

Anaerobic rumen fungi as a feed additive in ruminants: a review

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Abstract

The demand for meat and dairy products is increasing globally and to meet the growing demand for animal products, it is necessary to increase livestock productivity. The use of antibiotic growth promoters (AGPs) in livestock is banned in many countries which has led to lower feed efficiency issues and impaired growth rate. Recently, more emphasis has been given to the manipulation of the rumen ecosystem to enhance the utilization of feedstuff to improve production of ruminants by feeding direct-fed microbials (DFM). Ruminant diets are mostly composed of lignocellulosic feeds which can be breakdown by anaerobic rumen fungi (ARF) by penetrating the cell walls of plants through their rhizoids. ARF can be used as DFM to increase the digestibility of the fibrous feed and overall feed efficiency of the ruminants, thereby enhancing animal performance. For ruminants, ARF as DFM has been successfully used to improve fiber digestion in the rumen, feed efficiency, daily gain and milk production. ARF has applications as silage additives during the ensiling process for improving ensiling quality, preservation and rumen degradation. Recently, the use of novel silage additives such as ARF and their enzymes has been suggested to pre-cleave fibrous structures effectively in silages. The challenges in the application of ARF as feed additives are administration methods and large-scale cultivation. Further research is needed to develop economically feasible industrial-scale production of anaerobic fungal probiotics.

Keywords: Anaerobic rumen fungi; Fiber digestibility; Probiotic; Silage additive

Introduction

The demand for meat and dairy products is increasing globally due to rising incomes, population growth and changing dietary preferences. To meet the growing demand for animal products, it is necessary to increase the livestock productivity. As concerns are growing about the use of antibiotic growth promoters, more emphasis has been given to the positive manipulation of the rumen microbial ecosystem. This positive manipulation by feeding direct-fed microbials (DFM) enhances feedstuff utilization for improved production efficiency in ruminants (Punia et al., 2015).

Ruminant diets are mostly composed of lignocellulosic feeds, especially in developing nations where agricultural waste is a staple of ruminant feed. Rumen is a specialized chamber in the ruminant stomach that contains a diverse population of microorganisms, including bacteria, protozoa, anaerobic fungi (AF) and methanogens (Matthews et al., 2019). Rumen fermentation is of utmost importance for fibrous feed digestion and utilization in ruminants. Recently, attention has been focused on improving the digestibility of lignocellulosic feed by increasing the number or activity of ruminal lignocellulolytic organisms which leads to positive manipulation of rumen fermentation. Similarly, improving the lignocellulosic forage silage quality by using microbial additives for better preservation and digestibility (Muck et al., 2018).

Anaerobic rumen fungi (ARF) can penetrate and break the cell walls of plants through their rhizoids which enable easy access to other microbes to the cell wall. They also have the most potent cell wall degrading enzymes in the rumen microbial ecosystem (Kamra and Singh, 2017). ARF enables the utilization of low-quality fibrous feed, particularly at a time when feed production would be limited due to climate change, less farmable land due to rapid urbanization.

ARF can be used as DFM to increase the digestibility of the fibrous feed and overall feed efficiency of the ruminants, thereby enhancing animal performance. ARF has applications as silage additives during the ensiling process for the improvement of ensiling quality, aerobic stability and utility value of silage (Wang et al., 2019).

Current perception of anaerobic rumen fungi in ruminant nutrition

In ruminants, there is a necessity for high fermentation capacity to develop efficient production systems. A greater production level depends on the microbial activity in the rumen to transform organic matter (OM) into precursors of meat and milk (Castillo-Gonzalez et al., 2014) This has increased the interest of animal nutritionists and microbiologists in the evaluation of various strategies to alter the rumen microbial population to enhance ruminant production efficiency, performance and health.

ARF and their effects on the host animal remain largely unknown, despite recent advances in understanding of how archaea and bacteria affect the host function (Kittleman et al., 2014; Henderson et al., 2015; Wallace et al., 2019). Therefore, including ARF in ruminant feeding would make it possible to capture the rumen microbiota holistically and understands how it affects nutrition.

