UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

VIRGINIAMYCIN VIA MINERAL SUPPLEMENTATION AND SWARD HEIGHT IN GROWING NELORE YOUNG BULLS

João Paulo Ramos Costa

Zootecnista

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Orientador: Prof. Dr. Euclides Braga Malheiros

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

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: VIRGINIAMYCIN VIA MINERAL SUPPLEMENTATION AND SWARD HEIGHT IN GROWING NELORE YOUNG BULLS.

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Jaboticabal, 02 de fevereiro de 2016

DADOS CURRICULARES DO AUTOR

JOÃO PAULO RAMOS COSTA – nasceu em Montes Claros, interior do estado de Minas Gerais em 1984. Em 2004, começou sua graduação em Zootecnia na Universidade Federal de Minas Gerais, UFMG, Campus Montes Claros. Durante o seu período de graduação ele esteve envolvido com várias atividades de pesquisa. Em 2009 recebeu seu diploma e em 2010 mudou-se para Jaboticabal, onde obteve o grau de Mestre em 2012 também em Zootecnia. Durante sua vida acadêmica focou nas áreas de pesquisa em nutrição de ruminantes, manejo de pastagens e suplementação, com o objectivo de melhorar a utilização dos recursos basais de pastagem tropical usando suplementos, manejo de pastagens e aditivos alimentares.

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SUMMARY

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

C E R T I F I C A D O

Certificamos que o Protocolo nº 024583/12 do trabalho de pesquisa intitulado **"Virginiamicina via suplemento mineral em recria de novilhos em diferentes intensidades de pastejo: desempenho, perfil metabólico e metabolismo do nitrogênio",** sob a responsabilidade do Prof. Dr. Euclides Braga Malheiros está de acordo com os Princípios Éticos na Experimentação Animal, adotado pelo Colégio Brasileiro de Experimentação (COBEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 08 de novembro de 2012.

Jaboticabal, 08 de novembro de 2012.

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VIRGINIAMYCIN VIA MINERAL SUPPLEMENTATION AND SWARD HEIGHT IN THE GROWING NELORE BULLS

ABSTRACT

The objective was evaluate the effect of virginiamycin (VM) via mineral supplement and two swards height (SH) on performance, blood metabolites, ruminal fermentation, ruminal microorganisms and microbial N production. The trial was divided in two experiments. Chapter 1: Eighty Nellore young Bulls (initial BW = 258 ± 15 kg) were blocked by weight and randomly assigned into 16 paddocks (5 animals/paddock) arranged in a 2 × 2 factorial design: mineral supplement without VM (WVM) or with VM and two SH [15 and 35 cm (15SH and 35SH, respectively)]. The animals receiving VM had higher ADG ($P < 0.01$). As expected, the 35SH had higher ADG ($P < 0.01$). VM had no effect on mineral supplement intake by BW (MSIBW) or mineral supplement intake by animal (MSIA) ($P = 0.49$ and $P = 0.55$, respectively) and 15SH showed a greater MSIBW and MSIA ($P < 0.01$ and $P < 0.01$, respectively). Blood albumin was greater in VM ($P < 0.01$) and tended to be greater ($P = 0.07$) in 35SH. The blood urea concentration tended to decrease in VM ($P = 0.06$) and was higher ($P < 0.01$) in 15SH. Plasma NEFA and calcium concentrations were greater ($P = 0.007$ and $P = 0.038$, respectively) in VM. Chapter 2: Twelve Nellore steer (initial BW = 334 ± 47) ruminally cannulated were used in three 4 × 4 balanced Latin square were assigned in the same treatment arrangement cited above. The DMI was greater ($P < 0.01$) in 15SH than in 35SH. Ammonia-N tended to be higher in VM ($P = 0.07$). None ruminal bacteria proportion were affected ($P > 0.10$), except *R. flavefaciens* that tended to increase in

35SH (P = 0.08), while total protozoa counts increased (P = 0.03) and the *Entodinium* genus tended to increase (P = 0.05) in VM. The VM increased uric acid (P < 0.01) and tended to increase the purines derivatives excretion (PD; P = 0.074), absorbed purines (AP; P = 0.07), microbial N flow (NMIC; P = 0.07) and the PD: creatinine ratio index [PDC index (P = 0.07)]. Therefore, the VM effect seems to be more related to increasing the N microbial flow to the intestine caused by incresing of ruminal protozoa than the ruminal fermentation.

Key words: feed additive, *Brachiaria brizantha* cv. Marandu, tropical grass, ruminal protozoa, blood albumin, microbial N flow

VIRGINIAMICINA VIA SUPLEMENTO MINERAL E ALTURA DO DOSSEL NA RECRIA DE TOURINHOS NELORE

RESUMO

O objetivo foi avaliar o efeito da virginiamicina (VM) via suplemento mineral e altura pastos (AP) sobre o desempenho, parâmetros sanguíneos, fermentação ruminal, microorganismos ruminais e produção de N microbiano. O estudo foi dividido em dois experimentos. Capítulo 1: Oitenta tourinhos Nelore (inicial BW = 258 ± 15 kg) foram blocados por peso e distribuídos aleatoriamente em 16 piquetes (5 animais / piquete) arranjados em um fatorial 2 × 2: suplemento mineral sem VM (SVM) ou com VM e AP [15 e 35 cm (15AP e 35AP, respectivamente)]. Os animais que receberam VM tiveram maior GMD (P <0,01). Como esperado, o 35AP apresentou maior GMD (P <0,01).

A VM não afetou a ingestão de suplemento mineral por PV (ISPV) nem por animal (ISA) (P = 0,49 e P = 0,55, respectivamente) e 15AP mostrou um maior ISPV e ISA (P <0,01 e P <0,01 , respectivamente). Albumina sanguínea foi maior em VM (P <0,01) e tendeu ser maior (P = 0,07) em 35AP. A concentração de ureia no sangue tendeu a diminuir em VM (P = 0,06) e foi maior (P <0,01) em 15AP. A concentrações de NEFA e de cálcio foram maiores (P = 0,007 e P = 0,038, respectivamente) em VM. Capítulo 2: Doze Touros Nelore (PV inicial = 334 ± 47 kg) canulados no rúmem foram designados no mesmo tratamento citados acima em três quadrados latino balanceado 4 × 4. O CMS foi maior no 15AP (P <0,01) do que em 35AP. A amônia-N tendeu a ser maior em VM (P = 0,07). A proporção de nenhuma das bactérias ruminais foram afetados (P > 0,10),

excepto *R. flavefaciens* que tendeu a aumentar em 35AP (P = 0,08), enquanto que a contagem de protozoários totais aumentou (P = 0,03) e o género *Entodinium* tendeu a aumentar (P = 0,05) em VM. A VM aumento do ácido úrico (P <0,01) e tendeu a aumentar a excreção derivados de purinas (PD; P = 0,074), as purinas absorvidas (AP; P = 0,07), fluxo de N microbiano (NMIC; P = 0,07) e o índice de PDC (P = 0,07)]. Portanto, o efeito da VM parece estar mais relacionada ao aumento do fluxo de N microbiano para o intestino provocado pelo aumento dos protozoários ruminais do que a fermentação ruminal.

Palavra-chave: adidtivo alimentar, *Brachiaria brizantha* cv. Marandu, pasto, protozoários ruminais, albumina, fluxo de N microbiano

CHAPTER 1 – GENERAL CONSIDERATIONS

The half part of beef cattle production cames from tropical and subtropical áreas of the world (JANK et al. 2005). In Brazil, which makes part of it, is one of the most important producer of beef cattle because it has the largest commercial herd and is the bigger beef exporter of the globe (ABIEC, 2011). However, its productivities indexes still remain poor when compared with other countries (ALVES, 2011). Besides, in the next 35 years the food demand is expected to increase about 70 percent (FAO, 2013) and it pushes the beef agribusiness to improve their systems. Therefore, new strategies of nutritional and pasture management are nedded to improve the beef cattle industry.

In Brazil, the cultivated pasture area cover about 97 million of ha representing 60 percent of total pasture area in the country (IBGE, 2006) and the major genus cultivated is the *Brachiaria brizantha* cv. Marandu once that it represents 70 percent of the seed sold in the 90's (ANDRADE, 2001). This way, about 97 percent of the beef cattle are fed on pasture and therefore, the pasture management presents a great importance in beef cattle production in Brazil.

In the past 30 years the research of pasture management was strongly performed in tropical and subtropical regions of the world including Brazil. Several criteria of pasture management were studied, but the sward height (SH) has shown to be an easy and efficient way to apply and monitoring the pasture management in continuous stocking (DA SILVA et al., 2013). Therefore, now a day this criterion is widely used in field conditions for several genus and varieties of grasses.

For marandu palisade grass, the SH stabilished previously can range from 20 to

40 cm (SBRISSIA, 2004) and each height promote differents grazing intensities, consequently several structural characteristics of the canopy, chemistry composition of forage consumed and the animal performance also change with the SH target (DA SILVA et al., 2013; BARBERO et al., 2015). For exemple, when the SH increases, consequently the canopy structure also changes decreasing the portion of leaf and increasing the portion of stem and dead material.

On animal performance, the increasing of SH also increases the dry matter intake (DMI) and consequently the average daily gain (ADG) increases as well. According to HODGSON (1990), the limitation of DMI for the SH is a direct response of the bite mass (BM). It means that short SH decreases the BM and as the animal have a limited range of bite rate (BR), the DMI is also limited by the canopy structure. On the other hand, while the ADG decreasing with decreases of SH, the animal gain per ha increases because often there is an increment in the stocking rate (SR). This manner, although the shorter SH presents less weight gain than the taller, in the short SH there are more animals per ha (MOTT, 1960). Thus, greater animal gain can be found in tall SH and greater gain per area can be found in short SH.

In regarding the chemistry composition, the SH also promotes a great modification in the proportion of the nutrients of the pasture and their availability. It is manly caused for the turnover of leaves and tissues, which are higher in short SH when compared with taller SH (SBRISSIA et al. 2010). This way, the replacement of tissues is made continuously and than higher crude protein (CP) and digestibility content are found. In the other side, in the same situation, lower NDF and ADF content are also frequent (FLORES et al. 2008; DA SILVA et al. 2013; BARBERO et al. 2015).

How could be noticed, the SH of pasture management can modify several variables in a forage-based system, mainly animal performance. Others management tools are used to intensify the animal production as well. For example: the fertilization and irrigation that can increase the herbage mass production and hence increases the carrying capacity. The pasture supplementation that can increases the energy or other nutrient intake and the use of antibiotics growth promoters (AGP), which often improves the efficiency of digestive processes into the rumen and hence the productive efficiency (OWENS et al., 1991; MORAIS et al. 2011).

The AGP has widely been used since the 70 years to enhance the health and animal production in several countries (BRETSCHNEIDER et al 2008). The AGP can be divided in two groups: ionophores and non-ionophores. In the group of non-ionophore, one of the most known is the virginiamycin (VM).

The VM is natural antibiotic produced by *Streptomyces virginiae* and presents effect mainly against gram-positive bacteria (COCITO, 1979; NAGARAJA and TAYLOR, 1987). According to COCITO, (1979), this effect is due the irreversible link of the two VM factors to the ribossomal units of the bacterial cell and it inhibits the protein synthesis causing the bacteriostatic or even bactericidal effect.

Once VM has effect against gram-positive ruminal bactéria (NAGARAJA and TAYLOR, 1987), in a feedlot experiment durring the diet adaptation, COE et al., (1999) found that the antibiotic decreased the *Lactobacillus* and *Streptococcus bovis* count into the rumem. How is well know, those bacterias are the major responsible for the latic acid production into the rumem (NAGARAJA and Miller, 1989). Thus, controlling those microrganisms the pH can be kept high enough. In fact, it did happen when COE et al.,

(1999) compared VM with monensin/tylosin. The VM showed a greater pH up to 12 h after feed and the latic acid also remained low. This way, VM show to be a great tool on prevention of beef cattle acidosis in feedlots.

As was presented above, VM has been shown to be a great antimicrobial compound against gram-positive bacterias. However, in an in vivo study it did not happen with the main fibrolytic ruminal bacteria (*Ruminococcus albus, Ruminococcus flavefaciens*) (GUO et al., 2010). Therefore, the VM effects seem not be as the same as in in vitro study (NAGARAJA and TAYLOR, 1987) with this group of bacteria.

As in ruminal bacteria, VM also affected the ruminal protozoa population. The anti-protozoa effect of additives is desired because protozoa have been related to efficiency of N use into the rumen due the predation behavior of protozoa against ruminal bacteria, which reduces the N flow to the intestine (WALLACE and MCPHERSON, 1987).

From all references we found, 66.7% of the experiments VM decreased the amount of total protozoa into the rumen and 33.3% remain unchanged (Figure 1). However, an important point to be raised is related the method used to collect the ruminal fluid in the cited studies. All experiments that were observed effect of VM against ruminal protozoa, the ruminal fluid were collected using a stomach tube. As we know, the protozoa have the chemotaxis (WILLIAMS and COLEMAN, 1992) and how this method there is not options to change the sampling point, the ruminal fluid could be less representative. Besides, the method allows only liquid sampling, which once again could be a problem once that the particles attached protozoa is a significant component of total rumen protozoa (HOOK et al. 2012). This way, the method of ruminal fluid

sampling could bias these results. On the other side, the studies where the sampling method was via cannula, the results have been shown that VM did not change the protozoa population. Thus, the effect of VM against protozoa seems to be related with the method of ruminal sampling.

