Anaerobic rumen fungi and fungal direct-fed microbials in ruminant feeding

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ABSTRACT. The article discusses the importance and role of fungal direct-fed microbials (DFM) and anaerobic rumen fungi (ARF) in ruminants and their metabolic pathways of plant fibre decomposition, as well as rumen fermentation processes. ARF classification, enzymatic activity, impact on rumen metabolism, as well as fungal DFM and application of their metabolites in ruminant feeding are presented. The research area concerning ARF in ruminant feeding is gaining interest as the subject is still poorly elucidated. The latest research in the field of ruminant physiology and nutrition has pointed to a significant impact of ARF and fungal DFM on nutrient degradability, fermentation profile in the rumen, and animal performance. Although, the proportion of fungi in the total rumen microorganisms is low (approximately 8% of rumen biomass), they play a crucial role in generating a series of enzymes utilising difficult-to-debase compounds, such as cellulose, hemicelluloses and xylose. Although enzymes secreted by ARF are not able to degrade lignin, they can solubilise lignocellulosic complexes and expand the surface area for enzymatic activity. This leads to a better utilisation of fibrous feeds, increased nutrient digestibility, and enhanced rumen fermentation. A comprehensive description and discussion of these issues in the review provides an in-depth look at the role of ARF and the potential use of ARF as DFM in the ruminant nutrition in a-broad perspective.

Introduction

Ruminants, due to the unique structure of the digestive tract, especially their four-compartment stomach with the rumen and symbiosis with rumen microorganisms, are able to utilise fibrous feedstuffs to a much greater extent than monogastric animals (Ferdeș et al., 2020; Li et al., 2021). The nutritional value of forages is relatively low, thus their importance in monogastric production and the agri-food industry is strictly limited. Therefore, ruminants play a major role in the conversion of nutrients in fibre-rich feedstuffs into the nutrients derived from animal products, such as milk or meat,

which are a good source of protein and energy for humans (Gill et al., 2010). Compounds classified as crude fibre (CF), especially lignocellulosic complexes, are resistant to native enzymes of animal digestive tracts, but can be utilised by complex enzyme systems secreted by rumen microorganisms (Yue et al., 2007). Microbial processes in the rumen are responsible for supplying energy in the form of volatile fatty acids (VFA) and microbial proteins to the host animal (Li et al., 2021). An enormous microbial diversity has been found in the rumen that includes bacteria, protozoa, fungi and archaea. In the nutrition of livestock, such as cattle or buffalo, fungal direct-fed microbials (DFM) are used to improve

growth rates as well as to accelerate the production output (McAllister et al., 2011; Ferdeș et al., 2020; Li et al., 2021). Currently, anaerobic fungi are of interest to many researchers, both microbiologists and animal nutritionists, due to their ability to generate a range of enzymes capable of utilising lignified walls of the plants (Haitjema et al., 2014; Puniya et al., 2015). This review discusses the important role of fungal DFM and anaerobic rumen fungi (ARF) in ruminants and their metabolic pathways involved in the degradation of compounds classified as plant fibre, as well as in rumen fermentation processes.

Material and methods

A systematic review of peer-reviewed articles published in the Pubmed (www.ncbi.nlm.nih.gov; last accessed on April 2022), ISI Web of Science (www.webofknowledge.com; last accessed on April 2022), Google Scholar (www.scholar.google.com; last accessed on April 2022), and ScienceDirect (www.sciencedirect.com; last accessed on April 2022) databases was performed covering last 30 years, including older, primary, and original references. More than 50% of the references were from the past 10 years. Studies were selected if they reported comparisons of rumen microbiology with rumen physiology in the context of ARF or fungal DFM. A total of 122 studies from 75 different journals and books, and other scientific reports met the criteria and were included in the present review.

The review is divided into the following sections: description of the rumen ecosystem, characteristics and classification of ARF, description of rumen metabolism, carbohydrate metabolism, and description of the selected fungal DFM in ruminant feeding.

