EVALUATING THE EFFECT OF COPPER DEFICIENCY ON PINK EYE SUSCEPTIBILITY AND THE USE OF ENSILED JOHNSONGRASS AS WAYS TO IMPROVE BEEF CATTLE PRODUCTIVITY IN THE SOUTHEAST

by

CAMILA SOUSA DA SILVA

(Under the Direction of Robert Lawton Stewart, Jr.)

ABSTRACT

Minimizing costs of feeding and prevention of diseases in cattle herds can determine the success of beef cattle production. Therefore, two studies were undertanken to determine whether copper deficiency increases the susceptibility to acquire pink eye, a common and highly-infectious disease in cattle herds; and to investigate the use of Johnsongrass (*Sorghum halepense*) as an alternative ensiled forage. In experiment 1, copper deficiency was experimentally created over 94 days and animals were challenged with *Moraxella bovis* at the end of feeding period. In experiment 2, Johnsongrass was harvested at four maturity stages (3 weeks, boot, flower, and dough) to evaluate the effects on yield and quality. Our findings suggest that copper status plays a role on activation of the imune response. In addition, it was demonstrated that Johnsongrass can be ensiled, but quantity and quality is balanced by harvesting Johnsongrass before the flower stage.

INDEX WORDS: Copper, Pink Eye, Johnsongrass, Maturity Stages

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CAMILA SOUSA DA SILVA

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CAMILA SOUSA DA SILVA

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|---|---|
| Major Professor: | R. Lawton Stewart, Jr |
| Committee: | D. W. Hancock |
| | B. C. Credille |

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
August 2018

## DEDICATION

I dedicate this thesis to my parents, Cosma and Lucinaldo, my greatest examples of persistence and courage; and to my true friends, who made me feel closer to home by their constant presence throughout this challenge.

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# CHAPTER 1

# INTRODUCTION

Cattle production represents the most important agricultural activity in the United States. According to the United States Department of Agriculture (2016), this business generated $78.2 billion in cash receipts in 2015. Currently, the cattle inventory is approximately 94 million head and produced over 11 million kg of beef in 2017 (National Agricultural Statistical Services, 2018a, 2018b). Typically, this production comes from animals produced in a cow-calf system, where calves are initially raised in pasturelands and sent later to feedlots to be finished and slaughtered.

In both grazing and feedlot systems, nutrition and health are the most critical and costly factors in cattle production (Parker Gaddis et al. 2014; Martello et al. 2016). The United States Department of Agriculture (2018) estimated that in 2017 the total operating costs per cow in a cow-calf system was $560.00 and the expenses related to feed and animal health were $368.00 and $24.50, respectively. Hence, feed and health represent 70% of the total operating costs of the animal. Therefore, it is always desirable to find new alternative forage crops to help decrease the costs associated with feeding and prevent diseases in the herd to enhance profitability.

In regard to feeding, the genus *Sorghum* sp., part of the Poaceae family, contains species that have been cultivated for grain production or inclusion in animal diets as an alternative energy source to corn (Adewakun et al. 1989; Cattani et al. 2017; Wood et al. 2011). With its introduction and popularization in that sector, Sorghum (*Sorghum bicolor* (L.) Moench) became one of the most produced crops around the world. The Crop Production Report released by the United States Department of Agriculture (USDA National Agricultural Statistics Service, 2017) showed that the

sorghum area planted for all purposes (grain, forage, ethanol) and harvested for grain in the country between 2014 and 2016 was over two million hectares, and the area designated to silage production surpassed four million acres.

In addition to grain and forage Sorghum, there is another Sorghum species that is widely grown and of great importance for its impact as a weed and potentially alternative forage source: Johnsongrass (*Sorghum halepense* (L.) Pers.). Johnsongrass is primarily known for its weediness in row crops and hay fields and resilience to eradication practices, traits that make it a major weed in 53 countries around the world (Hartzler et al., 1991). In the United States, the occurrence of Johnsongrass has been reported in all states except Minnesota (USDA Plants Database, 2017).

A survey undertaken by McWhorter (1993) from 1976 through 1991 in Arkansas, Mississippi, and Louisiana revealed that about 75% of soybean fields in Arkansas were infested by Johnsongrass, whereas Mississippi and Louisiana had about 90% of soybean fields invaded. Infestation in cotton fields varied from 55 to 90% in the three states. At that time, these numbers represented an estimated reduction in average annual value of harvested cotton and soybeans of $5.8 ± 1.9 million and $23.7 ± 0.6 million, respectively, in the studied states. Though the introduction of varieties of alfalfa and row crops tolerant of glyphosate has greatly reduced the threat, Johnsongrass is still a significant weed in hay fields.

Johnsongrass is native from the Mediterranean region and was first introduced in the U.S. in the 1830's as a potential forage crop. However, with its wide dispersion and losses brought to other crops, producers and researchers turned their efforts to discover best management practices for eradicating it from fields rather than using it as forage. However, Johnsongrass characteristics justify the attempts of incorporating it into forage systems and animal production: it is a perennial

warm-season grass widely adapted in the US, palatable to cattle, has an adequate nutritive value, and can be grazed with proper management (Rankins and Bransby, 1995).

According to Heath et al. (1985), hay production is one of the primary uses of Johnsongrass. Nonetheless, adopting this method for conserving forage in the Southeast region can be challenging due to the high humidity occurring on the months when forage yield and drying conditions allow for hay production. For Johnsongrass, the morphological characteristic is a troublesome factor, since its plants have a thick culm (0.5-2.0 cm in diameter; Warwick and Black, 1983) that tends to retain moisture and prolongs the drying time normally required for proper hay curing.

Consequently, alternative methods for conserving Johnsongrass for use as a forage may be helpful. A common process employed to conserve forage and possibly successful for Johnsongrass is baled silage, or baleage. In baleage production, the forage is cut with the same machinery used for hay, wilted until moisture content reaches about 50-60% and wrapped in plastic to allow fermentation (Burns and Fisher, 2012).

Studies exploring the use of Johnsongrass as either fresh or conserved forage are limited. Rude and Rankins Jr. (1993) were able to demonstrate the use of Johnsongrass in the form of silage as a forage source in sheep diets, but no work has been done to evaluate Johnsongrass silage for cattle feeding. Since eradication of Johnsongrass from hay and row crop fields is difficult, its utilization as silage would enable its use as forage as an alternative to controlling it. This could result in reduced losses in major crops and ultimately increase the amount of high quality conserved forage produced in the Southeast.

Besides trying to minimize the costs associated with animal feeding, producers and industry often aim to find means to avoid the dissemination of diseases that can affect animal

performance, such as infectious bovine keratoconjuctivitis (IBK). Infectious bovine keratoconjuctivitis, popularly known as "pink eye", is a widespread disease that has significant impact on the productivity of cattle herds. The pain and discomfort provoked by pink eye leads to decreased body weight gain in affected animals and increased costs with medications to treat the disease (Ali et al., 2012; Snowder et al., 2005).

Infectious bovine keratoconjunctivitis is mainly associated with eye infections caused by *Moraxella bovis*, a gram-negative bacterium. However, two other bacterial species have been indicated as etiological agents of this illness in cattle, *Moraxella bovoculi* and *Moraxella ovis* (Loy and Brodersen, 2014). There are many ways by which IBK can be transmitted. These include direct contact, nasal and ocular discharges, and mechanical vectors. The most important vector is the face fly (*Musca autumnalis*), able to harbor *M. bovis* on their legs for up to three days (Gerhardt et al., 1982; Kopecky et al. 1986). After cattle are exposed to at least one of these means of transmission, *M. bovis* will rely on a mechanical irritation of the cornea and intrinsic mechanisms that allow attachment, survival, and multiplication of its cells to the bovine ocular tissue.

A large number of factors predispose animals to IBK including age, breed, season, mechanical irritation (dust, grass, weeds, etc.), eye pigmentation, concurrent pathogens, environment, and host immune response (Ali et al., 2012; Senturk et al., 2007; Snowder et al., 2005). In addition to these factors, it has been suggested that trace mineral deficiencies, such as copper (Cu) deficiency, can play a critical role in IBK due to its role in the immune response to invading pathogens (Postma et al. 2013, 2016). The importance of Cu to immune function is probably the most investigated role of Cu in the organism of different species. It has been reported, for instance, that neutrophils (cells from defense system) are provided with Cu-containing enzymes to protect the body against infections and, at the same time, avoid oxidative destruction of their

cells caused by over-production of oxidizing compounds (such as $H_2O_2$) that are used to kill pathogens (Cerone et al., 1998).

Despite the complexity of understanding the role of Cu on immune response, it has been shown that Cu deficiency impairs the function of innate and adaptive immune systems (Arthington et al. 1996; Cerone et al. 1998; Postma et al. 2016). In relation to the immune defense mechanisms utilized in specific areas as the eyes, the first barrier to prevent microbial invasions is the eyelid closure and washout by tears. At the same time, the epithelium itself acts as a barrier because of its intercellular tight junctions and constant renewal of its cells. Besides those actions, the local antibodies secreted (immunoglobulin G - IgG, immunoglobulin A - IgA), the complement system, the tear film, and other attributes of the immune system are used to initiate an immune response in ocular tissues (Gilger, 2008). However, Postma et al. (2013) could not detect a bactericidal activity of lachrymal secretion and the complement system in Cu-deficient bovines infected by IBK in vitro. This outcome suggests that the mechanism underlying ocular immunity in Cu-deficient bovines needs further investigation.

Based on the ideas introduced, this thesis explores the aims of two different studies. The first study, described in Chapter 2, evaluated the effects of Cu status of beef cattle on susceptibility to IBK infection in vivo. Our hypothesis is that copper deficiency is a key player in IBK through immunosuppression. The second study, presented in Chapter 3, assessed nutritive value, fermentation characteristics, and parameters of fermentation *in vitro* of Johnsongrass ensiled at four maturity stages. We hypothesize that Johnsongrass can be conserved as silage, but maturity stage has a considerable impact on quality of ensiled Johnsongrass.

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CHAPTER 2

REVIEW OF THE LITERATURE

## The Relationship Between Copper Status and Health of Cattle

### *Infectious Bovine Keratoconjuctivitis (IBK or Pink Eye)*

Infectious bovine keratoconjuctivitis (IBK), popularly known as pink eye, is a widespread disease that has significant impact on cattle herds. The pain and discomfort provoked by IBK leads to impairment of body weight in animals infected and increased costs with medications to treat the disease (Ali et al., 2012; Snowder et al., 2005).

Pink eye is mainly associated with eye infections by *Moraxella bovis*, a gram-negative bacterium. However, two other bacterial species have been indicated as etiological agents of this illness in cattle, *Moraxella bovoculi* and *Moraxella ovis*. In the United States, the occurrence of those bacteria has been identified in 29 of 30 states evaluated for a pink eye diagnosis in cattle herds from 2010 to 2013, with a total of 282 herds tested. Each one of the 29 states had at least one *Moraxella* sp. detected (Loy and Brodersen, 2014).

There are many ways by which pink eye can be disseminated but direct contact with nasal and ocular discharges and mechanical vectors are the two of the most important. The main vector of IBK is the face fly (*Musca autumnalis*), able to harbor *M. bovis* on their legs for up to three days (Gerhardt et al., 1982; Kopecky et al. 1986). After exposure to these means of transmission, the pathogenicity of *M. bovis* on the organism will be determined by two major factors: the presence

of cell components called fimbriae (type IV pili) on the bacteria's surface and the release of β-hemolytic cytotoxins.

The pili are filamentous appendages composed of repeating and homologous polypeptides that allow *M. bovis* to attach on the cornea surface, preventing the bacterium from being washed away by lacrimal secretations and by the mechanical action of blinking. Thus, this adherence process is the earliest event leading to colonization, infection and manifestation of the disease. The pilliated form of *M. bovis* shows much greater adherence to the cornea tissue than non-pilliated strains, which implies a higher capability of causing infection (Ruehl et al., 1988; Ruehl et al., 1993).

Following attachment, the release of hemolysin will be fundamental to the pathogenicity of *M. bovis*, providing citotoxic activity against neutrophils and corneal epithelial cells (Frank and Geber, 1981), and thus being linked to the cornea damage and ulceration (Beard and Moore, 1994). The mechanism by which hemolysins are able to harm bovine erythrocytes is termed pore-forming cytolysis, wherein hemolysins create transmembrane pores that promote rapid leakage of ions K+ from the target cells followed by cell swelling and onset of the cytolysis (Clinkenbeard and Thiessen, 1991), including monolayer disruption, cell detachment and finally lysis (Gray et al., 1995).

In order to grow on the eye surface, *M. bovis* needs iron to support RNA and DNA synthesis. However, the extracellular content of iron in the host is usually low, because most of the iron is the intracellular fraction incorporated into hemoglobin molecule for oxygen transport and ferritin for iron storage, and the extracellular iron content is bound to the protein-transporters transferrin (Tf) and lactoferrin (Lf). Through those proteins, extracellular iron is kept at levels lower than those necessary to microbial growth. Thus, to acquire iron from the host cells, *M. bovis*

is provided with iron ligants that catches iron directly from Tf and Lf, and so provides this element for its cell multiplication (Fewick et al., 1996; Yu and Schryvers, 2002).

Along with those virulence factors, *M. bovis* produces enzymes that degrade molecules from host cells and may contribute to corneal ulceration. Frank and Geber (1981) investigated hydrolytic enzymes of *Moraxella bovis* and found that all 13 strains of *M. bovis* studied were fibrinolytic, produced hemolysin, and are capable of damaging tissue lipids, mucopolysaccharides, and matrix proteins by producing enzymes that include C4 esterase, C8 esterase–lipase, C14 lipase, phosphoamidase, hyaluronidase, phosphatase, leucine and valine aminopeptidases and gelatinase. Although all the strains were unable to produce collagenase (that would degradate collagen, one of the main corneal components), ulceration observed in IBK appears to be associated with collagen release from epithelial cells, fibroblasts, and neutrophils as a consequence of the cell damage caused by enzymes from *M. bovis*.

Prieto et al. (2013) found that the persistence of *M. bovis* is also associated to the formation of a biofilm, which they defined as a self-produced polymeric matrix within which the bacteria embeds itself. The biofilm allows *M. bovis* to adhere to and grow on the eye and/or nasal cavities of cattle and persist in these niches.

Clinical signs of IBK are seen hours post-infection in one or both eyes and they become more severe within three weeks. The first signs are observed as an increase in lacrimation, photophobia, corneal opacity, and conjunctivitis. As the infection becomes more severe, the animals show worsened blepharospasm, sensitivity to touch, ocular purulent discharge, swelling, and corneal ulceration. Within 1-3 days after ulceration, the healing process may start with vascularisation into the cornea area and it is completed in approximately 70 days, leaving a scar

of 6-8 mm diameter. Cornea ulceration can also result in prolapse, leading to a permanent blindness (Angelos, 2015; Beard and Moore, 1994; Weech and Henshaw, 1983).

A large number of factors predispose the incidence of pink eye, among them age, breed, season, mechanical irritation (dust, grass, weeds, etc.), eye pigmentation, concurrent pathogens, environment, strain of *M. bovis*, and host immune response. For instance, calves are more susceptible to the infection than older cattle, with high level of susceptibility and/or exposure extending to 130 days of age (Ali et al., 2012; Senturk et al., 2007; Snowder et al., 2005); Hereford is the breed more likely to be infected, what appears to be related to eyelid pigmentation. However, all breeds are susceptible to IBK. There is also evidence of a maternal effect on susceptibility to IBK, since crossbred calves whose dams are Hereford have higher incidence than calves of an Angus breed dam (Snowder et al., 2005).

A higher number of cases correspond to an increase in temperatures and UV radiation. In the spring, there is a sharp increase in the number of infected animals, then a peak of reported cases during the summer and a decline in the fall. This pattern is strongly similar to the cycle of the *M. autumnalis* (Krafsur and Moon, 1997; Krafsur et al., 1999; Snowder et al., 2005; Weech and Renshaw, 1983). IBK outbreaks normally indicate an association between *M. bovis* and other bacteria, although their interaction in the clinical development of pink eye has not always been proven. However, Schnee et al. (2015) could demonstrate a high prevalence of *Mycoplasma bovoculi* (up to 92.5%) in IBK-affected herds at the acute stage of the disease, suggesting an interaction between *Moraxella* sp. and this microorganism.

Different strains of *M. bovis* show differing abilities to cause pink eye as well. While the strains FLA-64, EPP-63, HTy-65, WSE-64, IBH-65 and HIM-63 have the strongest capacity of producing keratoconjunctivitis, others do not appear to induce the disease (Pugh and Hughes,

1970). Finally, the host immune response is essential to determine whether the body will have enough strength to fight against the establishment of the pathogen and development of the disease.

*The role of the immune system on host defense*

The immune system, whose basic function is to protect the body against infections, consists of two main components: the innate (non-adaptive) and the acquired (or specific) system. The non-adaptive defense represents the first line of defense for immunity of the body, which is present from birth and does not depend on prior contact with an infectious agent. This immunity is formed by natural barriers, such as mucous membranes and skin, pH, $O_2$, secreted fatty acids (chemical barriers), and they are capable of retarding the establishment of an apparent infection. Some other effective agents of the innate system are blood proteins (for instance, interferons), proteins from the complement system, phagocytic cells (neutrophils and macrophages), other leucocytes (including natural killer (NK) cells), acting in phagocytosis, pinocytosis and inflammatory response towards invasion by a foreign agent (Bonham et al., 2002; Coico and Sunshine, 2015).

On the other hand, the acquired system requires a previous exposure to or immunization with certain substance or antigen in order to provide immunity. Its cells are represented by T cells (which participate in what has been called "cell-mediated immunity") and B cells (humoral immunity). The group of T cells can be divided into cytotoxic T cells, which express molecules called CD8+ on their membranes, and helper T cells, express CD4+ molecules. While helper T cells assist the production of antibodies, enhancing it, cytotoxic T cells have the ability of killing target cells when direct contact between these cells is made. Both subgroups can interact with cells from the innate system through cell-molecules surfaces to recognize and trigger an immune response against antigens (Coico and Sunshine, 2015).

Other important characteristics are used to define innate and adaptive systems. The innate system is designated as antigen nonspecific, because it can protect the body from invasion by a large spectrum of micro-organisms. On the contrary, the cells from adaptive system are programmed to respond only to and to memorize those antigens to which they were previously exposed, guaranteeing a rapid response in case of second-time infection. Plus, this specificity makes the cells distinguish themselves from foreign molecules, preventing them from creating an autoimmune response.

*Copper, importance and functions*

The role of copper in animal nutrition was first identified by Hart et al. (1928), when they pointed out that an inorganic element extracted and isolated from liver was the substance that permitted iron to be used for the synthesis of hemoglobin, preventing anemia in farm animals, especially pigs.