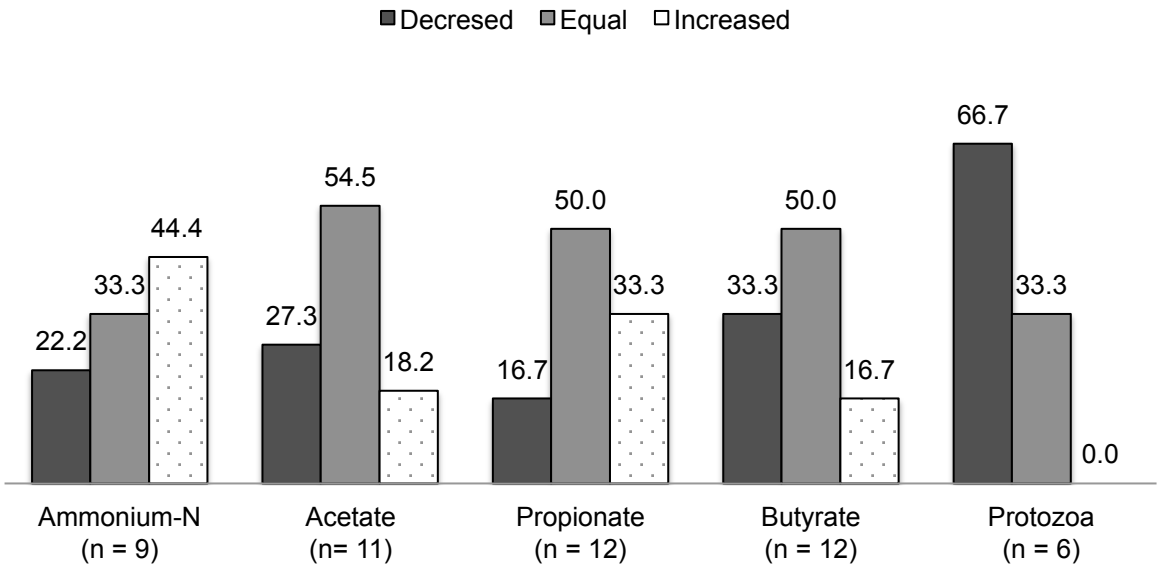

Figure 1. Percentage of results of studies that evaluated the VM effects against the control in ruminal fermentation parameters and protozoa counts. References: Nagaraja et al., 1995a**; Nagaraja et al., 1995b**; Nagaraja et al., 1987*; Coe et al., 1999; Golder et al., 2014; Murray et al., 1992**; Ives et al., 2002; Godfrey et al., 1995**; Van Nevel et al., 1984*; Van Nevel and Meyer, 1990*; Van Nevel and Meyer, 1992[+]*; Van Nevel and Meyer, 1992*; Salinas-Chavira, 2009; Valentine et al., 2000. * = in vitro studies; ** = stomach tube method of ruminal fluid sampling. [+] = same study.

Ammonium is a great indicator of protein degradation into the rumen (TAMMINGA, 1979). The Figure 1 shows that VM decreased ammonium concentration in only 22.2% of the studies and those, which remain unchanged or increased represent 77.8% of total. All experiments where there was a reduction in ammonium were performed in in vitro study. However, in in vivo studies the effects were differents. In a

feedlot experiment, IVES et al. (2002) found no difference between the VM treated group and the control. In the same way, COE et al., (1999) and GOLDER et al., (2014) did not find any difference between the VM treated and control. In another study where sheep where fed with forage based diet, supplemented with barley and receiving VM, NAGARAJA et al. (1995b) found a increment of ammonium concentration in ruminal fluid. As the study above, MURRAY et al., (1992) and GODFREY et al., (1995), also found an increment of ammonium in VM treated sheep. This way, VM seems to remain the ruminal ammonium concentration unchanged or even increased.

Another effect of VM in ruminal fermentation is the increasing of molar proportion of propionic acid and decrease the proportion of acetate and butyrate, which according to WOLIN (1960), means the most efficient pathway of carbohydrates fermentation into the rumem. According with the references herein researched, only 33.3% of the studies shown that VM in fact increased the propionate proportion and 50% remain unchanged, which means that 66.7% of the studies the VM did not change or change negatively the concentration of propionate in the ruminal fluid. Therefore, VM seems to change not much the VFA's.

Although VM have not been shown a large variation in ruminal parameters results, its effect in cattle performance is well know. ROGER et al. (1995), evaluated seven dose-response feedlot studies and found that VM improves the ADG and feed/gain ratio some of those. As the same way, SALINAS-CHAVIRA et al. (2009) shown that VM increased the G:F, which indicate an improvement of energy use. Therefore, the VM has improved feedlots cattle performance. On the other hand, few experiments using VM in pasture have been performed. So far, only one manuscript was

found and its outcomes follow the same behave as in feedlots, which VM enhancing the ADG as well (FIEMS et al., 1992). Thus way, in pasture conditions VM shows to improve the animal performance as well.

So far, we have seen that VM does not change much the ruminal fermentation parameters. However, something in ruminant metabolism is supposed to be changing as a response of VM once that the animal performance changes as we saw in the literature above-cited. A great hint is the enhance of microbial nitrogen production or flow to intestine (NMIC) and efficiency of microbial protein synthesis (EMPS) once that according to ROGERS et al. (1997), in a study with ionophore there was an enhance of microbial N flow to intestine and how it is well known, the increasing in the N flow to the intestine improves the metabolizable protein once that 64% comes from microbial CP (NRC, 2001). Besides, the use of ionophores have shown to increase the N retention in the animal body (MUNTIFERING et al., 1980), which means a improvement in the efficiency of N utilization and N availability that according to DETMMAN et al. (2014), it increases the portion of total N that can be used for anabolic purposes and enhances the overall efficiency of the utilisation of metabolisable protein. This way, the improvement of N metabolism as the N flows to the intestine can be a great way that takes account to animal performance improvement.

Another matter regarding the use of AGP in grazing system is the limitation due the labor to provide the additive in grazing condition (DAVENPORT et al., 1989). This way, the use of supplement as carrier is a great alternative to provide the AGP. However, its use should be careful once that the additive should not limit the intake as happened with ionophore (HORN et al., 2006). First, because the limitation of intake

could lead to decrease the consumption of mineral or nutrients provided by the supplement. Another matter is related to the dose intake of AGP, once that limiting the supplement also limits the consumption of the right dose of the AGP, which could does not promotes the intended effect on animal performance. Therefore, the AGP should not limit the supplement intake and ensure the consumption of the right dose of additive.

As we have seen, the effect of VM in performance is well known. However, the metabolic affects that cause it seems to remain unknown because there is a large variation in results mainly on ruminal fermentation. Thus, the use of blood metabolites could help to understand more about of the VM effect in the animal metabolism. The blood metabolites have been used to assess the nutritional status, which means the quantification of extent to which cattle are affected by nutrition. The proteic status has been evaluated using the plasm protein as: Total protein, which is the sum of albumin and globuinas. The albumin is the labile protein and its blood level can increases with high protein intake (LAW et al., 2009) or in period of under feed privation it can be used to provide aminoacids to the liver and other organs (ECKERSALL, 2008).

Another proteic blood metabolite is urea. Blood urea in ruminants differently of nonruminants has two sources: the ammonia produced into the rumen as result of protein degradation by ruminal microrganisms that reach the liver and is transformed into urea to be excreted or go back to the rumen via saliva or as product of deamination in the liver as a results of muscle mobilization (GONZALES et al., 2000; CHIMONYO et al., 2002). Blood urea still can be used as tool to analyze the protein-energy ratio into the rumen once that when there is an excess of N relative to energy, ruminal concentration of ammonium increases (HAMMOND, 2006).

In regard the blood metabolites related to energy metabolism, glucose has a moderate diagnostic value on assessment of nutritional status in cattle (NDLOVU et al., 2007). Undernutrition can reduce blood glucose and it is linked to the concentration and production of propionate that is its main precursor (REYNOLDS et al., 2003). Therefore, the glucose is a good indicator of propionate production into the rumen.

Lipids are important in the assessment of nutritional status of cattle. Among them, the non-esterified fatty acids (NEFA). The NEFA is a direct result of lipid catabolism, which means mobilization of fat to use as source of energy (ADEWUYIL et al., 2005). However, even under well nutrition, O'MARA et al. (2000) found that blood NEFA concentration increasing with the increses of protein intake. No explanation was found of it, but according to ØRSKOV et al., (1987), the increment of RUP can stimulate the body fat mobilization in dairy cattle to use in milk production. Therefore, NEFA can be changed by protein intake beyond malnutrition.

After fat mobilization, NEFA can be used as a source of energy in some tissues and can be transformed into beta-hydroxybutyrate (BHA) by the liver, which is much sensitive than NEFA and mensure the extention of the fat mobilization and indicates the negative energy balance (AGENAS et al., 2006). However, the major part of BHA comes from the butyric acid produced into the rumen by the microrganisms (AGENAS et al., 2006). Therefore, BHA is a response of NEFA blood levels and the VFA profile.

In non-ruminants the use of VM as an additive had shown to improve the calcium and phosphorus disgestibility (AGUDELO et al., 2007). However, none ruminants study was found in regarding this matter. Therefore, de use of blood metabolites could help understand if VM plays the same role in non-ruminants.

To promote a normal tissue growth and several others functions in animal body is imperative that minerals must be maintained in suitable levels in the blood (UNDERWOOD and SUTTLE, 1999). Calcium is the most abundant mineral in animal body and is responsible for several functions further structural component as membrane permeability and muscle contraction (INGVARTSEN and ANDERSEN, 2000). Calcium blood levels hardly decreases in grazing beef cattle, first because this mineral is abundant in warm-season grasses and the calcium metabolism is mediated by a strong endocrine control (GONZALEZ, 2000). However, its blood levels can be increased by the blood albumin once that about 45% of calcium is carried by this blood protein through the blood stream and the deficiency in protein and low albumin blood levels also decreases calcium absorption (ROSOL et al., 1995).

Phosphorus is envolved in every metabolic reaction, energy transference and efficient use of feed once that is required for ruminal microorganism to play a role of cellulose digestion and microbial protein synthesis (INVARTSEN and ANDERSEN, 2000). Phosphorus is mediated for the same endocrine system metabolism as calcium, however, it suffers a greater variation in blood when compared with calcium. Its blood level is affected by the calcium concentration in the diet, which must have a Ca:P ratio of 2:1 to 3:1 and the concentration of phosphorus in the diet (GONZALES, 2000). Therefore, blood phosphorus levels is more related to diet phosphorus and calcium content in the diet.

Magnesium is the third more important mineral in the animal metabolism. It plays differents roles in the animal body as in many enzymatic reactions and as a cofactor to adenosine triphosphatases (LAIRES et al., 2004). Magnesium does not have a

homeostatic control, for that reason its blood levels is linked to the diet concentration and for sodium, calcium and phosphorus concentration into the rumen, besides the potassium in soil, which is absorbed instead to magnesium. This way, the grass magnesium content decreases (GONZALES, 2000). Thus, magnesium blood levels seem to change in function of the feed content and the presence of other minerals in the rumen.

Therefore, our hypothesis is that the offering VM via mineral supplement improves animal performance even in both short and tall SH, improves the blood metabolites, does not limit the supplement intake, does not change the ruminal fermentation, the ruminal microorganisms and increases the microbial N flow to intestine.

The objective of this study was to evaluate the effect of VM via mineral supplement and SH on performance, blood metabolites, ruminal parameters, ruminal microorganisms and microbial N production of young Nellore Bulls.

1. LITERATURE CITED

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CHAPTER 2

O artigo a seguir está redigido conforme normas de publicação do *Journal of Animal Science*.

Running head: V**irginiamycin via mineral supplement**

Effect of virginiamycin via mineral supplement and sward height on growing Nellore young Bulls: Performance and blood metabolites[1]

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ABSTRACT

The objective was to evaluate the effect of virginiamycin (**VM**) via mineral supplement and two swards height (**SH**) on performance and blood metabolites in Nellore young Bulls. Eighty Nellore young Bulls (initial BW = 258 ± 15 kg) were blocked by weight and randomly assigned into 16 paddocks (5 animals/paddock) arranged in a 2 × 2 factorial design: mineral supplement without VM (**WVM**) or with VM and two SH [15 and 35 cm (**15SH** and **35SH**, respectively)]. Young Bulls receiving VM had higher ADG ($P < 0.01$) and final BW ($P = 0.05$). As expected, the 35SH had higher ADG and final BW ($P < 0.01$; $P = 0.05$, respectively) while the 15SH had higher Stocking rate and gain per hectare (GPH) ($P < 0.01$; $P < 0.01$, respectively). The use or not of VM did not influence the mineral supplement intake by BW [**MSIBW**(g/100 kg of BW/Day); $P = 0.49$] and mineral supplement intake by animal [**MSIA**(g/animal/Day); $P = 0.55$], however, animals grazing at 15SH showed higher MSIBW and MSIA ($P < 0.01$ and $P < 0.01$, respectively) than those at 35SH. Blood albumin showed higher concentration in cattle fed VM ($P < 0.01$) and tended to be higher ($P = 0.07$) in 35SH. The blood urea concentration tended to decrease in VM ($P = 0.06$) and was higher ($P < 0.01$) in 15SH. Similarly, plasma NEFA and calcium concentrations were higher ($P < 0.01$ and $P = 0.04$, respectively) in animals that received VM. Therefore, VM increases the animal gain, does not limit the mineral supplement intake and increases albumin, NEFA and blood calcium. The SH modifying the performance and the blood metabolites.

