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Review / *Derleme*

Rumen physiology: microorganisms, fermentation and manipulation

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Abstract: Ruminants are unique mammals that can convert the energy in roughage to edible products for humans. Hence, rumen fermentation has been excessively on the scope of researchers for long years. Advances in rumen fermentation are a vital concern to provide food with good quality for the growing population of man. This review focuses on physiology of rumen fermentation and the recent advances in the field.
Keywords: Fermentation, manipulation, methane, rumen, ruminant.

Rumen fizyolojisi: mikroorganizmaları, fermantasyonu ve manipülasyonu

Özet: Ruminantlar, kaba yemlerin içerdiği enerjiyi insanların değerlendirebileceği ürünlere dönüştürebilen benzersiz memelilerdir. Bu nedenle, rumen fermantasyonu yıllardan beri bilim insanlarının yoğun bir şekilde ilgisini çekmiştir. Rumen fermantasyonun geliştirilmesi nüfusu hızla artan insanlığın kaliteli gıda ihtiyacının karşılanmasında hayati öneme sahiptir. Bu derlemede rumen fermantasyon fizyolojisi ve bu alandaki son gelişmeler ele alınmıştır.
Anahtar sözcükler: Fermantasyon, manipülasyon, metan, rumen, ruminant.

Introduction

Plants capture and collect solar energy in their structural components and seeds via synthesizing organic compounds. The biological process of this energy conversion from the sun to the biological compounds is called photosynthesis (52, 78). It is the only way for life to survive on the earth with a few exceptions such as; some microorganisms gather energy from the oxidation of some inorganic matter instead of the sun and several rare sea creatures live on the ocean floor next to the hydrothermal vent. The most common organic compound synthesized via sunbeam is cellulose in the world (79). The dilemma about the cellulose is that neither man nor any other mammalian can degrade the cellulose properly except ruminants that diverge from monogastric mammalians with their unique continuous fermentation capability. Mammals other than ruminants cannot benefit from the chemical bond energy collected in the beta-1,4-glucosidic bonds of cellulose found in plant cell walls due to the lack of the cellulose-degrading enzymes in their gastrointestinal tract (10).

In ruminants as pregastric mammals, the rumen, reticulum, and omasum are pregastric compartments before the glandular stomach. The rumen, found only in ruminants, is a vast fermentation compartment in which nutrients are continuously digested by microorganisms. The reticulum filtrates the well-confined particles from larger ones and liquid to deliver throughout the omasum. After this, further digestion processes occur in the abomasum. The rumen is a highly developed continuous fermentation environment with up to 200 liters volume where synchronized contractions mix the rumen content at a constant temperature (~ 37-39 °C) and pH (between 5.5 and 7.0) and where plenty of microorganisms that each species has a different mission on the fermentation process (79). These properties of the rumen provide ruminants a unique advantage in degrading cellulose, thereby converting low-quality roughage to meat and milk. Hence, humans can use solar energy captured and converted into cellulose and subsequently degraded and resynthesized as digestible and healthy compounds through rumen fermentation. Ruminants' genetic revolution advanced

humans to use roughage as quality feed. Therefore, humanity is dependent on ruminants' continuous fermentation system until developing a method to convert roughage into digestible compounds conventionally. Therefore, understanding the ruminal fermentation mechanism is essential to meet the food demand of rapidly growing population of humans. This review aims to expand the process of ruminal fermentation in consideration of new literature to develop novel methods to enhance livestock performance and mitigate the environmental impacts of ruminants.

The rumen as a continuous fermenter

In mammals, the digestive system following the stomach is very similar, whereas, in the ruminants, the stomach is quite different from the other species. The ruminant digestive system consists of forestomaches; the rumen, reticulum, omasum, and abomasum, which is the equivalent of the stomach, found in monogastric animals. The rumen is a fermentation sac with a volume of up to 200 liters. According to Russell et al. (79), calculating the total volume of the rumen capacity of the world's domesticated ruminants, the rumen is the "world's largest commercial fermentation process" with 100 billion liters of total volume.

In the rumen, the enzymes of microorganisms dramatically alter the composition of the forage via the fermentation processes. Fermentation is done enzymatically and mechanically by symbiotic microorganisms in the rumen, not by the ruminant's own enzymes. This phenomenon provides ruminants the advantage of benefiting from nutrients such as cellulose, hemicellulose, lignin, and pectin, which are the most abundant in nature and other mammals and humans cannot digest or digest poorly (70). Robert E. Hungate pointed out rumen microbial ecosystem for the first time and revealed that the microbial interrelation with each other and the host is essential for other inventions to advent food and various sectors (41). Hungate has been recognized as the father of modern anaerobic microbial ecology due to his studies developing systematic anaerobic culture methods (59).

