**UNIVERSIDADE ESTADUAL PAULISTA – UNESP**

**CÂMPUS DE JABOTICABAL**

# DIETS AND LAMB MEAT INFLUENCED BY MICROBIAL INOCULANT AND AMYLOLYTIC ENZYME

**Erika Christina Lara**

Zootecnista

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# DIETS AND LAMB MEAT INFLUENCED BY MICROBIAL INOCULANT AND AMYLOLYTIC ENZYME

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Campus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Zootecnia

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## DADOS CURRICULARES DO AUTOR

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*"Talvez não tenha conseguido fazer o melhor,*

*mas lutei para que o melhor fosse feito.*

*Não sou o que deveria ser, mas Graças a Deus,*

*não sou o que era antes".*

*Marthin Luther King*

*DEDICO*

*Aos meus pais Jackson Lara e*

*Maria Cristina, e minha queria avó Hilda Tuxen,*

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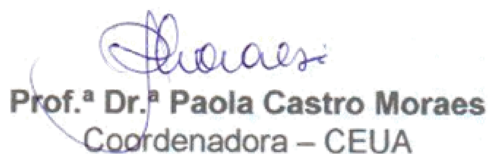

**Prof.ª Dr.ª Paola Castro Moraes**
Coordenadora – CEUA

# DIETS AND LAMB MEAT INFLUENCED BY MICROBIAL INOCULANT AND AMYLOLYTIC ENZYME

**ABSTRACT -** This trial aimed to evaluate the effects of diets containing corn silage inoculated with *Lactobacillus plantarum* and *Bacillus subtilis* and supplemented or not with amylase on the apparent digestibility, ruminal fermentation and microbial protein synthesis of wethers as well as, the growth performance and meat quality of lambs. For that, two studies were carried out and in both studies the animals received one of four treatments (diets): 1) Corn silage uninoculated and without amylase added to TMR; 2) Corn silage uninoculated and amylase added to TMR; 3) Corn silage inoculated with $1\times10^5$ CFU LP [MA 18/5U] and $1\times10^5$ CFU BS [AT553098] without amylase added to TMR; 4) Corn silage inoculated with $1\times10^5$ CFU LP [MA 18/5U] and $1\times10^5$ CFU BS [AT553098] and amylase added to TMR. The enzyme utilized was amylase at the rate of 2 g of the product / kg of dietary dry matter (DM) (602 dextrinizing unit (DU)/kg of dietary DM). Amylase supplementation on the diet containing uninoculated silage increased ($P$=0.045) dry matter (DM) intake of wethers compared with wethers fed uninoculated silage without amylase supplementation (1,311 vs. 1,066 g/d), but not differed from others treatments. The apparent digestibility of DM, OM, CP, NDF and GE increased ($P$<0.01) in wethers fed with inoculated silages or supplemented with amylase, without interaction among inoculants and amylase. Wethers fed diets containing uninoculated silage and supplemented with amylase showed higher propionic acid and lower acetic acid proportion, with low acetic:propionic acid ratio, consequently. Microbial N supply tended to be higher ($P$=0.097) in wethers fed uninoculated silage with amylase supplementation and inoculated silage without amylase (8.01; 8.05 g/d). However, no effect was verified on the efficiency of microbial N synthesis. In the second study, lambs fed inoculated silage had higher NDF intake ($P$=0.019) than lambs fed uninoculated silage (266.5 vs 245 g/d). Lambs fed inoculated silage had higher average daily gain ($P$=0.019) when compared with lambs fed uninoculated silages (232.5 vs. 211.5). The inoculation of silage increased ($P$<0.05) the content of saturated fatty acid (SFA) and decreased ($P$<0.05) the unsaturated fatty acid (UFA) (47.55 vs. 46.21% and 52.44 vs. 53.79%, respectively) and consequently decreased the UFA:SFA ratio. The amylase supplementation at moment of feeding trended ($P$<0.10) to decrease the values of PUFA:SFA ratio (0.14 vs. 0.16). The association of amylase in diets containing inoculated silage did not provided positive responses on the digestibility and microbial N supply of wethers and did not alter the carcass and meat quality of lambs. Inoculation of silage with *L. plantarum* and *B. subtilis* improved the average daily gain of lambs when was not associated with amylase supplementation.

**Keywords:** amylase, animal performance, bacterial inoculants, digestibility, fatty acid profile

**DIETAS E CARNE DE CORDEIRO INFLUENCIADAS POR INOCULANTE MICROBIANO E ENZIMA AMILOLÍTICA**

**RESUMO** - Objetivou-se neste trabalho avaliar os efeitos de dietas contendo silagem inoculada com *Lactobacillus plantarum* e *Bacillus subtilis* e suplementadas ou não com amilase sobre a digestibilidade aparente, fermentação ruminal e síntese de proteína microbiana em carneiros, assim como o desempenho e qualidade de carne de cordeiros. Para tanto, dois estudos foram conduzidos, no quais os animais receberam um dos quatro tratamentos (dietas): 1) silagem de milho não inoculada sem adição de amilase na mistura total da ração (MTR); 2) silagem de milho não inoculada e amilase adicionada na MRT; 3) silagem de milho inoculada com $1 \times 10^5$ UFC de *L. plantarum* e $1 \times 10^5$ UFC de *B. subtilis,* sem adição de amilase; 4) silagem de milho inoculada com $1 \times 10^5$ UFC de *L. plantarum* e $1 \times 10^5$ UFC de *B. subtilis* e amilase adicionada na MRT. A enzima utilizada foi a amilase numa taxa de aplicação de 2 g de produto / kg de matéria seca (MS) da dieta (602 unidade dextrinizante (UD) / kg de MS da dieta). A suplementação com amilase em dietas contendo silagem não inoculada aumentou ($P$=0,045) o consumo de matéria seca dos carneiros quando comparados com aqueles alimentados com silagem não inoculada sem suplementação com amilase (1,311 vs. 1,066 g/d), mas não diferiu dos outros tratamentos. A digestibilidade aparente da MS, MO, PB, FDN e EB aumentou ($P$<0,01) nos carneiros alimentos com silagem inoculada ou suplementados com amilase, sem interações entre os tratamentos. Os animais alimentados com dietas contendo silagem não inoculada e suplementados com amilase apresentaram alta proporção de ácido propiônico e baixa de ácido acético, e consequentemente baixa relação de aceitoc:propiônico. A síntese de proteína microbiana tendeu a ser maior ($P$=0,097) nos carneiros alimentados com silagem não inoculada e suplementados com amilase e também nos que receberam dieta contendo silagem inoculada sem suplementação com amilase (8,01; 8,05 g/d, respectivamente). Entretanto, nenhum efeito foi verificado na eficiência de síntese de proteína microbiana. No segundo estudo, cordeiros alimentados com silagem inoculada apresentaram maior consumo de FDN ($P$=0,019) do que aqueles alimentados com silagem não inoculada (266,5 vs. 245,0 g/d). Cordeiros que receberam dieta contendo silagem inoculada apresentam maior ganho de peso diário ($P$=0,019) quando comparados àqueles alimentados com silagem de milho não inoculada (232,5 vs. 211,5). A inoculação da silagem aumentou ($P$<0,05) o conteúdo de ácidos graxos saturados (AGS) e diminuiu ($P$<0,05) o conteúdo de ácidos graxos insaturados (AGI) (47,55 vs. 46,21% e 52,44 vs. 53,79%, respectivamente), e consequentemente, diminuiu a relação UFA:SFA. A suplementação com amilase no momento da alimentação tendeu (P<0,10) a diminuir a relação AGPI:AGS (0,14 vs. 0,16). O uso de amilases em dietas contendo silagem

de milho inoculada não resultou em respostas positivas na digestibilidade e síntese de proteína microbiana de carneiros, bem como não alterou as características de carcaça e qualidade de carne de cordeiros. O uso de dietas contendo silagem de milho inoculada com *L. plantarum* e *B. subtilis* e não suplementadas com amilase, aumentou o ganho de peso de cordeiros.

**Palavras chave:** desempenho animal, digestibilidade, enzimas amilolíticas, inoculantes bacterianos, perfil de ácidos graxos

# CHAPTER 1 - GENERAL CONSIDERATIONS

## 1. INTRODUCTION

Intensive production systems rely on cereal grain starch as the primary source of energy (DiLorenzo et al., 2011) that can comprises approximately 50 and 75% of the energy value of corn silage and grain, respectively (Nocek and Tamminga, 1991; NRC, 2001). The starch digestibility in ruminants, in general, is not considered a production limiting factor, however is not 100% efficient and can occur fermentation in the large intestine (energy-inefficient) and starch losses in feces.

Additives that increase the starch utilization by ruminants directly or indirectly through changes in ruminal fermentation pattern are an applicable alternative that is gaining traction in the animal nutrition sector. However, it should be borne in mind that prior to the adoption of these technologies, some management strategies and a more specific knowledge of the feed offered to animals may help to improve the starch utilization without changes in the final cost of the diet.

There are several ways to achieve greater digestibilities of the starch, being: 1- corn hybrid choice (Vitreousness, Ngonyamo-Majee, et al., 2008), 2-harvest time (Maturity, Ferrareto and Shaver, 2012), 3- ensiling time (Der Bedrosian et al., 2012; Young et al., 2012), 4- grain processing (Mc Allister et al., 1990) and lastly the adoption of technologies, among them the mainly are the microorganisms, with probiotic effect or not (Wiryawan and Brooker, 1995) and amylolytic enzymes (Tricarico et al., 2008).

Amylase supplementation (Rojo et al., 2005; Mendonza et al., 2013; Nozière et al., 2014), may increase the total number of viable bacteria, increasing fiber digestion and improving the ability of rumen bacteria to ingest and degrade feed and secondary metabolites. It could also increase the amount of crude protein (CP) available for microbial metabolism, which may increase fiber digestibility and the metabolizable energy (ME) density of the diet and providing greater intake of nutrients for metabolism and animal production (Tricarico et al., 2008; Salem et al., 2015).

Silage inoculant such as *B. subitilis* is another source of enzyme (i.e., amylase, xylanase and ferulic acid esterase; Kang et al., 2009) that may act on

complex polysaccharides and provide more soluble carbohydrates to be fermented by LAB within the silo (Phillip and Fellner, 1992) and enhanced the amylase degradability. This microorganism is little studied as silage inoculant, however has antagonistic activity against phytopathogenic fungi (Schisler et al., 2004; Angonese et al., 2009) and can control the growth of yeasts and molds (Basso et al., 2012; Lara et al., 2015).

Other important silage inoculant is the *L. plantarum* that aims to accelerate the decline in silage pH with consequent decreases of the proteolysis and prevention of the growth of spoilage microorganisms (Filya, 2003; Addah et al., 2014). In addition, *L. plantarum* may survive and show probiotic effect in the rumen (Weinberg et al., 2003; 2004a; 2004b) improving the nutrient digestibility (Addah et al., 2012; Lara et al., 2015) and consequently the animal performance (Aragon et al., 2012; Nishino, 2015).

Conversely, any manipulation of diet may alter the action of microorganisms in the rumen and interfere in the fatty acid profile of the meat. On the other hand, the use of different nutritional strategies should not negatively alter the physical and sensorial properties of the meat, altering important aspects to the consumer such as color, tenderness and flavor.

However, the results are often inconsistent and even when the increases ruminal or total starch digestibility are verified, is not always present improvement in animal performance (Rojo et al., 2005; Lee-Rangel et al., 2010; DiLorenzo et al. 2011) and meat quality. In addition, at the moment, few studies have been evaluated the effect of use amylases, moments before feeding, in diets (total mixed ration) with inoculated silage on metabolism, performance and meat quality of lambs.

Thus, our goal in this study was to evaluate diets containing inoculated corn silage with *L. plantarum* combined with *B. subtilis* and supplemented or not with amylase on the apparent nutrient digestibility, ruminal fermentation, nitrogen utilization and microbial protein synthesis of wethers and growth performance and meat quality of lambs.

## 2. LITERATURE REVIEW

## 2.1. Microbial inoculants

### 2.1.1. Classification and effect on fermentation and aerobic stability of corn silage

The whole-crop corn has desirable characteristics for ensilability, such as high productivity, adequate dry matter (DM) (30-35%) and fermentable carbohydrates content, low buffer capacity and adequate microbial fermentation (ZOPOLLATTO; SARTURI, 2009). However, compared with corn silage produced in temperate climates, the tropical corn silages may have a low nutritive value (lower starch and higher fiber concentration) and to be susceptible to aerobic deterioration (ADESOGAN, 2010; BERNARDES; ADESOGAN, 2012).

Poor silage quality has a major impact on production costs, especially when it represents more than 50% of the daily intake for ruminants (PARAGON et al., 2004). Thereby, microbial inoculants have been added to silages to improve fermentation efficiency (WEDDEL et al., 2002; KUNG et al., 2003) and mitigate the aerobic deterioration problem (WILKINSON; DAVIES, 2012), ensuring better nutritive value of silages.

First-generation silage inoculants contain homolactic and facultative heterofermentative lactic acid bacteria ($LAB^{Ho}$ and $LAB^{fh}$) which accelerate the decline in silage pH as a result of an increase in the production of lactic acid (ADDAH et al., 2014). This rapid decline in pH prevents the growth of spoilage bacteria, yeasts and molds as well as stops respiration by forage plant cells, conserving the sugars in silage (ADDAH et al., 2014).

*Lactobacillus plantarum* is the gram-positive, with probiotic properties and aero tolerant $LAB^{fh}$ commonly used in silage (OHASHI; USHIDA, 2009). During the anaerobic conditions of ensilage, *L. plantarum* quickly dominate the microbial population producing mainly lactic acid and a little quantity of acetic acids (CONTRERAS-GOVEA et al., 2013). This way, it can contribute to improve silage fermentation by increasing the silage fermentation rate (i.e., higher ratio between lactic and acetic acid), reducing proteolysis and forage protein deamination, and improving nutrients preservation and silage quality through a more efficient use of water soluble carbohydrates (WSC) (HENDERSON, 1993; ADDAH et al., 2011).

However, LAB$^{ho}$ and LAB$^{hf}$ often have no effect or can even make the aerobic stability of silages worse (MOON, 1983; MUCK; KUNG, 1997; FILYA et al., 2002;) because lactic acid can be easily oxidized by yeasts when the silage is exposed to air (PAHLOW et al., 2003). To work around this issue, a second-generation of inoculants was developed. *Lactobacillus buchneri* are heterofermentative LAB (LAB$^{he}$) and degrades some of the lactic acid into acetic acid, which inhibits the growth of yeasts and molds, improving aerobic stability of silage at feed-out (REICH; KUNG, 2010; WILKINSON; DAVIES, 2012).

Another microorganism that has been proposed as silage additive to enhance the aerobic stability of silage by the yeast and mold growth control is the *Bacillus subtilis* (PHILLIP; FELLNER, 1992; BASSO et al., 2012; LARA et al., 2015). A number of *Bacillus* species are antagonists of phytopathogenic fungi and can be used in biological control programs (WILHELM et al., 1998; WULFF et al., 2002; SCHISLER et al., 2004; ANGONESE et al., 2009;). In addition, *Bacillus* may maintain their viability when stored for long periods (PETRAS; CASIDA, 1985), because of their capacity to produce spores resistant to acid and oxygen (HOSOI et al., 2000).

The association of the 1$^{st}$ and 2$^{nd}$ generation of silage inoculants has been used for manufacture formulas of inoculants that may be an alternative strategy to decrease fermentation losses and protein degradation by producing greater quantities of lactate and enhancing the aerobic stability of silages in response to acetic acid production (DRIEHUIS et al., 2001; FILYA, 2003; HUISDEN et al., 2009). Phillip and Fellner (1992) found that inoculation of high-moisture corn ear with *B. subtilis* and *L. plantarum* could improve its aerobic stability. In tropical condition the use of *B. subtilis* alone or associated with *L. plantarum* decreased the yeasts and molds content and improved the aerobic stability of corn silage (LARA et al., 2015).

Besides the effects on fermentation and aerobic stability, some researches verified changes in the nutritive value of silages and with the advancement of the microbiology studies, a third generation of inoculants was introduced in the market. The mainly microorganism insert in this category was the *L. buchneri* based on their ability to produce fibrolytic enzymes (i.e., ferulate esterase enzyme) with the potential to increase forage digestibility (NSEREKO et al. 2008; KANG et al. 2009; ADDAH et

al., 2012). Likewise, *B. subtilis* can be considered a third-generation inoculant, because of their ability to produce a range of enzymes (i.e., amylase, xylanase and ferulic acid esterase (FAE)) (PRIEST, 1977; DONAGHY et al., 1998).

Therefore, the use of LAB and *B. subtilis* can go beyond improving the characteristics related to fermentation and aerobic stability of silages. However, the mode of action of these microorganisms and the variables involved in the whole process are not completely characterized.

### 2.1.2. Ruminal fermentation and animal performance

Inoculation of barley silage with *L. plantarum, L. buchneri* and *L. casei* in *in vitro* conditions increased the digestibility of neutral detergent fiber (NDF) after 24 and 48 h of incubation in the rumen (ADDAH et al., 2012). Similarly, inoculation of corn silage with different strains of LAB improved ruminal fiber digestibility after 48 h of incubation in steers (NSEREKO et al. 2008). A previous *in vitro* study reported that LAB, mainly in presence of starch, may compete with other microorganisms in the rumen (lactate-producing such as *Ruminobacter amylophilus* and *Streptococcus bovis*) for readily fermentable substrate resulting in less lactic acid, causing a higher ruminal pH, which in turn could be favorable for fibrolytic bacteria in the rumen (WEINBERG et al., 2007).

Increases on the metabolizable energy content in diets containing grass silage with *L. plantarum* inoculation was observed with consequent shift in the pattern of rumen fermentation towards increase propionic acid and decrease acetic acid (KEADY; STEEN, 1994). The propionic acid can be used to synthesize glucose and may offer an energetic benefit to the ruminant host (ØRSKOV, 1977).

The opposite was observed by Fellner et al. (2001), with higher concentration of acetate in the rumen fluid of steers fed high moisture corn inoculated with *L. plantarum* and *Enterococcus faecium*. However, no effect was verified in the ruminal parameters of lambs fed baled triticale–Hungarian vetch silage inoculated with *L. plantarum* or *L. Buchneri* (KELES; DEMIRCI, 2011).

In tropical conditions*, B. subitillis* combined with *L. plantarum*, improved *in vitro* dry matter digestibility (IVDMD) and organic matter digestibility (IVOMD) of corn silages (LARA et al., 2015), however the opposite was observed by Basso et al. (2013) in similar conditions. In addition, as previously described, *B. subtilis* and some

LAB can produce antimicrobial substances, such as bacteriocins, that can influence the profile of ruminal microorganisms when the animal is fed with inoculated silage (Muller et al., 1996; Yildirim, 2001; GOLLOP et al., 2005). However, researches are required because these bacteriocins can also act against beneficial LAB (OHMOMO et al., 2000).

Researches suggesting that some microorganisms in silage inoculants may remain active in the rumen and act synergistically when combined with other bacterial species, providing favorable shifts in rumen microbial ecology that improve DM and NDF digestibility (WEINBERG et al., 2003; 2004; 2007; LETTAT et al., 2012). This probiotic effect can characterize a fourth generation of silage inoculants, that besides to improve silage quality, digestibility, and aerobic stability, could alter the microbial ecology within the gastrointestinal tract of ruminants to benefit health and/or production efficiency (DUNIERE et al., 2015).

The *L. plantarum* MTD-1 strain can survive and stabilize pH conditions in the rumen (WEINBERG et al., 2004) and inhibit growth of detrimental microorganisms such as Gram-positive *Micrococcus luteus* and Gram-negative *Pseudomonas aeruginosa* (GOLLOP et al. 2005). Mohammed et al. (2012), reported greater relative population size of the same strain of *L. plantarum* in rumen fluid of cows fed inoculated alfalfa silage compared to those fed untreated silage.

However the results found in the literature suggest that the effect of inoculants depends on the forage properties on which are applied, diet composition and the methods of laboratory collection and evaluation, where differences due to liquid and solid phases of the rumen as well as cow-to-cow variations can be greater as compared to the effect of inoculation (MOHAMMED et al. 2012).

Nowadays, it is known that silage inoculants have improved digestibility and animal productivity without obvious shifts in fermentation products (MUCK 1993; WEINBERG; MUCK, 1996; ADESOGAN et al. 2009). A review by Weinberg and Muck (1996) showed that in 30% of the studies using inoculants containing LAB for cattle was verified improvement of feed intake, live-weight gain, energy feed efficiency, and milk production.

The most common use of microorganisms with potential probiotic effect is with direct-fed application that can offer benefits to livestock nutrition and health by

modifying the microbial ecology of the digestive tract (BRASHEARS et al., 2005). According to McAllister et al. (2011), the use of these direct-fed microorganisms enhance milk production and can exclude zoonotic pathogens from the intestinal tract. However, as previously reported, the microorganisms contained in the silage inoculants may survive and remain active within the rumen (WEINBERG et al., 2003; 2004), providing microbial biomass directly to the animal and interacting with ruminal microorganisms to improve ruminal functionality and growth performance (WEINBERG et al., 2004; MOHAMMED et al., 2012).

One LAB strain silage extensively studied has been the *L. plantarum* MTD-1, that has improved animal performance in those fed inoculated silage with this strain, regardless of fermentation patterns (NISHINO, 2015). On the other hand, inoculation of alfalfa with multiple strains of *L. plantarum* failed to cause improvements in DM intake, crude protein or fiber digestibility and milk production (RIZK et al., 2005). However, in recent study, Aragon et al. (2012) reported increase of 6.14% in DM intake and 100 g/d in average gain of young beef cattle fed corn silage inoculated with microbial additive.