Key words: feed additive, *Brachiaria brizantha* cv. Marandu, supplemet intake, blood albumin, NEFA, blood calcium

1. INTRODUCTION

Virginiamycin (**VM**) is a natural antibiotic that has been used as an additive on livestock production. According to Nagaraja et al., (1987) its effect is against gram-positive ruminal bacteria , which causes bacteriostatic or even bactericidal effect (Boon and Dewart, 1974). Previous studies, have shown that VM enhanced the ruminant performance (Murray et al., 1992; Silva et al., 2004; Salinas-Chavira, et al., 2009; Nuñez et al., 2013). These studies, however, have been performed in feedlots with high grain diets (Silva et al., 2004; Salinas-Chavira et al., 2009; Nuñez et al., 2013) or supplementation with sources of non-structural carbohydrate (Fiems et al., 1992). Thus, information of VM effects in performance when the pasture is the unique source of N and energy remains unknown, mainly when the sward height (**SH**), which is a criterian of pasture management, generate different animal gain (Da Silva et al., 2013; Barbero et al., 2015).

Moreover, the use of additives in this situation is limited due to the labor for administration (Davenport et al., 1989), which is necessary a carrier. Thus, the mineral supplement is an alternative of providing. However, its use should be careful once that the additive should not limit the intake and ensure the right dose consumption of additive (Bretschneider et al., 2008). Additionally, once the VM effect is supposed to change the ruminal fermentation (Nagaraja et al., 1995a) and some ruminal products are blood metabolites precursors (Agenas et al., 2006; Reynolds et al., 2003), these variables can provide information of the VM effects that contribute to animal gain. Therefore, we hypothesized that VM increases the animal performance even in short and tall SH, does not affect the mineral intake and improves blood metabolites. Thus, we aimed evaluate the effects of VM via mineral supplement and SH on performance and blood metabolites of Nellore young Bulls.

2. MATERIALS AND METHODS

Animals were cared for in accordance with acceptable practices and experimental protocols reviewed by the Brazilian College Experimentation (COBEA- Colégio Brasileiro de Experimentação animal) guidelines and approved by the Ethics, Bioethics and animal Welfare Committee (CEBEA- Comissão de Ética e Bem Estar Animal) of the FCAV- UNESP (Protocol number 024583/12).

The study was conducted from December 2012 through May 2013 at the Research Unit of the Agência Paulista de Tecnologia dos Agronegócios (APTA), Colina, Sao Paulo, Brazil.

Animals and experimental design

Eighty Nelore young Bulls (initial BW = 258 ± 15 kg) were used in the study. The animals were grouped into four blocks according its BW. The twenty heaviest were randomized to block I, the next twenty heaviest were randomized to block II, so on. There were two categories of animal within the experiment: testers (described above) and "put-and-take" animals (Mott and Lucas, 1952). Where the testers were used to evaluate the blood metabolites and performance per animal. The put-and-take animals were considered in the evaluation of animal gain per area, the stocking rate, supplement intake and still used to be added to or removed from as needed so that the sward height is maintained. Each block had four paddocks (16 paddoks in total) that were considered the experimental unit (4 paddock/treatment combination) and received five testers animals. Paddocks were assigned to receive, in a 2 × 2 factorial arrangement, 1 one of the 4 treatments combination of mineral supplement without (**WVM**) and with **VM** (V-Max, Phibro Animal Health, Ridgefield Park, NJ) and 15 and 35 cm of sward height (**15SH** and **35SH**) of pasture management. The SH criteria was choose because it has been shown that provide diferentes canopy structure and consequently differents DMI and animal gain (Da Silva et al., 2013; Barbero et al., 2015). Fifteen d before the beginning of the experimental period the

animals were treated against ecto and endoparasites by administration of ivermectin (Ivomec, Merial, Paulínea, BR).

Pasture management and supplement

The continuous stocking method of pasture management was used at the paddocks (1 ha) of *Brachiaria brizantha* cv. Marandu. The SH target were achieved 45 d before the beginning of the experiment using put-and-take animals (differents from de experiment) and also the SH targets were maintained using the same animals (Mott and Lucas, 1952). The sward height was measured each every 10 days using a sward stick. One hundred of points were taken and averaged per paddock. After, whether necessary animals were taken or added to keep the target SH. In late January, February and April all paddocks received 45 kg of N_2 and 15 kg of K_2O ha^{-1}. The supplement was provided daily at 1400 h in a covered and suspended mineral buckets with 20 cm of edge to avoid get lost the supplement by wind and rain. Before, the refusals were taken and oven dried at 55°C during 72 h to determine the supplement DM. As a virginiamycin carrier was used a complete commercial supplement (Calcium 14%, P 3%, Na 11.4%, Mg 0.25%, S 0.8%, Mn 0.102%, Zn 1700 ppm, Cu 510 ppm , Co 30.6 ppm, I 30.6 ppm, Se 8.5 ppm and 800 ppm of virginiamycin) and water were also offered for ad libitum consumption throughout the experiment. The concentration of virginiamycin was calculated to offer 40 mg/100 kg of BW based in a consumption of 50 g/100 kg of BW of mineral supplement.

Sampling and calculation

Forage sampling were taken four times (each 28 days). The sample were manually separated the leaf blade, stem and dead material and were oven drying at 55°C during 72 h to estimate the forage mass, botanical composition and forage allowance and at 22 and 24 d were made forage sampling by hand-plucked (Sollenberger and Cherney, 1995) methodology to simulate the diet

consumed by the animals to analize the chemistry composition. Pasture samples were collected using the double-sampling method according to Wilm et al. (1944). At d 0 the animals were weighed and ranked by initial BW and distributed within the experimental unit. At 14 d (14 d of adaptation) and each 28 d all animals were weighed for supplement intake adjustements. All animals were submited to water and feed restriction 16 h after the weighing to determine the shrunk BW. The ADG was determined as Final BW – initial BW and divided by number of experimental days (112 d) using the weight of 14 and 126 d of experiment. The stocking rate was calculated dividing the sum of BW of all animals (including the put-and-take animals) by the animal unit [AU- 450 kg of BW (Sollenberger et al., 2005)] multiplied by the paddock size. All the animal input and output were recorded to take into account in the calculation of the number of animal per day and BW per paddock per day and use in the subsequent calculations. The gain per ha (**GPH**) was obtained multiplying the ADG by the number of animals per day in each paddock and by 112. The supplement intake was measured daily during whole experimental period and was calculated as mineral supplement intake per BW (**MSIBW**) and mineral supplement intake per animal (**MSIA**) and were obtained according with the calculations [1] and [2] respectively.

$$\text{MSIBW} = ((\text{Provided} - \text{Refusal})/(\text{BW}_\text{d} \,/\, 100)) \times 1000 \qquad [1]$$

$$\text{MSIA} = ((\text{Provided} - \text{Refusal})/\text{number of animal per day}) \times 1000 \qquad [2]$$

In which MSIBW is expressed in grams per 100 kg of BW per day, MSIA is expressed in grams per animal per day, $\mathbf{BW_d}$ is the body weight per day within the paddock that was obtained summing the BW of all animals inside the paddock per day. The blood samples were taken via jugular venipuncture using commercial blood collection tubes (Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ) at d 14, 70 and 126. After, tubes were centrifuged at 5,000 rpm

for 15 minutes. Then, the serum and plasma were transferred to plastic tubes and stored at -20°C for later analysis. Vacutainer tubes without anticoagulant were used to obtain the serum to determine Albumin, Urea, Total Proteins, Calcium, Phosphorus and Magnesium. Vacutainer tube with sodium fluoride + EDTA were used to obtain the plasma to determine Glucose, NEFA and beta-hydroxybutyrate (**BHA**).

Chemical analysis

Forage samples were oven dried at 65°C for 72 h, ground in a knife mill (Wiley; A. H. Thomas, Philadelphia, PA) to pass through a 1-mm screen, and analyzed for DM (105°C for 12 h) and ash (500°C for 5 h) according to AOAC [1990 (DM, method number 934.01)]. The ADF concentrations were determined using the method 973.18 modified for use in an Ankom 200 fiber analyzer **(**Ankom Technology Corp., Fairport, NY; AOAC, 2006), and NDF (method for use in an Ankom 200 fiber analyzer, Ankom Technology Corp.; Van Soest et al., 1991) without sodium sulfite and corrected for residual ash. Lignin was measured after hydrolysis of the cellulose in ADF residues in a 72% H_2SO_4 (Van Soest and Roberson, 1985). Forage residue of NDF and ADF was recovered and analyzed for CP and ash for subsequent calculation of TDN according to Valadares Filho et al., (2010). Ether extract was measured according to AOAC [1996 (Gold fish, method number 945.16)]. The estimated *in vitro* true digestibility of dry matter (IVTDMD) of the forage was performed using *in vitro* assay with 0.5 g samples weighted in bags (ANKOM®-F57) and incubated in the apparatus "Daisy-II Fermenter" for 48 h (ANKOM® Technology Corp. Fairport, New York, USA) and subsequently analyzed for NDF (Tilley and Terry, 1963). The CP was estimated multiplying the N concentration measured using a Leco F528 N analyzer (LECO Corporation, St. Joseph, MI) by 6.25. The CP fractions were estimted according to Orskov and McDonald, (1979).The results of chemical composition are described in

Table 1.

Blood analysis

Concentrations of total proteins (colorimetric-biuret), albumin (Colorimetric-Green Bromcresol), urea (UV enzymatic), calcium (Colorimetric-cresolphthalein), phosphorus (UV-Daly and modified Ertingshausen), magnesium (Colorimetric-Magon sulfonated), glucose (enzymatic-colorimetric Trinder) were measured by a diagnostic test kit (Labtest Diagnostica SA, Lagoa Santa, MG, Brazil) and BHA (UV enzymatic) and NEFA (colorimetric) using a diagnostic kit test (RANDOX Laboratories Ltd., Ardmore, Diamond Road, Crumlin, Co. Antrim, United Kingdom). All blood metabolites were determined using an automatic spectrophotometer (LabMax Plenno, Labtest Diagnostics SA, Lagoa Santa, MG, Brazil).

Statistical analysis

Supplement intake and blood metabolites were analyzed as repeated measurement in a randomized complete block design with four blocks and four replicates and the performance variables were analyzed only as a randomized complete block design. The paddock was assigned as an experimental unit to supplement consuption variables. The five testers animals belonging each paddock were assigned as an experimental unit of performance variables and blood metabolites. The model statement used contained the fixed effects of virginiamycin, sward height and their interaction. Those variables, which were evaluated over the time, had the time effect included in their models. Block was considered as a randomized effect. All analysis was performed using the MIXED procedure (SAS 9.2 version, Inst. Inc., Cary, NC). In repeated measurements, data were tested for the best covariance structure that had the lowest Aikaike information criterion scores and then included in the REPEATED statement to proceed the analysis. Differences between means were determined using the T test. Significance was

declared at $P < 0.05$ and tendencies were determined if $P > 0.05$ and ≤ 0.10.

3. RESULTS

Performance

The ADG and final BW were greater ($P < 0.01$; $P = 0.05$, respectively; Table 2) in animals fed with VM, while that 35SH showed greater ADG and less final BW ($P < 0.01$ and $P < 0.01$, respectively) when compared with 15SH. The stocking rate and GPH were greater ($P < 0.01$ and $P = 0.04$ respectively) in 15SH and Regarding the supplement intake, neither MSIBW nor MSIA had no effect of VM ($P = 0.49$ and $P = 0.55$, respectively; Table 2). On the other hand, MSIBW and MSIA were greater in 15SH ($P < 0.01$ and $P < 0.01$; Table 2) when compared with 35SH. A virginiamycin × Time and SH × Time interaction were detected for both MSIBW and MSIA (all showed $P < 0.01$; Figure 1, 2, 3 and 4). In the Figure 1 and 2 as was showed in the main effect of mineral supplement intake (Table 2), the averages of both WVM and VM were pretty close during the whole experimental period and once more the result shows that virginiamycin did not affect the intake.

Blood metabolites

Serum albumin concentration was greater in VM ($P < 0.01$) when compared with WVM and tended to ($P = 0.07$) be greater in 35SH (Table 3). The blood urea concentration tended to decrease in animals that received VM ($P = 0.06$; Table 3), while was greater ($P < 0.01$) in 15SH when compared with 35SH. Blood glucose concentrations were similar ($P = 0.69$) between VM and WVM. Plasma NEFA concentration was greater ($P < 0.01$; Table 3) in animals receiving VM when compared with WMV. The same way as NEFA, blood calcium level was greater ($P = 0.04$; Table 3) in animals that received VM. All blood metabolites shown effect on time ($P < 0.01$; Table 3) except NEFA ($P = 0.31$, Table 3). However, as there was not interaction with the

main effect the results were not shown and discussed.