Anaerobic rumen fungi

Ruminant gastrointestinal tracts have been evolved with microbial environment which is conducive to microbial degradation of fibrous structures of plants. Rumen fungi are obligate anaerobes (Bauchop, 1979) and have chitin in their cell walls. In the mid-1970s, Orpin discovered that rumen zooflagellates, which were previously identified are actually fungal zoospores (Orpin, 1975). Anaerobic fungi were classified in 2007 as an independent phylum called Neocallimastigomycota. Anaerobic fungi are the sole members of the phylum Neocallimastigomycota. Anaerobic fungal species have been classified into eighteen genera based on their morphology of rhizoids, growth type and zoospore flagellum. The six genera of anaerobic fungi that have been described are *Anaeromyces*, *Caecomyces*, *Neocallimastix*, *Cyllamyces*, *Orpinomyces*, and *Piromyces* (Li et al., 2021).

The population of the fungi in the rumen range between 10^4 to 10^5 /ml rumen contents (Patra, 2012). The cell wall of anaerobic fungi has chitin as the main structural component. Instead of mitochondria, ATP-generating organelles (hydrogenosomes) are found which produce H_2 (Saye et al., 2021). Fungal biomass makes up 8-12% of the total microbial biomass in the rumen (Gunturu et al., 2019). The most common approaches for maintaining and isolating of AF are roll-tube technique (Joblin, 1981) and anaerobic bacterial cultivation technique (Hungate, 1969). These methods are used routinely along with growth and enumeration procedures of Theodorou et al. (1995) for the bench scale culture of anaerobic fungi. A characteristic of anaerobic rumen fungi related to ruminant nutrition is their ability to extensively colonize and break the lignin-containing cell walls of feed particles.

Life cycle of anaerobic rumen fungi

Neocallimastigomycota members exhibit an alternating lifecycle where the flagellated zoospores produced within one or more sporangia in non-motile stage (vegetative) which bursts to release the new generation. The motile stage is characterized by zoospores which colonize nutrient-rich environments via chemotactic response to the water-soluble carbohydrates and phenolic acids. Then these zoospores attach themselves to plant biomass, encyst and germination occurs. Then rhizoids will develop and these rhizoids physically penetrate the plant material which allows breakdown of plant material by enzymes and supplying

nutrients that support the development of multinucleate sporangia. In the presence of suitable inducers, the next generation zoospores will develop and mature in the sporangium, followed by the release of zoospores by the dissolution of the sporangial wall (Gruninger et al., 2014).

There is growing interest in using ARF and its enzymes as DFM and silage inoculants to improve digestibility of low quality fibrous feeds and feed efficiency in ruminants due to their capability of colonizing and degrading recalcitrant plant structures.

Role of anaerobic rumen fungi in ruminants

Forage is the most important feed source for ruminant feeding, but its high fiber content is one of the limiting factors. Among the rumen microbes, ARF has most efficient fibrolytic activity that helps to utilize roughages by breaking the lignocellulosic bonds. (Rabee et al., 2019).

Mechanism of action of anaerobic rumen fungi as fiber degraders

ARF attacks the plant cell walls physically and enzymatically which contributes to ruminal fiber degradation. The rhizoids of ARF physically penetrate the plant cell wall and release polysaccharidases against structural carbohydrates (Agustina et al., 2020). ARF enzymatically degrades plant cell walls using a diverse group of extracellular hydrolytic enzymes such as cellulases, hemicellulases, pectinases and esterases. Cellulases contain endoglucanases, exoglucanase and β-glucosidase. These enzymes act in synergy to convert cellulose to glucose (Punia et al., 2015). ARF uses different carbon sources as substrates and produces metabolites mainly formate, acetate, ethanol, lactate, CO₂ and H₂. Different proportions of metabolites formed from different carbon sources.

