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Methanogenesis in animals with foregut andhindgut fermentation: a review

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Abstract. Methane is the main greenhouse-gas contributor to global warming in the livestock sector; it is generated by anaerobic fermentation in the different sections of the gut, and the methane concentration differs significantly among species. Methane is produced only by certain types of microorganisms called methanogens. The species composition of methanogenic archaea population is largely affected by the diet, geographical location, host and the section of the gut. Consequently, methane production, either measured as total grams emitted per day or per bodyweight mass, differs greatly among animal species. The main difference in methanogenic activity among different gut sections and animal species is the substrate fermented and the metabolic pathway to complete anaerobic fermentation of plant material. The three main substrates used by methanogens are CO_2, acetate and compounds containing methyl groups. The three dominant orders of methanogens in gut environments are Methanomicrobiales, Methanobacteriales and Methanosarcinales. They normally are present in low numbers (below 3% of total microbiome). The present review will describe the main metabolic pathways and methanogens involved in CH_4 production in the gut of different host-animal species, as well as discuss general trends that influence such emissions, such as geographical distribution, feed composition, section of the gut, host age and diurnal and season variation. Finally, the review will describe animal species (large and small domestic ruminants, wild ruminants, camelids, pigs, rabbits, horses, macropods, termites and humans) specificities in the methanogen diversity and their effects on methane emission.

Additional keywords: digestive compartments, emission, methane production, methanogens, microbiota.

Introduction

Global warming is one of the main challenges in the world. The current predictions indicate that global temperatures will increase between 1^{O}C and 6^{O}C during the 21st century (IPCC 2015), primarily as a result of the accumulation of greenhouse gases in the atmosphere. Methane contributes to 16% of the greenhouse- gas emissions and is particularly aggressive due to its high warming potential, which is 21 times greater than that of carbon dioxide (Scheehle and Kruger 2006). Approximately 36% of the total methane emissions come from natural sources such as wetlands, oceans, termites and wild ruminants, while the remaining 64% originate from anthropogenic sources, including fossil-fuel use, livestock farming, landfill and rice agriculture (Bousquet *et al.* 2006). This human activity has promoted a two- fold increase on the methane concentration over the past 150 years, because its emissions have burdened the natural sinks on Earth (IPCC 2015). Enteric fermentation in farm animals is, after fossil-fuel use, the second-most important source of methane, representing 27% of the anthropogenic methane emissions (Bousquet *et al.* 2006). Hence, there is a need for understanding the complexity of the methanogenesis process in the gut and the methane-producing archaea, so as to develop cost-effective methane-mitigation strategies (Buddle *et al.* 2011). The present paper aims to provide a comprehensive review on the main biochemistry mechanisms in the methanogenesis, a description of the key microbes involved in this process and a selection of the main drivers that modulate the gut methanogenesis, such as the section of the gut, animal age, geographical location, diet or animal species considered.

Methanogenesis and methanogens

Methane is generated in the gastrointestinal tract as the end product of anaerobic respiration by a specialised group of microorganisms, the methanogens (Janssen and Kirs 2008). All methanogens are strictly anaerobic archaea belonging to the *Euyarchaeota* phylum and obtain most of their energy from methanogenesis. The methanogenesis pathway is complex and requires unique coenzymes and membrane-bound enzyme complexes (Hedderich and Whitman 2006). Although methanogens are phylogenetically diverse, they can use only a limited number of substrates. These substrates are restricted to the following three mayor types: CO_2, acetate and compounds containing methyl groups (Liu and Whitman 2008). Most organic compounds such as carbohydrates and volatile fatty acids (VFA) are not substrates for methanogens and must be

processed by other microbes (bacteria, protozoa or fungi) prior to their utilisation by methanogens. Thus, in all gut environments, most of the available energy is used by non-methanogenic organisms.

The first type of substrate used by methanogens is carbon dioxide (CO_2), because most methanogens can reduce CO_2 to methane, with H_2asthe primaryelectrondonor(hydrogenotrophic way). Many hydrogenotrophs can also use formate as an electron donor by the activity of the formate dehydrogenase (Table 1). In hydrogenotrophic methanogenesis, CO_2 is successively reduced to methane through formyl, methylene and methyl, forming C-1 moiety in which methyl coenzyme M reductase catalyses the last step of this metabolic route (Hedderich and Whitman 2006). Two methanogen species can also utilise carbon monoxide (CO) as reductant for methanogenesis from CO_2, by using CO dehydrogenase. However, growth with CO is slow and the generation time is more than 200 h for *Metanothermobacter thermoautoprophicus* and 65 h for *Metanosarcina barkeri* (Liu and Whitman 2008). Also *Methanosarcina acetivorans* can use CO for growth but by an entirely different and unconventional pathway (Rother and Metcalf 2004). Some hydrogentotropic methanogens can also oxidise secondary alcohols (i.e. propanol, butanol and cyclopentanol) and few species can use ethanol as an electron donor (Liu and Whitman 2008). These species represent an anomaly in the general rule, which is that methanogens cannot directly metabolise organic compounds.

The second type of substrate mainly used by methanogens is methyl group-containing compounds such as methanol, methylamines and methylsufides. These methyl groups are transferred to a cognate corrinoid protein and, subsequently, entered into the methanogenesis pathway via coenzyme M, to be further reduced to methane (Ferguson *et al.* 2000). Activation and transfer of the methyl group requires a substrate-specific methyltransferase. Methylotrophic methanogens are limited to the order Methanosarcinales, except for *Methanosphaera* species (order Methanobacteriales). In the methylotrophic methanogenesis, three methyl groups are reduced to methane for every molecule of CO_2 formed, aspect which is considered an imbalance. Nevertheless, *Methanomicrococcus blattioca* and *Methanosphera* species are obligated methylotrophic and hydrogenotrophic methanogens that are specialised in reducing methyl groups with hydrogen (H_2).

The third type of substrate is acetate, which is highly abundant in most anaerobic fermentations. As a result, up to two-thirds of the biologically generated methane is derived from acetate (Liu and Whitman 2008). Surprisingly, only two genera are known to use acetate for methanogenesis, namely,

Methanosaeta and *Methanosarcina*. They conduct an aceticlastic reaction that catalyses acetate, oxidising the carboxyl-group to CO_2 and reducing the methyl group to methane (Fig. 1).

