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Review

Holistic View and Novel Perspective on Ruminal and Extra-Gastrointestinal Methanogens in Cattle

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Abstract: Despite the extensive research conducted on ruminal methanogens and anti-methanogenic intervention strategies over the last 50 years, most of the currently researched enteric CH₄ abatement approaches have shown limited efficacy. This is largely because of the complex nature of animal production and the ruminal environment, host genetic variability of CH₄ production, and an incomplete understanding of the role of the ruminal microbiome in enteric CH₄ emissions. Recent sequencing-based studies suggest the presence of methanogenic archaea in extra-gastrointestinal tract tissues, including respiratory and reproductive tracts of cattle. While these sequencing data require further verification by culture-dependent methods, the consistent identification of methanogens with relatively greater frequency in the airway and urogenital tract of cattle plus increasing appreciation of the microbiome-gut-organ axis highlight the potential interactions between ruminal and extra-gastrointestinal methanogenic communities. Thus, a traditional singular focus on ruminal methanogens may not be sufficient, and a holistic approach which takes into consideration of the transfer of methanogens between ruminal, extra-gastrointestinal, and environmental microbial communities is of necessity to develop more efficient and long-term ruminal CH₄ mitigation strategies. In the present review, we provide a holistic survey of the methanogenic archaea present in different anatomical sites of cattle and discuss potential seeding sources of the ruminal methanogens.

Keywords: methanogens; enteric methane emission; cattle; microbiome; holistic; extra-intestinal microbial communities

1. Introduction

Global warming is projected to have major consequences on food security worldwide, exacerbating the expected increase in food demand by 70% to 100% by 2050 due to the population growth [1–3]. Anthropogenic greenhouse gas (GHG) emissions, particularly CH₄, are major contributors to global warming. Methane has a global warming potential approximately 28 times greater than CO₂ [2]. The agricultural sector is considered to be one of the major sources of CH₄ emissions, with ruminant animals, particularly domestic ruminants being significant contributors [4,5]. Ruminal methanogens are responsible for over 87% of total CH₄ emissions from ruminants and about 26% in comparison to other sources of CH₄ production in the environment [3,6]. Methane is produced in the rumen during the normal fermentation process by methanogenic archaea which use either CO₂ and hydrogen (H₂), methylamines or methanol, or acetate and H₂ to produce CH₄ [7]. Other microorganisms residing within the rumen such as bacteria, protozoa, and fungi can provide methanogens with excessive H₂, either directly or indirectly, and thereby promote methanogenic activity [8,9]. Although utilizing excess H₂ benefits ruminal fermentation by preventing H₂ build-up and feedback inhibition, ruminal CH₄ production represents 2%- 12% of gross energy loss [10].

Therefore, there is an impetus need for developing approaches to mitigate CH₄ emissions from ruminant livestock to reduce energy loss from animals while improving environmental health.

Over the last five decades, ruminal methanogens and anti-methanogenic intervention strategies have been extensively explored as a means of mitigating CH₄ emissions [11]. These strategies involve alterations in animal management, dietary composition, and ruminal fermentation, as well as direct inhibition of the growth and metabolic activity of methanogens using anti-methanogenic compounds and substances [12,13]. However, most of these approaches have shown limited efficacy due to the complex nature of animal management and ruminal physiology [14,15]. Additionally, recent research has focused on reducing CH₄ emissions from ruminants by genetic selection and manipulation of the ruminal microbiota, and the latter has become an active area of research due to advances in next-generation sequencing technologies [16]. While genomic selection could provide a long-term solution to CH₄ emissions [17–19], the impact of the selected anti-methanogenic traits on ruminal nutrient metabolism, fermentation, and the microbial community is yet to be defined. Recent identification of a distinctive ruminal microbiota between cattle and sheep with high and low CH₄ emitting phenotypes [20] suggest that the manipulation of the ruminal microbiota to mitigate livestock CH₄ production may provide long-lasting solutions.

While most of the current research has focused on characterizing the taxonomic diversity and abundance of methanogenic archaea in the rumen and the potential role of the ruminal microbiome in methanogenesis, emerging evidence suggests that microbial communities associated with extra-ruminal sites of animals including the reproductive and respiratory tracts and mammary glands may harbor methanogenic archaeal species [7,21–24] and these extra-gastrointestinal methanogens may interact with ruminal methanogens. Some of the methanogenic taxa (e.g., *Methanobrevibacter* spp.) have been reported to be shared as core taxa across ruminal, respiratory, and reproductive tract-associated microbial communities in cattle [21,25]. This, coupled with increased appreciation of the microbiome-gut-organ (respiratory/reproductive) axis [25,26] highlight the possible existence of interactions between methanogenic archaea in the rumen with methanogens and microbiomes present in extra-gastrointestinal organs. Such interactions between the rumen and other organs may be responsible for seeding the rumen or other organs with methanogenic species and/or influencing metabolic activities of the ruminal methanogens. The potential seeding of the rumen with methanogens by methanogens present in the reproductive tract is further supported by the recent identification of methanogenic archaeal species in fetal fluid and intestines of bovine fetuses [21,27]. Thus, these new developments point out that focusing solely on the methanogens present in the rumen and developing CH₄ mitigation strategies targeted at only ruminal methanogens could be too narrow of an approach. The ruminal and extra-gastrointestinal methanogens and their interactions on ruminal CH₄ emissions should be considered. In this review, we first provide a holistic survey of the methanogenic archaea present in different anatomical sites of cattle. We then discuss potential seeding sources of the ruminal methanogenic archaea in cattle. In addition, we highlight some challenges and future research directions associated with studying ruminal and extra-gastrointestinal methanogens in cattle.

2. General Overview of Methanogens

Methanogens are a diverse group of microorganisms that produce CH₄ as a metabolic byproduct of their energy conservation processes [28,29]. They are commonly present in a variety of environments including the digestive tracts of animals, predominantly ruminants, wetlands, and other anaerobic environments [11,30]. Methanogens are classified in the domain archaea and are phylogenetically diverse [31]. Methane production is an essential part of the global carbon cycle, accounting for approximately 18% of anthropogenic GHG emissions [32].

