STATE UNIVERSITY OF MARINGÁ
AGRICULTURAL SCIENCES CENTER

EFFECT OF S*ACCHAROMYCES CEREVISIAE* STRAIN CNCM I-1077 ON THE RUMINAL DEGRADABILITY OF FORAGES FROM SOUTH AMERICA

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Thesis presented to the Graduate Program in Animal Science of the State University of Maringá in partial fulfillment of requirements for the degree of MASTER OF SCIENCE IN ANIMAL SCIENCE, major: Animal Production.

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“Ain't about how fast I get there,
Ain't about what's waiting on the other side,
It's the climb”
Jon Mabe and Jessi Alexander

To my mother, who always struggled to give me the best education

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BIOGRAFY

AMANDA CAMILA DE OLIVEIRA POPPI, daughter of Nilceia de Oliveira and Edson Carlos Poppi, was born in Maringá, Paraná, on March 8th, 1995.

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In March of 2017 she joined the Graduate Program in Animal Science at the State University of Maringá. In April of 2019 she submitted to the examining board in order to receive the title of Master’s in Animal Science.

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ABSTRACT

The effect of live yeast *Saccharomyces cerevisiae* strain CNCM I-1077 (SC) on the ruminal degradability of forages commonly found in dairy diets in South America was evaluated. Four non-lactating rumen-cannulated Holstein cows were housed in a tie-stall barn and randomly assigned to two treatment sequences: Control-SC-Control or SC-Control-SC, in a switchback design, with three 30d periods. Cows in the SC treatment were supplied with 1×10^{10} cfu of yeast daily via rumen cannula. The *in situ* degradability of DM and NDF was measured in 15 forage samples collected in Brazil, Argentina, and Peru, and included corn silage (n = 5), tropical grass silage (n = 2), sugarcane silage (n = 2), oat silage (n = 2), ryegrass silage (n = 2), alfalfa silage (n = 1) and alfalfa hay (n = 1). Forages were assigned to three groups: corn silages, tropical grasses (sugarcane silages and tropical grass silages) and temperate grasses and alfalfas (oat silages, ryegrass silages, alfalfa silage and alfalfa hay). Each forage was incubated in the rumen for 12, 24 and 36 h after feeding. Rumen fluid was collected from the ventral sac for measuring yeast count, pH, ammonia and VFA. Cows supplemented with SC had higher counts of live yeasts in rumen fluid, showed a trend of higher ruminal pH and lower ruminal ammonia concentration. Acetate to propionate ratio was higher in the rumen fluid of animals receiving SC. There was no interaction between forage group and yeast supplementation for the *in situ* degradability. However, SC accelerated the DM and NDF degradation, as noticed by higher disappearance of DM and NDF at 12 and 24 h of incubation. Therefore, live yeast supplementation is a strategy to improve rumen function and increase the nutritive value of forages grown in tropical and subtropical areas.

I. INTRODUCTION

Feeding is the costliest factor of animal production and might represent almost 80% of the total production costs in dairy and beef operations (USDA, 2018). Hence, the efficiency of converting feedstuffs in human foods, such as milk and meet, have a high impact on animal production systems. Since ruminant diets typically contain a certain amount of forage, fiber digestibility is a crucial point in ruminant nutrition.

Cellulose and other structural polysaccharides present in the plant cell wall are the major source of energy for herbivorous animals fed forage-based diets, due to the symbiosis between these animals and microorganisms present in the rumen (Weimer, 1992). The main fermentation products of these components are volatile fatty acids (VFA), mainly acetate, propionate and butyrate, as well as gases, carbon dioxide and methane. In addition, the protein deamination process performed by some microorganisms can produce ammonia, microbial protein, VFA and carbon dioxide (Bergman, 1990).

The action of the microorganisms on plant degradation is dependent on the quality and accessibility to the plant cell wall matrix. These factors are related to the maturity, genetics, chemical and physical composition of tissues (Akin, 1989). Thus, lower quality plants have lower ruminal degradability and are not used efficiently for animal production. In this way, the use of feed additives such as probiotics may be a strategy to enhance feed efficiency, animal performance and health (Chaucheyras-Durand et al., 2008).

1. Literature Review

1.1. Forage Quality

Forage quality is a relative term to describe the degree to which forage meets the nutritional requirements of a specific kind and class of animal (Allen et al., 2011). Hence, quality is associated to animal response and, for instance, can be measured by weight gain and milk yield. Since animal performance is strongly related to intake of digestible nutrients, forage quality is mainly a function of intake and digestibility (Paterson et al., 1994).

Because cell wall is the single largest component of forages, fiber content and digestibility are primary determinants of forage quality. The plant cell wall is a complex matrix of polymers that surrounds every plant cell. Walls provide the physical support required for plants to grow and serve as a barrier from attack by pathogens and insects. While all cell walls share basic chemical characteristics, marked differences exist among plant tissues in terms of cell wall concentration, composition, and structural organization (Jung, 2012).

1.1.2. Factors affecting ruminal digestibility

There are several factors that affect the structure and quality of the forage plant, which may be due to environmental factors and factors inherent to the plant itself. Factors such as soil quality, temperature, solar radiation, water availability, cultivars and maturity can affect the characteristics of the same plant species (Ball et al., 2001).

1.1.2.1 Chemical Composition

Lignin and polysaccharides (cellulose, hemicellulose and pectin) are the main compounds of the plant cell wall matrix, in addition to proteins, phenolic compounds, water and minerals (Åman, 1993). Those polymers can be divided into two categories based on their associations with other compounds and availability to the animal: those that have some covalent attachment to core lignin and are not completely digested in the rumen and those that are poorly covalently attached to core lignin and largely fermentable in the rumen (Van Soest, 1994).

1.1.2.1.1. Cellulose

Cellulose is a homopolymer formed by β-D-glucose 1 → 4 bonds which build long chains with high degree of polymerization and high molecular weight. These chains can bind through hydrogen bonds forming cellulose microfibrils, which has great value for the availability of this molecule to microbial enzymatic hydrolysis during ruminal degradation (Iiyama et al., 1993; Delmer and Amor, 1995). Cellulose, in its majority, is found in combination with other components of the plant wall, such as hemicellulose and lignin. Cellulose can be separated into two fractions, the potentially digestible and the indigestible, can be found in several plant constituents and their amount varies between them and between species (Giger-Reverdin, 1995; Pereira, 2013).

