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Review

The Potential of Novel Gene Editing-Based Approaches in Forages and Rumen Archaea for Reducing Livestock Methane Emissions

Udaya Subedi [1,2], Kazi Kader [1], Kethmi N. Jayawardhane [2], Hari Poudel [1], Guanqun Chen [2], Surya Acharya [1], Luiz S. A. Camargo [3,*], Daniela Matias de C. Bittencourt [4,*] and Stacy D. Singer [1,*]

[1] Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, Lethbridge, AB T1J 4B1, Canada
[2] Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB T6G 2P5, Canada
[3] Embrapa Dairy Cattle, Juiz de Fora 36038-330, MG, Brazil
[4] Embrapa Genetic Resources and Biotechnology, Brasília 70770-917, DF, Brazil
* Correspondence: luiz.camargo@embrapa.br (L.S.A.C.); daniela.bittencourt@embrapa.br (D.M.d.C.B.); stacy.singer@agr.gc.ca (S.D.S.)

Abstract: Rising emissions of anthropogenic greenhouse gases such as carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) are a key driver of climate change, which is predicted to have myriad detrimental consequences in coming years if not kept in check. Given the potency of CH_4 in terms of trapping heat in the atmosphere in the short term, as well as the fact that ruminant production currently contributes approximately 30% of anthropogenic emissions, there is an impetus to substantially decrease the generation of ruminant-derived CH_4. While various strategies are being assessed in this context, a multi-faceted approach is likely required to achieve significant reductions. Feed supplementation is one strategy that has shown promise in this field by attenuating methanogenesis in rumen archaea; however, this can be costly and sometimes impractical. In this review, we examine and discuss the prospect of directly modulating forages and/or rumen archaea themselves in a manner that would reduce methanogenesis using CRISPR/Cas-mediated gene editing platforms. Such an approach could provide a valuable alternative to supplementation and has the potential to contribute to the sustainability of agriculture, as well as the mitigation of climate change, in the future.

Keywords: climate change; CRISPR/Cas; environmental sustainability; greenhouse gas emissions; ruminant livestock production

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1. Introduction

Anthropogenic greenhouse gas (GHG) emissions, which are mainly driven by energy production, industrial processes, transport, waste management and forestry/agriculture, have been rising dramatically in recent years [1]. These gases function to trap heat energy, thereby increasing global temperatures, and are thus the main drivers of climate change. Indeed, it has been predicted that temperatures could increase by up to 5.7 °C globally by the end of the 21st century compared to temperatures in the years 1850–1900 in a scenario of very high GHG emissions [2]. While the yields of many crop species will decline dramatically with even incremental temperature increases in certain regions, further detrimental impacts will be incurred through escalations in the intensity and frequency of heat waves, droughts, flooding and soil salinization, as well as the worsening of disease and pest outbreaks [3–5]. Furthermore, such climate change scenarios would also lead to a series of additional devastating effects, including increased risks of wildfires, shifts in biodiversity and the extinction of species [6,7]. As a result of this, moderating anthropogenic GHG emissions is becoming a fundamental

priority globally, with 131 countries intending to, or having already adopted or announced plans for net zero emission targets in the coming decades [8].

Of all GHGs emitted due to anthropogenic causes, carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) comprise the highest proportions, making up approximately 75%, 18% and 4% of the total volume, respectively [9]. Although the amount of CH_4 emitted is substantially less than CO_2 and it is a relatively short-lived pollutant, it has a global warming potential that is 34-times higher than that of CO_2 on a per unit mass basis over a 100-year time scale [10]. While global CH_4 emissions derive from multiple sources, approximately 30% of anthropogenic CH_4 arises from ruminant production [11], primarily due to inefficiencies in the enteric fermentation of insoluble carbohydrates (mainly lignin, cellulose and hemicellulose) in the rumen [12]. To limit global warming to 1.5 °C, it has been suggested that global agricultural CH_4 emissions need to be reduced by 24–47% by 2050 compared to 2010 [13], which is particularly challenging given the projection that the production of ruminant-derived meat needs to increase by 88% in that same time period to meet growing demand [14].

In the rumen, insoluble carbohydrates are broken down into soluble sugars, which then undergo anaerobic fermentation to yield volatile fatty acids, including acetate, propionate and butyrate. This fermentation process also leads to the generation of CO_2 and molecular hydrogen (H_2), which, at increased ruminal partial pressures, can inhibit microbial enzymes and thus has a detrimental effect on the rate of carbohydrate fermentation [15]. However, H_2 levels are typically kept in check by methanogens, which are a group of archaea that naturally occur in the rumen and use H_2 and CO_2 as substrates to produce CH_4 [16]. The resulting CH_4 is released into the atmosphere through eructation and breathing [17], and the amount produced is typically directly proportional to dry matter intake [16]. While this conversion of H_2 to CH_4 is beneficial in terms of rumen fermentation, it has a deleterious effect on the environment in the context of climate change and also represents a considerable loss for ruminant livestock producers, since approximately 2–15% of energy ingested is used for this process rather than for meat and milk production [18,19].

Over recent years, various methods have been assessed for their ability to mitigate CH_4 emissions from ruminant production systems, which would in turn reduce environmental impacts and economic losses for producers. For example, the utilization of diets with higher grain contents or concentrates have been found to lead to incremental reductions in CH_4 emissions from ruminants [20,21], as has the use of controlled rotational grazing [22] and animal breeding [23]. In addition, the administration of bacteriophages [24] and bacteriocins [25,26], as well as anti-methanogen vaccines [27], has also been suggested as a possible option in this context; however, their use is likely challenging due to the diversity of ruminal methanogens. In addition, their effectiveness in terms of reducing CH_4 emissions is still unclear, especially long-term in vivo [24].