Rumen ecosystem

The rumen is the main site of microbial fermentation in the digestive tract of ruminants. Plant structural carbohydrates, proteins and other organic materials present in the feedstuff are degraded by rumen microorganisms and converted into their monomers during anaerobic fermentation. In the mid-twentieth century, scientists believed that rumen fluid consisted only of bacteria with some populations of flagellate protozoa and ciliates (Liu et al., 2008), while in fact, the rumen environment comprises a diverse microbial population. The ruminal microbial bio-community consists 98% of Bacteria, 1% of Eukaryota, around 0.9% of Archaea, and finally less than 0.1% of viral origin. Symbiotic associations between these microbes can be found in severe anaerobic environments (Faniyi et al., 2019). The microbial ecosystem of the rumen consists of bacteria at an average concentration of 10^{10}, protozoa at a concentration of 10^6, and fungi at a concentration of 10^4 cells/ml (McSweeney and Mackie, 2012; Elekwachi et al., 2017); however, only 10–20% of the rumen microbial population has been identified (Sylvester et al., 2004). Approximately 300–400 different bacterial species have been found in rumen fluid (McSweeney and Mackie, 2012; Flad et al., 2020). Both bacteria and protists together account for 90% of the total microbial biomass and their activity is most influenced by the rumen physical and biochemical properties (McAllister et al., 2011). The major factors affecting the activity and growth of the ruminal microbial ecosystem are osmotic pressure, pH, temperature, buffering capacity and redox potential (Castillo-González et al., 2014; Dijkstra et al., 2020). The rumen temperature remains in the range of about 39.0–39.5 °C, but can raise up to 41 °C due to thermogenesis caused by the fermentation process (Wahrmund et al., 2012). The pH of the rumen depends on certain factors like animal sex, breed, age, weight, rate of saliva secretion, short-chain fatty acid production and absorption, variety and quantity of feed intake, especially forage: concentrate ration, size of feed particles and exchange of bicarbonate and phosphate by the rumen epithelium (Aschenbach et al., 2011). The optimal pH should be in the range of 5.5–7.0, while the intracellular pH of bacterial cells is close to 7.0, and tends to decrease in the acidic environment of the rumen (Krause and Oetzel, 2006). Microbial enzymes are very sensitive to pH changes, as acidic pH inhibits the proliferation of rumen microorganisms. This may occur as a result of imbalance of intracellular hydrogen ions. The end products of fermentation, such as CO_2, H_2 and VFA are used by methanogens as a substrate. Methane synthesised in the rumen prevents partial H_2 pressure from rising to a level that could inhibit the activity of microbial enzymes involved in electron transfer reactions, especially the NADH (nicotinamide adenine dinucleotide NAD + hydrogen H) dehydrogenase complex. As a result, NADH accumulation may reduce the effectiveness and rate of rumen fermentation (Morgavi et al., 2010). Broadway et al. (2015) found that yeast (single-celled fungi) supplementation enhanced ruminal pH and VFA contents, reduced methane generation, and increased the total count of microbes and cellulolytic

bacteria. Yeast, as a natural feed additive, has also been demonstrated to stimulate the development of lactic acid-utilising microorganisms and minimize lactic acid accumulation in the rumen (Marden et al., 2008), eliminate oxygen, and increase the abundance of the rumen microbiota (Chaucheyras-Durand and Durand, 2010). Live yeast supplements in the feed have recently been shown to promote rumen fibre decomposition in cattle grazing on tropical grasslands (Sousa et al. 2018).

Characteristics and classification of anaerobic rumen fungi

Nowadays, filamentous fungi are considered the primary colonisers and degraders of plant fibre in most terrestrial environments. However, before the study of Orpin (1975), who first assumed that some flagellate protozoa found in the rumen were the zoosporic stage of anaerobic *Chytridiomycete*, fungi were considered non proliferative in an anaerobic environment. Subsequent studies have shown that some of the fungi are micro-aero tolerant and adhere to particles of feeds through a rhizoid system (Denman et al., 2008; Castillo-González et al., 2014). The potential ability to break down plant fibre, secrete certain enzymes and resist pH changes in the rumen are the unique features of ARF that have made them particularly important in recent years and paved the way for new scientific approaches (Hess et al., 2020). Although the proportion of fungi in the total pool of rumen microorganisms is low, accounting for about 8% of rumen biomass, they play a key role in the degradation of ingested feed (Firkins et al., 2007; Nam and Garnsworthy, 2007; Jenkins et al., 2008; Edwards et al., 2017; Gruninger et al., 2018). The composition of ruminant diet, especially with high fibre content, may favour the development of fungal population in the rumen (Saye et al., 2021).

The taxonomy of anaerobic fungi is quite primitive and takes into consideration the morphological and fermentation characteristics of these microorganisms (Teunissen et al., 1993). Basically, three genera of monocentric fungi can be distinguished (zoospore with a single sporangium): *Neocallimastix* includes fungi that have numerous flagellate zoospores (7–30 flagella) and form a highly branched rhizoid; *Piromyces* species, uniflagellate, sometimes bi- or quadri-flagellate with a rhizoid of differing size and number of wings; *Caecomyces*, uniflagellate, sometimes bi- or quadri-flagellate, generate a bulbouls instead of filamentous rhizoid (Gruninger et al., 2014; Edwards et al., 2017). Moreover, many types of anaerobic polycentric fungi have been already identified in the rumen. These fungi have the ability to generate a very pervasive armed rhizoid which accommodates both nuclei and sporangia (Faniyi et al., 2019). Two genera of polycentric fungi have been reported: *Orpinomyces*, which has numerous flagellated zoospores (Hess et al., 2020; Saye et al., 2021), and *Anaeromyces*, which generates zoospores with a single flagellum (Gruninger et al., 2014). However, Sirohi et al. (2013) reported that the polycentric species of the genus *Orpinomyces* were prevalent (about 48%) in the rumen fluid of calves fed a high-fibre diet. The taxonomy of ARF is presented in Table 1.