Methanogens are classified within the archaeal phyla *Euryarchaeota*, *Crenarchaeota*, and *Korarchaeota*. They are further subdivided into several orders and families based on their phylogenetic relationships and metabolic pathways [33,34]. Recent research has revealed the diversity of methanogens in different environments, with new genera and species being identified through metagenomic analysis and other non-culture based techniques [35,36]. High-throughput sequencing

techniques have enabled the discovery of novel methanogen species and lineages from diverse environments, such as the animal gut and reproductive tracts, rice paddies, and deep-sea sediments [11,37,38]. For example, a recent study using metagenomic analysis identified a new genus of methanogen from the digestive tract of a giant panda, which was found to be closely related to *Methanobrevibacter smithii*, a common human gut methanogen [39–41].

Methanogens are believed to be one of the earliest forms of life on Earth, with evidence suggesting that they were present over 3.5 billion years ago [42]. Their unique metabolic capabilities are thought to have evolved in response to the anaerobic conditions of the early Earth, where they played a significant role in the carbon cycle [43]. Based on the substrates used to produce CH₄, methanogens can be classified as hydrogenotrophic, acetoclastic and methylotrophic methanogens [44,45]. The hydrogenotrophic class uses H₂ for the reduction of CO₂ into CH₄. Examples of such methanogens are *Methanobrevibacter*, *Methanobacterium*, and *Methanomicrobiales*. The rumen is mainly inhabited by hydrogenotrophic methanogens [46]. The second class, acetoclastic methanogens which are most commonly present in freshwater sediments and anaerobic digestors, but use alcohols such as ethanol or 2-propanol as electron donors to produce CH₄ [41,42,47–49]. The methylotrophic class which is predominant in freshwater and wetland soils [50–52] relies on methyl groups such as methanol and methylamines to produce CH₄, and encompasses the order *Methanococcales* and *Methanosarcinales*. Of note, hydrogenotrophic methanogens are the focus of the present review as they are the main class of methanogens involved in enteric CH₄ emissions from cattle. The other two classes of methanogens will be briefly discussed as potential environmental sources that could seed ruminal methanogens in cattle.

3. Main Methanogenic Species in the Rumen

The methanogenic community in the rumen of ruminant animals is dominated by two main phyla: Euryarchaeota and Crenarchaeota [53]. The Euryarchaeota phylum is the most abundant and taxonomically diverse group of methanogens in the rumen. This phylum includes the orders *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanocellales*. The Crenarchaeota phylum contains the order *Thermoproteales* and is present in some ruminants but is less abundant as compared to the Euryarchaeota phylum [54] (Table 1).

Table 1. Methanogenic archaeal species present in the rumen and feces of cattle.

Phylum	Family	Genus	Species	Sequencing method	Host	Reference
Rumen						
Euryarchaeota	Methanobacteriaceae	Methanobrevibacter	<i>ruminantium, smithii, gottschalkii, boviskoreani, milerae</i>	16S rRNA Sequencing V4	Korean native cattle	Lee et al. 2013
Euryarchaeota	Methanobacteriaceae	Methanobrevibacter	<i>ruminantium, smithii, gottschalkii, boviskoreani, milerae</i>	16S rRNA Sequencing V6-V8	Sheep	Snelling et al. 2014
Euryarchaeota	Methanobacteriaceae	Methanobrevibacter	<i>ruminantium, smithii, gottschalkii, boviskoreani, milerae, thaueri</i>	16S rRNA Sequencing V4	Dairy cow	Danielsson et al. 2017
Euryarchaeota	Methanobacteriaceae	Methanobrevibacter	<i>ruminantium</i>	16S rRNA Sequencing V4	Beef heifers	Samat et al. 2021
Euryarchaeota	Methanobacteriaceae	Methanosphaera	Not reported	16S rRNA	Ruminants	Hodet et al. 2018
Euryarchaeota	Methanobacteriaceae	Methanosphaera	<i>stadtmanae, cuniculi</i>	16S rRNA Sequencing V4	Breeding bulls	Koziol et al. 2022
Thermoplasmata	Methanomassiliicoccaceae	Methanomassiliicoccus	<i>luminyensis</i>	qPCR	Sheep, Cow	Guindo et al. 2020
Euryarchaeota	Methanospirillaceae	Methanospirillum	<i>hungatei and lacunae</i>	16S rRNA Sequencing V4	Dairy cow	Tamsin Lyons et al. 2018
Euryarchaeota	Methanobacteriales	Methanobacterium	<i>mobilis, formicicum, barkaeri</i>	16S & 18S	Grazing cattle	Jarvis et al. 2000
Euryarchaeota	Methanobacteriaceae	Methanomicrobium	<i>mobile</i>	16S rRNA Sequencing V1-V2, V2-V3, culturing	Sheep	Belanche et al. 2019
Euryarchaeota	Methanosarcinaceae	Methanosarcina	<i>barkeri</i>	16S rRNA Sequencing, culturing	Dairy cow	Lambie et al. 2015
Euryarchaeota	Methanocorpusculaceae	Methanocorpusculum	Not reported	16S rRNA Sequencing V3-V4	Dairy cow	Dingxing Jin et al. 2017
Nitrososphaerota	Nitrososphaeraceae	Nitrososphaera	Not reported	16S rRNA Sequencing V3-V4	Dairy cow	Dingxing Jin et al. 2017
Thermoproteota	Cenarchaeaceae	Cenarchaeum	Not reported	Shotgun metagenomics	Dairy cattle	Ngetich et al. 2022
Nitrososphaerota	Nitrosopumilaceae	Nitrosopumilus	Not reported	Shotgun metagenomics	Dairy cattle	Ngetich et al. 2022
Korarchaeota	Korarchaeales	Korarchaeota	Not reported	Shotgun metagenomics	Dairy cattle	Ngetich et al. 2022
Nanoarchaeota	Nanoarchaeaceae	Nanoarchaeum	Not reported	Shotgun metagenomics	Dairy cattle	Ngetich et al. 2022