4. DISCUSSION

Performance

Our findings show that VM increases the ADG in both SH. In other words, VM increses the animal gain in high and low rate of animal gain. It is important because in areas where the pasture management provides different SH and concenquently rate of gain, the use of VM is able to improve the animal performance. Looking in our results, VM showed a gain of 119 g per day per animal and an increment of 13.8% in ADG when compared with the WVM treatment. In addition, the difference of ADG between the VM and WVM is 13.5 kg in the experimental period (112 d), if this gain is projected over a year the final difference could reach around 43 kg, witch is a great difference of gain. Although, the literature presents only one reference about the effect of VM in grazing trials, our findings agree with the results of Fiems et al., (1992) which used an energetic supplement as a carrier for VM and found an increment of 13.1% in ADG when compared with the control treatment. Thus, our findings confirm that virginiamycin increases the grazing cattle weight gain. In regarding to SH, the greater ADG in 35SH is linked to the structural characteristics of the SH. As can be noticed, the forage mass and forage allowance (Table 1) were higher in 35SH than 15SH and it means according to Poppi et al. (1987) and Barbero et al., (2015), how greater is the forage availability, greater will be the ADG. The main explanation of it, according to Hodgson (1990), is related to the bite mass that is influenced for the SH. In other words, greater amount of forage in each bite increases the intake and thereby increases the ADG. On the other hand, lowers ADG are expected in lower SH (Mott, 1960; Da Silva et al., 2013). To achieve the SH target is needed to increase the number of animals within the paddock and than the stocking rate is increased. As a response of increment of

stocking rate, the forage availability decreses and bite mass also decreases and resulting in a less DMI (Hodgson et al., 1994 and Da Silva et al., 2013) and increses the GPH (Mott, 1960).

In this study we observed that VM did not affect the intake of mineral supplement and it is an important matter to be raised. First, because the limitation of mineral supplement intake caused by the additive could decrease the intake of minerals and impact on animal performance once that the mineral intake could not be achieved at the level of animal requirement. So, the performance could be affected negatively. Another matter is related to the additive dose intake. If the additive limits the mineral supplement consumption, as it has been noted with ionophores (Thonney et al., 1981; Horn, 2006), the own additive intakes could be lower than the dose required to be active and promote the intended effect. In fact, in our study the treatment of 35SH showed less mineral supplement intake than 15SH and the animal gain (Table 2) showed similar outcome (0.126 vs. 0.111 g of ADG for 15SH and 35SH, respectively) when compared VM and WVM within SH. Which does not make sense once the additive intake was different (38.6 vs. 20.9 mg/100 kg of BW for 15SH and 35SH, respectively). However, if we compare the weight gain of VM as a percentage of the WVM treatment the 15SH showed 18.05% and 35SH showed 10.87% of additional weight gain. It means that the additive plays its effect proportional to the VM's dose (38.6 vs. 20.9 mg/100 kg of BW for 15SH and 35SH, respectively), where higher dose had higher additional weight gain as a porcentage of the control's gain . Thus, it suggest that VM plays effect on animal gain proportionally to the additive dose intake.

According to Tait and Fisher (1996), the supplement intake is variable in cattle and in fact, it was observed in the beginning of the current experiment. As can be seen at the Figure 1, the SD of both VM and WVM showed great variation from 1 to 56 d, which indicates the overconsumption of additive. According to Bretschneider et al. (2008), the high intake of

additive could become a problem if the additive has a high toxicity, once that it was already observed intoxications of herds fed with ionophores. However, according to FDA (1994), VM has high level of safety for cattle feed. This way, the wide variation in mineral supplement intake is not a problem when the additive has low toxicity.

We observed that the SH, in fact, affected the mineral supplement intake. The higher mineral supplement intake in 15SH suggested that the intake was motivated for the forage intake. As explained early, short SH often promote less DMI (Da Silva et al., 2013), consequently, the mineral intake that comes from the forage will be lesser either. Although McDowell, (1996) consider that ruminant does not have the ability to identify theirs mineral deficiency, our findings shows that there is a relationship among the mineral intake from the forage and the provided for the mineral supplement, which means the mineral intake provided for the forage did not reach the cattle requirement in the 15SH. Therefore, cattle sought by more mineral supplement in 15SH. More about this matter is discussed in the blood metabolites.

As we have seen, the mineral supplement intake was not affected for the VM and was affected for SH.

Blood metabolites

In our outcomes we saw that VM increased the serum level of albumin, which is labile protein reservation (Eckersall, 2008). According to Eckersall (2008), albumin is often increased by several situations as illness, dehydration and crude protein consumption. In our case, where the animals were healthy and had water *ad libitum*, the albumin increment probably happened because there was higher amino acid absorption in the intestine and than the liver produced more albumin. This behavior was noticed by Law et al., (2009) in an experiment where the CP diet content was increased and also the serum albumin concentration increased. Furthermore, as can

be seem in the Table 4 (Chapter 2), the increased the flow of NMIC to the intestine and it probably increased amino acid absorptions and consequently more albumin produced at the liver.

The blood urea is an indicative of the protein metabolism in ruminants, mainly indicating a energy protein ratio into the consumed diet (Hammond, 1992). In the current study, when the animals were treated with VM there was a tendence of decreases the blood urea, which indicates the improvement of the energy protein ratio and utilization of dietary N (Nousiainen et al., 2004). This way, VM seems to improved the use of N into the rumen by the microbes, and than less ammonia-N arrived and became into urea by the liver. Differently of VM, the SH shown a great difference between 15SH and 35SH. This may have been caused by the wide difference in the CP fraction A in the forage (44.2 vs. 36.3% for 15SH and 35SH, respectively; Table 1). As is well-known, the fraction A is a source of NPN that is fastly transformed into ammonia by the microorganisms within the rumem (Orskov and McDonald, 1979; Sniffen et al., 1992). In fact, it did happen in the current experiment (17.9 vs. 11.1 mg/dL for 15SH and 35SH, respectively; Table 2, Chapter 2) and than the ammonia reached the liver and was transformed into urea. This hypothesis can be reinforced whether looking closely to the blood protein results. Once the high levels of blood proteins indicates high protein absorption in the intestine (Law et al., 2009), the higher CP forage content in 15SH (Table 1) was still insufficient to change their concentration. Therefore, the high level of blood urea is due the great portion of CP fraction A on 15SH.

According to Ndlovu et al., (2007), blood glucose has a moderate diagnostic value in the assessment of nutritional status in cattle. However, glucose can vary in situation where its precursor, the propionate, which is derived from the diet, is depressed thus the rate of glucose synthesis is also decreased (Reynolds et al., 2003). Supporting these results, propionate outcomes (Table 2) did not show difference between VM and WVM.

Our outcames shown that NEFA was incresed by VM. According to Adewuyi et al., (2005), high levels of NEFA means mobilization of fat from adipocytes, which it was unexpected because NEFA levels use to increases when the animal is a negative energy balance (NEB). In the other words, the intake of energy should be lower than the requirement and than it probably did not happen once the animals that received VM showed a great ADG, this way the energy intake was above the requirement. The higher blood NEFA level in VM group might be a consequence of the increasing of N flow to intestine as can be noticed in the Table 4 (Chapter 2). The same behaviour was found in a study with dairy cows where was provided high and low RUP supplements, which in consequence increased the flow of protein to the small intestine that increased the blood albumin and also increased the NEFA blood concentration as well (O'Mara et al., 2000). They did not find an explanation for it, but according to Ørskov et al., (1987), the increment of RUP can stimulate the body fat mobilization in dairy cattle to use in milk production. In the current experiment, in fact, it did happen as well, however, the NEFA mobilized probably was used as a source of energy in the muscle (Adewuyi et al., 2005), once that NEFA can be used for this purpose.

Only blood calcium among the minerals was affected for VM treatment. The VM increased the blood calcium concentration and it might be related with the blood albumin concentration, once that part of blood calcium is transported through the bloodstream bound to albumin (McDowell, 2003). Its mean that increasing protein in the diet also increases albumin in blood that, in turn, increases calcium absorption and its blood levels (Rosol et al. 1995). Although calcium does not have a great assessment value of animal mineral status because is mediated by a strong endocrine control (Ndlovu et al., 2007), its levels in blood indicates a better absorption in the intestine (Rosol et al. 1995).Therefore, the increment of blood calcium probably was

mediated by the increases of blood albumin.

In summary, offering VM via mineral supplement increases the animal gain without limiting the mineral supplement intake, which it is a great characteristic of feed additive carrier. Although the SH promoted differences in mineral supplement intake, where 15SH had higher consumption than 35SH, the difference did not affect the VM effects on BW gain. The 35SH showed a higher BW gain than 15SH. Regarding the blood metabolites, VM increases the blood albumin, NEFA and blood calcium. This way, Nellore young bulls receiving VM via mineral supplement improves their BW gain in low and high gain rate (SH).

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Table 1. Botanical composition, forage allowance and chemical composition of marandu grass[1]

Item	WVM[1]		VM	
	15SH	35SH	15SH	35SH
Botanical composition, kg/DM/ha^{-1}				
Forage mass	3895 ± 889[2]	10050 ±1435	3901 ± 887	9624 ± 1675
Leaf blade	1217 ± 360	2261 ± 585	1245 ± 333	2201 ± 587
Dead material	1438 ± 469	4127 ± 1086	1425 ± 428	3728 ± 928
Stem	1240 ± 293	3940 ± 988	1229 ± 244	3694 ± 895
Leaf /Stem	1.02 ± 0.3	0.61 ± 0.2	1.10 ± 0.3	0.61 ± 0.1
Forage allowance, kg of DM/kg of BW	1.46 ± 0.4	5.02 ± 1.1	1.42 ± 0.3	5.08 ± 1.1
Leaf allowance, kg of DM/kg of BW	0.47 ± 0.2	1.10 ± 0.4	0.45 ± 0.2	1.18 ± 0.4
Chemical composition				
CP[3], %	19.5 ± 2.9	15.8 ± 2.5	20.3 ± 2.5	16.0 ± 2.5
A[4], % of CP	43.9 ± 9.6	35.5 ± 9.1	44.6 ± 9.8	37.2 ± 11.9
B, % of CP	42.9 ± 10.4	46.4 ± 9.0	40.9 ± 9.8	45.6 ± 12.5
C, % of CP	13.2 ± 3.3	18.1 ± 2.3	14.5 ± 0.8	17.3 ± 2.9
NDF[5], %	55.2 ± 2.8	58.2 ± 3.1	54.3 ± 2.9	57.9 ± 3.3
ADF[5], %	29.1 ± 2.1	29.2 ± 3.4	27.9 ± 2.3	28.2 ± 3.3
ADL[6], %	6.8 ± 2.2	5.2 ± 2.2	6.1 ± 1.8	5.2 ± 1.9
Ether extract[7], %	5.7 ± 1.6	5.9 ± 1.4	6.2 ± 1.8	5.9 ± 2.2
Ash[8], %	9.5 ± 0.8	6.2 ± 0.4	7.2 ± 0.8	6.4 ± 0.5
TDN[9], %	65.3 ± 4.4	69.4 ± 4.1	67.0 ± 4.0	69.2 ± 4.4
IVTDMD[10], %	78.5 ± 3.1	74.8 ± 4.3	79.1 ± 2.8	75.8 ± 3.2

[1]Treatments: WVM= mineral supplement without virginiamycin; VM= mineral supplement with virginiamycin; 15SH= sward height of 15 cm ; and 35SH= sward height of 35 cm.

[2]Means ± SD were obtained from four samples collection on every each 28 days.

[3]CP = Total N by combustion assay (Leco 2000; Leco Instruments, Inc., St. Joseph, MI)

[4] CP fraction A, B and C according to Orskov and McDonald, (1979).

[5]NDF and ADF = Ankom-220 Fiber Analyzer (ANKOM Technology, USA).

[6]ADL = acording to Van Soest and Robertson (1985).

[7]AOAC (1996).

[8]AOAC (1990).

[9]TDN = Predicted from Valadares Filho et al., (2010) model.

[10]IVTDMD = in vitro truth dry matter digestibility according to Ankom Daisy^{II} (ANKOM Technology, USA).

Table 2. Effect of mineral supplement without (WVM) or with virginiamycin (VM) and sward height (SH) on performance and mineral supplement intake

Item	WVM		VM		SEM	*P*-value[1]		
	15SH	35SH	15SH	35SH		VM	SH	VM × SH
Stocking Rate, AU[2]	6.21	4.59	6.35	4.31	0.29	0.73	<0.01	0.42
Initial BW, kg	253	253	253	253	5.01	0.79	0.43	0.43
Final BW, kg	334	376	347	385	10.8	0.05	<0.01	0.72
ADG, kg/day	0.698	1.021	0.824	1.132	0.03	<0.01	<0.01	0.82
GPH[3], kg/ha	754	696	876	712	36.6	0.17	0.04	0.27
MSIBW[4], g/100 kg of BW/Day	41.8	29.2	48.2	26.1	1.79	0.49	<0.01	0.06
MSIA[5], g/animal/Day	122	96.2	143	86.2	9.96	0.55	<0.01	0.09

[1]VM = effect of virginiamycin; SH = effect of sward height and VM × SH = effect of the virginiamycin and sward height interaction; 15SH= sward height of 15 cm and 35SH= sward height of 35 cm.