Despite their limited number of substrates, methanogens are highly diverse and their classification includes five orders (Methanobacteriales, Methanomicrobiales, Methanosarcinales, Methanococcales and Methanopyrales), which differ in more than 82% of their 16S rRNA sequence identity, although recently more orders (i.e. Methanocellales and Methanomassiliicoccales) have been included. These orders have some common features such as their capacity to fluoresce blue-green as a result of the presence of the coenzyme F_{420} (Ashby *et al.* 2001) or the absence of peptidoglycan in the cell wall, but also differ on several biological aspects related to the structure of the cell wall, core lipids or substrates used.

(1) Methanobacteriales. Most members of Methanobacteriales use CO_2 and H_2 (hydrogenotrophic), but several species can also use formate, CO or secondary alcohols as electron donors. Most genera are rods of variable length (0.6–25 mm) that form filaments. Their cell-wall composition includes pseudomurein and their cellular lipids contain chaeol, archaeol and hydroxyarcheol as core lipids. Within this order, the main genera of interest in the gut methanogenesis are *Methanobacterium*, *Methanobrevibacter* and *Methanosphaera* (Hook *et al.* 2010).

(2) Methanomicrobiales. Members of this order use CO_2 and H_2 (i.e. they are hydrogenotrophic); most species can use formate and some can also use secondary alcohols as electron donors. The genera in this order vary in motility and shape from cocci to rods and sheathed rods. Most species have protein cell walls and some have glycoproteins. The cellular lipids contain archaeol and caldarchaeol. There are three families, with Methanomicrobiaceae being the only one of interest in the gut methanogenesis (Liu and Whitman 2008).

(3) Methanosarcinales. Members of this order are the only methanogens with cytochromes, which are membrane-bound electron carries that play a role in the oxidation of methyl groups to CO_2. Thus, species in the order Methanosarcinales have the widest substrate range, including a methyl group (methylotrophs), acetate (aceticlastic) or CO_2 and H_2 (hydrogenotrophic). Cytochromes are membrane-bound electron carries that play a role in the oxidation of methyl groups to CO_2. They all are non-motile and vary in morphology from cocci to pseudosarcinae and sheathed rods. Most species have protein cell walls and the cellular lipids contain archaeol, hydroxyarchaeol and caldarchaeol. The main genera of

interest in gut methanogenesis are the following: *Methanosaeta* is a specialist that uses exclusively acetate, even when it is available at low concentrations (5–20 mM), due to the presence of a high-affinity adenosine monophosphate. On the contrary, *Methanosarcina* is more of a generalist and prefers methanol and methylamine to acetate, unless it ispresent at high concentrations (1 mM), due to its low-affinity acetate kinase (Jetten *et al.* 1992). These microbes are particularly relevant on the methanogenesis from manure.

(4) Methanococcales. Hydrogenotrophs in this order produce methane by using CO_2, formate and H_2 as electron donors. Cells are small cocci (1–3 mm) and motile because of flagella. Cell wall has S-layer proteins and lacks glycoproteins and carbohydrates. All species of *Methanococcales* have been isolated from marine habitats and have no relevance in gut methanogenesis.

(5) Methanopyrales. This order is represented by a single hydrogenotrophic species, *Methanopyrus kandleri*. It is motile, rod-shaped, its cell wall contains pseudomurein and lipids contain archaeol. This hypertermophilic species inhabits only marine ecosystems (Liu and Whitman 2008).

(6) Methanocellales. This is a recently described order of methanogens that was initially identified in rice-field soil (Sakai *et al.* 2008). Methanocellales bacteria are unique among methanogens in their tolerance to O_2 stress (Yuan *et al.* 2009; Angel *et al.* 2011) and their adaptation to low H_2 partial pressures (Sakai *et al.* 2009).

(7) Methanomassiliicoccales. Methanomassiliicoccales is phylogenetically distant from all other orders of methanogens and is related to non-methanogenic archaea such as Thermoplasmatales (Tajima *et al.* 2001). All culture- based studies have agreed on a common methanogenic pathway relying on the obligate dependence of the strains on an external H_2 source to reduce methyl compounds into methane. Methanomassiliicoccales constitutes one of the three dominant archaeal lineages in the rumen (Janssen and Kirs 2008) and, in some ruminants, it represents half or more of the methanogens (Wright *et al*. 2007). This order is broadly distributed, and not limited to digestive tracts of animals, but is also retrieved in rice paddy fields, natural wetlands or freshwater sediments (Borrel *et al.* 2014).

(8) Others. Culture-independent approaches have helped discover the existence of methane-metabolising microorganisms outside the phylum Euryarchaeota. These findings comprise

uncultured methanogens belonging to phyla Bathyarchaeota (Evans *et al.* 2015) and Verstraetearchaeota (Vanwonterghem *et al.* 2016), both from the branch TACK (Proteoarcheota; Spang *et al.* 2017).

Methanogens are generally present in the gut in low numbers compared with the rest of microorganisms (<3%; Morgavi *et al.* 2010), possibly because they outcompete with sulfate-reducing, denitrifying and iron-reducing bacteria for H_2 when electron acceptors (other than CO_2) are present in the system (O_2, NO_3^-, Fe^{3+} or SO_4^{2-}). This situation probably occurs because these compounds are better electron acceptors than is CO_2, or thermodynamically more favourable. However, in gut fermentation systems, CO_2 is rarely a limiting factor. In addition to methanogens, homoacetogenic bacteria are also able to reduce CO_2 for energy production. These microbes reduce CO_2 by using H_2 or other substances such as sugars, alcohols, methylated compounds, CO and organic acids during the acetogenesis process. Nonetheless, acetogenesis by H_2 is thermodynamically less favourable than is methanogenesis. This reason may explain the predominance of methanogens in most gut environments, whereas the activity of homoacetogens contributes to explain the importance of acetate as an end product of the gut microbial fermentation. On the contrary, homoacetogens outcompete methanogens in specific gut environments, such as in the hindgut of termites and cockroaches, possibly as a result of their metabolic versatility and lower O_2 sensitivity (Liu and Whitman 2008).