From this discovery on, many other studies had been performed and proved that copper was essential to many biological functions. For example, there are several copper-dependent enzymes, of which the ones with greatest importance are ceruloplasmin, cytochrome oxidases, and monoamine oxidase. Ceruloplasmin is the carrier for copper transport involved in iron (Fe) metabolism and participates in oxidative reactions and acute phase response during inflammation (Halliwell, 1991). Cytochrome oxidades are involved in electron transportation, such as cytochrome c oxidase (CcO), the terminal complex of the respiratory chain that catalyzes the reduction of molecular oxygen to water producing free energy used to translocate protons across the membrane (Steininger et al., 2016). Lastly, monoamine oxidase oxidizes amines to aldehydes for cross-linkage in the formation of collagen and for deamination of norepinephrine and serotonin

(Fisher, 1975). On a larger scale, copper plays a critical role in animal reproduction, growth and immune response.

The first finding regarding the relationship between copper and female reproduction dates from 1936, when it was discovered that ovulation could be induced by intravenous injection of copper salts (Fevold et al., 1936). Since then, it has been shown that copper induces ovulation by stimulating the hypothalamus to release gonadotropin-releasing hormone (GnRH), which acts on pituitary cells to release luteinizing hormone (LH) in female rats either *in vitro* (Hazum, 1983; Gajewska et al., 2016) or *in vivo* (Kozhwski et al., 1990). Also, copper is a powerful releaser of GnRH from isolated hypothalamic granules (Burrows and Barnea, 1982).

Because of the copper-related enzyme function, the status of this trace element in females can affect fetus development as well. De Hann et al. (1994) showed that the activity of superoxide dismutase 1 (SOD1), a copper-dependent enzyme that protects the cells against oxidative damage (Al-Gubory et al., 2016), is high during early lung development and increases strongly by the time of birth, suggesting that the antioxidant system prepares itself to protect the lungs cells from oxidative damage caused by high oxygen inputs at birth. In addition, Graham et al. (1994) supported that link finding that fetal Cu increases as fetal size increases, and Cu concentration in the fetus's liver is less than or equal to Cu concentration in maternal liver through gestation.

In male reproduction, copper appears to be beneficial to semen quality and thus fertility due to its involvement in antioxidant function, although the effects are clearly dose-dependent. Tabassomi and Alavi-Shoushtari (2013) observed that adding 0.032 mg $L^{-1}$ of copper sulfate (CuSO4) to semen extenders might help to preserve the semen upon freezing processes, considering parameters as progressive motility, viability, membrane integrity and total antioxidant

capacity. On the other hand, higher copper concentrations (0.064 mg $L^{-1}$) showed to be detrimental to spermatozoa.

Copper is normally used as a growth promoter in diets of pigs and for poultry industry (Scott et al., 2016; Zhao et al., 2014), which is a well-established concept and would be associated with an antibacterial action of copper in the intestine (Shurson et al., 1990). However, this growth-promoting action might be related to a relationship between copper and hormones involved in growing performance. Wang et al. (2016) investigated the effect of dietary copper on serum growth-related hormones levels and growth performance of pigs and found that average daily gain and serum growth hormone (GH), insulin (INS), and insulin-like growth factor 1 (IGF-1) concentrations were increased significantly in the pigs fed on diets with 100, 150, 200, 250, and 300 mg/kg of copper sulfate, concluding that the improvement of growth was related to these hormones.

In ruminants, the growth-promoting effects of copper supplementation are controversial. In cattle, Dias et al. (2013) performed a meta-analysis of data from 12 different studies and detected that dietary copper does not alter the growth performance of animals at growing-finishing stages, whereas Felix et al. (2012) reported an improvement of 6% in feed efficiency in growing cattle under copper supplementation and fed with dried distiller grains. In goats, Zhang et al. (2008) suggested that the addition of 10 mg of copper per kg of dry matter to a basal diet would enhance the growth performance of cashmere goats.

The importance of copper to immune function is probably the most investigated role of copper in the organism of many species. It has been reported, for instance, that neutrophils (cells from defense system) are provided with copper-containing enzymes to protect the body against infections and, at the same time, avoid oxidative destruction of their cells caused by over-

production of oxidizing compounds used to kill pathogens (Cerone et al., 1998). In their study, Cerone et al. (1998) were able to confirm this assumption, observing that the intracellular content of copper was 72% lower in bovines with molybdenum-induced copper deficiency compared to non-deficient animals in addition to decreased $H_2O_2$ formation, what would be indicative of less enzyme activity.

Complementary results were found by Genther and Hansen (2014), who submitted forty steers to a depletion period of trace minerals by using a control diet (supplemental Cu, Mn, Se, and Zn) and a deficient diet (no supplemental Cu, Mn, Se, or Zn plus Fe and Mo as antagonists). The results pointed that the ability of neutrophils to kill bacteria and the activity of superoxide dismutase were impaired by the trace mineral deficiency.

In addition, copper status may affect lipid metabolism and impact meat fat profile, as demonstrated by Correa et al. (2012) who tested two sources and two levels of copper supplementation in Nelore beef cattle. In general, the copper supplementation changed the fatty acid profile of meat, with a higher proportion of unsaturated fatty acids over saturated fatty acids, and a reduction of cholesterol in the *Longissimus dorsi* muscle. As detailed in Engle (2011), these modifications would be related to an enhanced activity of specific enzymes acting on fat modification and cholesterol biosynthesis plus biohydrogenation of saturated fatty acids.

*Copper deficiency*

The first cases of copper deficiency were reported by Becker et al. (1965) referring to grazing animals in Florida. Nowadays, copper deficiency in cattle is a rather common disorder worldwide and represents a nutritional problem that may cause great economic losses on the cattle production.

Deficiency of copper is usually seen in animals on grazing systems, as indicated in the cases mentioned above, due to low levels of copper in the forage and high levels of its antagonists, mainly molybdenum (Mo), sulfur (S), and iron (Fe; Dermauw et al., 2013). The presence of these elements in the diet is one of the factors able to cause this disorder, thus characterizing a secondary deficiency. Graham (1991) explains that cases of nutrient deficiencies can be divided into primary and secondary. Primary deficiencies are brought out due to an inadequate dietary intake of the determined nutrient, whereas secondary or conditioned deficiencies occur in response to an impaired absorption, retention, or distribution of certain element in the body, which is related to a pre-existing condition or interaction between nutrients (in this case, minerals).

The interaction of copper with Mo and S promotes the synthesis of thiomolybdates, substances produced from Mo-containing compounds and S sources (sulphides resulting from S reduction by rumen bacteria, sulphates or sulfur amino acids) that bind to Cu in the rumen and renders it unavailable. If not enough copper is present in the rumen, the thiomolybdates will be absorbed through the rumen wall and small intestine and will bind to Cu-containing molecules, including enzymes, diminishing their activity and thus leading to deficiency (Gould and Kendall, 2011). This problem is very likely to occur, since the minerals are supplied from different sources (water, soil, supplements, forage) and the content of those antagonists is commonly high, especially for animals in grazing systems.

The USDA's National Animal Health Monitoring System (NAHMS) generated a report in 1991 that gives an overview of the mineral content of forages in the United States. A total of 352 forage samples from 18 states were collected, analyzed, and classified as showing that copper had an adequate level in 36% of the samples, but antagonists such as iron and molybdenum were marginal or high in content for 28.7% and 57.8% of the samples, respectively, indicating potential

risks of reduced copper availability induced by these elements. Although the production systems have become intensified over time, these results still represent the current scenario of mineral deficiencies led by inadequate balancing of trace minerals in cattle diets, especially for cattle produced in systems where the forage is incorporated as a main component.

Consistent interactions among Cu, Mo, and S were found by Dias et al. (2013), whose analysis showed that Dietary Cu had a positive effect on liver Cu of growing-finishing cattle, whereas Mo had a negative effect. Dietary Mo and S impaired average daily gain, but average daily gain was favored by an increased Cu:Mo ratio. Pogge et al. (2014) also observed that the absorption and retention of copper was less for steers fed a high-S diet for 28 days, and they suggest that this type of diet may limit growth and production of cattle in a long-term.

The NRC (2000) established that the requirements for copper may range from 4 mg/kg of diet to 15 mg/kg, but the variation depends on the dietary concentration of Mo and S. However, 10 mg Cu/kg diet should be adequate for cattle when the diet does not contain more than 0.25% of sulfur and 2 mg Mo/kg. In addition to dietary levels of Mo and S, some other factors have to be considered when Cu requirements are intended to be met. Simental cattle show a reduced ability to absorb dietary Cu in comparison to Angus cattle (Fry et al., 2013; Mullis et al., 2003), which implies a higher requirement for the Simental breed. Also, copper is more available in concentrate diets than in forage diets, which means that the requirement established for copper may be reduced for animals fed high concentrate (NRC, 2000). García-Vaquero (2011a) showed that cattle raised in intensive systems where diets were mainly based on concentrate feeds, copper supplementation was not justified. These authors showed that copper supplementation, under such production conditions, promoted high liver copper levels in 90% of the animals and 50% of the herd had liver concentration associated with copper toxicity.

The liver is the main site of copper storage in the body, and thus, liver copper concentration is usually used to detect a copper deficiency. Liver copper levels of less than 20 mg/kg DM are an indication of copper deficiency (Legleiter and Spears, 2007; Underwood, 1981). Measurements of copper concentrations in the liver can be made by liver biopsies, which is currently the most reliable method to determine copper status of animals.

Several symptoms are pointed out as an indicative of copper deficiency, including anemia, reduced growth, depigmentation and changes in the growth and appearance of hair, cardiac failure, bones that fragile and easily fractured, diarrhea, low reproduction characterized by delayed or depressed estrus (Underwood, 1981), and impaired immune response (as mentioned before), although Legleiter and Spears (2007) did not observe the occurrence of depigmented hair, rough hair coats, diarrhea, or leg abnormalities in steers and heifers, even with animals considered severely deficient in copper (liver copper achieving 2.3 mg/kg DM).

*Relationship Between Copper and the Immune System*

Due to the documented association between mineral nutrition and health, the relationship between copper and immune function is probably is the most investigated role of copper in the organism of many species, but there are still many questions to be answered. Despite the complexity understanding the role of copper in the immune response, researches have shown along the years that copper deficiency impairs the function of both innate and adaptive systems, although the majority of recent studies tend to evaluate the effect of copper status on immunity in association with other trace minerals. Thus, the association between copper itself and the immune system, especially in cattle, needs to be better investigated.

Among the changes caused by copper deficiency, there is evidence of reduced intracellular copper levels, Cu,Zn-SOD activity , $O_2$ and $H_2O_2$ production and increased lipid peroxidation in macrophages from copper-deficient bovines (Cerone et al., 2000). In addition, copper nanoparticles induce recruitment of macrophages to a site of infection, inhibit nitric oxide production, and modulate pro-inflammatory cytokines activity (Arancibia et al., 2016).

Cytokines are soluble mediators produced by cells from the innate and adaptive systems that, generally speaking, trigger and amplify the immune response (Coico and Sunshine, 2016). According to Oliveira et al. (2011), cytokines are necessary mediators to drive the local inflammatory response to the infection and injury locations, allowing a proper healing. In macrophages, inflammatory cytokines promote adherence and rolling of circulating monocytes (macrophages' precursors) along the blood vessels before their extravasation, maturation to macrophages and further migration to sites of inflammation (Arancibia et al., 2016; Gibson et al., 2016).

Interesting findings were described by Schafer et al. (2015) regarding cytokines activity in lambs experimentally infected with *Haemonchus contortus* and parenterally treated with a combination of copper and zinc. Infected and treated group (ITG) had a significant increase in serum levels of IL-1, IL-6, TNF-α and IFN-y on days 28 and 42 post-infection compared to the infected group with no copper-zinc treatment (IG). However, on 56 and 70 days post-infection, the treated group had lower levels of these cytokines than infected non-treated animals. These observations support that the infection triggered the production and release of pro-inflammatory cytokines and the initial administration of copper and zinc was unable to oppose these effects, but the second dose allowed observing a more positive response against the parasite through a

reduction of clinical manifestations as well as the content of these inflammatory markers in the animals.

Cerone et al. (1995) demonstrated that Cu deficiency also alters the production of immunoglobulins. In their study, serum production of immunoglobulins IgG in copper-deficient bovines challenged with *Brucella abortus* vaccine was reduced, and antibody titers to this pathogen were decreased by the deficiency. The authors considered that the copper deficiency have impacted negatively the activity of superoxide dismutase (SOD), leading to a smaller respiratory burst into the cells and failure of antigen presentation to lymphocyts T. This would be crucial to the immunity of an offspring generated from a copper-deficient animal, because IgG is the only class of immunoglobulin able to cross the placenta (Coico and Sunshine, 2016).

Later, by testing neutrophil functionality in copper deficient bovines, Cerone et al. (1998) supported the assumptions made in the previous study. Even though the number of neutrophils was not altered by the deficiency, the intracellular copper content decreased 72% in deficient animals, dropping from 0.116 µg Cu/6 x $l0^7$ cells in control group to 0.032 µg Cu/6 x $10^7$ cells in the deficient. Moreover, copper deficiency also reduced SOD activity and respiratory burst, which was determined by the amount of $H_2O_2$ production after cell activation. According to the authors, the lesser SOD activity may have impaired the dismutation of $O_2$ to $H_2O_2$ (which is one of the most important products to damage and kill pathogenic cells during phagocytosis) and this chain of events can cause adverse effects.

Complementary results were found by Senthilkumar et al. (2009), who reported that enzyme activity (SOD and ceruloplasmin) in sheep is enhanced by copper supplementation. Furthermore, copper supplementation modified humoral immune response, in which antibody titers against *Brucella abortus* antigen was greater for supplemented lambs. Plus, copper

supplementation increased the total Ig, IgG and IgM concentration against chicken RBC from 14 to 35 day of post sensitization.

It is also reported in the literature an alteration of lymphocyte populations in the spleen of mice by induced copper deficiency. Lukasewycz et al. (1985) showed that copper deficiency promoted a large increase in the number of B cells when splenomegaly occurred (increase in spleen size and splenocyte number), whereas total T cells remained at similar levels to animals in control group. Moreover, even when spleen size was small in copper-deficient animals, there was a high B-cell compartment, relative to that of T cells, suggesting some alteration in lymphocyte differentiation linked to copper deficiency.

In conditions where copper accumulates in the body to the point that it reaches a toxic level, there is a modification in lymphocyte populations as well, because high copper levels can cause cell alterations that may lead to apoptosis, the process where the cell programs its death. Thus, besides detecting cell apoptosis, Mitra et al. (2013) found that in spleen and thymus of copper treated mice CD4+ cells decreased and CD8+ cells increased significantly in the spleen and thymus, what might indicate accentuated apoptosis in copper-supplemented animals.

Spleen and thymus are two of the component organs of the immune system, and they are referred as the secondary and primary lymphatic organs. The primary organs are those involved in maturation of T and B cells into antigen-recognizing lymphocytes, and they are represented by bone narrow and spleen. After maturation, these cells migrate to the secondary organs (lymph nodes and spleen) and are activated (proliferate and differentiate) (Coico and Sunshine, 2016).

As mentioned earlier, the first barrier to prevent microbial invasions is the eyelid closure and washout by tears. At the same time, the epithelium itself acts as a barrier because of its intercellular tight junctions and constant renewal of its cells. Besides those actions, the local

antibodies secreted (IgG, IgA), the complement system, the tear film and other attributes of the immune system are used to initiate an immune response in ocular tissues (Gilger, 2008). Regarding IBK infections, Weech and Henshaw (1983) have suggested that immunity is more related to production of local antibacterial defense factors than to systemic factors. However, Postma et al. (2013) could not detect a bactericidal activity of lachrymal secretion and the complement system in copper deficient bovines infected by IBK. This outcome and other reports in the literature suggest that the mechanism underlying ocular immunity in copper deficient bovines needs further investigation.

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## Utilization of Johnsongrass (*Sorghum halepense*) As Ensiled Forage

*Characteristics of Johnsongrass (Sorghum halepense (L.) Pers)*

Johsongrass can be found in subtropical areas around the world, as it development is favored by hot and humid climates. Its prevalence has been mostly detected in disturbed lands for agricultural purposes, ditches, and wastelands (Gould, 1951). A detailed morphological description of Johnsongrass was given by Warwick and Black (1983). Overall, they characterize *S. halepense* as a perennial grass with fleshy rhizomes, with long and thick erect culms (0.5-2.5 m tall, 0.5-2.0 cm in diameter); leaves with hairless surface, prominent veins, and leaf sheath that release a waxy secretion; a ligule distinguished by a sheath collar of a fine membrane with a hairy fringe; the inflorescence is initially a compact panicum that later opens and spreads in branches, displaying a pyramidal shape. At initial stages of development, young plants may be mistaken with other sorghum types and corn. Seeds are small and remain enclosed by reddish-brown to black glumes marked with fine lines on the surface.

Johnsongrass seed exhibit dormancy at a certain level, a feature that contributes for survival and ability to overcome adverse conditions throughout the years, ultimately ensuring perpetuation of that specie (Krenchinski et al., 2015). Egley and Chandler (1978) state that Johnsongrass seed can remain viable in the soil for 25 years. Because this can be a real obstacle from a practical standpoint and for experimental procedures in germination studies, seed dormancy in major weeds has become a target of research in the past few years, as an attempt to acquire more information on best practices to break dormancy in specific weeds and utilize it to intensify the success obtained on suppressing their growth in the field (Mohammadi et al., 2013; Krenchinski et al., 2015).

Several methods have been reported to break dormancy in Johnsongrass seed effectively, including chemical treatment with sulfuric acid and potassium nitrate solutions, mechanical

scarification using sandpaper, soaking seeds in distilled water, glume removal, and the association between some of these. Light and temperature are also critical in dormancy breakage and seed germination (Mohammadi et al., 2013; Krenchinski et al., 2015).

Johnsongrass grows in various types of soil textures but performs best in well-drained clay soils with high water holding capacity and a soil pH within 5 to 7.5 (Newman, 1993; Ball et al., 2007). Fertility requirements are medium. Despite not being adapted to poorly drained clay soils, it can withstand short periods of flooding and saline soils (Rocateli and Manuchehri, 2017).

The active growth period is in the summer and fall. As a perennial, its growth ceases when soil temperatures fall below 16ºC, after spreading its seeds. In the following season, when soil temperature returns to 16ºC, new shoots from rhizomes will sprout. Seeds require a higher temperature for germination, around 21ºC (Rocateli and Manuchehri, 2017). Thus, the ability to overwinter and grow back rapidly under favorable conditions is mainly associated with the rhizome production. Alex et al. (1979) explains that shoots arising from buds on rhizomes would be larger on emergence and grow more rapidly than shoots arising from germinating seeds because of their initially greater physical size and supply of stored food. Although the production of new rhizomes is triggered by minimum temperatures of 15 – 20ºC, buried rhizomes can withstand winter temperatures and remain alive as long as the temperatures fluctuate above -3.5ºC (Hull, 1970).