Table 1: Anaerobic rumen fungal enzymes and their functions

Enzymes	Types	Functions
Cellulases	Endoglucanases Exoglucanase β-Glucosidase	Endo-glucanase internally breaks the linear cellulose chains. At these nick sites, exo-glucanase then act to release cellobiose, which is then hydrolyzed to glucose monomers by β-glucosidase
Esterases	p-Coumaroyl esterase Feruloyl esterase Acetyl esterase	They cleave phenolic acid residues which loosens the structures of cell wall, Acetyl group from xylose moieties is removed by acetyl xylan esterases
Hemicellulases	Xylanase Mannase	Degrade linear polysaccharide xylan to xylose complex (xylobiose, xylo oligosaccharides, xylose), Degrade mannose
Pectinases	Endocellular pectin-lyase Polygalacturonase	Degrades pectic substances
Proteases		Cleaves peptide bonds of proteins into smaller polypeptides or single amino acids

(Bhagat et al., 2023)

Anaerobic rumen fungal carbohydrate-active enzymes (CAZymes) can form multi-enzyme complexes called cellulosomes. Cellulases and other related enzymes tie together as an assortment to form cellulosomes. Cellulosomes are assembled around a scaffoldin which is decorated with cohesion domains. Cohesions interact with cellulase enzyme encoded dockerin domains which facilitates assembly of cellulases onto the scaffoldin (Haitjema et al., 2017). Anaerobic fungal cellulosomes are not species specific and so it theoretically enables cellulosome synthesis from different donors (Nagy et al., 2007). Anaerobic fungal cellulosomes can also be found in the form of free multi-enzyme complexes in the extracellular matrix (Gilmore et al., 2020).

Nutrient availability through anaerobic rumen fungi

Anaerobic fungi has the potential of contributing protein source to the host by production of proteolytic enzymes as well as microbial protein synthesised in the rumen. But still, there is a need for extensive research for evaluating actual fungal protein reaching the intestine. ARF involves in the ruminal biohydrogenation of linoleic acids and thus produces conjugated linoleic acids (CLA) (Hartinger and Zebeli, 2021). ARF greatly enhances the host's nutrient profile, which should be considered while evaluating the nutrients required for ruminants (Hess et al., 2020).

Anaerobic rumen fungal applications in animal nutrition

ARF are potent fiber degraders and produces enzymes that are potential for developing probiotics, natural products (Swift et al., 2022) and have industrial applications such as biogas production (Flad et al., 2020).
As a source of probiotic

The use of antibiotic growth promoters (AGPs) in livestock is banned in many countries which has led to lower feed efficiency issues, impaired growth rate and incidence of certain diseases. DFMs have been considered one of the alternatives to AGPs in livestock nutrition. ARF cleaves lignocellulosic bonds which leads to

much better utilization of fibrous feeds and increased digestibility of nutrients. ARF as DFM has been used to improve ruminal health, feed efficiency, milk yield, and daily weight gain in ruminants (Krol et al., 2022).

As a source of silage additive

Silages comprise the main dietary part of ruminant feeding which provides energy, structural fiber and nutrients. Silages with fewer sugars and more recalcitrant fibrous structures reduce the nutritive value and ruminal degradability of forages. ARF seems promising in improving the preservation and nutritive quality of silage due to their higher oxidative enzymatic function and possess rhizoids that penetrate the plant cell walls. Adding ARF or their cellulases during ensiling can break down plant cell walls which reduce crude fiber content of silages and enhance nutritional quality thereby improving forage utilization by ruminants (Ravi et al., 2021). Cellulases and free cellulosomes of ARF have the potential to significantly increase cellulolytic activity. Regarding the application of ARF in ruminant nutrition, this would offer the opportunity to culture of anaerobic fungi and use their spent culture medium which consists of CAZymes and cellulosomes as silage and feed additives.