In anaerobic gut environments, a proportion of the feed is degraded to fermentation products, as a result of the cooperation of multiple microbial groups. Plant structural carbohydrates, proteins and other organic polymers contained in feeds ingested by the animal are degraded to their monomer components by the so called primary anaerobic fermenters (i.e. bacteria, fungi and protozoa). These monomers are then further converted into VFA, lactate, ammonia, CO_2 and H_2 by both the primary fermenters and other microbes that do not have the capacity to hydrolyse complex polymers by themselves (secondary fermenters or syntrophs). Finally, methanogens catalyse the terminal step in the anaerobic fermentation by converting these latter substrates (CO_2, H_2, acetate) to methane. However, the activity of the syntrophs is favourable only at low H_2partial pressures; thus, the H_2-scavenging conducted by the methanogens is vital to maintain H_2 pressures below 10 Pa (Hedderich and Whitman 2006). The interaction and inter-dependency between H_2-producing microorganisms (i.e. bacteria, protozoa and fungi) and H_2-consuming methanogens is named interspecies H_2 transfer.

Factors affecting methanogenesis

(a) *Geographical distribution*

Methanogen diversity can be affected by the inter-animal variation, diet, geographical region, gut sampling and methodology used (Wright *et al.* 2007; Jeyanathan *et al.* 2011). There are some reports on the geographical distribution of methanogens, most of them by Wright and colleagues (Wright *etal.* 2004, 2006, 2007), suggestingthatbothdietandgeographical location of the mammal host may play an important role in moulding the methanogen population. In a large experiment, Henderson *et al.* (2015) analysed 742 individual animals from around the word, including 32 ruminant species, and a wide range of diets, and found *Methanobrevibacter gottschalkii* and *M. ruminantium* species in almost all samples, accounting for 74% of all rumen archaea. The most abundant methanogens found were *Methanosphaera* sp. and two Methanomassiliicoccaceae-affiliated groups, which accounted for 15% of the archaeal community. *Methanomicrobium* has been reported as an abundant (>5%) methanogen in Asia, but it is not universally prevalent. Thus, nearly 90% of the rumen methanogens belonged to the five most dominant groups. These observations suggest that rumen methanogens are much less diverse than are rumen bacteria in terms of taxonomy, but also in the range of substrates they use (Sharp *et al.* 1998). This universality, together with their limited diversity compared with bacteria (Seedorf *et al.* 2015), opens the possibility of developing small-molecule inhibitors to be used as successful methane (CH_4)-mitigation agents across the globe, as described before (Hristov *et al.* 2015).

(b) *Effect of feed composition*

Composition of diet influences CH_4 production in ruminants, since fermentation in the rumen depends entirely on the activity of microorganisms, which require a variety of nutrients, energy, nitrogen and minerals (Moss 1994). Thus, the quality of the diet exerts a clear effect on the activity of the rumen microbiota and, hence, production of metabolites, such as CH_4, in the rumen. Enteric CH_4 emission is highly reliant on diet composition and tends to decrease with a high protein content in ruminants, while the reverse occurs when fibre content is increased (Johnson and Johnson 1995; Kurihara *et al.* 1997). When dairy cows were fed on high-forage diets, CH_4production (per kilogram of dry-matter intake, DMI) was 35% higher than when cows were given on high-concentrate feed (Kurihara *et al.* 1997), because the amount of fermented cellulose contributed to CH_4 production more than did the amount of other carbohydrate components (Moe and Tyrrell 1979). Methane production from high-concentrate feed is lower than that from high-forage diet fed at near maintenance (Lovett *et al.* 2003). It is well known that forage type and quality affect the activity of rumen microbes, and,

thus, the CH_4 generation from the rumen. Kurihara *et al.* (1995) showed that CH_4 production from cows fed Italian ryegrass hay was lower than that from cows given maize silage. Forages that are highly digestible stay in the rumen for a shorter time, due to the high passage rate, while forages of lower digestibility stay in the foregut comparatively longer and, consequently, lead to higher CH_4 emissions. Methane emissions from animals being fed on leguminous forages is reported to be lower than those from animals feeding on grasses, because legumes promote a higher intake and production from the animals (Ramírez-Restrepo and Barry 2005).

Several studies have been conducted on the effect of dietary fibre (DF) on the CH_4 produced in the gastrointestinal tract of pigs (Jørgensen *et al.* 1996; Varel and Yen 1997; Bindelle *et al.* 2008). DF stimulates microbial species within the complex cellulolytic methanogens (Miller and Lin 2002). Moreover, the reductive activity (H_2) released during the fibre degradation is used by methanogens to reduce CO_2 to CH_4 (Seradj *et al.* 2014). However, such relationship is masked by the promotion of sulfate-reducing bacteria in the hindgut (Lin and Stewart 1997), which compete with methanogens for the substrate (H_2); such competitive relationships between both communities do occur (Ward and Winfrey 1985), although it has been scarcely addressed in the existing literature (Lin and Stewart 1997).

Utilisation of DF is very dependent on the microbiota already present in the hindgut, and, hence, on the development of the GI tract. Both the concentration and synthesis rate of CH_4 are low in caecum and the proximal colon, but they increase gradually in the posterior segments of the hindgut (Jensen and Jørgensen 1994). On the basis of their results, Jensen and Jørgensen (1994) hypothesised that only small net amounts of H_2 were produced in caecum and proximal colon, even though they found a higher microbial activity in these segments and also taking into account that H_2 production is an obligate contributor of anaerobic fermentation in the hindgut.

Pigs fed diets high in neutral detergent fibre tend to emit more CH_4, but no relevant changes in methanogen concentrations have been detected (Seradj *et al.* 2018); indeed, the link between abundance of methanogens and CH_4 formation in the hindgut ecosystem remains unclear and some results would confirm previous assays conducted by Cao *et al.* (2013, 2016), which showed that the availability neutral detergent fibre improved the diversity, but not abundance, of methanogens.

The extra CH_4 production from aceticlastic archaea may also bias the relationship between fibre availability and CH_4 generation. Presence of the aceticlastic methanogens has been demonstrated previously (Smith and Ingram-Smith 2007); both *Methanosarcina* and *Methanosaetas* species use acetate as a substrate for methanogenesis, but only the latter is known to be specific for acetate utilisation.

Crude protein supply does not seem to alter CH_4 emissions in pigs (Seradj *et al.* 2018), in line with other studies in which no significant differences were detected by lowering the crude protein content in the diet (Le *et al.* 2009; Atakora *et al.* 2011; Osada *et al.* 2011). However, the synchronic competition between methanogens and some ammonia-degrading species cannot be discarded, such as *S. Ruminantium* (Saengkerdsub and Ricke 2014). This competitive mechanism has been described in human gastrointestinal tract where some methanogenic species (*Methanobrevibacter smithii*) compete against the prominent saccharolytic species *B. thetaiotaomicron* for NH_4 assimilation through an upregulation of the ATP-dependent glutamine synthetase–glutamate synthase pathway (Samuel *et al.* 2007). *S. ruminantium* also possesses these ammonia-fixating pathways (Ricke and Schaefer 1996).

