Effect of Some Feed Additives on *In Vitro* Rumen Digestibility

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Abstract
Researchers have recently focused on studies involving the improvement of feeds and their digestibility using different feed additives. These studies include feed additives such as probiotics, prebiotics, enzymes, organic acids, and phytobiotics. Feed additives added to feeds at appropriate levels increase the amount and quality of animal products, digestibility of feeds, feed consumption, and feed utilization. Various methods have been developed to determine digestibility, which is one of the determinants of the quality of feeds, accurately and reliably. Since chemical analyses do not reveal the actual value of feed or feed additives, various digestive experiments such as *in vivo, in situ,* and *in vitro* digestibility have been developed and used. *In vivo* experiments are reliable, but they have disadvantages of being cumbersome and expensive. Consequently, *in vitro* experiments have been preferred more recently due to easier execution in a short time span at cheaper costs, lesser requirement of feed material, and controlled experimental conditions. This review is aimed to highlight the available information regarding the use of feed additives in *in vitro* rumen digestion experiments.

Keywords: *In vitro* digestibility, Feed additive, Ruminant.

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1. Introduction

The increase in the world population has increased the need for animal origin protein. Ruminants are in a unique position to meet the increasing demand for animal origin protein (Badhan et al., 2018). With the increase in the world population, agricultural land is constantly decreasing (Karakuş et al., 2019). Therefore, it requires an increase in productivity of animals, the addition of new feedstuffs, and increasing the nutritive value of existing feedstuffs (van der Poel et al., 2020). One possible way is the use of feed additives (Wenk, 2000). Recently, researchers have focused on investigating the use of feed additives as digestive system regulatory agents (Tawab et al., 2021; Ogunade & McCoun, 2021; Seifdavati et al., 2021).

The development of methods that allow accurate and reliable determination of digestibility, which is one of the factors that determine the feed quality, has been the focus of researchers' interest for many years. Different methods such as *in vivo, in vitro*, and *in situ* are used to determine the feed values of the feeds used in ruminant feeding. Although the results of *in vivo* studies are generally more reliable, *in vitro* methods are preferred more recently due to disadvantages of *in vivo* studies such as difficult, expensive, high workload, long time required, difficulty in experiment conditions control, and the need for more feed samples (Adesogan, 2005; Kılıç & Sarıçiçek, 2006; Tassone et al., 2020). Some *in vitro* digestion techniques are RUSITEC (Rumen Simulation Technique) (semi-open system), Daisy Incubator (closed system), Tilley Terry's two-stage digestion process, Goering and Van Soest (1970) method, enzyme method, and gas production techniques.

The RUSITEC system is a semi-continuous closed incubation model that obtains values very close to the results obtained in *in vivo* animal experiments. It basically consists of fermenters kept in water baths with a temperature of 39ºC and perforated inner containers placed in them (Szarkowski & Breckenrıdgee, 1977).

Goering and Van Soest (1970) developed neutral detergent fiber (NDF) and acid detergent fiber (ADF) methods to estimate the digestibility of components associated with feed ingredients. The NDF enables the determination of cell wall components (hemicellulose, cellulose, lignin) whereas, ADF determines the residues of cellulose and lignin (Kellems & Church, 2010).

In the Daisy Incubator device, a closed in vitro system, four independent jars each equivalent to a rumen environment are used. Nutrient analysis (NDF, Dry matter (DM), Organic matter (OM)) is carried out for the samples remaining in the bags after incubation (Tassone et al., 2020). The ANKOM Daisy Incubator method (ANKOM Technology, Macedonia, NY, US) enables the incubation of more than one sample also, automates the incubation environment, and eliminates manual handling in sample processing (Adesogan, 2005; Tassone et al., 2020).