Some studies conducted under tropical climate reported effects of adding $LAB^{ho}$ and $LAB^{fh}$ on ruminal fermentation characteristics of beef cattle, dairy cows, and lambs fed corn silage, along with increased ADG (average daily gain) in beef cattle and lambs (ZANETTE et al., 2011; BASSO et al., 2014; ANDRADE et al., 2016; RABELO et al., 2016). Likewise, Nkosi et al. (2009, 2010) found higher DM intake in the lambs fed inoculated silage than animals fed untreated silage and reported an increase on daily weight gain of 21.8 g/d (14.1%) and 35.0 g/d (22.7%) in lambs fed with corn silage inoculated *Pediococcus pentosaceus*, *L.* plantarum and *L. buchneri* and *L. buchneri* alone compared to animals fed untreated silage respectively (NKOSI et al., 2009).

The improvement on the performance and animal production fed inoculated silages may be due to the increase on the fiber digestibility (WEINBERG et al., 2004; NSEREKO et al., 2008). McAllister et al. (1998) verified increase in the DM and OM (organic matter) digestibility in lambs fed alfalfa silage inoculated with LAB and Aksu, et al. (2004) and Kamarloi & Yansari (2008) also observed enhances in the DM and

NDF digestibility in lambs and steers fed corn silage inoculated with LAB, respectively.

Although one of the possibilities for increasing silage digestibility is due to better preservation of nutrient during the fermentation process and conservation of a greater proportion of digestible nutrients (McDONALD; HENDERSON; HERON, 1991), these increases may also be due to the interacting among microorganisms present in inoculants and ruminal microorganisms, enzyme production from some microorganisms and effect of bacteriocins and antimicrobials substances, as described previously. However, little is known about the effect of these microorganisms on the rumen and the causes for increase in performance are difficult to explain (Muck, 2010).

A bacterium little studied as silage inoculant is the *B. subtilis*, which is a microorganism that has probiotic properties and is largely used in monogastric researches. For ruminants, *B. subtilis* has been utilized as direct-fed microbial additive and has shown great results. Garcia (2008) evaluated the weight gain of 38 calves receiving milk replacer with different doses of *B. subtilis*, and verified greater weight gain in the animals that consumed the largest dose of the probiotic.

The high sporulation capacity and spore resistance combined with the high production of a range of enzymes and antimicrobial compounds favor the use of *B. subtilis* as a silage inoculant. In addition, the high amylase production capacity from *B. subtilis* can improve the efficiency of silage utilization, in particular for corn silage, either by increasing the fermentation rate due to the degradation of the starch to the WSC (water soluble carbohydrate) (PAHLOW et al. 2003) or increasing corn grain digestibility (CROSBY et al., 2012).

## 2.2. Starch Digestibility of corn

Corn grains represent about 42% (37 to 47%) of DM of the whole corn plant, according to Pereira et al. (2012), when evaluating eight hybrids corn harvested at different stages of maturity. Thus, the starch, the main constituent of the grains, becomes a key nutrient for the energy supply readily available to ruminants. According to Giuberti et al. (2014), corn silage and high moisture corn silage may

contain around 20% to 60% of starch on the DM and this content may be influenced by the maturity of the plant at harvest.

Thus, the search for increased starch utilization of whole plant silages has been the goal of many researches aimed at improving ruminant performance and reducing feed costs, especially during periods where grain prices are relatively high (FERRARETO; SHAVER, 2012).

The use of additives that increase the use of starch directly or indirectly, through changes in ruminal fermentation pattern, is an alternative that is gaining strength in the animal nutrition sector. However, it should be borne in mind that prior to the adoption of these technologies, some management strategies and a more specific knowledge of the feed to be used may help to improve the use of starch without greater aggregation in the final cost of the animals diet.

There are several ways to achieve greater digestibilities of the starch, being: 1) corn hybrid choice, 2) harvest time (% of DM), 3) storage time, 4) grain processing, and lastly 5) the adoption of technologies such as enzymatic additives. According to Pereira (2014), currently, maize hybrids with hard textured grains, in which the high density vitreous endosperm predominates, are predominant in the Brazilian seed market. However, the correlation between vitreous and rumen degradability of corn grain is linear and negative (PHILIPPEAU; MICHALET-DOUREAU, 1997; CORREA et al., 2002).

Correa et al. (2002), cited by Pereira (2014), evaluated the relationship between vitreousness and starch digestion of Brazilian and North American corn grains at the mature maturation stage, using *in situ* ruminal incubations. The rumen starch degradability was 77.4% in the North American hybrids and 48.5% in the Brazilian hybrids. It is worth mentioning that among the Brazilian hybrids evaluated, there was the most farinaceous material available in the seed market, in addition to high vitreous material. The most degraded Brazilian hybrid had less degradability than the less degraded American hybrid. The relationship between vitreousness and rumen starch degradability was linear and negative ($r^2$ = 0.75), demonstrating that when farinaceous seeds are not widely available, as in Brazil, aiming for less vitreous endosperm, even if it is less than desired, may increase the proportion of the starch being degraded in the rumen.

On the other hand, the timing of the harvest can help at the ideal point of better degradability of the starch. The early harvesting can reduce the negative effect of grain hardness on the degradability of corn grain in the rumen, and the opposite can be verified when the harvest is done late, penalizing the rumen degradability of the grains (CALESTINE et al., 1998; PEREIRA et al., 2004). Some researches evaluating the maturity stage of corn plant at harvesting reported a decreased starch degradability of corn silage in the rumen when the maturity of harvest plants increases (BAL et al., 1997; PHILIPPEAU; MICHALET-DOREAU, 1997).

The time of storage is another important point and several studies have reported that ruminal DM digestion from high moisture corn and starch digestion from corn silages is relatively low at initial harvest, but increases with the time of ensiling because of natural proteolytic mechanisms that occur in the silo (KUNG JR., 2013). Benton et al. (2005) ensiled high moisture corn (HMC) for up to 298 days at various moistures and sampled the material every month. In all cases, *in situ* digestion of DM increased as days of storage increased.

Philippeau & Michalet-Doreau (1998) hypothesized that degradation of the protein-starch matrix during ensiling was responsible for increases in ruminal starch digestion with ensiling. Solubilization of prolamins by acids and alcohols was hypothesized at one time to be the driving factor of this result, but recent evidence suggests that proteolytic activity on zein proteins better explains this phenomenon (HOFFMAN et al., 2011).

It is known that the particle size reduction of grains, either by the animal or by mechanical processing of the grain prior to feeding, generally increases starch digestibility. Improved digestibility occurs by breaking down barriers that prevent enzymatic access to nutrient components, preservation, isolation of specific parts, improved palatability or detoxification of feed (MCALLISTER et al., 1990; POND et al., 1995).

According to McAllister et al. (2006), the processing of cereal grains, whether by grinding, rolling, pelleting, tempering (i.e., addition of water prior to rolling), steam rolling (i.e., exposure to steam prior to rolling) or steam flaking (i.e., longer duration of exposure and higher grain temperature), breaks down recalcitrant barriers such as

the hull, pericarp and protein matrix and allows microbes access to the starch harbored within endosperm cells.

Steam rolling and steam flaking expose grain to moisture and heat, and when the temperatures are above 80ºC, a portion of the starch in grain is gelatinized (MCALLISTER et al., 2006).This procedure allows an increase in ruminal degradability in relation to the whole grain, however, in comparison to the grinding, ruminal degradability is lower (REIS et al., 2014).

Another method of grain processing is the ensiling of high moisture grain, which increases the grain starch digestibility due to embrittlement and breakage of the protein matrix that surrounds the starch granules in the endosperm. In addition, the starch may also undergo the gelatinization process, increasing its susceptibility to enzymatic attack (REIS et al., 2014).

Increased ruminal degradation of the starch provided by the processing increases the availability of rapidly fermentable energy in the rumen and may increase the production of microbial protein and total volatile fatty acids (NOCEK; TAMMINGA, 1991). However, adverse effects due to higher availability of starch may occur, such as reduced digestibility of fibrous carbohydrates, forage and dry matter intake, and ruminal acidosis (MC CARTHY et al., 1989).

In addition to the alternatives described above, which aim to improve the efficiency of the use of starch by ruminants, studies point to the use of technologies, such as the application of amylolytic enzymes in animal diets. These enzymes are derived from the metabolism of microorganisms such as fungi and bacteria, and can be used as additives in order to aid in the digestion of starch and better utilization of nutrients by the animals.

### 2.3. Exogenous enzymes in ruminant nutrition

Researches involving the use of exogenous enzymes in ruminant nutrition have increased significantly in recent years and have aroused interest in the productive sector regarding the adoption of strategies to improve efficiency and animal production and consequently to decrease the cost of producing meat and milk. This is due to the advances in enzymatic biotechnology observed in recent decades, which has brought relevant and accurate information about the production,

activity and performance of the enzymes, as well as reducing the costs of production on an industrial scale

The first researches were in the 1960s with evaluations mainly on monogastric nutrition. For ruminants, it involved initially the application of exogenous enzymes in the silage process and later applied at fed directly. However, the vast amount of microorganisms involved in the fermentation process of silage and rumen, the complexity of the degradation of plant structural carbohydrates by ruminants and the variety of enzymatic products used, results in difficulty to understand the action of these enzymes in the respective environments, becoming a limiter to reach a conclusion or standardization of the use of enzymes for ruminants.

In addition, the enzymatic products have been applied in different dosages with a wide variety of experimental conditions. Different animals are used in the most diverse categories and fed with varied diets. Enzymes have been provided in different ways (sprayed on wet forage, added to the concentrate or sprayed into the total mixed rations, as dry powder or incubated directly into the rumen), and most studies are not provided with specific information regarding the enzymatic activity and conditions of the culture and production of the enzymes (BEAUCHEMIN; HOLTSHAUSEN, 2011).

### 2.3.1. Characterization, source and enzymatic activity

Enzymes are natural biocatalysts produced by living cells that elicit specific chemical reactions (GURUNG et al., 2013). Within the context of additives for ruminants, enzymes are used to catalyze degradation reactions, in which the substrates (foods) are digested in their chemical components (simple sugars, amino acids and fatty acids). These smaller molecules are then used for cell growth of the rumen or silage microorganisms, as well as by host animal (McALLISTER et al., 2001).

According to Adeola & Cowieson (2011), the world market of enzymes for food is dominated (about 90%) by Carbohydrases and phytases, with the carbohydrase being the most important group used as an additive in ruminant diets. However, proteases have also been studied, tested and used more frequently for ruminant nutrition.

Carbohydrases are enzymes that catalyze reactions of carbohydrate degradation, in other words, reduce carbohydrates of high molecular weight in residues of lower molecular weight and low complexity. Among them, the main classes are fibrolytic and amylolytic enzymes. In the case of proteases, these enzymes are involved in the degradation of long peptide chains and the formation of lower molecular weight peptides and amino acids (LEHNINGER, 1982). Amylolytic and proteolytic enzymes are commonly used in silage inoculants, and they are particularly useful for cereal silages, reducing the negative effect of the starch-protein matrix on starch digestion in ruminants (OWENS et al., 1986; HOFFMAN et al., 2011).

Despite of high amount of enzyme products used for ruminant nutrition, these mainly come from only four species of bacteria (*Bacillus subtilis*, *Lactobacillus acidophilus*, *L. plantarum*, and *Streptococcus faecium*, spp.) and three species of fungi (*Aspergillus Oryzae*, *Trichoderma reesei* and *Saccharomyces cerevisiae*) (MUIRHEAD, 1996). Although the source of the microorganism among the enzyme products is generally similar, the type and activity of the enzymes produced may vary widely, depending on the selected lineage, the substrate for growth and the culture medium used (CONSIDINE & COUGHLAN, 1989; GASHE, 1992; LEE et al., 1998).

The enzyme production occurs by metabolization of nutrients present in a specific culture medium, followed by enzymatic synthesis and secretion. The enzymatic activity is measurement directly in the extract produced, from divergent methodologies, either through the disappearance of a defined substrate or the generation of a product from enzymatic reactions catalyzed by the enzyme, per unit of time (McALLISTER, et al., 2001). According to Beauchemin, et al. (2003), enzymatic activities are mostly measured through conditions defined in industrial protocols, which consider the optimal conditions (pH and temperature) for enzymatic dosing, those where the highest activity is verified. However, when used such as ruminant additives, the condition of acting is different and may present low activity.

### 2.3.2. Amylolytic enzymes

Starch is the main constituent of the endosperm cells and is formed by a mixture of two polysaccharides: 20-30% amylose and 70-80% amylopectin

(ROONEY; PFLUGFELDER, 1986). Amylose is a linear chain polymer composed of glucose units linked, mainly by α- 1,4 glycosidic bonds, with α- 1,6 bonds less expressive. Amylopectin is the higher molecular weight polymer composed of small linear chains of glucose with numerous highly branched at α- 1,6 bonding sites (STEVNEBO et al., 2006).

Amylases are able to hydrolyze the glycosidic bonds of the starch molecule and, according to their mode of action, can be divided into two categories: endoamylases and exoamylases. The endoamylases (eg, α-amylases) hydrolyze the α-1,4 bonds within the amylose and amylopectin chain, releasing oligosaccharides of various sizes having α-configuration (eg, maltodextrins) (REDDY et al., 2003). Exoamylases (eg, glucoamylase or amyloglucosidase) hydrolyze α-1,4-type glycosidic linkages by removing successive glucose units from the non-reducing end of the chain, releasing D-glucose molecules into β-conformation. These enzymes also hydrolyze the α-1,6 bonds and some α-1,3 type bonds, however, at a lower rate (MICHELIN et al., 2008; KUMAR; SATYANARAYANA, 2009).

*Aspergillus oryzae* is the major microorganism used to extract amylase enzyme (TRICARICO et al., 2008). Mostly, the cultivation to obtain exogenous enzymes, in the majority, is by submerged fermentation (SmF), because allows better control of the conditions during fermentation. However, the Solid State Fermentation (SSF) method develops a tight contact with the insoluble substrate, therefore, achieving higher substrate concentration for fermentation and involves relatively little liquid when compared with SmF, downstream processing from SSF is theoretically simpler and less expensive (SUJANI; SERESINHE, 2015).

### 2.4. Use of amylolytic enzymes in ruminant nutrition

Exogenous enzymes have played a key role in improving the efficiency of meat and egg production by changing the nutritional profile of the ingredients. They degrade specific components of the diet and allow greater use of nutrients increasing feed efficiency (BARLETTA, 2011). The use of exogenous enzymes for ruminants has been directed primarily to the use of fibrolytic enzymes with the purpose to increase ruminal fiber digestion and improve ruminant production (BEAUCHEMIN et al., 1995).

Feeding supplemental amylases to ruminant has not been extensively studied. According Tricarico et al. (2007), the general perception is that starch digestion by ruminants is extensive and does not generally limit production as the incomplete or slow fiber digestion does. In addition, rapid digestion of excessive amounts of starch in the rumen may lead to ruminal acidosis (OWENS et al., 1998) representing a potential concern for inclusion of exogenous amylases in ruminant diets.

However, because of its hydrolytic action, supplemental α-amylase may increase the availability of starch hydrolysis products in the rumen and alter the ruminal fermentation process (TRICARICO et al., 2008). Recent several studies has shown that amylase application at feeding improved ruminal starch digestion in lambs and dairy cows (ROJO et al., 2005; MENDONZA et al., 2013, NOZIÈRE et al., 2014) and performance of sheep fed grain based diets (ROJO et al., 2001; MORA-JAIMES et al., 2002; BUENDIA et al., 2003).

When exogenous amylases are used as feed additive, it has been shown that proteases present in the rumen may degrade most of the diet-added amylase (HRISTOV et al., 1998). However, some polysaccharide-degrading enzymes have been shown to be resistant to degradation by ruminal proteases (BEAUCHEMIN et al., 1995). In addition, Morgavi et al. (2000) conducted studies evaluating the direct-fed enzyme addition and showed greater enzyme stability and permanency time in the rumen due to formation of enzyme-substrate complex, making difficult the action of microbial proteases.

#### 2.4.1. Ruminal Fermentation

The use of alpha amylase may increase the availability of products resulting from the starch hydrolysis in the rumen like oligosaccharides (which go from glucose to maltoheptaose), altering the ruminal fermentation process by the supply of substrates more easily hydrolysable by the ruminal microorganisms and increasing the ruminal starch digestibility (TRICARICO et al., 2008). Starch digestion in the rumen is more beneficial than post-ruminal digestion because ruminal digestion also increases the microbial protein outflow from the rumen where it is absorbed in the small intestines (DEFRAIN et al., 2005) and improves the animal production.

Rumen microbial protein represents a major source of amino acids to the ruminant animal and its production depends on a range of factors that can be

grouped under four major areas: substrate availability, DM intake, rumen dilution rate, intra-ruminal recycling of ammonia and pH of rumen contents (BOWEN, 2003). According to Russel et al. (1992), the microbial growth is a function of the amount of energy from ruminal fermentation and is maximized when the starch and protein rates are synchronized.

In addition, data from the literature indicate that there is a strong positive correlation between dry matter intake (DMI) and microbial growth (CLARK et al., 1992; GOMES et al., 1994; DJOUVINOV; TODOROV, 1994). However, it has been suggested that diets containing mainly silages are the cause of the low efficiency of microbial protein production (BEEVER, 1993), most likely due to the low pH and the non-synchrony of the energy and protein supply. This is caused because the ensiling process causes a reduction in the readily fermentable OM available to the rumen microbes and at the same time increases the readily available N due to extensive protein degradation (OWENS, 2003). Due this fact, the association of silage with readily fermentable carbohydrates is important to increase the efficiency of microbial production.

Crosby et al. (2012) and Rojo et al. (2001) verified on *in vitro* studies that the use of exogenous amylases increased sorghum and maize nutrient digestibility and ruminal starch digestibility, respectively. However, *in vivo* conditions, they did not verify the increase in the starch digestibility when amylases were used as supplement in diets for dairy cattle (CHEN et al., 1995; HRISTOV et al., 2008; GENCOLU et al., 2010) and finishing beef cattle (TRICARICO et al., 2007). These differences in responses between studies could be explained by differences in enzyme preparation or diet composition.

Chen et al. (1995) reported no effects of enzyme (amylase and protease mixture) application in steam flaked sorghum grain on starch digestibility or milk production, but observed increase on the OM, drude protein (CP) and NDF total tract digestibility. In the same direction, Gencoglu et al. (2010), evaluating the amylases addition on reduced-starch diet, observed increase on the DM and nutrient digestibility, except for starch, which was similar. The respective authors presented several possible explanations for the lack of difference in starch digestibility when exogenous amylase was added to the reduced-starch diet: 1) starch digestibility was

not affected ruminally (TRICARICO et al., 2005) or post-ruminally, 2) starch digestibility was increased ruminally (KLINGERMAN et al., 2009), but post-ruminal compensatory starch digestion (TAYLOR; ALLEN, 2005) resulted in similar total tract starch digestibilities, and 3) starch digestibility was increased ruminally, but hindgut fermentation (FIRKINS, 1997) resulted in similar total tract starch digestibilities.

Conversely, supplementation with a thermostable α-amylase from *Bacillus licheniformis* increased ruminal starch digestion in lambs (MORA-JAIMES et al., 2002; ROJO et al., 2005). In an experiment evaluating the effect of an exogenous amylase preparation on digestion of low and high starch diets in dairy cattle, Nozière et al. (2014) observed increase on the starch ruminal digestion (75% to 81%) when amylases were used, without effects in the total-tract starch digestion. They also reported increase in the true ruminal digestibility of OM, but no effect on the microbial N flow to the duodenum and none or small changes on selected fibrolytic and amylolytic bacteria and on the microbial community in general.

Tricarico et al. (2008) reported in a review that despite expecting improvements in ruminal digestibility of starch, when α-amylase from *Aspergillus oryzae* was used, that was not always verified. They suggested that those enzymes increase production of oligosaccharides from amylose and amylopectin that can be utilized by amylolytic or none amylolytic bacterial species thereby modifying bacterial populations and VFA production and these changes promoted increases on the molar proportions of butyrate and acetate at the expense of propionate.

In the same review, they evaluated the rumen bacterial growth when α-amylase was added into the medium containing starch and verified that the *Butyrivibrio fibrisolvens, Streptococcus bovis, Megasphaera elsdenii* and *Selenomonas ruminantium* grew rapidly. The opposite was reported by Cotta (1988) who observed none or slow growth of those bacteria in the presence of starch.

Changes in the rumen fermentation products was observed when α-amylase was added in diets for lactating cows and steers, where the enzyme addition decreased the molar proportion of propionate in the rumen and increased the molar proportions of acetate and butyrate just for lactating cows (TRICARICO et al. 2005). However, the effects on ruminal fermentation are not always evaluated and related to the changes in the digestibility and production of the animals, thus further studies

must be carried out in order to have a recommendation for the inclusion of amylases in ruminant diets.

### 2.4.2. Animal performance

It is hypothesized that α-amylase supplementation may represent an alternative for manipulating ruminal starch fermentation and thereby improves performance of ruminants. However, the mechanism of action of amylases and interactions with the different diets and animal categories has confused and preventing the generation of decisive information about the use of these enzymes and their effect on milk yield and weight gain of the ruminants.

A review about the use of exogenous enzymes to improve ruminant production was made by Meale et al. (2014) and they showed results from 28 publications with dairy cattle and 11 with beef cattle. From this publication, we selected and added research's that evaluated only the use of amylases (primary activities) for dairy cattle, beef cattle and small ruminants (Table 1).