(c) *Sections of the digestive tract*

The rumen is considered to be the main and most important fermentation chamber in the ruminant animal, whereas microbial breakdown of substrates in the caecum and colon are considered less relevant. However, hindgut fermentation may become relevant in situations in which substrate degradability in the rumen is decreased (Hoover 1978; Demeyer *et al.* 1996). Depending on the diet profile and the type of animal, between 3% and 14% of starch and between 17% and 35% of fibre ingested can arrive to the large intestine undigested and become available for fermentation (Immig 1996). In this scenario, reductive acetogenesis may become more efficient than methanogenesis, due to three main factors. The first one is the absence of rapidly fermentable carbohydrates; the hindgut is depleted with respect to readily available and easily fermentable carbohydrates, which are the major substrates for acetogenic bacteria in the rumen on a low- fibre diet. This depletion might favour the alternative pathway where acetogenic bacteria use the metabolic H_2 to reduce CO_2 to acetic acid. As methanogens are more limited in their growth substrates than are acetogenic bacteria (Jones 1991), a carbohydrate-depleted rumen or hindgut would enable a microbial environment where both a non-methanogenic and a methanogenic pathway can occur simultaneously, or even a microbial habitat where acetate instead of CH_4 is the H_2 sink. The

second factor is the absence of protozoa (Belanche *et al.* 2015); methanogenic bacteria isolated from the rumen clearly show a somatic symbiotic association with ciliate protozoa (Belanche *et al.* 2014), allowing for a more efficient interspecies H_2 transfer from H_2-producing protozoa to methanogens (Newbold *et al.* 2015). Ciliates can degrade less accessible plant material, thus providing a more sustainable H_2 source for the methanogens (Stumm *et al.* 1982). Finally, the presence of high amounts of free amino-acids from undigested proteins, enzymes, epithelial cells and peptides from cell lysis can initiate reductive acetogenesis (Demeyer *et al.* 1993).

As is known for ruminants, hindgut fermentation in monogastric animals differs from rumen fermentation, with a substantially lower CH_4 production and the presence of reductive acetogenesis or dissimilatory sulfate reduction (Table 1). Sulfate reduction and methanogenesis seem to be mutually exclusive, while methanogenesis and reductive acetogenesis may occur simultaneously in the hindgut. Although acetogenic bacteria have been isolated from the bovine rumen, methanogenesis prevails in the forestomachs.

Since saturation of fatty acids is performed after the ileal–caecal junction (Jørgensen and Just 1988), inclusion of unsaturated oils (such as soy-bean oil) in a basal diet reduce can reduce the amount of CH_4 excreted by pigs, since both metabolites compete for the available H_2 (Christensen and Thorbek 1987). Besides, gut microbial composition in pigs includes acetogenic bacteria (Graeve *et al.* 1990) and sulfate- reducing bacteria (Butine and Leedle 1989). It is accepted that sulfate-reducing bacteria have a higher substrate affinity for H_2 than do methanogenic bacteria when sulfate is available, and, thus, CH_4 emission happens only in the absence of or under limiting sulfate scenario (Lovley *et al.* 1982; Lupton and Zeikus 1984). Moreover, acetogenic bacteria are less competitive than are methanogenic bacteria for available H_2 (Prins and Lankhorst 1977), and, hence, acetogenic bacteria can become active only when both sulfate-reducing bacteria and methanogenic bacteria are less competitive in the H_2 uptake.

Nevertheless, acetogenesis seems to be significant in the caecum and proximal colon of pigs, where pH conditions change, because pH is an important factor in modulating the rate of H_2 uptake (Gibson *et al.* 1990). Both sulfate-reducing and methanogenic bacteria in human faeces have been shown to be pH sensitive when incubated *in vitro*, and it seems that they prefer neutral or slightly alkaline conditions, whereas acetogenic bacteria reach their maximum capacity at an acidic pH; pH in the caecum and proximal colon of pigs is usually between 5.0 and 5.5, while, in the distal colon, it

increases almost up to neutrality (pH 6.5–7.0). In these conditions, acetogenesis appears to be the dominating H_2 sink in the upper segments of the large intestine (caecum and proximal colon), while methanogenesis should be the dominant in the distal colon. Previous studies (Butine and Leedle 1989) have corroborated this hypothesis, showing that the concentration of methanogenic bacteria in pigs was more than 30 times higher in colon than in caecum. Moreover, in a study of Robinson *et al*. (1989), the production of CH_4 was significantly higher (9-fold) in colonic than in caecal samples.

(d) *Host age*

Studies performed by Skillman *et al.* (2004) showed that the establishment of *Methanobrevibacter* populations in young lambs occurred earlier and in a more stable way than did the establishment of *Methanobacterium* populations, which frequently appeared or disappeared as the rumen developed. At 7 weeks after birth, only *Methanobrevibacter* spp. were present in lambs as the detectable methanogens. These results are in accordance with previous reports that *Methanobrevibacter* spp. is the most prevalent methanogen in adult ruminants (Miller 1995; Sharp *et al.* 1998). Studies by Su *et al*. (2014) with piglets of 1–14 days of age showed that the age of the piglets significantly influenced the diversity of methanogens, mainly being dominated by the genus *Methanobrevibacter*. From 1 to 14 days of age, *M. smithii* abundance increased significantly, and that of *M. thaueri* and *M. millerae* decreased significantly. The substitution of *M. smithii* for *M. thaueri*/*M. millerae* did occur in a shorter time in Yorkshire piglets than in Meishan piglets.

In rabbits, methanogenesis appears to be almost absent before weaning, but it starts to increase afterwards (Marounek *et al.* 1999). Comparison of methanogenic activity and archaeal detection between young and adult rabbits lead to non- conclusive results. Using dot-blot hybridisation with 16S rRNA gene-targeted oligonucleotide probes, Bennegadi *et al.* (2003) showed that the archaeal abundance was higher before weaning, while Piattoni *et al*. (1995) reported that, *in vitro*, CH_4 production was negligible before weaning, started to be measurable ~32 days of age and increased further with age. Belenguer *et al.* (2011) detected that only a low proportion of rabbits (2 of 16, 70–80 days of age) produced a significant volume of CH_4 *in vivo*. Nevertheless, despite the proved existence of methanogenic archaea in the rabbit caecum, only some rabbits seem to display an important CH_4 production, which might be related to a potential genetic effect, as suggested by Piattoni *et al*. (1995).