Furthermore, a range of different diet supplements have shown promise with regard to decreasing CH_4 emissions from ruminants, including lipids, synthetic halogenated compounds, algae, short-chain nitrocompounds, nitrates, ionophores such as monensin, plant secondary compounds and essential oils [28–40]. For example, supplementation with red macroalgae *Asparagopsis* spp. has been shown to reduce CH_4 emissions by upwards of 95% in vitro [41,42] and up to approximately 25–98% in ruminants *in vivo* [40,43–46]. This occurs due to the fact that these macroalgae accumulate low-molecular-weight halogenated compounds (predominantly the brominated halomethane, bromoform) [41] that inhibit the methyl-coenzyme M reductase (MCR) enzyme, which catalyzes the rate-limiting and final step of the methanogenesis pathway, in rumen archaea [47]. Similarly, approximately 20–60% reductions in CH_4 emissions have also been reported when feed was supplemented with 3-nitrooxypropanol, which also inhibits the final step in methanogenesis [17], without interfering with milk or meat production [35,36,48–53]. Although these approaches have shown various degrees of success, the use of additives tends to be costly [54] and at times impractical, with possible safety and environmental impacts, which limit their use [46,55–57]. As such,

the direct genetic manipulation of forages/feed and/or the methanogens themselves to elicit these same effects could provide valuable alternative approaches.

In this review paper, we comprehensively examine and discuss the potential of CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein)-based gene editing platforms in plants and microbes as novel options for reducing CH_4 emissions from ruminants. The development of these, and other, strategies could conceivably contribute to a reduction in the impact of milk and meat production on CH_4 emissions, and thus climate change, in the future.

2. CRISPR/Cas Gene Editing

As a means of increasing the pace and precision of genetic improvements, and with the additional advantage of potentially mitigating concerns encompassing the use of transgenic organisms, highly specific gene editing platforms that allow for the provision of mutations at pre-defined genomic loci have been rapidly gaining popularity over recent years [58]. While several gene editing systems have been developed to date [59–61], CRISPR/Cas has become the tool of choice as a result of its low cost, superior flexibility, ease with which one can multiplex and overall simplicity. The CRISPR/Cas gene editing platform is derived from bacterial or archaeal adaptive immune systems that protect mainly against bacteriophages through the genomic integration of small viral genetic fragments, which are then transcribed into small RNAs that serve as recognition molecules. These small RNA molecules avert subsequent bacteriophage invasion by guiding Cas nucleases to homologous viral DNA or RNA, which leads to their cleavage [58]. In its simplest form as a gene editing tool, the platform requires the introduction of a class 2 Cas nuclease, which elicits a double-stranded break (DSB) in DNA at a specific pre-defined chromosomal locus, as well as a single guide RNA (sgRNA) that directs Cas to the target site within the host genome, into host cells. These sgRNAs typically comprise a chimeric RNA consisting of an approximately 20 nt customizable CRISPR RNA (crRNA) that is complementary to the selected target loci and serves as a guide for Cas, as well as a fixed trans-activating CRISPR RNA (tracrRNA), which is necessary for Cas recruitment. In virtually all cases, crRNAs must be designed to anneal immediately upstream of a protospacer adjacent motif (PAM) sequence, which is a necessity for cleavage by the Cas nuclease. In the case of Cas9 from *Streptomyces pyogenes*, which is the most commonly used Cas nuclease (class 2, type II) for gene editing, the PAM consists of 5′–NGG–3′ [62], or less often, 5′–NAG–3′ [63], with the DSB generally occurring approximately 3 nt upstream [62].

The DSBs incurred by the Cas nuclease are then repaired by the host's inherent DNA repair mechanisms, including either non-homologous end joining (NHEJ) or homology-directed repair (HDR; Figure 1). In the case of NHEJ-mediated gene editing, which is error-prone and the predominant DSB repair pathway in the somatic cells of higher plants [64], small insertions or deletions (indels) are typically incurred in the target gene that can disrupt its function. Since these mutations occur through the host's own DSB repair mechanisms, in many cases, they can be indistinguishable from those transpiring spontaneously or through conventional breeding approaches [58]. Conversely, HDR-mediated gene editing, which is highly accurate and a major DSB repair pathway in many archaea [65], requires the presence of a DNA repair template and can be used to elicit the insertion of heterologous DNA or gene replacements [66,67].

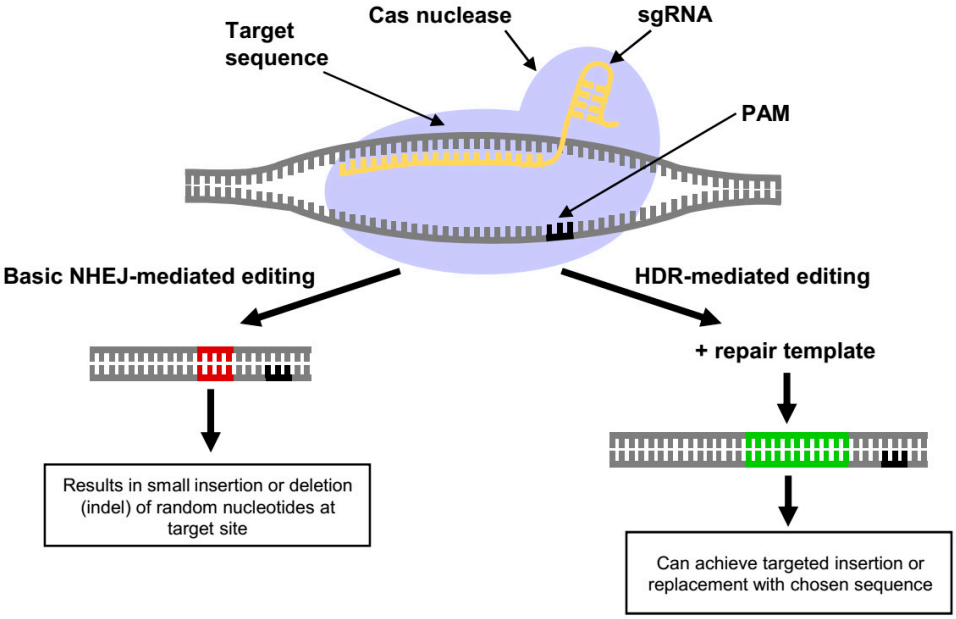

Figure 1. CRISPR/Cas-mediated gene editing platforms achieved through the targeted elicitation of double-stranded DNA breaks and NHEJ or HDR repair. HDR, homology-directed repair; NHEJ, non-homologous end joining; PAM, protospacer adjacent motif; sgRNA, single guide RNA.