**Table 1.** Summary of exogenous amylases (primary activity) effects on production traits and total tract apparent digestibility of nutrients in ruminants.

| References[1] | Design (n)[2] | Product | Effects[3] | | | |
|---|---|---|---|---|---|---|
| | | | MY | DM | ADG | TTD |
| ***Dairy cattle*** | | | | | | |
| 1 | CR (36) | Digest M[6] | - | - | NR | ↑CP[13] |
| 2 | CRB (24) | Amaize[7] | - | - | NR | NR |
| 3 | LS (20) | Amaize | - | NR | NR | NR |
| 4 | LS (4) | Amaize | NR | - | NR | ↑DM, OM and CP |
| 5 | LS (28) | Amaize and Experimental[8] | ↑[11] | ↑ | NR | ↑DM, OM, CP and NDF[11] |
| 6 | CR (45) | RR[9] | - | - | NR | NR |
| 7[4] | LS (4) | RR | - | - | NR | -[14] |
| ***Beef cattle*** | | | | | | |
| 8[5] | CRB (120, 96, 56) | Amaize | NR | - | ↑[12] | NR |
| 9 | CRB (32) | RR | NR | - | - | - |
| ***Small ruminants*** | | | | | | |
| 10 | LS (3) or CR (15) | Experimental | NR | ↓ | - | ↑DM and OM |
| 11 | CR (36) | Termozyme[10] | NR | - | - | ↑DM |

[1]1: Chen et al., 1995; 2: DeFrain et al., 2005; 3: Tricarico et al., 2005; 4: Hristov et al., 2008; 5: Klingerman et al., 2009; 6: Ferrareto et al., 2011; 7: Nozière et al., 2014; 8: Tricarico et al., 2007; 9: DiLorenzo et al., 2011; 10: Rojo et al., 2005; 11: Crosby et al., 2006.
[2]Experimental design: CR = Completely randomized; CRB = Completely randomized block; LS = Latin square; n = number of animals.
[3]↑ = increase; ↓ = decrease; - = no statistically effect; NR = not reported; MY = Milk yield (kg/d); DMI = Dry matter intake; ADG = Average daily gain; TTD = Total tract nutrients digestibility.
[4]Amylase added on 300 g of ground concentrate fifteen minutes before each meal.
[5] The experiment was divided into three phases.
[6]Loveland Industries Inc., Greeley, CO.
[7]Alltech Inc., Nichlasville, KY.
[8]Experimental preparation.
[9]DSM Nutritional products Ltd., Basel, Switzerland.
[10] Enmex, Mexico.
[11]Milk yield and digestibility increased only by low level of an experimental amylase enzyme.
[12]Quadract increase observed in experiment 2.
[13]Interaction with the grain processing.
[14]Increased ruminal digestibility of starch by 6.5%.

Improvements in milk yield in dairy cattle supplemented with exogenous amylases were not verified in almost all selected papers, except for Klingerman et al. (2009), which observed an increase in milk yield. The respective authors evaluated two enzyme preparations with different amylases activities and found higher DM intake and digestibility of DM, OM, CP and NDF, and increased milk yield when cows were supplemented with the product that contained the lowest enzymatic activity. In addition, the two enzymes remained stable in the rumen and showed substantial activity at pH 5.1 to 6.3, which can be found in the rumen of high-producing dairy cows.

Feeding supplemental amylases to finishing beef cattle has not been extensively studied. Tricarico et al. (2007) carried out two experiments in order to evaluate the effect of the amylase addition in different forages (alfalfa hay or cottonseed hulls) and corn processing (cracked or high-moisture corn). They observed a quadratic effect on the weight gain of the animals only in the experiment with different corn processing added to different doses (0.580 and 1.160 DU/kg of DM) of exogenous amylases. The respective authors concluded that although the results suggest that effects of *α*-amylase supplementation are not predictable with different forages sources or corn processing methods, there is potential for increased weight gain and improved carcass characteristics in finishing beef cattle fed this supplement.

DiLorenzo et al. (2011), also evaluated the effect of the amylase addition on corn processed in two ways (dry-rolled or steam-flaked) and found that the supplementation with exogenous α-amylase at 600 KNU/kg dietary DM did not affect

nutrient digestibility or performance by feedlot steers. However, apparent total tract digestibility of the OM tended ($p<0.01$) to increase with amylase supplementation in steers fed steam-flaked diets and the apparent total tract starch digestibility tended ($P<0.01$) to decrease with amylase supplementation in steers fed dry-rolled diets. The authors suggest that the tendency for an interaction between supplemental amylase and corn processing method for total tract starch digestion deserves further investigation.

As previously mentioned Rojo et al. (2005) verified increase on total tract digestion and decrease in the intake of DM and OM in lambs fed high sorghum diets and supplemented with exogenous amylases, respectively. However, gain and feed conversion were not affected by exogenous amylase. Similarly, Crosby et al. (2006) also found no improvements on sheep performance when amylases were added to the high grain diet. However they related increased in the DM digestibility and explained the lack of effect in the animal performance due to great variation in the intake, which could be associated to problems of subacute acidosis, which require further studies.

### 2.4.3. Variability of results

One of the main obstacles to conducting research with exogenous enzymes in diets for ruminants is the inconsistency of results. These inconsistencies are multifactorial, and can be attributed to four main factors: enzyme characteristics (i.e., differences in enzyme preparations, enzymatic activities, units of activity added, pH, and temperature effects on activity), forage (i.e., DM, type, maturity), animal (i.e., species, physiological state) and management (i.e., diet, mode of enzyme application, application rate, interaction time of enzymes applied to feed; BEAUCHEMIN et al., 2003).

In general, the enzymes used in animal diets are mixtures of different polysaccharidases, glucosidases, ligninases and proteases, which generally require different conditions to show maximum effect (VAHJEN; SIMON, 1999) than that found in the rumen. Recently, the endoglucanase and xylanase activities of 18 exogenous fibrolytic enzyme (EFE) from five companies at pH 3, 4, 5, 6, and 7 under constant temperature (39°C) or at 20, 30, 40, and 50°C under a constant pH of 6

were compared (ARRIOLA et al., 2011). They observed that 78 and 83% of the 18 EFE exhibited optimal endoglucanase and xylanase activities at 50°C, and 77 and 61% had optimal activity at pH 4 to 5, respectively. Conditions that were different than that found in the ruminal conditions.

Temperature of 50ºC and pH between 4,8 and 5,3 are recommended for cellulases from *Trichoderma spp*. (GHOSE, 1987; WOOD; BHAT, 1988). However, these conditions do not reflect the *in vivo* situation (SABATIER; FISH, 1996), such as rumen temperature of 39°C and pH between 5.8 and 6.8. Slominsky et al. (1993) identified this problem and, although higher enzymatic activities were found at pH 5.2 to 7.5, the recommendation was based on the conditions under which the enzymes should act (intestinal tract of monogastric).

Feed enzymes are often offered to end users in a variety of concentrations either as liquid products (often for post-pellet application) or dry products (ADEOLA; COWIESON, 2011). In both is necessary to remove some unwanted constituents, such as proteases and add some stabilizers and preservatives. Applying fibrolytic exogenous enzymes in a liquid form onto feeds prior to consumption can have a positive effect on animal performance (RODE et al., 1999; SCHINGOETHE et al., 1999; KUNG et al., 2000; YANG et al., 2000). Therefore, a minimum amount of moisture is required for the performance of the enzymes and if the enzyme preparation is in the powder form, it is necessary to add water or powdering the enzymes in the moisture forage.

The measurement of the enzymatic activity is another point to be studied, because the methodology used will depend on the enzyme studied and the laboratory assay. Activities of enzymes for use in the feed industry are most commonly measured by the generation of a product from the biochemical reaction catalyzed by the enzyme and are expressed as the amount of product produced per unit time (McALLISTER et al., 2001). However, the final unit of enzyme activity is not always the same in all commercial products. Therefore, is important to express the enzymatic activity in International Units (IU), where an IU is defined as the amount of enzyme required to release 1 mg of reducing sugars.

In addition, there is a lack of information related to the enzymatic characterization of commercial products, which makes difficult to understand the

possible effects of the enzymes on animal production. For example, early studies included amylase in combination with other enzyme activities, none of which were characterized (BURROUGHS et al., 1960). In some studies, amylase activities only represented a minor component of primarily microbial (McGILLIARD; STALLINGS, 1998) or predominantly fibrolytic preparations (McALLISTER et al., 1999; HRISTOV et al., 2000), while other studies used undefined preparations (CHEN et al., 1995) or thermostable alpha amylase from *Bacillus licheniformis* (ROJO et al., 2001; MORA-JAIMES et al., 2002, ROJO et al., 2005) with the specific objective of increasing starch digestion in sorghum.

The method of enzyme application also influences the performance and stability of the enzymes and, consequently, the animal production responses. These responses has been shown to differ among dry forage, fresh forage and silage (BEAUCHEMIN et al., 1995; FENG et al., 1996), and if the enzyme is applied to the total mixed rations (BEAUCHEMIN et al., 1999; YANG et al., 2000), concentrate (McALLISTER et al., 1998; RODE et al., 1999) or in the supplement (BOWMAN et al., 2002).

Exogenous enzymes should also be more effective when applied to high-moisture feeds such as silages than to dry feeds, because of the importance of water for enzymatic hydrolysis (BEAUCHEMIN et al., 1999). However, one study has demonstrated that enzyme application to the concentrate was more effective than application to the TMR (YANG et al., 2000). Conversely, Sutton et al. (2003) reported favorable responses when enzyme was added to the TMR mixture, due primary to the improved intake of digestible organic matter where, in the same study, they found some significant effect when enzyme was applied to the concentrate or directly infused to the rumen.

Provide enzymes before feeding is important to form linkages with the substrates that protect them from ruminal degradation and, this way, it is possible to increase the digestibility through different mechanisms such as: direct hydrolysis, improved acceptability, alterations in intestinal viscosity and changes in digestion site (BEAUCHEMIN et al., 2003; LOURES, 2004; MARTINS, 2006).

The level of enzymatic application, on other words, how many units of enzyme activity should be added per kilogram of DM to be ingested, is another important

factor that influences the animal response. The use of moderate levels of enzymes in ruminant feed can cause beneficial ruptures in the surface structure of feed before or after ingestion (NSEREKO et al., 2002). However, elevated levels of enzymes may be less effective than lower levels by decreasing microbial adhesion and limiting digestion, while optimal levels are dietary dependent (BEAUCHEMIN et al., 2003). In this sense, the amounts of enzymes normally used as additives in ruminant feed vary from 0.5 to 2.0 mg / g (or g/kg) of the total diet, based on MS (BEAUCHEMIN et al., 2003). However, these quantities are dependent on the specific characteristics of each product, such as type of activity of the enzymes present.

Therefore, new researches should be done to clarify the mechanisms of action of different enzymes used in ruminant nutrition to maximize animal performance. In addition, attention should be given to the characteristics of each enzyme prior to application as an additive for ruminants, and all information about the enzymatic dosage method, enzymatic activity, method and application dose should be included in all researches performed.

### 2.5. Changes in meat quality by the use of enzymatic and microbial additives

Meat quality has been a factor of extreme importance for the consumer and a critical point for the meat industry in the 21$^{st}$ century. With the increase in the demand for high quality meat, the meat industry must produce and supply consistently quality meat with tasty, safe and healthy for the consumer (LEMOS et al., 2017). For this, it becomes necessary to understand the factors that control the physical and sensorial properties of the meat and also to understand the characteristics that involve the nutritional quality of meat.

The most important physical properties of meat are temperature, pH, color, tenderness and weight loss by cooking (DABÉS, 2001). From these, pH is the most relevant factor, as it influences water holding capacity, weight loss by cooking, shear force, tenderness, succulence, flavor aroma and color of meat (DEVINE et al., 1993; BRESSAN et al., 2001). On the other hand, the fat and meat color are the most important factors for consumers (RIPOLL et al., 2008; CALNAN et al., 2016) and apart from tenderness, when eating (JELENÍKOVÁ et al., 2008).

Lately, the quantity and quality of fat and meat are also important because affect consumer health (MARTEMUCCI; D'ALESSANDRO, 2013; LEMOS et al. 2017). Sheep meat, as well as ruminants meat in general, is rich in saturated and monounsaturated fatty acids (SFA) with small amounts of polyunsaturated (SINCLAIR et al., 1982), being saturated fat the most harmful to human health.

This fact is due to the biohydrogenation of unsaturated fatty acid (FA) from the diets conducted by ruminal microorganisms before their absorption. However, this process is important because decreases the unsaturated fatty acid concentration, which are toxic to ruminal microorganisms, and contribute to the removal of $H^+$ ions from the ruminal environment, avoiding their accumulation. In addition, incomplete biohydrogenation allows the formation of intermediates of this process, such as conjugated linoleic acid (CLA), which is a promising for the future since current studies show that in addition to anticarcinogenic, antiarterosclerosis, antithrombotic, hypocholesterolemic, immunostimulatory also reduces fat and prevents diabetes (BARBOSA, 2013).

The saturated fatty acids (SFA) most found in sheep are myristic (2.04% - 3.65%), palmitic (20.88% - 24.22%) and stearic (11.89% - 15.09%); The monounsaturated (MUFA) are palmitolenic (2.23% - 2.54%) and oleic (31.74% - 45.23%) and the polyunsaturated (PUFA) are linoleic (4.73% -10.39%), linolenic (0.43% - 2.84%) and arachidonic (1.14% - 6.79%; PÉREZ et al., 2002), but these proportions vary, mainly, according to the diet. In addition, there are two main groups of PUFA: omega-3 (ω-3) and omega-6 (ω-6), that are considered essential of the human and their ratio (ω6:ω3), as well as, the PUFA:SFA ratio, have been often used to analyze the nutritional value of oils and fats and indicate the cholesterol-lowering potential.

Higher ω-6 intake associated with the lower ω-3 intake can cause physiological changes that initiate a pro-inflammatory and pro-thrombotic state with increasing in spasms in the blood vessel, vasoconstriction and blood viscosity, favoring the emergence of these diseases (PATTERSON et al., 2011). However the ideal ω6:ω3 ratio varies and while some suggested values from 4:1 to 5:1 (FREITAS; KIETZER, 2002; JONES; KUBOW, 2003), the Japan Society for Lipid Nutrition recommends the ratio of 4:1 for healthy adults and 2:1 to prevent chronic diseases in

the elderly. Likewise, atherogenicity and thrombogenicity indexes are used as measures to evaluate and compare the quality of different foods and diets (ARRUDA et al., 2012).

In ruminants, the FA composition of the meat is influenced by a greater extent of dietary factors (OLIVEIRA et al., 2013). It is possible to increase the PUFA in the diet by manipulating feed formulations, reflecting an increase in the proportion of PUFA in the meat and milk of ruminant animals (JAKOBSEN, 1999; FERNANDES et al., 2008). In this context the factors related to nutrition that can determine greater or lower variation of meat composition are: different proportions of concentrates and forage, as well as grazing or feedlot system, different sources of forage and concentrates (OLIVEIRA et al., 2013), supplementation with carbohydrates, proteins and lipids and use of feed additives.

The FA profile in the meat will depend mainly of the quantity and proportion of VFA´s absorbed from the rumen. Acetate is the main precursor for the FA synthesis in adipose tissue, and higher amounts of this acid in the plasma increase the formation of FA in adipocytes (POLIZEL et al., 2008). The β-OH-butyrate is the second most used precursor and is derived from the breakdown of butyrate in the liver (PALMIQUIST; MATTOS, 2006), but are metabolized preferentially in the mammary gland of lactating animals (VERNON, 1981). Propionate and lactate are almost totally captured by the liver, because they are gluconegogenic, and a minimal amount can be related to lipogenesis in adipose tissue.

The principal fatty acids synthesized from acetate in bovine and ovine adipose tissue slices are palmitic, stearic and oleic; small amounts of myristic and palmitoleic acids are formed but negligible amounts of shorter-chain length fatty acids were detected. However, the use of propionate as a precursor is associated with increased formation of odd and branched chain fatty acids (VERNON, 1981).

Glucose is the source of carbon less used for FA synthesis in adipose tissue, but it influences the formation of adipocytes, stimulates the activity of enzymes responsible for uptake of plasma FA (Lipase Lipoprotein) and increases the supply of glycerol-3-phosphate needed for FA esterification in adipose tissue. In addition, increases in dietary glucose concentration and the fermentation of carbohydrates in

VFA's in the rumen increases the rate of FA synthesis, indicating that body fat deposition is directly related to the net energy supply (SMITH; CROUSE, 1984).

Addition of glucose increased by 3-10-fold the rate of fatty acid synthesis from acetate in ovine and bovine adipose tissue. Glucose also stimulated the rate of fatty acid synthesis from β-OH-butyrate, but not from lactate or pyruvate. Abomasal or intravenous infusion of glucose increased the rate of fatty acid synthesis from both glucose and acetate, without necessarily increasing plasma glucose concentration (VERNON, 1981). Thus, the manipulation of diets for ruminants and use of additives may have a direct effect on the FA synthesis and composition.

Silage inoculants are generally used to improve the fermentation pattern and the silage stability of the feed-out period, has been show interesting results, with increasing in dry matter intake, feed efficiency and weight gain of the animals (ARAGON et al., 2012; BASSO et al., 2014; RABELO et al., 2016). This result may be mainly due to changes in the nutritive value of the inoculated silage, which provide better preserved silage with higher energy value and can increase rumen fermentation, microbial protein synthesis and animal production (MUCK, 2010).

In addition, some microorganisms develop and produce metabolites as bacteriocins that are stable in the silo and may alter the ruminal microorganisms (CALLAWAY et al., 1997; YILDIRIM, 2001). These metabolites can also alter the biohydrogenation processes in silage and possibly the rumen, with impact mainly on the gram-positive bacteria (KLAENHAMMER, 1993) and modify the VFA's profile in the rumen. As previously mentioned, *Bacillus subtilis* is also a microorganism that produces bacteriocins and antimicrobial substances (LANNA FILHO et al. 2010), but its effect on the quality of sheep meat when used as silage inoculant has not yet been studied.

Rabelo et al. (2016) evaluating the influence of *L. buchneri* as silage additive in the meat quality, indicated increase on the concentration of UFA and MUFA in the meat of beef cattle and increase of the PUFA and omega-6 when inoculated silage was associated with diet containing 60% of concentrated. However, no difference was detected in sensory parameters and meat color. Fugita et al. (2012) did not observe effect of bacterial-enzymatic inoculant containing homofermentative LAB strains on carcass and meat quality of Nelore x Angus steers fed corn silage.

Exogenous enzymes used as additives for ruminants have also been extensively studied with the objective of acting on the degradation of complex components of the diet, altering the fermentative profile of the rumen and consequently the animal production. Amylases are carbohydrases responsible for degrading amylose and amylopectin to lower molecular weight compounds that will be degraded and absorbed rapidly by ruminal bacteria. This process can change the microbial profile and the final products of the ruminal fermentation. Furthermore, as mentioned above, it may increase the energy supply to the animal by increasing the fatty acid synthesis with or without modification of the fatty acid profile of the meat.

Another important issue is that diets with high content of rapidly fermentable carbohydrates can contribute with decrease of the rumen pH and reduction of lipolysis (DOREAUS; FERLLAY, 1994), which is a precondition for the biohydrogenation (LATHAM et al., 1972). These changes result in changes in the ruminal microbiota influencing the fermentation standard and the final products (HOLANDA et al., 2011; KOZLOSKI, 2011). According to Bauman & Griinari (2003), pathways towards producing t10-18:1 instead of vaccenic acid are favored. Thus, these diets also could be an influence on fatty acid flow to the duodenum, and thus interfere with fatty acid muscle deposition (SCOLLAN et al., 2014).

## 3. RESEARCH OBJECTIVES

The objectives of this research was to investigate the effects of *L. plantarum* and *B. subtilis* as silage inoculant in diets supplemented or not with α-amylase moments before feeding on 1) the apparent digestibility, ruminal fermentation, nitrogen balance and microbial protein synthesis of wethers, and 2) growth performance, quality and fatty acid profile of lambs meat.

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# CHAPTER 2

The paper was written following the guidelines for authors of *Animal Feed Science and Technology*, with exception of tables and figures position.

**Apparent digestibility, ruminal fermentation and microbial protein synthesis of wethers fed diets containing inoculated corn silage and supplemented with amylolytic enzyme**

**Abstract**: This study was conducted to determine the effects of diets containing corn silage inoculated with *Lactobacillus plantarum* (LP) combined with *Bacillus subtilis* (BS) and supplemented or not with amylase on the apparent digestibility, ruminal fermentation, nitrogen utilization and microbial protein synthesis of wethers. Animals received one of four treatments (diets): 1) Corn silage uninoculated and without amylase added to total mixed ration (TMR); 2) Corn silage uninoculated and amylase added to TMR; 3) Corn silage inoculated with $1 \times 10^5$ CFU LP [MA 18/5U] and $1 \times 10^5$ CFU BS [AT553098] without amylase added to TMR; 4) Corn silage inoculated with $1 \times 10^5$ CFU LP [MA 18/5U] and $1 \times 10^5$ CFU BS [AT553098] and amylase added to TMR. Eight Dorper × Santa Ines crossbred wethers were used in a replicated 4 × 4 Latin square with four experimental periods of 16-d each consisting of 10 d for diet adaptation and six days for daily recovering of feed offered, orts and faeces from each wether. Amylase supplementation on the diet containing uninoculated silage increased ($P$=0.045) dry matter (DM) intake of wethers compared with wethers fed uninoculated silage without amylase supplementation (1,311 vs. 1,066 g/d), but not differed from others treatments. The apparent digestibility of DM, OM, CP, NDF and GE increased ($P$<0.01) in wethers fed with inoculated silages or supplemented with amylase, without interaction among inoculants and amylase. Wethers fed diets containing uninoculated silage and supplemented with amylase showed higher propionic acid and lower acetic acid proportion, with low acetic:propionic acid ratio, consequently. Microbial N supply tended to be higher ($P$=0.097) in wethers fed uninoculated silage with amylase supplementation and inoculated silage without amylase (8.01; 8.05 g/d) when compared to wethers fed inoculated silage with amylase supplementation (6.80 g/d), but did not differ from uninoculated silage without amylase supplementation (7.01 g/d). However, no effect was verified on the efficiency of microbial N synthesis.

