THE EFFECTS OF A COMBINATION OF SODIUM BENZOATE, POTASSIUM SORBATE, AND SODIUM NITRITE ON THE COMPOSITION OF THE MICROBIAL COMMUNITY, FERMENTATION, AND AEROBIC STABILITY OF WHOLE-PLANT CORN SILAGE AND HIGH MOISTURE CORN

by

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A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal and Food Sciences

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ABSTRACT

During silage fermentation, lactic acid bacteria produce lactic acid that lowers the pH of the forage mass leading to a generalized inhibition of microbial activity when coupled with anaerobic conditions. If the pH is not low enough undesirable fermentations can occur. For example, yeasts can produce ethanol and decrease dry matter recovery, and enterobacteria can increase proteolysis, producing NH_3-N. When silage is exposed to air, lactate-assimilating yeasts can initiate an aerobic spoilage process by oxidizing lactic acid and producing CO_2, H_2O, and heat. The degradation of lactic acid results in an increase in silage pH and allows the growth of aerobic bacteria and molds that furthers the spoilage process. Spoiled silage is undesirable because it is lower in nutritional value than fresh silage and when fed to cows, can affect animal intake, production, and health. Various silage additives have been evaluated to improve the aerobic stability of silages. Specifically, the use of chemical additives may be warranted especially for silage produced under challenging conditions such as short-term ensiling, air-stress during storage, and warm temperatures at feed-out, or to overcome the risks brought by the high numbers of yeasts present in silage. Although it is assumed that such silage additives exert their effect by altering the microbial communities in silage, the types of microorganisms that are impacted positively or negatively by the additives are usually not

known. Such knowledge would be important for attempts at improving additives or even developing novel additives. High-throughput sequencing (HTS) is a useful tool to analyze microbial communities because it enables the identification of unculturable, new and low-abundance microorganisms. Therefore, the objective of this study was to evaluate the effects of a chemical additive containing sodium benzoate, potassium sorbate, and sodium nitrite on the composition of the microbial community of corn silage and high moisture corn by HTS, and to analyze its ability to improve fermentation and aerobic stability under challenging conditions in North America. The effects of the additive were tested in a total mixed ration made with corn silage treated before or after ensiling (Experiment 1, Chapter 2), in corn silage ensiled for short-periods (Experiment 2, Chapter 3), in corn silage subjected to air-stress during storage (Experiment 3, Chapter 4), and in high moisture corn subjected to air-stress during storage and warm temperatures at feed-out (Experiment 4, Chapter 5). Microbial and chemical attributes of the ensiled materials were analyzed with respect to the chemical composition, concentration of organic acids and alcohols, and numbers of lactic acid bacteria, yeast, and molds. The aerobic stability of the silages was also determined. The composition of the bacterial and fungal communities was analyzed by sequencing of the ITS1 region present in fungi and the V4-V5 region of the 16S rRNA gene present in bacteria. In Experiment 1, the additive not only reduced the total numbers of yeasts in silage but also reduced the relative abundance of the lactating-assimilating yeasts *Candida tropicalis*, greatly improving aerobic stability. The additive was also useful in improving the stability of the total mixed rations when applied to the silage before or after ensiling. In

Experiment 2, the additive reduced the concentration of NH_3-N and markedly improved the stability of corn silage after short periods of storage (1 and 4 d), probably due to fungistatic (rather than fungicidal) effects. In Experiment 3, in corn silage stored for 63 d, the additive reduced the concentration of NH_3-N. The low dose of the additive did not reduce the total numbers of yeasts, but markedly improved aerobic stability. In non-air-stressed silos, the additive reduced the relative abundance of the lactate-assimilating yeasts *Candida tropicalis*. In air-stressed silos, it overcame the effects of air by inhibiting specific microorganisms that were stimulated by oxygen, such as *Pichia*. In Experiment 4, the additive reduced the relative abundance of Enterobacteriales and decreased proteolysis in high moisture corn. Moreover, it reduced the numbers of yeasts and lowered the ethanol production, increased dry matter recovery, and markedly improved the aerobic stability, overcoming the adverse effects of air-stress during storage and high temperatures at feed-out. Overall, the effects of the additive on the numbers of yeasts varied among experiments. Still, the additive improved aerobic stability in all Experiments. Therefore, the additive containing sodium benzoate, potassium sorbate, and sodium nitrite has the potential to be applied to improve the aerobic stability of total mixed rations, of corn silage that must be fed very soon after ensiling, of corn silage subjected to air-stress during storage, and of high moisture corn challenged with air during storage and that needs to be fed during warm days.