Feces							
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanosphaera</i>	<i>stadtmanae, cuniculi</i>	16S rRNA Sequencing V4	Breeding bulls	Koziol et al. 2022	
Euryarchaeota	<i>Methanospirillaceae</i>	<i>Methanospirillum</i>	<i>hungatei and lacunae</i>	16S rRNA Sequencing V4	Dairy cow	Guindo et al. 2020	
Euryarchaeota	<i>Methanocorpusculaceae</i>	<i>Methanocorpusculum</i>	Not reported	16S rRNA Sequencing V3-V4	Sika deer, Dairy cow	Tamsin Lyons et al. 2018	
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>smithii, millaerae, labreanum, aggregans</i>	PCR	Sheep	Dingxing Jin et al. 2017	
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>smithii, millaerae, labreanum, aggregans, thaueri</i>	PCR	cow	Dingxing Jin et al. 2017	
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>boviskoreoni, millerae, olleyae, ruminantium, wolini</i>	16S rRNA Sequencing V3-V4	Beef	Dingxing Jin et al. 2017	
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	Not reported	16S rRNA Sequencing V3-V4	Beef	Ngetich et al. 2022	
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanosphaera</i>	<i>cuniculi</i>	16S rRNA Sequencing V3-V4	Beef	Ngetich et al. 2022	
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanosphaera</i>	Not reported	16S rRNA Sequencing V3-V4	Beef	Ngetich et al. 2022	
Euryarchaeota	<i>Methanobacteriaceae</i>	Not reported	Not reported	16S rRNA Sequencing V3-V4	Beef	Ngetich et al. 2022	
Euryarchaeota	<i>Methanosarcinaceae</i>	<i>Methanosarcina</i>	<i>mazei</i>	16S rRNA Sequencing V3-V4	Beef	Guindo et al. 2020	
Halobacterota	<i>Methanomicrobia</i>	<i>Methanocorpusculum</i>	Not reported	16S rRNA Sequencing V3-V4	Beef	Guindo et al. 2020	
Korarchaeota	<i>Korarchaeales</i>	<i>Korarchaeota</i>	Not reported	Shotgun metagenomics	Dairy cattle	Webb et al. 2023	
Nanoarchaeota	<i>Nanoarchaeaceae</i>	<i>Nanoarchaeum</i>	Not reported	Shotgun metagenomics	Dairy cattle	Webb et al. 2023	
Nitrososphaerota	<i>Nitrososphaeraceae</i>	<i>Nitrososphaera</i>	Not reported	16S rRNA Sequencing V4	Dairy cow	Webb et al. 2023	
Nitrososphaerota	<i>Nitrososphaeraceae</i>	<i>Nitrososphaera</i>	Not reported	16S rRNA Sequencing V3-V4	Dairy cow	Webb et al. 2023	
Nitrososphaerota	<i>Nitrosopumilaceae</i>	<i>Nitrosopumilus</i>	Not reported	Shotgun metagenomics	Dairy cattle	Webb et al. 2023	
Thermoplasmata	<i>Methanomassiliicoccaceae</i>	<i>Methanomassiliicoccus</i>	<i>luminyensis</i>	qPCR	Sheep, Cow	Guindo et al. 2020	
Thermoplasmata	<i>Methanomethylophilaceae</i>	<i>Methanomassiliicoccus</i>	Not reported	16S rRNA Sequencing V3-V4	Beef	Webb et al. 2023	
Thermoproteota	<i>Cenarchaeaceae</i>	<i>Cenarchaeum</i>	Not reported	Shotgun metagenomics	Dairy cattle	Webb et al. 2023	

Methanobrevibacter is the most abundant genus of ruminal methanogens in cattle and it belongs to Euryarchaeota phylum [54]. Multiple species of *Methanobrevibacter* including *M. ruminantium*, *M. smithii*, and *M. gottschalkii* have been reported in the rumen of cattle other ruminant species (Table 1). The *Methanobrevibacter* spp. produce CH₄ from H₂ and CO₂, and they can also utilize formate and acetate as alternative substrates [55]. Another common archaeal genus found in the rumen is *Methanosphaera* [56], and species within this genus consume H₂ and CO₂, as well as methanol and methylamines to produce CH₄ [56]. Multiple species within *Methanosphaera* (e.g., *M. stadtmanae* and *M. cuniculi*) have also been detected in the rumen, and believed to contribute to the ruminal CH₄ production [57,58]. Guzman and colleagues identified *Methanomicrobiales mobile*, *Methanococcales votae*, and *Methanobrevibacter* spp. from the gastrointestinal tract of neonatal dairy calves sampled within five minutes of birth [59]. *Methanomassiliicoccus*, within the phylum Euryarchaeota, is a relatively recent discovered genus from the rumen and feces of ruminants [57]. This genus is unique in a way that it produces CH₄ through the reduction of methanol and methylamines, rather than H₂ and CO₂ [57]. *Methanomassiliicoccus* has been suggested as a potential target for inhibiting ruminal CH₄ emissions due to its ability to outcompete hydrogenotrophic methanogens [60]. Also, *Methanospirillum*, classified within the order *Methanomicrobiales* and the phylum Euryarchaeota, has been found in the rumen and feces of cattle and sheep [61]. Additionally, *Methanospirillum hungatei* and *Methanospirillum lacunae* are also present in the rumen [62]. Overall, the rumen is home to taxonomically and metabolically diverse methanogenic archaeal species.

4. Pro-and Anti-Methanogenic Ruminal Microorganisms

Methanogens undergo methanogenesis; an energy-intensive process that requires specific substrates and environmental conditions [63]. Syntrophic bacteria are among the most extensively studied microorganisms that enhance the activity of methanogens in the rumen. Syntrophic bacteria, such as *Syntrophomonas* spp. and *Smithella* spp., form symbiotic associations with methanogens, by degrading complex organic matter to simpler compounds that methanogens can utilize [57,64]. Examples of syntrophic bacteria are *Syntrophomonas wolfei*, *Syntrophobacter fumaroxidans*, and *Pelotomaculum thermopropionicum*. In this relationship, syntrophic bacteria oxidized short chain fatty acids (SCFAs) to produce H₂ and CO₂, both of which are used by methanogens to produce CH₄ [65].

In addition to syntrophic bacteria, some other bacterial species may enhance the activity of methanogens through various mechanisms in the rumen. For example, exopolysaccharide-producing bacterial species can promote the aggregation of methanogens, creating microenvironments favoring methanogenesis [55]. Other bacteria species that produce secondary metabolites including formic acid and ethanol can facilitate methanogenesis [66]. Furthermore, *Pelobacter* spp. and *Bacteroides* spp. can promote methanogenesis by enhancing the growth of specific methanogenic species [67].