Although culture and breath of CH_4 measurement-based assays have reported that colonisation of the human gut by methanogens does not begin until 2–3 years of age (Rutili *et al.* 1996), methanogens have been detected using molecular techniques during the first months of life (Palmer *et al.* 2007). Some studies have suggested that there may be an increase in the concentration of methanogens in the human colon during the ageing process, as is the case for the rat, in which the faecal concentrations of methanogenic archaea increase with age (Maczulak *et al.* 1989).

(e) *Diurnal and seasonal variation*

A variety of studies have reported that the CH_4 fluxes in wetlands show marked seasonal variation during the growing season (Whalen 2005; Song *et al.* 2009), but to the authors' knowledge, there is not much information regarding the variation of enteric CH_4 production, derived directly from diurnal and seasonal effects. Moreover, most of the experiments have been conducted under controlled conditions on animals kept in pens on constant rations and the observed effect in uncontrolled conditions may be highly influenced by the dietary effect. Evans *et al*. (2009) studied the methanogen populations in the foregut of the wallaby (*Macropus eugenii*) in individuals sampled in May (Australian autumn) and November (Australian spring), so as to investigate the response to a change in the natural diet. In the former group (individuals sampled in Australian autumn), *Methanobrevibacter*-relatedmethanogens werethemost abundant (91.6%), and consisted exclusively of *M. gottschalkii*. The second group (methanogens from the Thermoplasmatales-affiliated group) represented only 6.3%. Surprisingly, the opposite structure was observed in individuals from the same colony sampled in spring, with Thermoplasmatales-affiliated methanogens representing 91.7% and *Methanobrevibacter*-related methanogens only 6.2%. However, it is unknown whether this variation in the methanogens is indirectly linked to the inherent seasonal variation in the host diet.

CH_4 production in different species

(a) *Domestic ruminants*

Ruminants are the livestock animals with, by far, the greatest CH_4 emissions both per unit of DMI (21–38 L/kg) and by bodyweight (0.40–0.76 L/kg BW), with minor differences between large (cattle, buffalo and bison) and small (sheep, goat and deer) ruminants (Table 1). In the rumen, the main methanogenic pathway is the hydrogenotrophic one that uses CO_2 as a carbon source and H_2 as the main electron donor (Hungate 1967). Formate can be also considered as a relevant electron donor used by a large population of rumen hydrogenotrophic methanogens and may be

responsible for up to 18% of the CH_4 generated in the rumen (Hungate 1967). Other sources for CH_4 production are methylamines and methanol, which are mainly used by methylotrophic methanogens of the order Methanosarcinales and *Methanosphaera* spp. from the order Methanobacteriales (Liu and Whitman 2008). The full contribution of these substrates to methanogenesis has not been fully studied, and, although it has been considered minor (Morgavi *et al.* 2010), it appears to become more relevant when other routes are inhibited (Poulsen *et al.* 2013); this may explain the poor correlation between the observed reduction in CH_4 production and the abundance of most common rumen hydrogenotrophic methanogens (Karnati *et al.* 2009; Tekippe *et al.* 2011). Another way to produce methane in the rumen is the aceticlastic pathway, which uses acetate as a substrate, but this pathway appears to be limited to the members of the order Methanosarcinales (Liu and Whitman 2008) and is very much driven by the H_2 partial pressure, as described below.

Due to the capital importance of H_2 in rumen fermentation (Hungate 1967), the role of methanogens (H_2 utilisers) in both rumen functioning and animal performance is essential, although their contribution to the formation of rumen microbial biomass is not significant (Janssen and Kirs 2008). Mechanisms to remove the free H_2help reduce the inhibitory effect of H_2on the microbial degradation of plant material, and, thus, improve the rate of fermentation (Wolin 1979; McAllister and Newbold 2008). The overall pool of H_2 in the rumen is limited, and the dissolved H_2 concentration comprises 0.014–6.8% of its maximal solubility at 39^{O}C and one atmospheric pressure (0.1–50 mM). The rate and amount of CH_4 production is, therefore, determined by the rate and amount of H_2passing through the H_2-dissolved pool (Janssen 2010). The apparent K_m (half saturation constant in the Michaelis-Menten equation for substrate kinetics) for H_2 in the rumen for CH_4 formation is nearly 1.4 mM; when the H_2 concentration in the rumen increases, the rate of methanogenesis does not necessarily increase proportionally, and, in consequence, CH_4 production per unit feed seems to decrease with an increasing passage rate and starch content of the plant material. Although the available data on H_2 concentration in the rumen are limited, the few available studies have indicated that animals fed on forage diets have a lower H_2 concentration (0.2–1.3 mM) than those fed on grain diets (up to 28 mM; Hungate 1967; Barry *et al.* 1977; Hillman *et al.* 1985).

Therefore, it can be speculated than dissolved H_2 concentrations in the rumen appear to be higher when animals are fed readily digestible feed, reaching the higher concentrations directly after feeding. Moreover, H_2 concentration is higher under these conditions, which also promote increased

passage rates, decreased CH_4 formation, and a shift to propionate production. Summarising, factors involved in the increased passage rate of feed from the rumen also reduce the amount of CH_4 generated per unit of digested feed, increase the proportion of propionate as a fermentation end product, and increase the concentration of H_2 in the rumen (Janssen 2010). Some practical feeding strategies that have been shown to result in an effective decrease in CH_4 production are (Hristov *et al.* 2013) (1) increasing diet digestibility (i.e. forage quality, grain processing, diets with a high proportion of concentrate) and (2) inclusion of lipids in the diet.

Another key point to consider is the different enzymes used to ultimately produce CH_4 within the methanogenic archaeal population. Across the different metabolic pathways, methanogenic archaea use more than 30 different enzymes to yield CH_4. To our knowledge, both coenzyme M and methyl-coenzyme M are distinctive of methanogenic archaea (Balch and Wolfe 1979), aswellastheenzymesresponsibleoftheformationof methyl-coenzyme M and methyl-coenzyme M reductase. The rest of enzymes and coenzymes involved in methanogenesis are also present in sulfate-reducing archaea (Vorholt *et al.* 1997; Klenk *et al.* 1998).