**Keywords:** amylase, digestibility, microbial inoculant, performance, starch

## 1. Introduction

Intensive production systems rely on cereal grain starch as the primary source of energy (DiLorenzo et al., 2011) that can comprises approximately 50 and 75% of the energy value of corn silage and grain, respectively (Nocek and Tamminga, 1991; NRC, 2001). The starch digestibility in ruminants, in general, is not considered a production limiting factor, however is not 100% efficient and can occur fermentation in the large intestine (energy-inefficient) and starch losses in feces.

Additives that increase the starch utilization by ruminants directly or indirectly through changes in ruminal fermentation pattern are an applicable alternative that is gaining traction in the animal nutrition sector. However, it should be borne in mind that prior to the adoption of these technologies, some management strategies and a more specific knowledge of the feed offered to animals may help to improve the starch utilization without changes in the final cost of the diet.

There are several ways to achieve greater digestibilities of the starch, being: 1- corn hybrid choice (Vitreousness, Ngonyamo-Majee, et al., 2008), 2-harvest time (Maturity, Ferrareto and Shaver, 2012), 3- ensiling time (Der Bedrosian et al., 2012; Young et al., 2012), 4- grain processing (Mc Allister et al., 1990) and lastly the adoption of technologies, among them the mainly are the microorganisms, with probiotic effect or not (Wiryawan and Brooker, 1995) and amylolytic enzymes (Tricarico et al., 2008).

Amylase supplementation (Rojo et al., 2005; Mendonza et al., 2013; Nozière et al., 2014), may increase the total number of viable bacteria, increasing fiber digestion and improving the ability of rumen bacteria to ingest and degrade feed and secondary metabolites. It could also increase the amount of crude protein (CP) available for microbial metabolism, which may increase fiber digestibility and the metabolizable energy (ME) density of the diet and providing greater intake of nutrients for metabolism and animal production (Tricarico et al., 2008; Salem et al., 2015).

Silage inoculant such as *B. subitilis* is another source of enzyme (i.e., amylase, xylanase and ferulic acid esterase; Kang et al., 2009) that may act on complex polysaccharides and provide more soluble carbohydrates to be fermented by LAB within the silo (Phillip and Fellner, 1992) and enhanced the amylase

degradability. This microorganism is little studied as silage inoculant, however has antagonistic activity against phytopathogenic fungi (Schisler et al., 2004; Angonese et al., 2009) and can control the growth of yeasts and molds (Basso et al., 2012; Lara et al., 2015).

Other important silage inoculant is the *L. plantarum* that aims to accelerate the decline in silage pH with consequent decreases of the proteolysis and prevention of the growth of spoilage microorganisms (Filya, 2003; Addah et al., 2014). In addition, *L. plantarum* may survive and show probiotic effect in the rumen (Weinberg et al., 2003; 2004a; 2004b) improving the nutrient digestibility (Addah et al., 2012; Lara et al., 2015) and consequently the animal performance (Aragon et al., 2012; Nishino, 2015).

However, the results are often inconsistent and even when the increases ruminal or total starch digestibility are verified, is not always present improvement in animal performance (Rojo et al., 2005; Lee-Rangel et al., 2010; DiLorenzo et al. 2011). In addition, at the moment, few studies have been evaluated the effect of use amylases, moments before feeding, in diets (total mixed ration) with inoculated silage on metabolism and performance of wethers.

Thus, our goal in this study was to evaluate diets containing inoculated corn silage with *L. plantarum* combined with *B. subtilis* and supplemented or not with amylase on the apparent nutrient digestibility, ruminal fermentation, nitrogen utilization and microbial protein synthesis of wethers.

## 2. Material and Methods

The protocol used in the present study was in accordance with the Brazilian College of Animal Experimentation (COBEA – Colégio Brasileiro de Experimentação Animal) guidelines and was approved by the Ethics, Bioethics and Animal Welfare Committee (CEBEA – Comissão de Ética e Bem Estar Animal) of the FCAV-UNESP/Jaboticabal campus, Brazil (Projected number 1.754/15).

### *2.1. Crop harvest and silage preparation*

A flint corn hybrid (2B710, Dow AgroSciences Cravinhos, São Paulo, Brazil) was planted on December 18, 2013, at the São Paulo State University (UNESP) School of Agricultural and Veterinarian Sciences, Jaboticabal, located at

21°14'14.04" S and 48°17'27.92" W and harvested on March 24, 2014 at approximately 344 g/kg of dry matter (DM) at a stubble height of 20 cm using a pull-type JF 90® forage harvester (JF Agricultural Machinery, Itapira, Sao Paulo, Brazil). Forage was chopped to a length of cut settings of 10 mm without kernel processing. Corn forage was treated with water (0.7 L/t; Untreated) or with $1 \times 10^5$ CFU/g of fresh forage of LP MA [18/5U] (Lallemand Animal Nutrition, Goiânia, GO, Brazil) combined with $1 \times 10^5$ cfu/g of fresh forage of *BS* [AT553098] (FATEC Nutrição e Saúde Animal, Arujá, SP, Brazil). The inoculants were dissolved in water (2 L/t) and then sprayed on fresh forage during the silo filling. The application rate of the inoculant was determined in accordance with previous studies carried out in our lab in tropical conditions (Basso et al., 2012 and 2014; Lara et al., 2015).

Two stack silos were filled in two consecutive days with approximately 20 tons each treatments (inoculated or not) and compacted with a Wheel lounder (Cat®, Peoria, Chicago, USA) at a packing density of 580 ± 66 kg/m$^3$ (fresh matter basis). To avoid possible cross contamination, the uninoculated forage was ensiled first, followed by the inoculated forages. The silos were sealed with black-on-silver polyethylene film and covered with a layer of soil. Twelve fresh samples were taken to characterize the chemical composition of the corn plant at silo filling.

Silos were stored at an ambient temperature (22.1°C ± 2.5) for 170 d. The silage was removed at a rate of approximately 10.0 ± 4.0 cm/d from the silo face using a fork. Ten silage samples were collected weekly during all period of the experiment and it was made a composite sample that was stored at -20°C for subsequent analyses to characterize the corn silages (Table 1).

**Table 1.** Chemical composition, fermentation and microbial profile of uninoculated and inoculated corn plants prior to ensiling and after silos were opened[1] (n = 10).

| Item[2] | Corn plant | Corn silage | |
|---|---|---|---|
| | | Uninoculated | Inoculated |
| ***Chemical composition, g/kg of DM*** | | | |
| DM | 344 ± 0.32 | 330 ± 3.59 | 332 ± 3.25 |
| OM | 897 ± 1.19 | 951 ± 3.48 | 960 ± 1.73 |
| EE | 16.5 ± 0.45 | 19 ± 0.99 | 18 ± 1.00 |
| CP | 75.0 ± 0.85 | 87 ± 0.54 | 84 ± 0.51 |
| Starch | NM | 238 ± 1.98 | 218 ± 2.05 |
| NDF | 533 ± 4.22 | 480 ± 8.38 | 483 ± 9.19 |
| ADF | 238 ± 2.85 | 258 ± 5.05 | 258 ± 3.53 |
| Hemicellulose | 302 ± 3.22 | 240 ± 4.19 | 244 ± 3.36 |
| Cellulose | 207 ± 1.86 | 218 ± 3.93 | 222 ± 3.92 |
| Lignin | 28.5 ± 0.94 | 24 ± 1.53 | 23 ± 1.19 |
| CHO | 875 ± 2.52 | 842 ± 4.07 | 855 ± 2.37 |
| NFC | 382 ± 5.81 | 378 ± 10.33 | 392 ± 9.29 |
| GE, MJ/kg of DM | NM | 17 ± 0.29 | 18 ± 0.40 |
| ***Fermentation profile, g/kg of DM*** | | | |
| Lactic acid | NA | 15.1 ± 1.69 | 21.8 ± 2.10 |
| Acetic acid | NA | 14.2 ± 0.62 | 12.8 ± 0.84 |
| Propionic acid | NA | 1.1 ± 0.12 | 0.3 ± 0.04 |
| Butyric acid | NA | 0.7 ± 0.13 | 0.6 ± 0.06 |
| L:A | NA | 1.1 ± 0.18 | 2.0 ± 0.15 |
| pH | 4.40 ± 0.12 | 3.86 ± 0.03 | 3.74 ± 0.04 |
| $NH_3$-N[3] | 37.5 ± 5.13 | 44 ± 2.98 | 54 ± 4.32 |
| ***Microbiological profile, CFU/g of fresh silage*** | | | |
| Lactic acid bacteria | NM | 6.00 ± 0.13 | 5.99 ± 0.09 |
| Yeasts | 5.42 ± 0.14 | 6.35 ± 0.48 | 5.78 ± 0.46 |
| Molds | 4.50 ± 0.17 | 5.60 ± 0.20 | 5.15 ± 0.10 |

[1]Corn silage uninoculated or inoculated at ensiling with 1 × $10^5$ cfu/g of fresh forage of *L. plantarum* MA 18/5U (Lallemand Animal Nutrition, Goiânia, GO, Brazil) combined with 1 × $10^5$ cfu/g of fresh forage of *B. subtilis* AT553098 (FATEC Nutrição e Saúde Animal, Arujá, Sao Paulo, Brazil).
[2]DM = dry matter; OM = organic matter; EE = ether extract; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; CHO = total carbohydrates; NFC = non-fiber carbohydrates; GE = gross energy; L:A = lactic acid:acetic acid ratio.
[3]NH3-N = g/kg of total nitrogen.
NM = not measured
NA = not applicable

*2.2. Treatments and feeding*

Four diets were evaluated: (1) Corn silage uninoculated and no amylase added to TMR; (2) Corn silage uninoculated and amylase added to TMR; (3) Corn silage inoculated with $1 \times 10^5$ CFU *Lactobacillus plantarum* [MA 18/5U] and $1 \times 10^5$ CFU *Bacillus subtilis* [AT553098] and no amylase added to TMR; (4) Corn silage inoculated with $1 \times 10^5$ CFU *Lactobacillus plantarum* [MA 18/5U] and $1 \times 10^5$ CFU *Bacillus subtilis* [AT553098] and amylase added to TMR. Diets were composed of 400 g/kg of DM corn silage and 600 g/kg of DM of concentrated (Table 2). Wethers and lambs were fed to meet their requirements to gain 300 g/d based on previous studies reported by NRC (2007), in which those animals have a similar growth curve.

**Table 2.** Composition of the experimental diets (g/kg on a dry matter basis)[1].

| Item[2] | Uninoculated | Inoculated |
|---|---|---|
| ***Ingredient proportions*** | | |
| Corn silage | 400.0 | |
| Ground corn | 479.6 | |
| Urea | 12.0 | |
| Soybean meal | 84.0 | |
| Comercial Premix[3] | 24.4 | |
| ***Chemical composition*** | | |
| DM | 669 | 675 |
| OM | 942 | 947 |
| EE | 21 | 20 |
| CP | 151 | 150 |
| Starch | 371 | 363 |
| NDF | 257 | 258 |
| ADF | 126 | 126 |
| Cellulose | 108 | 109 |
| Hemicellulose | 156 | 157 |
| Lignin | 13 | 12 |
| CHO | 763 | 769 |
| NFC | 514 | 520 |
| ME, MJ/kg of DM | 10.5 | 10.5 |

[1]Corn silage uninoculated or inoculated at ensiling with $1 \times 10^5$ cfu/g of fresh forage of *L. plantarum* [MA 18/5U] (Lallemand Animal Nutrition, Goiânia, GO, Brazil) combined with $1 \times 10^5$ cfu/g of fresh forage of *B. subtilis* [AT553098] (FATEC Nutrição e Saúde Animal, Arujá, Sao Paulo, Brazil).
[2]DM = dry matter; OM = organic matter; EE = ether extract; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; CHO = total carbohydrates; NFC = non-fiber carbohydrates; ME = Metabolized energy (ME = total apparent digestibility of gross energy × 0.82).

[3]Composition: NaCl = 4.2 g/kg of DM; Phosphate = 4.2 g/kg of DM; Limestone = 15.4 g/kg of DM; Ca = 120%; P = 40%; Na = 282%; Cl = 448%; K = 857%; Mg = 17%; S = 22%; Fe = 123mg; Co = 10 mg; Se = 0.43mg, Vit.A = 5,671UI; Vit.D3 = 501UI; Vit.E = 40mg.

A powdered concentrate from *Aspergillus oryzae* (Amaize, Alltech Inc.) containing primarily α-amylase activity was used in these studies. The α-amylase activity was determined according to the procedure described in the Food Chemicals Codex (1996). One α-amylase dextrinizing unit (DU) was defined as the quantity of enzyme required to dextrinize soluble starch at the rate of 1 g/h at 30°C and pH 4.8. The preparation used in this study contained 301 DU/g of product and was added to TMR at time of feeding delivery a rate of 2 g/kg of dietary DM of the product. Therefore, final concentrations of α-amylase fed to lambs and wethers were 602 DU/kg of dietary DM.

Eight Dorper × Santa Ines crossbred wethers, fitted with ruminal silicone-type cannulas (diameter 6.4 cm) were used in a replicated 4 × 4 Latin square. The initial body weight was 37.4 ± 2.2 kg (6 months) and they were housed in individual metabolism crates (1.2 × 0.5 m) equipped with individual feed, water containers and faeces and urine collectors. The animals were fed *ad libtum* (approximately 10% of orts) once a day (0700 h) with free access to drink water. The study consisted of four experimental periods of 16-d each consisting of 10 d for diet adaptation based on previous studies in our lab (Basso et al., 2014; Rabelo et al., 2016) and six days for measurements of intake, digestibility, N balance, microbial protein synthesis and ruminal fermentation.

*2.3. Feed intake, apparent total digestibility and urine collection*

On day 11-15, amount of feed offered, orts and faeces from each wether were recorded daily and then 10% sample was taken. At the end of each period, daily samples were pooled for each wether within the treatment, thoroughly mixed and stored at −20 °C for later analysis. Orts were used to calculate dry matter intake (DMI) daily, and its chemical composition was also taken in consideration when digestibility was calculated.

At the same time, total urine produced daily was collected in plastic vessels containing 100 mL of sulphuric acid solution (10%, v/v), to keep the final pH below 3, placed below the urine outlet in the metabolism crates. After each 24 h collection period, the total weight and volume of urine excreted were determined. A composite

sample was made from the 2 sampling days and two subsamples (10 mL) were collected and storage at - 20°C for later analysis of purine derivatives (PD) to estimate microbial synthesis. Other 10-mL urine sample was diluted with 40 mL of a 0.036 N $H_2SO_4$ solution before storage.

*2.4. Rumen samples*

On day 16, a 50 mL sample of ruminal fluid was collected from each wether before feeding (0 h), and at 3, 6 9 and 12 h post-feeding. Ruminal fluid was squeezed through four layers of cheesecloth and its pH was immediately measured using a pH meter (MA522 model, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). A volume of 1 mL of $H_2SO_4$ diluted with distilled water (1:1 v/v) was then added to the ruminal fluid to inhibit microbial activity. The resulting solution was stored at -20°C unil further analysis of volatile fatty acids (VFA) and $NH_3$-N.

*2.5. Microbial analyses*

For microbiological analyses, fresh silage samples (25 g) from each replicate were homogenized in 225 mL of autoclaved saline solution (0.85% NaCl) and the mixture was manually agitated for 1 min. An aliquot (1 mL) of this solution was transferred into tubes containing 9 mL of saline solution, and thereafter, 1 mL of this mixture was plated onto Petri plates after serial dilutions of $10^{-1}$ – $10^{-9}$. Man, Rogosa, and Sharpe (MRS) agar was used to count LAB by pour plate, whereas potato dextrose agar (PDA) was used to count yeasts and molds by spread-plate. Both MRS and PDA plates were incubated at 28°C according to the previous studies carried out in our lab (Basso et al., 2012; Lara et al., 2015; Rabelo et al., 2016). The LAB and yeasts were counted after 2 d, and molds were counted after 5 d, respectively. All microbiological data were $\log_{10}$ transformed.

2.6. *Chemical analyses*

A water extract was produced from fresh silage samples (Kung et al., 1984) using a Phillips Walita blender (Walita, Varginha, MG, Brazil), and silage pH was measured using a pH meter (model MA522, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). The VFAs, in fresh forage were measured in a gas chromatograph (GC2014, Shimadzu Corporation, Kyoto, Japan) equipped with a HP-INNOWax capillary column (30 m × 0.32 mm; Agilent Technologies, Colorado, USA).

At an initial temperature of 80°C for 3 min followed by a heating rate of 20°C $min^{-1}$ until a final temperature of 240°C was achieved. Lactic acid was determined using a colorimetric method (Pryce, 1969). The $NH_3$-N was measured by distillation (AOAC, 1996; method number 941.04). The pH and $NH_3$-N of ruminal fluid were analyzed as described for silage, whereas VFAs were analyzed in the supernatant after centrifugation at 4°C and 20,000 g for 30 min.

Samples of silage, concentrate, orts and feces were oven dried (55°C for 72 h) and processed in a knife mill before being ground through a 1-mm screen and analyzed for DM (105°C for 12 h) (AOAC 1996; method number 930.15) and ash (500°C for 5 h) (AOAC 1996; method number 923.03). Neutral detergent fiber (aNDFom) and acid detergent fiber (ADF) were determined in a Ankom 2000 Fiber Analyzer (Ankom Technologies, Macedon, NY, USA) without sodium sulfite following the procedures described by Mertens (2002) and AOAC (1996, method number 973.18), respectively. Lignin (sa) was sequentially measured after hydrolysis of the ADF residual in 72% $H_2SO_4$ (Van Soest and Robertson, 1985). Hemicellulose was calculated after sequential analyze on the same sample. Ether extract (EE) was measured according to the AOAC (1996; method number 920.39). The N concentration was determined by rapid combustion using a Leco F528 N analyzer (LECO Corporation, St. Joseph, MI, USA). The crude protein (CP) was calculated as total N × 6.25. Total carbohydrate (CHO) and non-fiber carbohydrate (NFC) concentrations were calculated as described by Sniffen et al. (1992). Starch was determined enzymatically according to Bach Knudsen et al. (1987). Gross energy (GE) was determined with a bomb calorimeter (PARR 6200; Parr Instrument Company, Milone, IL). The metabolized energy (ME) was estimated as 0.82 from apparent digestibility energy (DE) (NRC, 1985; 2007).

Microbial protein synthesis was calculated via urinary total excretion of purine derivates (allantoin + uric acid + xanthine + hypoxanthine) according to the technique of Fujihara et al (1987), as described by Chen and Gomes (1992) and calculated according to Pina et al. (2009). The efficiency of microbial N synthesis (EMNS) was expressed as grams of microbial N per Kilogram of digestible OM fermented in the rumen (DOMR; calculated as digestible OM intake × 0.65, according to the Agricultural Research Council (1984)).

*2.7. Calculations*

The apparent digestibility was calculated using the following equation:

$$\text{Apparent digestibility} = \left[\frac{(\text{Nutrient intake} - \text{fecal nutrient output})}{(\text{Nutrient intake})}\right] \times 100;$$

where intake and fecal output was expressed in grams.

To estimate microbial protein synthesis, the daily PD excretion and microbial N supply were calculated according to Chen et al. (1995) model, using the following equations:

$$\text{Daily PD} = 0.84 \times \text{AP} + \left[0.150 \times \text{BW}^{0.75 \times e^{(-0.25\text{AP})}}\right];$$

where daily PD is expressed in millimoles per day, AP is the absorption of microbial purines (mmol/d), 0.84 represents the recovery of absorbed purines as PD in urine, and the component within brackets represents the endogenous contribution, which decreases as exogenous purines become available for utilization by animal, and

$$\text{Microbial N} = (\text{AP} \times 70) \div (0.116 \times 0.83 \times 1{,}000) = 0.727 \times \text{AP};$$

where microbial N is expressed in grams N per day, the N concentration of purines is 70 mg N/mmol, the ratio of purine-N:total N is mixed ruminal microbes is taken as 11.6:100, and the digestibility of microbial purines is assumed to be 0.83.

*2.8. Statistical analyses*

Data of intake, digestibility, N balance and microbial protein synthesis from metabolism experiment were analyzed as a replicated 4 × 4 Latin square design and data of intake and growth performance from the growth performance study were analyzed as a randomized block design. The treatments were arrangement in a 2 × 2 factorial (corn silage inoculated or not with *L. plantarum* and *B. subtilis*, supplemented diet or not with amylase). All data were analyzed using the PROC MIXED procedure of SAS (version 9.0; SAS Inst. Inc., Cary, NC). The model included the fixed effect of treatment and Latin square or block design, and random effects of period, animal and error.