INTRODUCTION

Silage making is a process that aims to preserve the dry matter (DM) and nutritive value of fodder through fermentation and provide a feed that remains stable during storage and feed out that can be fed to ruminants throughout the year. Lactic acid bacteria (LAB) that naturally occur on the crop, ferment water-soluble carbohydrates (WSC) and produce organic acids. Lactic acid, notably, decreases silage pH and with anaerobic conditions, creates an inhospitable environment that curtails undesirable microorganisms and excessive microbial metabolism that can cause poor fermentations and/or spoilage (Pahlow et al., 2003). Specifically, whole-plant corn is the primary forage source that is ensiled and for dairy cattle in the United States (Erdman et al., 2011). Other feed sources, such as high moisture corn (HMC), can also be used as part of the diet of beef or dairy cattle. HMC is produced by ensiling of corn kernels before drying and is an alternative to feeding dry corn (Lardy, 2016).

Ironically, efficient silage fermentations (e.g., one that is dominated by homolactic acid bacteria) do not always result in silages that are stable when exposed to air (Muck and Kung, 1997). The reason for this finding is that often, such fermentations produce limited amounts of antifungal compounds. Thus, when air penetrates a silage mass during storage or feed out, high numbers of lactate-assimilating yeasts are able to consume lactic acid and increase silage pH (Woolford, 1990) resulting in spoiled silage.

Silages that remain stable for long periods of time (e.g. multiple days) after initial exposure to air are said to have good aerobic stability, a desirable characteristic for high quality silages. The speed of spoilage is increased when temperatures during feed out are high, greatly reducing the aerobic stability of silage (Ashbell et al., 2002). Under specific weather or logistics conditions, producers cannot wait several weeks for silage to cure and must feed out silage immediately or very soon after harvest. This short-term ensiled forage can have low aerobic stability due to incomplete fermentation.

Various types of additives can be applied to the silage to stimulate fermentation, inhibit undesirable fermentations such as ones caused by *Clostridia*, enterobacteria, and yeasts, and/or improve aerobic stability (Bolsen et al., 1996). Chemical additives such as potassium sorbate and sodium benzoate have antifungal properties and can increase the aerobic stability of silage, while sodium nitrite can reduce the chances of undesirable fermentations (Knicky and Spörndly, 2011). Different chemicals have diverse modes of action. For this reason, various chemicals can act synergistically and display a broader application range and greater efficacy when applied in combination than alone (Stanojevic et al., 2009).

Several studies have evaluated the effectiveness of a chemical additive containing potassium sorbate, sodium benzoate, and sodium nitrite (Salinity, Göteborg, Sweden) on improving the aerobic stability of silages (Knicky and Spörndly, 2011; Da Silva et al., 2015; Knicky and Spörndly, 2015; Kung et al., 2018). However, most studies were done in Europe (Knicky and Spörndly, 2011; Knicky et al, 2014; Knicky et al, 2016; Knicky and Spörndly, 2015; Seppala et al, 2016). Soil properties, climate, and crop varieties

found in the United States are different from the ones encountered in Europe, therefore, there is a need to evaluate the effects of this chemical additive under North American conditions. Additionally, more studies assessing the efficacy of the additive in whole-plant corn silage and HMC, especially under challenging conditions such as short ensiling times, air stress during storage, and high temperatures during feed-out are needed.

In the past, the impact of additives on silage microbial communities was analyzed PCR based methods, but improvements in high throughput sequencing (HTS) have made it possible to characterize microbial populations genetically. Not only is this approach more comprehensive than culture-based methods, but unculturable, new and low-abundance microorganisms are being detected (Muck, 2013; Kumar et al., 2015). Analysis of the composition of the bacterial and fungal communities of silage using HTS can help to boost the understanding of the microbiology of silage made with different crops, at diverse locations and climates, and treated with different additives. Also, HTS can be a helpful tool to deepen the knowledge on the microbiome of silages at different stages of the ensiling process (Duniere et al., 2017).

Chapter 1

LITERATURE REVIEW

Whole-Plant Corn Silage and High Moisture Corn

Silage is largely produced around the world to provide high-quality feed uniformly throughout the year. Silage is used to feed animals when forage production is low, especially during the winter or dry season, and to preserve forage that accumulates due to high production and low animal consumption (Muck and Shinners, 2001).