Besides temperature, soil depth has a great influence on rhizome concentration in the soil profile and their survival (Horowitz, 1972). The greatest concentration of Johnsongrass rhizomes is found in the upper 15 cm of depth, and shoot emergence decreases with deeper burial. However, a higher content of stored carbohydrates in winter rhizomes is associated with deeper soil layers, between 30 to 45 cm deep (Horowitz, 1972; Hartzler et al., 1991). The assumption is that

overwintering rhizomes found in the deepest layers decay and have their reserves used once they start being replaced (Hull, 1970).

Factors affecting the aggressiveness of Johnsongrass relative to other species are related to direct competition for sunlight, moisture and minerals (Lolas and Coble, 1982), and the fact that Johnsongrass can harbor some important infectious agents and pests of row crops (such as maize dwarf mosaic virus (MDMV) in corn) as observed by Ghosheh et al. (1996). This same experiment demonstrated that plant origin (disseminated by rhizomes or seeds) could also have an effect on Johnsongrass interaction with other species. Johnsongrass established by rhizomes showed a strong ability to compete with corn and decrease its yield, whereas seedlings had no effect on corn grain yields (Gosheh et al. 1996). Similar responses were obtained by Mistkas et al. (2003), whose work also aimed to study how Johnsongrass established from seeds or rhizomes interferes in corn development.

*Nutritive Value of Johnsongrass for Animal Feeding*

Sorghum plants are commonly grown where moisture is a limiting factor for other species used as an energy source, mainly corn. Fewer studies have examined the forage potential of Johnsongrass relative to several other plants contained its genus, so reports of Johnsongrass-fed animals are scarce. However, there is practical and scientific evidence that it could be incorporated into ruminant diets rather than exterminated from infested fields.

Aumont et al. (1995) provide thorough results indicating the nutritive value of Johnsongrass among 22 other tropical species used as fodder in the Caribbean (Table 2.1). Comparing the tested species, *S. halepense* was classified as one of four with the highest in vitro dry matter digestibility and had the highest energy content.

Similar values were obtained for some of those nutrients by Dillard et al. (2011) evaluating the impact of commercial fertilizer, broiler litter, or grass-legume mixture on Johnsongrass in Alabama (USA). For one of their treatments, consisting of plots where only commercial fertilizer was applied and no clover had been blended, the chemical composition was 10.2% of crude protein, 63.2% NDF, and 36.3% ADF. Additionally, the authors present values of 0.17, 0.85, 37.6 and 4.5 mg/kg for phosphorus (P), potassium (K), zinc (Zn), and copper (Cu) respectively. In the same study, relative feed value (RFV) was also predicted and reported to be 87.9, which is similar to an RFV produced by a medium-quality alfafa hay. In their study, no differences were observed among treatments (control Johnsongrass-clover mixture or fertilizers sources).

Johnsongrass can be grazed or produced as conserved forage, most frequently hay. When utilized for grazing, Rankins and Bransby (1995) were able to show that Johnsongras can sustain average daily gains of up to 0.55 kg for 87 days with steers on a continuously stocked system. It can also be utilized in pastures for co-grazing of goats and sheeps (Animut et al., 2005). Johnsongrass is considered highly palatable in an early stage of growth, but there is a risk of toxicity from fresh-consumed plants (USDA Planta Database, 2017) associated with the synthesis of secondary compounds in leaves and rhizomes that represent an important mechanism of competition with other plants.

Liu et al. (2011) were able to isolate seven compounds in subterraneous parts of Johnsongrass, being three of them phenols (p-hydroxybenzoate, p-hydroxybenzaldehyde, and p-hydroxybenzoic acid), in addition to three types of flavonoids (apigenin, luteolin, and diosmetin), and dhurrin, a cyanogenic compound. In the same study, these metabolites manifested inhibitory activity on the shoot and root growth of lettuce plants (*Lactuca sativa* L. var. *angustata*), besides inducing defects as swelling root tips, discoloration, and a lack of root hairs, which according to

the authors might influence the absorption capacity of the plant and retard its growth and further development.

Dhurrin in both Johnsongrass and forage sorghum (*Sorghum bicolor* (L.) Moench) is a major problem faced by producers when trying to feed these plants to ruminants. When animals ingest and crush leaves and culms of dhurrin-containing plants, this compound is hydrolyzed and prussic acid, or HCN, is quickly produced. HCN is absorbed into the bloodstream and inhibits the transport of oxygen to cells, causing cellular asphyxiation and sudden death of animals (Hoveland and Monson, 1980; Sher et al., 2012). Under conditions of drought, frost, and imbalanced nutrients in the soil, accumulation of dhurrin is even more stimulated. Sher et al. (2012) showed a strong positive relationship between HCN content and N fertilization levels in different varieties of sorghum cultivated in a dry region, with annual rainfall of 538 and 278 mm over the two consecutive years of the study.

Besides the issues associated with poisoning by prussic acid, animals consuming fresh Johnsongrass can suffer with nitrate toxicosis. Certain plants, including Johnsongrass and other sorghum types, tend to accumulate nitrate if submitted to stress (especially drought) and excessive fertilization. The nitrate molecule is converted into nitrite by rumen microbes, but with excessive intake nitrites can accumulate, cross the rumen wall, enter the bloodstream and bind to hemoglobin to form met-hemoglobin (met-Hb), a poor transporter of oxygen in the body (Sidhu et al. 2011). Diets containing above 1% of nitrates are highly toxic (Nicholson, 2011).

The accumulation of nitrates is equally a critical point when feeding Johnsongrass as hay because nitrate levels do not reduce in forage conserved as hay or cut and fed as greenchop, different from the decreased levels of HCN observed in hay or harvested forage. Deaths caused by nitrate poisoning had been reported 6 months after forage had been cut and fed as hay (Nicholson,

2011). Ensiling, on the other hand, can reduce nitrate levels by more than 30% (Nicholson, 2011). Although Johnsongrass can represent a good deal for hay production, its thick stems are a negative point for hay production, as they reduce hay drying rates. In areas of hot and humid summers and frequent rainfall events, as typically occurs in Georgia, this is a fact that needs to be taken into consideration.

Baleage production has become a common practice in recent years and would represent a more suitable solution for utilizing Johnsongrass as conserved forage. In the baling process, forage is harvested at higher moisture level (60% of DM) and bales are wrapped in plastic for fermentation to occur. This technique allows producers to harvest forage within shorter intervals compared to hay, increases quality, and reduces storage losses (Pruitt and Lacy, 2014). These advantages were confirmed by McCormick et al. (2011), who evaluated forage conservation efficiency and lactation response to bahiagrass conserved as outside-stored hay, barn-stored hay and baleage. There was a little difference between initial and final dry matter content of baleage (0.5%), while the outside-stored hay the dry matter content actually decreased from 83.6 to 79.7%. Fiber concentration (NDF and ADF) were higher for outside-stored hay compared to barn-stored hay and baleage, and resulted in a decrease of 12.8% in net energy for lactation. Milk yield was lower for cows consuming outside-stored hay, but it did not differ between barn-stored hay and baleage.

The improvement in quality associated with baleage could be particularly important for Johnsongrass, since sorghum-type plants have greater ADF and ADL levels (Prostko et al. 1998), but no results evaluating the impact of baling process on Johnsongrass quality are available.

*Impact of Maturity on Forage Quality*

The effect of maturity stages on forage quality and consequently on ruminant production is well established. In fact, maturity stage at harvest is considered the most important factor determining forage quality (Atis et al. 2012). As stated by Rinne et al. (1997), advanced maturity is associated with poor quality forage, which is attributed to low digestibility and limitation of intake. At a cellular level, that effect is a response to an increase in the proportion of cell wall carbohydrates in the plant that simultaneously alter chemical composition and digestion characteristics (Van Soest, 1994).

Some of the parameters employed to evaluate forage quality include the analysis of fiber fraction (NDF - neutral detergent fiber, ADF - acid detergent fiber), ADL (acid detergent lignin), and RFV (relative feed value), the last one being an estimation of overall forage quality since it takes into account intake and dry matter digestibility based on ADF (Rohweder et al., 1978). Nonetheless, because ADF is not the most adequate predictor for dry matter digestibility, RFV has been largely replaced by a newest index, relative forage quality (RFQ; Moore and Undersander, 2002, Grev et al. 2017, Neu et al. 2017). Relative forage quality predicts the voluntary intake of available energy when that forage is fed as the sole source of energy and protein. As such, RFQ is a superior index for estimating forage quality and comparing different forages while providing a more accurate prediction of animal performance (Moore and Undersander, 2002).

As a plant grows from vegetative phase to maturity, NDF, ADF, and lignin content increase, whereas more soluble fractions (e.g. protein) tend to decrease, as validated by the findings of Yari et al. (2014). They found decreased leaf portion, leaf:stem ratio, CP and nitrogen to OM ratio (N:OM) in alfalfa hay when plant growth advanced from early bud to late bud and early flower stages, whereas NDF, ADF, and lignin increased. The same pattern is usually followed by

other species and in fresh or conserved forage (Snyman and Joubert, 1996, Rinne et al. 1997, Alstrup et al. 2016).

Changes in composition related with plant maturity represent a dilemma in forage production because mature plants have lower quality but higher yields (Carmi et al., 2006), therefore it is crucial to find the break point where forage yield continues to increase, but quality starts to decline. When one chooses to produce conserved forage as silage, harvest time and its effect on dry matter will be decisive because proper dry matter content is necessary for successful ensiling (Carmi et al., 2005; Miron et al., 2005, 2006).

Yahaya et al. (2002) studied the effect of different moisture contents at ensiling on silo degradation and digestibility of structural carbohydrates of orchardgrass (*Dactylis glomerata* L.) Forage was harvested at early flowering stage and ensiled with different moisture contents: low (40%), medium (65%), and high (76%). All silages tested were considered of satisfactory quality, but the content of hemicellulose, cellulose, pectin and gross energy and water soluble carbohydrates decreased with higher moisture content in the silage. DM losses, although small and within acceptable range (2-3%), increased as moisture content increased. DM digestibility did not change due to elevated moisture, but the digestibility of crude protein was lower in silage with 76% of moisture as a result of higher production of ammonia as percentage total nitrogen due to extended hydrolysis and proteolysis of protein during fermentation. Based on these observations, the authors concluded that wilting forage (increasing dry matter content) before ensiling may reduce these losses and improve digestibility. This is the process adopted for baleage production. Comparing it to the work above mentioned, moisture level of baleage would range between the first two treatments (40-60% moisture, 60-40% DM).

The relationship between plant maturity and dry matter content appears to have a greater effect on sorghum plants. A minimal dry matter content of 24.7% is required for suitable ensilage conditions according to Castle and Watson (1973), but a lower dry matter content has been observed for different varieties of forage sorghum in four maturity stages (panicle emergence stage, milky stage, dough stage, and physiologic maturity stage) in study undertaken by Atis et al. (2012). They discuss that delaying of harvesting time through PM stage might be useful to ensure the fermentation of silage because of higher dry matter accumulation, but since part of this dry matter content is related with the deposition of lignin in thickening stems, forage sorghum should be harvested at the dough stage. Those recommendations have not been defined for Johnsongrass in regard of ensiling process. Despite the most common practice is to cut Johnsongrass in the boot stage (CIAT and FAO, 2016), it has not been defined if the effect of maturity on quality of ensiled Johnsongrass will follow that same trend.

Rude and Rankins (1993) evaluated Johnsongrass and bermudagrass as alternatives to corn for ensiling with poultry litter. In their experiment Johnsongrass was harvested at the boot stage. Despite the fact that the Johnsongrass and poultry litter mixture resulted in silage with lower nutritive value compared to corn+poultry litter, Johnsongrass still produced acceptable digestibilities, was palatable, had adequate crude protein content for all types of cattle with addition of up to 20%, and would be an economical option, since the stands could be utilized for ensiling at lower cost compared to corn and yields of 2-5 tons of dry matter per acre.

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Table 2.1. Nutrient composition of *S. halepense* (Johnsongrass) samples collected across the Caribean region[1]

| Nutrient | Concentration |
|---|---|
| Organic matter, % | 91.2 |
| Crude protein, % | 10.8 |
| Acid detergent fiber, % | 34.5 |
| Neutral detergent fiber, % | 58.6 |
| Gross energy, MJ/kg DM | 18.2 |
| Digestible energy, MJ/kg DM | 11.4 |
| Net energy for maintenance, MJ/kg DM | 5.28 |
| In vitro digestibility, % | 63.6 |
| PDIA[2], g/kg DM | 39.6 |
| PDIME[2], g/kg DM | 48.7 |
| PDIMN[2], g/kg DM | 29.8 |
| Voluntary DM intake by sheep, g/kg MBW | 54.0 |

[1]Adapted from Aumont et al. (1995). [2] PDIA - Protein digestible in the small-intestine supplied by rumen-undegraded dietary protein; PDIME - Protein digestible in the small intestine supplied by microbial protein from rumen fermented OM; PDIMN - Protein digestible in the small intestine supplied by microbial protein from rumen N.

# CHAPTER 3

# EFFECT OF COPPER STATUS ON SUSCEPTIBILITY TO INFECTIOUS BOVINE KERATOCONJUNCITIVIS (IBK) IN BEEF CATLE

[1]Silva, C. S. da, F. Maia, D. Seidel, M., B. C. Credille, S. Czerwinski, D. Hurley, and R. L. Stewart, To Be Submitted to *Journal of Animal Science*

## Abstract

Infectious bovine keratoconjunctivitis (**IBK**) or pink eye is a highly-contagious infection that affects the eyes of cattle and brings economic losses worldwide. Copper (**Cu**) is one of the microminerals that are critical to adequate immune function, being associated with the ability of the body to fight against invasions of pathogens such as *Moraxella bovis*, the IBK-causing bacteria. Thus, the objective was to assess the effect of copper status on immune response to IBK infection in beef cattle. Thirty heifers were assigned to ten pens receiving either a copper-sufficient diet (**Cu+**) or copper-deficient diet (**Cu-**) for 94 days. At the end of this period, animals were challenged with *M. bovis*. Blood and tears samples were collected three days before challenge (**D -3**), on the day of the challenge (**D 0**), and two days post-challenge (**D 2**). Liver samples were collected only on D 0. Complete blood cell count and production of reactive oxygen species (**ROS**) by neutrophils were evaluated. The hepatic concentrations of Cu and Mo indicated that Cu deficiency was successfully created in the Cu- group ($P < 0.05$). No visual signs of IBK were detected, but the number of circulating leukocytes tended to decrease from D 0 to D 2 regardless of diet ($P = 0.0680$), whereas the numbers of segmented neutrophils tended to increase ($P = 0.0886$). Moreover, Cu- heifers had an increase in band neutrophils from D -3 to D 2 ($P = 0.0120$) and tended to have higher monocyte numbers ($P = 0.0602$) compared to Cu+ animals. Lymphocyte numbers significantly decreased from D -3 to 2 in both groups. The endogenous production of ROS declined from D -3 to D 2, but it increased when neutrophils were stimulated with *M. bovis* and *Staphylococcus aureus* ($P < 0.05$). Despite the lack of visual signs of IBK, these changes suggest that *M. bovis* induced activation of an acute inflammatory response and Cu status appears to play a role in recruitment of these cells to the site of infection. Additional studies are needed in

order to elucidate the mechanisms underlying the activation of immune response in cattle challenged with *M. bovis* in vivo.

## Introduction

Infectious bovine keratoconjuctivitis, popularly known as "pink eye", is a widespread disease that has significant impact on the productivity of cattle herds. The pain and discomfort provoked by pink eye leads to decreased body weight gain in affected animals and increased costs with medications to treat the disease (Ali et al., 2012; Snowder et al., 2005).

Pink eye is mainly associated with eye infections by *Moraxella bovis* (Loy and Brodersen, 2014), which can be disseminated among animals by direct contact, nasal and ocular discharges, and by the face fly (*Musca autumnalis*). The fly is can harbor the bacterium in its legs for up to three days (Gerhardt et al., 1982; Kopecky et al. 1986). A large number of factors predispose animals to pink eye, including the host immune response (Ali et al., 2012; Senturk et al., 2007; Snowder et al., 2005). The ability of the body to fight against invading pathogens is, in turn, closely related to the mineral status of the animal. Copper is one of the trace minerals that have been associated to immunocompetence (Cerone et al. 1998; Prohaska and Brokate, 2001; Arancibia et al. (2016). For instance, Cerone et al. (2000) have demonstrated that copper deficiency reduced intracellular copper levels and increased lipid peroxidation in bovine macrophages, as well as impaired their ability to convert reactive oxygen species (ROS) into less harmful compounds. In addition, Arancibia et al. (2016) have recently shown that copper nanoparticles induced the recruitment of macrophages to a site of infection and modulated pro-inflammatory activity of soluble mediators known as cytokines in the innate and adaptative systems

In regard to IBK, little is known about the effects of copper deficiency on immune response of cattle. Postma et al. (2013, 2016) could not detect a bactericidal activity of lachrymal secretion and the complement system in copper-deficient bovines infected by IBK in vitro, but saw that the activity of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) was lower in Cu-deficient animals compared to a Cu-supplemented group. These outcomes suggest that the mechanism underlying ocular immunity in copper deficient bovines needs further investigation. Thus, the objective of this study was to evaluate the effect of copper status on susceptibility to IBK infections in beef cattle in vivo.

## Material and Methods

*Animals and Experimental Diet*

The study was carried out from December 2017 to March 2018 at the UGA Beef Research Unit located in Eatonton, GA. Thirty crossbred Angus heifers with average BW of 272 kg were initially sorted by BW and randomly assigned to ten pens (three heifers per pen) with free access to water. Each pen was considered as an experimental unit and was randomly assigned to one of two treatments: a copper-sufficient diet (Cu+) or a copper-deficient diet (Cu-). Each diet was composed of bermudagrass hay as roughage and a supplement containing dried distillers grains (DDG) and mineral mix (Tables 3.1 and 3.2). The Cu- diet was formulated to induce copper deficiency through the addition of Mo (0.88 g/animal/day) as a Cu antagonist. Animals were fed on the two diets for 94 days, which gives sufficient time for inducing copper deficiency (Bass, 2016).

*Pink Eye Challenge*

After the 94-d feeding period, animals on both treatments were challenged with of *Moraxella bovis* Tifton I (Angelos et al. 2007). Prior to the challenge, *M. bovis* was cultured for 7 days in poor iron media, yielding a final concentration of 4 x $10^{10}$ cells/ mL of media. An aliquot of 100 mL was taken to the field in the morning of the challenge and kept on ice at all times. Prior to challenge, all Cu+ and Cu- heifers had their eyes examined by an ophthalmologist to assure that the corneas had no previous damage. Following examination, the corneal surface was anesthetized with lidocaine HCl 2% (Vet One, MWI Animal Health) directly instilled onto the eye. Then, the right eye was gently scratched by using a wire brush, vertically and horizontally across the ocular globe (providing the mechanical irritation required for the bacteria to install), and sterile swabs were immersed into the inoculum, swirled for a few seconds, and used to wipe the bacteria-containing liquid onto both right and left eyes. The animals were monitored over 10 days for visual detection of pink eye infection (i.e. sensitivity to light, ocular discharge, and initial ulceration). In addition, it had been established that animals would be treated once corneal ulceration had been detected.