1.1.2.1.2. Hemicellulose

Hemicellulose is a heteropolysaccharide that is found in the cell wall. Hemicellulose is characterized by several units of amorphous sugars linked by different types of bonds. Their chains have a lower degree of polymerization when compared to cellulose (and not as resistant to solubilization and hydrolysis) but are commonly found associated to lignin by covalent bonds. They occur in various structural types and are divided into four subgroups: xylan, γ-glycan, xyloglycan, and mannan, being named according to the predominant monosaccharide (Giger-Reverdin, 1995; Ebringerová et al., 2005).

1.1.2.1.3. Pectin

Pectin is a polymer formed by complex polysaccharides, found in the middle lamella, and has the function of hydrating and cellular adhesion. In addition, pectin can play a role on the firmness of the cell, but it depends on the orientation, proprieties and connections among cellulose and pectic substances. Its content decreases from the primary to secondary wall, in the direction of plasma membrane. Grasses have a low pectin content when compared to legumes. It is one of the components of the cell wall that has low molecular weight and is highly digestible. Pectin is a non-fiber carbohydrate,

due to its solubility in neutral detergent (Van Soest, 1994; Thakur et al., 1997; Lempp, 2013).

1.1.2.1.4. Lignin

Lignin is a phenolic polymer composed of highly branched phenylpropanoids, unique to vascular land plants (Adler, 1977). Lignin is deposited on the cell wall during the secondary wall formation to confer thickening and protection, it is generally related to the indigestible fraction of the forages (Jung and Deetz, 1993). The denomination is used to describe groups of polymers with three aromatic alcohols (p-coumaril, coniferil and synapil). The terms "core" and "non-core" are used to differentiate the types of lignin found in forages (Jung, 1989; Susmel and Stefanon, 1993).

Core lignin generally has two or more bonds between phenolic monomers units, has high molecular weight and it is highly condensed. On the other hand, non-core lignin has a low molecular weight, a covalent bond on the phenolic compound and is generally bound to the hemicellulose fraction in the secondary cell wall (Jung, 1989; Van Soest, 1994). According to Hartley (1972) the p-coumaric acid, generally related to less digestible materials, has a higher concentration in non-core lignin, which possibly demonstrates that this type of lignin has a greater effect on animal nutrition. However, Wilson (1994) believes that this division presents little importance for the study of digestibility since both types have an effect on fiber degradability.

1.1.2.2. Morphology

Forages are complex organisms that consist of leaf, stem, inflorescence, and root and its cell walls differentiate structurally and chemically according to their functions within the plant. Thus, densely clustered, thick-walled and lignin-rich cells can be found in tissues that have function linked to lift, whereas thin-walled and lignin-free cells may be related to biochemical processes of carbon assimilation (Wilson, 1994; Paciullo, 2002).

Three forms of vegetal cell wall are found: primary, secondary and tertiary. The primary wall has a thickness of approximately 0.2 μm and its development occurs during the cell growth, and may be the only wall to develop, as in the parenchyma. The

secondary wall develops internally to the primary wall after complete cellular expansion and gives the cell protection to tension and compression due to its lignification, being able to reach a thickness of 5 μm. Finally, the tertiary wall is located inside of the secondary wall and is characterized as being membranous and thin (Wilson, 1993).

According to Akin (1989), tissues can be classified as: quickly digested, partially or slowly digested and nondigestible. Some plant tissues can be rapidly degraded by ruminants as result of no physical barrier to digestion. Other tissues can vary in digestibility, showing partial or no resistance to ruminal microorganisms and this difference may be a result of stressful situation or even maturity (e.g. high temperature and hydric stress) increasing lignin and phenolic complexes. Forages with large proportions of sclerenchyma and xylem cells in leaf blades, and epidermis, sclerenchyma ring (grasses) or interbundular cells (legumes), and xylem in stems have generally low rates of digestion, showing that these tissues generally form structural barriers, being nondigestible for ruminants (Akin, 1989).

In tropical forage leaves, the tissues that have fast digestion are mesophilic and phloem, the epidermis and parenchymatic sheath of the bundles have an intermediate digestibility, and the xylem and sclerenchyma are not accessible. In temperate forage leaves, in addition to the mesophyll and the phloem, the epidermis has a high rate of digestion, while the parenchymatic sheath of the bundles can be rapidly digested depending on its species, and as in the tropics, the xylem and the inner sheath of the bundles are indigestible. For grasses, the epidermis and ring of sclerenchyma are nondigestible, the parenchyma can be rapidly degraded or depending on its maturity and the phloem is rapidly degraded. Finally, in legumes the mesophyll is rapidly degraded in leaflets and vascular tissues in general are indigestible. In legume steam, the digestibility of the parenchyma is dependent on its maturity, and xylem is not accessible for ruminant digestibility (Akin, 1989).

Strongly related, the anatomical characteristics of the plant and its nutritional value are shown as good indicators of food quality, where the proportion of tissues and thickness of the cell wall are the main characteristics that affect animal use. The lignified and highly fibrous tissues have low digestibility (Allinson and Osbourn, 1970; Carvalho and Pires, 2008). The difficulty of lignin degradation can be related to several factors, such as the physical impediment caused by the binding of lignin with polysaccharides that may hind the access of the enzymes, hydrophobicity caused by lignin polymers that

limit the action of fibrolytic enzymes, and a possible toxic effect of lignin components on ruminal microorganisms (Jung and Deetz; Susmel and Stefanon, 1993). Jung (1989) reported that there was a negative correlation between lignin core and in vitro fermentation. The p-coumaric acid is esterified in the core-lignin, where in experiments using its free form, its presence reduced activities of cellulolytic microorganisms, decreased bacterial growth rate and reduced fungal activity. Beyond that, the ferulic acid is primarily esterified in hemicellulose, and at experimental levels it was correlated with decrease in degradation in vitro. It was also observed that cinnamic acids had a significant reduction in digestibility. However, the toxicity caused by these acids is unlikely due to their low concentration in forage and ruminal environment and the bacteria have detoxification mechanisms (Paciullo, 2002).

The main limitation of forage lignification apparently is due to its physical impediment to the action of the hydrolytic enzymes at the carbohydrate center of reaction, where the concentration, ramification and association with other carbohydrates of the lignin cause negative effects on its degradation (Jung and Deetz, 1993). Moreover, the thickness of the cell wall is a physical factor that inhibits the digestion, where the greater the thickness of the secondary wall, the smaller is the access of the microorganisms and the longer is the time necessary for its complete digestion (Carvalho and Pires, 2008).

