is less evident, however, in the first weeks of life, when the rumen community is highly dynamic and variable across individuals (24). These observations have given rise to the principle of microbial programming of the rumen microbiota—dietary or management interventions in early life that will imprint a desirable and persistent microbial pattern on the rumen, before the microbiota becomes fully established—as a means of improving ruminant production (24). Accordingly, recent years have seen renewed interest in the patterns of microbial colonization of the rumen during the first days and weeks of life (22, 89, 90). There is evidence that dietary interventions during early life may have long-lasting effects on rumen microbial composition (91– 93), but few long-term studies have been conducted to date. To effectively discern the optimal time for manipulation/intervention, the temporal sequence of rumen microbial colonization, and the factors that influence it, must be fully defined. Recent data suggest that the first three weeks of life may be a crucial window to manipulate a colonizing rumen microbiome (93a). However, studies encompassing the entire life cycle of the animal will be necessary to establish what, if any, is the ideal time frame for manipulation to most robustly improve host performance.

3.2. Negative Environmental Impacts of the Lower-Gut Microbiome

Augmenting the production of CH_4 and other greenhouse gases in the rumen, the lower-gut microbiota also plays important roles in CH_4 and waste nitrate production. Previous work has shown the presence of methanogens in the GIT of dairy calves at birth, with their abundance differing among 0- and 3-day-old calves (94). Zhou and colleagues (95) also showed that *Methanobrevibacter* was the main methanogenic taxon in the ileum of 3- to 4-week-old dairy calves. The presence of methanogens in the neonatal gut suggests that these archaea, and their metabolites, might play an important role in the early stages of intestinal development, and possibly methane emissions in the hindgut.

Although less formidable than the rumen, up to 10% of enteric methanogenesis in cattle occurs in the cecum, resulting in a loss of dietary energy that can reach 12% (96). Therefore, reducing methane synthesis in the hindgut regions may also reduce overall enteric GHG production and improve production efficiency. Accordingly, there is increasing interest in the composition and functional dynamics of the methanogenic community in the hindgut. From a compositional

perspective, the hindgut archaea differ from those in the rumen, with *Methanobacteriales* reported to be the dominant group in the cecum (96). While the relationship between nutritional management strategies and total methane output from the rumen has been studied in depth, knowledge of the relationship between methanogenesis in the lower gut and host production remains limited. Thus, future studies on the relationship between nutritional manipulation, intestinal methanogen colonization, and methane release will be of significant benefit to ruminant animal production.

In addition to CH_4, other waste components of feces and urine [including urea, nitrate (NO_3^-), nitrite (NO_2^-), ammonia, and hydrogen sulfide] are also of concern to producers and consumers. In human studies, a large proportion of dietary NO_3^- is typically absorbed in the upper intestinal tract, with approximately one-third of daily nitrate absorption occurring in the lower intestine (97). The gut microbiota is postulated to play important roles in nitrate utilization and conversion, as it has been previously suggested that *Escherichia coli* possesses genes encoding NO_3^- and nitrite reductase enzymes (98). Moreover, whereas *E. coli* was thought to convert NO_3^- to nitrite and subsequently to ammonia, *Lactobacillus acidophilus*, *Lactobacillus plantarum* species, and *Bifidobacterium longum* subsp. *infantis* were shown to generate large amounts of lactic acid, providing conditions appropriate for nitrite disproportionation to NO in vitro (99). Although most work to date is derived from in vitro studies, there is no doubt of the significant role of the gut microbiome in NO_3^- conversion. However, studies of the lower-gut microbiome and the composition of N compounds in fecal waste are limited in cattle, and such studies should be carried out to fully determine the contribution of the hindgut microbiome to the environmental impacts of ruminant production.