Effects of supplementation of anaerobic rumen fungi as direct-fed microbials in ruminants

Effect on feed digestibility

A key problem in tropical areas of developing countries is lack of supply of good quality and quantity of feed to support higher animal productivity. Anaerobic gut fungi can be used as DFM as they have a unique ability to degrade recalcitrant plant structures by mechanical and enzymatic action and also have potential for improvement of fermentation in rumen.

Hillaire and Jouany (1989), has reported the probiotic capability of ARF for ruminants as they observed wheat straw degradation rate increased by 15% with the administration of *Neocallimastix* isolate. Fungus-free calves dosing with *Neocallimastix* sp. show 35% higher forage intake (Theodorou et al., 1990). Further Gordon and Phillips (1993) reported that 40% increase in fibrous feed intake with the administration of *Neocallimastix* sp. SLI in fungus-free sheep. Paul et al. (2006) conducted an *in vitro* experiment to assess the effect of different strains of anaerobic fungi (*Piromyces* sp, *Anaeromyces* sp, *Orpinomyces* sp) inclusion to mixed rumen microflora of buffalo. The results showed that *piromyces* sp. showed the highest effect on the apparent digestibility, true digestibility and neutral detergent fiber (NDF) digestibility of lignocellulosic feed. Supplementation of *piromyces* sp. increases volatile fatty acids and enzymatic activities like carboxy methyl cellulase, xylanase, esterases and protease.

Paul et al. (2011) has reported that supplementation of buffaloes with anaerobic fungal liquid culture with a dose of 200 ml of about 10^6 thallus forming units (TFU) per ml and encapsulated culture improved the dry matter (DM), organic matter (OM) digestibility and neutral detergent fiber (NDF) which results in improvement of total digestible nutrients (TDN) of the ration. The results of encapsulated culture were mostly comparable to liquid culture, although effects were much less long lasting with encapsulated culture. The development of encapsulated ARF has promising applications at field level and commercially. *In vitro* dry matter digestibility (IVDMD) in cattle rumen fluid improved by anaerobic rumen fungi administration and *Orpinomyces* sp. shows higher fiber digestibility of lignocellulosic feed than *Anaeromyces* sp (Sirohi et al., 2013).

The digestibility of fibrous feed enhances due to the increase of anaerobic fungal rhizoid growth which led to the rupture of the physical structure of the cell wall and may be due to the increased amount of fibrolytic enzymes. ARF can degrade fibrous feeds and so there is a great scope to use an efficient fungal strain with high fibrolytic activity to improve fibrous feed utilization.

Tannins present in many feeds and forages are anti-nutritional for ruminants and inhibit rumen microbial growth. According to studies, *in vitro* degradation (12%) of condensed tannins improved by the addition of *Piromyces* sp. FNG5. This ARF isolate can tolerate up to 20 g/L tannic acid concentrations which is more than the amount of tannic acid that is theoretically expected in the rumen when fed a diet of high tannin content plants diet exclusively (Paul et al., 2006). ARF can tolerate or degrade tannin or phenolic monomers, so they can be used as DFM to reduce the adverse effects of anti-nutritional effects of tannins in ruminants.

Effect on growth rate, feed efficiency and rumen fermentation

Growth rate and feed efficiency of buffalo calves were significantly improved on drenching of 250 ml of ARF culture ($\approx 10^6$ tfu/ml) every 4th day (Sehgal et al., 2008). Supplementation of fungal culture with the dose rate of 1420×10^6 zoospores/mL in complete wheat straw-based feed block in buffaloes results in significant improvement in daily gains with nearly similar feed intake. Feed efficiency was improved due to increased body weight gain with similar feed intake (Kumar et al., 2015). Several studies have shown that the administration of ARF has improved the growth rate and feed efficiency of ruminants (Manikumar et al., 2003; Dey et al., 2004). The increased growth performance in calves is due to improved digestibility of nutrients particularly that of CF, NDF, ADF and improved DCP and TDN. ARF has high fibrolytic activity and proteolytic activity which increases the digestibility of feed. Improved nutrient utilization of feeds increases the growth rate.