(b) *Wild ruminants*

Methane production from wild ruminant is quite a hard task to be estimated, basically due to the scarce data on their populations and their voluntary feed-intake level. Crutzen *et al*. (1986) believed that wild ruminants mostly comprise deer and moose, and, as they live entirely on forage and herbs close to maintenance levels, it was assumed that ~9% of the gross energy (GE) intake is lost as CH_4. The few studies (Lawler 2002; Hansen 2012) conducted on muskoxen and Norwegian reindeer demonstrated a lower CH_4 production (2.0–3.2% and 5.1–7.6% of GE intake, for muskoxen and Norwegian reindeer respectively) than that of conventional domestic ruminants such as cattle (Woodward *et al.* 2001; 8.6–10.8% of GE).

Salgado (2017) also documented that *Methanobrevibacter ruminantium* and *M. olleyae*, together with *M. smithii*, *M. gottschalkii*, *M. millerae* and *M. thaurei*, were the most abundant methanogens found in these two species of wild ruminant (muskoxen and Norwegian reindeer) where the relative abundance of *M. ruminantium* and *M. olleyae* take lead when animals consume forage-based diets (lichen-based), compared with pelleted concentrate. The same author (Salgado 2017) also concluded that there was a trend between an increase in *M. ruminantium* and *M. olleyae* and a low CH_4 output in the Norwegian reindeer and muskoxen.

(c) *Camelids*

As foregut fermenters, camelids are physiologically similar to ruminants (although present some differences, such as having a three-chambered stomach, compared with a four-chambered stomach of the ruminants), but they are evolutionarily distant from them; moreover, they are known to have a higher productivity on poor-quality vegetation and a lower production of enteric CH_4. On average, camelids produce approximately one-third less CH_4 per unit of DMI than do their large ruminant counterparts (Table 1). Gut methanogens have been studied in different camelid species with distinct evolutionary lineages (St-Pierre and Wright 2013). In the forestomach of alpacas fed a mixture of timothy, clover and rye, supplemented with fresh fruits, *Methanobrevibacter*-related methanogens appeared to be the most abundant archaea, especially those related to *M. millerae* (St-Pierre and Wright 2012). Hindgut methanogen populations in Bactrian camels were described by Turnbull *et al*. (2012), using faecal samples from animals maintained in captivity. According to that study, *Methanobrevibacter*- related archaea were also the most highly represented group, but, in contrast to the alpaca forestomach, 92.6% of Bactrian faecal 16S rRNA gene sequences were not assigned, although they were related, to *Methanobrevibacter* spp.

(d) *Pigs*

Contrary to ruminants, methanogenesis promotes unsubstantial losses of digestible energy in pigs (Christensen and Thorbek 1987). In a recent study of Jørgensen *et al*. (2011), the average CH_4 production by growing pigs was estimated to be 0.39% of the GE or 0.47% of digestible energy, which is similar to the value for all classes of pigs (0.6% of GE) assumed in the report on emission of greenhouse gases from Danish agriculture (Mikkelsen *et al.* 2011). However, these values can be variable, because both conditions in the fermentation compartment (von Heimendahl *et al.* 2010) and symbiotic microbiota, including methanogens (Cao *et al.* 2016), vary intensely among individuals. Luo *et al*. (2012) studied the diversity of methanogens in Landrace (lean) and Erhualian (obese) pigs. *Methanobrevibacter* was the most abundant genus in both breeds, and *Methanosphaera* the second- most abundant methanogen in Landrace pigs. They also indicated that Landrace pigs have a significantly higher concentration of methanogens than do the Erhualian pigs. Recent studies by A.Seradj, J. Balcells and G. de la Fuente (unpubl. data) also suggest the existence of a breed effect, with pure Duroc animals being higher CH_4 producers than commercial animals, based on Landrace and Large White breeds. This higher production was not followed by an increase in the archaea population in the same animals, in accordance with Luo's work. In both cases, the

representative genus was *Methanobrevibacter.*

(e) *Rabbits*

Methanogenic microorganisms have been described in the caecum of adult rabbits and are diverse. Michelland *et al.* (2010) showed differences between the archaeal community present in the rumen of cows and that present in soft and hard faeces of rabbits. Studies of Kušar and Avguštin (2010) suggested that the methanogenic community that inhabits the rabbit's caecum is exclusive, with low complexity and few dominant species, mostly being monopolised by *Methanobrevibacter* sp.

In rabbits, the utilisation of nutrients in the caecum is similar to that observed in other herbivores, but the VFA profile shows a predominance of acetate, followed by butyrate and then by propionate (Gidenne *et al.* 2008), in comparison to the fermentation pattern present in the rumen, where propionate is normally present at a higher concentration than is butyrate. This rabbit-specific VFA pattern seems to be related to the microbiota composition instead of the types of the nutrient entering the caecum (Adjiri *et al.* 1992). Further *in vitro* studies have confirmed the differences in the acetic to propionic ratio from caecum (5.45) and that from rumen contents (2.39) when similar substrate is fermented. Same VFA profile was also observed when comparing wild and domestic rabbits, although lower acetate and higher butyrate proportions were observed in wild rabbits (Abecia *et al.* 2012). This increase in the butyrate concentration can be explained by an increase in butyrate-producing bacteria in the rabbit; these bacteria are well represented across several *Clostridium* clusters (Pryde *et al.* 2002). The greater acetate molar proportion is directly related to the major abundance of acetate-producing bacteria in the rabbit's caecum (Morvan *et al.* 1996).

Competition among the three main H_2-consuming organisms, namely, methanogenic archaea, acetogenic bacteria and sulfate- reducing bacteria, has been already described inthe large intestine. Sulfate-reducing bacteria have a higher substrate affinity for H_2 than do methanogenic archaea, and, thus, have an initial competitive advantage (Gibson *et al.* 1990), but their growth largely depends on sulfate availability. Reductive acetogenesis is an alternative pathway for H_2 disposal, although, theoretically, the relative substrate affinities of methanogens for H_2 ought to promote methanogenesis in a competitive environment (Macfarlane and Gibson 1997). Thus, methanogenesis should generally dominate H_2-dependent acetate production in anaerobic ecosystems; however, the significant production of acetogenesis in rabbits could be related to the higher acid sensitivity of both sulfate-reducing bacteria and methanogens (Gibson *et al.* 1990).