Whole-plant corn silage is the primary forage source fed to dairy cattle in the United States because of high nutrient yields and because it's low buffering capacity allows it to ensile easily compared to many other types of forages (Erdman et al., 2011), especially legumes. According to the Crop Production 2015 Summary (USDA, 2016), 126,894 million tons of the corn harvested in 2015 in the United States were destined to whole-plant silage production, totaling 7.1% of all the corn harvested (USDA, 2016). Over the last decade in the United States, the rise in corn prices and the diminished supply of corn grain has increased the reliance on corn silage and corn residues to feed cattle (Klopfenstein et al., 2013). Shaver and Kaiser (2011) surveyed farms in the

Wisconsin area and reported that forage comprised 50% to 60% of the TMR dry matter, with whole-plant corn containing 40% to 70% of the forage dry matter (DM).

High-moisture corn (HMC) is produced by harvesting corn before the kernels dry, usually at more than 24 % moisture content, followed by ensiling (Lardy, 2016). High moisture corn can be fed to dairy cows as an alternative to dry corn. Some of the advantages of HMC compared to dry corn are the eradication of drying costs, reduction of field losses, prolongation of the harvesting season, and potentially higher ruminal starch digestibility. High moisture corn can be harvested after corn silage and before dry corn, prolongating the harvesting season (Swanson, 2009). The starch:zein protein (prolamin) matrix content in corn grain is negatively correlated with starch digestibility, as the prolamin matrix hinders the access of ruminal microorganisms to starch. The prolamin matrix content increases with maturity and decreases with time of ensiling due to proteolytic processes (Hoffman et al., 2011). Therefore, HMC can have a higher starch digestibility compared to dry corn, because it is harvested in an early stage of maturity and because it undergoes the ensiling process. A major disadvantage of HMC is that because it has a relatively high population of naturally occurring yeasts (Kung et al., 2007) and because it has a low moisture content and low level of fermentable sugars, the extent of the fermentation is limited (Bucholtz, 2012) with lower concentration of volatile fatty acids (VFA) and higher pH than whole-plant corn silage, making it more difficult to stabilize during fermentation and aerobic exposure.

Silage Microorganisms

Lactic Acid Bacteria (LAB)

LAB are gram-positive facultative anaerobic bacteria that produce lactic acid as the primary product of carbohydrate fermentation. Some of the principal genera of LAB are *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus, Streptococcus, Enterococcus* and *Weissella*. According to their glucose metabolism, LAB can be divided into obligate homofermentative, obligate heterofermentative, and facultative heterofermentative (Holzapfel and Wood, 2012).

Homofermentative LAB produce lactic acid as the only product of glucose fermentation through the Embden–Meyerhof–Parnas pathway. This type of bacteria possesses the enzyme aldolase and produces 2 ATP per molecule of glucose. Obligate heterofermentative bacteria do not have the enzyme aldolase, but they produce phosphoketolase. Heterofermenters ferment glucose and pentose through the Pentose Phosphate pathway, producing lactate, CO_2, and acetic acid or ethanol. This pathway produces only 1 ATP per glucose. However, the conversion of acetyl-phosphate to acetate can produce additional ATP. The group denominated facultative heterofermentative possesses both aldolase and phosphoketolase enzymes and can ferment glucose by the Embden–Meyerhof–Parnas pathway and pentoses by the action of an inducible phosphoketolase (Fugelsang and Edwards, 2007).

Fast production of lactic acid by LAB is essential to make good quality silage. Lactic acid is a strong acid that reduces fresh corn pH from approximately 5.5 to 3.7 after

ensiling. The low pH inhibits growth of undesirable microorganisms that can cause undesirable fermentations from enterobacteria and clostridia (McDonald, 1982).

Yeasts

Yeasts are eukaryotic organisms that are usually unicellular. Dimorphic yeasts are exceptions, since they can form filaments in specific environmental conditions. Most yeasts belong to the subdivision of Ascomycota and Deuteromycota. Ascomycetes produce sexual spores, whereas the Deuteromycetes (imperfect fungi) do not have a sexual stage of reproduction. Only a few yeasts are part of the Basidiomycota, a group that comprises filamentous fungi that reproduces by structures called basidia (Reed and Nagodawithana, 2012).

Yeasts can metabolize sugars anaerobically and produce ethanol and CO_2, causing DM losses (McDonald et al., 1991). Under aerobic conditions, lactate-assimilating yeasts can initiate aerobic spoilage of silage because they oxidize lactic acid, producing CO_2, H_2O, and heat. The consumption of lactic acid by lactate-assimilating yeasts increases silage pH, which allows growth of other microorganisms, such as aerobic bacteria and molds that cause further spoilage (Duniere et al., 2013).