While some bacteria promote methanogens, there are some bacteria in the rumen that can inhibit methanogenesis. Sulfate-reducing bacteria (SRB) such as *Desulfovibrio* spp. consume organic matter and produce hydrogen sulfide (H₂S). Both SRB and methanogens compete for H₂, as SRB require it to reduce sulfate to sulfide; while methanogens need H₂ to reduce CO₂ for CH₄ production. Because of this competition, the presence of SRB indirectly decreases methane production in the rumen [20]. Ruminal SRB utilize various forms of sulfur (S), including sulfate, sulfite, thiosulfate, and elemental S, as optional H₂ sinks [68]. As the end-product of the sulfate reduction pathway, H₂S can inhibit methanogenic activity, consequently reducing ruminal CH₄ production [69]. In addition, abundance of bacterial genera such as *Prevotella*, *Fibrobacter*, and *Ruminococcus* were found to be negatively correlated with methanogenic activity in dairy cows [14]. These bacteria that are involved in the degradation of fiber and the production of propionate can compete with methanogenesis for H₂. Fumarate-reducing and lactate-producing bacteria may also inhibit methanogens, which was suggested by a study where nitrate supplementation reduced CH₄ emission in grazing steers by promoting fumarate-reducing and lactate-producing bacteria [70]. Jenayathan and colleagues [12] also reported that direct-fed microbials comprising of *Propionibacterium* and *Lactobacillus* spp. were able to mitigate CH₄ emissions in sheep. The genus *Lactobacillus* has a long history of being used as a probiotic and has recently been proposed to mitigate ruminal CH₄ emissions [71]. Other genera such

as *Prevotella* and *Succinivibrio* have been negatively associated with ruminal methanogenesis [56,72,73]. The family *Succinivibrionaceae* is a key gut microbial member in the Tammar wallaby that produce only 20% of the CH₄ emissions of cattle [74]. This is because *Succinivibrionaceae* produce succinate through the fumarate-succinate pathway, which is an intermediate product of propionate, that are easily absorbed by the animal for energy [75].

Certain viral species can infect methanogens, called methanogenic viruses or methanophages. Methanophages against *Methanosarcina*, *Methanococcus*, and *Methanobacterium* [76–78] have been reported in methanogen abundant anoxic environments. Viruses that infect bacterial species can compete with methanogens for substrates [76] and have also been reported in methanogenic environments. Accordingly, it is plausible that viruses infecting methanogenic archaea or anti-methanogenic bacteria are present in the bovine rumen.

Methane produced in the rumen can be utilized by other ruminal community members. Methanotrophic archaea, also known as methanotrophs, are a group of archaea that can utilize CH₄ as the sole source of carbon and energy [79]. Under aerobic conditions, methanotrophs combine O₂ and CH₄ to form formaldehyde, which is then incorporated into organic compounds via the ribulose monophosphate pathway by type I methanotrophs (γ -proteobacteria) or the serine pathway by type II methanotrophs (α proteobacteria) [79]. The methanotrophic bacteria including *Methylobacterium*, *Methylomonas* and *Methylomicrobium* genera have been detected in the bovine rumen and have been shown to influence methanogens [80].

Fungi species have been shown to promote methanogenic activity by producing various enzymes such as cellulases and hemicellulases, and these enzymes can break down complex polysaccharides (cellulose and hemicellulose) into simpler compounds that can be utilized by methanogens [81]. Anaerobic fungi, such as *Neocallimastix* spp., and *Piromyces* spp., have been extensively studied for their ability to enhance the activity of methanogens in the rumen [81]. These fungi species are commonly found in the rumen and play a crucial role in the degradation of plant biomass [82]. As discussed above, it is apparent that the activities of methanogens in the rumen are interdependent on other microbes and their respective activities in the ruminal environment. Thus, understanding the interactions between the methanogens and other microorganisms within the rumen is important as such interactions could be harnessed for enteric methane mitigations from cattle.

5. Methanogens in the Reproductive Tract: Vagina, Uterus and Semen

Methanogen presence has been reported in both the lower and upper reproductive tracts of cattle (Figure 1, Table 2). The microbial community associated with the bovine vaginal tract has been relatively well characterized as compared to the cervical and uterine microbiota [83]. Overall, there is less species richness and community diversity in the vaginal microbiota as compared to the bovine gut, and this community has been shown to influence reproductive health and fertility of female cattle [83,84]. Although the archaeal members of the vaginal microbiome have not yet been as extensively characterized as the bacterial members, the presence of some methanogens in the cattle vagina have been reported from 16S rRNA gene amplicon-based studies. The *Methanobrevibacter* genus has been reported to be the predominant methanogen genus in the vaginal samples of Nellore cows [85]. We recently reported 6 amplicon sequence variants (ASVs) that were classified as *Methanobrevibacter* spp. (5 of these taxa were unclassified at species level and the remaining one was *M. ruminantium*) present from vaginal swabs of both virgin yearling heifers and pregnant beef heifers [21]. The *Methanobrevibacter ruminantium* taxa was most frequently identified from the vaginal swab samples with greater abundance than the other *Methanobrevibacter* taxa [21].

Table 2. Methanogenic archaeal species present in the reproductive and respiratory tracts of cattle.