Acetogens are more adapted to grow in a poor-substrate environment and also are more resistant to bile salts (Jezierny *et al.* 2007), which, in fact, gives them a competitive advantage in a digestive tract with a lower pH and a fast passage rate, compared with methanogens (Morvan *et al.* 1996); this might explain the lower concentration of methanogens in the caecum and colon of rabbits than in the gastrointestinal tract (GIT) of other host species. Similarly, there are no published studies on the presence of protozoa in rabbit species; protozoa play a central role in the H_2 metabolic transfer with the archaeal community in the rumen as well as bacteria concentration in the cell. This absence of protozoa could partially explain the higher concentration of bacteria present in the rabbit caecum than in the rumen (Abecia *et al.* 2004).

(f) *Horses*

Methane losses in horses range between 1.9% and 4.2% of GE, depending on the performance status of the animal (Kienzle *et al.* 2010). This is a lower amount than the predicted value for ruminants. However, CH_4 production by equids (horses, mules and asses) is considerable (up to 80 L per animal per day) compared with that for other monogastric animals. As it has been shown in the case of the rabbit, the main difference between hindgut and rumen fermentation is the predominance of reductive acetogenesis in the former, compared with the latter (Váradyová *et al.* 2000), which provides more energy in the form of VFA than does the methanogenic pathway. The study of Morvan *et al.* (1996) showed a higher concentration of acetogenic bacteria in the caecum than in rumen fluids of horses. Subsequently, some studies performed *in vitro* with semi-continuous culture systems using either equine caecum content (Zeyner *et al.* 2007) or equine faeces (Müller 2009) suggested that equine hindgut fermentation is more similar to bovine hindgut fermentation than to rumen fermentation, since the production of CH_4 remained low when rapidly fermentable carbohydrates were added to hay, even if the pH value within the fermenters was kept stable.

In 1996, archaea were identified in the horse caecum (Morvan *et al.* 1996), and after more than 20 years, the information available is still limited. The prokaryotic methanogen community was 10^4–10^6 cells/g fresh matter of equine caecal contents (Morvan *et al.* 1996). In a more recent study, the ratio of methanogenic archaea versus total bacteria (MA : TB) was measured by quantitative polymerase chain reaction (Dougal *et al.* 2012) and showed some differences between sections of the GIT, being greater in the right dorsal colon than in the caecum. Methanogenic archaea are affiliated with the orders Methanobacteriales, Methanomicrobiales and Methanoplasmatales. Recent data have reported different genera in the faecal ecosystem of the horse

(*Methanocorpusculum*, *Methanobrevibacter*, *Methanosphaera*, *Methanobacterium*, *Sulfolobus* and *Methanosarcina*; Fernandes *et al.* 2014; Lwin and Matsui 2014). The few studies focused on different regions of the horse GIT have shown that archaeal diversity may differ between faeces and colon, and, as also is the case for other species, faeces as the representatives of equine gut microbiome should be accepted with caution (Fliegerova *et al.* 2016).

(g) *Others species*

Macropods

Due to their geographical isolation, macropod marsupials have evolved separately from other major herbivore groups such as ruminants and camelids. Similarly to camelids, their low to undetectable levels of CH_4 emissions and their higher productivity on vegetation of poor quality than for ruminants have led to an increased interest in their microbiota composition (Kempton *et al.* 1976; von Engelhardt *et al.* 1978; Dellow *et al.* 1988).

Of particular interest is the gut system of some native Australian macropods such as kangaroos and wallabies. These marsupials exhibit foregut fermentation analogous to that of the rumen; however, they appear to emit minimal amounts of CH_4 compared with ruminants. The mechanisms behind this are poorly understood and could be physiological, such as body temperature, retention time of feed in the gut or host regulation of microorganisms in the gut (von Engelhardt *et al.* 1978). However, potentially, acetogenesis acts in concert with methanogenesis in these animals. Acetogens have been isolated fromeasterngrey(*Macropus giganteus*) andred (*Macropusrufus*) kangaroos (Ouwerkerk *et al.* 2009), as well as from the forestomach of the tammar wallaby (Gagen *et al.* 2014), and all isolates are potent hydrogenotrophs. The recently isolated tammar wallaby acetogen has also demonstrated mixotrophic capabilities, as well as the ability to grow and consume H_2 when in co-culture with a methanogen, with H_2 available at high partial pressures (e.g. >5 mM H_2; Gagen *et al.* 2014). Furthermore, when grown in co-culture with a methanogen, the tammar wallaby acetogen has been found to recycle H_2 generated from fermentative growth rather than release it for methanogenesis. Isolates such as these, with favourable metabolic characteristics, may be a contributing factor to lower CH_4 emissions in other gut ecosystems and could potentially be useful for strategies to reduce CH_4 emissions from ruminants and redirect the otherwise lost energy into acetate.

Humans

Human methanogenesis is mostly H_2-dependent for the reduction of both CO_2 and methyl compounds; thus, a reduction in H_2 concentration improves the fermentation and induces alternatives in the metabolic pathways of fermentative bacteria (Nakamura *et al.* 2010). Besides methanogens, this reduction is also performed by two types of hydrogenotrophic microorganism, namely, the reductive acetogenic bacteria (e.g. *Ruminococcus* spp.; Bernalier-Donadille 2010) and the sulfate- reducing bacteria (e.g. *Desulfovibrio* spp.; Rey *et al.* 2013). Hydrogenotrophic methanogenesis from CO_2 utilises 4 mol of H_2 and 1 mol of CO_2 per mol of produced CH_4, and, thus, efficiently decreases the gas partial pressure in the colon. Methanogenic archaea were discovered more than 30 years ago in the human digestive tract through the detection of CH_4 in the breath and two methanogenic species belonging to the order Methanobacteriales, namely, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, have been isolated (Gaci *et al.* 2014). *M. smithii* uses H_2 (or formate) to reduce CO_2 and *M. stadtmanae* uses H_2 to reduce methanol. *M. smithii* was also shown to compete efficiently for the nitrogenous nutrient pool (Samuel *et al.* 2007) and be capable to using different end products from the organic-matter degradation in the gut (Samuel *et al.* 2007). Moreover, it is important to highlight that almost all sequenced human GIT-associated methanogens possess the genes *mta*ABC encoding methyl-transferases required for methanol utilisation, highlighting the importance of this metabolism for gut methanogens. In the case of *stadtmanae*, it is clear that these genes are involved in methanogenesis (Fricke *et al.* 2006), but their role remains less clear for *Methanobrevibacter* spp.