Tilley and Terry's (1963) *in vitro* digestibility measurement technique consist of two stages. In the first stage, feed samples, rumen fluid, and the buffer solution are incubated under anaerobic conditions at 39 ºC for 48 hours that imitates the fermentation process in rumen. In the second stage, insoluble proteins are broken down by treatment with an acid-pepsin solution for 48 hours (pH = 2). The disadvantage of this method is that it takes a long time and is inconvenient (Baran et al., 2017).

One of the methods used to determine the nutritional value of feeds is the gas production technique. Hohenheim (Menke) gas production technique is the most widely used gas production technique of today. It is a feed evaluation method based on the measurement of gases (methane (CH_4), carbon dioxide (CO_2), etc.), which are the final products of fermentation (Menke et al., 1979; Kılıç & Sarıçiçek, 2006).

Ruminants digest structural carbohydrates (cellulose, hemicellulose) through anaerobic fermentation by cellulolytic microorganisms (bacteria, protozoa, and fungi) in the rumen (Badhan, 2018). However, ruminants cannot digest about 20-70% of the cellulose in the feed. Therefore, not all feed energy can be considered as net energy (Kocaoğlu Güçlü & Kara, 2010). Limited penetration of cellulolytic microorganisms into the plant cell, insufficient retention time of feed in the rumen, and limiting enzyme activity are the reasons for the restriction of cellulose digestion in the rumen (Badhan, 2018).

Low cellulose digestibility reduces the feed conversion ratio and performance of ruminant animals. Improving the cellulose degradability in rumen provides additional energy to ruminants and increases the nutritional value of feeds (Riberio et al., 2018). One of the ways to improve the cellulose degradability is the use of feed additives that should be evidence based. It requires the evaluation of the effects of feed additives on the ruminal digestibility of nutrients. Studies on the use of alternative feed additives such as probiotics (bacteria, fungi, and yeast), prebiotics, enzymes, organic acids, and phytogenics in ruminants have increased in recent years. The purpose of this review is to evaluate the effects of feed additives that have been used in *in vitro* digestion experiments of ruminants in recent years.

2. Enzymes

Enzymes being bioactive proteins are biological catalysts that carry out chemical reactions by acting on specific substrates (Gado et al., 2017). Dietary supplemental enzymes increase the nutrient utilization in the digestive system and neutralize the undesirable antinutritional factors in feeds. Enzyme preparations used in ruminants are obtained from bacteria (*Lactobacillus plantarum*, *Bacillus subtilis*, *Lactobacillus acidophilus*, and *Streptococcus faecium*), fungi (*Trichoderma longibrachiatum, Aspergillus oryzae,* and *Trichoderma reesei*), and *Saccharomyces cerevisiae* yeast (McAllister et al., 2001).

Studies involving the use of exogenous enzymes in ruminant diets date back to the 1960s. However, the inconsistent results obtained, the unknown mechanism of action of enzymes, and high production costs have limited the developments in this field. With the recent advances in biotechnology, reduction of enzyme production cost, and better-defined commercial enzyme products, the knowledge of potential use of exogenous enzymes to increase feed utilization in ruminants has evolved (Sujana & Seresinhe, 2015).

Recently, it has been reported that exogenous fibrinolytic enzymes increase the digestibility of feeds in *in vitro* rumen digestion studies (Kondratovich et al., 2019; Li et al., 2019; Santoso et al., 2020). Factors such as the mode of action of exogenous enzymes, the type of animal used, compatibility between exogenous enzymes, and rumen microflora affect the enzyme activity. Abdi & Kılıç (2018) reported that lignin peroxidase enzyme increased the lignin digestibility of wheat straw, soy straw, and sorghum straw. Kondratovich et al. (2019) reported an improvement in the digestibility of corncob, especially sorghum grain, sorghum grain, wheat straw, cottonseed hulls, rape, soybean hulls, and alfalfa following supplementation of *Trichoderma reesei* extract containing xylanase. It was observed that cellobiohydrolase enzyme produced from *Pichia pastoris* yeast originating from *Lentinula edodes* fungus increased the cellulose digestibility of rice, wheat, and corn straws (Li et al., 2019). Santoso et al. (2020) revealed that increasing cellulase doses increased the *in vitro* dry matter, *in vitro* organic matter, and NDF digestibility of silage made from agricultural waste (Table 1).