Ruminants are born functionally monogastric. Consequently, fermentation occurs by the consumption of forages which leads to a mature rumen function under the effect of volatile fatty acids (VFAs) released as the end products of fermentation, and hormones such as insulin, insulin-like growth factor IGF-1, and epidermal growth factor EGF (74). The rumen is covered with the stratified squamous epithelium with leaf-like papillae. However, the absorption of VFAs, nitrogenous compounds and minerals, and bicarbonate secretion occurs in the rumen (28, 83). The rumen and the reticulum perform the same function indeed. The reticulum anatomically shapes differently since covered with honeycomb-like internal mucosa to perform its critical function of transferring the digested, confined, and denser particles into the omasum. Therefore, the reticulum acts functionally in coordination with the rumen, ensuring that only the digested particles of the rumen content are allowed to be transferred to the omasum via its two-stage motility synchronized with rumen and omasum (17). The critical function of the rumen is; (*i*) to ensure optimal conditions such as temperature, pH, and osmotic pressure which all of them are required for effective fermentation and (*ii*) provide the nutritional presence required by microorganisms to maintain the fermentation and (*iii*) ensure the microorganism adsorption onto particles by stirring. Indeed, in this symbiotic relationship between ruminants and microorganisms, forages are digested by microorganisms, as the rumen provides the optimum conditions for increasing the microbial population by removing VFAs that lead to lower pH in the rumen if VFAs' levels are high (28).

The rumen consists of two parts in which the feed is collected according to the particle size and specific weight. Dorsal sac where gas and roughages with a lower specific weight due to being rougher and undigested are collected and ventral sac where confined and digested roughage with higher specific weight, are sedimented. Rumen pH ranges from 5.5 to 7.0, depending on the feed composition. The temperature in the rumen is between 38-40 °C, which is a proper temperature for rumen microorganisms providing effective fermentation (74).

The feed ruminant consumed, digested by rumen microorganisms and degraded into its essential components. Consequently, novel organic compounds and vitamin B_{12} are synthesized to form the structural components for the growth of the microbial population. Profile of rumen microorganisms varies depending on the content of the diet and correspondingly, an alteration in the composition of rumen microorganisms results in both intermediate and end products generated by fermentation and digestion of feed in the rumen (30, 95). The rumen content differs in free-living ruminants compared to the ruminants in the intensive stock farming. High-yield breeds are used in the intensive farming due to economic concerns. Hence, the feed with a high proportion of concentrates meaning high in digestible carbohydrates, raw proteins, and energy, is provided for high yield (63). Ruminants consume carbohydrates, cellulose, hemicellulose, lignin, pectin, vegetable wax, cutin, suberin, and starch. They also consume short-chain carbohydrates such as soluble sugars, protein, non-protein nitrogen compounds, fats, minerals, vitamins, bicarbonate, and phosphate comes in water and saliva. In the process of ruminal fermentation, fat, cellulose, hemicellulose, pectin, starch,

and soluble sugars are converted into VFAs by microorganisms called microbial degradation. As a result, the structural elements of the forage converted to VFAs, carbon dioxide, and hydrogen in the rumen (26). VFAs are essential for supply approximately 80% of the animal's energy needs. VFAs synthesized in rumen consist of roughly acetic acid (45-70%), propionic acid (15-40%), butyric acid (5-20%), and iso-acids (2-5%) derived from protein degradation (22, 47). The most critical volatile fatty acids for the ruminant are acetic and propionic acids. Acetic acid is used by cells as a direct energy source and is converted into long-chain fatty acids to form milk fat. Propionic acid is used in glucose synthesis in the liver. Butyric acid mainly provides the energy requirements of the rumen epithelium. There are other products such as formic acid, lactic acid, CO_2, ethanol, and ammonia in the rumen. Hydrogen produced by microorganisms and retained by reducing NAD^+, $NADP^+$, and FAD^+, is released into the rumen by re-oxidation of these co-factors, thus fermentation continuity is maintained. However, when hydrogen density is high in the rumen, the synthesis of volatile fatty acids is affected due to suppressed re-oxidation of NADH, NADPH, and FADH (58). Methanogens use hydrogen and CO_2 found in the rumen in the production of methane (CH_4). The entire methane is excreted with eructation. Although methane formation is an undesirable phenomenon due to energy loss, it is essential to remove hydrogen from rumen content and necessary for the continuity of fermentation (60).

Approximately 12% of the feed's gross energy is lost due to methane synthesis (46). Such losses are considerable for intensive production systems. In addition, microorganisms convert the raw protein taken with feed into amino acids, peptides, a small number of volatile fatty acids, and ammonia (84). A significant amount of ammonia is used as a source of nitrogen to synthesize protein-building compounds for the growth of microorganisms. Some ammonia is also absorbed from the rumen and converted into urea in the liver. A little proportion of urea is then moved back to the rumen through rumen epithelium, while the other part is brought back to the rumen by saliva. Another notable amount of ammonia is also excreted with urine through the kidneys and discarded. The latter is considerable nitrogen loss as up to 25% of nitrogen taken with nutrients is excreted in this way. However, this excretion rate decreases in feeding with poor quality roughages (48).