Less frequently used CRISPR/Cas-based platforms also exist that make use of modified Cas enzymes (either catalytically inactive or with only nickase activity) fused to enzymes or domains with other functional capacities. These modified Cas enzymes are then guided to a selected genomic locus by the sgRNA without provoking a DSB [68]. Such platforms include, for example, cytosine and adenine base-editor systems, which allow for C-to-T or A-to-G substitutions [69,70], prime editing, which enables small, specific nucleotide changes to be made at a target locus [71,72], and epigenetic editing, which allows for the regulation of transcriptional activity [73,74]. While all of the aforementioned gene editing tools allow for the theoretical removal of the editing machinery once the edit has been achieved, other CRISPR/Cas platforms also exist that would necessitate the persistent presence of a Cas- and sgRNA(s)-encoding transgene in the host to maintain the desired outcome, including those resulting in transcriptional modulation through the direct activation or repression of target gene expression [75,76], or the cleavage of single-stranded RNA rather than double-stranded DNA [77,78]. While all of these platforms have value, those that require the enduring presence of a transgene would likely be more difficult to implement in an agricultural context due to regulatory constraints, as discussed below.

Many countries have recently modernized, or are in the process of modernizing, regulatory policies to incorporate gene editing technology into their frameworks. In the majority of countries, these updated processes apply across plants, microorganisms and animals, while in other countries, such as Canada, discussions are ongoing regarding regulatory procedures for microorganisms and animals. Gene editing-mediated improvements in organisms achieved using site-directed nucleases (SDN) such as Cas can typically be divided into three classes, including those involving the provocation of point mutations or indels through NHEJ (SDN-1; typically used for gene knock-out or knock-down), the introduction of short DNA insertions or base pair modifications using an external DNA repair template and HDR (SDN-2), and the insertion of longer DNA sequences such as a transgene (SDN-3). In terms of updated regulatory guidelines, organisms that have been modulated using SDN-1 gene editing approaches are not automatically subjected to regulation in countries such as the United States, Canada, Brazil, Argentina, Israel, Nigeria, Australia and Japan, for example [79]. Organisms bred using an SDN-2 approach, on the other hand, are only necessarily regulated in a small subset of these countries, such as Australia [80]. Finally, organisms in which an SDN-3 approach was used for improvement

are typically considered to be 'genetically modified' (GM) in most countries, and are thus subject to regulatory processes [58,79]. In contrast, the European Union, New Zealand and South Africa currently consider all forms of gene-edited organisms to be GM, regardless of the approach used [79]. This global asynchrony will almost certainly be problematic in terms of trade going forward. In addition, gene editing technologies are advancing at such a rapid rate that even updated policies may not capture recent progress in the field. As such, there is a push towards the further harmonization of regulatory guidelines that are sufficiently flexible to encompass innovations in this area [58].

3. The Genetic Modulation of Forages for Reduced CH_4 Emissions

3.1. CRISPR/Cas Gene Editing in Forages

While progress has been made in terms of enhancing various traits, including biomass production, stress tolerance and nutritional quality, using conventional breeding approaches in forage crops [81], the outcrossing and polyploid nature of many of these species complicates this process. As such, biotechnology has the potential to further our ability to elicit timely improvements in such crops. Although transgenic approaches, including over-expression and RNAi-mediated down-regulation, comprise the most common biotechnological strategies used in forages to date [54,82–85], issues related to public perception, as well as the exorbitant cost and time associated with regulatory processes, impedes the implementation of such plants in the field [58]. In contrast, most countries in which regulatory policies have been modernized to take into account gene-edited varieties have opted to treat edited plants lacking foreign DNA as they would conventionally bred plants, since the mutations elicited are derived from the plant's own DNA repair mechanisms [58]. As such, the use of CRISPR/Cas is becoming increasingly popular in many crop species, including forages [86–88]. However, in its simplest and most commonly used form in plants, which depends upon the generation of NHEJ-mediated indels within coding regions, it is mainly limited to reducing or knocking out gene function [58]. Although several promising CRISPR/Cas-mediated NHEJ-based systems have shown promise for the up-regulation of gene expression or translation in plants, including the disruption of repressor elements within target gene promoters [89] or upstream open reading frames within 5′ untranslated regions of a target gene [90], they are far more complicated to apply and require the presence of the necessary regulatory elements. Therefore, negative regulators of selected pathways, which are often less well-characterized than positive regulators, typically must be identified and targeted to elicit desired phenotypic outcomes using CRISPR/Cas-mediated gene editing.

3.2. The Potential of Forage Lipid Modulation as a Means of Reducing CH_4 Emissions from Ruminants

Feed supplementation in the form of vegetable oils or oilseeds has shown great promise for its ability to persistently reduce enteric CH_4 emissions [91–95]. The ability of lipids to reduce CH_4 emissions has been suggested to occur, at least in part, through the hydrogenation of unsaturated fatty acids, which provides an alternative sink for H_2, as well as an inhibitory effect on methanogen activity and protozoal numbers [19,91,96]. In addition, since the amount of CH_4 produced correlates with the quantity a ruminant eats, increasing the proportion of lipids in feed also reduces emissions through an augmentation in caloric density and consequent decrease in dry matter intake [97,98].

Unfortunately, the vegetative tissues of forage crops contain only very small amounts of lipids (typically 1.5–3% dry weight [DW]), which varies somewhat depending on the species [99], time of season and stage of development [99–102]. In addition, the drying of herbage, which is required for the production of hay, has been found to reduce total fatty acid contents even further [99,103]. Since dietary supplementation with vegetable oils can be prohibitively costly and impractical for producers [16], and there is also a competitive need for oilseeds for human consumption [104], it has been difficult to implement the use of such supplements on a large scale. Given that a 1% increase in the lipid content of livestock feed can reduce CH_4 emissions by 3.5% to 5.6% [96,105], and lipids also provide

more energy per unit than the other main components of plant cells (carbohydrates and proteins), the direct manipulation of forage crops themselves to accumulate lipids would be an ideal scenario in terms of economic viability for producers.