Kumar et al. (2015) has reported that the administration of zoospores of *Neocallimastix* sp. GR-1 broth culture (1420×10^6 zoospores/ mL) in calf diet results in lower rumen pH which might be due to the enhanced nutrient digestibility and total volatile fatty acids (TVFA). Zoospore count increases with supplementation of

Neocallimastix sp. GR1 in the rumen of calves which in turn facilitated hydrolytic activities and the breakdown of fibrous structures to improve nutrient digestibility.

Effect on milk yield

Supplementation of lactating buffalo diet with *Orpinomyces* sp. C-14 and *Piromyces* sp. WNG-12 (250 ml/ lactating animal/ week) results in higher average milk yield in *Piromyces* administration than *Orpinomyces* administration. The increased milk yield indicates a better utilization of the straw-based diet in buffaloes supplemented with anaerobic fungi. ARF degrades the lignocellulose of straw which increases the digestible energy availability to the animal and this might have been responsible for enhancing microbial protein and milk yield in lactating animals (Saxena et al., 2010).

Anaerobic rumen fungal enzymes as feed additives

Ruminal fiber digestion improved with live ARF additives but adding ARF-derived enzymes did not affect the fermentation pattern (Lee et al., 2000). This suggests that rumen microbes may rapidly degrade fungal enzymes or that these enzymes could reduce the growth or activity of ruminal microbes. This emphasizes the importance of adding viable cultures of ARF as feed additives to ruminant feed.

Effect of anaerobic rumen fungi as silage additives

Good silage quality is characterized by optimum silage pH which is required for effective fermentation and preservation. Lee et al. (2015) reported that at 30 days of rice straw ensiling with anaerobic fungi (10^6 TFU/ml), shows significantly lower pH values than control group while there was no difference at 120 days of ensilation of ARF. The decreased pH values is due to the accumulation of volatile fatty acids (VFA). Fungal treatment improved rice straw silage (RSS) *In situ* ruminal DM disappearance. Silage pH decreases by ensilation of anaerobic fungi isolates to fibrous forage due to increased VFA which were produced by the breakdown of carbohydrates by the enzymatic activity of fungi (Bolaji, 2019).

Similarly, ensiling different lignocellulosic forages with *Orpinomyces* and *Neocallimastix* fungal species improves silage quality. This was indicated in terms of enhancement in soluble fraction i.e., crude protein, decrease in fibre content, pH, increase in metabolites and total antioxidant content (TAC) with minimal nutrient loss (Idowu et al., 2020). The ensiled forages inoculated with ARF showed improved CP and fiber reduction, indicating that the fungal inoculants are showing proteolytic and hydrolytic enzymatic action. This concludes that supplementation of the isolated ARF as silage inoculants improved the silage quality of the forages.

Ensilation of *Piromyces* sp. CN6 CGMCC 14449 fungus (FU) with 10^5 TFU/g and compound enzyme (EN) 0.033 mg/g (containing cellulase and xylanase) increased the lactate, crude protein (CP) and water-soluble carbohydrate (WSC) contents, whereas reduced the acetate, ADF and NDF contents in silage after 30 days of ensiling. This indicates that more homolactic fermentation is produced in silages treated with the fungus and enzymes. ARF decompose lignocellulosic biomass into more WSCs due to its fibrolytic enzymes. Similarly, ensilation with anaerobic rumen fungal inoculant improved *in vitro* digestibility of dry matter (IVDMD), NDF and ADF in silage after 30 days of fermentation (Wang et al., 2019). This demonstrates that the anaerobic gut fungi had effects comparable to compound enzyme on reduction of NDF and ADF during ensilation. The texture of straws can be destroyed by fungal enzymes which facilitates the breakdown of straw by rumen microbes and enzymes.

Harteringer et al. (2022) has reported that ensilation of grass silages with ARF and mixed ruminal fluid improved the quality of silages. ARF inoculation reduces the pH of the silage due to enhanced VFA production. *In situ* ruminal fiber degradability improved with the supplementation of grass silage with ARF supernatant but not influenced by ensilation of mixed ruminal fluid. This concludes that these additives improved the silage quality, whereas ARF supernatant only improved ruminal fiber degradability and therefore may be used to improve silage quality and fiber utilization in ruminants.