Data of pH, $NH_3$-N and VFA analysis of variance included repeated measures overtime and the fixed effect treatments, time and treatment × time interaction, and

random effects of period, animal and error. Heterogeneous autoregressive, autoregressive Moving Average and banded were the best covariance structures for the data as these had the lowest Aikaike information criterion score.

Homogeneity of the data was verified using the UNIVARIATE procedure of SAS. Studentized residuals were plotted against the predicted values using the plot procedure to analyze data for outliers. Differences among means were determined using the PDIFF, which differentiates means based on Fisher's *F*-protected least significant difference test. Significance was declared at $P \leq 0.05$ and a trend at $P \leq 0.10$.

## 3. Results

### *3.1. Feed intake and apparent total digestibility of wethers*

There were interactions ($P<0.05$) between inoculation silage and amylase supplementation for DM and starch intake, with a tendency ($P<0.10$) of interaction on OM, NDF, GE, DE and ME intake (Table 3).

Wethers fed with inoculated silage and supplemented with amylase showed lesser intake of OM, NDF, GE and starch than wethers fed uninoculated silage supplemented with amylase, but it did not differ from uninoculated and inoculated silage without amylase supplementation. Likewise, the intakes of DM, OM and starch were greater in wethers fed uninoculated silage and supplemented with amylase when compared with wethers fed uninoculated silage without amylase supplementation, which not differ from diets containing corn silage inoculated and supplemented or not with amylase.

The apparent digestibility of DM, OM, CP, NDF and GE was 3.69, 3.57, 4.33, 9.14, 4.41%, respectively, higher in wethers supplemented with amylase (Table 3; $P<0.05$). Likewise, inoculated silages promoted an increase of 3.71, 3.85, 7.68, 11.20, 5.01% of the DM ($P=0.083$), MO, CP, NDF and GE digestibility ($P<0.05$), respectively, when compared wethers fed uninoculated silages. The starch digestibility was not affected by inoculation or amylase supplementation at moment of wethers feeding.

**Table 3.** Intake and coefficients of total tract apparent digestibility of nutrients of wethers fed diets containing corn silage (Uninoculated or Inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| Item[1] | Uninoculated | | Inoculated | | SEM | *P*-value[2] | | |
|---|---|---|---|---|---|---|---|---|
| | No Amylase | Amylase | No Amylase | Amylase | | S | A | S × A |
| ***Intake of nutrients, g/d*** | | | | | | | | |
| DM | 1,066b | 1,311a | 1,263ab | 1,128ab | 0.05 | 0.930 | 0.540 | 0.045 |
| OM | 1,021b | 1,238a | 1,201ab | 1,073b | 43.71 | 0.719 | 0.904 | 0.088 |
| CP | 183 | 203 | 195 | 175 | 7.15 | 0.665 | 0.850 | 0.114 |
| EE | 26 | 29 | 28 | 27 | 0.86 | 0.869 | 0.486 | 0.179 |
| NDF | 275ab | 310a | 306ab | 268b | 10.73 | 0.787 | 0.936 | 0.074 |
| Starch | 383b | 519a | 449b | 403b | 21.99 | 0.470 | 0.200 | 0.014 |
| GE, MJ/kg of DM | 20ab | 23a | 22ab | 20b | 0.81 | 0.702 | 0.924 | 0.089 |
| DE, MJ/kg of DM | 12.8 | 16.6 | 16.1 | 15.2 | 0.65 | 0.467 | 0.289 | 0.088 |
| ME, MJ/kg of DM | 10.5 | 13.6 | 13.2 | 12.5 | 0.53 | 0.467 | 0.289 | 0.088 |
| ***Coefficients of total tract apparent digestibility*** | | | | | | | | |
| DM | 0.714 | 0.741 | 0.732 | 0.779 | 0.009 | 0.083 | 0.024 | 0.530 |
| OM | 0.737 | 0.758 | 0.762 | 0.812 | 0.008 | 0.026 | 0.042 | 0.388 |
| CP | 0.649 | 0.685 | 0.698 | 0.789 | 0.014 | 0.001 | 0.005 | 0.180 |
| EE | 0.896 | 0.897 | 0.901 | 0.957 | 0.013 | 0.190 | 0.250 | 0.280 |
| NDF | 0.396 | 0.471 | 0.491 | 0.600 | 0.024 | 0.001 | 0.020 | 0.660 |
| Starch | 0.967 | 0.969 | 0.964 | 0.969 | 0.003 | 0.770 | 0.500 | 0.830 |
| GE | 0.689 | 0.728 | 0.734 | 0.783 | 0.010 | 0.011 | 0.022 | 0.782 |

[*]Corn silage uninoculated or inoculated at ensiling with 1 × $10^5$ cfu/g of fresh forage of *L. plantarum* [MA 18/5U] (Lallemand Animal Nutrition, Goiânia, GO, Brazil) combined with 1 × $10^5$ cfu/g of fresh forage of *B. subtilis* [AT553098] (FATEC Nutrição e Saúde Animal, Arujá, Sao Paulo, Brazil).
[1]DM = Dry matter; OM = Organic matter; CP = Crude protein; EE = Ether extract; NDF = Neutral detergente fiber; GE = Gross energy; DE = Digestible energy; ME = Metabolized energy.
[2]S, silage; A: amylase; S×A: interaction between silage and amylase.

### *3.2. Ruminal parameters*

The treatments did not alter the ruminal pH, $NH_3$-N and total VFA. There were interactions ($P$<0.05) between silages and amylase supplementation in the acetic acid and propionic acid proportion, as well as in acetic acid:propionic acid ratio (Table 4, Figure 1). Wethers fed uninoculated silage and supplemented with amylase had lower acetic acid and higher propionic acid proportion (65.66 and 24.14), respectively. Conversely, when amylase was associated with inoculated silage,

wethers showed higher acetic acid and lower propionic acid proportion (24.14 and 19.75), respectively.

Wethers fed uninoculated silage and supplemented with amylase had lower acetic:propionic acid ratio in ruminal fluid. On the other hand, the inoculation of silage tended ($P$=0.094) to enhance the butyric acid proportion in the rumen fluid (11.21 vs 11.24).

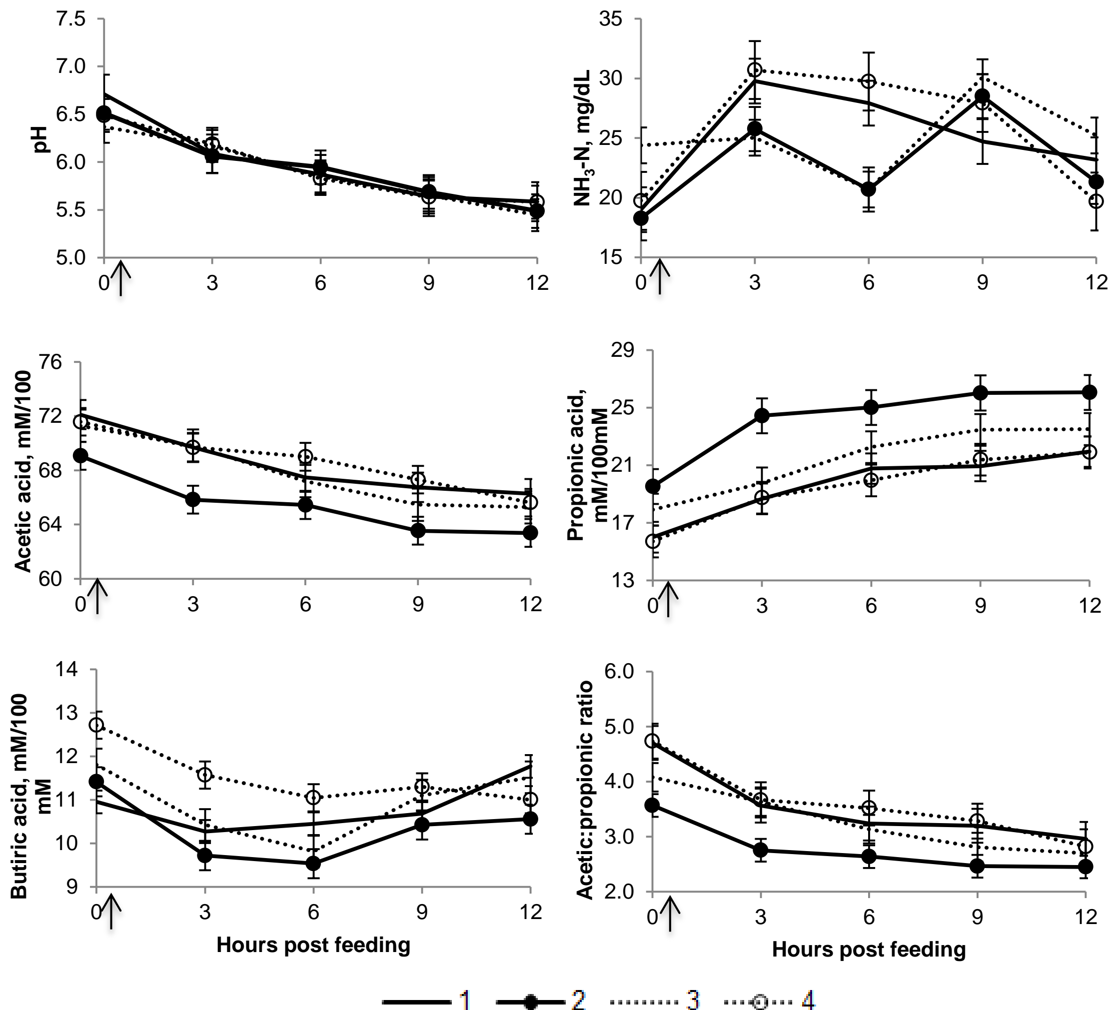


**Figure 1.** Ruminal parameters of wethers fed diets: 1) Corn silage uninoculated and no amylase added to TMR, 2) Corn silage uninoculated and amylase added to TMR, 3) Corn silage inoculated with 1×10$^5$ CFU *Lactobacillus plantarum* [MA 18/5U] and 1×10$^5$ CFU *Bacillus subtilis* [AT553098] and no amylase added to TMR, 4) Corn silage inoculated with 1×10$^5$ CFU *Lactobacillus plantarum* [MA 18/5U] and 1×10$^5$ CFU *Bacillus subtilis* [AT553098] and amylase added to TMR. ↑Feeding. Vertical bars are SEM.

**Table 4.** Ruminal parameters of wethers fed diets containing corn silage (Uninoculated or Inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| | Uninoculated | | Inoculated | | SEM | P-value[1] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No Amylase | Amylase | No Amylase | Amylase | | S | A | S × A | T | A × S × T |
| pH | 5.98 | 5.94 | 5.91 | 5.96 | 0.04 | 0.462 | 0.895 | 0.234 | <.0001 | 0.352 |
| $NH_3$-N, mg/dL[2] | 24.92 | 22.92 | 25.25 | 25.08 | 0.77 | 0.231 | 0.294 | 0.375 | <.0001 | 0.145 |
| Total VFA, mM/L | 78.43 | 79.26 | 71.82 | 78.63 | 1.67 | 0.274 | 0.246 | 0.365 | <.0001 | 0.255 |
| ***Molar proportion, mM/100 Mm*** | | | | | | | | | | |
| Acetic acid | 68.34ab | 65.66b | 67.98ab | 68.48a | 0.32 | 0.124 | 0.173 | 0.047 | <.0001 | 0.953 |
| Propionic acid | 19.64b | 24.14a | 21.20ab | 19.75b | 0.38 | 0.139 | 0.111 | 0.002 | <.0001 | 0.995 |
| Butyric acid | 11.20 | 10.23 | 10.90 | 11.58 | 0.14 | 0.094 | 0.923 | 0.133 | <.0001 | 0.406 |
| Acetic:propionic acid ratio | 3.49a | 2.79b | 3.30ab | 3.56a | 0.08 | 0.083 | 0.183 | 0.005 | <.0001 | 0.809 |

[*]Corn silage uninoculated or inoculated at ensiling with $1 \times 10^5$ cfu/g of fresh forage of *L. plantarum* [MA 18/5U] (Lallemand Animal Nutrition, Goiânia, GO, Brazil) combined with $1 \times 10^5$ cfu/g of fresh forage of *B. subtilis* [AT553098] (FATEC Nutrição e Saúde Animal, Arujá, Sao Paulo, Brazil).
[1]S, silage; A: amylase; S×A: interaction between silage and amylase; T, time; E×S×T, interaction among amylase, silage and time.
[2]$NH_3$-N: Ammonia nitrogen

All variables from ruminal fermentation showed time effect (*P*<.0001). The first peak of $NH_3$-N production was observed after 3 hours of feeding. Acetate concentrations decreased and propionate concentrations increased throughout the day. Animals fed uninoculated silage with amylase supplementation at time of feeding showed higher propionic acid and lower acetic acid production in the rumen during all the time of evaluation.

*3.3. The N utilization and ruminal microbial N synthesis*

Interaction between silage and amylase supplementation was observed on N feces (Table 5, *P*<0.001). Wethers fed inoculated silage and supplemented with amylase had lower N excretion on the feces. The N excretion by urine was lower in wethers fed inoculated corn silage and supplemented with amylase, however was statistically significant. The treatments did not alter the N intake, N retained and N absorbed, as well as the relations between N retained and N intake or N absorbed.

**Table 5.** N utilization and ruminal microbial N synthesis of wethers fed diets containing corn silage (Uninoculated or Inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| | Uninoculated | | Inoculated | | SEM | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| | No Amylase | Amylase | No Amylase | Amylase | | S | A | S × A |
| ***N utilization, g/d*** | | | | | | | | |
| N Intake | 29.0 | 32.5 | 31.1 | 27.9 | 1.14 | 0.526 | 0.935 | 0.102 |
| N Urinary | 7.27 | 7.98 | 7.68 | 6.51 | 0.42 | 0.501 | 0.765 | 0.239 |
| N Feces | 8.89a | 9.96a | 9.63a | 6.42b | 0.52 | 0.013 | 0.049 | <0.001 |
| N Retained | 13.8 | 14.6 | 13.8 | 14.8 | 0.85 | 0.954 | 0.567 | 0.924 |
| N Absorved | 19.7 | 22.5 | 21.5 | 21.4 | 0.84 | 0.855 | 0.404 | 0.396 |
| N Ret/N Intake | 0.45 | 0.43 | 0.44 | 0.53 | 1.95 | 0.195 | 0.265 | 0.143 |
| N Ret/N Absorved | 0.65 | 0.63 | 0.64 | 0.68 | 2.15 | 0.605 | 0.828 | 0.349 |
| ***Ruminal microbial N synthesis*** | | | | | | | | |
| Microbial N supply, g/d | 7.01ab | 8.01a | 8.05a | 6.33b | 0.30 | 0.571 | 0.522 | 0.025 |
| DOMR,[2] g/d | 324b | 409a | 400a | 378ab | 17.3 | 0.434 | 0.275 | 0.074 |
| EMNS, kg DOMR[3] | 20.88 | 20.88 | 20.69 | 16.97 | 1.00 | 0.317 | 0.364 | 0.363 |

[*]Corn silage uninoculated or inoculated at ensiling with 1 × $10^5$ cfu/g of fresh forage of *L. plantarum* [MA 18/5U] (Lallemand Animal Nutrition, Goiânia, GO, Brazil) combined with 1 × $10^5$ cfu/g of fresh forage of *B. subtilis* [AT553098] (FATEC Nutrição e Saúde Animal, Arujá, Sao Paulo, Brazil).
[1]S, silage; A: amylase; S×A: interaction between silage and amylase.

[2]DOMR: digestible OM fermented in the rumen
[3]EMNS: efficiency of microbial N synthesis.

There was interaction (*P*=0.025) between silage and amylase supplementation on Microbial N supply (Table 5). The microbial N supply was lower (*P*=0.025) in wethers fed inoculated silage supplemented with amylase. Likewise, the DOMR tended to be lower (*P*=0.074) in wethers fed inoculated silage and supplemented with amylase and wethers fed uninoculated silage with no amylase supplementation. However no differences were observed on EMNS in wethers fed with inoculated silage and supplemented with amylase.

## 4. Discussion

Wethers fed uninoculated silage and supplemented with amylase had a higher DMI, but not differing from animals fed inoculated silage with or without amylase supplementation. Intake of low-fiber diets is controlled by the animal's energy demand (Van Soest, 1994; Mertens, 2010), however, in this study the digestible energy intake did not justify the weight gain of the animals, since the gain was below 300 g/d (formulated diet). Thus, since the diets contained similar proportions of NDF and NFC, the increases on the DMI likely arised from increased digestibility caused by inoculation or amylase supplementation. According to Blaxter and Wilson (1962) and Mertens (2010), the increase in digestibility has been shown increasing the rate of passage and, consequently, positively increases the intake of the animals.

On the other hand, nutrient digestibility increased when amylase was added on the both silage, except for starch. The highest intake and digestibility of non-starch nutrients when amylase was added to the diet is supported by the theory of Tricarico et al. (2008) that the exogenous amylase (i.e., amylases from *A. oryzae*) increases the availability of starch hydrolysis products in the rumen that can be utilized by amylolytic or none amylolytic bacterial species thereby modifying bacterial populations and VFA production, providing greater intake of nutrients for metabolism and animal production. According to Beauchemin et al. (2004), the increase of enzymatic activity in the rumen can increase the ruminal hydrolytic capacity, enhancing the digestibility of all components of the diet, rather than act just on the specifics targets of enzymes.

Agreement with results found in this study, Chen et al. (1995) also reported no effects of enzyme (amylase and protease mixture) application in steam flaked sorghum grain on starch digestibility, but observed increase on the OM, CP and NDF total tract digestibility. Likewise, previous studies did not verified increase on the starch digestibility when amylases were used as supplement in diets for dairy cattle (Hristov et al., 2008; Gencoglu et al., 2010) and finishing beef cattle (Tricarico et al., 2007).

Higher DMI and digestibility of DM, OM, CP and NDF also were found when two enzyme preparations with different amylases activities were added to the TMR for dairy cattle (Klingerman et al., 2009). Similarly, Tricarico et al. (2007) related high DMI in growing heifers when were supplemented with α-amylase from *A. oryzae*, however with enzyme activity around 950 DU/kg of DM consumed. Otherwise, Rojo et al. (2005) verified increase on total tract digestion and decrease on intake of DM and OM in lambs fed high sorghum diets and supplemented with exogenous amylases, respectively.

The lack of effect in starch digestibility when exogenous amylase was added to the diet can be attributed by the three facts cited by Gencoglu et al. (2010): 1) starch digestibility was not affected ruminally (Tricarico et al., 2005) or postruminally, 2) starch digestibility was increased ruminally (Klingerman et al., 2009), but postruminal compensatory starch digestion (Taylor and Allen, 2005) resulted in similar total tract starch digestibilities for the treatments, and 3) starch digestibility was increased ruminally, but hindgut fermentation (Firkins, 1997) resulted in similar total tract starch digestibilities for the treatments.

Increases on the nutrient digestibility was also verified with inoculation of silage, which may be due the some microorganisms in silage inoculants may remain active in the rumen and act synergistically with others ruminal bacterial species (probiotic effect; Weinberg, 2003; 2004a; 2004b; 2007). In addition, is verified enzyme production (Priest, 1977; Donaghy et al., 1998; Nsereko et al., 2008; Kang et al. 2009; Addah et al., 2012) and effect of bacteriocins and antimicrobials substances from some microorganisms as *B. subtilis* (Wilhelm et al., 1998; Wulff et al., 2002; Schisler et al., 2004; Angonese et al., 2009), which can modify the ruminal fermentation pattern.

Our results agree with some *in vitro* studies that reported increases on the NDF (Nsereko et al., 2008; Addah et al., 2012), DM and OM digestibility (Lara et al., 2015). Conversely, Basso et al. (2014) found no effects on the nutrients digestibility when lambs were fed with corn silage inoculated with *L. plantarum* and *L. buchneri*. Rowghani et al. (2008) evaluated the effects of *L. plantarum* and *P. acidipropionici* in corn silage and also found no effect on the digestibility in sheep. These contrasting results could be due to differences among bacterial strains and application rates and differences in the magnitude of the intake responses as well as differences among animals and their inherent passage rates (Mertens and Ely, 1982).

This inconsistency of results may be due to the vast amount of microorganisms involved in the fermentation process of silage and rumen, the complexity of the degradation of plant structural carbohydrates by ruminants and the variety of enzymatic products used, made it difficult to understand the action of these enzymes in the respective environments, becoming a limiter to reach a conclusion or standardization of the use of enzymes for ruminants.

Improvements in DM and DE intake, as well as digestibility with addition of inoculants and amylase, was unable to alter the EMNS. The lower EMNS and microbial N supply was observed in wethers fed inoculated silage and supplemented with amylase. The values of EMNS observed in this present study are within the range of values (14 to 49 g/kg of DOMR) reported by the Agricultural Research Council (1984).

It is know that the quantity of microbial protein produced in the rumen varies with the quantity of available N and energy (SCA, 1990; AFRC, 1993; NRC, 1996), so the availability of larger amounts of sugars, due to their rapid hydrolysis capacity, increases the quickness of energy release, which may not be compatible with the best use of N in the diet. Some studies concluded that the increase on ruminal digestibility of starch may result in a higher contribution of metabolizable protein (amino acids that reach the blood) due to higher microbial growth, increasing animal production (Ferraretto et al., 2013). In this study, despite the improvement in nutrient digestibility, the starch digestibility was not altered, which resulted in the lack of effect in the EMNS. Nozière et al. (2014) reported increase in ruminal digestibility of OM and starch but did not effect on the microbial N flow to the duodenum and none or

small changes on selected fibrolytic and amylolytic bacteria on the microbial community in first-lactation cows.