The life cycle of anaerobic fungi switches between an immobilized thallus, bearing one or many sporangia and a motile zoospore stage. Zoospores, depending on the genus of fungi, have a single or a bunch of flagella (7–30) positioned on their surfaces (Gruninger et al., 2014). These zoospores are attracted to freshly ingested fodder, probably by means of chemotaxis (Orpin, 1975). In *Neocallimastix* zoospores, four different receptors for glucose, sucrose, sorbitol and mannose were found (Teunissen et al., 1993). After attaching to feed particles, zoospores

Table 1. Taxonomy of anaerobic rumen fungi (Sayers et al., 2020)

Superkingdom: **Eukaryota**				
Clade: **Opisthokonta**				
Kingdom: **Fungi**				
Phylum: **Chytridiomycota**				
Class: **Neocallimastigomycetes**				
Order: **Neocallimastigales**				
Family: **Neocallimastigaceae**				
Genus:				
Neocallimastix sp.:	**Piromyces sp.:**	**Caecomyces sp.:**	**Orpinomyces sp.:**	**Anaeromyces sp.:**
N. frontalis,	*P. communis,*	*C. communis,*	*O. bovis,*	*A. mucronatus,*
N. patriciarum,	*P. mae,*	*C. equi*	*O. joyonii*	*A. etegans*
N. hurleyensis,	*P. dumbonica,*			
N. variabilis	*P. minutus,*			
	P. spiralis,			
	P. rhizinjiata			

encyst and form an encapsulation, then germinate, and at the end of germination form a large, vegetative form called fungal thallus with sporangium and rhizoid. A fully developed sporangium contains approximately 88 mononucleotide zoospores formed by continuous division of a single fungal nucleus present in the body of a new zoospore. The rhizoid is a structure that adheres to the growth substrate and penetrates it when found at the appropriate time (Vinzelj et al., 2020). The life cycle of cultivated fungi varies between 24–32 h, while zoosporogenesis has been reported to only occur about 8 h after germination (Orpin, 1975; Wubah and Kim, 1996). The actual time of the fungal growth cycle in the rumen has not yet been investigated but it is estimated to be similar to the mean withholding time of the digesta in the rumen, i.e. approximately 8–12 h (Hess et al., 2020). The lifespan is longer if fungi have the ability to generate multiple zoospores, as these zoospores from a single sporangium and may be able to replace other spores washed out before the completion of the life cycle. Some of the fungi have the ability to grow vegetatively without generating zoospores. However, the vegetative growth of fungi in the rumen is not yet fully understood, but it has been observed that nitrate-nitrogen would be used for the vegetative development of fungus in an acidic environment (Kamra and Singh, 2017). It has been found that ARF occur in all parts of the ruminant gastrointestinal tract. The resting phase is an important stage in the ARF life cycle. At this phase, fungi can survive outside the alimentary tract when exposed to oxygen and dry periods, and anaerobic fungi in this form have been found in faeces (Trinci et al., 1994). ARF are able to colonize the digestive tract of ruminants a few days after the animal is born. In lambs born after cesarean section, no ARF are detected at least until 6 months of age; however, if they are kept with ARF-positive animals, the colonization usually occurs after the second week of life of newborns. Agüero et al. (2016) found that microbial-derived compounds administered to pregnant mice differentiated gut-specific innate lymphoid cells in the foetus. The mode of action of these compounds has not yet been understood and it is not known whether the microorganisms, their metabolites or microbes in combination with secreted compounds impact the foetus. Possible vectors of ARF transfer from mother to newborn animal include maternal saliva, feed, faeces, infected dust particles, and rumen aerosols generated during rumination or belching processes. The ability of ARF to colonize the alimentary tract

of such young pre-ruminant animals indicates that most of them are able to metabolize milk sugar (lactose) and plant CF in animals' diet is not necessary for most of them to colonise the gastrointestinal tract (Trinci et al., 1994; Joshi et al., 2018). It has also been demonstrated that ARF such as *Neocallimastix* and *Piromyces* can be transferred between species (Duarte and Huynen, 2019).

ARF and rumen metabolism

Due to the ability to secrete a wide array of enzymes, ARF, which are part of the rumen ecosystem, participate in the efficient utilisation of various compounds contained in the ruminant diets (Hartinger and Zebeli, 2021). Removal of ARF from the rumen of sheep by chemical treatments decreased dry matter (DM) intake by 50–70% (Gordon and Phillips, 1993). The formation of large ARF colonies is strictly related to feeding ruminants a diet rich in forages, such as silage, hay or haylage, whose passage rate through the rumen is slow. The size of feed particles is also a very important issue: smaller particles, which pass through the rumen quickly, provide a less favourable environment for ARF proliferation due to their limited ability to attach to feed particles (Sayers et al., 2020). It was also observed that concentrate feeds and oils included in the diet decreased the density of the ARF population (Orpin, 1975; Obispo and Dehority, 1992). Obispo and Dehority (1992) reported that animals fed once, twice or three times a day did not demonstrate any significant differences in ARF concentration.