Termites

The genus *Methanobrevibacter* (Methanobacteriales) is the most abundant methanogen colonising the hindgut of lower termites (Ohkuma *et al.* 1999; Shinzato *et al.* 2001); in contrast, higher termites present a more diverse methanogenic community, mainly composed of Methanobacteriales, Methanomicrobiales and Methanosarcinales (Miyata *et al.* 2007). Some studies based on phylogenetic analyses have shown that these groups are also present in wood-feeding cockroaches (Hara *et al.* 2002) and scarab beetle larvae (Egert *et al.* 2003); however, aceticlastic methanogenesis could not be verified in any of these animals. It can be hypothesised that a shorter retention time in the digestive tract might prevent or hinder the colonisation of slow-growing aceticlastic methanogens (Liu and Whitman 2008).

Conclusions

The present paper is a comprehensive review that has highlighted the similarities and differences between the methanogenic composition and metabolic pathways used across foregut- and hindgut-fermenting animals. Foregut fermenters (ruminants and camelids), on average, produce between 3.65 and 5.44 times more CH_4 than do hindgut fermenters (pigs, rabbits, horses and ostriches; Seradj *et al*. 2018). This is explained by differences in the diet, digestive physiology and more diverse metabolic routes in hindgut to direct H_2 produced from plant-component fermentation. Deeper understanding of the key microbial groups and pathways will be necessary to develop future methane- mitigation strategies.

Conflicts of interest

The authors declare no conflicts of interest.

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Table 1. Description of the enteric methane emissions, most common methanogens and methanogenic pathways for the main livestock species

BW, bodyweight; CH_4, methane; CO_2, carbon dioxide; DMI, dry-matter intake; H_2, hydrogen

Host species	CH_4 produced in L/kg DMI (L/kg BW in parentheses)	Most abundant microorganisms	Most probable pathway	Reference
Large ruminants (cattle, bison, buffalo)	26–38 (0.56–0.76)	*Methanobrevibacter gottschalkii* *Methanobrevibacter millerae* *Methanobrevibacter smithii* *Methanobrevibacter thaueri* *Methanobrevibacter ruminantium* *Methanobrevibacter olleyae* *Methanosphaera stadtmanae* *Thermoplasmata*	Use H_2 to reduce CO_2 to CH_4 or reduce methyl groups derived from methanol or methylamines	Patra *et al*. (2017) Seedorf *et al.* (2015) Kelly *et al*. (2016) Henderson *et al.* (2015)
Small ruminants (sheep, goat, deer)	21–32 (0.40–0.71)	*Methanobrevibacter gottschalkii* *Methanobrevibacter millerae* *Methanobrevibacter smithii* *Methanobrevibacter thaueri* *Methanobrevibacter ruminantium* *Methanobrevibacter olleyae* *Methanosphaera stadtmanae* *Thermoplasmata*	Use H_2 to reduce CO_2 to CH_4 or reduce methyl groups derived from methanol or methylamines	Patra *et al*. (2017) Seedorf *et al.* (2015) Kelly *et al*. (2016) Henderson *et al.* (2015)
Camelids	16–24 (0.21–0.33)	*Methanobrevibacter millerae* *Methanobrevibacter ruminantium*	Use H_2 to reduce CO_2 to CH_4	Dittmann *et al*. (2014) St-Pierre and Wright (2013)
Pigs	2.3 (0.04–0.08)	*Methanobrevibacter ruminantium* *Methanobrevibacter wolinii* *Methanosphaera stadtmanae*	Use H_2 to reduce CO_2 to CH_4	Cao *et al.* (2016) Gong *et al*. (2018) Jensen (1996)
Rabbits	2.93 (0.13)	*Methanobrevibacter smithii*	Use H_2 to reduce CO_2 to CH_4	Franz *et al*. (2011)
Horses	6.1 (0.11–0.15)	*Methanocorpusculum labreanum* *Methanobrevibacter smithii* *Methanobrevibacter gottschalkii*	Use H_2 to reduce CO_2 to CH_4	Crutzen *et al.* (1986) Jensen (1996) Lwin and Matsui (2014)
Ostriches	11.6 (0.01–0.16)	*Methanocorpusculum spp* *Methanobrevibacter spp*	Use H_2 to reduce CO_2 to CH_4	Frei *et al*. (2015) Swart *et al*. (1993) Videvall *et al*. (2018)
Humans	0.07–6.67 (0.0006–0.06)	*Methanobrevibacter smithii* *Methanosphaera stadtmaniae*	Use H_2 to reduce: (1) CO_2 to CH_4 (2) methanol to CH_4	Gaci *et al.* (2014) Crutzen *et al.* (1986) Sahakian *et al*. (2010)
Macropods	4.9–11.24 (0.08–0.14)	*Methanobrevibacter gottschalkii* *Methanosphaera stadtmanae*	Use H_2 to reduce: (1) CO_2 to CH_4 (2) methanol to CH_4	Madsen and Bertelsen (2012) Evans *et al.* (2009) Klieve *et al*. (2012)

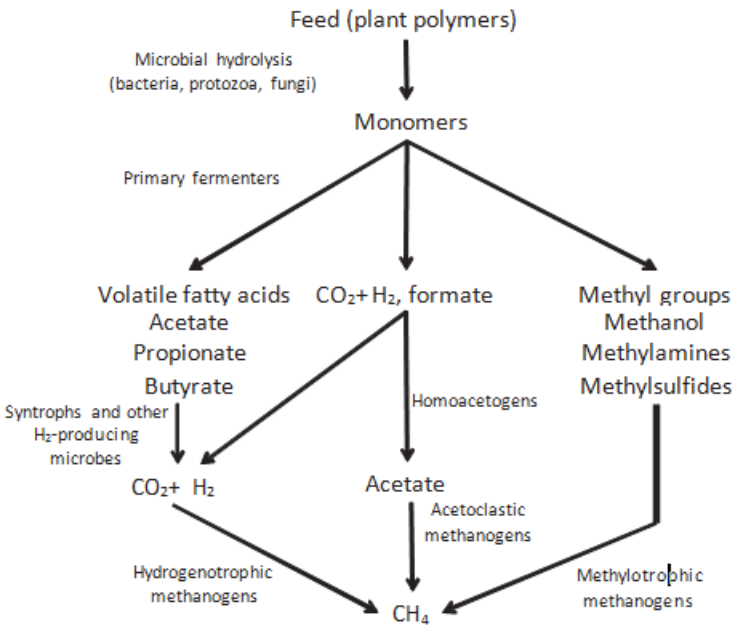

Fig. 1. Schematic anaerobic fermentation of organic matter to methane. The main substrates and microbial groups catalysing the reactions are indicated.