Phylum	Family	Genus	Species	Sequencing platform	Host	Reference
Vagina						
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>ruminantium</i>	16S rRNA Sequencing V3-V4	Beef heifers	Amat et al. 2021
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>ruminantium</i>	16S rRNA Sequencing V3-V4	Beef heifers	Winders et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>wolini</i>	16S rRNA Sequencing V3-V4	Beef heifers	Winders et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>not reported</i>	16S rRNA Sequencing V3	Beef cattle	Laguardia-Nascimento et al., 2015
Uterus						
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>ruminantium</i>	16S rRNA Sequencing V3-V4	Beef heifers	Winders et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>wolini</i>	16S rRNA Sequencing V3-V4	Beef heifers	Winders et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanosphaera</i>	<i>not reported</i>	16S rRNA Sequencing V3-V4	Beef heifers	Winders et al. 2023
Semen						
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanosphaera</i>	<i>stadtmanae, cuniculi</i>	16S rRNA Sequencing V4	Beef bulls	Koziol et al. 2022
Thermoplasmatota	<i>Methanomassiliicoccaceae</i>	<i>Methanomassiliicoccus</i>	<i>luminyensis</i>	16S rRNA Sequencing V4	Beef bulls	Koziol et al. 2022
Crenarchaeota	<i>Nitrososphaeraceae</i>	Not reported	<i>Not reported</i>	16S rRNA Sequencing V3-V4	Beef bulls	Webb et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobacterium</i>	<i>mobilis,formicum,bryantii,</i>	16S rRNA Sequencing V4	Beef bulls	Koziol et al. 2022
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>boviskoreoni, millerae,olleyae, ruminatum, wolini</i>	16S rRNA Sequencing V3-V4	Beef bulls	Webb et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>Not reported</i>	16S rRNA Sequencing V3-V4	Beef bulls	Webb et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanosphaera</i>	<i>cuniculi</i>	16S rRNA Sequencing V3-V4	Beef bulls	Webb et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanosphaera</i>	<i>Not reported</i>	16S rRNA Sequencing V3-V4	Beef bulls	Webb et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Not reported</i>	<i>Not reported</i>	16S rRNA Sequencing V3-V4	Beef bulls	Webb et al. 2023
Euryarchaeota	<i>Methanosarcinaceae</i>	<i>Methanosarcina</i>	<i>mazei</i>	16S rRNA Sequencing V3-V4	Beef bulls	Webb et al. 2023
Thermoplasmatota	<i>Methanomethylphilaceae</i>	<i>Methanomassiliicoccus</i>	<i>Not reported</i>	16S rRNA Sequencing V3-V4	Beef bulls	Webb et al. 2023
Halobacterota	<i>Methanomicrobia</i>	<i>Methanococpusculum</i>	<i>Not reported</i>	16S rRNA Sequencing V3-V4	Beef bulls	Webb et al. 2023
Halobacterota	<i>Methanomicrobia</i>	<i>Methanococpusculum</i>	<i>Not reported</i>	16S rRNA Sequencing V3-V4	Beef bulls	Cojkic et al.2021
Milk						
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>ruminantium, smithii, milerae</i>	Shotgun metagenomics	Dairy cow	Ekman et al. 2020; Guindo et al. 2020
Euryarchaeota	<i>Methanocorpusculaceae</i>	<i>Methanocorpusculum</i>	<i>Not reported</i>	Shotgun metagenomics	Dairy cow	Ekma et al.2020
Nasopharynx						
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>ruminantium</i>	16S rRNA Sequencing V3-V4	Beef heifers	Winders et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>wolinii</i>	16S rRNA Sequencing V3-V5	Beef heifers	Winders et al. 2023
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>Not reported</i>	16S rRNA Sequencing V4	Beef cattle	Amat et al. 2019
Udder teat						
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanobrevibacter</i>	<i>ruminantium, smithii, milerae</i>	Shotgun metagenomics	Dairy cow	Ekman et al. 2020; Guindo et al. 2020
Euryarchaeota	<i>Methanobacteriaceae</i>	<i>Methanosphaera</i>	<i>stadtmanae, cuniculi</i>	Shotgun metagenomics	Dairy cow	Ekma et al.2020
Nitrososphaerota	<i>Nitrosopumilaceae</i>	<i>Nitrosopumilus</i>	<i>Not reported</i>	Shotgun metagenomics	Yak calves	Guo et al. 2023
Nanoarchaeota	<i>Nanoarchaeaceae</i>	<i>Nanoarchaeum</i>	<i>Not reported</i>	Shotgun metagenomics	Yak calves	Guo et al. 2023

While there are no culture-based studies reporting the isolation and identification of methanogens in the vaginal tract of cattle, methanogenic archaeal species have been cultured and isolated from the vaginal swabs [86] and urine specimens [87] of women with urinary tract infections. Belay et al. [86] cultured two different *Methanobrevibacter smithii* strains from the vaginal swabs of women diagnosed with bacterial vaginosis using traditional anaerobic culturing technique. Briefly, vaginal swabs were enriched in a medium containing various salts and additives, and yeast extract and tryptone in anaerobic serum tubes pressurized with H₂-CO₂ and supplemented with cysteine hydrochloride and Na₂S. 9H₂O. Methane production in the serum tube was monitored using gas chromatography. Following enrichment, the cultures that produced CH₄ gas were plated on medium with 1% Gelrite to isolate methanogens. Two methanogen isolates were isolated and taxonomically identified as *Methanobrevibacter smithii* PS and ALI based on the morphological, cultural, and immunological features. Likewise, Grine and colleagues [87] isolated *Methanobrevibacter smithii* strains from the urine samples of women suffering from urinary tract infections using a Hungate culture tube containing SAB medium, a versatile medium that supports the growth of most of methanogen species [88]. Based on their methanogenic archaeal culturing results from 383 urine specimens (61% of them from women) prospectively collected for diagnosing urinary tract infection, these authors suggest that *M. smithii* is part of the urinary microbiota of some individuals. Thus, both sequencing and culturing-based results obtained from bovine and human studies discussed above highlight that the microbial community in the bovine lower reproductive tract could harbor methanogenic archaea. The physiological function of methanogens in the lower reproductive tract of cattle remains to be explored even though some studies have suggested potential association of methanogens with reproductive health [21,85,89,90]. In the human vaginal tract, methanogens are known to play a significant role in prevention of acid accumulation (increase in vaginal pH) which disrupts the vaginal microbiota in bacterial vaginosis patients [86,87,91].

The *in-utero* environment has long been viewed as sterile as the cervix was thought to prevent ascending bacteria from the lower reproductive tract into the uterus (Wang et al., 2018). However, culture-independent high-throughput sequencing technologies have enabled the identification of commensal microbiota presence in the bovine uterus during both pregnancy and after parturition [93]. The potential role of the uterine microbiome in reproductive health, conception, and embryo development is increasingly appreciated [83,94,95]. Within the bovine uterine microbial community, methanogenic archaeal species have been reported. A sequencing-based study identified several methanogens in both pregnant and non-pregnant cows, including *Methanosphaera stadtmanae*, *Methanobrevibacter ruminantium*, and *Methanobacterium congolense* [85] (Table 2; Figure 1). Our lab has also identified *Methanobrevibacter ruminantium* and *Methanobrevibacter wolini*, which accounted for 0.05% of the total microbiota present in the uterus of virgin beef heifers (21-months old) [25]. We also observed greater abundance of *Methanobrevibacter ruminantium* (ASV330) in the uterine microbiota of beef cows that became pregnant than those remained open following artificial insemination, suggesting positive association of *Methanobrevibacter ruminantium* with fertility [96]. In contrary, methanogens have been shown to be present in uterus of cows with uterine infections, suggesting a potential role in pathogenesis [97]. The functional and taxonomic characteristics of methanogens in the bovine uterus, like those found in the vagina, are yet to be fully explored.