On the other hand, in this study lower N excretion was observed in the urine of the wethers fed inoculated silage and supplemented with amylase, with a consequent increase in the retained N / absorved N and retained N / intake N ratio, which proves that no accumulation of N in the rumen with subsequent absorption and excretion in the urine occurred. In addition, lower N excreted in feces was also verified, which may indicate that there was efficiency in the capture of N in the rumen, but with no effects on the EMNS and animal production.

When wethers were fed with uninoculated silage and supplemented with amylase, as well as animals fed inoculated silage without amylase supplementation, better values of microbial N supply were found. This may be due the higher DMI and DE intake by wethers, evidencing that the effects of inoculated silage and amylase supplementation are better when used in the isolated form in wethers diets. Salem, et al. (2015) reported improve on the nutrient digestibility, nitrogen balance and N utilization, as well as microbial protein production, when sheep was fed with a mixture of enzyme preparation. Basso et al. (2014) found greater microbial N supply in lambs fed corn silage inoculated with *L. buchneri* and justified the improvement by the possible survival of the bacteria of the inoculant in the rumen or by the increase of DMI.

The treatments did not alter the pH, NH3-N and total VFA content, however, diets containing uninoculated silage and supplemented with amylase provided lower acetic acid and higher propionic acid proportion in all period of evaluating. Consequently, the acetic:propionic ratio was lower in wethers rumen fluid fed at the same treatment. The opposite was observed when the wethers were fed diet containing inoculated silage and supplemented with amylases, where higher acetic acid and lower propionic acid proportions were verified.

The propionic acid is the main end product of starch fermentation and can be used to synthesize glucose and may offer an energetic benefit to the ruminant host (Ørskov, 1977). Nozière et al. (2014) also found similar response, with no effects on pH, $NH_3$-N and total VFA production when amylase was added to the high starch diets, however, was observed high propionic acid and lower acetic acid proportions.

No change in the VFA profiles following amylase supplementation also was verified by DeFrain et al. (2005) and Hristov et al. (2008). These responses are contrary to the theory of Tricarico et al. (2008) in which the α-amylase supplementation from the *A. oryzae* did not increase the ruminal starch digestibility, but alters the ruminal fermentation with high molar proportion of butyric and acetic acid and decrease on the propionic acid.

The differences in the end products of rumen fermentation in the various studies evaluated may be due to the use of different enzymatic products, which contains enzymes from different microorganisms as well as variable enzymatic activity and due to the different ways of enzyme applying to the diet. Each enzyme has an optimal pH and temperature of action and the effect will depend of these factors together with the form of application and the enzyme level used. In the present study, the amylase had an optimum pH around 4.8, which is low when compared with ruminal pH, since a pH close to 5.0 is due to acute acidosis and less than 5.5 of subclinical acidosis (Nagajara and Town, 1990). However, in the present study, the pH found in the rumen ranged of 5.4 to 6.1, which was relatively low due to the high proportion of total VFA that may have favored the performance of the amylases, when enzymes were used without the association with the inoculated silage.

In addition, negative responses or the lack of effect on the use of amylases associated with inoculated silage may have resulted from the fact that the bacteria from the inoculants used produce a range of enzymes that may have competed with the amylases added at the time of feeding, decreasing their hydrolytic effect. In addition, the enzymes were added in powder form only a few minutes before feeding, which would lead to a longer time for enzyme activation, since they are more effective when applied in liquid form (Morgavi et al., 2000; Wallace et al., 2001). This fact means that the amylases may have been consumed by the greater amount of bacteria and proteases found in the rumen of the animals fed with the inoculated silage. To this can be confirmed, we suggest more study about microbiology population in the rumen.

## 5. Conclusions

The supplementation diet with amylase improved the intake and digestibility of nutrients, with no effects on efficiency of microbial N supply and performance of lambs. The association of amylase in diets containing inoculated silage did not provide positive responses on the digestibility and microbial N supply.

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# CHAPTER 3

The paper was written following the guidelines for authors of Meat Science, with exception of tables and figures position.

## Growth performance, carcass characteristics and meat quality of lambs fed diets containing inoculated corn silage and supplemented with amylolytic enzyme

**Abstract:** This study aimed to investigate the effects of diets containing inoculated corn silage with *Lactobacillus plantarum* (LP) combined with *Bacillus subtilis* (BS) and supplemented or not with amylase on growth performance, carcass characteristics, commercial cut yield, quality and fatty acid profile in lamb meat. Forty non-castrated male feedlot Lambs (Texel × Dorper) received one of four treatments (diets): (1) Corn silage uninoculated and without amylase added to TMR; (2) Corn silage uninoculated and amylase added to TMR; (3) Corn silage inoculated with $1\times10^5$ CFU LP [MA 18/5U] and $1\times10^5$ CFU BS [AT553098] without amylase added to TMR; (4) Corn silage inoculated with $1\times10^5$ CFU LP [MA 18/5U] and $1\times10^5$ CFU BS [AT553098] and amylase added to TMR. The highest ADG was observed in lambs fed inoculated silage when compared with lambs fed uninoculated silage (232.5 vs. 211.5 g/d, Table 3). The G:F ratio was similar between treatments ($P$>0.05). There was no effect of diets containing inoculated silage and supplementation with amylase on the chemical composition and carcass characteristics of lambs meat. The inoculation of silage increased ($P$<0.05) the content of saturated fatty acid (SFA) and decreased ($P$<0.05) the unsaturated fatty acid (UFA) (47.555 vs. 46.205 % and 52.445 vs. 53.795 %, respectively) and consequently decreased the UFA:SFA ratio. Inoculation of silage with L. plantarum and B. subtilis improved the average daily gain of lambs when was not associated with amylase supplementation. Diets containing inoculated corn silage and supplemented with amylase did not change the carcass and meat quality traits with few effect on the meat fatty acid profile.

**Keywords: amylase, carcass characteristics, fatty acids, feedlot**

### 1. Introduction

The total demand for animal products in developing countries is expected to more than double by 2030 (FAO, 2015), driven by economic and population growth. At the same time, there is a growth of the interest for quality and health foods with low impact on human health and disease, giving rise to the market of the "natural

foods". This is due to the fat content of the meat and its composition in fatty acids (FA), together with the standards of modern life (stress and sedentary lifestyle), are associated with disorders in human health, such as obesity, hypertension and heart problems (Maia et al., 2012).

Despite meat to be a concentrated nutrient source, previously considered essential to optimal human growth and development (Higgs, 2000), the sheep meat, as well as that of ruminants in general, is rich in saturated and monounsaturated FA with small amounts of polyunsaturated (Sinclair, Slattery & O´Dea, 1982), being saturated fat the most harmful to human health. However, nutritional strategies can be used to modify the content of the different FA in the ruminant musculature, making the meat healthier (Andrae et al., 2001).

The biohydrogenation is the process responsible for transforming the unsaturated FA present on ruminant diets into saturated FA, being fundamentally a bacterial process (Jenkins et al., 2008). In addition, the fatty acid profile in the meat will depend mainly of the quantity and proportion of VFA's absorbed from the rumen. Thus, any manipulation in the diet may alter the action of microorganisms in the rumen and interfere in the fatty acid profile of the meat. However, the use of different nutritional strategies should not negatively alter the physical and sensorial properties of the meat, altering important aspects to the consumer such as color, tenderness and flavor.

Animals in feedlot systems rely on cereal grain starch as the primary source of energy (DiLorenzo et al., 2011) that can comprises approximately 50 and 75% of the energy value of corn Thereby, the adoption of technologies to improve the starch utilization has been studied, among them, the use of microorganisms, with probiotic effect or not (Wiryawan and Brooker, 1995), such as silage inoculants (i.e., *L. plantarum*) that are used to improve the fermentation process and reduce dry matter (DM) losses (McDonald et al., 1991).

Some microorganisms (i.e., *L. plantarum* MTD-1) can survive and act in the rumen as probiotic (Weinberg, Muck & Weimer, 2003; Weinberg et al., 2004) and produce a range of enzymes (Donaghy, Kelly & McKay, 1998; Priest, 1977; Kang et al., 2009) as well as bacteriocins (Lanna Filho, Ferro & Pinho, 2010) (i.e., *B. subtilis*), that can modify the ruminal microflora due to competition or inhibition of

microorganisms within the rumen (Weinberg, Chen & Gamburg, 2004; Weinberg et a., 2007).

However, diets with high content of rapidly fermentable carbohydrates can contribute with decrease of the rumen pH and reduce of Lipolysis (Doreaus & Ferllay, 1994), which is a precondition for the biohydrogenation (Latham, Storry & Sharpe, 1972). However, none study evaluating the effects of the use of inoculated silage associated with the exogenous amylases added in the total mixed ration (TMR) on the carcass characteristics, quality and fatty acid profile of lamb meat was carried out.

Thereby, the objectives of this study were to evaluate diets containing corn silage inoculated with *L. plantarum* and *B. subtilis* and supplemented or not with amylase on growth performance, carcass characteristics, commercial cut yield and meat quality of lambs.

## 2. Material and Methods

The protocol used in the present study was in accordance with the Brazilian College of Animal Experimentation (COBEA – Colégio Brasileiro de Experimentação Animal) guidelines and was approved by the Ethics, Bioethics and Animal Welfare Committee (CEBEA – Comissão de Ética e Bem Estar Animal) of the FCAV-UNESP/Jaboticabal campus, Brazil (Projected number 1.754/15).

### *2.1. Crop harvest and silage preparation*

A flint corn hybrid (2B710, Dow AgroSciences Cravinhos, São Paulo, Brazil) was planted on December 18, 2013, at the São Paulo State University (UNESP) School of Agricultural and Veterinarian Sciences, Jaboticabal, located at 21°14'14.04" S and 48°17'27.92" W and harvested on March 24, 2014 at approximately 344 g/kg of dry matter (DM) at a stubble height of 20 cm using a pull-type JF 90® forage harvester (JF Agricultural Machinery, Itapira, Sao Paulo, Brazil). Forage was chopped to a length of cut settings of 10 mm without kernel processing. Corn forage was treated with water (0.7 L/t; Untreated) or with $1×10^5$ CFU/g of fresh forage of LP MA [18/5U] (Lallemand Animal Nutrition, Goiânia, GO, Brazil) combined with $1×10^5$ cfu/g of fresh forage of *BS* [AT553098] (FATEC Nutrição e Saúde Animal,

Arujá, SP, Brazil). The inoculants were dissolved in water (2 L/t) and then sprayed on fresh forage during the silo filling. The application rate of the inoculant was determined in accordance with previous studies carried out in our lab in tropical conditions (Basso et al., 2012 and 2014; Lara et al., 2015).

Two stack silos were filled in two consecutive days with approximately 20 tons each treatments (inoculated or not) and compacted with a Wheel lounder (Cat®, Peoria, Chicago, USA) at a packing density of 580 ± 66 kg/m$^3$ (fresh matter basis). To avoid possible cross contamination, the uninoculated forage was ensiled first, followed by the inoculated forages. The silos were sealed with black-on-silver polyethylene film and covered with a layer of soil. Twelve fresh samples were taken to characterize the chemical composition of the corn plant at silo filling.

Silos were stored at an ambient temperature (22.1°C ± 2.5) for 170 d. The silage was removed at a rate of approximately 10.0 ± 4.0 cm/d from the silo face using a fork. Ten silage samples were collected weekly during all period of the experiment and it was made a composite sample that was stored at -20°C for subsequent analyses to characterize the corn silages (Table 1).

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**Table 1.** Chemical composition, fermentation and microbial profile of uninoculated and inoculated corn plants prior to ensiling and after silos were opened[1] (n = 10).

| Item[2] | Corn plant | Corn silage | |
|---|---|---|---|
| | | Uninoculated | Inoculated |
| ***Chemical composition, g/kg of DM*** | | | |
| DM | 344 ± 0.32 | 330 ± 3.59 | 332 ± 3.25 |
| OM | 897 ± 1.19 | 951 ± 3.48 | 960 ± 1.73 |
| EE | 16.5 ± 0.45 | 19 ± 0.99 | 18 ± 1.00 |
| CP | 75.0 ± 0.85 | 87 ± 0.54 | 84 ± 0.51 |
| Starch | NM | 238 ± 1.98 | 218 ± 2.05 |
| NDF | 533 ± 4.22 | 480 ± 8.38 | 483 ± 9.19 |
| ADF | 238 ± 2.85 | 258 ± 5.05 | 258 ± 3.53 |
| Hemicellulose | 302 ± 3.22 | 240 ± 4.19 | 244 ± 3.36 |
| Cellulose | 207 ± 1.86 | 218 ± 3.93 | 222 ± 3.92 |
| Lignin | 28.5 ± 0.94 | 24 ± 1.53 | 23 ± 1.19 |
| CHO | 875 ± 2.52 | 842 ± 4.07 | 855 ± 2.37 |
| NFC | 382 ± 5.81 | 378 ± 10.33 | 392 ± 9.29 |
| GE, MJ/kg of DM | NM | 17 ± 0.29 | 18 ± 0.40 |
| ***Fermentation profile, g/kg of DM*** | | | |
| Lactic acid | NA | 15.1 ± 1.69 | 21.8 ± 2.10 |
| Acetic acid | NA | 14.2 ± 0.62 | 12.8 ± 0.84 |
| Propionic acid | NA | 1.1 ± 0.12 | 0.3 ± 0.04 |
| Butyric acid | NA | 0.7 ± 0.13 | 0.6 ± 0.06 |
| L:A | NA | 1.1 ± 0.18 | 2.0 ± 0.15 |
| pH | 4.40 ± 0.12 | 3.86 ± 0.03 | 3.74 ± 0.04 |
| $NH_3$-N[3] | 37.5 ± 5.13 | 44 ± 2.98 | 54 ± 4.32 |
| ***Microbiological profile, CFU/g of fresh silage*** | | | |
| Lactic acid bacteria | NM | 6.00 ± 0.13 | 5.99 ± 0.09 |
| Yeasts | 5.42 ± 0.14 | 6.35 ± 0.48 | 5.78 ± 0.46 |
| Molds | 4.50 ± 0.17 | 5.60 ± 0.20 | 5.15 ± 0.10 |

[1]Corn silage uninoculated or inoculated at ensiling with 1 × $10^5$ cfu/g of fresh forage of *L. plantarum* [MA 18/5U] (Lallemand Animal Nutrition, Goiânia, GO, Brazil) combined with 1 × $10^5$ cfu/g of fresh forage of *B. subtilis* [AT553098] (FATEC Nutrição e Saúde Animal, Arujá, Sao Paulo, Brazil).
[2]DM = dry matter; OM = organic matter; EE = ether extract; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; CHO = total carbohydrates; NSC = non-fiber carbohydrates; GE = gross energy; L:A = lactic acid:acetic acid ratio.
[3]$NH_3$-N = g/kg of total nitrogen.

## 2.2. Treatments and feeding

Four diets were evaluated: (1) Corn silage uninoculated and no amylase added to TMR; (2) Corn silage uninoculated and amylase added to TMR; (3) Corn

silage inoculated with $1\times10^5$ CFU *Lactobacillus plantarum* [MA 18/5U] and $1\times10^5$ CFU *Bacillus subtilis* [AT553098] and no amylase added to TMR; (4) Corn silage inoculated with $1\times10^5$ CFU *Lactobacillus plantarum* [MA 18/5U] and $1\times10^5$ CFU *Bacillus subtilis* [AT553098] and amylase added to TMR. Diets were composed of 400 g/kg of DM corn silage and 600 g/kg of DM of concentrated (Table 2). Wethers and lambs were fed to meet their requirements to gain 300 g/d based on previous studies reported by NRC (2007), in which those animals have a similar growth curve

A powdered concentrate from *Aspergillus oryzae* (Amaize, Alltech Inc.) containing primarily α-amylase activity was used in these studies. The α-amylase activity was determined according to the procedure described in the Food Chemicals Codex (1996). One α-amylase dextrinizing unit (DU) was defined as the quantity of enzyme required to dextrinize soluble starch at the rate of 1 g/h at 30°C and pH 4.8. The preparation used in this study contained 301 DU/g of product and was added to TMR at time of feeding delivery a rate of 2 g/kg of dietary DM of the product. Therefore, final concentrations of α-amylase fed to lambs and wethers were 602 DU/kg of dietary DM.

*2.3. Animal Study*

*2.3.1. Nutrients intake and growth performance*

On September 10, 2014 forty lambs non-castrated males (Texel x Dorper), with average initial body weight of 23.9 ± 4.7 kg (three months) were blocked by weight into 10 groups. Lambs were fed *ad libitum* (approximately 10% orts) twice daily (0700 and 1600 h) and provided with free access to water. Orts were weighed daily before morning feeding to estimate intake. Samples of offered feed and orts were collected twice weekly and stored at - 20°C for later analyses.

Lambs were housed in individual wooden pens (0.5 $m^2$) with slated floored, each fitted with a feed and water container in a well-ventilated covered barn. Lambs were adapted to diets for 15 d with a gradual increase in concentrate levels during this period. During the days 1-3 of the adaptation period lambs were fed only corn silage. Thereafter, concentrate was included by 10% in the diet for each three subsequent days of adaptation until reach the forage:concentrate ratio of 40:60.

**Table 2.** Composition of the experimental diets (g/kg on a dry matter basis)[1].

| Item[2] | Uninoculated | Inoculated |
|---|---|---|
| ***Ingredient proportions*** | | |
| Corn silage | 400.0 | |
| Ground corn | 479.6 | |
| Urea | 12.0 | |
| Soybean meal | 84.0 | |
| Comercial Premix[3] | 24.4 | |
| ***Chemical composition*** | | |
| DM | 669 | 675 |
| OM | 942 | 947 |
| EE | 21 | 20 |
| CP | 151 | 150 |
| Starch | 371 | 363 |
| NDF | 257 | 258 |
| ADF | 126 | 126 |
| ME, MJ/kg of DM | 10.5 | 10.5 |
| ***Fatty acid profile (%)*** | | |
| C12:0 Lauric | 0.29 | 0.33 |
| C14:0 Myristic | 0.32 | 0.37 |
| C15:0 Pentatonic | 0.06 | 0.06 |
| C16:0 Palmitic | 16.90 | 16.00 |
| C17:0 Heptadecanoic | 0.23 | 0.33 |
| C18:0 Stearic | 3.06 | 3.05 |
| C20:0 Arachidic | 0.78 | 0.77 |
| C22:0 Behenic | 0.37 | 0.34 |
| C23:0 Tricosanoic | 0.10 | 0.07 |
| C24:0 Lignoceric | 0.52 | 0.49 |
| C16:1 Palmitoleic | 0.20 | 0.20 |
| C17:1 *cis-10*-Heptadecenoic | 0.11 | 0.14 |
| C18:1 *n-9* Oleic | 28.90 | 28.71 |
| C18:1 *n-7* Vaccenic | 1.06 | 1.45 |
| C20:1 *n-9* 5-Eicosanoic | 0.37 | 0.36 |
| C18:2 *n-6* Linoleic | 41.60 | 40.84 |
| C18:3 *n-3* Linolenic | 0.20 | 0.16 |
| C18:3 *n-6* γ-Linolenic | 4.93 | 6.33 |
| SFA[4] | 22.63 | 21.81 |
| UFA[5] | 77.37 | 78.19 |
| MUFA[6] | 30.64 | 30.86 |
| PUFA[7] | 46.73 | 47.33 |

[1]Corn silage uninoculated or inoculated at ensiling with 1 × $10^5$ cfu/g of fresh forage of *L. plantarum* [MA 18/5U] (Lallemand Animal Nutrition, Goiânia, GO, Brazil) combined with 1 × $10^5$ cfu/g of fresh forage of *B. subtilis* [AT553098] (FATEC Nutrição e Saúde Animal, Arujá, Sao Paulo, Brazil).
[2]DM = dry matter; OM = organic matter; EE = ether extract; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; CHO = total carbohydrates; NSC = nonstructural carbohydrates; GE = gross energy.

[3]Composition: NaCl = 4.2 g/kg of DM; Phosphate = 4.2 g/kg of DM; Limestone = 15.4 g/kg of DM; Ca = 120%; P = 40%; Na = 282%; Cl = 448%; K = 857%; Mg = 17%; S = 22%; Fe = 123mg; Co = 10 mg; Se = 0.43mg, Vit.A = 5,671UI; Vit.D3 = 501UI; Vit.E = 40mg.
[4]Saturated fatty acids: C12:0, C14:0, C15:0, C16:0, C18:0, C20:0, C22:0, C23:0, C24:0.
[5]Unsaturated fatty acids: C16:1, C17:1 *cis-10*, C17:1, C18:1 *n-9*, C18:1 *n-7*, C18:2 *n-6*, C18:3 *n-3*, C18:3 *n-6*, C20:1 *n-9*.
[6]Monounsaturated fatty acids: C16:1, C17:1 *cis-10*, C17:1, C18:1 *n-9*, C18:1 *n-7*, C20:1 *n-9*.
[7]Polyunsaturated fatty acids: C18:2 *n-6*, C18:3 *n-3*, C18:3 *n-6*.

The initial and final body weights (BWs) were measured after 16-h fast and average daily gain (ADG) was calculated by subtracting initial BW from the final BW and dividing the difference by the trial duration of time that the lambs taken to be slaughtered with 35.0 ± 1.8 kg of body weight (slaughter criteria).

*2.3.2. Slaughter and carcass traits data collection and sampling procedures*

Lambs were slaughtered by cerebral concussion (TEC 10 PC) followed by jugular and carotid venesection with subsequent evisceration and removal of the head and ends of the limbs, according to procedures characterizing humane slaughter. After the slaughter, the initial pH (pHi) was measured on the left carcass of each animal in triplicate on the *Longissimus* between the 12$^{th}$ and 13$^{th}$ ribs using a pH-meter (Testo® 205) equipped with a penetrating electrode.