ARF, thanks to the secretion of specific enzymes with glucohydrolytic, amylolytic, cellulolytic and/or xylanolytic activity, may grow on a number of structural carbohydrates that form a huge part of the ruminant diet. This characteristic allows efficient utilisation of polysaccharides (cellulose, xylan, starch), disaccharides (cellobiose, sucrose, maltose, lactose) and monosaccharides (glucose, xylose, fructose) (Breton et al., 1990; Trinci et al., 1994; Hanafy et al., 2017; Joshi et al., 2018; Li et al., 2021). Some of the fungal species, e.g. *O. communis, N. frontalis* or *P. joyonii* have a strong ability to digest structural polysaccharides compared to cellulolytic bacterial species (Bernalier et al., 1992). The activity of these enzymes is regulated by the availability of substrates, depending on their phylogenetic origin or rhizoid structure. Lignin, which is closely related to structural carbohydrates in plant cell walls, is not degraded or utilised by ARF, but may be solubilised by polysaccharide-debasing enzymes secreted by

ARF (Teunissen et al., 1993). After fragmentation of fibrous plants in the rumen, ARF penetrate plant tissues, thereby increasing surface area for enzymatic activity (Dagar et al., 2011). A close relationship has been found between lignocellulose and ARF, which have a strong ability to debase plant particles and dissolve plant lignin (Cheng et al., 2009; Jin et al., 2011). Enzymes produced by ARF and their functions are presented in Table 2.

S. cerevisiae has been proved to suppress proliferation of lactatogenic and methanogenic microorganisms, as well as support the settlement of fibrolytic microorganisms in the rumen (Welty et al., 2019). These fungal DFM prevent a decrease in rumen pH and increase fibre degradability in the rumen by promoting the proliferation of fungi, such as *N. frontalis* or lactate-metabolising bacteria like *Selenomonas ruminantium* and *M. elsdenii*, which compete for glucose with *Streptococcus bovis*, and thus prevent rumen acidosis (Yang et al., 2016; Welty et al., 2019). One of the beneficial effects of yeast on rumen fermentation is their ability to scavenge excess oxygen in the rumen and to optimize the rumen environment for the proliferation of anaerobic microorganisms (Elghandour et al., 2014; 2017).

Table 2. Enzymes produced by anaerobic rumen fungi and their functions

Enzyme	Function	Fungi	References
Xylanases	Degrade linear polysaccharide xylan to xylose complex (xylobiose, xylose, heterodisaccharides of xylose, xylooligosaccharides); break down hemicellulose.	*T. longibrachiatum* *N. frontalis* *N. patriciarum* *Aspergillus* sp. *Orpinomyces* sp. *S. cerevisiae*	Beg et al. (2001); Leng et al. (2018); Liu et al. (2008); Mäkelä et al. (2018); Martın et al. (2002); Paul et al. (2006; 2010)
Cellulases: endoglucanases, exoglucanase, β-glucosidase, exocellulase	Break down cellulose into monosaccharides such as β-glucose or shorter polysaccharides and oligosaccharides. Exoglucanase releases cellobiose, which is in turn hydrolysed by β-glucosidase to glucose monomers. β-glucosidase catalyses hydrolysis of glycosidic bonds to terminal non-reducing residues in β-D-glucosides and oligosaccharides, releasing glucose. Exocellulase is involved in hydrolysis of cellulose and related polysaccharides, acts at the end of polysaccharide chain.	*Aspergillus* sp. *Neocallimastix* sp. *Piromyces* sp.	Atanasova-Pancevska and Kungulovski (2008); Comlekcioglu et al. (2010); Jasani et al. (2016); Leng et al. (2018); Mäkelä et al. (2018); Paul et al. (2006; 2010); Teunissen et al. (1993); Wang et al. (2011)
Protease	Catalyses proteolysis, breakdown of proteins into smaller polypeptides or single amino acids by cleaving peptide bonds within proteins.	*Neocallimastix* sp. *Piromyces* sp. *A. niger*	Lopes et al. (2011); Paul et al. (2006); Paul et al. (2010)
Esterases: acetyl esterase, acetyl xylan, esterases, feruloyl esterase	Cleave phenolic acid (p-coumaric and ferulic acid) residues from lignin hemicellulose or lignin xylan complexes, loosening cell wall structures, thereby allowing access to previously protected polysaccharides. Deacetylation of xylans and xylo-oligosaccharides. Hydrolases acting on carboxylic ester bonds.	*Neocallimastix* sp. *Piromyces* sp. *T. reesei* *S. commune* *A. pullulans* *A. niger*	Elghandour et al. (2014); Lange et al. (2019); Marvin-Sikkema et al. (1993); Paul et al. (2006); Paul et al. (2010)
Chitinases	Morphogenetic and autolytic function, these ARF are not able to utilise chitin as a carbon source.	*Orpinomyces* sp. *Anaeromyces* sp. *C. sympodialis* sp. nov.	Cheng et al. (2009); Novotná et al. (2008)
Pectinases: protopectinases, depolymerases, pectinesterase	Protopectinases degrade insoluble protopectin and give rise to highly polymerised soluble pectin. Depolymerases catalyse hydrolytic cleavage of α-(1-4)-glycosidic bonds in galacturonic acid moieties of pectic substances. Pectin esterases catalyse de-esterification of methyl ester linkages of galacturonan backbone of pectic substances.	*A. niger* *S. cerevisiae* *T. reesei*	Gainvors et al. (1994); Kopečný and Hodrová (1995); Murad and Azzaz (2011)

Carbohydrate metabolism

From a nutritional point of view, dietary carbohydrates can be classified in two main groups: the first group that can be easily degraded or absorbed includes: non-structural carbohydrates, non-fibre carbohydrates, and watersoluble carbohydrates, while the second group of carbohydrates that exhibit resistance against gastrointestinal tract enzymes includes: crude fibre (CF), neutral-detergent fibre (NDF) and acid-detergent fibre (ADF).