Recent sequencing-based studies revealed that there are diverse and dynamic microbial communities residing within the bull reproductive tract [98]. Methanogenic archaeal species are identified as commensal microbiota associated with bull semen and the abundance of methanogens in the semen may have an association with bull fertility. For example, Koziol and colleagues identified *Methanobrevibacter*, *Methanosphaera*, and *Methanomassiliicoccus* as the predominant methanogens present in the semen of breeding beef bulls [99] using 16S rRNA gene amplicon sequencing. Likewise, *Methanocorpusculum* spp. was identified in bovine bull semen, and the relative abundance of this methanogenic genus was inversely correlated with seminal commensal genera *Ruminococcaceae* and *Rikenellaceae* RC9 Gut group [100]. Our research group detected more than a dozen methanogenic archaeal taxa (Listed in Figure 1; Table 2) from semen samples collected at three

Figure 1. Presence (purple color) or absence (blue) of methanogenic archaeal species reported in different anatomical sites of cattle. This figure was generated based on the data presented in the references listed in Tables 1 and 2.

However, evidence derived from human studies suggests that the lower respiratory tract may harbor methanogenic archaea. *Methanobrevibacter* spp. (*oralis* and *smithii*) have been cultured and isolated from sputum, trachea-bronchial, and broncho-alveolar samples collected from humans [108].

Methanogenic archaeal cell presence in the upper respiratory tract of cattle raises an important question about the survival mechanisms of methanogens in such an O₂ rich environment given that archaeal species involved in methanogenesis are believed to be strict anaerobes. It is not uncommon to identify anaerobic bacterial species some of which are associated with the ruminal commensal microbiota such as *Ruminococcus* and *Thermodesulfovibrio* (sulfate reducing bacteria) in the upper respiratory tract of cattle [109]. Anaerobic bacteria are predominant components of the upper respiratory tract, and mixed anaerobic-aerobic agents are often responsible for respiratory infections in humans [110]. Thus, despite that some of the methanogenic species in the nasopharyngeal and lung tissue samples of cattle can be transient and from eructation and inhalation of ruminal gas cap, the aerosols generated from the dust and soil particles [106,111,112], the airway mucosal surface of the cattle can harbor methanogens as part of their commensal microbiota. These airway specific methanogens could have evolved to adapt and survive in the microenvironments along the respiratory tract, especially those parts with reduced O₂ levels. One of the potential methanogen-promoting factors in the airway can be associated with biofilm forming bacterial cells, as biofilms can provide localized anaerobic conditions. Biofilms can form in different areas of the respiratory tract [113]. In addition, the presence of oxygen-utilizing bacteria in the respiratory tract may support the growth of methanogens by consuming O₂ and thereby creating anaerobic pockets where methanogens can persist [114,115]. Future culture-dependent studies are needed to isolate methanogens from the respiratory tract and explore metabolic features of these methanogens, and their interactions with the methanogens present in the rumen.

7. Methanogens in the Udder

The mammary gland is another site that has been reported to harbor relatively diverse and rich microbial communities [116]. Distinctive and site-specific microbial communities are present in various niches of the udder including the teat apex, teat canal, milk, and colostrum [116]. Some of these communities encompass archaeal members (Table 2; Figure 1). Guo and colleagues characterized the archaeal community shared between the maternal rumen and milk in grazing yak calves. They identified two archaeal phyla and 11 different common archaeal genera [60]. The primary archaeal phyla found on the skin of the teat were *Euryarchaeota* (76.8%) and *Thaumarchaeota* (23.1%) with dominant genera being *Methanobrevibacter* (61.7%) and *Candidatus nitrocosmicus* (12.5%). Similar findings have been reported for the skin of dairy cows, where *Methanobrevibacter* and *Methanosphaera* were identified as the primary genera [117]. Species within *Methanobrevibacter*, including *M. ruminantium*, *M. smithii*, *M. millerae*, and some *Methanocorpusculum* spp. have also been detected in the bovine milk [23,60,117]. No data is available regarding the presence of methanogenic archaea in bovine colostrum. However, *Methanobrevibacter oralis* and *M. smithii* species have been found in human colostrum [118], suggesting that methanogenic archaea may also be present in the cattle colostrum.

Similar to the respiratory tract, the presence of methanogens on teat skin and udder raises an important question regarding the strict anaerobic nature of these methanogens. *Methanobrevibacter* and *Candidatus nitrocosmicus* are known to be strict anaerobes and are typically unable to survive with exposure to ambient air for more than 10 minutes [119]. Therefore, their survival on the teat skin would be unlikely unless there is a wound present that creates an anaerobic environment suitable for methanogens. Further, research is needed to investigate the mechanisms and conditions that allow for the presence of methanogens on the skin and explore their potential role in the overall microbial ecology of the teat as related to milk production.

8. Potential Seeding Sources of the Ruminal Methanogens

As illustrated in the Figure 2, there are multiple potential seeding sources for the ruminal methanogenic archaea in cattle, and these sources could be the microbial communities present in the different anatomical sites of bovine body, and other external sources which will be discussed in detail below.