Table 1: Some studies examining the effect of enzymes on *in vitro* digestibility recently

Animal Species	Enzyme	Feed used (Substrate)	*In vitro* digestibility method	Dose	References
Bull	*Lignin peroxidase*	wheat straw, soybean straw, sorghum straw	ANKOM Daisy II Incubator	1 enzyme unit for every 10 grams of straw	Abdi & Kılıç, 2018
Beef steers	*Trichoderma reesei* fermentation extract; exogenous xylanase	corncobs, sorghum grain, wheat straw, cotton burrs, corn stalks, cottonseed hulls, grape pomace, soybean hulls, LQ alfalfa, sorghum stalks, sorghum dry distillers' grains.	ANKOM Daisy II Incubator	0.75 µL/g	Kondratovich et al., 2019
Beef steers	Recombinant cellobiohydrolase produced by *Pichia pastoris*	rice straw, wheat straw, corn straw	Gas production technique	8 ug enzyme for every 20 mg straw	Li et al., 2019
Cattle	Cellulase	agricultural wastes (feed silage containing oil palm frond and rice crop residue)	Tilley and Terry (1963) technique	1 ml/kg, 2 ml/kg, 3 ml/kg, 4 ml/kg	Santoso et al., 2020

3. Probiotics

Probiotics are non-pathogenic and non-toxic living microorganisms that can have a beneficial effect on host animals at appropriate doses, proliferate as implanted in the intestines, are not absorbed from the digestive tract, and show antagonistic effect against pathogenic microorganisms (Arowolo & He, 2018). In addition, probiotics are naturally occurring live microorganisms that improve the digestive system of live animals (Elghandour et al., 2015). Probiotics have a potential substitute for antibiotics and widely used in the feed industry (Hassan et al. 2019). Probiotics commonly used in ruminants include yeast (*S. cerevisiae*) and bacterial species such as *Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Propionibacterium, Megasphaera*, and *Prevotella* (Arowolo & He, 2018).

Live *S. cerevisiae* is one of the most common and effective probiotics used in ruminant nutrition because it stabilizes the rumen environment for adequate functioning of microbial flora, particularly fibrolytic bacteria (Arowolo & He, 2018). Adding *S. cerevisiae* directly to sorghum straw or pre-incubation for 72 hours increased gas production, DM, and NDF digestibility (Elghandour et al., 2014). Ogunade and McCoun (2021) examined the effect of red clover-orchardgrass hay on *in vitro* digestibility by adding *S. cerevisiae* and *Aspergillus*-based enzyme to beef cattle diets. Compared to the control group, there was no difference in *in vitro* digestibility in the experimental group containing *S. cerevisiae* and the experimental group containing *Aspergillus*-based enzyme. However, *in vitro* dry matter and NDF digestibility increased in the experimental group containing *S. cerevisiae* and

Aspergillus-based enzymes (Table 2).
The use of bacterial probiotics as ruminant feed additives can increase DM consumption, cellulose digestibility, and growth performance (Hassan et al., 2019). *Rhodopseudomonas palustris* is a gram-negative photosynthetic bacterium that is common in nature (Chen et al., 2020). Chen et al. (2020) concluded that *Rhodopseudomonas palustris* promoted the growth of rumen microorganisms and maintained the anaerobic environment of rumen required for microbial fermentation. Hassan et al. (2019) reported that the powder and liquid forms of *Ruminococcus flavefaciens* bacteria increase *in vitro* dry matter digestibility (Table 2). Seifdavati et al. (2021) investigated the the effect of wastes of wheat straw, wheat bran, and urea (WU) treated with probiotic (PR) and organic acids (OA) on ruminal gas production and digestibility of pumpkin by-product (PB) silage. They reported an improvement in DM and OM digestibility with increasing level of PB and probiotic supplementation.