4. REVEALING THE TRUE BOVINE GUT MICROBIOME: ARTIFACTS AND CHALLENGES

As we have recently described (14), development of next-generation sequencing and other omics technologies in the last decade has allowed the study of host-associated microbial communities in ruminants at a depth never before possible. Today, researchers can use a variety of approaches to discern metataxonomic, metagenomic, metatranscriptomic, metabolomic, and metaproteomic profiles of a microbial community and identify patterns or changes related to a biological state of interest. However, high-throughput sequencing efforts are subject to a range of biases, including method of sample collection (100), method and duration of sample preservation prior to analysis (101), choice of nucleic acid extraction protocol (102, 103), and sequencing technology (104). Furthermore, a large variety of bioinformatic tools have been developed for the analysis of high-throughput sequencing data in recent years but have not been widely compared for their consistency. Finally, although these technologies represent powerful approaches to generate large, high-quality data sets, the best strategy for analysis of these data to draw meaningful and biologically sound conclusions remains a point of debate. In this section, commonly used approaches for analyzing omic data are summarized, and we draw on the literature to propose more robust methods for best-practice statistical analysis of large omics data sets for studies of the ruminant gut microbiome.

Data sets generated using omics technologies are inherently compositional and are constrained in a mathematical space known as simplex space, where the features [e.g., operational taxonomic units (OTUs) or amplicon sequence variants (ASVs), genes, proteins] in each sample are assigned proportions of a unit of measurement, varying between 0 and 1 (105). Unlike the simplex space, the Euclidean space does not exhibit constraints between 0 and 1 but can accept any real number along its dimensions (106). Thus, the analysis of microbiome data requires statistical methods that account for the simplex structure of compositional data sets, which excludes standard statistical approaches (including Pearson correlations, principal component analysis, and linear regression)

that use the assumptions of the Euclidian space (105, 106–109). However, these traditional statistical methods are still commonly used by the scientific community to analyze microbiome data sets.

Pearson (107) first identified the original problem in analyzing compositional data in 1897, when he realized that the count values per feature in compositional data are not independent, with the value of one feature necessarily restricting the value of at least one other feature (106). Later, it was discovered that this property can lead to negative correlation biases and false univariate inferences observed in compositional data, rendering invalid any correlation- or covariance-based methods (105, 110). An easy analogy to explain this distortion is the see-saw effect, in which a change in the abundance of one feature results in a biased correlation between the other features (one goes up, another goes down). This bias is caused by the spurious relationship between absolute abundance in the environment and the relative abundance after sequencing, which is not equivalent in compositional data sets because the number of reads obtained from a sample is determined by the capacity of the instrument and not by the actual number of molecules of DNA in the environment (109, 111). Therefore, compositional data sets are very different from data sets composed of ordinary numbers that can take any value, and treating high-throughput sequencing data as compositional is rather intuitive if the researcher considers that the number of counts in such data sets reflects the proportion of counts per feature per sample multiplied by the sequencing depth (106, 112).

Variation in sequencing depth (the total number of counts observed) among biological samples/replicates is another significant confounder of the analysis that should be carefully addressed, as abundance issues arise around the variation in the number of sequences obtained for each sample. A technique commonly used to account for sequencing depth variation in amplicon sequencing studies is rarefying or subsampling the read counts of each sample to a defined level across samples, but this approach excludes less abundant features, leading to a loss of precision in the results (113). If the researcher instead chooses to use the entire data set (without rarefying), they must employ a transformation or scaling method (e.g., trimmed mean of M values, TMM) to account for the magnitude of sequence depth between samples (114, 115). The identification of differentially abundant taxa associated with a given phenotype or treatment should not involve the use of models that apply Poisson distribution because it is too restrictive to deal with overdispersion (116). To address the overdispersion problem, researchers have proposed the use of negative binomial distributions, although it tends to increase the false-discovery rate arising from the compositional nature of microbiome data sets (108, 112, 116). Thus, the data analyst should be careful while analyzing microbiome data, as it exhibits a compositional structure that must be taken into consideration in the statistical analysis. Some alternative techniques to investigate this type of data have been developed in recent years and are discussed in the next section.