Other characteristics that may be related to forage quality are the anatomical characteristics that show the proportion and disposition of lignified and non-lignified tissues within the plant, as well as physiological characteristics such as efficiency in the carbon cycle. With increasing forage age, more lignified the components are and there are lost in the nutritive value within foliar sheaths and stems, as they increase the parenchyma tissue, and can be affected by the environment and the species (Lempp, 2013).

Epidermall cells, such as cell rich of silica and bulliform cells, have negative effects on cell degradation. Silica confers stiffness to the cell and bulliform cells are more resistant to ruminal degradation and occupy large space in the leaf blade. In addition, the epidermis may present cuticle and cutin that resist colonization of the microorganisms (Wilson, 1993; Paciullo, 2002; Lempp, 2013).

Although grasses have a lower lignin content, they have a lower rate of degradability when compared to other species. One of the plausible explanations is that there are lignin binds through xylose and arabinose covalently to the hemicellulose,

hampering its ruminal degradation (Jung, 1989). Compared to the C3 and C4 plants, the first one has a greater advantage in relation to its qualitative potential, because it has a lower elongation of stem coarseness, slides with lower proportion of lignifiable tissues, lower levels of neutral detergent fiber (NDF) and lignin. In addition, C4 plants exhibit Girder cells, which cause thickening of well-developed veins and parenchymal cells, thereby decreasing their rate of degradation (Paciullo, 2002; Lempp, 2013).

1.1.3. Ways to Improve Forage Degradability

Although there are intrinsic factors in plants that hinder access and degradability by the ruminal microorganisms, there are ways to reverse them by using different genotypes of forages, plants with different maturities, exogenous substances capable of cleaving cell walls (e.g. chemicals, enzymes), and supply of additives able to enhance the ruminal environment and potentialize the action of fibrolytic microorganisms.

Several studies have been carried out with the aim of improving the forage composition through genetic selection and manipulation. The composition can be altered by modifying the concentration and composition of lignin, by the quality of the protein, decreasing anti-nutritional factors and thereby increasing its nutritional value (Casler, 2004). In addition, with the advancement of maturity the fiber content in the plant is increased, making it less digestible (Raymond, 1969). Salazar et al. (2010), in an experiment carried out at the Agronomic Institute in Campinas-SP, evaluating the effect of 15 maize hybrids at different maturity stages (harvested with 90, 120 and 150 days post-germination), observed that there was an increase in lignin deposition at maturity, and there was a difference between the hybrids used, suggesting a great variability among the genetic groups and maturity.

Exogenous substances may also be used to improve forage digestibility. Exogenous enzymes can be used at the time of feeding or during the ensiling process, hydrolyzing the cell wall in readily fermentable sugars for silo and rumen microorganisms (Adesogan, 2005). Alkalizing agents (sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$), anhydrous ammonia (NH_3) and calcium oxide (CaO)) partially solubilize the hemicellulose and damage the hydrogen bonds, increasing fiber digestion (Oliveira et al., 2002; Andrade et al., 2007; Mota et al., 2010).

Another way of changing forage degradability is by manipulating the ruminal environment. Due to the importance of ruminal digestion, the manipulation of fermentation is a tool that allows making the system more efficient, for instance by increasing the transformation of fibrous compounds into nutrients for the synthesis of meat and milk (Wallace, 1994; Arcuri and Mantovani, 2006; Mantovani and Bento, 2008).

Among additives used for ruminants, pre- and probiotics, which normally contain live strains of microorganisms, inactivated microorganisms or microbial cell fractions, may potentially benefit the indigenous microbiota (Martin and Nisbet, 1992). Benefits on gut bacteria population and animal immune response have been reported (Rose, 1987). In addition, biological additives do not generate residues into the final products, being an interesting alternative to the traditional additives.

1.2.Yeast effect on the Ruminal Environment

1.2.1. Yeast Characterization

Yeasts are eukaryotic cells, belonging to the *Fungi* kingdom with nuclear membrane and cell walls. Measuring between 3 and 10 μm, they have the capacity to produce energy and soluble forms of nutrients from any organic matter source, being denominated heterotrophic (Bennett, 1998). Through enzymes, yeasts digest proteins, glucose and lipids, and can absorb amino acids and monosaccharides from their cell membrane. They are considered facultative anaerobes, which, in the presence of oxygen convert sugars into carbon dioxide and energy and when absence produce ethanol (Walker and White, 2005).

A widespread use of yeast in animal production is in the form of active dry yeast products (ADY), which preserve the viability and metabolic activity of the cell and have a high concentration of viable cells (> 10 billion cfu/g). There are about 500 different yeast species with morphological, metabolic and reproductive differences. *Saccharomyces cerevisiae* stands out in the production of beverages, food and animal use, being the most common strain currently in use (Chaucheyras-Durand et al., 2008).

1.2.2. Yeast Effects on Ruminal Environment

Studies have shown that the use of *Saccharomyces cerevisiae* assists in ruminal metabolism, increases the total number of viable bacteria and cellulolytic bacteria, besides stimulating lactate-consuming bacteria in the rumen, resulting in a greater degradation of fiber, greater synthesis of microbial protein and higher animal performance (Rose, 1987; Chaucheyras-Durand et al., 2008).

1.2.2.1 Ruminal pH

Diets of high-producing ruminant animals often contain a high proportion of concentrate, low proportion of forages and physically effective NDF and smaller particle size, causing a low chewing rate A reduced chewing activity and diets with high content of readily fermentable substrates can cause an accumulation of acids (e.g. VFA and lactic acid) produced by ruminal microorganisms and a reduction in ruminal buffering capacity, causing a drop in pH (Plaizier et al., 2008). Prolonged ruminal acidity causes detrimental in consumption and nutrient degradation. In addition, some microorganism's species, such as cellulolytic microorganisms, are sensitive to ruminal acidity. Low ruminal pH is associated with lower fiber degradability and diseases such as ruminites, liver abscess, lameness, inflammations, diarrheas and milk-fat depression (Russell et al., 1979; Dijkstra et al., 2012).

In a study carried out by Bach et al. (2007), daily supplementation of *Saccharomyces cerevisiae* strain CNCM I-1077 at 10^{10} CFU/d, led to higher ruminal pH (6.05 vs. 5.49). Thrune et al. (2009) reported that the same yeast strain resulted in a shorter time in subacute acidosis. Similar results were found by Nocek et al. (2002) and Chung et al. (2011). In contrast, McGinn et al. (2004) evaluating ruminal parameters in addition to commercial yeasts (1g/d) did not find differences for ruminal pH.. Possenti et al. (2008) comparing the inclusion of yeast in cattle's diet (10 g/d) did not find significative differences for ammonia concentration among the treatments and pH was more stable in the control treatment (without yeast).