*Sample Collection*

Animal weights were recorded at the beginning of the experimental period and on the challenge day. Feed samples were collected at the end of the experimental period. Blood and tear samples were collected on day 3 prior to challenge (D -3), on the day of the challenge (D 0), and on days 2 and 4 post-challenge (D 2, 4). Samples from days -3 and 0 were considered as baselines. Blood was collected using 8.5 mL vacutainer tubes containing acid citrate dextrose (ACD) as an anticoagulant (BD Diagnostics, USA), and it was slowly inverted five times and kept in cooler

with no ice until it was transported to the lab. Blood was tested for complete blood cell count (CBC), and production of reactive oxygen species (ROS). Blood collected for CBC was submitted to the UGA Veterinary Diagnostic Laboratories (Athens, GA) for analysis. Tears were collected using micro-hematocrit capillary tubes (41A2502 - Kimble Chase, USA) by positioning the tube onto the lacrimal lake in the lower eyelid.

Liver samples were collected only on the day of the challenge. Liver samples were obtained through biopsy. The hair between the $9^{th}$-$11^{th}$ intercostal spaces of the right hemithorax and level with the greater trochanter of the femur was clipped with a #40 surgical blade. Next, the skin was cleaned with 4% chlorhexidine scrub and 70% isopropyl alcohol, and the skin over the biopsy site was anesthetized with five mL of 2% lidocaine hydrochloride injected subcutaneously. Following anesthesia, a #15 scalpel blade was used to make an incision through the skin, where a 14-gauge, 15 cm bevel tip biopsy instrument (1406 PGI SuperCore Semi-Automatic Biopsy Instrument) was inserted approximately 6 cm deep. This procedure allowed the collection of 10-12 mg of liver tissue and was repeated, if necessary, to obtain an optimum amount of sample (15 mg) for analysis. The liver tissue was transferred from the apparatus into 8-mL vacutainer tubes with a 19-gauge hypodermic needle and immediately placed on ice for transportation. Liver samples were stored at -20$^{o}$C until the end of the sampling period. Thereafter, the samples were shipped to the Veterinary Diagnostic Laboratory at Michigan State University for analysis of content of trace minerals (Cu, Mn, Se, Zn, Mo, Fe, and Co).

*Preparation and Recovery of leucocytes for Assessment of Cell counts, Cell viability, and ROS production*

Reactive oxygen species was measured from the amount of reactive oxygen species produced by leucocytes, which were obtained by preparation of buffy coats from whole blood.

Upon arrival, the blood was centrifuged for 5 min at 2000 rpm and the plasma was removed. Then, the buffy coat layer was collected with a 10-mL disposable pipette and transferred to a 50-mL conical centrifuge tube, to which 20 mL of sterile water was added to lyse erythrocytes. Tubes were vortexed for 30-40 s and received 20 mL of 2X phosphate buffer solution (PBS) to reestablish osmotic conditions. The tubes were vortexed one more time and spun a second time. After centrifugation, the supernatant was discarded, the cells were suspended with 10 mL of 1X PBS and extra 40 mL of PBS solution was added to bring the volume to 50 mL. After mixing the contents, 50 µL of cell suspension were transferred to a 1.5 mL microcentrifuge tube containing 450 µL of trypan blue (Trypan blue solution, Mediatech, Inc, Manassas, VA). Lastly, the microcentrifuge tubes were vortexed, and 10 microliters of the mix were placed in a hemocytometer for assessment of cell count and viability. At the end of the process, cell concentration was adjusted to $3x10^6$ cells/mL of sterile PBS for use in the ROS production assay.

The production of ROS was quantified through the conversion of colorless dihydrorhodamine-123 (DHR-123) to fluorescent rhodamine-123 (RHO-123). The leucocyte suspension adjusted in the previous step was placed in 96-well flat bottom plates. Each well received 100 microliters and four wells were used as replicates per animal. Plates were divided into four sections (one per animal). Individual columns per section were assigned to the following samples (Fig. 3.1):

1) Endogenous ROS production (columns 1, 2, 3, 8) - RPMI medium, which represented the background sample; medium plus DHR-123, used as dye control; cells in medium only and DHR-123.

2) Induced ROS production (columns 4, 5, 6, 7, 9, 10, 11, 12) - *Moraxella bovis* diluted at 1:50 plus DHR-123; *Staphilococcus aureus* whole killed antigen diluted at 1:100 plus DHR-123;

*S. aureus* antigen diluted at 1:200 plus DHR-123; and cells plus DHR-123 and phorbol myristal acetate (PMA) at $10^{-7}$ M, which is used to induce maximal cellular enzyme activity (Hurley et al. 2006).

Except for column 1, the volume of DHR-123 added to the wells was 10 µL. In addition, *S. aureus* was used as a standard control for comparison with the capacity of generation of ROS by *M. bovis*. After adding those samples, the plates were incubated at 37 º C for 60 minutes. At the end of the incubation, fluorescent conversion of the dye was assessed by a fluorescent plate reader with 485nm excitation filter and 538nm emission. The ROS produced was reported as arbitrary fluorescent units (AFU), and the data acquired from the plate reader was transferred to an Excel spreadsheet.

*Statistical Analysis*

ROS and CBC data were analyzed as repeated measures using PROC MIXED procedure of SAS, in which days of sampling and diets were treated as fixed effects and pens as random effects. The mineral concentrations in the liver were analyzed using the PROC GLM procedure. Means were compared using Tukey's HSD test and differences were considered significant at $\propto = 0.05$.

## Results and Discussion

*Liver Mineral Concentrations*

The concentration of trace minerals in the liver was impacted by the copper level in the diets ($P < 0.05$), as shown in Table 3.3. The concentration of Fe and Cu in the Cu+ group was

significantly higher ($P < 0.05$) than in the Cu- heifers, whereas Mo and Mn were lower. There was also a tendency of lower Co levels in Cu+ heifers ($P = 0.0861$).

The most significant changes were in liver concentrations of Cu and Mo, as expected by the composition of the diets. All animals receiving the Cu- diet had low storage of Cu and reached a high concentration of Mo in the liver (8.58 - 14.62 µg of Mo/g of dry tissue). Concentrations of Cu and Mo are within a normal range when Cu varies from 90 to 350 µg/g of dry tissue and Mo from 0.5 to 5 µg/g (Corah, 1995). Although the Cu + group showed a significantly higher liver concentration of Fe and lower of Mn, both groups had adequate hepatic levels of these minerals. Normal hepatic concentrations of Fe range from 160 to 1000 µg/g of dry tissue; for Mn, these concentrations vary from 9 - 21 µg/g of dry tissue (Corah, 1995). Hepatic levels of Co were above the adequate range in both groups, which is between 0.07 µg/g and 0.3 µg of Co/g of dry tissue. However, hepatic level of Co is not a reliable indicator of the status of this trace mineral in the animal (Corah, 1995). The concentrations of Cu and Mo in the liver indicate that copper deficiency was successfully created within the 90 days of feeding. Our results are in agreement with Genther and Hansen (2014) who could experimentally create Cu-deficient steers for further evaluations in 89 days of feeding. However, they could identify a moderate Cu deficiency within 71 days.

*Complete Blood Cell Count (CBC)*

The results of CBC show an effect of day on the numbers of the majority of white cells circulating in the blood (Table 3.4), but none of the heifers had visible signs of pink eye. The concentration of leucocytes tended to decrease from D 0 to D 2 regardless of diet ($P = 0.068$). This trend follows a tendency of increment in the numbers of segmented neutrophils ($P = 0.0886$) and a significant increase in the number of band neutrophils associated with day of evaluation and diet ($P = 0.0120$). In the Cu+ heifers, no band neutrophils were detected on D -3 or D 2 of the *M. bovis*

challenge. However, circulating band neutrophils were detected in Cu- heifers, with more elevated concentrations on D -3 and D 0 (0.03 x $10^{-3}$ cells/µl) compared to D 2 (0.01 x $10^{-3}$ cells/µl). On D 2, the concentration of band neutrophils was similar ($P$ = 0.6106) between treatments.

Along with differences observed in leukocytes and neutrophils, there was a significant decrease ($P$ = 0.0016) in lymphocyte concentrations from D -3 and D 0 to D 2, as well as an increment in monocyte concentrations from baseline to two days after challenge ($P$ = 0.026). Furthermore, there was a trend ($P$ = 0.0602) for Cu- animals to show a higher concentration of monocytes than the Cu+ group (0.43 x $10^{-3}$ cells/µl versus 0.23 x $10^{-3}$ cells/µl, respectively). No significant effects of diet, day or day x diet interaction were detected on the values of eosinophils ($P \geq$ 0.5195).

All heifers showed leucocyte numbers within the normal range of 8 – 18 x $10^{-3}$ cells/µl, and segmented neutrophils representing between 15 - 45% of the total number of white blood cells, which is the interval accepted for healthy animals (Latimer, 2011; Jones and Alisson, 2007). Nonetheless, the general changes detected in blood cell count before and after inoculation of the animals with *M. bovis* indicate that the immune system triggered an acute inflammation process, possibly due to the experimental practices employed in an attempt to induce keratoconjunctivitis, but also because of the invasion of the pathogen. In both groups, the number of segmented neutrophils tended to be greater two days after the challenge compared to the baseline, but the biggest evidence of activation of an acute inflammatory response is the identification of band neutrophils in the blood of Cu- animals (Jones and Alisson, 2007).

Neutrophils are the most important cell member of the innate immunity with the primary function of phagocytosis and killing of invading microbes at sites of infection to where they are recruited. They also eliminate transformed cells, amplify and modulate acute inflammation, and

are involved in regulation of granulopoiesis (production of granulocytes and generation of mature segmented neutrophils; Latimer, 2011). When an acute inflammation is present and there is an increased demand for segmented neutrophils, immature band neutrophils may be released from the bone marrow (where production and maturation of these cells occur) into the blood circulation and directed to the site of infection or cell/tissue injury (Jones and Alisson, 2007). For instance, Baydar and Dabak (2014) detected a dramatic difference in the numbers of band neutrophils in cows with mastitis and traumatic reticuloperitonitis (RPT) compared to a healthy group of animals.

In normal conditions, the percentage of band neutrophils in relation to the total number of white blood cells is between 0 and 2% (Kramer, 2000). Baydar and Dabak (2014) reported that band neutrophils represented 22.6% and 10.6% of the total population of leucocytes in the mastitis and RPT groups, respectively. In the present study, the presence of band neutrophils in the blood of Cu- animals (although with less intensity) suggests that Cu deficiency may have impaired the functionally of mature neutrophils in the acute inflammatory response. When endothelial cells sense infection and tissue damage at specific sites, they release the pro-inflammatory cytokines tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6) that will activate and induce changes on vascular endothelial cells, leucocytes, and bone marrow, promoting the recruitment and local delivery of cells to the tissue to fight the infection and repair tissue damage (Abbas et al. 2012). In addition, the pro-inflammatory cytokine interleukin-12 (IL-12) is released by resident macrophages to amplify innate immunity and mediate the response of adaptive immunity (Abas et al. 2012; Jimbo et al. 2017).

These early events of immune response were demonstrated in vitro by Jimbo et al. (2017) who have shown that the incubation of bovine neutrophils with *Mycoplasma bovis* stimulated the release of TNF and IL-12 by neutrophils. Another important finding was reported earlier by

Gengelbach et al. (1996), who studied the effect of copper deficiency on the immune response of calves challenged with bovine rhinotracheitis virus (IBRV) two days after weaning. Deficient animals who also had Mo included in their diet showed lowest plasma levels of TNF at weaning and tended to have lowest plasma levels of TNF five days after the challenge. Taking these results together, we can suggest that the initial stress and *M. bovis* challenge induced the release of pro-inflammatory cytokines and promoted acute inflammatory reactions from D -3 to 0 in both groups. However, the effect of *M. bovis* on neutrophils may have been more deleterious in the Cu- animals.

Jimbo et al. (2017) also reported that *Mycoplasma bovis* induced apoptosis (programmed cell death) of neutrophils, causing a reduction of at least 50% in this cell population. Apoptosis is triggered by a number of factors, including the accumulation of reactive oxygen species (ROS) inside the cell that act, among other roles, as signaling molecules for apoptosis pathways (Simon et al. 2000; Schieber and Chandel, 2014). Cerone et al. (1998) noted that Cu deficiency impaired the activity of Cu-superoxide dismutase (Cu-SOD), which keeps superoxide anions from accumulating and harming the cell or inducing apoptosis. Therefore, based on those studies, our results could indicate that Cu-deficient neutrophils are more prone to undergo apoptosis when challenged with *M. bovis*. This could explain why the demand for neutrophils was intensified to the point that the immature forms migrated from the bone marrow to site of infection/injury (in this case, the eye).

As it occurs with neutrophils, monocytes are released into the blood circulation and directed to sites of inflammation, where they mature and become macrophages. The increased mobilization of monocytes from D -3 to 2, which tended to be higher in the Cu- animals ($P$ = 0.0916), may be linked to the changes in neutrophil populations and it is in accordance with the natural, continued migration, and prolonged action of these cells when neutrophil recruitment stops

(Abbas et al. 2012). Besides having phagocytic function, macrophages are the link between the innate immunity and the adaptive immune response: after a microbial agent is ingested by activated macrophages, they will destroy it, generate peptides from microbial proteins, and present them to T lymphocytes to initiate the adaptive immune response (Abbas et al. 2012).

No changes were detected in the number of lymphocytes from D -3 to 0, but the values were lower two days after the *M. bovis* challenge. This early elevation of lymphocytes numbers followed by a decline between D 0 and 2 happened concomitantly to the recruitment of monocytes, suggesting that the colonization of the ocular tissue by *M. bovis* was effectively prevented and explaining the lack of visual manifestation of pink eye in the heifers. The main mechanisms used by the immune system to block the invasion in this study were likely direct elimination of the bacterium by macrophages or, if the bacterium was able to overcome this first defense, "neutralization" of bacterium by B lymphocytes through antibody production, which would avoid the establishment of the infection (Mittrucker et al. 2000; Lyashchenko et al. 2017).

One important factor that needs to be taken into account when looking into these results is that the lack of effect of diet on most of the parameters analyzed as well as the absence of visual signs of pink eye in the animals of this study could be because *Moraxella bovis* may have been weakened in the field.

Prior to transportation and experimental practices, the bacterium was kept in ideal conditions in the laboratory and appeared to have fully developed the mechanisms necessary for its installation in the cornea (such as pilli formation) (Postma et al. 2008), which should have been aided by the mechanical irritation of the eyes of all animals before inoculation. If this was the case, the effect of the diets on the immune competence of the heifers were minimal. The factors that

might have impaired the ability of *M. bovis* to cause infection in our study will need further investigation.

*Reactive Oxygen Species (ROS)*

The endogenous production of ROS is presented in Fig. 3.2. The production of ROS (AFU) in media, media+DHR-123 and cells only were lower on D 2 post-challenge in comparison to the baseline, 3 days before the challenge ($P < 0.05$). This result suggests that oxidative stress was induced on the pre-challenge day, probably as an effect of handling and consequent stress on the animals. However, the decline in ROS produced by the cells on the day of the challenge and even more two days post-challenge (Fig. 3.2-B) suggests that animals became adapted to the sampling practices and, as far as synthesis of free radicals is concerned, stress caused by handling had minimal impact on the oxidant activity of cells.

In contrast, the induced ROS production by different bacterial stimulants (*M. bovis*, *S. aureus*) increased from D -3 to D 2 ($P < 0.005$; Fig. 3.3), but no diet or interaction between day and diet was observed. There was no effect of day, diet, or a day x diet interaction when PMA was used as stimuli ($P > 0.05$). The same response was observed when ROS was corrected for endogenous ROS (response ratio - RR; Fig. 3.4). The increment in ROS produced by neutrophils supports the suggestion of the previous discussion, and indicates that ability of neutrophils to produce ROS was not impaired. Moreover, neutrophil numbers tended to increase from D -3 to 2, which corroborates with changes observed in ROS.

Reactive oxygen species are generated as a product of aerobic metabolism from the electron transport chain or through activation of NADPH oxidase. During inflammatory reactions, neutrophils and macrophages produce a considerable amount of ROS by the enzymatic action of

the NADPH oxidase complex. These include superoxide anion ($\cdot O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxy radical ($OH\cdot$) (Sordillo and Aitken, 2009; Sordillo et al. 2009). The superoxide anion is dismutated to $H_2O_2$ by Cu-SOD. Then, another enzyme, myeloperoxidase, employs $H_2O_2$ in the oxidation of halogens (such as $Cl^-$) and produces hypochlorous acid or other hypohalous acids, which are highly toxic antimicrobial agents (Pereira et al, 2001; Rinaldi et al. 2007).

Superoxide dismutase is actually a family of enzymes that can have different metal elements coupled to Zn in its structure. One of them is the Cu-dependent enzyme Cu,Zn-SOD (Pratt et al. 2015), which raises the possibility of having its function altered by the Cu status of the body. As a consequence, the ability of phagocytic cells to perform their function and/or avoid oxidative cellular damage may be dictated by the functionally of the enzyme (Arthington et al. 1996; Cerone et al. 1998b; Postma et al. 2016). The activity of Cu,Zn-SOD has not been measured in this present study, but there is supporting evidence that ROS would be accumulated in the Cu deficient neutrophils due to reduced activity of the enzyme and conversion of superoxide to the less toxic components, which would induce the activation of apoptosis pathways previously mentioned. Postma et al. (2016) tested the activity of SOD of neutrophils isolated from Cu-deficient cattle and incubated with *Moraxella bovis* and found that the activity of the enzyme was lower in Cu-deficient animals compared to the Cu-supplemented group. Studies conducted earlier (Prohaska and Brokate, 2001; Cerone et al. 1998) also showed the suppressing effects of Cu deficiency on SOD activity. Evaluating this enzyme in further studies certainly is an important step towards a better understanding of the effects of *Moraxella bovis* on immune cells and development of signs of pink eye infections in vivo.

## Conclusions

Heifers challenged with *Moraxella bovis* did not develop clinical signs of IBK infection in the present study, but the alterations observed in leucocyte populations, especially in band neutrophils, lymphocytes, and monocytes, may indicate activation of an inflammatory response against invasion of that pathogen. Moreover, Cu status appears to play a role in recruitment of these cells to the site of infection, which might be a result of ROS accumulation inside of effector cells. Additional studies will need to be performed to elucidate the mechanisms involved in the activation of immune response in cattle challenged with *M. bovis* in vivo.