Table 2: Some studies examining the effect probiotics on *in vitro* digestibility recently

Animal Species	**Probiotic**	**Feed used (Substrate)**	***In vitro* digestibility method**	**Dose**	**References**
Cow	*S. cerevisiae*	sorghum straw	Goering and Van Soest (1970) technique	4 mg/g DM, 8 mg/g DM, 12 mg/g DM	Elghandour et al., 2014
Beef steers	*S. cerevisiae* *Aspergillus*-based enzyme extracts	red clover–orchardgrass hay	Gas production technique	Control (CON) (basal diet without additive) *S. cerevisiae* (SC) (CON+15 g/day of live SC), *Aspergillus*-based enzyme extracts (ABE), (CON+5 g/day of ABE) SC+ABE (15 g/d of live SC + 5 g of ABE).	Ogunade & McCoun, 2021
Cow	*Rhodopseudomonas palustris*	Total Mix Ration	Gas production technique	0, 1.3, 2.6, 3.9, 5.2×10^6 cfu/ml	Chen et al., 2020
Lamb	*Ruminococcus flavefaciens*	concentrate feed (%60) + rice straw (%40)	Gas production technique	powder (20 g) or liquid (10 ml)	Hassan et al., 2019
Sheep	Probiotic*	pumpkin by-product (PB) silage	ANKOM Daisy II Incubator	% 0.1	Seifdavati et al., 2021

*Probiotic includes dried *Lactobacillus plantarum*, sugar, dried mushrooms, di-potassium phosphate, glycine, magnesium sulfate, sodium aluminum oxide, sodium erythorbate.

4. Prebiotics

Prebiotics is a form of substrates used selectively by health-beneficial host microorganisms. Prebiotics have benefits such as inhibition of pathogenic microbes in the digestive system, activation of the immune system, and vitamin synthesis (Cherry et al., 2019). Prebiotics contain carbohydrates that produce essential fatty acids. Prebiotics can improve the nutrient digestibility and feed efficiency (Singh et al., 2017).
Crude glycerin is a residue from biodiesel production. Glycerin is fermentable into propionate in the

rumen. Increased propionate concentration in the rumen will reduce H availability and consequently methane production. Seankamsorn et al. (2020) reported that the addition of 21% crude glycerin to TMR diets with 2% chitosan supplementation increased ruminal propionate concentration and reduced methane production without adversely affecting gas kinetics or nutrient digestibility (Tablo 3). Chitosan is a biopolymer found in the exoskeletons of crabs and shrimp. Chitosan can also reduce methane production by changing the volatile fatty acid (VFA) profile in the rumen. Goiri et al. (2010) reported that the use of chitosan increased the propionate ratio in the rumen of sheep diet without affecting nutrient digestibility. Li et al. (2018) evaluated the effect of bioactive polysaccharides and oligosaccharides on ruminal fermentation, feed digestibility, and biohydrogenation. They reported that the xylooligosaccharides (XOS) group increased the DM, NDF, and ADF digestibility within 24 hours whereas no change was noted after 48 hours of incubation compared with control and glucose (GLU) groups. APS (Astragalus polysaccharide), LBP (*Lycium barbarum* polysaccharide), FOS (fructooligosaccharide) or COS (chitooligosaccharide) supplementation had no effect on nutrient digestibility. Singh et al. (2020) reported that the inclusion of mannan oligosaccharides in aflatoxin-contaminated feed partially improved the *in vitro* rumen fermentation parameters (Tablo 3).
Inulin belongs to a class of carbohydrates known as fructans. Inulin is a natural fructooligosaccharide linked to β (2-1) commonly found in plants (Singh et al., 2017). The 2,1- link inulin protects the upper part of the gastrointestinal tract from digestion. It has been reported that the use of high-dose inulin (300 mg/L) in perennial rye decreases *in vitro* dry matter digestibility, while low-dose inulin (100 mg/L) increases *in vitro* dry matter digestibility (Tablo 3).