Rumen microorganisms and their role in ruminal fermentation

Rumen harbors microorganisms, bacteria, protozoa, fungi, bacteriophages, and even parasites. The total microbial biomass consists of bacteria (~ 40-50%), protozoa (40%), and fungi (8%) (88). Although bacteriophages and parasites represent a negligible percentage, they still have potential effects on the microbial diversity and consequently the performance of rumen fermentation (29, 31). Even though various factors influence the microbial diversity of the rumen, the most critical factor is the composition of feed, which alters the rumen environment by influencing the microbial diversity (50). Indeed, all biochemical flow is based on the composition of feed, the diversity of rumen microorganisms, and the environmental conditions of the rumen unless an external factor that might affect the rumen. It has known that the rumen environment has a strong adaptation capability to the feed provided and the other external changes due to the rumen microorganisms with their high adaptation and the buffering capacity of rumen and saliva. Thus, although it is easy to alter the rumen fermentation and microbial diversity by manipulating the composition of feed and adding some additives, the rumen microorganisms still adapt to the new conditions a while after providing different feed and additives (58). Therefore, discovering a persistent method to manipulate rumen fermentation has recently been an essential purpose for researchers.

Rumen bacteria

Rumen bacteria can be divided into four essential subpopulations according to their place into the rumen: 1) those attached to the rumen epithelial cells which are less than 1% of the total rumen microbes, 2) those attached to feed particles (~ 70-80% of the total rumen bacteria) (50), 3) those suspended in the ruminal fluid (~ 20-25% of total rumen bacteria) (56), and 4) those attached to the surface of protozoa or fungal sporangia (97). It is estimated that more than 3.000 species of bacteria inhabited the rumen (2). A considerable proportion of the rumen bacteria consists of obligate anaerobe (3). It is thus principal to maintain the rumen O_2 concentration below 0.5% to survive the rumen anaerobic bacteria. Facultative anaerobes, which represent a small amount of the bacterial population, are essential to maintain the rumen environment absolutely anaerobe conditions by consuming oxygen arisen from feed intake. Those bacteria require a small amount of oxygen to maintain their vitality. Gram-negative bacteria form the majority of the rumen bacterial population. On the other hand, ruminants fed with concentrates show a rapid increase in the number of Gram-positive bacteria (9). The changes in feed composition consequently cause alterations in the composition of rumen microorganisms. Furthermore, sudden changes in feed composition may have adverse effects on the protozoa and bacterial species, which may lead to several pathological problems. The higher proportion of the concentrates in the feed increases

especially the Gram-positive rumen bacteria, methanogenic archaea with greater methane production capacities compared to the other species, hyper-ammonia producing bacteria that produce ammonia 20 folds faster than the other species, and lactic acid producers (12). Gram-positive bacteria also produce butyrate, acetate, lactate, format, and hydrogen, whereas Gram-negative bacteria produce propionate and succinate, which are much more suitable for ruminants (22, 92). Fiber-associated bacteria degrade plant ingredients and have an essential role in rumen fermentation. Rumen bacteria are also classified according to their function (14).

Cellulolytics: Rumen environment's essential bacteria are the cellulolytic bacteria such as *Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Ruminococcus flavefaciens, Ruminococcus albus, Clostridium cellobioparum, Clostridium longisporum, Clostridium lochheadii,* and *Eubacterium cellulosolvens* (14). *Bacteroides (Fibrobacter) succinogenes, Ruminococcus albus, and Ruminococcus flavefaciens* that Robert Hungate (40) isolated for the first time are the most important among the cellulolytic bacteria of the rumen. The latter three species have different binding sides and specificities. Thus, they do not compete for the adhesion side of the plants. Cellulolytic bacteria of the rumen require adhering to cellulose for their cellulolytic activity. These species have a wide range of fibrolytic enzymes called glycoside hydrolases (GHs) for cellulose degradation (13).

Hemicellulolytics: *Prevotella ruminicola, Eubacterium xylanophilum, Eubacterium uniformis* are non-cellulose fiber degrading bacteria of the rumen (14), and they have more than a hundred glycoside hydrolases (13, 93). Most of the cellulolytics are also capable of hemicellulose degradation (65).