Anaerobic fungi obtain energy from carbohydrate fermentation (Orpin, 1975) using only the glycolysis pathway to catabolise phosphoenolpyruvate or glucose pyruvate (Marvin-Sikkema et al., 1993). In this process, carbon is converted in the cytoplasm to lactate, formate, succinate, and ethanol or transported to hydrogenosomes, where the end products are acetate, CO_2 and H_2 (Marvin-Sikkema et al., 1993). The quantity and composition of fermentation gases varies between genera. In general, polycentric anaerobic fungi produce less lactate compared to monocentric organisms (Gordon and Phillips, 1993). However, some studies reported that *Piromyces* present in the rumen did not generate any lactate (Ho et al., 1996). Some ARF species were reported to produce an endo-pectin lyase and polygalacturonase (Gordon and Phillips, 1993). Starch and glycogen can be fermented by anaerobic fungi, while some isolates of *Piromyces*, *Anaeromyces* and *Caecomyces* neither ferment these polymers nor their component – disaccharide maltose (Gordon and Phillips, 1993). *Pecoramyces ruminantium* is the only species that does not use lactose, while it readily utilises mannose and inulin (Hanafy et al., 2017; Joshi et al., 2018). It has been reported that anaerobic fungi can digest plant material with a similar decomposition rate of monomeric sugar-containing cellulose as well as hemicellulose: glucose, xylose, galactose and arabinose, while the last two were not fermented by fungi (Joshi et al., 2018). Nevertheless, Joshi et al. (2018) reported a large variation in substrate utilisation between *Liebetanzomyces polymorphus* strains G1SC and G6SC, *A. mucronatus* strain BF2 (Breton et al., 1990), *P. communis* strain SM5 (Trinci et al., 1994) and *P. ruminantium* strains C1A and S4B (Hanafy et al., 2017). All the aforementioned species of fungi successfully utilised polysaccharides (cellulose, xylan, starch), disaccharides (cellobiose, sucrose, maltose) and monosaccharides (glucose, xylose, fructose).

Fibre-degrading microorganisms play a crucial role in rumen ecology. In the rumen, the most im-portant role of ARF is the decomposition of various structural carbohydrates and polysaccharides that are resistant to the action of enzymes of other microorganisms present in ruminal fluid. Due to the time-limited presence of easily degradable sugars in the rumen, ARF are only able to utilise them in small quantities. Therefore, the main substrates for ARF during rumen fermentation are complex polysaccharides, such as cellulose, hemicelluloses, pectin and other storage polymers like starch and inulin, whose passage rates through the rumen are slower. All ARF genera have the ability to degrade cellulose, which is the main constituent of ADF in the rumen, but isolates such as *Piromyces, Caecomyces* and *Anaeromyces* encounter some complications depending on the crystallinity of cellulose (Gordon and Phillips, 1993). Xylan, the basic unit of hemicellulose, is utilised by all fungi, however, some isolates grow poorly on this substrate. Anaerobic fungi produce a variety of enzymes for cleaving polysaccharides found in the rhizomycelium and many others secreted to the rumen environment. The presence and enzymatic activity of fungi is limited to certain surfaces and vary depending on their life cycle (Gerbi et al., 1996). The secretion of enzymes depends on the fungi growth phase and type of incubator. Fibrinolytic enzymes are suppressed by the presence of sugar monomers in a sequence derived from polysaccharide breakdown – cellulases by glucose, and xylanases by xylose and arabinose (Flad et al., 2020). Ruminal fungi have the ability to hydrolyse xylans and cellulose by secreting degrading enzymes. Fungal zoospores quickly adhere to feed particles through chemotaxis and split zones of lignified tissues by mechanical activity, while the remaining non-lignified plant tissues are rapidly debased (Wang et al., 2011). The first reports of the presence of acetylxylan esterases in fungal cellulolytic and hemicellulolytic systems of *T. reesei, A. niger, S. commune*, and *A. pullulans* were presented by Biely (1985). Acetylxylan esterase produced by various fungi and bacteria is one of the enzymes required for the hydrolysis of native xylans and complete hydrolysis of galactoglucomannans. Ferulic acid esterase and acetyl esterase hydrolyse cross-linkages between lignin and polysaccharides in the cell walls via phenolic compounds, thereby allowing access to protected polysaccharides (Yue et al., 2007). Fungal esterases were reported to exhibit highly specific activities towards acetylated glucuronoxylan (Várnai et al., 2014; Nagpal et al., 2020).

When the ruminant diet consist of lignin-rich plants, ruminal fungi such as *N. frontalis* play a vital role in digesting lignocellulosic complexes and making cellulose in these compounds available for bacterial species (Borneman et al., 1992). Endoglucanase has the ability to convert cellulose into glucose. Initially, endoglucanase cuts internally the linear cellulose chains. The major cellulosedecomposing enzymatic complex comprises various endoglucanases and cellobiohydrolases in combination with β-glucosidase. Likewise, xylanases can also be found in high molecular weight complexes containing endo-acting as well as exo-acting enzymes that have been recognized and characterised (Wood and Wilson, 1995). Exoglucanases, usually cellobiohydrolases, are of significant importance to cellulolytic systems; these enzymes can cleave a cellulose polymer from the reducing or non-reducing end of the polysaccharide chain. Exoglucanase releases cellobiose, which can readily be converted by β-glucosidase to glucose monomers (Atanasova-Pancevska and Kungulovski, 2008; Comlekcioglu et al., 2010).