Plants contain a wide range of lipids, the composition of which diverges extensively among tissues. Unlike oilseeds, where the vast majority of lipids are in the form of the storage molecule triacylglycerol (TAG) [106], leaf lipids primarily comprise galactolipids (mainly monogalactosyldiacylglycerol [MGDG] and digalactosyldiacylglycerol), sulfolipids (mainly sulphoquinovosyldiacylglycerol) and phospholipids (mainly phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidylglycerol) that largely function as membrane constituents [107]. While only a very small proportion of TAG accumulates in plant photosynthetic tissues (generally less than 1% of total lipids) [108], it has been suggested that it plays an important role in protecting cells against oxidative damage and lipotoxicity by sequestering toxic free fatty acids [109], as well as in stress responses [110] and, to a small extent, as an energy store for periods of darkness [111].

The biosynthesis of the various lipid classes in both leaves and seeds begins with a common phase termed *de novo* fatty acid biosynthesis, which occurs in plastids and is initiated with the conversion of acetyl-coenzyme A (CoA) into malonyl-CoA via the catalytic action of acetyl-CoA carboxylase [112]. In photosynthetic tissues, the resulting malonyl-CoA is then used for the ultimate production of 16:0-, 18:0- and 18:1-acyl carrier proteins, which are then either utilized within the chloroplast for the production of various glycerolipids in the 'prokaryotic' lipid biosynthetic pathway, or are converted to free fatty acids, activated to CoA esters and exported out of the plastid for use in the 'eukaryotic' pathway that takes place on the endoplasmic reticulum (ER), where a small amount of TAG is generated in addition to other glycerolipids (Figure 2) [106]. In all plant species, a proportion of glycerolipids produced in the eukaryotic pathway in the ER are transferred back to plastids, where they serve as precursors for galactolipid biosynthesis [113]. In the majority of plants, this pathway is used exclusively for the biosynthesis of galactolipids, and due to the substrate preferences of enzymes utilized in the eukaryotic pathway, this results in a relatively high proportion of 18-carbon unsaturated fatty acids in the galactolipid fraction (these species are often referred to as '18:3 plants'). In contrast, a subset of plant species also synthesizes a proportion of galactolipids through the prokaryotic pathway in plastids, which results in a higher proportion of 16-carbon unsaturated fatty acids in their galactolipids (these species are often referred to as '16:3 plants') [113,114].

Unfortunately, with the exception of preliminary evidence suggesting that incremental increases in shoot lipid content of alfalfa (*Medicago sativa* L.) and sainfoin (*Onobrychis viciifolia* Scop.) may be possible through ethyl methanesulfonate (EMS) mutagenesis [115], progress has been very limited in this context using conventional breeding approaches, at least in part because little genetic variability exists in this trait [99]. In contrast, substantial gains in vegetative lipid contents have been achieved in plants using transgenic approaches, largely through the combinatorial manipulation of multiple genes within lipid biosynthetic and degradation pathways, as well genes involved in the formation of lipid droplets and carbon availability. For example, TAG levels upwards of 30% DW have been achieved in tobacco (*Nicotiana tabacum*) leaves, which is akin to levels typically present in oilseeds [116]. However, this level of lipid augmentation is not desirable in a forage crop, since lipids at levels higher than approximately 6–7% of dietary dry matter limit the amount of organic matter that is fermented in the rumen and/or feed intake, which can have a negative impact on livestock performance and quality [117]. Smaller increases in total fatty acids have been achieved in forage species or their close relatives, such as ryegrass (*Lolium perenne*), sorghum (*Sorghum bicolor*) and *Lotus japonicus*, through the over-expression of *DIACYLGLYCEROL ACYLTRANSFERASE 1* (*DGAT1*)/*CYSTEINE OLEOSIN*, *WRINKLED 1* (*WRI1*)/*DGAT2a*/*OLEOSIN-L*, and *MYB73*, respectively [118–120]. In addition, it has been shown that the aforementioned ryegrass lines, which exhibited approximately 6.0–6.4% total fatty acids on a DW basis (compared to approximately 3.7% in wild-type plants), led to 10–15% reductions in CH_4 production using in vitro assessments [121], which points to the potential usefulness of such an approach. However, the

resulting plants are transgenic and are thus subject to strict regulatory policies, which hinders their implementation [58].