[2]AU = animal unit (450 kg of BW).

[3]GPH = gain per hectare.

[4]MSIBW = mineral supplement intake per BW.

[5]MSIA = mineral supplement intake per animal.

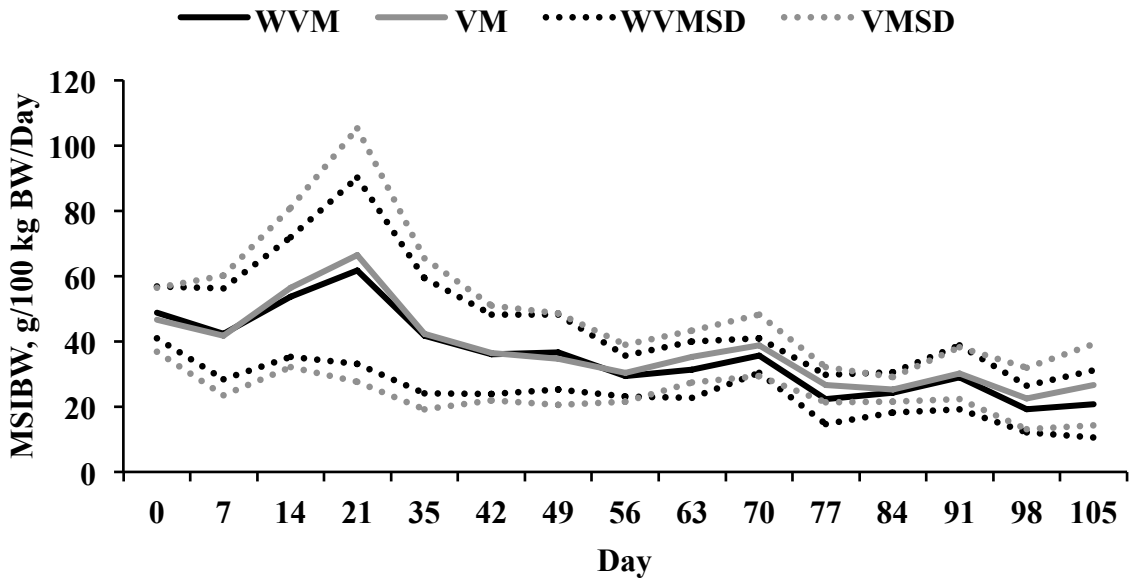

Figure 1. Effect of virginiamycin × Time interaction on mineral supplement intake. There was effect of virginiamycin × Time ($P < 0.001$). MSIBW= mineral supplement intake per BW; WVM = without virginiamycin; VM = with virginiamycin; WVMSD = without virginiamycin standard deviation and VMSD = with virginiamycin standard deviation.

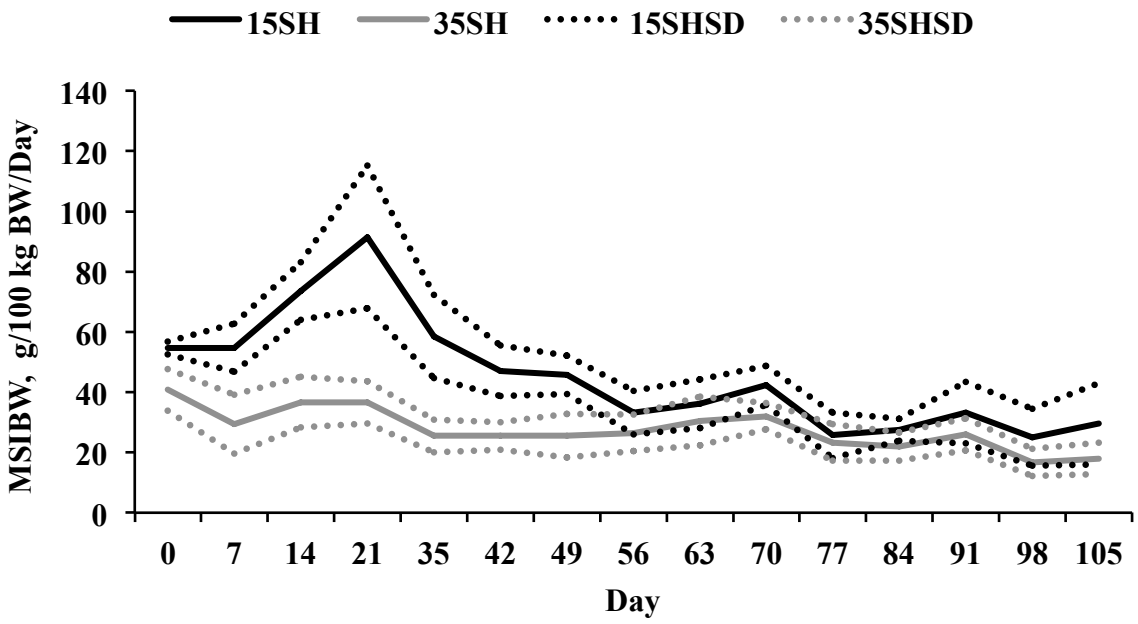

Figure 2. Effect of sward height × Time interaction on mineral supplement intake. There was effect of sward height × Time ($P < 0.001$). MSIBW = mineral supplement intake per BW; 15SH = 15 cm of sward height; 35SH = 35 cm of sward height; 15SHSD = 15 cm of sward height standard deviation and 35SHSD = 35 cm of sward height standard deviation.

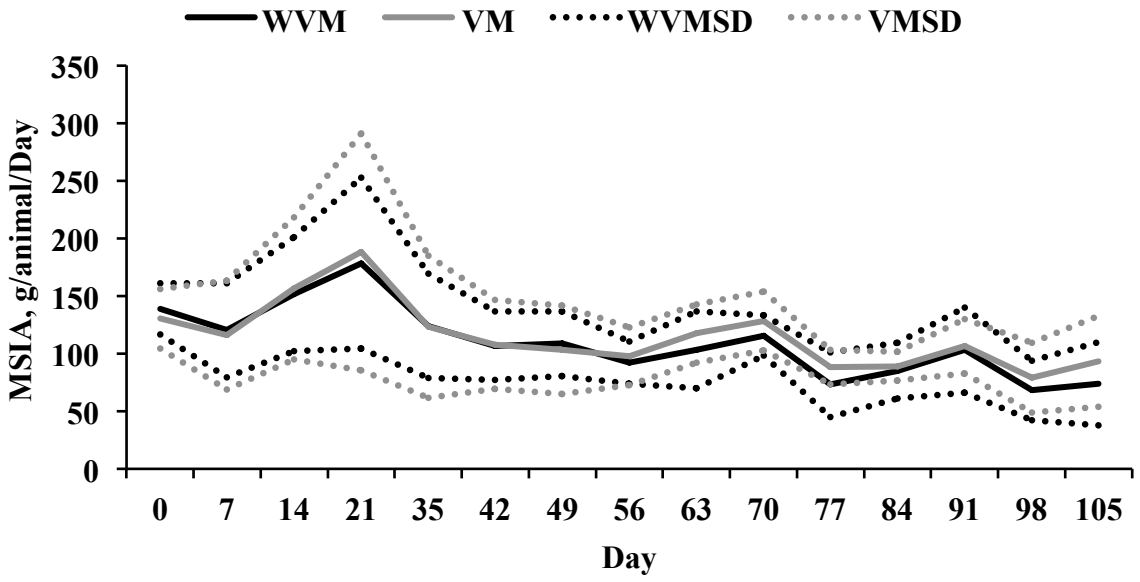

Figure 3. Effect of virginiamycin × Time interaction on mineral supplement intake. There was effect of virginiamycin × Time ($P < 0.001$). MSIA = mineral supplement intake per animal; WVM = without virginiamycin; VM = with virginiamycin; WVMSD = without virginiamycin standard deviation and VMSD = with virginiamycin standard deviation.

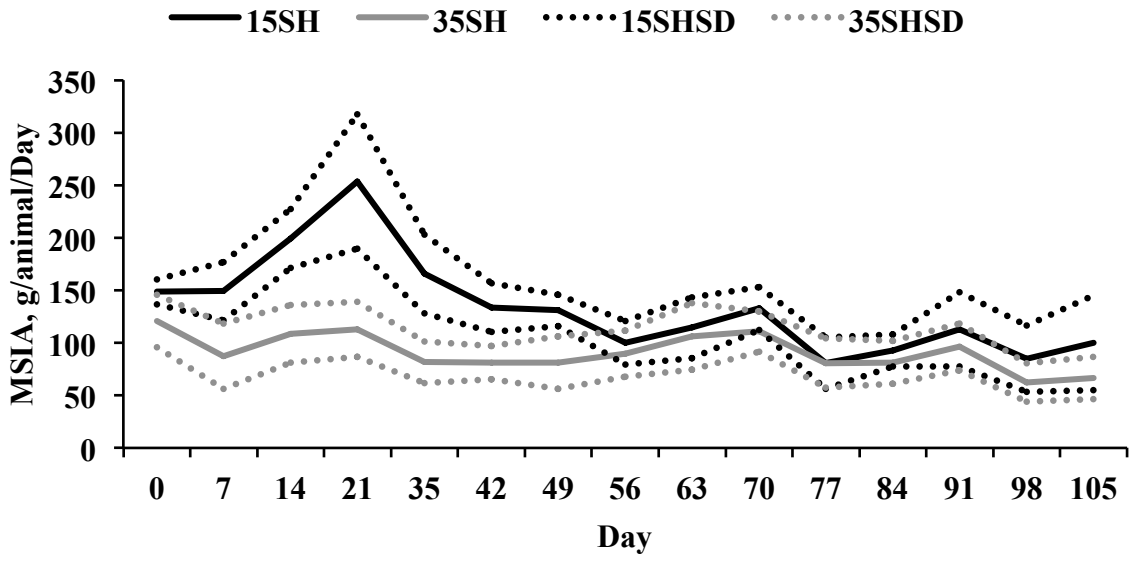

Figure 4. Effect of sward height × Time interaction on mineral supplement intake. There was effect of sward height × Time ($P < 0.001$). MSIA = mineral supplement intake per animal; 15SH = 15 cm of sward height; 35SH = 35 cm of sward height; 15SHSD = 15 cm of sward height standard deviation and 35SHSD = 35 cm of sward height standard deviation.

Table 3. Effect of mineral supplement without (WVM) or with virginiamycin (VM) and sward height (SH) on blood metabolites

Item	WVM		VM		SEM	*P*-value[1]			
	15SH	35SH	15SH	35SH		VM	SH	VM ×SH	T
Albumin, g/dL	2.74	2.81	2.86	2.96	0.03	<0.01	0.07	0.76	<0.01
Total protein, g/dL	8.87	8.66	8.91	8.76	0.21	0.73	0.36	0.88	<0.01
Urea, mg/dL	40.1	33.7	38.7	32.2	1.19	0.06	<0.01	0.99	<0.01
Glucose, mg/dL	75.5	77.3	72.8	82.6	2.88	0.69	0.09	0.23	<0.01
NEFA[2], mmol/L	0.40	0.39	0.47	0.48	0.02	<0.01	0.89	0.65	0.31
BHA[3], mmol/L	0.15	0.15	0.16	0.15	0.01	0.76	0.59	0.67	<0.01
Calcium, mg/dL	8.57	8.93	8.98	8.97	0.10	0.04	0.10	0.08	<0.01
Phosphorus, mg/dL	7.60	7.53	7.62	7.22	0.12	0.42	0.17	0.33	<0.01
Magnesium, mg/dL	2.11	2.32	2.18	2.13	0.10	0.68	0.59	0.38	<0.01

[1]VM = effect of virginiamycin; SH = effect of sward height; VM × SH = effect of the virginiamycin and sward height interaction and T = time effect; 15SH= sward height of 15 cm and 35SH= sward height of 35 cm

[2]NEFA = Non esterified fatty acids.

[3]BHA = beta-hydroxybutyrate.

CHAPTER 3

O artigo a seguir está redigido conforme normas de publicação do *Journal of Animal Science*.

Running head: Virginiamycin via mineral supplement

Effect of virginiamycin via mineral supplement and sward height in growing Nellore young Bulls: Ruminal fermentation, ruminal microorganisms and microbial N flow[1]

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ABSTRACT

The objective of this study was evaluate the effect of virginiamycin (**VM**) via mineral supplement and two swards height (**SH**) on ruminal fermentation, ruminal microorganisms and microbial N production. Twelve Nellore young bulls ruminally cannulated (initial BW = 334 ± 47) were assigned into three 4 × 4 balanced Latin square design to recieve one of those four combination of a 2 × 2 factorial treatments: mineral supplement without VM (**WVM**) or with VM and two SH [15 and 35 cm (**15SH** and **35SH**, respectively)]. Ammonia-N tended to be higher in VM ($P = 0.07$). The DMI was greater ($P < 0.01$) in 15SH than in 35SH. None of ruminal bacteria proportion were affected ($P > 0.10$), except *R. flavefaciens* that tended to increase in 35SH ($P = 0.08$), while total protozoa counts increased ($P = 0.03$) and the *Entodinium* genus tended to increase ($P = 0.05$) in VM. The VM increased uric acid ($P < 0.01$) and tended to increase the purines derivatives excretion (**PD**; $P = 0.07$), absorbed purines (**AP**; $P = 0.07$), microbial N flow (**NMIC**; $P = 0.07$) and PD: creatinine ratio index [PDC index ($P = 0.07$)]. Therefore, the VM effect seems to be more related to the increasing of forage N availability and the microbial N flow to the intestine than in ruminal fermetation.