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Table 3.1. Chemical composition of ingredients (bermudagrass hay, dried distillers grain, Cu+ mineral mix, and Cu- mineral mix) used in the formulation of copper sufficient and copper-deficient diets[1]

| Nutrient | Ingredient | | | |
|---|---|---|---|---|
| | Bermudagrass Hay | Dried Distillers Grain | Cu+ Mineral | Cu- Mineral |
| Dry matter, % | 92.1 | 88.1 | 96.1 | 95.6 |
| Crude protein, %DM | 8.7 | 29.3 | 0.7 | 1.2 |
| Neutral detergent fiber, %DM | 76.0 | 30.1 | 11.6 | 15.8 |
| Acid detergent fiber, %DM | 42.2 | 12.9 | 3.0 | 6.0 |
| Calcium, %DM | 0.41 | 0.12 | 19.24 | 15.64 |
| Phosphorus, %DM | 0.25 | 0.86 | 7.73 | 7.13 |
| Magnesium, %DM | 0.22 | 0.36 | 0.50 | 4.96 |
| Potassium, %DM | 1.23 | 1.26 | 0.63 | 2.20 |
| Sodium, %DM | 0.06 | 0.21 | 7.46 | 5.59 |
| Iron, ppm | 124 | 81 | 8650 | 7909 |
| Manganese, ppm | 92 | 20 | 2729 | 5015 |
| Zinc, ppm | 47 | 80 | 3831 | 5573 |
| Copper, ppm | 10 | 5 | 1223 | 22 |

[1] Cumberland Valley Analytical Services (Waynesboro, PA)

Table 3.2. Chemical composition of copper sufficient (Cu+) and copper deficient (Cu-) diets utilized to create different Cu status (Cu+, Cu-) in heifers

| Nutrient | Diet | |
|---|---|---|
| | Cu + | Cu - |
| Dry matter, % | 87.2 | 87.0 |
| Crude protein, %DM | 26.7 | 28.0 |
| Neutral detergent fiber, %DM | 29.5 | 33.4 |
| Acid detergent fiber, %DM | 11.5 | 13.0 |
| Calcium, %DM | 1.05 | 0.84 |
| Phosphorus, %DM | 1.16 | 1.13 |
| Magnesium, %DM | 0.35 | 0.61 |
| Potassium, %DM | 1.17 | 1.32 |
| Sodium, %DM | 0.66 | 0.45 |
| Iron, ppm | 447 | 410 |
| Manganese, ppm | 162 | 256 |
| Zinc, ppm | 233 | 349 |
| Copper, ppm | 72 | 8 |

[1] Cumberland Valley Analytical Services (Waynesboro, PA)

Table 3.3. Liver concentration (µg/g dry tissue) of trace minerals in heifers fed a copper sufficient (Cu+) diet or a copper deficient (Cu-) diet

| Mineral | Diet | | *P*-value[1] |
|---|---|---|---|
| | Cu + | Cu - | |
| Selenium (Se) | 1.654 | 1.570 | 0.1197 |
| Iron (Fe) | 407.870 | 354.910 | 0.0355 |
| Copper (Cu) | 228.168 | 22.904 | <0.0001 |
| Zinc (Zn) | 120.318 | 121.844 | 0.8708 |
| Molybdenum (Mo) | 3.648 | 11.692 | <0.0001 |
| Manganese (Mn) | 10.864 | 11.642 | 0.0375 |
| Cobalt (Co) | 0.412 | 0.476 | 0.0861 |

[1] Means of groups are statistically different at P<0.05 and tendencies were considered significant at P<0.10.

Table 3.4. Effect of copper status (copper sufficient - Cu+, copper deficient - Cu-) on complete blood cell count (CBC, cells x $10^{-3}$/µl) of heifers 3 days prior *M. bovis* challenge (D -3), on the day of the challenge (D 0), and 2 days post-challenge (D 2)

| Diet | Item | Day | | | P-value | | |
|---|---|---|---|---|---|---|---|
| | | D -3 | D 0 | D 2 | Day | Diet | Day*Diet |
| Cu + | Leucocytes | 13.13 | 13.41 | 12.24 | 0.0680 | 0.4241 | 0.4141 |
| | Platelets | 393.30$^{b}$ | 400.33$^{b}$ | 464.60$^{a}$ | 0.0009 | 0.7874 | 0.3151 |
| | Segmented neutrophils | 2.77 | 3.54 | 3.11 | 0.0886 | 0.1469 | 0.1688 |
| | Band neutrophils | 0.00$^{c}$ | 0.00$^{c}$ | 0.00$^{c}$ | 0.0120 | 0.0594 | 0.0120 |
| | Lymphocytes | 9.32$^{a}$ | 9.74$^{a}$ | 7.37$^{b}$ | 0.0016 | 0.7976 | 0.9004 |
| | Monocytes | 0.175$^{b}$ | 0.204$^{ab}$ | 0.411$^{a}$ | 0.0260 | 0.0602 | 0.0602 |
| | Eosinophils | 0.769 | 0.694 | 0.815 | 0.2505 | 0.8798 | 0.5195 |
| Cu - | Leucocytes | 12.38 | 12.87 | 11.90 | 0.0680 | 0.4241 | 0.4141 |
| | Platelets | 411.77$^{b}$ | 374.00$^{b}$ | 508.27$^{a}$ | 0.0009 | 0.7874 | 0.3151 |
| | Segmented neutrophils | 2.16 | 2.38 | 3.03 | 0.0886 | 0.1469 | 0.1688 |
| | Band neutrophils | 0.03$^{ab}$ | 0.03$^{a}$ | 0.01$^{c}$ | 0.0120 | 0.0594 | 0.0120 |
| | Lymphocytes | 9.18$^{a}$ | 9.18$^{a}$ | 7.24$^{b}$ | 0.0016 | 0.7976 | 0.9004 |
| | Monocytes | 0.432$^{b}$ | 0.416$^{ab}$ | 0.446$^{a}$ | 0.0260 | 0.0602 | 0.0602 |
| | Eosinophils | 0.659 | 0.653 | 1.09 | 0.2505 | 0.8798 | 0.5195 |

$^{a,b,c,}$ Means with different superscript letters across days or across diets are statistically different at $P<0.05$. Tendencies were considered significant at $P<0.10$.

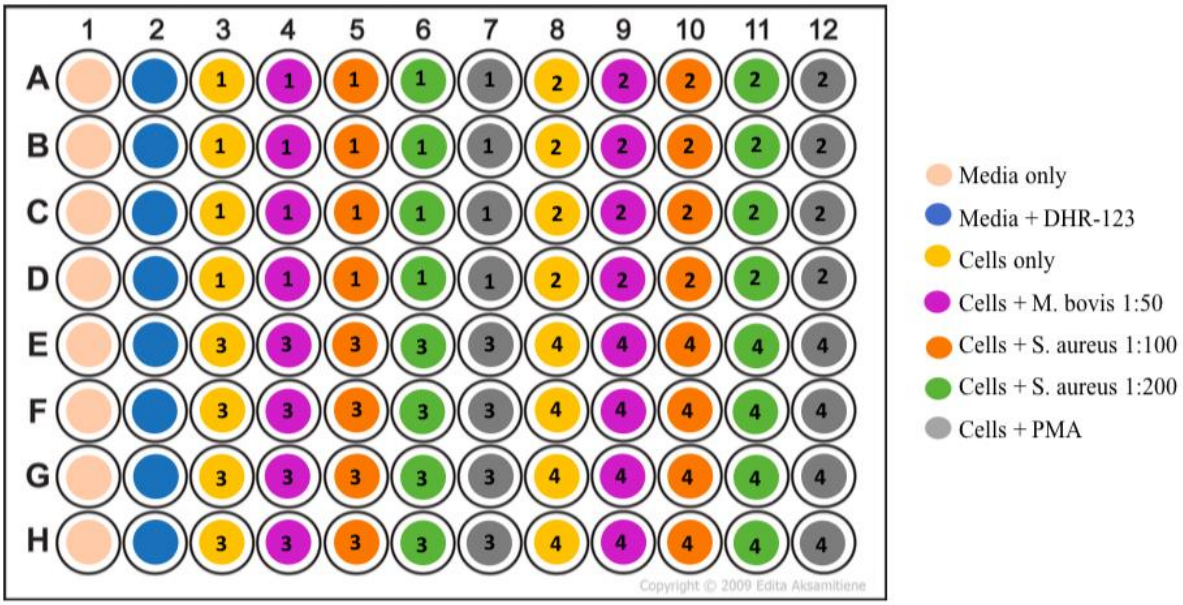


Figure 3.1. Layout of samples in 96-well microplates used in ROS analysis[1].

[1]Each plate contained samples from four animals and each well was replicated four times. Numbers within circles correspond to animals.

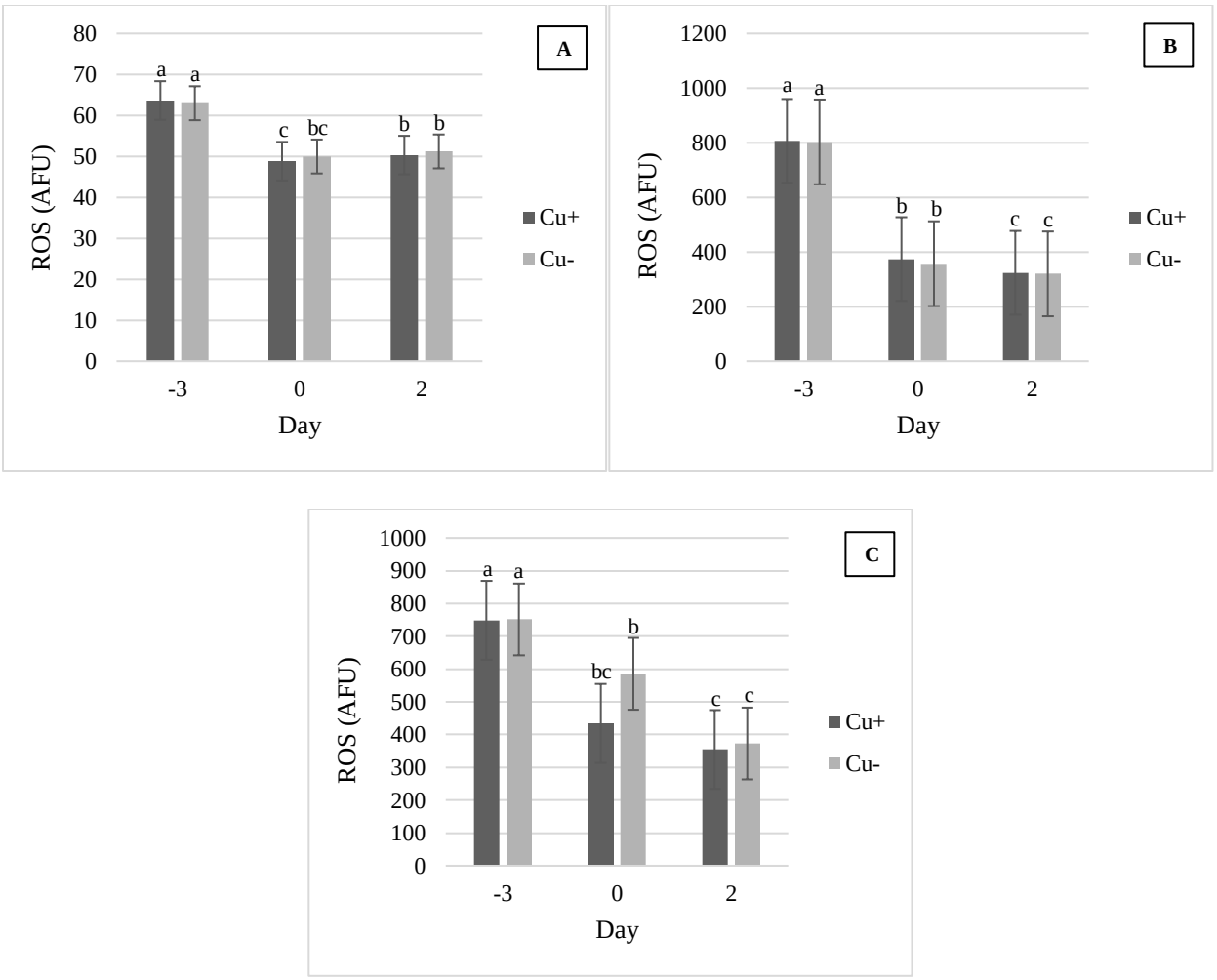


Figure 3.2. Endogenous ROS production (AFU) of neutrophils from Cu-sufficient and Cu-deficient heifers. A: Media only; B: Media + DHR-123; C: Cells only. Lowercase letters represent the statistically significant of day at $P < 0.001$. No effects of diet or day x diet interaction were detected ($P \geq 0.05$).

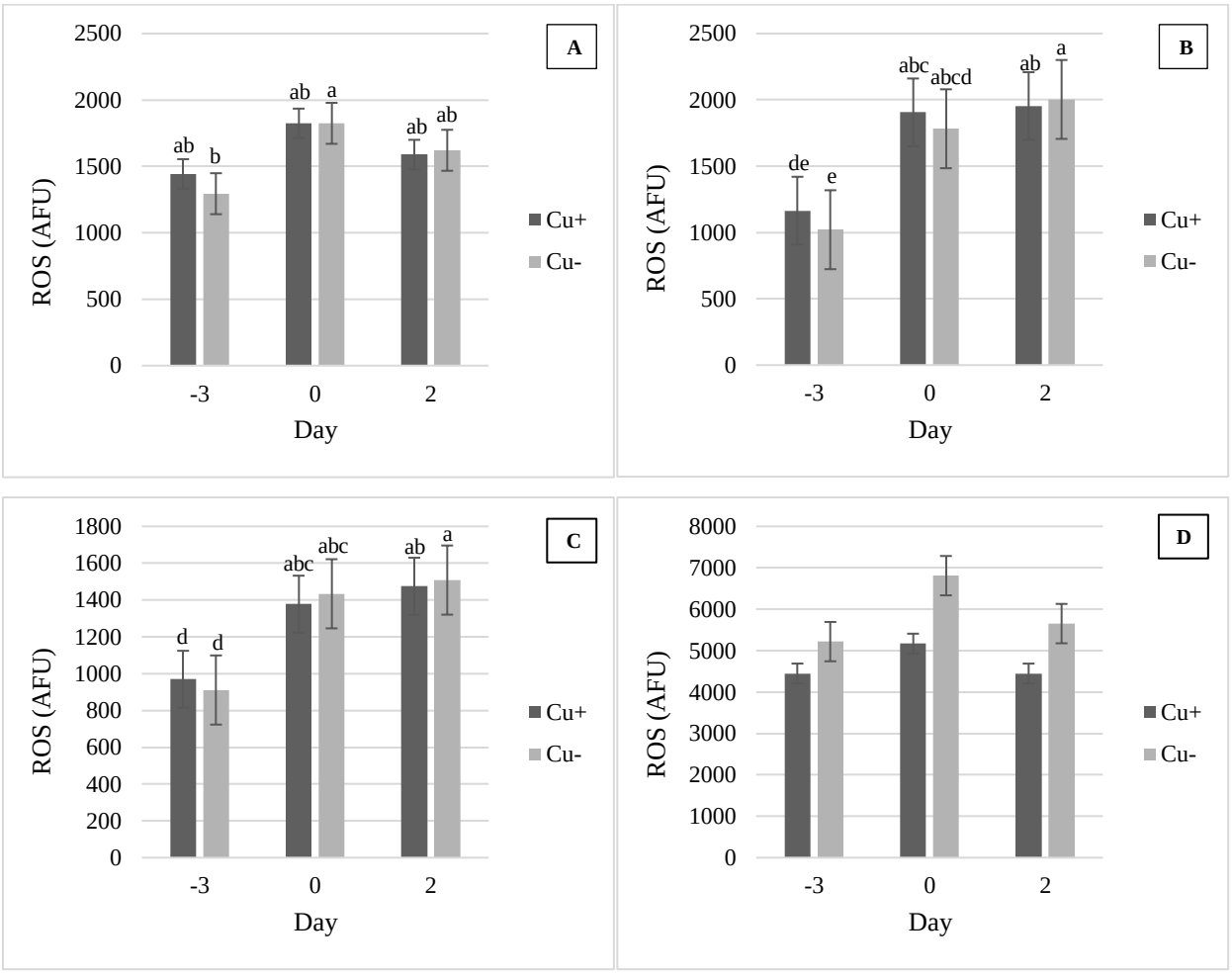


Figure 3.3. Induced ROS production (AFU) of neutrophils from Cu sufficient and Cu-deficient heifers. A: *M. bovis* diluted 1:50; B: *S. aureus* diluted 1:100; C: *S. aureus* diluted 1:200; D: PMA. Lowercase letters represent the statistically significant effect of day on *M. bovis* (P=0.0140), *S. aureus* 1:100 (P<0.0001) and *S. aureus* 1:200 (P<0.0001). No effects of diet or day x diet interaction were detected ($P \geq 0.05$).