Pectinolytics: *Treponema saccharophilum* and *Lachnospira multiparus* (14), mainly degrade the pectin. *Butyrivibrio fibrisolvens*, *Bacteriodes ruminicola*, *Lachnospira multiparus, Succinivibrio dextrinosolvens,* and *Streptecoccus bovis* are also pectinolytic. The three groups of bacteria above are the largest group within the bacterial population in the rumen. As fiber degraders, their population increases in roughage feed conditions, and their end products are substantially acetic acid and H_2. These three species together with the amylolytics are called primary rumen bacteria. The other bacterial groups are called seconders due to using the end products of the primary ones (22,85).

Amylolytics: *Streptococcus bovis, Ruminobacter amylophilus, Prevotella ruminicola* (14), and *Butyrivibrio fibrisolvens* (19) are mainly amylolytic bacteria. *Succinivibrio dextrinosolvens, Succnivibrio amylolytica, Selenomonas ruminantium, Bifidobacterium ruminantium, Lactobacillus acidophilus, Lactobacillus casei* and *Lactobacillus fermentum* are saccarolytic. On the other hand, rumen bacteria do not function strictly toward a single biochemical flow. Rather, a combination of different functions, as can be seen in the above-mentioned bacteria, each species has their own function such as *Prevotella ruminicola* and *Butyrivibrio fibrisolvens* are hemicellulolytic and pectinolytic, respectively. Amylolytic bacteria in the rumen are more resistant to lower pH values, while *S. bovis* is the most resistant to pH 4.5 (87). Amylolytics' growth is promoted when ruminant is fed with proportionally higher grain feed. Amylolytics are rapid-growing species with their growth rate (doubling time) from 15 minutes to 4 hours compared to cellulolytics. These species produce propionic acid, lactic acid, succinic acid, format, and CO_2 as end products. They are associated with the digestion of starch and soluble sugar (85).

Acetogens: A small proportion of rumen bacteria is described as acetogens because of their reductive acetogenesis capability from CO_2 and H_2. Acetogens in the rumen are *Acetitomaculum ruminis* and *Eubacterium limosum,* and they are expected to be more. Although their reductive acetogenesis capability can be proved *in vitro,* acetogens have not functioned as alternative H_2 sink in the rumen. Acetic acid synthesis yields less energy than methane synthesis. Hence, acetogens cannot thermodynamically compete with methanogens. Nevertheless, further studies are needed on whether the acetogens can be used as an alternative H_2 sink to mitigate methane production in the rumen with different conditions (45).

Proteolytics: *Prevotella ruminicola, Ruminobacter amylophilus, Clostridium bifermentans* and *Colostridium proteoclasticum* are the most common proteolytics (14). Although *Bacteroides ruminicola* is essential for proteolytic activities in rumen, it seems that there is a synergism between species such as *Butyrivibrio fibrisolvens, Selenomonas ruminantium,* and *Streptococcus bovis* to enhance this activity according to the variety of the bacterial population. Proteolytic activity represents a major loss of dietary amino acids for ruminant (91).

Acid and urea utilizers: *Megasphaera elsdeni* and *Anaerovibrio lipolytica* have a critical role when the rumen lactic acid production is high due to the high grain content feed (51). *Megasphaera elsdeni* also utilizes urea to ammonia (NH_3) and CO_2 (14).

Lipolytic: *Anaerovibrio lipolytica* ferments the glycerol to propionic acid and succinic acid; fructose, ribose, and lactic acid to acetic acid, propionic acid, and CO_2. The critical importance of *Anaerovibrio lipolytica* arise from its lactic acid utilizing capability in the ruminants fed with high grain feed. All fermentation processes produce a small amount of H_2 (85).

Rumen methanogens

Recent studies revealed that rumen methanogens constitute 2.8 to 4% of ruminal microorganisms (44) and belong to domain archaea (62). More than 90% of rumen archaea are member of genera; *Methanobrevibacter* (more than 60%), *Methanomicrobium* (up to 15%), and rest of the rumen archaea referred to rumen cluster C approximately 16% or *Thermoplasmatales* that function in the rumen is unknown. Although they are a small number of rumen microorganisms, the effects of rumen methanogen archaea on rumen fermentation are significant as they are the main CH_4 producers in the rumen. Interestingly, a considerable proportion of methane production in the rumen is attributed mainly not to the *Methanobrevibacter* or *Methanomicrobium*, which constitutes roughly 75% of the rumen archaea. Instead, it is attributed to the rumen cluster C archaea even though they are only 16% of rumen archaea, and their fundamental function and biochemical flow in the rumen has remained unknown yet (59).

Rumen protozoa

In addition to the bacteria, the obligate or facultative anaerobic protozoa with various species of ciliates and flagellates form another group of rumen microorganisms. Ciliata accounts for nearly half of the rumen biomass, while flagellates are much less. Some researchers consider protozoa for digestive functions in the rumen (74). In contrast, other researchers reported them as valuable as a nitrogen source to compensate the nitrogen-poor feed out of season only for wild ruminants fed primarily with poor quality forage (96). Protozoa digest nutrients, bacteria, fungi, and other protozoa. They serve a pivotal role in preventing rumen acidosis via rapidly removing carbohydrates that can be easily fermented. Moreover, they eliminate toxic compounds from plants and reduce the risk of heavy metal poisoning. However, protozoa are not essential for regular digestive functions in the rumen (65).