8.1. Within the Bovine Body

Ruminal methanogens have the potential to be seeded from the reproductive system of the cow, particularly from the vagina, during calving. Our research team observed that certain methanogenic taxa are present in both the rumen and reproductive tract (vagina and uterus) of beef cattle [21,25]. This may suggest that there could be methanogenic archaea transfer between the gastrointestinal and reproductive tracts within the same animal. Likewise, the methanogenic archaeal species associated with the bovine upper respiratory tract may also be involved in introducing the rumen with methanogenic species. Our recent research revealed the presence of methanogenic taxa shared by the rumen and nasopharynx as core taxa [21,25]. Another important seeding source for the ruminal methanogens could be the udder and mammary gland of female cattle. As discussed above, methanogens can be present in bovine teat and milk. When neonatal calves are nursing, it becomes a direct route for transferring methanogens into the calf gut [86]. Additionally, bull semen harbors methanogens, and thus it is highly likely that the sperm containing methanogens traveling through the female reproductive tract could potentially introduce methanogens into the uterus and reproductive system of the female [99]. Therefore, it would not be surprising to find the same genera of methanogens in both the sperm and milk and in the vagina, uterus, and gut of animals. In addition, diverse and dynamic bacterial microbiota has been reported to be present on the ocular surface of healthy newborn calves [98] and weaned beef calves [120], and cattle hooves are also home to commensal microbiota [98,121]. These studies have not reported the presence of methanogenic archaea in bovine eye and feet. However, until the absence of the methanogens from these sites is confirmed with studies using archaeal or methanogens specific amplicon sequencing or shotgun metagenomic sequencing, the possibility of the methanogenic archaeal exchange between ocular and hoof microbial communities with the bovine gut could not be ruled out.

8.2. Other Sources

There are several external sources that could introduce cattle rumen with methanogens (Figure 2). Among which, the pasture, soil and drinking water that cattle consume may transfer methanogens to cattle rumen [59,72]. Methanogens are commonly found in muddy soils [122,123]. The common and dominant genera of the soil associated methanogens include *Methanoregula*, *Methanobacterium*, *Methanosarcina*, *Methanolinea* spp. present in wetlands and water [124–126]. Presence of *Methanosarcina* and *Methanocella* spp. in cattle, sheep and swine grazing land [127], wet soils and desert soils [128] have been documented. *Methanosarcina* spp. have been identified as the major methanogen in pasture soils compacted by cattle [22,129] and arable soils [130]. Cattle are known to consume approximately 350 kg of soil per cow per year through geophagia which refers to the act of eating soil and dirt [131,132]. Thus, it is most likely that methanogens associated with soil can be transferred into the cattle rumen. Methanogens could be transmitted to cattle via farm dust and dust particles. A study was conducted by Bønløkke and colleagues to investigate the exposure of livestock farmers to archaea [133]. For this, they analyzed the number of 16S rRNA gene copies from archaea and bacteria present in the personal filter samples obtained from 327 farmers working on 89 Danish farms including cattle and chicken farms. Both archaea and bacteria were detected in all types of farm environments. *Methanobrevibacter* and *Methanosarcina* species were found to be dominant in aerosols from both pig and cattle farms [133]. The aerosols likely consisted of a mixture of nasal fluid from farmers and other farm workers, as well as dust from the soil. When these aerosols are inhaled by cattle, they could potentially transfer methanogens into the airway systems and ultimately to the cattle rumen. Farmers and farm workers could also be a source for introducing the cattle with

methanogens. In the human gut and other parts of the body harbor methanogens. *Methanobrevibacter smithii* and *Methanobrevibacter oralis* are found in the intestine and sputum, while *Methanobrevibacter smithii* has been reported in bronchoalveolar [108], milk [134], and urine [87]. *Methanospaera stadmagnae* [135] and *Methanobrevibacter oralis* are associated with periodontal disease [108,136]. The methanogens present in the human body can be first transferred into the environment through spit and urine, and then ultimately to cattle.

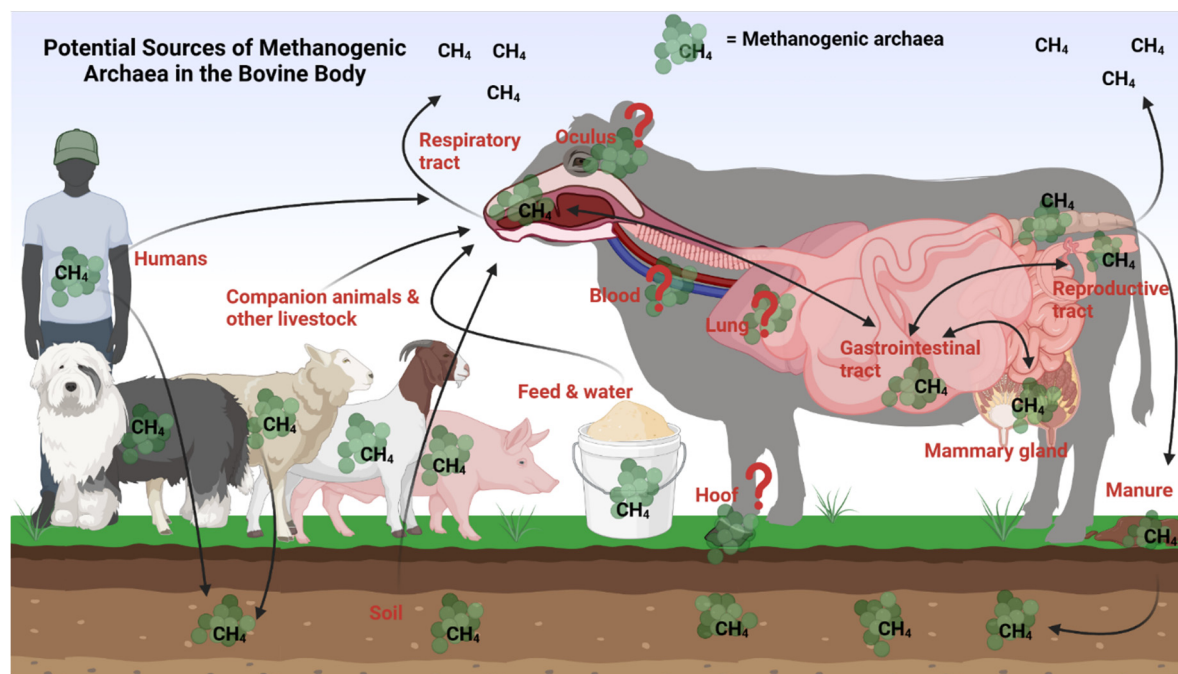


Figure 2. Holistic view of the bovine methanogens and potential sources of ruminal methanogens in cattle. Potential seeding sources of methanogenic archaea in the bovine body can include the soil, feed, water, farm laborers, other livestock species, or pets. Methanogens from the respiratory tract, reproductive tract, and mammary gland may seed the gastrointestinal tract, and vice versa. It is unclear if methanogens inhabit the oculus, blood, liver, lung, or hoof tissue, but the presence of other microorganisms at these sites could indicate the potential presence of methanogens here as well. Figure created using Biorender.com.