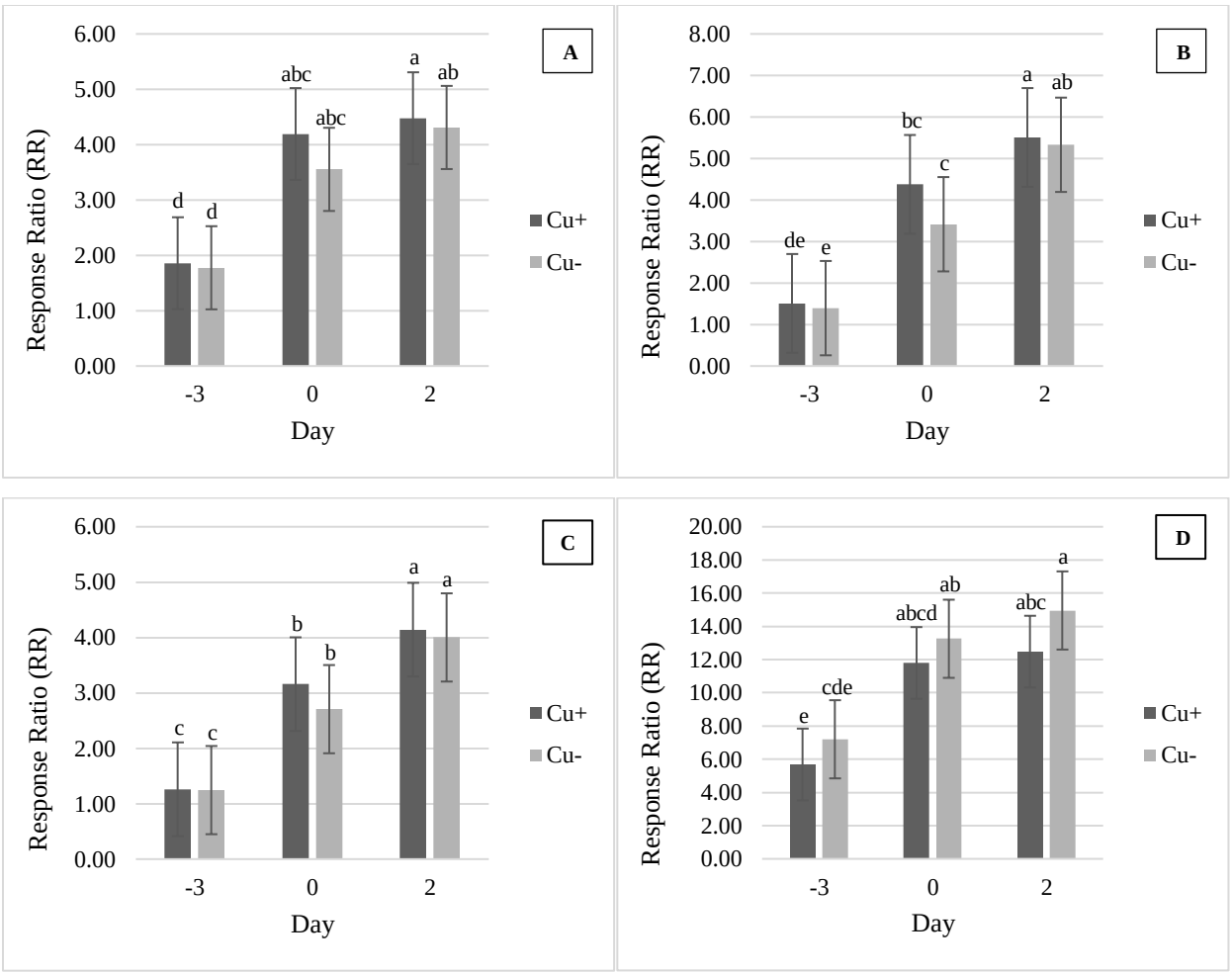


Figure 3.4. Induced ROS corrected for endogenous production (RR) of neutrophils from Cu sufficient and Cu-deficient heifers. A: *M. bovis* diluted 1:50; B: *S. aureus* diluted 1:100; C: *S. aureus* diluted 1:200; D: PMA. Lowercase letters represent the statistically significant effect of day on *M. bovis*, *S. aureus* 1:100, *S. aureus* 1:200, and PMA at P<0.0001. No effects of diet or day x diet interaction were detected ($P \geq 0.05$).

# CHAPTER 4

# IMPACT OF MATURITY STAGES ON YIELD, NUTRITIVE VALUE, FERMENTATION CHARACTERISTICS, AND PARAMETERS OF FERMENTATION IN VITRO OF ENSILED JOHNSONGRASS (*Sorghum halepense* (L.) Pers)

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[1]Silva, C. S. da, F. Maia, J. Lourenco, D. Seidel, T. R. Callaway, D. Hancock, and R. L. Stewart, To Be Submitted to *Journal of Animal Science*

**Abstract**

Johnsongrass (*Sorghum halepense*) is a non-native, invasive species that causes enormous losses in crop and hay fields, which could be minimized by using Johnsongrass as conserved forage. Two experiments were conducted to evaluate yield and quality of Johnsongrass ensiled at four maturities: 3 weeks, boot, flower, and dough. In Experiment 1, yield, botanical composition, nutritive value, and fermentation characteristics of Johnsongrass were measured. In experiment 2, Johnsongrass silage was incubated for 48 hrs for assessment of gas production, pH, in vitro dry matter digestibility (**IVDMD**) and volatile fatty acids (**VFAs**). The experimental area consisted of 16 plots divided into four blocks (2.74 m x 4.57 m), and treatment was randomly assigned to plot within block. In two cuttings from each maturity stage, the forage was dried to approximately 55% DM, and packed in mini-silos at a density of 0.24 kg/L. After 10 weeks, the silos were open and silage samples were frozen for further analysis. Data from both experiments were tested for the effects of maturity stage and harvesting time (first and second harvest). The results from experiment 1 showed an increase ($P < 0.0001$) in DM yield from 3-week stage to dough stage. The proportion of Johnsongrass declined in 3-week stage plots but increased in the flower stage ($P = 0.0010$). CP content of the silage was greatest for 3 weeks and boot in the first harvest, and greatest for 3 weeks in the second harvest ($P < 0.0001$). On the first harvest, ADF was lower in the 3 weeks and boot stage silages, but on the second harvest boot, flower, and dough did not differ ($P < 0.0001$). The highest value of TDN was observed when forage was packed at 3 weeks and the lowest values at the flower and dough stages ($P < 0.0001$), indicating that ensiling Johnsongrass after the boot stage, especially if later in the season, will decrease the amount of nutrients available in the silage. Despite the negative impact of late maturity on nutrient content, the analysis of fermentation characteristics indicates that Johnsongrass is suitable for ensiling. In

experiment 2, it was observed that production of gas, IVDMD, and production of VFAs *in vitro* were greater ($P < 0.05$) for Johnsongrass ensiled at earliest maturities. Overall, these results demonstrate that Johnsongrass can be successfully ensiled but, to balance quality and quantity, Johnsongrass should be ensiled before it reaches the flower stage.

## Introduction

The genus *Sorghum* sp., part of the Poaceae family, contains what are considered the oldest domesticated plants in current agriculture (Vinall, 1936). With their introduction and popularization in this sector, sorghum (*Sorghum bicolor* (L.) Moench) is among the most cultivated crops around the world, primarily for grain production and animal fodder. The Crop Production Report released by the United States Department of Agriculture (USDA National Agricultural Statistics Service, 2017) showed that the sorghum area planted for all purposes and harvested for grain in the country between 2014 and 2016 was over two million hectares, and the area designated to silage production surpassed one million hectares.

Johnsongrass (*Sorghum halepense* (L.) Pers.) is a member of the genus as well but known overall for its extreme ability of invading hay and row crop fields (mainly corn, soybean, and cotton) and resilience to eradication practices. This makes Johnsongrass a major weed in 53 countries around the world (Hartzle et al., 1993). In the United States, the occurrence of Johnsongrass has been reported in all states except Minnesota (USDA Plants Database, 2017).

Ilustrating these facts, a survey undertaken by McWhorter (1993) from 1976 through 1991 in Arkansas, Mississippi, and Louisiana revealed that about 90% of soybean fields in Mississippi and Louisiana had Johnsongrass, and 70 to 80% of soybeans fields in Arkansas; infestation in cotton fields varied from 55 to 90% in the three states. At that time, these numbers represent an

estimated reduction in average annual value of harvested cotton and soybeans of $5.8 ± 1.9 million and $23.7 ± 0.6 million, respectively, in the studied states.

Johnsongrass is native from the Mediterranean region and was first introduced in the U.S. in the 1830's to be utilized as a forage crop. With its wide dispersion and losses brought to other crops, producers and researchers turned their efforts to discover best management practices for eradicating it from fields rather than using it as forage. However, Johnsongrass characteristics justify the attempts of incorporating it into forage systems and animal production: it is a perennial warm-season grass widely adapted in the US, palatable to cattle; it has a adequate nutritive value; and it can be grazed with proper management (Rankins Jr. and Bransby, 1995).

According to Heath et al. (1985), hay production is one of the primary uses of Johnsongrass. Nonetheless, adopting this method for conserving forage in the Southeast region can be challenging due to the high humidity occurring on the months when forage yield and sunlight incidence are propitious for hay production. For this specie in particular, the morphological characteristic is one more troublesome factor, since Johnsongrass plants have a thick culm (0.5-2.0 cm in diameter; Warwick and Black, 1983) that increase the drying time normally required for proper hay curing.

As a result of these hay making issues, finding alternative methods for preventing issues caused by Johnsongrass by utilizing it as cattle feed may be promising. Another common process employed to conserve forage and possibly successful for Johnsongrass is baled silage, or baleage. In baleage production, the forage is cut with the same machinery used for hay, wilted until moisture content reaches about 55% and wrapped in plastic to allow fermentation (Burns and Fisher, 2012).

Studies exploring the use of Johnsongrass as either fresh or conserved forage are very scarce. Rude and Rankins (1993) demonstrated the potential of use of Johnsongrass in the form of

silage in sheep diets, but no work has been done to evaluate Johnsongrass silage in bovine diets. Since Johnsongrass is challenging to eliminate from many hay and row crop fields, its utilization as silage would avoid expenses designated to controlling it, reduce losses in major crops, and ultimately increase the amount of high quality conserved forage produced in the Southeast.

## Material and Methods

This study was divided into two experiments: Experiment 1 was carried out from May to December of 2017 to evaluate the effect of maturity stages on Johnsongrass yield, nutritive value, and parameters of fermentation of Johnsongrass silage. Experiment 2 was conducted in May of 2018 as an in vitro study where parameters of fermentation and in vitro dry matter digestibility (IVDMD) of Jonhsongrass silage were assessed.

### *Experiment 1*

#### *Location, Treatments, and Forage Management*

Plots were delimited in a preexisting stand of Johnsongrass located at the J. Phil Campbell, Sr. Research and Education Center in Watkinsville, GA (latitude 33.877º, longitude -83.424º). Weather information of this location (averages of minimum temperature, maximum temperature, and precipitation) for the months of field evaluations are provided in Table 4.1. The soil type of the research site is mostly classified as sandy loam and sandy-clay loam (Web Soil Survey, 2017). Soil samples were taken at the research site, composed, and sent to UGA Soil, Plant and Water Laboratory. Results showed a soil pH of 6.2 and mineral levels as follows: 17 kg/ha of P, 145 kg/ha of K, 1729 kg/ha of Ca, 222 kg/ha of Mg, 3 kg/ha of Zn, and 125 kg/ha of Mn. According

to these results, the research site had low levels of P, medium levels of K, and high levels of Ca and Mg; Zn and Mn levels were considered adequate.

Prior to designating plots, the area was mowed to a 7.5 cm stubble height, and the residue was removed to simulate a hay harvest (day 0). This area was divided into four blocks separated by alleys of 1.5 m. Blocks were subdivided into four plots of 4.57 x 2.74 m each, totalizing 16 experimental units. The experimental area was fertilized with 22.7 kg of N (in the form of urea) at plant emergence based on N recommendations for forage sorghum (Ball et al. 2007, Vendramini et al. 2010), since no recommended levels specific for Johnsongrass were found. Phosphorus and K were applied following recommendations given by the soil test report (23.0 kg/ha of $P_2O_5$, 90.8 kg/ha of $K_2O$). Plots within a block were randomly assigned to one of four treatments, which consisted of four maturity stages of Johnsongrass: 3 weeks, boot stage, flower, and dough stage. For the 3-week treatment, the first harvest occurred when plants reached approximately 60 cm in height and every 3 weeks thereafter. For boot, flower, and dough stage treatments, plots were observed approximately twice a week and harvest time was set based on morphological characteristics of plants at each stage. Plants were considered as being at the boot stage when an enlargement of the top-portion of the stem was observed due to the development and pre-emergence of the seedhead (inflorescence) enclosed in the stem. At the flower stage, the seed head had emerged, and the peduncle was fully elongated. The dough stage represented plants harvested at soft dough, when seeds were filled and could be squeezed between fingers but had little or no liquid present.

*Data Collection*

Prior to harvesting plots, botanical composition was first assessed subjectively by visual identification of different components in the plot. These included Johnsongrass, other grasses, legumes, and weeds, for which a percentage was attributed. Then, samples were randomly collected by tossing a square quadrat three times within each plot and clipping all forage contained within the quadrat at 7.5 cm with scissors. Different plant species identified in the quadrat were manually separated and similar components from the three quadrats were combined to represent an individual sample per component per plot. Samples were weighed fresh and after drying at 60ºC for 48 hrs for estimation of the botanical composition. Botanical composition was calculated as the amount of Johnsongrass, other grasses, legumes, and/or weeds in the plots in relation to the total sample mass collected (i.e. % Johnsongrass = (Johnsongrass (g) / Total sample mass (g)*100).

Plant maturity was assessed at each harvest for boot, flower and dough stages by collecting Johnsongrass tillers prior to harvest. In each plot, two 91 cm-grazing sticks (used in practical estimation of forage availability for grazing) were placed in parallel on the ground approximately 12 cm apart, and all tillers growing within that segment were collected. Tillers were ranked in development based on their characteristics (e. g. number of visible/palpable nodes, development of panicle, and hardness of seeds) and grades were used for determination of growth stage using the mean stage count (MSC) according to methodology proposed by Moore et al. (1991). Based on this method, the target MSC was 3.0 for boot stage, 3.5 for flower, and 4.3 for dough stage plots. The calculated MSC (based on grades of tillers) for boot, flower, and dough stage plots were 2.9, 3.2, and 4.0, respectively.

Next, forage was harvested using a push lawn mower with grass catcher attachment (Troy-Bilt, Valley City, OH) set to leave a stubble of 7.5 cm. When forage was cut at flower stage and

dough stage, a hedge trimmer (Husqvarna 122HD60) was used before the push mower to aid in the harvesting of stalks and maximize the amount of forage collected. All material collected from each plot was placed on an individual tarp (1.2 m x 1.8 m), which had been previously tared, and weighed on a digital hanging scale (CS25 Pentair, USA). Grab samples were taken for each plot, weighed initially and dried at 60°C for 48 hrs to determine DM percentage ((dry wt/wet wt) x 100)) and subsequent determination of dry matter yield.

*Silo Preparation*

Each treatment has two harvests used for silage, but the harvests did not align such that all treatments produced a harvest at the same time. So, the two harvests for each treatment closest to the end of July 31 were ensiled. Therefore, 32 mini-silos were prepared in total and consisted of 90 cm lengths of 10-cm diameter polyvinyl chloride pipes sealed with air-tight rubber caps at either ends. The target dry matter for packing was 55%. After plots were harvested, the forage was evenly spread on tarps to allow drying in the sun, and the weight of each tarp was monitored every 30-45 min. Drying time varied from plot to plot, from 1 to 4 hrs, depending on forage mass and weather conditions. The target density for each silo was 0.24 kg/L, which resulted in 2.16 kg of forage being packed at 55% DM.

Approximately 100 g of forage was used for determination of DM prior ensiling through the microwave technique (Gay et al., 2009). In this method, the forage material was placed in a microwave-safe container along with a mug filled with water (for trapping excess of moisture), and its weight recorded initially and after consecutively drying for 2 min. The final weight is recorded when the weight difference becomes less than 1 g. In the present study, the objective of

performing that procedure was to determine, by weight difference, how much the fresh forage from each plot had to dry in order to achieve appropriate moisture for ensiling.

Once the forage had reached adequate moisture, a small layer of forage was placed at the bottom of the silo and covered with a section of plastic. Then, the forage remaining was packed by putting in small amounts at a time and compacting it using a steel-stick. Once the silo was packed within 5 cm of the top of the silo, another layer of plastic was placed at the top, followed by an extra layer of forage, and the silo was closed. These extra layers on each end were used to ensure that fermentation and quality of packed forage would be minimally impacted by air that have entered the silo.

The silos were allowed to ferment outside under a covered structure for 10 weeks and monitored for four days post-packing, then weekly, to check for pressure buildup and potential leaks. After 10 weeks, the silos were opened, and their contents frozen at -20° C. Half of the Johnsongrass silage samples were transferred to paper bags and freeze-dried in a VirTis Freeze mobile lyophilizer (SP Industries, Warminster, PA) at -50° C for 24 to 48 hrs depending on the thickness and maturity of the sample. The freeze-dried samples were ground at 2 mm using a Model 4 Wiley Mill (Thomas Scientific, Swedesboro, NJ), then ground through a CT 193 Cyclotec Sample Mill (FOSS, Eden Prairie, MN) fitted with 1-mm screen for in vitro analysis. The remaining half of the frozen samples were shipped to Cumberland Valley Analytical Services (Hagerstown, MD) in quart-size reclosable freezer bags for analysis of fermentation characteristics.

*Experiment 2*

*In Vitro Fermentation*

The in vitro assay was performed in May of 2018. Samples were simultaneously analyzed for IVDMD, gas production, pH, and volatile fatty acid concentration. On the morning of the incubation, approximately 2 L of rumen fluid was collected from three cannulated crossbred Angus steers that were grazing a pasture of tall fescue (*Festuca arundinacea*) and white clover (*Trifolium repens*) for the previous months. The ruminal fluid was collected by grabbing the ruminal contents through the canula and straining it through a nylon paint strainer into a 1-L plastic bottle. The bottles were placed in a cooler with warm water for transportation to the lab.

Upon arrival to the lab, the bottles were inverted slowly to mix stratified layers of the fluid and the pH was measured with a portable pH meter (pH = 6.35). Ruminal fluid was mixed with a minimal medium described by Wells et al. (1997) at a 3:1 ratio (3 parts of medium, 1 part of ruminal fluid). The medium was prepared, adjusted to a final pH of 6.5, and saturated with $CO_2$ 24 hrs prior incubation. 10 mL of ruminal fluid-media mix were delivered to 20-mL crimp-top glass tubes containing 0.1 g of Johnsongrass silage samples at the different stages. Blank samples (containing only ruminal fluid-media mix) were also incubated. Five tubes per sample were used as replicates. The tubes were constantly bubbled with $CO_2$ before receiving the mix and immediately sealed with a rubber stopper and crimp thereafter. After sealing, the tubes were placed in an incubator at 39° C for 48 hrs and were gently shaken every 6 hrs throughout the incubation period.

When the 48-hour incubation was completed, the tubes were removed from the incubator and immediately placed on ice to stop fermentation. After 20 min, gas volume was measured from each tube using a 16-gauge needle coupled to a 60-mL disposable syringe. Next, the seal was

broken and pH was recorded. Samples for VFA analysis were obtained by filtering the content of the glass tubes into a previously weighed 10x50 Dacron bag, positioned on top of a V-shaped bottom plastic storage tube. Plastic storage tubes were immediately capped and stored at -20° C. The glass tubes were rinsed with deionized water to remove particles stuck to the tube walls. The residue present inside the bags after filtration was rinsed with tap water until the water from wash was clean. The bags containing the residue post-incubation were placed in a dryer at 60° C for 48 hrs and weighed at the end of this period for determination of dry matter degradation.