Rumen fungi

All fungi detected so far are obligatorily anaerobic with a narrow temperature optimum between 33-41 °C (74). The species of fungi, which belong to the genus *Neocallimastix*, *Piromyces*, *Orpinomyces*, *Caecomyces*, and *Anaeromyces* are inhabited in the rumen. Fungi vary broadly with nutrition and reach the highest amount of ruminal population 12 hours after feeding. Their mass increases to 8% of the total biomass in roughage feeding (88). Rumen fungi are crucial in the digestion of cellulose. In the absence of fungi, there is a significant decrease in the digestion of cellulose in the rumen. Fungi also facilitate bacterial colonization onto plant structure by degrading the plant cell wall and stimulating digestion. Some of the fungal species have reportedly shown proteolytic activity. Low rumen pH inhibits fungal reproduction. High levels of readily digestible sugars also reduce fungal generation. This explains the growing population of fungi in roughage-rich feeding (69).

Rumen viruses

Bacteriophages are viruses that infect rumen bacteria. Their number range from $2x10^7$ to $1x10^8$ per ml of rumen fluid. Although more than 100 different bacteriophages have been identified to date, this number can be expected to expand to a large number of bacteriophage species, given that bacteriophages are unique to all bacterial species. Biotechnological methods can develop bacteriophages specific to the type of bacteria, and there may be a potential for suppression of bacteria whose rate of reproduction in the rumen is to be decreased (49).

Rumen parasites

The first study that empirically demonstrates disease-driven increases in methane (CH_4) yield in livestock reveals interesting results. Gastrointestinal parasite infestations increase methane yield (g CH_4/kg of DMI) by up to 33% (29).

Manipulation of ruminal fermentation

A significant amount of greenhouse gases around the world is released due to agricultural activities that play a critical role in food production and the economy. However, global warming and loss of productivity due to greenhouse gas emissions arising from these activities have still been debated. While animal products account for 40% of the world's agricultural products, a significant proportion of this production comes from ruminants. Ruminants produce meat and milk, which are precious nutrients for humans, by digesting the plant's structural components that humans cannot digest. Humans provide life safety and healthy feed resources for the ruminants the whole year and ruminants provide essential nutrients for humans. This mutualistic cooperation with ruminants raised the world's ruminant population enormously while humans supply essential nutrients such as milk and meat (82). However, due to fermentation, ruminants release greenhouse gases such as methane (CH_4) and nitrous oxide (N_2O) which are essential contributors to global warming. In addition, the formation of nitrous oxide is significant during the processing of agricultural land used in ruminant nutrition. Since the 2000s, scientists have performed numerous studies to develop new methods for reducing methane gas emissions in livestock. In those studies, scientists have proposed various methods such as the

addition of ionophores, organic acids and plant essential oils to feed, immunization, modification of feed composition, rumen defaunation, alternative hydrogen (H_2) sinks, modification of the microbial rumen distribution, and animal breed replacement with the minimal methane-producing breeds (35).

Modification of the feed composition

The composition of the feed given to ruminants significantly affects the production of methane. Methane production can be reduced by up to 90% with the modification of the feed composition. Fermentation performance decreases with poor-quality roughage, lacking in vitamins, minerals, proteins, and energy, thus increases methane production. However, supplementation of minerals and nitrogen sources improves fermentation performance and consequently decreases methane production. Fresh roughage reduces methane production. Fresh alfalfa, oats, sorghum instead of hay and replacing the 30% wheat straw with fresh sorghum reduces methane production by 33% (37). Feeding with roughage rich in tannins, low fiber, a high rate of dry matter, and a shorter digestion time in rumen reduces methane production (4). Feeding with fast degradable roughage, slowly digestible starch instead of faster one, legumes instead of meadow, silage instead of fresh or dry grass, and even opting for corn silage with relatively slow degradable starch instead of meadow silage reduces methane production by up to 28% (6). Fermentation of starch promotes the production of propionic acid compared to feeds with low starch content. Propionic acid production decreases methane production by allowing the greater use of metabolic H_2 and suppressing the protozoa that are important H_2 suppliers for methanogens, by reducing rumen pH. Sugar digestion, on the other hand, leads to more methane production than starch. Since sugar can be dissolved in water, it is quickly fermented in the rumen and is mainly used to produce butyric acid. Butyric acid increases methane production when rumen pH is high and adequate metabolic H_2 is present (15). Methane production can be reduced by up to 90% when the concentrate feeds rate increases to 90%. On the other hand, in this case, the risk of subacute ruminal acidosis (SARA) should be considered. Feed with a ratio of 90% concentrates is not sustainable in ruminants (54).