Fungi demonstrate a preference for colonising highly lignified tissues, degrading them more efficiently than rumen bacteria, however there is no evidence of lignin fermentation (Moreira et al., 2000). It has been reported that fungi are unable to cleave ether bonds between phenolic moieties and polysaccharides, as well as internal phenolic bonds in the lignin complex (McAllister et al., 2011). Nevertheless, they generate feruloyl and p-coumaroyl esterases that cleave feruloyl and p-coumaroyl arabinoxylans *in vitro* (Borneman et al., 1992), and are thus can break down ester linkages between lignin molecules to hemicellulose. When acting on plant cell walls, these enzymes work synergistically with fungal enzymes, e.g. xylanase (Wubah and Kim, 1996). Considering the above, it seems likely that lignin is solubilised as polysaccharide lignin complexes that have been cleaved at the interface between phenolic and hemicellulose residues (Wubah and Kim, 1996; Comlekcioglu et al., 2010).

Due to their documented ability to decompose polysaccharides, ARF present in rumen fluid may positively affect the ensiling process of biomass, especially sugar- and water-rich green roughages, which are prone to rapid deterioration. At present, various silage additives, such as inoculants, enzymes, acids or combined preparations are used to enhance the ensiling process, improve silage quality and reduce nutrient losses. From a biological point of view, rumen fluid is a mixture of silage additives commonly used in feed industry: microorganisms, enzymes and acids debasing three-dimensional lignocellulosic complexes (Wang et al., 2011). Ren et al. (2021) reported that the application of rumen fluid (7 g/100 g wet material) in ensiling sweet sorghum biomass, significantly improved the preservation process, silage quality, while reducing nutrients losses. Moreover, ensiling bio-augmented with rumen fluid, changed the structure of lignocellulosic complexes, reduced crystallinity of polysaccharides and increased their availability.

Fungal direct-fed microbials (DFM) in ruminant feeding

ARF species are enriched with fungal DFM application in ruminants' diet for efficient utilisation of fibrous feeds in terms of increased feed intake, body weight gains and milk production, improved digestibility of low-quality forages and prevention of excessive lactate production when ruminants are fed diets with high concentrate feed contents (Wallace et al., 2001; Dey et al., 2004; Lesmeister et al., 2004; Thareja et al., 2006; Elghandour et al., 2014; 2017). Fungal DFM and their metabolites were shown to improve the rumen fermentation profile (Marvin-Sikkema et al., 1993; Elghandour et al., 2014; 2017) and exert beneficial changes in the activity and abundance of rumen microorganisms – increase total counts of rumen anaerobes and cellulolytic bacteria with fungal extract supplementation. In addition to well-characterised and commonly applied yeasts in the ruminant production, DFM include certain fungal species that can produce fibre-degrading enzymes in the ruminant diet: *T. longibrachiatum* (Nsereko et al., 2002), *A. niger, P. funiculosum* (Wallace et al., 2001), *Neocallimastix* sp. (Trinci et al., 1994; Cheng et al., 2009), *A. oryzae* (Nsereko et al., 2002) or *S. cerevisiae* (Galvão et al., 2005; Welty et al., 2019).

Saccharomyces cerevisiae

A number of positive effects of including *S. cerevisiae* in ruminant diets on animal growth and production have been demonstrated. By altering the rumen environment, yeasts improve fermentation efficiency, plant fibre degradability in the rumen, feed conversion ratio and DM intake. They also have been shown to improve the health of newborns and adult animals (Mao et al., 2013; Cömert et al., 2015; Arowolo and He, 2018). *S. cerevisiae* has also been proved to stimulate the proliferation of lactic acid bacteria through the synthesis of vitamins and organic acids. The application of *S. cerevisiae* culture