VFA analysis were performed according to methodology described by Henry et al (2015). Rumen fluid samples were defrosted at room temperature, transferred to 2.0 mL microcentrifuge tubes, and centrifuged at 10000 g for 15 min. 1.0 mL of the supernatant was collected from the tubes and mixed with 200 microliters of 25% metaphosphoric acid (5:1 ratio). These subsamples were kept at -20°C overnight. On the next day, subsamples were defrosted and centrifuged one more time at 10000 g. 500 μL of the supernatant were added to 1 mL of ethyl acetate (2:1 ratio) and shaken vigorously. Samples were allowed to settle for about 3 min or until the supernatant portion could be collected. The supernatant was transferred to 1.8 screw cap vials and analyzed in a gas chromatographer (GC-2010 Plus; Shimadzu Corporation, Japan) through a flame ionization detector and a capillary column (Zebron ZB- FFAP GC Cap. Column 30 m x 0.32 mm x 0.25 μm; Phenomenex Inc., Torrance, CA). The retention time for major volatile fatty acids (acetic acid, propionic acid, butyric acid) were 2.874, 3.305, and 3.826 min.

*Statistical Analysis*

Dry matter yield, botanical composition, nutrient content, and fermentation characteristics evaluated in experiment 1 were analyzed using PROC GLM procedure of SAS (SAS Institute Inc.,

Cary, NC). Plots were arranged in a randomized complete block design with four plots in each treatment, replicated four times. Harvest, maturity stage, and the interaction harvest x maturity were considered as the main effects. Data collected in experiment 2 were analyzed by PROC MIXED procedure of SAS having harvest and maturity stage as fixed effects and tube as the random effect. Least square means from both experiments were generated by LSMEANS and compared with Tukey's HSD test of multiple comparisons. Differences were considered significant at $\alpha < 0.05$.

## Results and Discussion

*Experiment 1*

The results of the cumulative DM yield are presented in Fig. 4.1. There was a significant effect of maturity stages on forage mass produced by Johnsongrass. Forage production was significantly greater ($P < 0.0001$) for Johnsongrass harvested at the dough stage (2930 kg/ha) compared to boot (2031 kg/ha), with flower intermediate (2570 kg/ha); all of these values were greater than the yields of Johnsongrass harvested at 3 weeks (747 kg/ha; $P < 0.0001$).

In the current study, it was seen that Johnsongrass produced less DM compared to sorghum species used for grain or forage production. Dry matter yield of *Sorghum* sp. has been reported as 12 to 20 t/ha (Miron et al. 2005; Amer et al. 2012; Bean at al. 2013), a four-fold increase compared to the highest DM yield of Johnsongrass in the current trial. In addition, the highest DM yield of Johnsongrass is substantially lower than the annual yield expected from bermudagrass (*Cynodon dactylon* (L.) Pers) hay under different irrigation levels or no irrigation. Zhou et al. (2014) reported that the 5-year average of expected yield of bermudagrass hay baled at 15% moisture was 6.84

t/ha without irrigation, 8.09 t/ha at medium irrigation level (1.52 cm/wk$^{-1}$), and 8.43 t/ha under high irrigation (3.05 cm/ wk$^{-1}$).

Botanical composition of Johnsongrass plots is summarized in Fig. 4.2. The percentage of Johnsongrass did not differ ($P > 0.05$) between the initial and final harvest for boot and dough stages. However, Johnsongrass decreased ($P = 0.0460$) from the initial to the final harvest in 3-week plots while in flower-stage plots it increased ($P = 0.0010$; Fig. 4.2.A). The changes in the 3-week treatment were the result of an increase ($P = 0.0151$; Fig. 4.2.C) of other grasses, since no statistical differences were observed in percentage of weeds ($P > 0.05$; Fig. 4.2.B). Species that fell into the "other grasses" category were plants that are considered forage species other than Johsongrass. These other grasses were predominantly smooth crabgrass (*Digitaria ischaemum* (Schreb.)). This shift in composition of species in 3-week stage plots indicate that the growth of Johnsongrass was suppressed by short harvest intervals preventing rhizome growth and seed production (Warwick and Black, 1983). In fact, the employment of continuous grazing or mowing has been often suggested as one of the few efficient methods for controlling infestations of Johnsongrass (Ceseski et al. 2017; Rocateli and Manuchehri, 2017). Except for the 3-week treatment, there was an average of over 60% Johnsongrass in all of the treated plots.

The nutritive value of Johnsongrass prior to ensiling is presented in Table 4.2. There was a harvest x maturity stage interaction ($P < 0.05$) for all parameters of nutritive value, however the results will be discussed within harvest. The lowest DM content was observed in the 3-week stage on the second harvest ($P = 0.0004$). For CP, lower values were detected in plots that reached the flower and dough stages and greater at the 3 weeks and boot stages within the first harvest. On the second cutting, the 3-week stage had the highest CP concentration ($P < 0.0001$). The first harvest also resulted in higher content of TDN for the 3-week and boot stages compared to flower and

dough. As with the CP values, the second harvest resulted in highest TDN for forage at the 3-week stage ($P = 0.0015$). In contrast, the flower and dough stages had the highest NDF and ADF values in comparison to the 3 weeks and dough stages within the first harvest, and lowest for 3-week in the second ($P < 0.0006$).

The negative impact of advanced plant maturity observed in this study is consistent with the changes previously reported in grain sorghum, forage sorghum, and sudangrass (*Sorghum bicolor* (L.) Moench ssp. *drummondii* (Nees ex Steud.) de Wet & Harlan] (Snyman & Joubert, 1996; Abdelhadi & Tricarico, 2009; Beck et al. 2013), which indicate that as plants growth progresses, protein concentration decreases while fiber content increases due to the deposition of lignified tissues as part of the structural support of the plant (Crowe et al. 2017). Mature Johnsongrass plants can reach 2.5 m tall and have stems of up to 2.0 cm in diameter (Warnick & Black, 1983), which reflects in the high NDF and ADF values found in the present study (around 70% and 44%, respectively).

Harvesting time and maturity stages had similar effects on the quality of ensiled Johnsongrass (Table 4.3). The DM content was lowest for boot stage in the first harvest and no differences ($P > 0.05$) were observed in 3 weeks, flower, and dough. On the second harvest, the boot stage had higher DM content compared to 3 weeks ($P < 0.0001$), while flower and dough were intermediate. Differently from the results obtained pre-ensiling, the CP content of the silage was greatest for 3 weeks and boot in the first harvest, and greatest for 3 week in the second harvest. This change was accompanied by an increase in NDF levels ($P<0.0001$). In the first harvest, ADF was lower in the 3 weeks and boot stage silages, but on the second harvest boot, flower, and dough did not differ ($P < 0.0001$). The NDF and ADF concentrations in Johnsongrass silage found in this study are comparable to values reported by Thomas et al. (2013) for silage made from forage

sorghum. However, with exception of the dough stage on the first harvest, Johnsongrass silage showed higher CP value compared to that of sorghum silages treated or untreated with inoculants, which were all below 7.6%.

The highest value of TDN was observed when forage was packed at 3 weeks and the lowest values at the flower and dough stages. Overall, these results indicate that ensiling Johnsongrass after the boot stage compromises the amount of nutrients available. In addition, it is important to note that the CP levels of all maturity stages remained constant after ensiling, except for Johnsongrass ensiled at the dough stage. This decrease in CP concentration post-ensiling in mature Johnsongrass may indicate that advanced maturity may promote proteolysis, where part of the protein fraction is degraded to non-protein nitrogen during wilting (Rotz and Muck, 1994; Ding et al., 2013). The changes in CP content from fresh to ensiled forage in this study corroborates with Zhang et al. (2016) who reported a 23% decrease in CP concentration in forage sorghum ensiled at the milk stage, which was related to proteolysis reaction before ensiling.

The fermentation characteristics of ensiled Johnsongrass are presented in Table 4.4. The interaction between harvesting time and maturity stage was significant ($P < 0.0001$) for all parameters of fermentation, except for the concentration of non-structural carbohydrates (NSC; $P = 0.1097$). When NSC content was compared across harvests, the second harvest showed a higher ($P < 0.0001$) concentration of NSC in the silage than the first (21% and 18.5%, respectively). Within treatments, the 3-week (21.5%) and boot stages (19.9%) showed greater NSC content compared to the dough (18.8%) and flower stages (18.8%; $P = 0.0008$).

The level of NSC in the silage is an important indicator of soluble carbohydrates in the pre-ensiled forage as well as quality of fermentation (Yang et al. 2006). According to Wanapat et al. (2013) cellulose and hemicellulose are broken down to simple sugars (non-structural

carbohydrates) during the ensilage process and those simple sugars are further utilized by certain groups of bacteria, such as *Lactobacillus* spp. (Dogi et al. 2013; Rahman et al. 2017), that generate organic acids as their final products (mainly lactic, acetic, butyric acid). Lactic and acetic acid are the most desirable organic acids: lactic acid will cause a quick drop in pH and inhibition of secondary fermentation by undesirable microorganisms, such as *Clostridia*, preserving quality along storage; and acetic acid enhances aerobic stability due to its antifungal properties that prevent yeast from becoming active when silage is exposed to air (Zhang et al. 2009).

The amount of lactic acid produced by the fermentation process in the first harvest was greatest ($P = 0.0240$) for Johnsongrass ensiled at boot and dough stages. On the second harvest, 3-week stage silage had the highest concentration of lactic acid ($P = 0.0240$; Table 4.4). Moreover, forage ensiled at 3 weeks and boot stages in the first harvest presented higher acetic acid values. Flower stage showed an intermediate concentration of acetic acid between the 3-week stage and dough stage in the first harvest ($P < 0.0001$). On the second harvest, acetic acid concentrations were similar among treatments. Butyric acid levels were undetectable in all samples.

Silage pH was lower ($P < 0.0001$) in the boot stage compared to flower stage within the first harvest; however, the boot stage had higher pH than 3 weeks, flower, and dough stages in the second harvest ($P < 0.0001$). Thus, the boot stage presented one of the lowest pH values but also the highest pH. The significant rise in pH observed from the first to the second in this particular treatment may be associated with inhibition of growth of lactic acid-producing bacteria by low moisture content (higher DM concentration) of that silage in the second cutting (Table 4.3). In addition, the negative impact of low moisture content on those microorganisms may explain the low production of total acids detected in that treatment.

As explored by Troller and Stinson (1981) and later demonstrated by Zheng et al. (2011), the growth of *Lactobacillus plantarum* can be completed suppressed by low moisture levels in their environment. Zheng et al. (2011) tested the effects of moisture content on microbial activity and the quality of silage produced from tomato pomace and sugar beet pulp and found that ensiling tomato pomace and sugar beet pulp at 10% and 30% moisture (90% and 70% of DM, respectively) did not support microbial activity and acidification of the silage. In the present study all moisture contents were above 30%, but it can be suggested that the higher DM of the boot-stage silage on the second harvest impaired the fermentation to some extent, whereas the lower DM in the boot stage on the first harvest favored fermentation.

The analysis of fermentation characteristics indicates that Johnsongrass can be utilized for ensiling. According to reference values of fermentation quality of silage provided by Ward and Ondarza (2008), pH values of the current study were near a desirable pH of approximately 4.0, with the exception of the boot-stage silage from the second harvest; nonetheless, pH values above 4.0 can be observed for dry silages (> 40% DM) as it has been adopted in this study. Lactic acid should range from 4-7% of DM, which were observed in boot and dough-stage silages on the first harvest and at 3 weeks on the second harvest. For acetic acid, acceptable levels are below 3%, as it was seen for Johnsongrass silages at all growth stages. Lastly, non-detectable levels of butyric acid indicate that minimum undesirable fermentation occurred in the silos.

*Experiment 2*

The total gas volume produced over 48 hrs of incubation is presented in Fig. 4.3. There was an interaction ($P$ = 0.0185) between harvesting time and maturity stages of Johnsongrass, so results will be discussed within harvest. On the first harvest, the most evident impact of maturity

on gas production occurred for Johnsongrass ensiled at the boot compared to the dough stage ($P = 0.0028$). On the second harvest, gas production did not differ among treatments ($P > 0.05$). The pronounced difference between boot and dough stages followed the general trends observed for nutritive value of fresh and ensiled Johnsongrass (Tables 4.2 and 4.3), suggesting that ensiling Johnsongrass early in its development will provide more substrates for degradation by rumen bacteria (which reflect in increased gas production), and consequently enhance nutrient utilization by the animal. These observations corroborate with the findings of Ribeiro et al. (2014) who investigated, among other traits, *in vitro* gas production of *Andropogon gayanus* grass harvested at different maturities and preserved as hay or silage. Those authors reported both hay and silage exhibited a decline in gas production as maturity of the plant advanced. According to these authors, a decrease in gas production occurs in response to a reduction in cell wall degradability in mature plants, explaining the difference in gas production between the boot and dough stages in Johnsongrass.

There was no significant effect of harvest, forage maturity, or their interaction on the pH *in vitro* ($P > 0.05$) and the average of pH across harvests and maturity stages was 5.94 (Table 4.5). The IVDMD, on the contrary, was affected by both harvest and treatments ($P = 0.0013$; Table 4.5). On the first harvest, the boot stage showed the highest digestibility along with the 3-week stage, which were both different from the digestibility of the dough-stage silage. The flower stage showed a value intermediate between the 3-week and dough stages. On the second harvest, there was a decline in digestibility from the 3-week to the dough stage ($P = 0.0013$). These differences are probably associated with the impact of advanced maturity on degradability of cell wall fractions (NDF, ADF, and lignin) of Johnsongrass, as pointed out earlier. Even though the digestibility of NDF and ADF was not measured in the current study, the 3-week and boot stage silages had the

lowest ADF and NDF values and overall highest digestibilities, suggesting that NDF and ADF concentration was the key factor affecting the digestibility of Johnsongrass.

One explanation for that change is that advanced maturity impaired the degradability of cell wall fractions as pointed out earlier. Because the 3-week and boot stages had the lowest ADF values, it is presumed that NDF and ADF concentration was the key factor affecting the digestibility of Johnsongrass even though the digestibility of the fiber fraction was not measured. Bean et al. (2013) reported a negative correlation of $r \leq -0.72$ between NDF, ADF, and lignin and true digestibility of sorghum classes cultivated for grain and forage yield.

Another possible reason for decreasing IVDMD with increased maturities is a limitation of CP in the substrate for cellulolytic bacteria to ferment structural carbohydrates of Johnsongrass at the latest stages of growth. Sampaio et al. (2009) demonstrated the effects of protein levels in the diet of animals offered signal grass (*Brachiaria decumbens* Stapf.) hay with an average 4.86% CP level on DM basis. They observed a linear positive relationship between the potentially degradable NDF and CP protein levels in the diet (5.19, 7.11, 8.60, 11.67 and 13.02 % on DM basis) and suggested that when CP level is above 7%, the enzymatic characteristics in the rumen do not limit the fiber utilization by microorganisms; on the contrary, the degradation process will depend on the substrate characteristics and enzymatic pool in the rumen when CP level is below 7%.

The total VFA production was impacted by harvest and maturity stages, but there were no significant interactions between these two effects ($P = 0.9644$). VFA production was greater for Johnsongrass ensiled at 3 weeks (33.21 m*M)* in comparison to flower (32.47 m*M)* and dough stages (31.78 m*M),* with intermediate value obtained in the boot stage (32.69 m*M*; $P < 0.0001$). The average of total VFAs produced from plants in the first cutting (33.12 m*M*) was highest compared to the second cutting (31.96 m*M*).

In regard to specific VFAs, there was a significant harvest x maturity stage interaction for acetate and results will be discussed within harvest. On the first harvest, acetate production was higher for Johnsongrass at the boot and flower stages in comparison to 3-week and dough stages. On the second harvest, the highest acetate value was observed in the 3-week stage. The boot stage showed higher acetate production than dough; the flower stage showed an intermediate value between the last two ($P = 0.0074$).

Acetate production generally increases to a certain level as maturity of forages progresses (Rinne et al. 1997; Vanhatalo et al. 2009; Sarmadi et al. 2016). However, as mentioned earlier, there has to be a minimum amount of protein available in the rumen (7%) for any degradation of substrate to occur in the rumen, which can suggest that the low crude protein level in dough-stage silage from the first harvest limited microbial activity in the rumen and structural carbohydrates were incompletely degraded. Nonetheless, it is worth mentioning that the differences observed in acetate production among treatments and across harvests would likely not promote different biological effects.

Propionate production was highest in Johnsongrass silage at the first harvest of the 3-week stage, but no statistical difference was seen on the second cutting when the 3 weeks, boot, and flower stages were compared ($P < 0.0001$). When looking at butyrate on the first harvest, the most apparent difference occurred with fermentation of silage at the 3-week stage, which showed the highest molar proportion of all comparisons. As a consequence, the best A:P ratio was observed with Johnsongrass from the first harvest ensiled at 3 weeks ($P = 0.0056$).

Butyrate production was affected by harvest and maturity stages ($P = 0.0136$). The first harvest resulted in higher proportion of butyrate in the 3-week stage and there was no difference between boot, flower, and dough. On the second harvest, the flower-stage silage yielded more

butyrate than the boot stage, but they both did not differ from 3 weeks and dough. Overall, the changes in individual VFAs corroborate with the greater concentration of protein and digestibility of DM at the earliest stages of growth, indicating that younger plants will promote best fermentation.

## Conclusions

It can be concluded that Johnsongrass can be ensiled and potentially used for cattle feeding. Harvesting and ensiling Johnsongrass before it reaches the flower stage will provide the best balance between yield, nutritive value, and quality of fermentation. In addition, 3-weeks and boot-stage silages will be more digestible and generate more energy in the form of VFAs for the animal. Therefore, producers can benefit from ensiling Johnsongrass in areas of high infestation.

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Table 4.1. Averages of minimum temperature, maximum temperature, and precipitation in Watkinsville, GA, between June and September of 2017

| Months | Minimum Temperature (°C) | Maximum Temperature (°C) | Precipitation (mm) |
|---|---|---|---|
| June | 19.2 | 29.0 | 7.62 |
| July | 21.3 | 32.4 | 2.54 |
| August | 20.6 | 30.3 | 2.54 |
| September | 16.5 | 28.0 | 5.1 |

Source: University of Georgia Weather Network (2018)

Table 4.2. Nutritive value of fresh Johnsongrass harvested in two periods (first, second) and at four maturity stages (3 weeks, boot, flower, and dough)

| | First Harvest | | | | Second Harvest | | | | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | 3 weeks | Boot | Flower | Dough | 3 weeks | Boot | Flower | Dough | H | MS | H x MS |
| DM | 93.6$^{abcd}$ | 93.4$^{d}$ | 94.1$^{abc}$ | 94.1$^{ab}$ | 93.0$^{e}$ | 93.9$^{abcd}$ | 93.6$^{abcd}$ | 94.1$^{a}$ | 0.1958 | <0.0001 | 0.0004 |
| CP | 14.6$^{a}$ | 13.5$^{ab}$ | 8.5$^{cd}$ | 7.2$^{d}$ | 13.9$^{ab}$ | 7.8$^{cd}$ | 9.1$^{c}$ | 7.2$^{d}$ | <0.0001 | <0.0001 | <0.0001 |
| TDN | 59.4$^{ab}$ | 58.6$^{abc}$ | 55.4$^{e}$ | 55.7$^{de}$ | 60.2$^{a}$ | 56.4$^{cde}$ | 57.7$^{bcd}$ | 55.9$^{de}$ | 0.4326 | <0.0001 | 0.0015 |
| NDF | 60.7$^{ef}$ | 63.3$^{de}$ | 69.2$^{ab}$ | 70.1$^{a}$ | 58.7$^{f}$ | 69.1$^{abcd}$ | 66.9$^{abcd}$ | 69.1$^{abc}$ | 0.8460 | <0.0001 | 0.0006 |
| ADF | 35.6$^{ef}$ | 37.7$^{e}$ | 43.4$^{abc}$ | 44.4$^{a}$ | 33.9$^{f}$ | 43.2$^{abcd}$ | 41.3$^{abcd}$ | 44.1$^{ab}$ | 0.5034 | <0.0001 | 0.0002 |

[1] H = harvest; MS = maturity stage.