Interaction with other microbes

Despite the research that has been done to determine how dosing of ARF cultures in ruminants affects the abundance of the microbial population, there hasn't been any evidence that suggests increased number of ARF in rumen leads to decrease in bacteria or protozoa or a decreased digestibility of feed (Li et al., 2021). The positive correlation between fungal and bacterial concentrations is mostly because ARF physically breaks the plant tissue through rhizoids which increases surface area for colonization and nutrient availability for other fibrolytic microorganisms. Rumen fungi are efficient at breaking down complex carbohydrates and they produce compounds such as acetate, that are preferred by bacteria. In turn, bacteria produce metabolic by-products such as formate and hydrogen, which are essential for the growth and activity of fungi. This mutualistic relationship improves overall fiber digestion.

Methanogens produce methane by microbial fermentation of feed from hydrogen and carbon dioxide in the rumen. Increased anaerobic fungi population in the rumen enhances hydrogen production which leads to increased methane production. The *in vitro* apparent digestibility of straw improved with *Piromyces* sp. supplementation with 10^6 TFU/ in buffalo rumen fluid (Paul et al., 2010). Addition of anaerobic fungal culture results in increase of absolute amount of methane production but it has no effect on methane production per unit of the truly digestible substrate because enhanced digestibility of straw simultaneously.

Challenges

Despite such promising findings about the effects of supplementation of ARF, the route and form of rumen fungi administration needs to be considered, too, as it is important for practical applicability. In research experiments, ARF was administered into the rumen in the form of fresh culture on a daily or weekly basis by oral drenching or with feed. Such difficult approaches are appropriate for academic studies only but challenging to implement at daily operations of livestock production.

In ruminants, feed additives are supplied mostly through diet and this may also be a convenient choice to supply ARF-based probiotics, as well. ARF is highly sensitive to oxygen and so precautions must be taken in order to use them as feed additives. ARF can be protected from harmful influences by encapsulation but it enabled protection from the air for only up to 12 h, thus indicating that further development work need to be done (Paul et al., 2011). ARF as an economically feasible feed additive of ARF can only become possible if the commercial scale production of an ARF feed additive cost is considerably lower than the economic return obtained by livestock farmer's. Techniques are currently not available for large-scale production of encapsulated ARF and would need to be developed.

Future prospects

ARF will play a significant role in the progress of novel strategies due to their ability to break open recalcitrant plant structures. It is currently unclear how feed components such as fiber, starch, nitrogen and lipid increase fungal population because underlying mechanisms are complex and are poorly understood. As a result, there remains great interest in developing a reliable feed-based strategy to increase the ARF population. Feed intake increases with ARF supplementation and if ARF increases feed intake in equines, the development of an equine ARF probiotic may allow for the substitution of part of concentrates with fibrous feed (Jullian and Grimm, 2017).

ARF resting structures might be an alternative approach to be used in ruminant nutrition. Advances in understanding the ARF resting phase biology would also facilitate the application of ARF in other areas where lignocellulosic biomass is utilised such as for production of biofuels and chemicals. As such, high priority research area for development should be the characterization of the resting phase of ARF.

Conclusions

Based on the above mentioned works ARF has been used successfully for improving rumen, fiber digestion, enhancing milk yield, efficiency of feed and daily body weight gain in animals. These anaerobic fungi have high fibrolytic activity than bacteria and so can be supplemented to increase the digestibility of lignocellulosic feeds and improve the forage silage quality for better preservation and digestibility as most of the tropical countries feed lignocellulosic forages to ruminants. Therefore, ARF can be used as DFM to increase the digestibility of lignocellulosic feeds and to enhance animal performance. However, further research is needed to develop economically feasible industrial scale production of ARF probiotics.

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