However, it is suggested that the effect of yeast on the maintenance of ruminal pH generally occurs with a decrease in lactate concentration, which may be related to substrate competition with lactate-producing bacteria, as well as to stimulate the growth of lactate-consuming microorganisms, as summarized by Chaucheyras-Durand et al.

(2008). Although there is a tendency to improve ruminal fermentation and pH stabilization, there is still no consensus on the use of yeast in ruminant production, and there are studies with different responses to this additive (Desnoyers et al., 2009).

The increase in ruminal bacterial cells is often observed with the use of live yeast, which diverts N ruminal to microbial protein synthesis, changing volatile fatty acids production and consequently raising the pH (Chaucheyras-Durand et al., 2008). Another effect that may be related to the action of living yeast is the stimulation of Entodiniomorphid protozoa, which competes with amylolytic bacteria per substrate, has a lower rate of starch fermentation and consume lactate. As facultative anaerobic organisms, yeast can consumes the oxygen present in the rumen, benefiting the ruminal metabolism, beyond providing nutrients for these other microorganisms (Brassard et al., 2006; Chaucheyras-Durand et al., 2008; Vohra et al., 2016).

1.2.2.2. Fiber digestibility

Ruminants have the ability to degrade forage cell wall components by symbiosis with ruminal microorganisms, which hydrolyze these molecules and produce energy, volatile fatty acids, gases, microbial protein, among other compounds (Weimer, 1998). However, in some situations, such as in different species, maturation and plant parts, this degradation is hampered by complex and not accessible structures, diminishing the use by the animal.

Chaucheyras-Durand et al. (2010) found out that the supplementation of yeast resulted in higher ruminal *in situ* degradation of DM and NDF in alfalfa hay, associated to a stimulation on anaerobic fungi and *B. fibrisolvens* growth. Similar results were found by Guedes et al. (2008) evaluating the supplementation of yeast on fiber degradation in corn silage samples with different quality (high and low degradability). Yeast supplied at 1 g/d had a greater benefit on the ruminal degradability of lower quality silage. Williams et al. (1991) evaluated the effects of live yeast for heifers and verified an increase of DM degradation with the inclusion of yeast, mainly at 12 h of incubation. The same results were reported by Bitencourt et al. (2011). On the other hand, Hadjipanayiotou (1997) evaluated the degradability of five feedstuffs (barley grain, soybean meal, barley straw, barley hay, alfalfa hay) in three rumen-fistulated goats, and concluded that the use of yeast did not affect diet digestibility and animal performance. Hristov et al. (2010)

measured the ruminal degradation and fermentation in dairy cows, and also did not observe differences with the use of the yeast.

The increase in fiber degradability has been not consistence among experiments. However, when observed, the higher degradability in the presence of yeasts may be due to its influence on the activity of fiber-degrading microorganisms in the rumen. Apparently, live yeasts may increase fungal colonization, polysaccharidase and glycoside-hydrolase activities, besides increasing and accelerating the proliferation of fibrolytic bacteria (Chaucheyras-Durand et al., 2008). The increase of these microorganisms may be due to growth factors related to these additives, in addition the oxygen consumption carried out by the yeasts and a higher rumen pH (Desnoyers et al., 2009; Vohra et al., 2016; Shurson, 2018).

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496

II Effect of *Saccharomyces cerevisiae* strain CNCM I-1077 on the ruminal degradability of forages from South America

(Manuscript style and form consistent with the Instructions for Authors of the Journal of Animal Science)

ABSTRACT

The effect of live yeast *Saccharomyces cerevisiae* strain CNCM I-1077 (SC) on the ruminal degradability of forages commonly found in dairy diets in South America was evaluated. We also examined if SC supplementation interacts with forage quality to affect ruminal fiber degradability. Four non-lactating rumen-cannulated Holstein cows were housed in a tie-stall barn and randomly assigned to two treatment sequences: Control-SC-Control or SC-Control-SC, in a switchback design, with three 30-d periods. Cows in the SC treatment were supplied with 1×10^{10} colony forming units (cfu) of yeast daily via rumen cannula. The *in situ* degradability of DM and NDF was measured in 15 forage samples collected in Brazil, Argentina and Peru, including corn silage (n = 5), tropical grass silage (n = 2), sugarcane silage (n = 2), oat silage (n = 2), ryegrass silage (n = 2), alfalfa silage (n = 1) and alfalfa hay (n = 1). Forages were assigned to three groups: corn silages, tropical grasses (sugarcane silages and tropical grass silages) and temperate grasses and alfalfas (oat silages, ryegrass silages, alfalfa silage and alfalfa hay). Each forage was incubated in the rumen for 12, 24 and 36 h after feeding. Rumen fluid was collected from the ventral sac for measuring yeast count, pH, ammonia, lactate and VFA. Cows supplemented with SC had higher counts of live yeasts in rumen fluid, showed a trend of higher ruminal pH and lower ammonia concentration. Acetate to propionate ratio was higher and lactate was lower in the rumen fluid of animals receiving SC. Contrary to our expectation, there was no interaction between forage

group and yeast supplementation for the *in situ* degradability. However, SC accelerated the DM and NDF degradation, as noticed by higher disappearance of DM and NDF at 12 and 24 h of incubation. Therefore, live yeast supplementation is a strategy to improve rumen function and increase the nutritive value of forages grown in tropical and subtropical areas.

Key words: cell wall, fermentation, live yeast, roughage, rumen degradability

INTRODUCTION

In high-producing ruminant diets, forages are included to provide physically effective fiber, to keep ruminal function and animal health (Mertens, 1997). Nevertheless, forages are also important source of nutrients, depending on their quality, which is mainly defined by the content of neutral detergent fiber (NDF) and its digestibility (NDFD) (Huhtanen et al., 2006). Moreover, the content and digestibility of NDF in diet may regulate feed intake, due to the physical filling of digestive compartments, and in turn, constrain the animal performance (Mertens, 1994; Allen, 2000).

Forage species, genotypes, growing environment, maturity and harvesting management affect forage composition and digestibility. Meanwhile, different strategies can be used to improve forage digestibility, such as the application of exogenous fibrolytic enzymes (Adesogan, 2005) and chemicals (e.g. sodium hydroxide, anhydrous ammonia, calcium oxide) (Klopfenstein, 1978), as well as the manipulation of the ruminal fermentation (Wallace, 1994). The use of pre- and probiotics in ruminant diets is an alternative to improve forage degradability via improvement of rumen fermentation, in addition to the benefits to animal health (Adesogan et al., 2019; Bach et al., 2019).