$^{a,b,c,d,e,f}$ Means followed by different lowercase letters within a row are statistically significant (P<0.05).

Table 4.3. Nutritive value of Johnsongrass silage prepared in two harvesting periods (first, second) and at four maturity stages (3 weeks, boot, flower, and dough)

| | First Harvest | | | | Second Harvest | | | | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | 3 weeks | Boot | Flower | Dough | 3 weeks | Boot | Flower | Dough | H | MS | H x MS |
| DM | 46.6$^{bc}$ | 31.3$^{d}$ | 49.5$^{abc}$ | 46.8$^{bc}$ | 42.7$^{bc}$ | 57.3$^{a}$ | 50.0$^{ab}$ | 49.8$^{ab}$ | 0.0003 | 0.0387 | <0.0001 |
| CP | 14.7$^{a}$ | 13.3$^{ab}$ | 8.2$^{cd}$ | 6.4$^{f}$ | 13.7$^{ab}$ | 7.6$^{cdef}$ | 8.0$^{cde}$ | 8.9$^{c}$ | <0.0001 | <0.0001 | <0.0001 |
| NDF | 56.3$^{de}$ | 59.2$^{d}$ | 67.5$^{b}$ | 71.7$^{a}$ | 55.1$^{e}$ | 66.6$^{bc}$ | 66.4$^{bcd}$ | 64.7$^{bcd}$ | 0.3752 | <0.0001 | <0.0001 |
| ADF | 35.8$^{ef}$ | 37.9$^{e}$ | 45.2$^{ab}$ | 46.9$^{a}$ | 34.9$^{f}$ | 43.3$^{bc}$ | 42.6$^{bcd}$ | 41.3$^{cd}$ | 0.0261 | <0.0001 | <0.0001 |
| TDN | 61.1$^{a}$ | 59.0$^{bc}$ | 55.5$^{efg}$ | 54.3$^{g}$ | 59.6$^{ab}$ | 56.6$^{def}$ | 57.5$^{cde}$ | 58.3$^{bcd}$ | 0.1206 | <0.0001 | <0.0001 |

[1] H = harvest; MS = maturity stage.

$^{a,b,c,d,e,f}$ Means followed by different lowercase letters within a row are statistically significant (P<0.05).

Table 4.4. Fermentation characteristics of Johnsongrass silage prepared in two harvesting periods (first, second) and at four maturity stages (3 weeks, boot, flower, and dough)

| | First Harvest | | | | Second Harvest | | | | *P*-value[2] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Item[1] | 3 weeks | Boot | Flower | Dough | 3 weeks | Boot | Flower | Dough | H | MS | H x MS |
| tVFA | $3.67^{bcd}$ | $6.77^{a}$ | $3.15^{cd}$ | $4.15^{bc}$ | $5.45^{ab}$ | $2.05^{d}$ | $3.25^{cd}$ | $3.40^{cd}$ | 0.0032 | 0.0065 | <0.0001 |
| Lac | $1.90^{d}$ | $4.27^{ab}$ | $1.87^{d}$ | $3.47^{abc}$ | $4.30^{a}$ | $1.47^{d}$ | $2.65^{cd}$ | $2.45^{cd}$ | 0.4028 | 0.0240 | <0.0001 |
| Ace | $1.77^{ab}$ | $2.50^{a}$ | $1.27^{bc}$ | $0.70^{c}$ | $1.11^{bc}$ | $0.59^{c}$ | $0.61^{c}$ | $0.92^{c}$ | <0.0001 | 0.0006 | <0.0001 |
| pH | $4.55^{bcde}$ | $4.23^{e}$ | $4.58^{bcd}$ | $4.35^{cde}$ | $4.40^{bcde}$ | $5.21^{a}$ | $4.60^{bc}$ | $4.70^{b}$ | <0.0001 | 0.0194 | <0.0001 |

[1] tVFAs = total volatile fatty acids (VFAs); Lac = lactic acid, % total VFAs; Ace = acetic acid, % total VFAs. [2] H = harvest; MS = maturity stage.

$^{a,b,c,d,e}$ Means followed by different lowercase letters within a row are statistically significant ($P<0.05$).

Table 4.5. Digestibility and production of volatile fatty acids (VFAs) *in vitro* of Johnsongrass silage prepared in two harvesting periods (first, second) and at four maturity stages (3 weeks, boot, flower, and dough)

| Item | First Harvest | | | | Second Harvest | | | | *P*-value[2] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 weeks | Boot | Flower | Dough | 3 weeks | Boot | Flower | Dough | H | MS | H x MS |
| IVDMD | 65.7$^{abc}$ | 68.8$^{a}$ | 59.0$^{cd}$ | 53.0$^{d}$ | 68.5$^{ab}$ | 56.9$^{d}$ | 56.8$^{d}$ | 52.8$^{d}$ | 0.0313 | <0.0001 | 0.0013 |
| Volatile Fatty Acids (m*M*)[1] | | | | | | | | | | | |
| Ace | 17.45$^{bc}$ | 17.82$^{a}$ | 17.74$^{a}$ | 17.30$^{bcd}$ | 17.57$^{ab}$ | 17.29$^{bcde}$ | 17.03$^{def}$ | 16.87$^{f}$ | <0.0001 | 0.0007 | 0.0074 |
| Prop | 8.40$^{a}$ | 8.00$^{b}$ | 7.67$^{bcd}$ | 7.55$^{cde}$ | 7.69$^{bc}$ | 7.47$^{cde}$ | 7.45$^{cde}$ | 7.23$^{e}$ | <0.0001 | <0.0001 | 0.1945 |
| IsoBut | 0.54$^{a}$ | 0.51$^{a}$ | 0.50$^{a}$ | 0.54$^{a}$ | 0.49$^{b}$ | 0.50$^{b}$ | 0.49$^{b}$ | 0.50$^{b}$ | 0.0008 | 0.1386 | 0.1390 |
| But | 5.22$^{a}$ | 4.93$^{bc}$ | 4.84$^{bc}$ | 4.83$^{bc}$ | 4.77$^{bc}$ | 4.71$^{c}$ | 4.99$^{b}$ | 4.76$^{bc}$ | 0.0196 | 0.1065 | 0.0136 |
| Val | 0.60$^{a}$ | 0.58$^{ab}$ | 0.55$^{c}$ | 0.55$^{c}$ | 0.56$^{bc}$ | 0.55$^{c}$ | 0.55$^{c}$ | 0.54$^{c}$ | 0.0004 | 0.0018 | 0.0711 |
| Cap | 0.30$^{a}$ | 0.21$^{a}$ | 0.33$^{a}$ | 0.30$^{a}$ | 0.22$^{b}$ | 0.21$^{b}$ | 0.19$^{b}$ | 0.14$^{b}$ | 0.0033 | 0.4834 | 0.1956 |
| A:P Ratio | 2.08$^{d}$ | 2.22$^{c}$ | 2.31$^{abc}$ | 2.29$^{abc}$ | 2.29$^{abc}$ | 2.31$^{ab}$ | 2.29$^{abc}$ | 2.34$^{a}$ | 0.0009 | 0.0008 | 0.0056 |

[1] Ace = acetate; Prop = propionate; IsoBut = isoburyrate; But = butyrate; Val = valeric acid; Cap = caproic acid; tVFAs = total volatile fatty acids (VFAs). [2] H = harvest; MS = maturity stage.

$^{a,b,c,d,e,f}$ Means followed by different lowercase letters within a row are statistically significant (P<0.05).

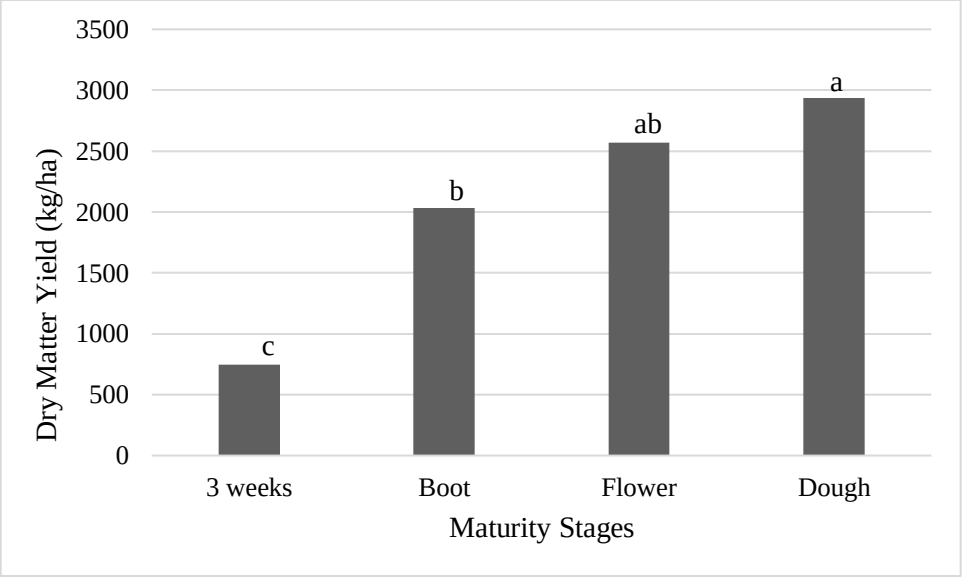


Figure 4.1. Total dry matter yield (kg/ha) of Johnsongrass harvested at four maturity stages (3 weeks, boot, flower, and dough). Bars identified by different lowercase letters are statistically significant ($P < 0.0001$).

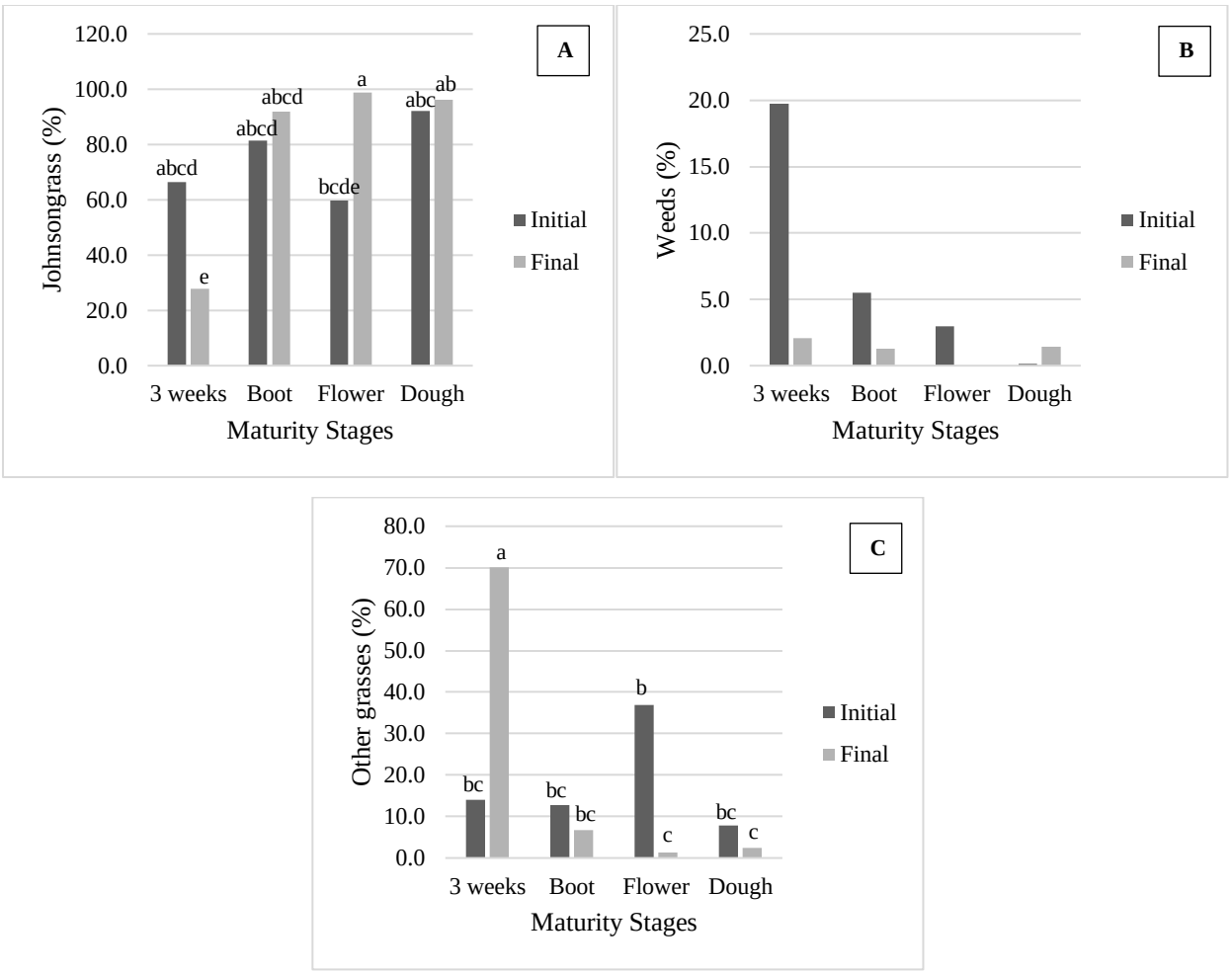


Figure 4.2. Initial and final proportion of Johnsongrass (A), weeds (B), and other grasses (C) in botanical composition of plots harvested at four maturity stages of Johnsongrass (3 weeks, boot, flower, and dough). Bars identified by different lowercase letters are statistically significant ($P < 0.05$).

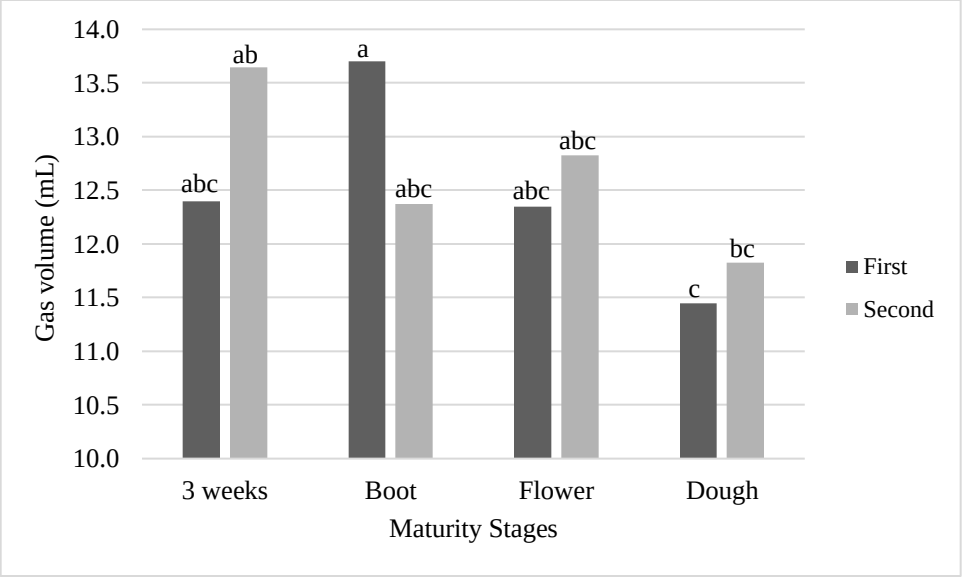


Figure 4.3. Volume of gas (mL) produced *in vitro* by Johnsongrass ensiled in two harvesting periods (first, second) and four maturity stages (3 weeks, boot flower, and dough). Bars identified by different lowercase letters are statistically significant ($P < 0.05$).

# CHAPTER 5

# CONCLUSIONS

The experimental model utilized to create Cu deficiency in the first study did not induce the appearance of clinical signs of IBK (such as sensitivity to light and ocular discharge) in the heifers. However, the findings of that research suggest that challenging the animals with *M. bovis* promoted an acute inflammatory response, which was revealed by the changes in different cell populations from baseline to two days post-challenge. Moreover, Cu deficiency appeared to affect the recruitment of phagocytic cells to sites of infection, which gives an indication of increased demanded for segmented neutrophils in Cu-deficient animals. The increased ROS production induced by *S. aureus* from D 3 to D 2 suggest that the activity of neutrophils was not impaired in neither of the groups, but highest ROS production might be one of the factors involved in differences observed in circulating band neutrophils of Cu-deficient animals. However, these effects need to be further investigated.

From a scientific perspective, the findings of the current research help to build the knowledge around biological factors that determine whether *M. bovis* will successfully overcome the immune defenses. In addition, these results can aid future researchers on developing best models for experimentally inducing clinical signs of IBK and investigating it in vivo. Moreover, if additional studies indicate that copper deficiency can affect the immunecompetence of cattle, producers will discover an extra tool for controlling IBK in the herd: mineral supplementation. In the long term, many areas of high IBK incidence would benefit from implementation of mineral nutrition programs that aimed to prevent IBK, enhancing profitability.

The results of the second study showed that Johnsongrass exhibited lower DM yield than other Sorghum species or bermudagrass hay, but resulted in silage with adequate nutrient content, especially at earlier stages of growth. Overall, Johnsongrass harvested at 3-weeks and boot stage had better nutrient content (more protein, less fiber) compared to harvests at flower and dough stages. Earlier stages were also more digestible and produced more VFAs *in vitro*. In addition, Johnsongrass was suitable for ensiling. However, the findings of that study suggest that controlling DM content of Johnsongrass at ensiling is critical for the success of the fermentation process. Ultimately, Johnsongrass can be used as an alternative forage for ensiling. In order to balance yield and quality, producers should harvest and ensile Johnsongrass at early maturities, before the flower stage.

By demonstrating the benefits of ensiling Johnsongrass, these findings will add valuable information to the scientific literature, which has extensively focused on testing different strategies to eradicate Johnsongrass. In the United States and many areas of the world where Johnsongrass was disseminated, producers have attempted to eliminate it without achieving much success. The results presented here demonstrate that producers can take advantage of infested fields by harvesting and ensiling Johnsongrass at early stages of growth rather than adopting eradication practices. Hence, Johnsongrass could be utilized as an alternative forage source in problematic areas.