Key words: feed additive, *Brachiaria brizantha* cv. Marandu, tropical grass, VFA, ruminal protozoa, microbial N flow

1. INTRODUCTION

The use of growth promoters became a great tool of improvement in livestock production wide world. The virginiamycin (**VM**), which is a natural antibiotic. It has been shown that VM improves animal performance (Murray et al., 1992; Salinas-Chavira, et al., 2009; Nuñez et al., 2013; Silva et al., 2004) due to the moderating starch fermentation (Murray et al., 1992), inhibitor of lactate production (Hedde et al., 1982), increment of molar proportion of propionate and anti-protozoa (Nagaraja et al., 1995b) and protein sparing effects (Ives et al., 2002). However, its effect in forage-based systems where the forage is the sole source of N and energy remains unknown once that most part of studies were carried out in feedlots or supplemented grazing ruminants. Moreover, these effects are inconsistent because some studies have been reported differents results (Salinas-Chavira et al., 2009; Coe et al., 1999), mainly its effect against gram-positive (Guo et al., 2010), which includes ruminal fibrolityc bacterias (*Ruminococcus albus, Ruminococcus flavefaciens*) once VM is suppoused to affect them and ciliate protozoa. In addition, none study was found reporting microbial protein production whith VM. According to Storm and Ørskov, (1983), the microbial N account for 50 to 80% of absorbable protein that arrives the small intestine besides it indicates the improvement of feed N availability, which consequently increases the supply of metabolizable protein to animal that can be used to anabolics purpose (NRC, 2001; Detmann et al., 2014). Therefore, we hypothesized that VM enhance the ruminal fermentation, modify profile of ruminal microorganisms and increases the microbial N flow to the intestine. Thus, we evaluated the effects of virginiamycin via mineral supplement and SH on ruminal fermentation, ruminal microorganisms and microbial N production.

2. MATERIALS AND METHODS

Animals were cared for in accordance with acceptable practices and experimental protocols reviewed by the Brazilian College Experimentation (COBEA- Colégio Brasileiro de Experimentação animal) guidelines and approved by the Ethics, Bioethics and animal Welfare Committee (CEBEA- Comissão de Ética e Bem Estar Animal) of the FCAV- UNESP (Protocol number 024583/12).

The study was conducted from December 2012 through May 2013 at the Research Unit of the Agência Paulista de Tecnologia dos Agronegócios (APTA), Colina, Sao Paulo, Brazil.

Animals and Experimental design

Twelve Nelore young Bulls ruminally cannulated fitted with silicone cannula were used in three 4 × 4 balanced Latin square. The animals were ranked according with the BW and assigned into the blocks used the Chapter 1, where the four heaviest animals were randomized into the block I (Latin square I), the next four heaviest animals were randomized into the block II (Latin square II) and the four remaining were randomized into the block III (Latin square III). The initial BW were 376 ± 47, 325 ± 28 and 300 ± 31 kg for Latin square I, II and III, respectively. Twelve paddocks (four paddocks per block) where the cannulated animals were maintained were assigned to receive, in a 2 × 2 factorial arrangement, one of the 4 treatments combination of mineral supplement without (WVM) and with VM (V-Max, Phibro Animal Health, Ridgefield Park, NJ) and 15 and 35 centimeters of sward height (SH) of pasture management. There were four periods with 28 days each and 14 d of adaptation. In the end of the period the cannulated animals were weighted and replaced of paddock according to previous randomizing. Fifteen d before the beginning of the experiment the animals were treated against ecto and endoparasites by administration of ivermectin (Ivomec, Merial, Paulínea, BR).

Pasture management and supplement

The paddocks measuring 1 ha were implanted with *Brachiaria brizantha* cv. Marandu and was used the continuous stocking method of pasture management. The SH target were achieved 45 d before the beginning of the experiment using different animals from the experiment and the SH were maintained using the method of put-and-take using contemporary animals. The sward height was measured each every 10 days using a sward stick. One hundred of points were taken and averaged per paddock. After, whether necessary were taken or added animals to keep the sward height target. In late January, February and April all paddocks received 45 kg of N_2 and 15 kg of K_2O ha^{-1}. The supplement was provided daily at 1400 h in a covered and suspended mineral buckets with 20 cm of edge to avoid get lost the supplement by wind and rain. Before, the refusals were taken and oven dried at 55°C during 72 h to determine the supplement DM. As a virginiamycin carrier was used a complete commercial supplement (Calcium 14%, P 3%, Na 11.4%, Mg 0.25%, S 0.8%, Mn 0.102%, Zn 1700 ppm, Cu 510 ppm , Co 30.6 ppm, I 30.6 ppm, Se 8.5 ppm and 800 ppm of virginiamycin) and water were also offered for ad libitum consumption throughout the experiment. The concentration of virginiamycin was calculated to offer 40 mg/100 kg of BW based in a consumption of 50 g/100 kg of BW.

Sampling and procedures

Were made four (each 28 days) forage sampling where the sample were manually separated the leaf blade, stem and dead material and were oven drying at 55°C during 72 h to estimate de forage mass, botanical composition and forage allowance and at 22 and 24 d were made forage sampling by handling plucking methodology to simulate the diet consumed by the animals to determine the chemistry composition. The pasture samples were collected using the double-sampling method according to Wilm et al. (1944).

Ruminal fluid collection was made at 25 d at 6, 12, 18 and 24 h. A 50 mL sample of ruminal fluid was collected from each animal and squeezed through 2 layers of cheesecloth and the pH was immediately measured. Subsequently, 1 mL of H_2SO (1:1) was added to the ruminal fluid and the solution was stored at –20°C for analysis of concentrations of ammonia N and VFA.

The DMI was calculated using chromium oxide [$Cr_2 O_3$ (Penning, 2004)] as a external marker to predict the fecal output and indigestible NDF (**iNDF**) as an internal marker to estimate the forage digestibility. The chromio oxide was provided from 14 to 24 d in portions of 10 g within the rumen via cannulae always at 1600 h. The first seven days were concidered the adaptaion period and at 21 to 24 d were collected feces once a day (first day at 0600, second at 1000, third at 1400 and fourth at 1800 h). Feces were collected from fresh samples on the ground right after the defecation avoiding grab soil particles. At 22 and 24 d were made forage sampling by hand-plucking methodology to simulate the diet consumed by the animals to determine the chemical composition and then use for calculation. The TDN was calculated according to Valadares Filho et al. (2010).

At 14 and 16 d samples of ruminal contents were collected (at 1600 h) via cannula to measure the protozoa and bacteria population respectively. To measure protozoa, five sub samples were taken (liquid and solid) from different regions of the rumen and then a pool was made and 40 g of solids was placed within a 100 mL tube, added more 10 mL of liquid content and placed within the ice. Immediately after collect, were added 50 mL of formalin solution [1:1- water: 370 mL/L formaldehyde (D' Agosto & Carneiro, 1999)] within the tubes and samples were stored at -20°C. Ruminal bacteria sampling was performed as the same way described for protozoa. Therefore, were collected 20 g of solid and 5 mL of liquid rumen content and placed within the ice. After, were added 25 mL of phosphate saline buffer (pH 7.4), stirred vigorously for 3 min,

and then the contents were filtered with a four layers of cheesecloth. The filtrate was subjected to centrifugation at 16,000 g for 10 min. The supernatant was discarded and the remaining precipitate was re-suspended in 10 ml of phosphate saline buffer, centrifuged at 16,000 g for 10 min and the supernatant was discarded. The precipitate was immediately stored at − 20 °C till the DNA extraction and analysis for real-time PCR.

At 28 d the cannulated animals were taken to individual pens (10 m^{-2}) at 1600 h to spot urine sampling (Chizzotti et al. 2008) to determine the purine derivatives (**PD**) (Fujihara et al., 1987) excretion and estimate the microbial protein synthesis. The urine was collected using a stick adapted with a plastic recipient (500 mL) at the tip. After collect, urine was placed in a plastic pot and stored at 4 °C. Immediately after sampling, subsamples (10 mL) of urine was diluted in 40 mL of a 0.036 *N* H_2SO_4 solution before storage at − 20 °C.

Chemical Analysis

Aliquots of strained ruminal content collected were thawed in the refrigerator over- night and centrifuged at 4°C and 20,000 × *g* for 30 min, and the supernatant was analyzed for VFA according to the methodology described by Palmquist and Conrad (1971) in a gas chromatograph (GC2014; Shimadzu Corporation, Kyoto, Japan) using a HP-INNO wax capillary column (30 m by 0.32 mm; Agilent Technologies, Loveland, CO) at an initial temperature of 80°C and a final temperature of 240°C. The pH of ruminal fluid was measured using a pH meter (DM-23-DC model; DIGIMED – Digicrom Analytic, Sao Paulo, Brazil) and ammonia-N was measured by a colorimetric method according to Chaney and Marbach (1962).

Forage and feces samples were oven dried at 65°C for 72 h, ground in a knife mill (Wiley; A. H. Thomas, Philadelphia, PA) to pass through a 1-mm screen (forage pooled from two hand-pukling

samples and feces pooled from four samples) and analyzed for DM and ash according to AOAC [1990 (method number 934.01)]. The NDF and ADF concentrations were determined using the method of Van Soest et al. (1991) in an ANKOM 2000 Fiber Analyzer (ANKOM Technologies, Macedon, NY) without sodium sulfite and corrected for residual ash. Lignin was measured after hydrolysis of the cellulose in ADF residues in a 72% H_2SO_4 (Van Soest and Roberson, 1985). Forage residue of NDF and ADF was recovered and analyzed for CP and ash for subsequent calculation of TDN. Ether extract was measured according to AOAC [1996 (Gold fish, method number 945.16)]. The estimated *in vitro* true digestibility of dry matter (IVTDMD) of the forage was performed using *in vitro* assay with 0.5 g samples weighted in bags (ANKOM®-F57) and incubated in the apparatus "Daisy-II Fermenter" for 48 h (ANKOM® Technology Corp. Fairport, New York, USA) and subsequently analyzed for NDF (Tilley and Terry, 1963). The CP was determined multiplying the N concentration measured using a Leco F528 N analyzer (LECO Corporation, St. Joseph, MI) for 6.25. The CP fractions were estimted according to Orskov and McDonald, (1979). The results of chemistry composition are described in Table 1. Indigestible NDF concentrations in forage and faces were determined as described by Krizsan and Huhtanen (2013). Samples were weighed (0.5 g) within F57 filter bags and placed within the rumen of a cannulated steer for 288 h and subsequently analyzed for NDF as described above. Faecal samples were evaluated for chromium oxide content by using atomic absorption (Williams et al. 1962).

Direct counts and protozoa identification were performed in a Sedgewick-Rafter counting chamber (Dehority et al. 1989). The samples were diluted with 200 ml/l glycerol and stained using Lugol's solution before counting the cells (D'Agosto and Carneiro 1999). DNA extraction was performed using "Fast spin kit for soil" from MP Bio® according to the manufacturer

instructions and quantified using Nano Drop instrument (Thermo Scientific, Wilmington, MA), based on UV spectroscopy. The amplifications were performed in triplicates and negative controls were run in the assay, omitting the total DNA. Real-time PCR was performed with Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster City, TX). Rox was used as a passive reference dye. Primers concentration were tested to determine minimum primer concentration giving the lowest threshold cycle (**Ct**) and to reduce nonspecific amplification before start the reaction. Were used the follow primers for qPCR: General bacteria [forward (5' CGGCAACGAGCGCAACCC' 3), reverse (5' CCATTGTAGCACGTGTGTAGCC' 3)], *Fibrobacter succinogenes* [forward (5'GTTCGGAATTACTGGGCGTAAA' 3), reverse (5'CGCCTGCCCCTGAACTATC'3)], *Ruminococcus flavefaciens* [(forward 5'CGAACGGAGATAATTTGAGTTTACTTAGG' 3), reverse, (5'CGGTCTCTGTATGTTATGAGGTATTACC'3)] according Denman and McSweeney (2006). *Ruminococcus albus* [forward (5'CCCTAAAAGCAGTCTTAGTTCG'3), reverse (5 'CCTCCTTGCGGTTAGAACA'3) Mosoni et al. (2007)]. *Selenomonas ruminantium* [forward (5'GGCGGGAAGGCAAGTCAGTC'3), reverse (5' CCTCTCCTGCACTCAAGAAAGACAG'3), Khafipour et al. (2009)]. *Prevotella* spp. [forward (5'GGTTCTGAGAGGAAGGTCCCC'3), reverse (5' TCCTGCACGCTACTTGGCTG'3), Stevenson and Weimer (2007)]. Conditions for PCR were 50 °C for 2 min; 95 °C for 10 min; 35 cycles of 95 °C for 15 s; and 60 °C for 1 min. Each conventional PCR mixture (12.5 μL) contained (final concentrations) 1× Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, TX), 600 nM of each primer, and 100 ng of metagenomic DNA. Specificity of amplified products was confirmed by melting temperatures and dissociation curves after each amplification. Amplicon specificity was performed via dissociation curve analysis of

PCR end products.