The carcasses were weighed to obtain the hot carcass weight (HCW) and transferred to a cold chamber at 4°C for 24 hours where they were hung by the gastrocnemius tendons on hangers fitted to keep them 17 cm apart. The cold carcasses were weighed to obtain the cold carcass weight (CCW). Carcass yield was calculated using HCW and CCW divided by body weight (BW) and then multiplying the result by 100. In the left half of each carcass commercial cut yield was measured by separating the carcass half into neck, shoulder, rib, loin and leg.

After 24 h post-mortem chill, final pH ($pH_{24h}$) was measured in triplicate on the *Longissimus lumborum* between the 12$^{th}$ and 13$^{th}$ ribs. For the color measurements, the muscle was air exposed for 30 minutes between the 12$^{th}$ and 13$^{th}$ ribs (loin) for proper myoglobin oxygenation as described by Cañeque and Sañudo (2000). Instrumental meat color expressed as L* (lightness), a* (redness), and b* (yellowness) according to CIELAB color system (Commission International de L'Eclairage, 1976) using a colorimeter, model Minolta CR-400, illuminant D65 (Minolta Camera Co., Osaka, Japan). The colorimeter was calibrated before

analyzing the samples against white and black standards. Triplicate readings were made for each sample and average values were recorded.

At 24 h post-mortem at 4°C, *Longissimus* muscles were removed from the two half-carcass, weighed, vacuum packaged, stored at -20°C for subsequent analysis. The left loins were used in the physical-chemical analyses and the right loins in the sensory analyses. *Longissimus* muscle area was traced on transparencies and measured later with a planimeter and RFT measurements were taken 3/4 the length ventrally over the longissimus muscle by using a digital paquimeter (Greiner, Rouse, Wilson, Cundiff, & Wheeler, 2003).

For the qualitative analysis, the muscle was thawed at 10 °C in a BOD incubator for 12 h, with the exception of the samples for determination of thawing loss (30 days after being frozen – TL30D), which remained in the BOD incubator at 5 °C for 16 h and were weighed before and after this period, according to the methodology described by Koohmaraie, Shackelford, and Wheeler, Koohmaraie, and Schackelford (1998).

*2.3.3. Water holding capacity*

To determine the water-holding capacity (WHC) of the meat, 2 g of the meat was placed on a filter paper between two acrylic plates, and a 10-kg weight was placed over those plates for 5 minutes. Then, the samples were weighed and the WHC was calculated by the following equation: WHC = final weight/initial weight x100, in accordance with Hamm (1986). The results were expressed in percentage of water retained in relation to the initial weight of the sample.

*2.3.4. Cooking weight loss*

Cooking weight loss (CWL) were calculated by the weight of thawed samples before and after cooking in a pre-heated industrial oven at 175°C until the internal temperature of the sample reached 71°C, which was then removed from the oven and cooled at room temperature. CWL was calculated by the equation: 100 – (weight of the baked sample × 100/raw sample weight), in percentage.

*2.3.5. Warner-Bratzler Shear force*

Warner-Bratzler shear force was obtained from the same samples used for the CWL using a texture meter (Texture Analyser TAXT2i; Stable Micro Systems Ltd.,

Godalming, UK), which measures the necessary pressure to cut the portion of the muscle. Six cylindrical samples per steak, with 1.27 cm diameter (Wheeler et al., 1995) free of visible fat and connective tissue, were obtained from the center of the cooked samples, with the direction of the muscle fibers parallel to the length. The cylinders were completely sheared perpendicularly to the muscle fibers with a Warner Bratzler blade of 1.016 mm at a speed of 300 mm/min. The results are expressed in kgf/cm2.

*2.3.6. Meat chemical composition*

Samples used for chemical analysis were lyophilizated for 72 h. All samples were then ground using a ball mill and analyzed for moisture, protein (AOAC, 1990; method number 920.87), ether extract (AOAC, 1990; method number 920.85;), and ash (AOAC, 1990; method number 924.05;).

*2.3.7. Fatty acid profile*

Samples of the transversal section were collected from the *longissimus*, freeze dried, and frozen for later lipid extraction and determination of free fatty acids (FFAs). Lipids were extracted using a mixture of chloroform–methanol (Bligh & Dyer, 1959), and the fatty acid methyl esters (FAME) were obtained by the ISO 5509 method (International Organization for Standardization, 1978) using n-hexane, methanol, and KOH. A mixed standard (C4–C24, Sigma-Aldrich, São Paulo, Brazil) was used to quantify individual fatty acids. Qualitative and quantitative measurements of fatty acid content were performed by chromatography using a Shimadzu gas chromatograph (model GC-14B with a Communication Bus Module CBM 102, Kyoto, Japan) equipped with a flame ionization detector (FID) and fused silica capillary column (Omegawax 250, Sigma-Aldrich, São Paulo, Brazil). The column was 30 m in length with a diameter of 0.25 mm and a film thickness of 0.25 μm (Supelco SP-24136, Sigma-Aldrich, São Paulo, Brazil). Helium was used as the carrier gas at a flow of 1 mL $min^{-1}$. Fatty acid analysis was performed by auto injection of 1 μL of each sample at a “split” at a division ratio of 1/100 and temperature of 250 °C. The temperature of the oven was programmed to remain at 100 °C for 2 min and then increase to 220 °C at 4 °C min−1 for 25 min, while the detector was maintained at 280 °C. The identification and quantification of fatty acid methyl esters were achieved by a

comparison with the retention times and concentrations of fatty acid methyl esters in the standard.

*2.4. Chemical Analyses*

A water extract was made from fresh silage samples as described by Kung et al. (1984) and silage pH was measured using a digital potentiometer (MA522 model, Marconi Laboratory Equipment, Piracicaba, São Paulo, Brazil). The volatile fatty acids (VFAs), in fresh forage were measured in a gas chromatograph (GC2014, Shimadzu Corporation, Kyoto, Japan) equipped with a HP-INNOWax capillary column (30 m × 0.32 mm; Agilent Technologies, Colorado, USA). At an initial temperature of 80°C for 3 min followed by a heating rate of 20°C $min^{-1}$ until a final temperature of 240°C was achieved. Lactic acid was determined using a colorimetric method (Pryce, 1969). The free fatty acids (FFAs) in silages were measured using the same procedure as described for meat samples. Ammonia N was measured by distillation (AOAC, 1996; method number 941.04).

Samples of silage and concentrate, were oven dried (55°C for 72 h) and processed in a knife mill before being ground through a 1-mm screen and analyzed for DM (105°C for 12 h) (AOAC 1996; method number 930.15) and ash (500°C for 5 h) (AOAC 1996; method number 923.03). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined in an Ankom 2000 Fiber Analyzer (Ankom Technologies, Macedon, NY, USA) without sodium sulfite following the procedures described by Mertens (2002) and AOAC (1996, method number 973.18), respectively. The NDF was determined using a heat stable amylase and corrected for ash and protein and ADF content was corrected for ash. Ether extract was measured according to the AOAC (1996; method number 920.39). The N concentration was determined by rapid combustion using a Leco F528 N analyzer (LECO Corporation, St. Joseph, MI, USA). The crude protein (CP) was calculated as total N × 6.25. Total carbohydrate (CHO) was calculated as described by Sniffen et al. (1992). Starch was determined enzymatically according to Bach Knudsen, Eggum, and Jacobsen (1987). Gross energy (GE) was determined with a bomb calorimeter (PARR 6200; Parr Instrument Company, Milone, IL). The metabolized energy (ME) was estimated as 0.82 from apparent digestibility energy (DE; NRC, 1985).

### *2.5. Statistical analysis*

Data were analyzed as a randomized block design and the treatments were arrangement in a 2 × 2 factorial (with or without inoculated with *L. plantarum* and *B. subtilis*, supplemented diet or not with amylase). All data were analyzed using the PROC MIXED procedure of SAS (version 9.0; SAS Inst. Inc., Cary, NC). The model included the fixed effect of treatment and random effects of block design and error.

Homogeneity of the data was verified using the UNIVARIATE procedure of SAS. Studentized residuals were plotted against the predicted values using the plot procedure to analyze data for outliers. Differences among means were determined using the PDIFF, which differentiates means based on Fisher's F-protected least significant difference test. Significance was declared at $P \leq 0.05$ and a trend at $P \leq 0.10$.

## 3. Results

### *3.1. Nutrients intake and growth performance*

There was no interaction between silage and amylase supplementation at moment of feeding on nutrients intake, growth performance and carcass traits of lambs (Table 3). Lambs fed inoculated silage had higher ($P$=0.019) NDF intake than lambs fed uninoculated silage (266.5 vs. 245 g/d, Table 3). Similarly, the highest ADG was observed in lambs fed inoculated silage when compared with lambs fed uninoculated silage (232.5 vs. 211.5 g/d, Table 3). The G:F ratio was similar between treatments ($P$>0.05).

**Table 3.** Nutrients intake, growth performance and carcass traits of lambs fed diets containing corn silage (Uninoculated or Inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| Item[2] | Uninoculated | | Inoculated | | SEM | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| | No Amylase | Amylase | No Amylase | Amylase | | S | A | S × A |
| ***Intake of nutrients, g/d*** | | | | | | | | |
| DM | 1,136 | 1,124 | 1,175 | 1,176 | 25.76 | 0.244 | 0.898 | 0.866 |
| OM | 1,063 | 1,055 | 1,106 | 1,083 | 21.01 | 0.270 | 0.619 | 0.826 |
| CP | 177 | 174 | 178 | 179 | 3.35 | 0.521 | 0.882 | 0.743 |
| EE | 25 | 23 | 23 | 23 | 0.45 | 0.364 | 0.734 | 0.249 |
| NDF | 250 | 240 | 272 | 261 | 7.02 | 0.019 | 0.254 | 0.928 |
| DE, MJ/kg of DM[3] | 13.2 | 13.8 | 14.4 | 15.4 | 0.33 | 0.003 | 0.067 | 0.631 |
| ME, MJ/kg of DM | 10.8 | 11.3 | 11.8 | 12.6 | 0.27 | 0.003 | 0.067 | 0.631 |
| ***Growth performance and carcass traits[4]*** | | | | | | | | |
| Final BW, kg | 35.6 | 36.1 | 35.7 | 35.4 | 0.18 | 0.478 | 0.787 | 0.204 |
| ADG, g/d | 213 | 210 | 244 | 221 | 0.01 | 0.023 | 0.153 | 0.264 |
| G:F | 187 | 196 | 205 | 193 | 0.01 | 0.362 | 0.817 | 0.207 |

[1]S, silage; A: amylase; S×A: interaction between silage and amylase.
[2]DM = Dry matter; OM = Organic matter; CP = Crude protein; EE = Ether extract; NDF = Neutral detergent fiber; GE = Gross energy.
[3]Based on the digestibility of energy from the metabolic study.
[4]BW = body weight; ADG = Average daily gain; G:F = Gain:Feed ratio (feed efficiency); HCW = Hot carcass weight; HCY = Hot carcass yield.

### *3.2. Carcass characteristics of lambs*

There was no effect and no interaction (*P*>0.10) between silages and supplementation with amylase in the carcass traits (Table 4).

The HCW was not affected by inoculation of silage and supplementation with amylase. However, lambs supplemented with amylase at moments of feeding, tended to show higher (*P*=0.059) HCY when compared with lambs without supplementation (48.4 vs. 47.5 Table 4).

**Table 4.** Carcass traits from lambs fed diets containing corn silage (Uninoculated or Inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| | Uninoculated | | Inoculated | | SEM | *P*-value[2] | | |
|---|---|---|---|---|---|---|---|---|
| ***Item[1]*** | No Amylase | Amylase | No Amylase | Amylase | | S | A | S × A |
| HCW, kg | 17.0 | 17.2 | 17.1 | 16.9 | 0.11 | 0.631 | 0.848 | 0.447 |
| CCW, kg | 16.6 | 16.7 | 16.7 | 16.3 | 0.108 | 0.500 | 0.647 | 0.295 |
| HCY, % | 48.9 | 47.3 | 47.9 | 47.7 | 0.24 | 0.450 | 0.059 | 0.129 |
| CCY, % | 47.0 | 45.8 | 46.7 | 46.3 | 0.290 | 0.870 | 0.166 | 0.469 |
| RFT, mm | 4.20 | 3.70 | 3.60 | 3.60 | 0.200 | 0.355 | 0.501 | 0.469 |
| LMA, $cm^2$ | 14.8 | 14.5 | 14.2 | 13.3 | 0.327 | 0.184 | 0.391 | 0.672 |

[1]HCW = hot carcass weight; CCW = Cold carcass weight; HCY = hot carcass yield; CCY = Cold carcass yield; RFT = Rib fat thickness; LMA = *Longissimus* muscle area.
[2]S, silage; A: amylase; S×A: interaction between silage and amylase.

### *3.3. Chemical composition of longissimus muscle*

There was no effect of inoculation of silage and diet supplementation with amylase on the chemical composition of lambs meat ($P$>0.10; Table 5).

**Table 5.** Chemical composition (%) of *longissimus muscle* from lambs fed diets containing corn silage (Uninoculated or Inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| | Uninoculated | | Inoculated | | SEM | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| ***Item*** | No Amylase | Amylase | No Amylase | Amylase | | S | A | S × A |
| Moisture | 74.5 | 74.0 | 73.1 | 74.9 | 0.446 | 0.771 | 0.480 | 0.190 |
| Ash | 0.86 | 0.86 | 0.90 | 0.82 | 0.020 | 0.952 | 0.268 | 0.232 |
| Ether extract | 2.22 | 2.07 | 2.47 | 2.26 | 0.110 | 0.304 | 0.403 | 0.890 |
| Crude protein | 22.4 | 23.1 | 23.5 | 22.0 | 0.363 | 0.958 | 0.562 | 0.129 |

[1]S, silage; A: amylase; S×A: interaction between silage and amylase.

### *3.4. Commercial cut weight and yield of lambs*

There was interaction (P<0.05) between inoculated silage and enzyme supplementation was observed for leg weight (Table 6). Lambs fed inoculated silage and supplemented with amylase had lower leg weight than lambs fed inoculated silage without enzyme supplementation and uninoculated silage with supplemented amylase, but did not differ from lambs fed uninoculated silage without supplementation. The inoculation of the silage and amylase supplementation at the moment before lambs feeding did not alter ($P$>0.05) the others commercial cut and yield of lambs carcass.

**Table 6.** Commercial cut weight and yield of lambs fed diets containing corn silage (Uninoculated or Inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| | Uninoculated | | Inoculated | | SEM | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| | No Amylase | Amylase | No Amylase | Amylase | | S | A | S × A |
| ***Commercial cut, kg*** | | | | | | | | |
| Neck | 0.64 | 0.67 | 0.59 | 0.63 | 0.017 | 0.176 | 0.385 | 0.881 |
| Shoulder | 1.83 | 1.83 | 1.82 | 1.74 | 0.02 | 0.289 | 0.340 | 0.340 |
| Rib | 2.04 | 1.92 | 1.98 | 2.01 | 0.040 | 0.820 | 0.569 | 0.379 |
| Loin | 1.06 | 1.09 | 1.04 | 1.01 | 0.018 | 0.157 | 0.945 | 0.340 |
| Leg | 2.81ab | 2.85a | 2.85a | 2.68b | 0.026 | 0.169 | 0.176 | 0.037 |
| ***Commercial cut yield, %*** | | | | | | | | |
| Neck | 7.40 | 7.77 | 7.59 | 7.64 | 0.186 | 0.656 | 0.287 | 0.917 |
| Shoulder | 21.9 | 21.8 | 22.3 | 21.6 | 0.209 | 0.793 | 0.391 | 0.447 |
| Rib | 24.4 | 23.5 | 24.2 | 24.9 | 0.317 | 0.366 | 0.888 | 0.268 |
| Loin | 12.7 | 13.0 | 12.5 | 12.5 | 0.147 | 0.260 | 0.680 | 0.584 |
| Leg | 33.6 | 33.9 | 33.7 | 33.3 | 0.255 | 0.616 | 0.941 | 0.494 |

[1]S, silage; A: enzyme; S×A: interaction between silage and amylase.

### *3.5. Meat traits and fatty acids profile*

There was no interaction ($P$>0.05) between silage and amylase supplementation for meat traits and meat color values and was not observed effect of silage or supplementation on the same parameters (Table 7). A tendency to higher initial pH ($P$=0.094) was verified in lambs fed inoculated silage when compared with lambs fed uninoculated silage (6.55 vs. 6.50).

**Table 7.** Meat quality from lambs fed diets containing corn silage (Uninoculated or Inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| | Uninoculated | | Inoculated | | SEM | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|---|
| | No Amylase | Amylase | No Amylase | Amylase | | S | A | S x A |
| ***Meat traits***[1] | | | | | | | | |
| pH | | | | | | | | |
| Initial | 6.5 | 6.5 | 6.6 | 6.5 | 0.030 | 0.094 | 0.517 | 0.644 |
| Final | 5.6 | 5.5 | 5.7 | 5.7 | 0.038 | 0.128 | 0.477 | 0.861 |
| TL, % | 4.2 | 4.8 | 5.0 | 4.6 | 0.295 | 0.603 | 0.888 | 0.470 |
| CWL, % | 27.0 | 28.6 | 29.4 | 27.7 | 0.550 | 0.490 | 0.980 | 0.138 |
| WHC, %[3] | 41.6 | 42.5 | 42.2 | 44.6 | 0.551 | 0.207 | 0.129 | 0.443 |
| WBSF, kgf[4] | 3.0 | 3.0 | 3.1 | 2.9 | 0.143 | 0.774 | 0.640 | 0.626 |
| ***Meat color***[2] | | | | | | | | |
| L* | 38.8 | 37.9 | 37.6 | 37.4 | 0.281 | 0.144 | 0.338 | 0.500 |
| a* | 16.7 | 17.2 | 16.0 | 16.8 | 0.223 | 0.231 | 0.113 | 0.666 |
| b* | 1.3 | 1.2 | 1.0 | 0.8 | 0.141 | 0.126 | 0.557 | 0.900 |

[1]TL = Tawing loss, CWL = Cooking weight loss, WHC = Water holding capacity, WBSF = Warner-Bratzler shear force
[2]L* = luminosity (0 = black and 100 = white); a* = index from green (−) to red (+); b* = index from blue (−) to yellow (+).
[3]S, silage; A: amylase; S×A: interaction between silage and amylase.

There was a trend (*P*=0.09) for interaction between silage and amylase supplementation for oleic content (Table 8). The meat from lambs fed uninoculated silage without amylase supplementation showed higher oleic content than meat from lambs fed inoculated silage without supplementation (41.96 vs. 40.19 %), but did not differ from meat of the lambs fed uninoculated and inoculated silage supplemented with amylase (41.42 and 41.28 %). The inoculation silage altered the content of the pentatonic fatty acid (*P*=0.023), where lambs fed inoculated silage had higher content of this fatty acid than animals fed uninoculated silage (0.266 vs. 0.240 %). Supplementation with amylase at moment before feeding trended to decrease some polyunsaturated fatty acids content as eicosadienoic (*P*=0.053), arachidonic (*P*<0.05) and docosatetraenoic (*P*<0.05).