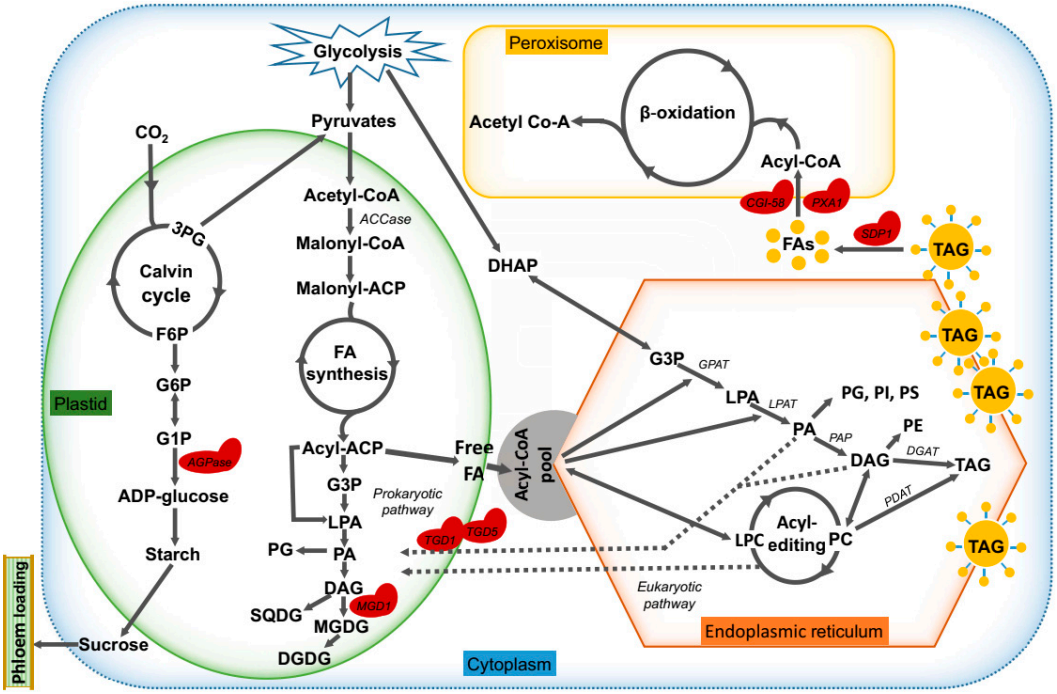

Figure 2. Simplified diagrammatic representation of lipid biosynthesis in the photosynthetic tissues of plants. Prokaryotic and eukaryotic pathways for the synthesis of lipids in photosynthetic tissues are indicated within the plastid and endoplasmic reticulum, respectively. Enzymes that have previously been reported to enhance foliar lipid content when their corresponding genes are knocked-down or knocked-out are indicated on a red background. Known reactions are denoted by solid gray lines, whereas putative events are denoted with dashed lines. Abbreviations: 3PG, 3-phosphoglycerate; ACCase, acetylCoA carboxylase; Acyl-ACP, acyl carrier protein; ADP-glucose, adenosine diphosphate glucose; AGPase, adenosine diphosphate glucose pyrophosphorylase; CGI-58, comparative gene identification-58; CO_2, carbon dioxide; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DGDG, digalactosyldiacylglycerol; DHAP, dihydroxyacetone phosphate; F6P, fructose-6-phosphate; FAs, fatty acids; G1P, glucose-1-phosphate; G3P, glyceraldehyde 3-phosphate; G6P, glucose-6-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPAT, lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcholine; Malonyl-ACP, malonyl acyl carrier protein; MGD1, monogalactosyldiacylglycerol synthase 1; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PG, phosphatidylglycerol; PXA1, peroxisomal ABC-transporter; SDP1, sugar-dependent 1; SQDG, sulfoguinovosyldiacylglycerol; TAG, triacylglycerol; TGD1/5, trigalactosyldiacylglycerol 1/5.

While there is growing interest in the use of gene editing approaches for the modulation of seed oil content [122,123], as of yet, relatively little progress has been made in terms of enhancing lipid levels in the vegetative tissues of plants using such methods. However, several negative regulators of shoot lipid accumulation have now been identified in plants [124–126], which largely function in pathways that compete with lipid/TAG biosynthesis or facilitate TAG turnover and have the potential to act as candidate targets for such endeavors in the future. While the down-regulation/mutation of genes encoding some of these regulators, such as sucrose-H+ symporter 2 (SUC2), has been found to lead to substantial phenotypic abnormalities [127], others have been shown to enhance vegetative lipid contents without substantial defects in biomass production, which makes them ideal targets for disruption via CRISPR/Cas.

One strategy that is being explored as a means of enhancing TAG contents in vegetative tissues involves reducing the proportion of substrates used for the biosynthesis of membrane lipids so that more are available for TAG production. For example, thylakoid lipid assembly in plastids requires a substantial proportion of fatty acids synthesized in both prokaryotic and eukaryotic pathways. In chloroplasts, phosphatidic acid (PA) is dephosphorylated through the catalytic action of PA phosphatase to generate diacylglycerol (DAG), which is a precursor of the predominant thylakoid membrane lipid MGDG. PA and DAG are also produced in the eukaryotic pathway on the ER, and it has been suggested that one or both of these molecules can then be transferred to chloroplasts by a group of trigalactosyldiacylglycerol (TGD) proteins, including TGD1-TGD5 located in plastid membranes [113,128]. Therefore, it is conceivable that reducing the amount of PA directed towards chloroplasts may concomitantly allow for increases in shoot TAG content. Indeed, in *Arabidopsis*, *tgd1* and *tgd5* mutants have been found to exhibit significant increases in TAG in vegetative tissues [127–129]. However, due to the importance of thylakoid membranes in plants, these mutants have been found to display a moderate reduction in growth [128,130]. A similar phenomenon was also observed in tobacco and *Arabidopsis* whereby the *MONOGALACTOSYLDIACYLGLYCEROL SYNTHASE 1* gene (*MGD1*), which encodes an enzyme that catalyzes the conversion of DAG to MGDG, was down-regulated or mutated [131,132]. Furthermore, the down-regulation of a *TGD5* homolog in *Medicago truncatula*, which is a close diploid relative of alfalfa, did not significantly impact shoot TAG accumulation in transient assessments using virus-induced gene silencing (VIGS) [126]. Taken together with the fact that increasing TAG at the expense of other lipids would likely not lead to increases in total lipid content, these findings indicate that such a strategy may not be suitable for CRISPR/Cas-based applications in forages.