Purine derivatives (**PD**) were analyzed as the sum of allantoin and uric acid. Allantoin was measured according to Young and Conway, (1942) and uric acid and creatinine were measured using diagnostic colorimetric test kits (Labtest Diagnostica SA, Lagoa Santa, MG, Brazil).

Calculation

The DMI was calculated according the follow equation [1]:

$$\text{DMI (kg/Day)} = (\text{FE} \times \text{iNDF}_\text{f})/\text{iNDF}_\text{fo} \quad [1]$$

In which the FE is the fecal excretion (kg of DM/Day) obteined dividing the provided chromium (g/Day) by the chromium concenration in feces (g/Day); iNDF_f and iNDF_fo is the indigestible NDF concentration in feces and in forage, respectively.

Relative quantification was used to determine species proportion. The results were expressed as a 16S rDNA ratio of general bacteria, according the equation [2]:

$$\text{Relative quantification} = 2^{-(\text{Ct target} - \text{Ct total bacteria})}, \quad [2]$$

In which Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold.

The total urinary volume was estimated according to Souza et al. (2010) using the ratio of creatinine concentration in the urine upon the daily creatinine excretion (**CE**) estimated by the equation [3] proposed by SILVA et al. (2012) as follow:

$$\text{CE} = 0.0345 \times (\text{SBW} \times 0.96)^{0.9491}, \quad [3]$$

in which CE is the daily creatinine excretion (g/Day) and **SBW** is the shrunk BW of the animals. The urinary volume was used only to predict the urea excretion. The estimation of the daily PD excretion was calculated using the purine derivatives: creatinine ratio index (PDC index) according to Chen et al., (2004), as described by equations [4] and [5] below:

$$\text{PDC index} = ([\text{PD}]/[\text{Creatinine}]) \times \text{BW}^{0.75}, \text{ and} \quad [4]$$

$$\text{PD excretion (mmol/d)} = \text{PDC index} \times 0.98, \quad [5]$$

in which [PD] and [creatinine] are PD and creatinine concentrations in urine, in mmol/L and 0.98 is the daily creatinine excretion in mmol/kg of $BW^{0.75}$ proposed by Chizzoti et al., (2008).

The purines absorbed (**PA**) and microbial N flow to the small intestine (**NMIC**) were calculated according to Verbic et al. (1990) and Chen and Gomes (1992) and modifications made by Barbosa et al., (2011) for Nellore cattle as follow in the equations [6] and [7]:

$$\text{PA} = [(\text{PD} - (0.30 \times \text{BW}^{0.75})]/0.80), \quad [6]$$

$$\text{NMIC} = (70 \times \text{PA}/0.93 \times 0.137 \times 1{,}000), \quad [7]$$

in which PA is the purines absorbed (mmol/day) and NMIC is the microbial N flow to the small intestine (g/day).

Statistical analysis

All variables were analyzed as triple 4 × 4 Latin Square. Only ruminal parameters variables were evaluated as repeated measurement, which had four sampling times. The protozoa datas were transformed to log (x + 1) for did not present normality. The model statement used contained the effect of VM, SH and their interaction, Latin Square, period and animal within

Latin square. The treatments and time (ruminal parameters) were considered fixed effect and Latin square, animal and period were considered randomized effect. All analysis were performed using the MIXED procedure (SAS 9.2 version, Inst. Inc., Cary, NC). In repeated measurements, data were tested for the best covariance structure that had the lowest Aikaike information criterion scores and then included in the REPEATED statement to proceed the analysis. Differences between means were determined using the T test. Significance was declared at $P < 0.05$ and tendencies were determined if $P > 0.05$ and ≤ 0.10.

3. RESULTS

Ruminal parameters

The Ruminal fluid pH was not affected by none of treatments ($P > 0.10$; Table 2). However, ammonia-N tended to be higher in VM ($P = 0.07$) and there was an increased ($P < 0.01$) in 15SH. Regarding the VFA, none of them was modified by VM ($P > 0.10$; Table 2). Total VFA's and acetate concentration tended to be higher ($P = 0.08$; Table 2) on 35SH when compared with 15SH and isobutyrate, isovalarate and valerate were higher ($P < 0.01$, $P < 0.01$ and $P = 0.02$, respectively; Table 2) in 15SH than 35SH.

Dry matter intake

Young bulls that received mineral supplement WVM had a similar DMI compared with VM (7.73 vs. 7.30 kg, respectively; P = 0.39). Differently, the SH showed effect (8.20 vs. 6.83 kg for 15SH and 35SH, respectively; P < 0.01), where the 15SH showed great DMI when compared with 35SH.

Ruminal microorganisms

Ruminal bacteria proportion were not affected by none of treatments ($P > 0.10$; Table 3),

except *R. flavefaciens* that tended to increase in 35SH ($P = 0.08$), while total protozoa counts increased ($P = 0.03$; Table 3) and the *Entodinium* genus tended to increase ($P = 0.05$) in animals that received VM.

Microbial nitrogen synthesis

Animals that received VM had higher ($P < 0.01$; Table 4) uric acid excretion and tended be higher ($P = 0.07$, $P = 0.07$, $P = 0.07$ and $P = 0.07$, respectively) in PD excretion, AP, NMIC and PDC index. Urea excretion was higher in animals that were in 15SH ($P < 0.01$).

4. DISCUSSION

Ruminal parameters

The ammonia-N in ruminal fluid is a indicative of protein degradation (Tamminga, 1979) and the effect of VM on it are variable according the previus references. In in vitro studies, in fact, VM shown a reduction on ammonia production (Van Nevel et al., 1984 and Van Nevel and Demeyer, 1990), while that in vivo studies, were not found differences in ruminal ammonia on steers in feedlot system (Ives et al., 2002) and there was even an increment of ammonia-N in sheep fed forage based diet supplemented with barley and receiving VM (Nagaranja et al., 1995b), which is in agreement with our findings that in vivo studies the effect of virginiamycin on ruminal ammonia seems to be unchanged or even increased. In regarding the results from SH, ammonia-N was higher in 15SH. This result is probably related to the high CP fraction A showed for 15SH (Table 1) and already explained previously in Chapter. 1 discussion.

Although VM did not affect the VFA's molar proportion, Nagaraja et al., (1995a) had shown the increment of propionate and decrease of acetate in rumen, which is the expected effect of an additive. However, in the current study no differences were found mainly for propionate

and acetate, which is in agreement with other studies (Coe et al., 1999; Valentine et al., 2000; Salinas-Chavira et al., 2009). The current study shows that even in a forage-based diet, where the forage was the unique source of protein and energy, the molar proportion of VFAs were not changed by VM, which complement the knowledge about VM ruminal action in grazing animals and agree with Salinas-Chavira et al., (2009) that the effect of VM on ruminal parameters are small and does not account for improvement in performance.

All branched-chain VFAs in the current study was changed by the SH, which report the effect of CP forage content and their fractions. According to Cotta and Hespell, (1986), when diets have high RDP, amino acids as leucine, isoleucine and valine are deaminated and theirs carbonic skeletons are used as energy by some groups of bacterias, which the end products are isobutyrate, isovalerate and valerate. Indeed, it did happen once that both the CP forage content (19.6 vs. 17.7%; Table 1) and CP fraction A (44.25 vs. 36.3%; Table 1) in 15SH were greater than 35SH. Additionally, which also could contributed in minor proportion to the greater levels of ammonia-N and consequently blood urea (Table 3, Chapter 1) in 15SH. In regarding to time effect, there was not interaction with the main effects and for that reason the results were not presented and discussed. So far, the outcomes of ruminal fermentation seems to be affected more intensely by the SH than by the VM.

Dry matter intake

The DMI in the current experiment was not affected by the VM, but for the SH. In fact, it was expected. In regarding the SH, however, the averages were the opposite (8.20 vs. 6.83 kg for 15SH and 35SH, respectively) that have shown for to Da Silva et al., (2013) and Barbero et al.,

(2015). According to them, in shorter SH the intake is lower than in the taller SH and as a consequence, the ADG is proportional. Looking to our performance results, the 15SH showed a ADG of 0.761 kg and 1.076 kg in 35SH. Therefore, the results of DMI obteinad herein does not agree with the ADG observed. This way, something has gone wrong in the DMI analysis. To confirm this approach we use the DMI prediction models from Valadares Filho et al., (2010), which was developed with tropical datas and the results are clear. Those animals in 15SH that gained 0.761 kg/Day should intake 6.41 kg of DM/Day and those kept in 35SH that gained 1.076 kg/Day should intake 7.05 kg of DM/Day. Thus, we believe that the iNDF used as an internal marker has a relation with the occurred problem. Usually, the values of iNDF of feces are lower in short SH and higher in taller SH (Barbero et al., 2015, datas were not shown in the manuscript, but were used to obtain the DMI) . However, it was the opposite in the current experiment, where the average of iNDF in feces were 45.8 vs. 35.5 % for 15SH and 35SH, respectively. This contrary behaviour can be explained by the previous experimental condition of the pasture, which had the heights above the criteria (50.2 ± 7.3 cm) at 45 d before the beginning of the experiment. For that reason there was necessity to trim the pasture at the SH desired. However, the remaining dead material (38.4% of the forage mass; Table 1) were pretty higher in both SH when compared with the outcomes from Barbero et al., (2015) and Da Silva et al., (2013) (15.5 and 9.4%, respectively). This way, the animals in 15SH probably consumed more dead material compared with those in 35SH once that according to Gontijo Neto, (2003), taller SH allows the animals select better quality components of the pasture (e.g. leaves) and avoid the consumption of dead material. As a consequence, there was a greater rich material iNDF intake in the short SH resulting in misguided results.

Ruminal Microrganisms

It has been well-known that VM has an anti-protozoal activity (Murray et al. 1992; Nagaraja et al., 1995a; Ngaraja et al., 1995b), however, in the current study was obtained an increment of 60% of *Entodinium* genus and 50% in total protozoa, which was fully unexpected. So far, this study is the unique that has shown the increment of protozoa in ruminants treated with VM. However, it is important emphasize that the current experiment the forage was the unique source of energy and protein, which is different from those studies cited above where the VM carrier or total diet ranged from 17 to 100% of concentrate. Thus, the diet could interfere at the VM effect in protozoa population. Additionally, a point to be raised is related to the ruminal sampling method used. In several *in vivo* studies (Murray et al. 1992; Nagaraja et al., 1995a; Nagaraja et al., 1995b) where the VM anti-protozoa effect were obtained, the sampling method was the stomach tube and the sample was basically liquid. As it is well-known, the protozoa, mainly entodinomorphids remain attached at the particles (Abe et al., 1981; Hook et al. 2012), as a consequence the composition of microorganism in liquid and solid phase can be different (Legay-Carmier and Bauchart, 1989) and could significantly underestimate the size of rumen protozoal population once that the attached protozoa is a significant component of total rumen protozoa (Hook et al. 2012). Besides, the protozoa has the capacity to move through the rumem compatiments chemotactically attracted to fine fiber particles and starch grains (Williams and Coleman, 1992), which could interefere in the sample once that the method does not allow to sampling in differents rumem sites. This way, the sampling method could have been biased the previuos experiments results. In our study the sampling was via cannula and were taken solid and liquid ruminal content from five different rumen regions in a 1:4 ratio. Therefore, we believe that the procedure could influence the results and to fortify this hypothesis, Ives et al., (2002) and Coe et al., (1999), reported no VM effect on ciliate protozoa population using sampling method

via cannula as well. The large increasing of total protozoa in VM treatments is mostly in a consequence of *Entodinium* increment, which increased about 77% in VM treatment. As a percentage of total, *Entodinium* is the most abundant ciliate protozoa. In our study its population was about 75% that it is pretty close the range of 80-98% proposed by Dehority, (2003) and according to Belanche at al., (2012), in an *in vitro* study this genus can participate from 70 to 75% of bacteria breakdown. For this reason, probably the ammonia-N had a increasing in VM treatment once that AA and ammonium-N is released within the medium by protozoa after bacterial lyses (Wallace and McPherson, 1987; Newbold et al., 1997). Thus, it seems that protozoa played an important role in VM effect in the current study.

The main cellulolytic rumen bacteria according to Russel and Hespell, (1981) are *F. succinogenes, R. albus* and *R. flavefaciens* and none of treatment effect was found in the current study in those bacteria. Although Nagajara and Taylor, (1987), had been shown the *R. albus* and *R. flavefaciens* VM susceptibility in an *in vitro* study, few studies have been performed in *in vivo* situations to verify the real effect of VM in the cellulolytic ruminal bacteria. Conversely, well as our findings, Guo et al., (2010), did not find any effect of VM neither in cellulolytic bacteria nor in *Ruminoccocus flavefaciens*, which seems that *in vivo* studies VM does not affect the cellulolytic bacteria proportion. It does not mean that the amount of bacteria was not affected by VM once the current analyze is qualitative and not quantitative.