Supplementing oil to the feed

Although the addition of oil to the feed varies according to the amount, form, and feed composition of the oil, it has been reported that methane production decreases by 5.6% for every 1% added oil (4). As a supplement it reduces the methane synthesis by up to 21% via hydrogenation of fatty acids by utilizing the H_2 present in the rumen, suppressing methanogenic archaea and cellulolytic bacteria, and decelerating the digestion of fibrous nutrients (24, 64). Long-chain fatty acids, especially linoleic acid, have a toxic effect due to the impairment of the cell integrity of Gram-positive bacteria such as *F. succinogenes*, *R. albus*, and *R. flavefaciens* (55).

Supplementing organic acid to the feed

Organic acids are not recommended for livestock since they are expensive and difficult to apply in grazing ruminants. However, researchers reported that organic acids (fumarate and malate) reduce the synthesis of ruminal methane. Organic acids increase the synthesis of propionic acid by using H_2 as an alternative biochemical pathway instead of methane synthesis. Thus, methane synthesis is reduced due to the lack of H_2 in the rumen (43, 61). It has been reported that methane gas production decreases to a negligible level of 1-2% with an 80-90% concentrate proportion of feed. However, in this case, the risk of subacute ruminal acidosis (SARA) arises. Further reduction in rumen pH may be prevented by an alternative H_2 sink created via organic acid supplementation. In this case, feeding with high concentrates, which radically reduces the methane synthesis, may become sustainable (1).

Supplementing antibiotics to the feed

Supplementing the feed with the ionophore group of antibiotics to increase the ruminant's yield also significantly decreased the greenhouse gas synthesis unto the 2000s. Ionophores reduce the H_2 synthesis by up to 30% via suppressing H_2 producers such as Gram-positive bacteria and ciliates rather than methanogen archaea (57). Although ionophores increase ruminant’s yield, they are not valuable for reducing methane production in advanced enterprises where ruminants feed with concentrates with high protein and energy to meet the high nutrition needs. In addition, the inhibition effect of the ionophore antibiotics on methane production is not constant (34). Although ionophores generally significantly reduce greenhouse gas emissions that arise from enteric fermentation, they have been banned in European Union Countries and Turkey since 2006 due to various concerns such as developing resistant microorganisms and food residues (23). Although it is not prohibited in other countries, the livestock and agriculture sector is forced to take alternative and even more effective measures against ionophores due to the reaction worldwide against the antibiotic additives (77).

Probiotic addition to the feed

Oeztuerk (68) reported a decrease in acetic acid/propionic acid (A/P) rate and improved fermentation performance in an *in-vitro* study 0.7% alive

Saccharomyces cerevisiae supplemented to the ruminant feed. It is reported that the addition of yeast to the diet reduces methane production by increasing the synthesis of propionic acid, reducing the number of protozoa, and increasing animal yield (11, 67, 72, 73). Lila et al. (53) reported that yeast supplementation accelerates the synthesis of acetic acid by acetogens and consequently suppresses methane synthesis through the consumption of metabolic H_2 in the rumen. Adding yeast to the feed also contributes to the stabilization of the rumen pH, thus ruminants fed with concentrates become more resistant to subacute rumen acidosis (SARA).

Adding enzymes to the feed

Enzymes such as cellulase and hemicellulase added to the feed are the concentrated fermentation products and accelerate fiber digestion. The low fiber content or easily digestible fibers in feed reduces methane production. Since the acceleration of fiber digestion shortens the duration of the ingesta's stay in the rumen, it reduces methane production (4). Accelerating fiber digestion also reduces the A/P ratio (27). On the other hand, enzyme supplementation has not affected fiber digestion in some studies. The effect of enzyme supplementation on fiber digestibility varies according to the composition of the feed. Therefore, it does not seem possible to recommend a single enzyme formula (4).

Alternative H_2 sinks

Adding unsaturated fatty acids, nitrates, and sulfate, organic acid precursors to the feed, reduces methane production by allowing H_2 to be consumed in an alternative and more competitive biochemical pathway. Although adding nitrates and sulfates reduces methane production, it is not a suitable way to recover energy lost by methane synthesis (90). In other H_2 sinks, the bio-energy potential of H_2 is used by the ruminant. However, adding short-chain fatty acids may utilize a very small proportion of H_2 from the rumen environment (20). Microbial biosynthesis is also the alternative way to use H_2 (42).