The supplementation of live yeasts, such as *Saccharomyces cerevisiae* strains, may increase the total number of cellulolytic bacteria, stimulate lactate consumption and decrease lactate production, increase rumen pH and reduce oxygen concentration in the rumen fluid, resulting in higher fiber degradation, greater synthesis of microbial protein and improved animal performance (Chaucheyras-Durand et al., 2008; Ondarza et al., 2010). However, the benefits of live yeast supplementation on ruminal degradability of forages grown in tropical and subtropical areas is seldom reported (Sousa et al., 2018).

The aim of this study was to evaluate the effect of live yeast *Saccharomyces cerevisiae* strain CNCM I-1077 on the ruminal degradability of DM and NDF of several forage samples commonly found in diets of dairy cows in South America and verify if the magnitude of improvement in DM and NDF degradation is dependent on forage quality.

MATERIAL AND METHODS

Forage Samples

Fifteen forage samples, including corn silage, tropical grass silage, sugarcane silage, alfalfa haylage and hay, ryegrass haylage and oat haylage were collected across South America (Brazil, Argentina and Peru). Samples were dried in a forced-air oven at 55ºC during 72 h at sampling site, packed in polyethylene bags and sent to the State University of Maringá. Information about collection sites and forage composition is shown in Tables 1 and 2.

Cows, Facilities and Experimental Design

Animal care and handling procedures were approved by the Ethics Committee for Animal Use of the Maringa State University (protocol number 8208090218 –

572 CEUA/UEM). Four non-lactating rumen-cannulated Holstein cows (two primiparous
573 and two multiparous; average 545 kg of BW) were housed in a tie-stall barn with rubber
574 beds, individual feedbunks and water bowls. The diet offered to the cows consisted of
575 65% of corn silage and 35% of concentrates (corn grain ground, soybean meal, wheat
576 bran and mineral-vitamin mix) and contained 12% of CP and 38% of NDF (DM basis).
577 Every morning, diet ingredients were mixed and fed as a total mixed ration (TMR) at
578 08:00 h, after removing the refusals from the previous day. The amount of TMR was
579 adjusted daily to allow at least 10% as orts.

580 The experimental treatments were: 1) control (Ctrl) and 2) live yeast
581 supplemented at 1×10^{10} cfu/d per cow (SC; *Saccharomyces cerevisiae* strain CNCM I-
582 1077; Lallemand Animal Nutrition, Aparecida de Goiânia, GO). The live yeast was
583 diluted in 250 mL of distilled water at 40°C and dosed directly into the rumen, through
584 the rumen cannula, every morning immediately before TMR distribution. Cows
585 receiving the control treatment were also dosed with 250 mL of distilled water at 40°C
586 to avoid ruminal oxygen stress bias between treatments. The treatments were compared
587 in a switchback design, with three 30-d periods, being 19 d of adaptation and the last 11
588 d of sampling. There were two treatment sequences: Ctrl-SC-Ctrl or SC-Ctrl-SC. Cows
589 were paired on parity and randomly assigned to each treatment sequence.

590

591 ***In situ Degradability***

592 From d 20 to d 30 of each period, two 5-d runs were performed for measuring
593 the *in situ* disappearance of DM and NDF of the 15 forage samples (8 or 7 forages
594 assigned to each run randomly). Dry forage samples were ground in a Wiley mill with a
595 5-mm screen and weighed in woven *in situ* bags (10 × 20 cm; 50 µm porosity; Ankom
596 Technology, Macedon, NY, USA). Approximately 5 g was placed in each bag. Each

feed was incubated in triplicate for 12, 24 and 36 h after feeding. Two blank bags were included in each time point. Before the incubation, the bags were soaked in warm water (39°C) for 20 min. Bags were inserted in reverse order and recovered all together. Immediately after removing, bags were submerged in cold water (0°C) for 5 min and washed in a washing machine (three cycles, followed by a final spin). Washed bags were dried in forced-air oven at 55°C for 72 h, weighed, and their contents were ground through a 1-mm screen using a Wiley Mill for measuring NDF concentration.

Sampling of Feed, Feces and Rumen Fluid

Samples of diet ingredients were collected from d20 to d30 of each period and subsequently composed by period. The apparent digestibility of DM, NDF and NDS were determined using indigestible NDF (iNDF) as internal marker (Huhtanen et al., 1994). Fecal grab samples were collected every 8 h, from d20 to d24 in each period and composed by cow. Samples were oven-dried at 55°C for 72 h and ground (1-mm screen; Wiley mill) for analyzes of DM, ash, NDF and iNDF.

On d30 of each period, rumen fluid was collected from the ventral sac at 0, 2, 4, 8 and 12 h after feeding for measuring pH (pH meter model Tec5, Tecnal® Piracicaba, Brazil), ammonia, lactate and VFA. Yeast count was measured in samples collected at 0, 2 and 8 h.

Laboratory Analyses

Samples of forages, ration and feces were analyzed for DM (method 934.01; AOAC, 1990), NDF, assayed with a heat stable amylase and expressed inclusive of residual ash (Mertens, 2002), ash (method 942.05; AOAC, 1990) and iNDF, by *in situ* incubation for 288 h (Huhtanen et al., 1994). Neutral detergent solubles were calculated

as NDS = 100 – ash – NDF. Ration was also analyzed for CP by Kjeldahl procedure (method 984.13; AOAC, 1990). Forage samples were additionally analyzed for CP, ADF, assayed sequentially and expressed inclusive of residual ash, and ADL, determined by solubilization of cellulose with sulphuric acid and expressed inclusive of residual ash (Van Soest, 1967). Hemicellulose was calculated as NDF – ADF and cellulose as ADF – ADL.

Ruminal volatile fatty acids were determined by gas chromatography (GCMS QP 2010 plus, Shimadzu, Kyoto, Japan) using a capillary column (Stabilwax, Restek, Bellefonte, PA; 60 m, 0.25 mm ø, 0.25 μm crossbond carbowax polyethylene glycol). Ammonia (Chaney and Marbach, 1962) and lactate (Pryce, 1969) were determined by colorimetric methods. Yeast was enumerated in malt extract agar (M137, Himedia®, Mumbai, India) acidified to pH 3.5 with lactic acid. The plates were incubated aerobically for 2 d at 30ºC. The number of colony forming units (cfu) was expressed as $\log_{10}$ cfu/mL.