4.1. Alternative Techniques to Study Microbiome Data

To circumvent the issues outlined above, alternative statistical methods have been developed to replace the standard statistical approaches in the analysis of compositional omics data. In this context, it is advised to carry out the identification of differentially abundant features and microbial signatures using Analysis of Composition of Microbiomes (ANCOM) (111) and *MixMC* (117), which are detailed below, or other similar approaches. This section is not comprehensive, and we direct the reader to a recent article by Gloor and colleagues (112), which covers these approaches in a level of detail beyond the scope of the present review.

4.1.1. Analysis of Composition of Microbiomes. ANCOM is a statistical procedure that compares the Aitchison's log-ratio of the abundance of each taxon with the abundance of all

remaining taxa individually (111). In this method, differential tests (e.g., Mann–Whitney U, ANOVA, ANOVA with Linear Mixed Effect Models, Friedman, Kruskal–Wallis, and Wilcoxon tests) are calculated on each log-ratio to reveal differences in the relative abundance of a taxon between two ecosystems (**Supplemental Figure 1**). These differential tests are used to accept or reject the null hypothesis of equality for the abundance of taxa across groups for the condition of interest (e.g., diet). For each taxon, ANCOM computes the number of tests performed and obtains a count random variable W that represents the number of null hypotheses that need to be rejected. The final significance of each test for a taxon is determined using Benjamini–Hochberg (118) algorithms to control the false discovery rate. To deal with the sparsity of the data, ANCOM uses an arbitrary pseudo count value of 0.001 to replace the zero counts and calculate the log-ratios. For drawing inferences regarding taxon abundance in the ecosystem, ANCOM has been suggested as a reliable method to control the identification of false positives and has been incorporated recently into the QIIME 2 pipeline (119). A recent study evaluating seven statistical methods for differential abundance testing (edgeR, DESeq, DESeq2, Wilcoxon rank-sum test, Voom, metagenomeSeq, and ANCOM) (114) suggested that the novel methodology implemented in ANCOM based on log-ratio transformations of count data, as defined by Aitchison (105), was the most effective approach to control false discovery rates. ANCOM was recently implemented in a bioinformatic pipeline developed by our research group (104) and showed reliable results while detecting differentially abundant taxa identified by Kraken (120) and Mothur (121) from a rumen metatranscriptome data set, thus allowing the robust assessment of active microbial taxa and their contributions to cattle FE.

4.1.2. *MixMC*. *MixMC* (117) is a multivariate statistical framework that takes into account the inherent characteristics of microbiome data (sparsity and compositionality) to identify microbial signatures associated with the phenotype or condition being studied, and it is currently implemented as the R package *mixOmics* (122). Before data are centered log-ratio (CLR) transformed and analyzed via sparse partial-least-squares discriminant analysis (sPLS-DA models), preprocessing and normalization (e.g., total sum scaling) steps are performed to account for uneven sequencing depths across samples and the sparsity of the data set (**Supplemental Figure 2**). Using this approach, *MixMC*, sPLS-DA is employed in conjunction with CLR transformations to project the data from a simplex space to a Euclidian space and includes a multilevel decomposition approach for repeated measure designs that are commonly encountered in microbiome studies (117). This is an appropriate analytical step toward detecting subtle differences when high inter-subject variability is present due to sampling being repeatedly performed on the same subjects and in multiple habitats (117, 123, 124). To account for subject variability, the data variance is decomposed into within-subject variation (owing to habitat) and between-subject variation while handling the compositional structure of microbiome data appropriately (117). The scientific community has used this method extensively to investigate differences in gut microbial signatures in Crohn's disease patients versus in healthy controls (125), as well as bacterial signature variations in the fecal microbiota of HIV-infected individuals (126). Although the analytical approaches discussed here have been useful in dealing with the inherent characteristics of microbiome data sets (e.g., composition and sparsity), improvements in data interpretation while comparing across studies are still needed, especially for ruminant-related research.