Microbial nitrogen synthesis

In the current study the microbial N flow to intestine was increased for VM. This way, there was a N supply to small intestine. It represents 36% of increment of N compounds flow to the intestine, which impact in the metabolizable protein uptake once that 64% of it comes from microbial CP (NRC, 2001). According to Detmann et al., (2014), the increment of microbial N

supply often enhance the N forage availability since this increment also increases the portion of total nitrogen that can be used for anabolic purposes besides to enhance the overall efficiency of utilization of metabolizable protein. Thus, it seems that VM enhance the N availability and consequently, more N can be used in maintenance and production. None literature was found reporting VM effect in NMIC. But, regarding to ionophores, which have a similar effect in ruminal microorganisms, showed an increment of N flow to duodenum as well (Rogers et al., 1997). However, in the referred study the increment of N compounds flow to intestine was attributed the lower bacteria predation by protozoa, consequently increased the bacteria yield. In our study, we suggest the opposite once the total protozoa count increased in VM treatment (17.6 vs. 26.4 protozoa $\times 10^4$/mL for WVM and VM, respectively; Table 3) and despite the protozoa have been considered the lesser part (11.9% of total N from protozoa; Sylvester et al., 2005) of N microbial flow toward intestine, the proportion of protozoa in the current study could be greater once that the increasing of forage in the diet also increases the proportion of N flow from protozoa to intestine (Sylvester et al., 2005), which means that in forage-based diet the contribution of N from protozoa to the intestine supply could be greater once that the diet was only forage. This way, we suggest that it account as the major contributor to the ADG found in the current study. As a consequence of the effect of VM in NMIC flow to intestine we also understand that it is the main contributor to the increasing of blood albumin as discussed earlier in the Chapter 1. once that according to Law et al., (2009), the increasing of N supply to intestine in this case via N dietary intake also increases the AA absorption hence the albumin production.

Regarding the urea excretion, the 15SH showed higher excretion than 35SH. Certainly, the greater amount of urea excretion was due the higher CP content in forage in 15SH or rather by the higher CP fraction A found as shown earlier (44.25 vs. 36.30% of N for 15SH and 35SH,

respectively; Table 1) that was transformed in ammonia within the rumen (17.9 vs. 11.1 mg/dL for 15SH and 35SH, respectively; Table 2) and than transformed in urea and hence excreted as urea in urine.

In summary, VM via mineral supplement did not affect the ruminal parameters. On the other side, protozoa was increased by VM as well as NMIC flow to the intestine. Therefore, the VM effects seems to be more related to the supply of N microbial to the intestine and hence to the animal.

5. LITERATURE CITED

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Table 1. Botanical composition, forage allowance and chemical composition of marandu grass[1]

Item	WVM[1]		VM	
	15SH	35SH	15SH	35SH
Botanical composition, kg/DM/ha^{-1}				
Forage mass	3895 ± 889[2]	10050 ±1435	3901 ± 887	9624 ± 1675
Leaf blade	1217 ± 360	2261 ± 585	1245 ± 333	2201 ± 587
Dead material	1438 ± 469	4127 ± 1086	1425 ± 428	3728 ± 928
Stem	1240 ± 293	3940 ± 988	1229 ± 244	3694 ± 895
Leaf /Stem	1.02 ± 0.3	0.61 ± 0.2	1.10 ± 0.3	0.61 ± 0.1
Forage allowance, kg of DM/kg of BW	1.46 ± 0.4	5.02 ± 1.1	1.42 ± 0.3	5.08 ± 1.1
Leaf allowance, kg of DM/kg of BW	0.47 ± 0.2	1.10 ± 0.4	0.45 ± 0.2	1.18 ± 0.4
Chemical composition				
CP[3], %	19.5 ± 2.9	15.8 ± 2.5	20.3 ± 2.5	16.0 ± 2.5
A[4], % of CP	43.9 ± 9.6	35.5 ± 9.1	44.6 ± 9.8	37.2 ± 11.9
B, % of CP	42.9 ± 10.4	46.4 ± 9.0	40.9 ± 9.8	45.6 ± 12.5
C, % of CP	13.2 ± 3.3	18.1 ± 2.3	14.5 ± 0.8	17.3 ± 2.9
NDF[5], %	55.2 ± 2.8	58.2 ± 3.1	54.3 ± 2.9	57.9 ± 3.3
ADF[5], %	29.1 ± 2.1	29.2 ± 3.4	27.9 ± 2.3	28.2 ± 3.3
ADL[6], %	6.8 ± 2.2	5.2 ± 2.2	6.1 ± 1.8	5.2 ± 1.9
Ether extract[7], %	5.7 ± 1.6	5.9 ± 1.4	6.2 ± 1.8	5.9 ± 2.2
Ash[8], %	9.5 ± 0.8	6.2 ± 0.4	7.2 ± 0.8	6.4 ± 0.5
TDN[9], %	65.3 ± 4.4	69.4 ± 4.1	67.0 ± 4.0	69.2 ± 4.4
IVTDMD[10], %	78.5 ± 3.1	74.8 ± 4.3	79.1 ± 2.8	75.8 ± 3.2

[1]Treatments: WVM= mineral supplement without virginiamycin; VM= mineral supplement with virginiamycin; 15SH= sward height of 15 cm ; and 35SH= sward height of 35 cm.

[2]Means ± SD were obtained from four samples collection on every each 28 days.

[3]CP= Total N by combustion assay (Leco 2000; Leco Instruments, Inc., St. Joseph, MI)

[4] CP fraction A, B and C according to Orskov and McDonald, (1979).

[5]NDF and ADF= Ankom-220 Fiber Analyzer (ANKOM Technology, USA).

[6]ADL= acording to Van Soest and Robertson (1985).

[7]AOAC (1996).

[8]AOAC (1990).

[9]TDN= Predicted from Valadares Filho et al., (2010) model.

[10]IVTDMD= in vitro truth dry matter digestibility according to Ankom Daisy[II] (ANKOM Technology, USA).

Table 2. Effect of mineral supplement without (WVM) or with virginiamycin (VM) and sward height (SH) on VFA's, ammonium-N and pH

	WVM		VM		SEM	*P*-value[1]			
Item	15SH	35SH	15SH	35SH		VM	SH	VM ×SH	T
pH	6.53	6.47	6.56	6.50	0.14	0.56	0.22	0.96	<0.01
Ammonia-N, mg/dL	17.3	10.8	18.6	11.3	1.43	0.07	0.01	0.42	<0.01
Total VFA, m*M*	91.7	95.3	92.5	92.8	5.16	0.28	0.08	0.25	0.07
Acetate	64.2	67.8	64.7	65.4	3.56	0.28	0.08	0.25	0.07
Propionate	16.1	16.4	15.9	16.3	1.01	0.68	0.47	0.95	0.01
Butyrate	8.52	8.50	8.81	8.50	0.54	0.61	0.53	0.59	0.03
Isobutyrate	0.68	0.67	0.78	0.66	0.03	0.38	<0.01	0.32	0.56
Isovalerate	1.32	1.11	1.38	1.18	0.10	0.11	<0.01	0.97	<0.01
Valerate	0.78	0.75	0.81	0.75	0.04	0.32	0.02	0.77	<0.01
Acet:Prop	4.01	4.15	4.11	4.04	0.09	0.80	0.78	0.58	<0.01

[1]VM= effect of virginiamycin; SH= effect of sward height; VM × SH= effect of the virginiamycin and sward height interaction and T= effect of time; 15SH= sward height of 15 cm and 35SH= sward height of 35 cm

Table 3. Effect of mineral supplement without (WVM) or with virginiamycin (VM) and sward height (SH) on ruminal bacteria and protozoa population

	WVM		VM		SEM	*P*-value[1]		
Item	15SH	35SH	15SH	35SH		VM	SH	VM × SH
Bacteria[2], %								
F. succinogenes	0.005	0.004	0.005	0.007	0.0012	0.34	0.79	0.31
R. albus	0.002	0.003	0.002	0.003	0.0007	0.95	0.27	0.94
R. flavefaciens	0.002	0.004	0.002	0.004	0.0009	0.71	0.08	0.92
S. ruminantium	0.067	0.084	0.042	0.110	0.0017	0.98	0.13	0.38
Prevotella spp.	11.8	9.82	11.3	20.4	3.13	0.27	0.64	0.21
Protozoa[3], × 10^4/mL								
Entodinium	11.5	13.8	21.8	18.8	2.56	0.05	0.86	0.27
Epidinium	1.16	0.80	1.31	0.40	0.35	0.23	0.69	0.20
Dasytricha	0.91	1.04	1.31	1.60	0.24	0.83	0.61	0.33
Isotricha	0.66	0.69	0.79	0.82	0.22	0.31	0.65	0.78
Charonina	0.32	0.26	0.39	0.56	0.19	0.31	0.03	0.08
Metadinium	0.14	0.13	0.10	0.17	0.08	0.73	0.70	0.33
Ostracodinium	0.34	0.18	0.05	0.26	0.09	0.41	0.45	0.12
Eremoplastron	0.58	0.45	1.27	1.14	0.31	0.27	0.84	0.45
Polyplastron	0.07	0.32	0.19	0.13	0.11	0.59	0.76	0.44
Eudiplodinium	0.37	0.16	0.33	0.28	0.10	0.75	0.23	0.11
Diplodinium	0.18	0.80	0.49	0.21	0.25	0.37	0.71	0.43
Total	16.4	18.8	28.2	24.6	3.10	0.03	0.93	0.37

[1]VM = effect of virginiamycin; SH = effect of sward height and VM × SH = effect of the virginiamycin and sward height interaction; 15SH= sward height of 15 cm and 35SH= sward height of 35 cm.

[2]Relative quantification = $2^{-(\text{Cttarget}-\text{Cttotal bacteria})}$.

[3]Protozoa direct counts.

Table 4. Effect of mineral supplement without (WVM) or with virginiamycin (VM) and sward height (SH) on purine derivatives, urea excrecion and microbial N flow

	WVM		VM		SEM	*P*-value[1]		
Item	15SH	35SH	15SH	35SH		VM	SH	VM × SH
Allantoin, mmol/day	54.2	65.6	75.4	70.5	6.21	0.16	0.71	0.36
Uric acid, mmol/day	11.4	12.2	17.3	14.7	0.86	<0.01	0.48	0.17
PD[2], mmol/day	67.4	78.5	93.5	87.5	6.61	0.07	0.78	0.36
AP[3], mmol/day	54.4	68.9	87.5	80.6	8.23	0.07	0.74	0.36
Urea excretion, g/day	111	74	115	69	5.15	0.82	<0.01	0.32
NMIC[4], g/day	29.9	37.8	48.1	44.3	4.51	0.07	0.74	0.36
PDC index[5]	68.7	80.1	95.4	89.3	6.72	0.07	0.77	0.36

[1]VM = effect of virginiamycin; SH = effect of sward height and VM × SH = effect of the virginiamycin and sward height interaction; 15SH= sward height of 15 cm and 35SH= sward height of 35 cm.

[2]PD = total urinary excretion of purine derivatives (allantoin + uric acid).

[3]AP = absorbed purine derivatives.

[4]NMIC = microbial N flow to the small intestine.

[5]PDC index = (PD\Creatinine) × $BW^{0.75}$

CHAPTER 4 – PRACTICE IMPLICATIONS OF USE OF VIRGINIAMYCIN IN GRAZING SYSTEM

As noted in the two chapter written previously, when VM is offered via mineral supplement, in fact, does increase the ADG. Therefore, the VM works well in a grazing system of growing beef. However, the use of this kind of technology must be done in farms companies that already have a great pasture management which allows the intake of material of good chemistry composition once that the VM becomes the forage nutrients more available. This way, to be successful in this matter, the first step is have a productive and well managed pasture.

When we are talking about a technology to be applied in an operation, a important point to be raised is the cost of implementation which also is a great concern about the use of additive. This way, some matter related to the use of VM in grazing system are discussed below.

At farms which already have a structure of mineralization as mineral buckets, the higher cost would be only to buy the additive once would not be necessary to build this structure and as the additive is added into the mineral supplement, the cost of offering (fuel, tractor and staff) remain the same, as nothing else is necessary to spend.

Based in the idea that the sole cost is the aquisition of VM, here was done a calculation to show the cost and the income to apply this technology. Using the datas from the current experiment we obtained that the cost to add the VM in the mineral supplement was R$ 0.08 per day per animal (Bulls consuming 115 g per day per animal in VM treatment) and the difference of gain from control was 11 kg from the

control treatment in the end of the experimente (112 d). Considering the @ prices of R$ 148.67 and carcass yield of 50%, the income promoted by VM was R$ 54.51 minus R$ 8.96, which is the cost of inclusion of VM in the supplement, we have a profit of R$ 45.55. Therefore, the use of VM in this experiment resulted in a profit of R$ 45.55 for each animal in the end of the period. It is important to emphasise that this calculation only provides information for the additional cost of using VM. A more accurate calculation must be done using the whole cost involved in the growing season as animal aquisition, cost of staff between others.

As discussed earlier in the Chapter 1, another important matter is regarding the mineral supplement offering. To obtain a great animal performance with whatever feed additive provided via mineral supplement, the mineral buckets must has an easy access for cattle often visits, enough linear space of edge (4 cm per 450 kg of BW), covered to avoid rain and sun, a wide edge to protect from wind and located in a dry and clean place where the animals often pass. This manner, the use of additive will be well successful.

In summary, VM enhances the beef cattle performance and has a great economic viability. Therefore, the use of VM is a great tool of improve the beef cattle production in grazing system.