Table 3: Some studies examining the effect prebiotics on *in vitro* digestibility recently

Animal Species	Prebiotic	Feed used (Substrate)	*In vitro* digestibility method	Dose	References
Dairy cattle	Crude Glycerin, Chitosan	Total Mix Ration (TMR)	Gas production technique	crude glycerin (0, 10.5, and 21% of TMR), chitosan (0, 1, and 2% DM of substrate)	Seankamsorn et al., 2020
Goat	Bioactive polysaccharides and oligosaccharides (POSs)	Basal diet	Rumen Simulation Technique (RUSITEC)	10 g/kg diet	Li et al., 2018
Sheep	Inulin	corn silage, perennial ryegrass, vetch/oat hay	ANKOM Daisy II Incubator	0, 100, 200, 300 mg/l	Salman et al., 2017
Buffalo	mannan oligosaccharides	Basal feed	Gas production technique	0, 0.05, 0.1, 0.2%	Singh et al., 2020

5. Organic Acids

Organic acids formed by the oxidation of an aldehyde group include lactic acid, formic acid, oxalic acid, malonic acid, malic acid, acetic acid, succinic acid, aspartic acid, citric acid, pyruvic acid, fumaric acid, and their salts. Organic acids are used in the feeding of farm animals to increase the acidity of feeds and prevent feed spoilage, maintain the balance between pathogens and beneficial microorganisms (probiotics) in the digestive system, improve the digestion and absorption of nutrients, stimulate growth, and to promote health (Kocaoğlu Güçlü & Kara, 2010).
Zhang et al. (2017) reported that treatment of drooping wild ryegrass (Elymus nutans Griseb.) silage with 3 g/kg formic acid increased cumulative gas production but did not affect *in vitro* rumen digestibility. Harder et al. (2015) concluded that citric acid or lactic acid treatment can be used to counteract the adverse effects of fiber digestion in grains. Genç et al. (2020) revealed that 0.1% fumaric acid treatment increases the *in vitro* organic matter digestibility of either tea factory waste or tea tree leaves as potential alternative feed sources for ruminants.

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Table 4: Some studies examining the effect organic acids on *in vitro* digestibility recently

Animal Species	Organic acid	Feed used (Substrate)	*In vitro* digestibility method	Dose	References
Cow	Formic acid	drooping wild ryegrass (*Elymus nutans* Griseb.) silage	Gas production technique	3 g /kg	Zhang et al., 2017
Cow	Citric acid Lactic acid	barley	Rumen Simulation Technique (RUSITEC)	50.25 g/L Citric asid, 76.25 g/L Lactic acid	Harder et al., 2015
Dairy Cattle	Fumaric acid Malic acid	leaves of *Robinia pseudoacacia* (Black locust, acacia), *Prunus laurocerasus* (cherry laurel), *Quercus cerris* (oak), *Camellia sinensis* (tea factory wastes, TFW	ANKOM Daisy II Incubator	0.1%, 0.2% or 0.3% Fumaric acid or Malic acid	Genç et al., 2020

6. Phytobiotics

Aromatic plants produce various essences to multiply, survive, and to protect themselves. These extracts are obtained from plants by steam distillation or extraction method. Phytobiotics that have aromatic activity are called plant extracts obtained from aromatic and spicy plants. The obtained herbal extracts are called essential oils or vegetable-essential oils. It is used to eliminate metabolic disorders, increase appetite, stimulate digestion, and benefit from its antimicrobial, antifungal, and antioxidant properties (Yeşilbağ, 2007).