4.2. Current Challenges When Comparing Results Across Studies

Although next-generation sequencing resulted in an explosion of publications exploring the microbial diversity in various ecosystems in the last decades, interpretation of the data generated

across multiple studies is still hampered by the lack of standardization in the bioinformatic and statistical procedures employed by the different research groups. One instance of this problem appeared when the rumen microbiome of efficient cattle was compared across studies to find consensus microbial taxa and/or genes that could serve as global biomarkers for predicting ruminant FE and methane emissions. Huws et al. (25) reported that microbial gene correlations with RFI described by Shabat et al. (43) overlapped with those of Li & Guan (26) only in relation to a lower abundance of genes involved in amino acid metabolism in the rumen of feed-efficient animals. These results support the hypothesis that feed-efficient cattle excrete less urinary ammonia and exhibit improved rumen nitrogen use compared with inefficient cattle (17, 127). Notably, however, there were inconsistencies in the findings of Shabat et al. (43) and Li & Guan (26). Although this could be attributable to differences in experimental design [different cattle, method of sample collection, nucleic acid choice (DNA versus RNA)], the lack of a standardized approach to data analysis may also play a role, indicating that reliable comparisons across studies are currently impractical.

Some aspects of data analysis that are important to standardize include methods for OTU picking [which remains the most common approach to microbiome assessment using amplicon sequencing, though its use is declining with the emergence of amplicon sequence variant approaches (119)], the algorithms for taxonomy classification, cutoffs for taxa inclusion/exclusion, and especially the statistical methods used to analyze microbiome data (128). The statistical methods discussed above are robust and could serve as a generic model of data analysis that, if practiced correctly, could further standardize the interpretation of microbiome data and facilitate comparisons across studies. However, data analysis standardization is still a complicated process owing to the complexity and heterogeneity of the available data sets generated by a wide variety of omics platforms (129). One elegant approach that could overcome the hurdles posed by the different data sources and offer opportunities to harness the full potential of microbiome data is to integrate information generated by large-scale molecular omics platforms into multivariate models (25). The data integration using multivariate models could be applied to extract information generated across different omic platforms to gain a better understanding of the complex interplay between the microbiome and phenotypes measured at different layers of molecular assays (130). Although challenging, there are instances of success in the literature showing the benefits of integrating a varied array of data types generated from omics technologies in microbiome studies.

In terms of statistical methods, the first approaches that allowed data integration and enabled the identification of multi-omics molecular signatures were concatenation-based integration methods (131) and model-based integration methods (e.g., ensemble classifiers) (132). Concatenation-based integration combined multiple data sets into a single large data set with the aim of predicting a phenotype of interest (e.g., human cancer) (131). In contrast, model-based integration approaches developed a predictive model on each individual data set before combining the ensemble classifiers in the model predictions (e.g., using blood-based diagnosis of acute renal allograft rejection) (132). Despite this advance, there is still a need for more sophisticated integrative modeling methods that can identify multi-omics molecular signatures by differentiating features from information generated across multiple functional levels, aiming to discover multi-omic biomarker panels associated with biological phenotypes of interest. These methods are still in their infancy, and the continued technical and analytical advances in the field of molecular biology and statistics will likely offer opportunities to develop integrative methods that allow the standardization of the analytical workflow and consequently more reliable comparisons of results across studies.

The range of biases and variation in studies of the ruminant gut microbiome suggests an urgent need for comprehensive discussion between research groups internationally to standardize all

protocols, from sample collection and storage through to laboratory processing, sequencing, and data analyses. Steps have been taken in this regard in recent years, with the formation of international research consortiums like RuminOmics (**http://www.ruminomics.eu/**) and the Rumen Microbial Genomics Network (**http://www.rmgnetwork.org/**). Further expansion of these forums will allow for reliable comparisons of published literature, but in the meantime, scientists should remain reticent of these potential biases when comparing results obtained across different studies.