in dairy calf starter diets at a dose of 20 g/kg DM exerted a positive effect on DM intake and animal growth (Lesmeister et al., 2004). It was found that the addition of yeasts to the diet of neonatal ruminants enhanced early microbial colonisation of the rumen, increased VFA concentration and decreased the production of rumen ammonia nitrogen (Hassan et al., 2016). Moreover, Kumar et al. (1997) reported an increase in the number of rumen bacteria and rumen VFA profile in buffalo calves fed a diet with yeast culture. However, many authors found no significant differences in growth performance and rumen parameters in calves fed a diet supplemented with *S. cerevisiae* (Agarwal et al., 2002; Dey et al., 2004; Saxena et al., 2010). It was also confirmed that live dry yeast applied in newborn calves' nutrition decreased the duration and incidence of diarrhea (Agarwal et al., 2002; Galvão et al., 2005). Plata et al. (1994) reported that an increased number of rumen protozoa and better diet utilisation in steers fed a straw-based diet was the result of improved NDF degradability. The efficiency of *S. cerevisiae* administration in beef cattle nutrition depends on the type of diet, and more significant results were observed in animals fed a maize silage-based diet compared to grass silage (Wallace et al., 2001; Burdick Sanchez et al., 2014). This demonstrated the benefits of including *S. cerevisiae* in ruminant diets in terms of starch and sugar utilisation in the rumen, which was confirmed by an increase in live body weight by approximately 13% (Puniya et al., 2015). There are reports that *S. cerevisiae* improve the efficiency of dietary gross energy utilisation by increasing propionate concentration, contributing to total rumen VFA (Miller-Webster et al., 2002) and reducing methane biosynthesis in the rumen (Elghandour et al., 2015). It is assumed (Hristov et al., 2013; Vallejo-Hernández et al., 2018) that reduced methanogenesis in the rumen is a consequence of increased competition of acetogenic microorganisms with methanogens. These assumptions were also confirmed by De Ondarza et al. (2010) and Cakiroglu et al. (2010), who reported higher total VFA and propionate levels, as well as a reduced acetate to propionate ratio in the rumen of animals, whose diets were supplemented with *S. cerevisiae*. There is some evidence that the inclusion of *S. cerevisiae* to ruminant diets prevents excess lactic acid levels in rumen fluid by competing with lactogenic microorganisms, such as *M. elsdenii* and *S. ruminantium*, thereby limiting the occurrence of rumen acidosis. Malekkhahi et al. (2016) observed that the application of 10 g of active dry yeast improved ru-

men pH in primiparous cows with induced subacute rumen acidosis. Similarly, Pinloche et al. (2013) reported that supplementing a cattle diet with yeasts at a dose 5 g/day/head decreased the concentration of L- and D-lactate isomers by 58%, while increasing the concentration of VFA. Numerous studies confirmed the effect of yeast culture on the proportion of VFA and explained this fact as the influence of yeast culture on the growth of various species of rumen microbes, as well as their effect on rumen pH and lactic acid concentration. Sousa et al. (2018) reported that yeast supplementation increased total VFA, decreased acetate and increased propionate levels. Similar effects of *S. cerevisiae* addition were demonstrated by Arowolo and He (2018), except for the positive impact of supplementation with *S. cerevisiae* fermentation products on VFA concentration; these authors showed increased microbial protein synthesis and more efficient conversion of dietary nitrogen to milk nitrogen. The same effect was observed by Erasmus et al. (1992), who noted increased microbial protein synthesis and alteration of the amino acid profile in the duodenal digesta of dairy cows fed diets supplemented with yeast. The addition of *S. cerevisiae* in the amount of 56 g/day/head increased microbial protein synthesis by 9.3% (Hristov et al., 2013). Similarly the inclusion of 120 and 180 g/day/head of *S. cerevisiae* fermentation products in the cows' diet increased microbial protein synthesis by 12.84 and 9.67%, respectively (Zhu et al., 2017). Supplementation of milking cows' diets with *S. cerevisiae* increased milk yield by approx. 4%, which was associated with higher feed DM intake, and a more significant effect was found in cows in early lactation compared to middle and late lactation (Moreno, 2012).

Trichoderma longibrachiatum

T. longibrachiatum is a soil clonal fungus belonging to the family *Hypocreaceae* (genus *Trichoderma*). *T. longibrachiatum* has the potential to play a key role as a mycoparasitic and biocontrol agent (Sanchez et al., 2007). According to the literature (Morgavi et al., 2010), *Trichoderma* is often applied in the feed industry as one of the enzyme components, especially that enzymes of fungal origin are characterised by a lower, i.e. optimal, pH for the rumen environment of animals. It has also been noted that exogenous enzymes potentially exhibit enzymatic activity, limiting the rate of fibre hydrolysis (Morgavi et al., 2000). Aerobic fungi of the genus *Trichoderma* are common species used as a fungal source of enzymes in livestock nutrition.

Trichoderma spp. are able to release several fibre hydrolysing enzymes into the medium and degrade the substrates. The enzyme system of *Trichoderma* has a lower optimum pH than rumen fungal enzymes that synthesize cell-associated cellulose enzyme complexes (Morgavi et al., 2010). Nsereko et al. (2002) conducted an experiment in dairy cows fed a diet supplemented with fibrolytic enzymes (mainly cellulase and xylanase) from *T. longibrachiatum* and recorded an increase in the number of hemicellulose- and cellobiose-utilising microorganisms and cellulose degradation products in the rumen. The authors noted that the increased fibre digestion recorded in cows fed with the addition of *T. longibrachiatum* enzymes was a consequence of a higher number of rumen microorganisms, and concluded that this fungus-derived feed additive could increase the amount of microbial proteins available to ruminants. Moreover, it has been found that some compound in barley whole crop silage can reduce the activity of endo-1,4-β-xylanase derived from *T. longibrachiatum* by up to 50% without affecting cellulase activity (Nsereko et al., 2002). The same conclusions were drawn by Beauchemin et al. (2003), who reported that the efficiency of fungal enzyme incorporation into ruminant diets depended on dietary ingredients – generally the enzymes were more effective when added to dry feeds than those with a high moisture. Morgavi et al. (2000) have investigated the relationship between ARF activity and three different *T. longibrachiatum* enzyme complexes added to ruminant diets. The first enzyme preparation contained a dominant proportion of xylanase, the second one cellulase, and the third complex with intermediate proportions of these enzymes. The latter authors reported that all applied *T. longibrachiatum* enzyme complexes enhanced the enzymatic activity of rumen microorganisms and improved cellulose and xylan degradability, thereby increasing the efficiency of rumen feed degradation.