An alternative approach would be to enhance the availability of substrates for overall lipid biosynthesis in vegetative tissues by reducing the proportion of carbon used for the production of non-lipid-related compounds in plant cells, which would theoretically make additional carbon available for lipid biosynthesis. For instance, starch and oil biosynthesis pathways compete with one another for assimilated carbon precursors in plants, and the disruption of key enzymes involved in the starch biosynthetic pathway has been found to enhance lipid levels in vegetative tissues previously. In the starch biosynthetic pathway, the rate-limiting step comprises the conversion of glucose-1-phosphate (G1P) to adenosine diphosphate glucose (ADP-glucose), which acts as the glucosyl donor for starch synthases, via the catalytic action of ADP-glucose pyrophosphorylase (AGPase) [133]. In *Arabidopsis*, the mutation/down-regulation of the gene encoding the catalytic isoform of the small subunit of AGPase led to significant increases in vegetative TAG compared to the wild type [127,134], while its VIGS-mediated down-regulation in *M. truncatula* resulted in a significant elevation in total shoot lipid content upwards of 4.5% DW [126]. In addition, although there is some evidence that the mutation of these genes may lead to a slight reduction in growth and early flowering, at least under short days, in *Arabidopsis*, these alterations appear to be species and/or isoform-specific [127,135]. For example, rice, maize, barley and potato with disrupted AGPase activity have been found to display normal vegetative growth with morphological abnormalities only present in certain instances in starch-accumulating organs, such as grains or tubers [136–140]. Mutants deficient in AGPase also tend to exhibit substantial increases in soluble carbohydrate levels at the expense of starch [127,141,142], which may have the potential to reduce the incidence of ruminal acidosis [143] and further decrease CH_4 emissions [144]. However, such an inverse correlation between starch and lipid levels has not always been observed when this gene is disrupted/down-regulated on its own [142], and in many cases, it appears that this strategy instead provides an additive contribution to enhancements in vegetative lipid content when combined with modifications in a variety of other genes [127,134,142,145]. As such, further research on forage species is required to determine the suitability of such an approach for reducing CH_4 emissions and improving livestock health in the future.

Reducing the breakdown of TAG has also been demonstrated as a potential strategy for eliciting enhanced lipid accumulation in vegetative tissues. For instance, the mutation of *SUGAR DEPENDENT1* (*SDP1*), which encodes a patatin-like acyl-hydrolase domain-containing lipase that hydrolyzes TAG into glycerol and free fatty acids [146], has been found to enhance TAG content in *Arabidopsis* vegetative tissues [109,125,142]. Similarly, the mutation of *PEROXISOMAL ABC TRANSPORTER 1* (*PXA1*), which functions in the transport of fatty acids into peroxisomes for β-oxidation, as well as *COMPARATIVE GENE IDENTIFICATION-58* (*CGI-58*), which acts cooperatively with *PXA1* to facilitate lipid turnover [147], in *Arabidopsis* has also been found to lead to significant increases in TAG accumulation in vegetative tissues [124,147]. In line with this, the VIGS-induced down-regulation of *SDP1* and *PXA1* homologs in *M. truncatula* prompted up to 40% increases in total shoot lipid contents [126]. Furthermore, while both *sdp1* and *pxa1 Arabidopsis* mutants displayed detriments in seed germination and early post-germinative growth in the absence of sucrose, they are otherwise similar to the wild type in their growth following the onset of photosynthesis [146–148], and no obvious phenotypic effects have been noted in *cgi-58* mutants to date [124,147]. It is possible that the germination/post-germinative deficits apparent in *Arabidopsis sdp1* and *pxa1* mutants are specific to oilseed species due to the fact that these processes rely upon the breakdown of TAG as an energy source prior to the initiation of photosynthesis in young seedlings [146], and as such, these same consequences may not be apparent in other plant groups, such as legume or grass forages. Taken together, this suggests that all three genes may provide interesting candidates for CRISPR/Cas-mediated mutation in forages.

Furthermore, a combinatory approach involving the simultaneous disruption/down-regulation of genes encoding AGPase subunits and SDP1/PXA1 may provide even further increases in vegetative TAG/lipid contents as has been demonstrated in *Arabidopsis* and potato previously [142,149]. Similarly, the deletion of a region immediately upstream of *DGAT2*, including a non-essential photosynthesis-related gene and the *DGAT2* promoter, using CRISPR/Cas9 with dual sgRNAs in the *Arabidopsis sdp1* mutant allowed the *DGAT2* coding sequence to fall under the control of the more highly expressed upstream gene. This led to the up-regulation of *DGAT2* expression in leaves [150]. Since *DGAT2* encodes a key enzyme of acyl-CoA-dependent TAG biosynthesis [151], this novel NHEJ-mediated gain-of-function gene editing approach allowed for the doubling of total leaf lipid contents [150], which would be ideal in a forage crop for ruminant production. However, it is possible that these combinatorial approaches could be associated with a decline in biomass production [142,150], although in the latter case, this decrease appears to be minor and further research is required to determine whether it is significant under field conditions.

While all of these strategies hold promise for enhancing lipid content in forage shoots, which could feasibly mitigate CH_4 emissions from the ruminants that feed on them, there is some evidence that not only lipid content, but also lipid composition, may play a role in such an outcome. For example, medium-chain and unsaturated fatty acids appear to provide the greatest reduction in CH_4 emissions, in the latter case potentially through their ability to act as electron acceptors during biohydrogenation [152,153]. Therefore, it is likely that fatty acid compositional changes in edited genotypes also need to be taken into consideration in this context.

3.3. The Potential of Other Forage Traits to Reduce CH_4 Emissions from Ruminants

In addition to the modulation of forage shoot lipid content/quality, there is evidence that several other forage traits may also have potential in terms of mitigating CH_4 emissions from ruminants. For example, forages containing condensed tannins (CTs), which are polyphenolic secondary metabolites that bind proteins and thus slow down their degradation in the rumen and also function in plant defense and stress response mechanisms [154], have been shown to reduce CH_4 emissions in ruminants in vivo [155,156]. Although the mechanisms driving CH_4 mitigation in this case are not well-understood, they may derive, at least in part, from a decline in fiber digestibility, an ability to act as

a H_2 sink and/or the direct inhibition of methanogens [157,158]. However, results have been highly inconsistent among studies [154]; it is a phenomenon that has been suggested to be attributable to differences in CT composition occurring within and among species, as well as environmental effects [159–161] and the co-presence of other secondary plant metabolites [162]. Unfortunately, relatively little is currently known regarding such effects, and further research is necessary to advance our understanding of CTs in forage crops. Similarly, increasing the digestibility of forages has also been suggested as a possible means of mitigating CH_4 emissions from ruminants [163]. As is the case with CTs, however, the effect has been rather inconsistent among studies [164], and since enhanced digestibility tends to increase dry matter intake, CH_4 emissions have been found to increase in certain cases [163].