Apart from bovine, other animal species (e.g., sheep, chicken, pig and dogs) raised on the same farm with cattle could inoculating cattle rumen with methanogens. Guindo et al. [137] conducted a study where fecal samples were analyzed from pigs, dogs, cats, sheep, and horses using PCR-sequencing targeted methanogens. Seven different species of methanogens including, *Methanobrevibacter smithii*, *Methanobrevibacter millerae* and *Methanomassiliicoccus luminyensis*, some of which are known to be present in the human digestive tract. *Methanobrevibacter smithii* were present in all the animal species studied [137]. Specifically, *Methanobrevibacter smithii* was present 50% of cases in pigs, 25% of cases in dogs, 16.7% of cases in cats, and 4.2% in both sheep and horses [137]. These findings indicate that *Methanobrevibacter smithii* is core archaeon shared by multiple animal species and humans, and it could be transferred between human and different animal species.

9. Challenges Associated with the Ruminal Methanogens and Future Directions

In vitro culturing techniques allow researchers to isolate and study specific microorganisms in a controlled environment and can provide insights into the physiology and ecology of these organisms. Isolation and culturing of methanogens from the rumen is a particularly challenging task due to their strict anaerobic nature and growth requirements for special culturing media, culturing apparatus, and H₂ gas supplementation, all of which have hindered the progress of isolation, and metabolic and

genomic characterization of the methanogenic archaea associated with ruminal CH₄ production [138,139]. While recent advances in culture-independent high-throughput sequencing techniques have enabled characterization of ruminal microbiota and the potential involvement in methanogenic activity, the information is mostly limited to the bacterial population of the ruminal microbial ecosystem as most of the sequencing is based on 16S rRNA gene amplicon sequencing, which is more specific to bacteria and captures only a small fraction of the archaeal population. Thus, to understand the complete taxonomic characterization of the methanogens and other archaeal populations in the rumen, and other extra-gastrointestinal sites of the bovine body, archaea targeting amplicon sequencing such as 16S rRNA V2-V3 primers and *mcrA* gene-based primers should be applied. To gain insights beyond the taxonomic properties of bovine methanogens, the metabolic features, and their interactions with other microorganisms such as fungi, bacteria and viruses, shotgun metagenomic sequencing should be performed. Also, comparative genomics on methanogenic isolates from different cattle body sites (e.g., respiratory, reproductive, ruminal and udder) should be performed to understand the genomic adaptive features of the methanogens from different anatomical sites and identify the seeding sources of the ruminal methanogens. Given the several external sources including the feed, soil and water consumed by cattle, and other farm animals and farmers who could exchange methanogens with cattle rumen and other body sites, comprehensive survey of the methanogens present in these external sources and their interactions with ruminal methanogens in cattle warrants further research.

While these culture-independent sequencing methods can provide taxonomic composition and functional features of the methanogenic archaeal members and their interactions with other microbial partners in a particular niche, one of the limitations of these sequencing techniques is that they could not provide information on the viability of the methanogenic archaeal cells. Considering the anaerobic nature of methanogens and their presence in non-anaerobic body sites including respiratory and lower reproductive tracts of cattle, it is critical to use culture-dependent techniques to isolate methanogens from these sites and verify findings from metagenomic sequencing. It is plausible that some of the genomic DNA extracted from the samples associated with nasopharynx, lung tissue and vagina of cattle might be originated from transient and dead methanogens. Nevertheless, identification of methanogenic archaeal genera and species with relatively high abundance, and consistently over multiple sampling timepoints in bovine respiratory and reproductive tracts presented above highlights that the methanogenic archaea could colonize and survive aerobic mucosal surfaces along the airway and urogenital tract of cattle. Thus, further research is warranted to isolate methanogens from extra-gastrointestinal tracts of cattle and explore the survival mechanisms of the methanogens in the environment where oxygen is present. Some anaerobic bacterial species can have evolved mechanisms that can either minimize the extent to which oxygen disrupts their metabolism [140,141] and/or rely on potential anaerobic and aerobic species co-existence mechanisms [142].

Emerging evidence from humans [141], bovine fetal fluids [21,27], the fetal intestine [21,27,143], as well as the human fetal lung [144], suggests that microbial colonization of calves may begin *in utero*. This, coupled with rodent studies which demonstrate that fetal metabolic and nervous system development is impacted by the maternal microbiota during pregnancy [145,146], highlight the potential and extended role, of the maternal microbiome in calf microbiome development. Recent studies reported the presence of methanogenic archaeal species in fetal fluids and fetal intestine at 4-8 months of gestation. Thus, these recent developments warrant a re-consideration of the timing and mechanisms involved in the first colonization of calf ruminal methanogens.

10. Conclusions

Methanogens are responsible for enteric CH₄ emissions in cattle, which contributes to a significant amount of dietary energy loss to the host and GHG emissions. The species associated with ruminal CH₄ production are not only present in the cattle rumen, but they could also present in extra-gastrointestinal organs of cattle such as the reproductive and respiratory tracts, udder, eye, and hoof. Recent sequencing-based studies revealed that certain methanogenic taxa are shared by the ruminal,

respiratory, and reproductive tracts of cattle as core taxa, suggesting potential interactions between the ruminal and extra-intestinal methanogens. The seeding of the rumen with methanogenic archaea could be from many possible sources within the bovine body (e.g., reproductive and respiratory tracts, and other microbial communities) and external sources such as pasture, soil, water, and farm animals. Therefore, a singular focus on the methanogens in the rumen may not be sufficient and a holistic approach which takes into consideration the transfer of methanogens between ruminal, extra-gastrointestinal, and environmental microbial communities. Taxonomic, genomic, and metabolic characteristics of the methanogenic archaeal species in the rumen and other bovine body sites should be investigated to gain more holistic insights into the methanogens in cattle. It is anticipated that the holistic understanding of the methanogens in the rumen and their interactions with the extra-gastrointestinal methanogens, and identification of the seeding sources (both within and external), and the colonization timing of the ruminal methanogens are important for the development of more effective CH₄ mitigation strategies in cattle.

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