Aspergillus sp.

A. niger is a widely known cosmopolite fungus which belongs to the family *Trichocomaceae* and genus *Aspergillus*, similarly to *A. oryzae*. It is also one of the sources of proteases, with the ability to excrete high amounts of enzymes in the process of solid state fermentation (Ooi et al., 2021). As reported by Kong et al. (2021), *A. oryzae* and *A. niger* have been used for decades in food science and production (Sadh et al., 2017) and their enzyme production have been optimized. Research in the field of nutritional quality of *A. oryzae* and *A. niger* has been fairly intensive (Sun et al., 2014; Altop et al.,

2018; Kong et al., 2021). The addition of *A. oryzae* to the diet was shown to increase ruminal fermentation due to an increase in the number of cellulolytic bacteria (Sallam et al., 2020). Similarly, esterase enzymes present in the extract from *Aspergillus* could have had a positive effect on fibre digestion (Damásio et al., 2013). Additionally, *Aspergillus* fermentation extracts can directly stimulate the rumen fungi, which improves fibre digestion. *A. oryzae* extract has been proven to contain malic acid, which stimulates rumen lactate utilisers (*S. ruminantium* and *M. elsdenii*) to use lactic acid (Waldrip and Martin, 1993; Desnoyers et al., 2009). The application of *A. oryzae* in diets of lactating cows can increase milk production, feed utilisation and heat stress tolerance (Higginbotham et al., 2004). The addition of *A. oryzae* fermentation extract to the diet of young calves can shorten the weaning period and increase the number of rumen bacteria and VFA levels (Beharka et al., 1991). The inclusion of *A. oryzae* in the calf ration has a positive effect on the apparent digestibility of DM, organic matter, crude protein, NDF and gross energy.

Anaerobic rumen fungi

There is clear positive correlation between ARF inclusion in ruminant diets and DM intake of feeds, especially fibrous forages of low nutritional value, and thus animal growth performance, rumen parameters and diet digestibility (Dey et al., 2004; Saxena et al., 2010; Kholif et al., 2014). ARF have been reported to colonise particles of fibrous feeds in the rumen and secrete a wide-array of enzymes cleaving linkages of non-digestible compounds contained in plant fibre (Dagar et al., 2011; Edwards et al., 2017). *Neocallimastix* sp. administered with buffalo calves' diet increased body weight gains and improved feed conversion ratio as well as nutrient digestibility (Thareja et al., 2006). Likewise, an increase in voluntary forage DM intake (approx. 35% and 40%) has been reported in early-weaned calves and ARF-deprived sheep fed a diet supplemented with *Neocallimastix* sp. (Denman et al., 2008; Cheng et al., 2009). A similar effects has been observed after *Piromyces* sp. inclusion to the diets of pre-ruminant and ruminant animals. It has been assumed that higher nutrient digestibility, better feed conversion ratio and growth performance as well as altered fermentation profile, found in several studies, is the result of enzymatic activity of *Piromyces* sp. that secrete, among others, cellulase, xylanase, feruloyl, acetyl esterases and carboxymethyl cellulase (Paul et al., 2006; Tripathi et al., 2007; Paul et al., 2010). Saxena et al. (2010) have recorded that

Orpinomyces sp. C-14 or *Piromyces* sp. WNG-12 (250 ml; 3–5 days of growth/animal/week) addition to the diet of milking buffalo cows led to an increase in milk yield and fat-corrected milk yield by about 6%. Incorporation of the aforementioned ARF species in the diet also improved digestibility of the main nutrients, such as DM, CF, NDF and ADF, as well as reduced ammonia nitrogen and pH value of the rumen fluid, while increasing total VFA and total nitrogen levels. *Orpinomyces* sp. has also been found to improve the growth and DM, CP, NDF and ADF digestibility in calves (Dey et al., 2004). The same tendency was observed by Lee et al. (2015) in sheep fed a diet supplemented with *Orpinomyces* strain KNGF-2.

Conclusions

In light of international regulations and consumer demands to withdraw growthaccelerating antibiotics and limit the use of therapeutic antibiotics, DFM offer an option. For ruminants, as stated in published articles, ARF and DFM have been successfully used to improve ruminal and gastro-intestinal health, enhance milk production, feed efficiency and daily gain in animals. The observed positive production effects, in which breeders are most interested, are the result of ARF participation in many complex decomposition processes occurring mainly in the rumen. The most important feature of ARF is the ability to secrete a variety of enzymes responsible for cleaving bonds in hard-to-digest compounds in fibre-rich ruminant diets. This contributes to a much better utilisation of these feeds and increased digestibility of nutrients. Additionally, rumen fungi have a positive effect on rumen fermentation and may decrease incidence of metabolic disorders, i.e. acidosis. However, further research is needed to develop methods for mass production of these fungal inocula for adoption as natural feed additives.

Conflict of interest

The Authors declare that there is no conflict of interest.

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