4. The Genetic Modulation of Rumen Methanogens for Reduced CH_4 Emissions

4.1. CRISPR/Cas Gene Editing in Archaea

The *S. pyogenes*-derived CRISPR/Cas9 platform has been shown to lead to the successful introduction of insertions (knock-in) and deletions (knock-out) through HDR with high efficiency in the methanogen, *Methanosarcina acetivorans*, without any evidence of off-target mutations [165]. Furthermore, as is the case in plants, once editing had been achieved in the target gene, plasmids bearing the gene editing machinery could successfully be removed from edited strains [165]. While this means that the resulting knock-out strains lacked foreign DNA, the same was not the case for knock-in strains, as a DNA fragment was inserted into the genome in this instance. The fact that transformation efficiencies were thousands of times higher when a DNA repair template was used corroborates that HDR is the preferred DNA repair mechanism in archaea, with NHEJ-mediated repair being extremely rare [65]. However, the co-introduction of NHEJ machinery from the closely related *Methanocella paludicola*, which is the only archaeal species in which a complete NHEJ complex has been detected thus far [166], also allowed for the efficient provision of targeted deletions without any need for a repair template [165].

Since most archaea encode adaptive CRISPR/Cas-based immune systems, another strategy for achieving genome editing is to make use of their endogenous apparatus. This means that only sgRNA(s) and DNA templates would need to be introduced to elicit HDR [167]. In line with this, endogenous class 1, type I and III CRISPR/Cas systems have been successfully used to introduce deletions, insertions and point mutations in the genome of the thermophilic archaeon, *Sulfolobus islandicus* [168], and it is feasible that a similar strategy would apply across all archaea that encode active CRISPR/Cas systems. To date, native CRISPR/Cas systems have been reported in multiple *Methanobrevibacter* strains, including *M. arboriphilicus* ANOR1 and *M. oralis* JMR01 [169,170], which suggests that such an approach would be valuable for the modulation of rumen methanogens.

4.2. Potential CRISPR/Cas-Based Approaches in Rumen Methanogens to Mitigate CH_4 Emissions

The microbial community in the rumen is one of the most diverse gut ecosystems in the animal kingdom, including archaea, bacteria, protozoa, fungi and a largely unknown virome [171]. Although gaps remain in our understanding of these communities at present, the recent introduction and integration of metagenomic, transcriptomic, proteomic and metabolomic techniques provides the possibility of acquiring a systems-level understanding of microorganism species composition in the rumen and the role of such associations on host metabolism [172–175].

Archaea are the second most abundant microbial community in the rumen, of which approximately 77% are hydrogenotrophic methanogens, which reduce CO_2 to CH_4 with H_2 as an electron donor, and 22% are methylotrophic methanogens, which instead make use of small methylated carbon compounds [176,177]. While the two most prominent groups of rumen methanogens are *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium*, with other genera such as *Methanomicrobium* contributing smaller proportions, an animal's diet can influence their abundance in the rumen [176,178]. Interestingly, the

methanogens *Methanobrevibacter* and *Methanomicrobium* can also form a symbiotic relationship with protozoa [179], allowing for the H_2 generated by fermenters to be utilized by methanogens, which in turn keeps H_2 concentrations low and favors the metabolism of the fermenters [180].

Recent studies have also revealed that rumen microbial population structure and function are influenced not only by nutritional factors, but also by host genetics [174,181]. For example, an examination of the association of host genetics with the phylogenetic and functional composition of the rumen microbiome led to the identification of 22 operational taxonomic units (OTUs) whose abundances were associated with rumen metabolic traits and host physiological traits with measurable heritability [182]. Similarly, genome-wide association analysis of a common set of single-nucleotide polymorphisms found in cattle genomes and corresponding rumen bacterial community composition led to the identification of OTUs, families and phyla with high heritability [183]. Although our understanding of how a stable rumen microbial community assembles itself and what factors affect microbial composition/function is still limited, these findings indicate that certain rumen microbial features are heritable.

The availability of the *M. ruminantium* genome sequence [184] and the recent prediction of its operome functional properties [185], along with the fact that the biochemistry of methanogenic pathways is well-known [186,187], are opening up new possibilities of using genetic strategies to modulate CH_4 production in the rumen. This highlights the possibility of further improving animal production efficiency and mitigating the emission of GHGs from ruminants using gene editing strategies on rumen archaea in vitro, and subsequently re-introducing them into the rumen (Figure 3). While such an approach holds promise, further research is essential to gain a better understanding of the key microorganisms involved in rumen methanogenesis, as well as their activities, to successfully manipulate rumen processes in this manner.

As is the case with plants, one of the biggest challenges of using gene editing in rumen methanogens to reduce CH_4 emissions is the identification of suitable target genes, which can be difficult due to the intricacy of the methanogenesis process. Indeed, targeting an inappropriate gene would not only be ineffective, but could also lead to the generation of less competitive methanogens in the rumen microbiome or even be lethal. Intriguingly, it has been shown that the transcript abundance of genes encoding enzymes involved in the hydrogenotrophic methanogenesis pathway is significantly lower in the rumen of sheep producing low CH_4 emissions than in those of sheep with high CH_4 emissions [188]. The largest transcriptional differences were apparent in genes that encode the key MCR enzyme [188], which is found in all methanogens and is essential for their growth [189]. Although such differences in the transcriptomes of rumen methanogens between ruminants with low and high CH_4 emissions could arise due to a variety of reasons, one can speculate that the down-regulation of particular genes involved in hydrogenotrophic methanogenesis could be a useful strategy to reduce CH_4 emissions. Unfortunately, deletions introduced through HDR-mediated CRISPR/Cas in genes encoding an MCR and a heterodisulfide reductase (hdrED), which is another essential component of methanogenesis in methylotrophic pathways, in *M. acetivorans* yielded very few transformants, indicating the lethality of these genetic modifications [165]. In contrast, deletions in genes encoding monomethylamina-specific methyltransferases (mtmCB1 and mtmCB2), which enable methylamine utilization in the methylotrophic pathway, led to the production of thousands of transformants [165], which suggests that the knockout of methyltransferase genes may be a superior target choice in the context of reducing CH_4 production in the rumen, at least in the case of ruminant methanogens that utilize the methylotrophic pathway.