Adding secondary plant metabolites

Plant extracts are organic compounds that humans have used in various pathological situations for thousands of years. Plant extracts are not structural compounds of the plant. Secondary plant metabolites are the reproductive and defense system components, protecting the plant against insects, harmful animals, microorganisms, other plants, and even harmful sunrays. These metabolites consist of tannins, saponins, flavonoids, sulfurous organic compounds, and essential oils with inhibitor activity for many microorganisms. These compounds with selective antibacterial effects on Gram-positive bacteria significantly reduce methane synthesis in the rumen (75). Antimicrobial activity of plant extracts has not been well documented yet. However, some researchers reported that plant extracts affect microbial cells in several sites. Not all of these mechanisms are separate targets; some are affected as a consequence of another mechanism being targeted (7). However, it seems that all mechanisms are directly or indirectly connected to the primary effect of essential oils on the bacterial envelopes (81).

Impair the cell membrane: Plant extracts impair the phospholipid bilayer of the cell membrane. Essential oils could affect the biosynthesis of lipids, including unsaturated fatty acids, thus modifying the cell membrane structure due to the hydrophobic characteristic. EOs in the bacterial cell decrease the level of unsaturated fatty acids that are generally responsible for the membrane fluidity. For instance, thymol, carvacrol, and eugenol can increase saturated fatty acids and decrease C18 unsaturated fatty acids (66). Thymol binds membrane proteins by hydrophobic bonding and hydrogen bonding, and thus changes the permeability of the cell membrane. An important characteristic of plant extracts and their components is their hydrophobicity, which enables them to partition in the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (7).

Leakage of ions and other cell contents: Plant extracts may occur a leakage of ions and cell content. Ions and cell content leakage can be tolerated until a certain amount. On the other hand, extensive loss of cell contents or the exit of critical molecules and ions will lead to death (7).

Coagulation of the cytoplasmic content: It has been reported that cinnamon and oregano extracts exhibit a wide range of significant abnormalities including coagulation of the cytoplasmic content of *E. coli* and *S. aureus* (5).

Modulation of ion channels: Plant extracts studied in excitable cells and researches revealed that plant extracts affect ion channels and consequently lead to several actions including even death in cell, depending on the ion channel function (21).

Decreasing the cytoplasmic pH: A decrease in cytoplasmic pH (pH_{int}) and cell wall disruption was observed in cells treated with plant extracts, suggesting a possible mechanism of antibacterial action (32). The hypothesis of carvacrol mechanism of action on the cell membrane suggests that carvacrol binds H^+ and diffuses through the cytoplasmic membrane to the cytoplasm where it dissociates, releasing its proton. In the cytoplasm, carvacrol attaches a potassium ion (or another ion), which will be then transported across the cytoplasmic membrane to the external environment. Once outside the cytoplasmic

membrane a proton is again fixed on carvacrol, which releases the potassium ion. In its protonated (undissociated) form, carvacrol is ready to diffuse again across the cytoplasmic membrane and dissociates, releasing the proton into the cytoplasm (80). This proton transport mechanism is in accordance with the decrease in cytoplasmic pH (pH_{int}).

Compromising the genetic material of the cell: Plant extracts may compromise the genetic material of the cell. Thus, lead to mutations or death of the cell (36).

Cell lysis: Eeugenol, the main component of clove EO, has shown a broad antibacterial activity against both Gram-positive and Gram-negative bacteria. Eugenol can bind to proteins, inactivate enzyme activities (i.e. glycolytic enzymes), and cause cell lysis (18).

Plant extracts, which were commonly used until the middle of the twentieth century, have lost their popularity in developed countries after the widespread production of synthetic drugs that are economical, more specific, effective, and easily applicable. Until the 1980s, while most societies in other countries were still trying to provide treatment with secondary plant metabolites, interest in natural organic compounds in Western countries began to rise again, and it became debatable whether they were safer in terms of their side effects. However, since plant extracts contain numerous complex organic compounds, it is hard to collect them separately and reveal effective doses relative to synthetic compounds. With the increasing interest in plant extracts for half a century, the number of studies revealing the medical effectiveness of these plants is increasing rapidly (33). Plant extracts, tannins, saponins, and essential (etheric, volatile) oils can be added separately or mixed in different proportions. Nonetheless, producing commercial feed additives from plant extracts requires further researches to cope with challenges such as the rapid adaptation of ruminal microorganisms to the plant extracts, and their unpleasant smell.