**Table 8.** Fatty acids profile (%) of intramuscular fat in the *longissimus* muscle of lambs fed diets containing corn silage (Uninoculated or inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| | Uninoculated | | Inoculated | | SEM | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| ***Item*** | No Amylase | Amylase | No Amylase | Amylase | | S | A | S × A |
| ***Saturated*** | | | | | | | | |
| C10:0 Capric | 0.12 | 0.13 | 0.14 | 0.12 | 0.006 | 0.988 | 0.550 | 0.242 |
| C12:0 Lauric | 0.08 | 0.07 | 0.07 | 0.08 | 0.004 | 0.577 | 0.628 | 0.275 |
| C14:0 Myristic | 2.22 | 2.25 | 2.35 | 2.24 | 0.061 | 0.633 | 0.721 | 0.579 |
| C15:0 Pentatonic | 0.24 | 0.24 | 0.27 | 0.26 | 0.007 | 0.023 | 0.570 | 0.615 |
| C16:0 Palmitic | 25.2 | 26.1 | 26.2 | 26.4 | 0.190 | 0.104 | 0.152 | 0.361 |
| C17:0 Heptadecanoic | 0.89 | 0.93 | 0.89 | 0.92 | 0.019 | 0.863 | 0.316 | 0.935 |
| C18:0 Stearic | 16.5 | 17.2 | 17.2 | 16.7 | 0.310 | 0.867 | 0.829 | 0.366 |
| C20:0 Arachidic | 0.08 | 0.09 | 0.09 | 0.09 | 0.002 | 0.170 | 0.179 | 0.284 |
| ***Monounsaturated*** | | | | | | | | |
| C14:1 Myristoleic | 0.09 | 0.09 | 0.09 | 0.08 | 0.004 | 0.676 | 0.591 | 0.858 |
| C16:1 Palmitoleic | 1.45 | 1.52 | 1.60 | 1.40 | 0.042 | 0.879 | 0.454 | 0.131 |
| C17:1 *c-10* Heptadecenoic | 0.51 | 0.50 | 0.50 | 0.50 | 0.012 | 0.725 | 0.917 | 0.881 |
| C18:1 *n-9* Oleic | 42.0a | 41.4ab | 40.2b | 41.3ab | 0.246 | 0.051 | 0.559 | 0.090 |
| C18:1 *n-7* Vaccenic | 2.76 | 2.61 | 2.60 | 2.53 | 0.060 | 0.274 | 0.325 | 0.733 |
| C20:1 *n-9* 5-Eicosanoic | 0.09 | 0.08 | 0.08 | 0.08 | 0.002 | 0.601 | 0.414 | 0.414 |
| C24:1 *n-9* Nervonic | 0.20 | 0.16 | 0.17 | 0.15 | 0.008 | 0.186 | 0.090 | 0.465 |
| ***Polyunsaturated*** | | | | | | | | |
| C18:2 *n-6* Linoleic | 4.60 | 4.20 | 4.71 | 4.31 | 0.136 | 0.701 | 0.156 | 0.986 |
| C18:2 *c-9 t-11* CLA | 0.47 | 0.47 | 0.47 | 0.53 | 0.019 | 0.395 | 0.454 | 0.454 |
| C18:3 n-6 ϒ-Linolenic | 0.07 | 0.06 | 0.07 | 0.06 | 0.003 | 0.848 | 0.294 | 0.612 |
| C18:3 *n-3* Linolenic | 0.20 | 0.21 | 0.21 | 0.20 | 0.005 | 0.729 | 0.729 | 0.539 |
| C20:2 Eicosadienoic | 0.04 | 0.03 | 0.04 | 0.04 | 0.001 | 0.174 | 0.053 | 0.616 |
| C20:3 *n-6* Eicosatrienoic | 0.15 | 0.12 | 0.13 | 0.12 | 0.006 | 0.348 | 0.100 | 0.297 |
| C20:4 *n-6* Arachidonic | 1.67 | 1.21 | 1.35 | 1.15 | 0.075 | 0.205 | 0.032 | 0.382 |
| C20:5 *n-5* Eicosapentaenoic | 0.05 | 0.04 | 0.05 | 0.06 | 0.005 | 0.383 | 0.903 | 0.354 |
| C22:4 *n-6* Docosatetraenoic | 0.15 | 0.12 | 0.14 | 0.11 | 0.007 | 0.289 | 0.029 | 0.690 |
| C22:6 n-3 Docosahexaenoic | 0.03 | 0.02 | 0.03 | 0.02 | 0.002 | 0.537 | 0.133 | 0.682 |

[1]S, silage; A: amylase; S×A: interaction between silage and amylase.

An interaction (*P*<0.05) between silage and enzyme supplementation was observed for elongase activity (Table 9). Meat from lambs that were fed inoculated silage supplemented without amylase had elongase activity that were 1.38, 1.27 and 1.96 U lower than meat from lambs fed inoculated silage with amylase supplementation and uninoculated silage with or without amylase supplementation, respectively.

**Table 9.** Concentrations (%), ratios and indices of intramuscular fatty acids in the *longissimus* muscle of lambs fed diets containing corn silage (Uninoculated or Inoculated) and supplemented with amylase at moment of feeding (Amylase or No Amylase).

| | Uninoculated | | Inoculated | | SEM | *P*-value[2] | | |
|---|---|---|---|---|---|---|---|---|
| ***Item***[1] | No Amylase | Amylase | No Amylase | Amylase | | S | A | S × A |
| SFA | 45.37 | 47.04 | 47.56 | 47.55 | 0.325 | 0.035 | 0.182 | 0.177 |
| UFA | 54.63 | 52.96 | 52.44 | 52.45 | 0.325 | 0.035 | 0.182 | 0.177 |
| MUFA | 47.11 | 46.46 | 45.25 | 46.34 | 0.286 | 0.084 | 0.694 | 0.123 |
| PUFA | 7.52 | 6.5 | 7.19 | 6.74 | 0.234 | 0.926 | 0.132 | 0.551 |
| UFA:SFA | 1.21 | 1.13 | 1.11 | 1.11 | 0.015 | 0.031 | 0.148 | 0.162 |
| PUFA:SFA | 0.17 | 0.14 | 0.15 | 0.14 | 0.005 | 0.612 | 0.092 | 0.424 |
| Omega-3 | 0.30 | 0.27 | 0.30 | 0.34 | 0.013 | 0.224 | 0.788 | 0.187 |
| Omega-6 | 6.64 | 5.70 | 6.39 | 5.92 | 0.219 | 0.963 | 0.122 | 0.595 |
| Omega-6/Omega-3 | 21.15 | 21.04 | 22.14 | 20.4 | 0.622 | 0.868 | 0.376 | 0.436 |
| $\Delta^9$ Desaturase 16 | 5.43 | 5.48 | 5.74 | 5.12 | 0.132 | 0.932 | 0.290 | 0.215 |
| $\Delta^9$ Desaturase 18 | 71.79 | 70.67 | 70.1 | 71.22 | 0.431 | 0.520 | 0.999 | 0.212 |
| Elongase | 68.59a | 67.90a | 66.63b | 68.01a | 0.232 | 0.019 | 0.357 | 0.009 |
| Atherogenicity | 0.34 | 0.35 | 0.37 | 0.35 | 0.005 | 0.185 | 0.538 | 0.115 |
| Thrombogenic | 6.22 | 5.68 | 6.17 | 6.20 | 0.136 | 0.897 | 0.064 | 0.947 |

[1]SFA = Saturated fatty acids (Sum of C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0), UFA = Unsaturated fatty acids (Sum of C14:1, C16:1, C17:1 *cis-10*, C18:1 *n-9*, C18:1 *n-7*, C18:2 *n-6*, C18:2 *cis-9 trans-11*, C18:3 *n-6*, C18:3 *n-3*, C20:1 *n-9*, C20:2, C20:3 *n-6*, C20:4 *n-6*, C20:5 *n-5*, C22:4 *n-6*, C24:1 *n-9*, C22:6 *n-3*). MUFA = Monounsaturated fatty acids (Sum of C14:1, C16:1, C17:1 *cis-10*, C18:1 *n-9*, C18:1 *n-7*, C20:1 *n-9*, C24:1 *n-9*). PUFA = Polyunsaturated fatty acids (Sum of C18:2 *n-6*, C18:2 *cis-9 trans-11*, C18:3 *n-6*, C18:3 *n-3*, C20:2, C20:3 *n-6*, C20:4 *n-6*, C20:5 *n-3*, C22:4 *n-6*, C22:6 *n-3*). $\Delta^9$ desaturase 16 = 100[(C16:1cis-9)/(C16:1cis-9+C16:0)]. $\Delta^9$ desaturase 18 = 100[(C18:1cis-9)/(C18:1cis-9+C18:0)]. Elongase = 100[(C18:0+C18:1cis9)/(C16:0+C16:1cis9+C18:0+C18:1cis9)]. Atherogenicity = [(C12:0+(4 × C14:0)+C16:0)]/UFA. Thrombogenicity = [(C14:0+C16:0+C18:0)/[(0.5×ΣMUFA)+(0.5 × Σω6+(3 × Σω3)+(Σω3/Σω6)].
[2]S, silage; A: amylase; S×A: interaction between silage and amylase.

The inoculation of silage altered (*P*<0.05) SFA, UFA, and UFA:SFA ration of meat lambs and trended to decrease (*P*=0.084) the MUFA content when compared of meat lambs fed uninoculated silage (45.795 vs 46.785 %). Lambs fed inoculated silage showed higher SFA and lower UFA content than lambs fed uninoculated silage

(47.555 vs. 46.205 % and 52.445 vs. 53.795 %). Consequently, labs fed inoculated silage had lower value of UFA:SFA ratio.

The supplementation with amylase at moment of feeding trended ($P$<0.10) to decrease the values of PUFA:SFA ratio (0.14 vs. 0.16) as well as the thrombogenic index (5.940 vs. 6.195) of the meat when compared with lambs no supplemented with amylase, but no effect ($P$>0.05) was observed for atherogenicity index.

**4. Discussion**

Lambs fed uninoculated or inoculated silage, both with amylase supplementation, consumed lower NDF likely by sorting. Indeed, the consumed diet of lambs supplemented with amylase slightly had lower NDF content. Yet, orts of those animals were found to have a higher content of NDF suggesting the lambs sorted by consuming a higher amount of digestible particles in TMR. The NDF content of refusals was 261 and 250 g/d for diets with and without amylase supplementation. Otherwise, DMI was unaffected by amylase supplementation.

On the other hand, silage inoculation provided better NDF intake from lambs, without effects on the DMI, which may have changed the DOMR, since inoculation also increased DE and ME intake. Although the digestibility was not measured in this experiment, we can make a relation with the digestibility analyzed on Chapter 2, where the NDF digestibility was higher in the animals fed with inoculated silage, which provided improvements in the weight gain of the animals that received inoculated silage.

Our results agree with some *in vitro* studies that reported increases on the NDF (Nsereko et al., 2008; Addah et al., 2012), DM and OM digestibility (Lara et al., 2015), as well as improvements on daily weight gain in lambs (Nkosi et al., 2009; Basso et al., 2014) and steers (Rabelo et al., 2016) fed corn silage inoculated with $BAL^{fh}$ or $BAL^{hf}$.

The DM and nutrient intake is the main determinant of animal productivity and the intake allied to the digestibility, determine the amount of nutrients available for absorption and, consequently, for the animal production (NRC, 2001). Aragon et al. (2012) reported increase of 6.14% in DM intake and 100 g/d in average gain of young beef cattle fed corn silage inoculated with microbial additive. On the other

hand, Nkosi et al. (2009; 2010) found higher DM intake in lambs fed inoculated silage than animals fed untreated silage and reported an increase on daily weight gain of 22.7% in lambs fed with corn silage inoculated with *Pediococcus pentosaceus*, *L. plantarum* and *L. buchneri* and *L. buchneri* alone compared to animals fed uninoculated silage respectively.

On the other hand, the amylase supplementation did not affect the ADG. Similar results were found when amylase from *B. licheniformis*, were added to lambs diets, with increases on the DMI and digestibility of DM and starch and no changes in the ADG (Crosby et al., 2006). Rojo et al. (2005) evaluating the sheep responses by amylase addition also no found differences on ADG, however related increase on the ruminal starch digestibility. Thereby, results found in the literature mostly are inconsistent, and even when the enzymes increase ruminal or total starch digestibility, is not always result improvement in animal performance (Rojo et al., 2005; Lee-Rangel et al., 2010; DiLorenzo et al., 2011).

The inoculation of silage and the amylase supplementation had no effects on the carcass traits of lambs. The amylase added on the diets at moment of feeding did not alter the weight of carcass and consequently did not improve the carcass yield, since the last one is directly affected by carcass weight (Owens & Gardner, 2000). These results were expected by the fact that the supplementation of the diet with amylase had no effect on the average daily gain (ADG) and final body weight (BW) of lambs. However, lambs had consistent carcass yield for the animal category used in this study, which is specialized for meat production, around 40 – 50 % (Silva Sobrinho, 2001).

None of the treatments affected the *Longissimus* muscle area (LMA), what can be explained by the lack of effect on the final BW, since is the main factor that affects the LMA. The values of LMA around 13.3 to 14.8 $cm^2$ found in this study are higher than that found for crossbred Texel lambs in feedlot of 11.2 $cm^2$. Rib fat thickness (RFT) also was not affected by the treatments and their values classified the carcass as median, with a thickness between 2 and 5 mm.

No changing in carcass traits also was found when lambs were fed with diets supplemented with amylase from *A. awamori*, with values around 49.69% (Prado et al., 2015). Other study reported that the use of exogenous enzymes

(endoglucanases) added on the TMR prior to feeding did not alter the carcass characteristics of lambs (McAllister et al., 2000).

The inoculation of silage with *L. plantarum* and *B. subtilis* did not provided changes on the carcass traits of lambs, even though greater ADG observed in lambs fed inoculated silage. Higher ADG and no effect on carcass traits also was observed in lambs fed inoculated silage with *L.plantarum* and *L. buchneri* (Basso et al., 2014).

Several factors can affect carcass yield, especially feed, which is unquestionably one of the most important, especially dietary energy levels (Alves et al., 2003). The inoculation of the silage and the amylase supplementation improved the nutrients digestibility and the intake of DE and ME, as well as altered the microbial N supply (Chapter 2). With this, it was expected that greater carcass yield and RFT, however, the energy available from the diet did not provide the weight gain determined for the animals in this study, which was 300 g/d, thus it can be justified the lack of effects of the diets on the carcass traits.

The inoculation of corn silage and amylase supplementation did not alter the chemical composition of meat. According to Prata (1999), the chemical composition of the lambs meat shows values around 75% of moisture, 19% of crude protein, 4% of fat and 1.1% of ash, which are in according with this study, except for crude protein and fat, that showed higher and lower values (21.99 to 23.55% and 2.07 to 2.47, respectively). However, young animals show higher quantities of moisture and muscle, while lower fat content (Rebello, 2003). In addition, the chemical composition of the meat is influenced by several factors, like species, age, breed, sex and weight at slaughter (Russo et al., 1999; Bonagurio et al., 2004; Zeola et al., 2004).

The nutritional level, of which the animal is subjected, besides having a great influence on the weight and yield of the carcass, may change the proportion of commercial cuts (Sañudo & Sierra, 1993; Osório et al., 1995; Sainz, 1996) and the body tissues (Preston & Willis, 1974; Santos, 1999; Furusho-Garcia, 2001). However, in present study the feeding lambs with inoculated silage and supplemented with amylase did not cause any changes in the distribution of the components of the carcass and any commercial cut yield. The use of silage inoculated associated with amylase supplementation decreased leg weight, but did not alter the final yield of the same cut. This result can be explained by the lower weight of the carcass of lambs

fed with inoculated silage and supplemented with amylase, since the commercial cut weights are directly related to the carcass weight.

Meat quality was not affected by the inoculation of silage and amylase supplementation at moment of feeding. The final pH found in this study it is in agree with the related by Duarte et al. (2011) from 5.5 to 5.8 for unstressed animals. The final pH of the carcass is an important characteristic to be evaluated as it is responsible for changes in meat quality traits such as color (Jeremiah, Tong, & Gibson, 1991; Wulf, O'Connor, Tatum, & Smith, 1997), however no change was verified for color meat in this study. Coloration variables remained within previously reported normal ranges for lambs of 30.3 to 49.47 for L* and 8.24 to 23.53 for a*, except for b* that was lower than 3.30 to 11.10 for (Sañudo, Arribas & Silva Sobrinho, 2008).

Cooking weight loss and water holding capacity has positive correlation (Pardi et al., 1993) and in this study, the values found for these parameters were lower than that found in the literature for lambs. The lowest capacity of holding water of meat results in losses of nutritive value by the exudate and consequently in dry and low tender meat (Dabés, 2001). However the tenderness was not influenced and showed values around 3 kgf, that was less than 5 kgf, precondition for classification of tender meat (Tatum, Smith & Belk, 1999).

In a study evaluating the use of *L. buchneri* in corn silage, was related no effect of the inoculation on the meat traits of finishing beef cattle, being that inoculation did not affect negatively the meat traits (Rabelo et al., 2016). The lack of effect of the treatments on the meat traits in this study is expected because when lambs are slaughtered in early age, the diet cannot influence the meat and carcass quality (Frescura et al., 2005).

As discussed in Chapter 2, inoculation of silage and supplementation with amylase improved the digestibility and energy intake, in addition, there was an increase in the propionic acid in the rumen when amylase was added on the uninoculated silage. It is known that propionate is the main precursor of glucose, and the higher absorption of this acid can provide greater amounts of glucose and result in greater synthesis of fatty acids. However, in the present study no changes were

observed in the final carcass weight and fat content and few changes were observed in the fatty acid profile of meat.

The inoculation of silage increased the concentration of some FA as heptadecanoic (0.23 vs. 0.33%), vaccenic (1.06 vs. 1.45), linolenic n-6 (4.93 vs. 6.33, Table 3). Instead linoleic fatty acid decreased with inoculation with *L. plantarum* and *B. subtillis* (41.6 vs. 40.84). The UFA and PUFA was higher in the inoculated silage than uninoculated silage (78.19 vs. 77.37 and 47.33 vs. 46.73, respectively).

Studies have reported slight effects on the FA composition of grass silages by the use of additives like formalin, formic acid, or enzymes (Dewhurst and King, 1998; Shingfield et al., 2005; Arvidsson, Gustavsson & Martinsson, 2009). According Ogawa et al. (2005) the BAL population found in the silage has the capacity to biohydrogenate the linoleic and linolenic and this process will depends on the additive and the forage utilized. Alves et al. (2011) found few effects on the FA composition of the ryegrass and corn silages.

Lambs fed with inoculated silage had lower concentration of oleic acid, as well as UFA and MUFA, providing low UFA:SFA ratio. In addition, the inoculation increased the SFA, a disadvantage, since these may lead to an increase in cholesterol synthesis and favor the accumulation of low-density lipoproteins, which represents a risk factor for the occurrence of cardiovascular diseases (Moloney et al., 2001; Madruga et al., 2008).

Furthermore, the values of UFA:SFA ratio were lower than 0.4, which is recommended by the UK Department of Health for the healthy food. However, in ruminants the UFA:SFA ratio usually is low, around 0.1 (Scollan et al., 2001) and can varies of 0.06 to 0.15 depending of the biohydrogenation process in the rumen (French et al., 2000).

The content of oleic acid was low in lambs fed inoculated silage. However, the oleic and linoleic acids comprised around 85% of the total FA of the *Longissimus* muscle in this study. These respective FA has a positively impact meat health attributes, reducing the concentration of low-density lipoprotein (LD) cholesterol and raising the concentration of high-density lipoprotein (HDL) cholesterol in the blood (Fugita et al., 2012). The oleic acid is formed from stearic acid which is the most

abundant acid found in lamb meat (Diaz et al., 2005) and the lowest value of oleic acid can be associated by the high value of the stearic acid in the same treatment.

However, the proportion of fatty acids in terms of percentage is different from when expressed quantitatively (mg per g of meat or fat), and even when differences in the proportion of some fatty acids was observed, it should be noted that they are numbers expressed as percentages and differences in quantities are not always biologically significant.

A recent research found opposite results when finishing beef cattle were fed with corn silage inoculated with BAL, with increase on the oleic acid and decrease on the palmitic acid. In addition, the authors related increase on the UFA:SFA ratio and highlighted out that there is a potential to use silage inoculant to improve FA profile in the meat of beef cattle, however this must be more investigated to know the mechanisms of action from inoculant on the silage and on the meat (Rabelo et al., 2016).

The supplementation with amylase decreased some UFA and trend to decrease the PUFA:SFA ratio. Amylases are carbohydrases responsible for degrading amylose and amylopectin to lower molecular weight compounds that will be degraded and absorbed rapidly by ruminal bacteria and the high concentration of the VFA´s, resulted by the increases on the rate of fermentation, can be increase the FA synthesis and alter the FA profile in the meat. However in this study was not verified increase on the fat (ether extract) and the weight of the carcass and few changes was detected on the FA profile.

Animals fed with diets rich in rapidly fermentable carbohydrates may have an increase of the plasma insulin content and consequently increase on the lipogenesis with greater Δ9 – Desaturase (Sinclair, 2007), which is responsible for the increase of the synthesis of CLA on the animals tissues from VFA´s produced in the rumen (Dunshea et al., 2005; Aharoni et al., 2005; Nute et al., 2007; Sun et al., 2009). However, in this study the high propionate production in the rumen of animals supplemented with amylases (Chapter 2) did not affect the content of CLA, an important acid with anticarcinogenic and antiatherogenic properties. This can be explained by the fact of the propionate is almost totally captured by the liver and the

acetate is the main precursor of the FA synthesis in the tissue (Polizel Netoet al., 2008).

On the other hand, the use of amylase in diets with high concentrate levels may decrease the ruminal pH, which can decrease hydrogenase activity, producing less conversion of linoleic to stearic acid (Tove & Matrone, 1962). The pH of the rumen was relatively low in all lambs in this study however did not influence the biohydrogenation, since no higher percentages of UFA were found in the meat from animals supplemented with amylase.

Atherogenicity and thrombogenicity are related to pro- and anti-atherogenic acids, and low index values correspond to higher levels of anti-atherogenic fatty acids in fat, and, consequently, a greater preventive potential against coronary heart diseases (Ulbricht & Southgate, 1991). In this study the inoculation of silage did not affect this indexes, however when lambs were supplemented with amylase, the thrombogenic index was decreased.

Therefore, in summary the inoculated silage and the supplementation with amylase at moment of feeding showed few effects on the carcass and meat traits with some negative effects on the meat FA profile, with decrease on the some unsaturated acids and proportion of UFA, MUFA and UFA:SFA ratio. Nevertheless, little is known about how the inoculants act on the FA profile of both silages and meat, and almost no information exists about of the possible survival and performance of inoculants in the rumen of the animals, modifying the microbiota and the end products of the ruminal fermentation.

Similarly, in spite of the vast amount of studies evaluating the use of exogenous enzymes in ruminant feed, information about how this enzymes can change the meat FA profile is scarce, since that is unknown the exact effects on improving the digestion of feed and consequently the animal performance. Probably, due to the variability of the results, which can be attributed to four main factors: enzyme characteristics (i.e., differences in enzyme preparations, enzymatic activities, units of activity added, pH, and temperature effects on activity), forage (i.e., type, maturity), animal (i.e., species, physiological state) and management (i.e., diet, mode of enzyme application, application rate, interaction time of enzymes applied to feed) (Beauchemin et al., 2003).

## 5. Conclusion

Inoculation of silage with L. plantarum and B. subtilis improved the average daily gain of lambs when was not associated with amylase supplementation. Diets containing inoculated corn silage and supplemented with amylase did not change the carcass and meat quality traits with few effect on the meat fatty acid profile.

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