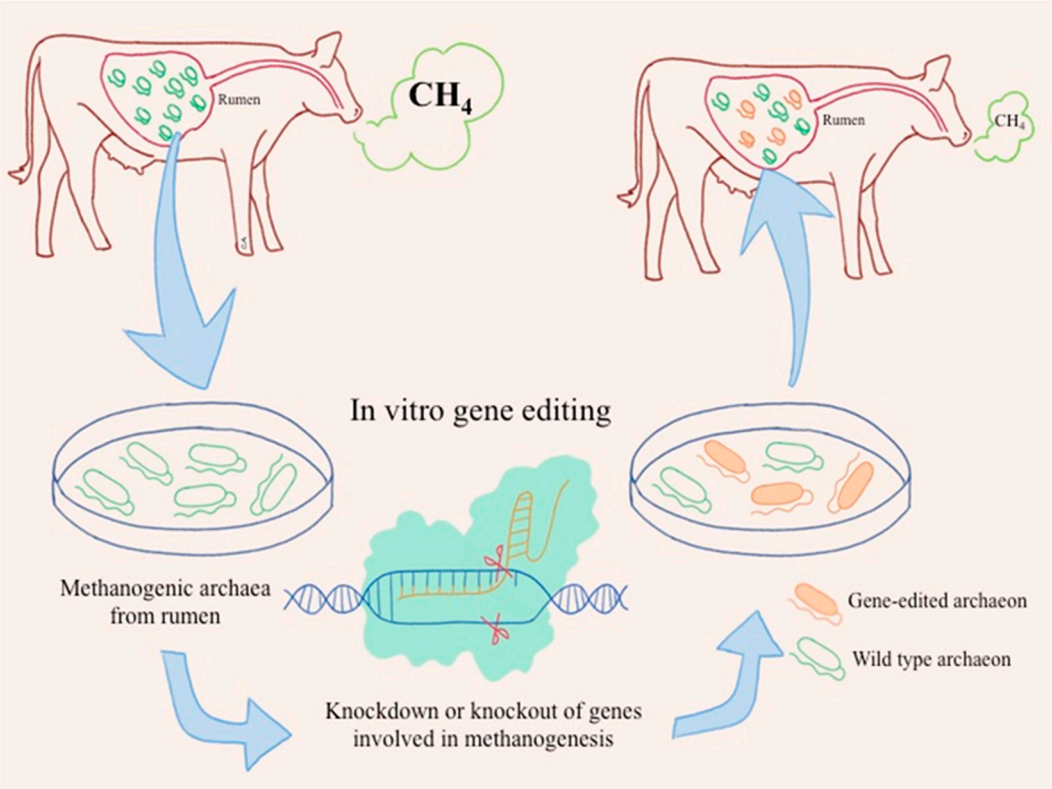

Figure 3. Possible in vitro approach for the gene editing of rumen archaea. Methanogenic archaea would be collected from the rumen and cultured in vitro, at which point genes involved in methanogenesis could be knocked down or knocked out using genome editing technologies such as CRISPR/Cas9. The gene-edited archaea would then be replicated in vitro and reintroduced into the rumen for repopulation. As long as the edited archaea were competitive within the rumen population, the expected result would be a reduction in the production of CH_4 from the ruminant.

In addition to knock-outs achieved through gene interruption, reductions in gene expression can also be achieved using a CRISPR interference system (CRISPRi), which is achieved without eliciting any break in DNA strands and may provide benefits when targeting essential genes such as *MCR*. For example, the expression of a target gene can be repressed using an inactive Cas9 (with no endonucleolytic activity; dCas9), which together with an sgRNA binds to the target DNA sequence of a protein-coding region to block RNA polymerase and transcript elongation [190,191]. In *M. acetivorans*, such a CRISPRi system has been stably integrated into the genome or converted into a replicating plasmid, resulting in up to a 90% reduction in target gene transcript abundance [192]. A similar, but alternative, approach to the use of dCas9 for CRISPRi is to make use of an endogenous Cas protein that targets RNAs rather than double-stranded DNA for cleavage, such as in the class 1 type III-B CRISPR/Cas system [193] that can be found in at least certain archaea [194]. This approach has been successfully applied in *Sulfolobus solfataricus* to down-regulate specific RNAs by taking advantage of an endogenous endonuclease along with an introduced guide crRNA and could theoretically also be applicable in other archaea [195,196]. However, with all types of CRISPRi, the editing machinery and/or gRNA are required long-term in the archaeal strain to maintain the knock-down effect, and the transgenic nature of the resulting strains in the rumen of live cattle could potentially hinder implementation, at least in the short term.

5. Conclusions

Given the considerable role of rumen methanogens in the emission of GHGs, it will be of paramount importance in coming years to target this process for improvement as a contri-

bution towards mitigating the worst climate change scenarios. While this will likely entail a multi-pronged approach involving livestock genetic factors and possible supplementation, the direct CRISPR/Cas-based manipulation of forages and rumen methanogens could conceivably provide two of a combination of strategies with additive effects to achieve such an outcome. As such, these approaches warrant further study to determine their efficacy in this context and also to strike a balance among effectiveness, methanogen or plant competitiveness, animal performance, consumer acceptance and ease of implementation. Although regulatory processes could conceivably impede the global adoption of gene-edited archaea and crops due to discrepancies among nations, it is likely that those generated using SDN-1 and/or SDN-2 approaches will be treated as non-GM in the vast majority of countries. Considering the opportunities that gene editing can offer to mitigate GHG emissions in an agricultural context, a regulatory alignment among countries will be of utmost importance to safely stimulate innovation and avoid further trade barriers in the future.

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