Statistical Analysis

Statistical analysis was performed using the MIXED procedure of SAS (version 9.4). The DM intake and apparent digestibility were compared using a model that included fixed effects of treatment, period, treatment × period and random effects of cow and cow × treatment. An autoregressive first order [AR(1)] covariance structure was defined and the effect of cow was the subject. Rumen fluid parameters (yeast count, ammonia, pH and VFA) were analyzed with the same model including the fixed effect of time and treatment × time.

For the *in situ* assay, forages were assigned to three groups: corn silages, tropical grasses (sugarcane silages and tropical grass silages) and temperate grasses and alfalfas

(oat silages, ryegrass silages, alfalfa silage and alfalfa hay).Outcomes were analyzed with the same model described above including the fixed effects of forage group and interaction between forage group and treatment. Differences between treatments were declared if $P \leq 0.05$ and trends considered if $0.05 < P \leq 0.15$.

RESULTS

The SC did not affect the DM intake (average 10.45 kg/d) and apparent digestibility of nutrients (Table 3). Cows supplemented with SC had higher counts of yeast in rumen fluid and showed a trend of lower ($P = 0.10$) ammonia concentration and higher ($P = 0.12$) ruminal pH (Table 4). There was an interaction ($P < 0.01$) between yeast supplementation and time after feeding for lactate concentration (Figure 1). Cows receiving SC had a lower lactate concentration in the rumen fluid, especially at 8 h after feeding.

Animals treated with SC had higher acetate:propionate ratio, and there was a trend for lower concentrations of propionate ($P = 0.12$) and valerate ($P = 0.15$) in the rumen fluid. The concentrations of acetate, iso-butyrate, butyrate, iso-valerate and total VFA did not differ between treatments.

There was no interaction between forage group and yeast supplementation for the *in situ* degradability (Table 5). The SC significantly increased the NDF and DM degradability at 24 h and tended to increase the ruminal degradability of DM and NDF at 12 h of incubation. No difference was observed for the *in situ* degradability of DM and NDF at 36 h of incubation.

672 **DISCUSSION**

673

674 Active dry yeasts have been widely used as feed additive to improve animal
675 performance and health (Chaucheyras-Durand et al., 2008). Cattle responses attributed
676 to live yeast supplementation are often associated with improved rumen function.
677 Reduced redox potential (by oxygen scavenging) (Marden et al., 2008), higher pH (by
678 decreasing lactic acid production and increasing utilization of lactic acid) (Williams et
679 al 1991; Chaucheyras et al., 1996; Chaucheyras-Durand et al. 2005; Guedes et al, 2008)
680 and greater availability of growth factors (e.g. organic acids and vitamins) (Jouany,
681 2006; Chaucheyras-Durand et al., 2008) have been associated with stimulation of rumen
682 microbiota (Newbold et al., 1996; Mosoni et al., 2007; Sousa et al., 2018), increased
683 microbial protein synthesis (Moya et al., 2018) and enhanced fiber degradation in the
684 rumen of animals fed live yeasts (Chaucheyras-Durand and Fonty, 2001; Guedes et al,
685 2008; Sousa et al., 2018). In the current trial, the most notable response was the greater
686 *in situ* degradability of NDF in forages incubated in cows receiving the SC.
687 In this study, animals fed SC had higher counts of yeasts, tended to have higher
688 pH values, lower concentrations of ammonia and lactate at a comparable concentration
689 of VFA in the rumen fluid. These findings indicate that SC might have stimulated the
690 growth of bacteria in the rumen (Harrison et al., 1988; Erasmus et al., 1992). Usually,
691 the increase in rumen pH in animals supplemented with SC is related to a lower
692 concentration of lactate and an increased activity of fibrolytic bacteria and fungi in the
693 ruminal digesta (Chaucheyras-Durand and Fonty, 2001; Desnoyers et al., 2009;
694 Chaucheyras-Durand et al., 2015). Although there was no difference in the content of
695 total VFA and most individual VFA, cows fed SC had a lower concentration of lactate
696 and higher acetate:propionate ratio, due to a trend of lower propionate concentration. In

697 the rumen, propionate is synthetized via succinyl-CoA and acrylyl-CoA pathways
698 (Russell and Wallace, 1988). Lactic acid produced by rumen bacteria or ingested with
699 fermented feedstuffs can be converted to propionate via acrylyl-CoA pathway by
700 lactate-fermenting bacteria, such as *Veillonella alcalescens*, *Megasphaera elsdenii* and
701 *Selenomonas ruminantium* (Mackie et al., 1984). Yeast supplementation has been
702 associated with either a decreased production and increased utilization of lactic acid
703 (Williams et al 1991; Chaucheyras et al., 1996; Chaucheyras-Durand et al. 2005;
704 Guedes et al, 2008). Although lactate concentrations were relatively low (< 1 mM)
705 indicating non-acidotic conditions among treatments, in this study, two peaks of lactate
706 were detected in the rumen fluid. The first occurred immediately after TMR feeding,
707 certainly by the intake of lactic acid present in the corn silage. At 8 h after feeding,
708 lactate concentration increased again, likely as an intermediate of ruminal fermentation,
709 which coincided with the highest concentrations of VFA and pH nadir (not showed).
710 However, this increase was mainly observed in the control cows. Then, SC decreased
711 lactate concentration mainly at 8 h after feeding. Hence, the lower concentration of
712 lactate is a plausible explanation to the lower concentration of propionate and higher
713 acetate:propionate ratio observed in cows supplemented with SC. Moreover, the greater
714 fiber degradation might have contributed to the greater acetate:propionate ratio in cows
715 receiving SC. Compared with species capable of fermenting non-fiber carbohydrates,
716 ruminal fibrolytic microorganisms generally lead to a higher proportion of acetate
717 among their fermentation end-products (Russell and Wallace, 1988; Wolin and Miller,
718 1988).