5. FUTURE PERSPECTIVES FOR STUDYING THE BOVINE GUT MICROBIOME: DRIVER OR PASSENGER?

In the course of this review, we endeavored to provide the reader with the current state of the art in terms of microbiome–host relationships in cattle, as well as their contribution to animal production. As a result of the technological advances seen in the last decade, the role of the gut microbiome as a critical facet of efficient and regenerative livestock production systems is above reproach. We know the rumen microorganisms are, in terms of both composition and function, associated with economically and environmentally pertinent traits like FE (42) and intensity of methane emission (75), and there is increasing evidence that the rumen microbiome may be subject to a degree of host genetic control (44). The intestinal microbiota are also closely associated with host metabolism (133), health (134), and immune system development (63). However, we must recognize that these associations are exactly that: only an indicator of a relationship. For all the advances in our knowledge of the mammalian gut organisms over the last 10–20 years, there remains scant evidence of any robust causal relationship between the gut microbes and host production traits, and research concerning the lower-gut microbial functions in ruminants is at an early stage. Moreover, the million-dollar question remains unanswered: What is the ideal gut microbiome? Can it even be determined if one exists? And if so, can a gut microbial community be modulated effectively enough to ensure the desired community becomes established? The vast functional redundancy among gut microorganisms makes it unlikely that the removal of a small number of microbial groups would have any lasting impact on community function or host metabolism (88). Conversely, to seed a more favorable microbiota, functional niches for these microbial groups to occupy would need to be available, so measuring the effectiveness of manipulation via functional changes rather than taxonomic changes is preferable. Several aspects must be considered if we are to first define the optimal gut microbiota and subsequently apply this knowledge to improve host nutrition and immunity, thereby maximizing the productivity and sustainability of agricultural systems.

Taking the next step forward in understanding the total extent to which the gut microbiome contributes to cattle production will likely require a reevaluation of research hypotheses, experimental approaches, and data analysis. Currently, an investigation to examine relationships between a host phenotype/genotype (e.g., RFI) and the resident microbiome will typically begin by asking one or more of the following questions: (*a*) Who is there? (*b*) How many of them are there? And (*c*) what are they doing? In short, such studies seek to identify the microbial taxa or genes responsible for the phenotype (135). The output of such a study, be it one using metagenomic, metaproteomic, or meta-metabolomic approaches, is usually a list of biomarker taxa, genes, or metabolites, associated with the phenotype/genotype of interest, but often lacking any clear biological relevance. Moreover, it is impossible to conclusively state whether these changes in microbial composition/function are a driver or a product of host divergence. A shift in thinking from associative to causal relationships between the microbe and host traits will be required for robust contribution of microbiome research to enhanced animal production strategies. The time

has come for microbiome research in ruminants to shift focus toward causal, mechanism-based studies, to conclusively identify microbial pathways that actively contribute to a host phenotype, which will in turn allow us to elucidate the optimum gut microbiome under any given condition. Weight is added to such a strategy by evidence that host genetics may also influence some members of the rumen microbiota (30, 44), though this has not yet been fully confirmed. If strongly defined heritable relationships between the host and the microbiome can be elucidated, it might be possible to target the host (e.g., via genetic selection) to optimize the microbiome, rather than vice versa, as is the current practice.

5.1. Integrative Analysis of the Bovine Gut Microbiome to Identify Causal Relationships Between Host and Microbe

Key to this will be a move from piecemeal evaluation of the microbiome—i.e., examining composition/function/metabolism separately—to viewing each aspect as an equally important cog in a complex machine. These approaches—microbiome-wide association studies (MWAS)—although complex, allow the whole microbiome to be linked as one dynamic system with pertinent host traits like FE and methane emission (i.e., by evaluating the metagenome, metaproteome, meta-metabolome, etc., as part of a single study) (136). Although nontrivial, MWAS would allow the whole microbiome to be linked as one dynamic system with pertinent host traits like FE and methane emission, ultimately offering an opportunity to predict phenotypic traits and discover new biological signatures (137). This will be particularly applicable in terms of early-life manipulation, as discussed above. It is unknown if host genetics influence colonization patterns in the rumen, but if this could, through multi-omic frameworks, be confirmed, it might be possible to select for a more favorable colonization pattern that is amenable to persistent manipulation via dietary or management interventions.