Akanmu & Hassen (2017) investigated the effects of plant extracts obtained from several medicinal herbs (Leaf fraction of *Piper betle*, *Aloe vera*, *Carica papaya*, *Azadirachta indica*, *Moringa oleifera*, *Tithonia diversifolia*, *Jatropha curcas,* and *Moringa oleifera* pods) with pure methanol on the *in vitro* rumen fermentation of *Eragrostis curvula* herb. They found that *in vitro* digestibility values of organic matter were superior to all extracts associated with the control group. Medjekal et al. (2017) reported that the *Zingiber officinale* plant increased methane and total gas but increased *in vitro* dry matter digestibility. Also, *Nigella sativa* and *Rosmarinus officinalis* plants have been shown to reduce *in vitro* dry matter digestibility while reducing methane gas production.

Zhou et al. (2020) reported that the use of oregano essential oil (13, 52, 91, and 130 mg/L) quadratically increased the DM, NDF, and ADF digestibility. Compared with the control group, the DM and NDF digestibilities were the highest in group supplemented with 52 mg/L oregano essential oil, while the ADF digestibility was the highest in group supplemented with 91 mg/L oregano essential oil. Therefore, it revealed that 91 mg/L oregano essential oil would be the most suitable inclusion rate for future *in vivo* studies. Tawab et al. (2021) concluded that a mixture of thyme and celery could increase ruminal fermentation in ruminant diets, reducing gas production without any adverse effect on nutrient digestibility. However, Khorsandi et al. (2019) found that using pomegranate (*Punica granatum* L.) by-products silage (PBS) at 180 g/kg DM had higher ruminal NDF digestibility against PBS used at 60 g/kg and 120 g/kg DM. However, they found that using PBS at 180 g/kg DM had higher ruminal NDF digestibility against PBS used at 60 g/kg and 120 g/kg DM.

Table 5: Some studies examining the effect phytogenic feed additives on *in vitro* digestibility recently

Animal Species	Phytobiotics	Feed used (Substrate)	*In vitro* digestibility method	Dose	References
Sheep	Eaf fraction of *Piper betle*, *Aloe vera*, *Carica papaya*, *Azadirachta indica*, *Moringa oleifera*, *Tithonia diversifolia*, *Jatropha curcas, Moringa oleifera* pods	Eragrostis curvula hay	Gas production technique	25, 50, 75 ve 100 mg/l distilled water	Akanmu & Hassen, 2017
Sheep	*Nigella sativa, Rosmarinus officinalis Zingiber officinale*	a mixture of alfalfa hay (500 g/kg), grass hay (400 g/kg) and barley grain (100 g/kg)	Gas production technique	50 mg DM of each plant/ 500 mg DM of the substrate	Medjekal et al., 2017
Sheep	oregano essential oil	same diet fed to donor sheep	Gas production technique	13, 52, 91, 130 mg/l	Zhou et al., 2020
Cow	thyme plus celery mix	60% concentrate feed mixture, 40% clover	Gas production technique	2.5 thyme + 2.5 celery g/kg DM, 5 thyme + 5 celery g/ kg DM, 10 thyme + 10 celery g/kg DM,	Tawab et al., 2021
Cow	Pomegranate (*Punica granatum L.*) by-products silage	Conventional dairy cows TMR	Gas production technique	0, 60, 120 and 180 g PBS/kg DM	Khorsandi et al., 2019

7. Conclusion

In recent years, digestibility measurement, which is of great importance for ruminant feeding due to its advantages being easy to apply and get results in a shorter time, is carried out by *in vitro* methods. The available literature is an evidence of many studies conducted so far in this domain except for organic acids. Many of these studies consist of alternative feed additives such as probiotics, prebiotics, enzymes, organic acids, and phytobiotics. Available *in vitro* studies suggest that these feed additives have positive effects on nutrient digestibility in a dose-dependent fashion. Further studies to comprehend the possible effect of organic acids on *in vitro* ruminal digestibility are suggested.

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