719 The main cellulolytic species found in the ruminal environment are
720 *Ruminococcus flavefaciens*, *R. albus* and *Fibrobacter succinogenes* (Bayer et al., 1998;
721 Forsberg et al., 2000). Chaucheyras-Durand et al. (2015) found out that SC

722 supplementation resulted in higher ruminal degradation of several feedstuffs and a
723 stimulation of ruminal populations of anaerobic fungi and fibrolytic bacteria, such as *B.*
724 *fibrisolvens* and *R. flavefaciens*. Jiang et al. (2019) examined effects of dose and
725 viability of supplemented *Saccharomyces cerevisiae* (strain YE1496) on ruminal
726 fermentation of dairy cows. They reported an increase in the relative abundance of some
727 ruminal cellulolytic bacteria (*Ruminococcus* and *Fibrobacter succinogenes*) but also of
728 amylolytic bacteria (*Ruminobacter*, *Bifidobacterium*, and *Selenomonas ruminantium*).
729 In that trial, adding live instead of killed yeast increased the relative abundance of
730 fibrolytics, such as *Ruminococcus* and *F. succinogenes* (Jiang et al., 2019). Sousa et al.
731 (2018) evaluating the SC supplementation in grazing cattle reported an increased
732 population of *R. flavefaciens*, especially during hottest periods of the year.
733 It has been claimed that forage quality can influence the SC effect on ruminal
734 degradation. Guedes et al. (2008) described a larger response to SC supplementation in
735 corn silages of lower NDF degradability than in corn silages with higher NDF
736 degradability *in situ*. Recently, Sousa et al. (2018) reported a higher relative benefit of
737 SC on NDF degradability in tropical forages of lower NDF degradability. However, the
738 absolute increase in NDF degradability (g/kg) reported by the authors was higher in
739 forages with higher quality, with higher increase in NDF degradability in Palisade grass
740 (+25 g/kg), Guineagrass (+ 23 g/kg) and corn silage (+ 26 g/kg) than in sugarcane silage
741 (+ 17 g/kg) and Bermudagrass hay (+ 19 g/kg). Since the SC benefits are mainly based
742 on increased fibrolytic activity by stimulation of bacteria and fungi (Chaucheyras-
743 Durand et al., 2015), it seems unlikely that plant tissues with greater recalcitrance would
744 be benefited more than a less lignified cell wall in response to SC supplementation, at
745 least under realistic digesta retention times.

In the current trial, there was no interaction between forage group and yeast supplementation for the *in situ* degradability. The SC supplementation increased the *in situ* degradability of DM at 12 and 24 h of incubation by 2.3%-unit and 2.8%-units, which represents a relative increase by 2.5% and 4.6%, respectively. Overall, the higher DM degradability was mainly due to an increase of NDF degradability at 12 and 24 h of incubation by 2.0%-unit and 2.94%-units, which represents a relative increase by 9.7% and 10.3%, respectively.

The NDF comprises different cell wall components. Therefore, NDF is not a homogeneous fraction or has uniform digestibility (Van Soest, 1994). Previous studies have indicated that NDF degradation is better predicted assuming that NDF is the sum of iNDF and potentially digestible NDF (pdNDF), and that pdNDF is represented by two digestible fractions, with rapidly and slowly degradable fractions, respectively (Ellis et al., 2005; Huhtanen et al., 2008; Raffrenato et al., 2019). Several reports have suggested that SC supplementation could accelerate the rate of fiber degradation, with a small or no SC effect for longer incubation times (William et al. 1991; Girard and Dawson, 1995; Callaway and Martin, 1997; Sousa et al., 2018). In the present study, it is likely that the degradation rate of NDF of the forage sources was faster when SC was fed. Meanwhile, no difference between control and SC was observed when the forage samples were incubated for 36 h. Those findings suggest that degradation of pdNDF fast pool is mainly favored by yeast supplementation.

CONCLUSION

Saccharomyces cerevisiae strain CNCM I-1077 improved rumen function and increased fiber and dry matter degradability, without interacting with forage group. Live

yeast supplementation is a strategy to improve the nutritive value of forages grown in tropical and subtropical areas.

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Table 1. List of forages sampled in the South America

ID	Type	Forage	Scientific name	Conservation	Local	State	Country	Assigned group
A	C4 grass	Corn	*Zea mays*	Silage	Saladillo	Buenos Aires	Argentina	Corn silage
B	C4 grass	Corn	*Zea mays*	Silage	Castro	PR	Brazil	Corn silage
C	C4 grass	Corn	*Zea mays*	Silage	Bela Vista de Goiás	GO	Brazil	Corn silage
D	C4 grass	Corn	*Zea mays*	Silage	Mandaguaçu	PR	Brazil	Corn silage
E	C4 grass	Corn	*Zea mays*	Silage	Arequipa	Arequipa	Peru	Corn silage
F	Legume	Alfalfa	*Medicago sativa*	Hay	Lunardelli	PR	Brazil	Temperate/Alfalfa
G	Legume	Alfalfa	*Medicago sativa*	Silage	Castro	PR	Brazil	Temperate/Alfalfa
H	C3 grass	Oat	*Avena sativa*	Silage	Arapoti	PR	Brazil	Temperate/Alfalfa
I	C3 grass	Oat	*Avena sativa*	Silage	Castro	PR	Brazil	Temperate/Alfalfa
J	C3 grass	Ryegrass	*Lolium multiflorum*	Silage	Castro	PR	Brazil	Temperate/Alfalfa
K	C3 grass	Ryegrass	*Lolium multiflorum*	Silage	Castro	PR	Brazil	Temperate/Alfalfa
L	C4 grass	Sugarcane	*Saccharum officinarum*	Silage	Nova Andradina	MS	Brazil	Tropical grass
M	C4 grass	Sugarcane	*Saccharum officinarum*	Silage	Agudos	SP	Brazil	Tropical grass
N	C4 grass	Tropical grass	*Panicum maximum* cv. Mombaça	Silage	São Miguel do Aragaia	GO	Brazil	Tropical grass
O	C4 grass	Tropical grass	*Panicum maximum* cv. Mombaça	Silage	Terenos	MS	Brazil	Tropical grass

Table 2. Chemical composition of the forage samples (% DM, unless otherwise stated)

Forage	DM (% as fed)	CP	Ash	NDF	Hemicellulose	ADF	Cellulose	ADL	iNDF[1]
A-Corn silage	27.2	7.77	5.94	53.6	26.0	27.5	23.8	3.72	17.7
B-Corn silage	34.0	7.45	3.93	43.1	25.3	17.7	15.6	2.11	13.3
C-Corn silage	25.5	4.58	2.58	59.0	25.6	33.4	28.4	4.96	20.5
D-Corn silage	29.3	7.60	3.43	40.3	20.0	20.3	17.7	2.59	13.8
E-Corn silage	32.3	8.96	10.4	60.1	24.6	35.4	29.6	5.81	16.7
F-Alfalfa hay	90.7	14.0	7.18	72.0	19.0	53.0	39.0	14.0	47.3
G-Alfalfa silage	53.6	15.8	8.75	54.2	16.2	38.0	28.8	9.29	29.6
H-Oat silage	21.7	7.12	7.72	61.1	24.7	36.4	30.3	5.98	26.2
I-Oat silage	29.0	9.44	8.77	66.1	26.6	39.5	35.5	3.84	17.5
J-Ryegrass silage	49.0	14.3	10.8	59.5	24.8	34.4	29.8	4.60	18.8
K-Ryegrass silage	54.5	16.9	12.1	51.9	21.6	30.3	27.1	3.19	12.2
L-Sugarcane silage	33.1	2.58	2.25	76.7	29.8	44.8	34.0	10.9	37.3
M-Sugarcane silage	24.3	2.49	2.43	80.5	31.1	49.4	38.9	10.5	40.4
N-Tropical grass silage	28.5	3.09	8.34	83.7	28.5	55.2	46.6	8.59	42.8
O-Tropical grass silage	39.0	4.29	7.24	81.3	32.5	48.8	42.1	6.66	38.2

[1]Indigestible NDF.