However, several hurdles remain to be overcome before such approaches can be widely implemented, including the large number of variables generated by different omic platforms (e.g., sequencing versus mass spectrometry) and the relatively low number of biological samples typical to such studies (138). The issue of low experimental power hampering the retrieval of statistically sound results, although prevalent throughout the biosciences (139), is particularly problematic in studies of large animals like cattle, as the costs involved in obtaining and caring for these animals are often major constraints of experimental sample size. Despite these difficulties, multi-omic methods have been developed and to date have been applied mainly in the field of cancer research, where information collected from various molecular components (e.g., gene expression, nucleotide sequences, protein abundances) of human tissue samples has revealed oncogenic molecular signatures and novel biomarkers associated with the disease (130, 137, 138). Such approaches have also been adapted and applied as MWAS in studies of the human gut microbiome (140). These methods can be broadly divided into unsupervised analyses (e.g., matrix factorization, Bayesian methods), which draw an inference across multi-omic data sets when samples are unlabeled, and supervised analyses (e.g., support vector machine, semidefinite programming), which consider the phenotype labels of samples groups (137). Although multivariate approaches (e.g., *MixMC*) have been successful in identifying signatures in microbiome data sets, as discussed previously, these techniques have limitations in distinguishing phenotypic groups of interest based on biomarkers present in multiple functional layers of high-dimensional multi-omics data. Owing to the massive amount of data available in public databases (e.g., the National Center for Biotechnology Information), such multivariate methods still need to be developed to reveal insights into the relationship between the microbial consortia and the different levels of omic data (e.g., metagenomics, metatranscriptomics, meta-metabolomics). The emergence of these new

statistical methods in the field of microbiology, in the coming years, will create unprecedented opportunities to discover biologically relevant signatures and biomarkers that predict phenotypic outcomes (e.g., high/low-methane-emitting cattle, early/late disease states) at multiple functional levels of complex biological systems, allowing elucidation of definitive, causal, host–microbe relationships in ruminants. As discussed in the previous section, there remains extensive incongruence among research groups globally in terms of experimental practices and downstream analyses, with microbiome studies biased by methodology choice at almost every experimental stage. This can manifest itself with divergent results in the literature from seemingly similar studies; for example, in one study of young calves, *Firmicutes* was reported to be the most predominant phylum during the first week of life (141); however, the phylum *Bacteroidetes* was the most abundant in another study (142). This discrepancy may arise from differences in calf management or sample collection method, as well as being due to technical differences discussed previously. This reinforces the need for standardization of experimental procedures, which will be critical in facilitating equitable comparisons of data across studies.

The field of meta-omic research remains in its infancy and has made great strides to date. However, microbiome research must begin to move from associative studies to those that aim to provide robust evidence of causal relationships between the animal and its resident microbiome. The capability to discern whether a shift in microbial composition/function is a driver or a product of diverging host phenotype will also be vital to conclude to what extent the microbiome contributes to host well-being and production. The first, and arguably the most critical, step in this direction, as it will facilitate equitable cross-study comparisons and meta-analyses, is the establishment of internationally standardized best-practice guidelines for studies of the bovine gut microbiome, from sample collection through to bioinformatic and statistical analysis. Expansion of the collaborative forums discussed above would provide an excellent starting point for such steps to be taken. Additionally, evaluating the whole spectrum of a microbiome in terms of its contribution to economically valuable traits via MWAS may offer a viable approach to improve the efficiency and sustainability of livestock systems via integration in breeding programs. For such efforts to be successful, it is absolutely critical to fully understand the mechanistic interaction between host and microbe throughout the life cycle of the animal, which calls for the implementation of large-scale, longitudinal studies. While the rumen microorganisms have been the major focal point of bovine gut microbiome research, the role of the hindgut microbiome in host health and production must be more closely evaluated, given its role in feed digestion and subsequent methane production, and this is further necessitated by the role of the lower gut as a source of foodborne pathogens and nitrate wastes. We propose that consideration of the whole gut microbiome should be paramount in research programs concerning animal productivity and health, which has huge potential to make valuable contributions to efficient and regenerative livestock production globally.

DISCLOSURE STATEMENT

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