Modifying the ruminal microbial distribution

It is possible to reduce methane production by regulating the distribution of microorganisms in the rumen through halogenated methane analogs (70), competitive microorganisms, specific microorganisms targeting rumen microorganisms, or immunization (25). One option is specifically to target methanogens by the antibiotics (64), bacteriocins (8, 69, 86), or bacteriophage (39). Another is to decrease H_2 production so that less H_2 is available for methane formation (45). It has been reported that methane production can be reduced with vaccines developed against methanogenic archaea (70). However, the variety of methane-microorganisms in the rumen is not limited to a particular strain. Instead, it varies according to the region and the composition of the feed. Therefore, it is pretty challenging to develop a vaccine, which can be used worldwide (94). McAllister and Newbold (58) reported that developing a vaccine targeting cell membrane components of methanogens could reduce methane production. The same researchers proposed reducing methane production by supplementing bacteriophages and microorganisms that produce bacteriocin and directing hydrogen to microorganisms that utilize H_2 to synthesize different fermentation products from methane such as propionic and acetic acid (58). Acetogenesis is, $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$ and $\Delta G = -8.8$ kJ. On the other hand methanogenesis is, $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ and $\Delta G = -67.4$ kJ. As can be seen in the biochemical flow, Gibbs Energy Changes (ΔG) indicates the less energy demand in methane synthesis. Negative ΔG indicates the energy release. Two different H_2 sinks given above suggest that methane synthesis provides more electron flow, which means more ATP, can be synthesized in the methanogen archaea (89). Acetogenesis, on the other hand, almost occurs without ATP production. Therefore, acetogens cannot compete with methanogens thermodynamically. However, further research is needed for these proposed methods. If acetogens successfully synthesized acetic acid from H_2 and CO_2 in the rumen instead of methane production, the animal's energy gain would be higher by up to 4-15%. This method is still not available in the field, as successful results have not yet been achieved in the hydrogen competition of acetogens with acetogenesis (38).

On the other hand, halogenated methane analogs such as aminochloral, chloroform, trichloroethylene, chloralhydrate, alpha-cyclodextrin, trichloroacetamide, 9, 10-anthraquinone, 2-bromoethane sulfonic acid, and bromochloromethane are useless as well as have some harmful side effects. In addition, methanogens can develop resistance to such chemicals. Therefore, their use in ruminant nutrition is not sustainable (70). A decrease in the number of protozoa in the rumen reduces methane production because methanogens on the surface of ciliates or in their endosymbionts are removed and H_2 production by protozoa is altered (38, 47). Although removing protozoa from the rumen reduces methane gas production, it is not the most effective option concerning the deterioration of fermentation in the rumen and a decrease in the animal's performance (38).

Improving the animal breed

Excluding low-yielding animals, keeping animals more efficient, and produce less methane gas are the recommended breeding methods for mitigating methane gas production. Since methane production is directly proportional to the number of animals, separating low-yielding animals from the herd and replacing them with more efficient animals reduces the amount of methane

production per unit yield. Many studies aimed at reducing methane production have revealed different results. Therefore, some researchers have suggested that animals produce less methane gas should be bred since this may be due to phenotypic and genetic properties, although some researchers report that this may not be the efficient solution (16, 25, 76). Thus, this should be further investigated whether methane production is related to animal breeds.

Conclusions

Humans, although are unique among the Earth's life forms in their ability to use language and high cognitive skills, they unfortunately, are not able to digest cellulose that is the most abundant plant structural component synthesized by the plants via biochemical conversion of the sunbeam. Ruminants, with their unique digestive system, on the other hand, can convert the energy in roughage to edible products for humans. Hence, rumen fermentation has been excessively on the scope of researchers for long years. Advances in rumen fermentation are a vital concern to provide food with good quality for the growing population of man. Ruminants digest roughage with microbial fermentation process by the microorganisms inhabited in the rumen. Nevertheless, agriculture contributes to nutrient and air pollution in several ways and livestock holds the bigger proportion of this contribution. The main pollutants of interest in relation to ruminant production systems are nitrogen (N) from nitrate leaching and ammonia emissions/deposition, minerals particularly phosphorus (P), greenhouse gases (GHG; carbon dioxide, CO_2; methane, CH_4 and nitrous oxide, N_2O), particulate matter and volatile organic compounds. Considering the rapid growth in the human population and, thus, the increasing number of ruminants to meet the amount of quality food demand, it is expected that greenhouse gas production and environmental pollution will be at a tremendous rate soon. Although the ionophore group antibiotics supplemented in the ruminant feed help reduce methane emissions, they have been banned in EU countries and Turkey since 2006. On the other hand, supplementing ruminant feed with ionophores leads to inconvenience although it is not restricted in other countries. Therefore, researchers have been performing intensive studies to reduce methane gas and nitrous oxide synthesis in the rumen for the last two decades. Various methods have been suggested that reportedly reduce methane production, such as modifying the feed composition, modifying rumen microorganisms via supplementing the feed with additives and microorganisms. Of these methods, plant extracts, which researchers have recently been studying intensively, present promising results in reducing methane production. Since plant extracts are a mixture of numerous organic compounds, each compound should be isolated and studied individually and in combination to reveal its effectiveness in methane mitigation. Organic compounds discovered with antimicrobial activities to decrease methane production in the rumen will also shed light on humanity's fight against various infections.

Conflict of Interest

The authors declared that there is no conflict of interest.

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