Table 3. Dry matter intake and apparent digestibility of nutrients in non-lactating cows supplemented or not with live yeast

Item	Treatment		SEM	P-value
	Control	Yeast		
DM intake (kg/d)	10.1	10.8	1.03	0.53
DM digestibility (%)	63.1	63.2	1.22	0.95
NDF digestibility (%)	43.7	44.9	2.27	0.72
NDS[1] digestibility (%)	82.1	82.8	1.03	0.65

[1]NDS: neutral detergent solubles.

Table 4. Yeast count, pH, ammonia and VFA in the rumen fluid of non-lactating cows supplemented or not with live yeast

	Treatment			P-value[2]		
Item	Control	Yeast	SEM[1]	T	H	T × H
Yeast count (log_{10} cfu/mL)	4.99	5.40	0.084	0.05	<0.01	0.37
Ammonia (mg/dL)	11.0	9.39	0.50	0.10	<0.01	0.72
pH	6.15	6.26	0.032	0.12	<0.01	0.71
Lactate (mM)	0.593	0.472	0.064	0.03	<0.01	0.04
Acetate (mM)	72.4	69.4	2.33	0.44	0.01	0.96
Propionate (mM)	26.2	23.3	0.94	0.12	<0.01	0.98
Butyrate (mM)	13.1	12.2	0.41	0.23	<0.01	0.88
i-Butyrate (mM)	1.49	1.37	0.127	0.55	0.99	0.97
i-Valerate (mM)	0.532	0.563	0.021	0.38	<0.01	0.79
Valerate (mM)	1.30	1.16	0.050	0.15	<0.01	0.63
Total VFA (mM)	115	109	3.4	0.27	<0.01	0.99
Acetate:Propionate	2.82	3.02	0.045	0.05	<0.01	0.42

[1]Standard error of the mean.

[2]T: effect of yeast supplementation, H: effect of hour after feeding, T × H: interaction between yeast and hour after feeding.

Table 5. Effect of live yeast and forage group on the ruminal degradability of DM and NDF

	Treatment									
	Control			Yeast				P-value[3]		
Item	Temp/Leg[1]	Corn silage[1]	Trop. grass[1]	Temp/Leg[1]	Corn silage[1]	Trop. grass[1]	SEM[2]	T	G	T × G
DM degradability (% DM)										
12 h	47.6	49.4	24.9	49.6	51.6	27.1	1.51	0.09	<0.01	0.99
24 h	56.0	56.7	31.0	59.4	59.7	32.9	1.53	0.03	<0.01	0.85
36 h	66.6	65.2	39.1	66.9	66.1	39.1	1.78	0.77	<0.01	0.97
NDF degradability (% NDF)										
12 h	19.4	10.8	8.96	21.8	12.7	10.5	1.49	0.08	<0.01	0.89
24 h	31.4	21.0	16.2	35.0	25.0	17.8	1.90	0.04	<0.01	0.70
36 h	46.1	35.5	26.0	47.3	36.6	25.2	2.34	0.78	<0.01	0.87

[1]Forage group: Temp/Leg - oat silages, ryegrass silages, alfalfa silage and alfalfa hay; Trop. grass - sugarcane silages and tropical grass silages; Corn silage - corn silages.

[2]Standard error of the mean.

[3]T: effect of yeast supplementation, G: effect of forage group, T × G: interaction between yeast supplementation and forage group.

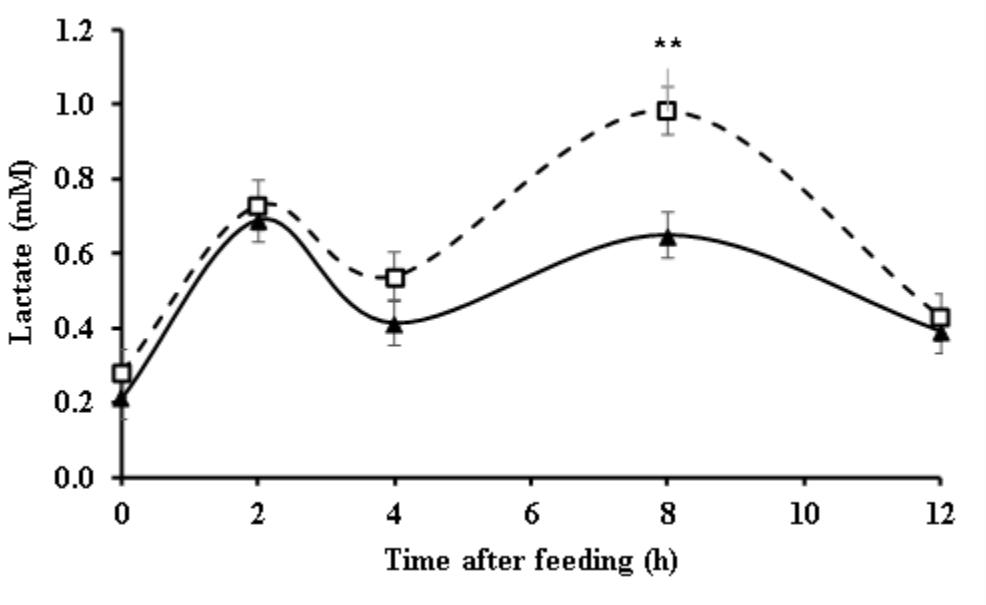

Figure 1. Ruminal lactate concentration in cows supplemented (▲) or not (□) with live yeast. P = 0.03 for treatment, P < 0.01 for time, P = 0.04 for treatment × time. ** P < 0.01 for Control vs. Yeast at 8 h after feeding.