Species of Candida, Pichia, Saccharomyces, and Issatchenkia are lactate-assimilating yeasts known to be associated with silage spoilage (Santos et al., 2017). Some authors evaluated the capacity of certain yeasts species to assimilate lactate. Strains of *Pichia anomala, P. kudriavzevii, P. manshurica, Candida kruzei, C. tropicalis, C.*

glabrata, C. quercitrusa, C. ethanolica, C. rugosa, Saccharomyces cerevisiae, Issatchenkia orientalis isolated from corn silage, and *P. anomala, C. kruzei,* and *I. orientalis* isolated from HMC showed the ability to assimilate lactate (Burmeister and Hartman, 1966; Carvalho et al., 2016; Santos et al., 2017; Wang et al., 2018).

Molds

Molds are mainly aerobes, and for this reason, their growth is associated with poor silo packing or sealing and with the final stages of aerobic spoilage. Molds from the soil, air, or plant tissues can contaminate forage. Mold proliferation can reduce silage nutritional value and palatability and produce toxins that can cause severe health problems to the animal (Fink-Gremmels, 1999). The main mycotoxin-producing fungi found in corn silage are *Fusarium*, *Aspergillus*, and *Penicillium*, which in certain conditions produce aflatoxin B1, deoxynivalenol, zearalenone, fumonisins, mycophenolic acid, and roquefortine C. These toxins are hazardous to cattle because they can cause reduced milk production, decreased performance, infertility, and paralytic effects (Driehuis et al., 2018).

Rumen microorganisms can degrade some mycotoxins, but other toxins are resistant to degradation in the intestinal tract and can cause health problems when reaching the bloodstream. Furthermore, some mycotoxins can affect the rumen microbial populations and reduce its detoxification capacity. Animal health problems associated with mycotoxins include metabolic and hormonal changes and inflammation (Fink-Gremmels, 2008).

Clostridia

Clostridia are gram-positive obligate anaerobic spore-forming bacteria. They are divided into three groups. The proteolytic strains produce ammonia and toxic compounds called biogenic amines. *Clostridium butyricum*-type bacteria are saccharolytic, and *C. tyrobutyricum* types mainly ferment lactate to butyric acid, H_2, and CO_2. The fermentation of lactate is undesirable because it increases silage pH, allowing for the growth of spoilage microorganisms (Muck, 2010).

Clostridial spores can survive in the intestinal tract of the cow and are shed in feces. Fecal contamination of the udder can contaminate the milk, causing the development of undesirable characteristics in milk itself and on dairy products. Even more harmful is the extremely pathogenic species *C. botulinum*, the causative agent of botulism (Lindström et al., 2010).

Clostridia grow fast in the early stages of fermentation. For this reason, to reduce the chances of clostridial fermentation, a quick drop in silage pH or low water activity are required and can be achieved by ensiling drier forage or by wilting. Another mechanism that prevents clostridial fermentation is the conversion of nitrates from the plant to nitrite and nitric oxide, which are effective in inhibiting clostridial growth (Gibson, 1965).

Enterobacteria

Enterobacteria are gram-negative facultative anaerobic bacteria. Some of the representatives of this group are *Salmonella, Escherichia, Enterobacter, Serratia,* and

Citrobacter. Enterobacteria have proteolytic activity and can form ammonia and toxic biogenic amines. These bacteria are also able to reduce nitrate to nitrite, and further convert nitrite to ammonia and nitrous oxide. Nitrite can be converted into nitric oxide that, when exposed to air, is oxidized to nitrogen oxides. These gases and their acids can cause severe health problems, but can efficiently inhibit clostridia (Driehuis and Elferink, 2000).

During early fermentation, enterobacteria that naturally occur in the crop compete with LAB for nutrients. As a pH below 5.0 inhibits enterobacteria, a fast drop in pH is essential to reduce the proliferation of these undesirable microorganisms. Therefore, if the pH drop is slow or if the silo is not completely anaerobic, enterobacteria can survive. Once the pH increases after silage is exposed to air at feed out, enterobacteria that survived in the silo can start to multiply and rapidly deteriorate the silage (Driehuis and Elferink, 2000).

Acetic Acid Bacteria

Acetic acid bacteria are gram-negative obligate aerobic bacteria. The principal genus of acetic acid bacteria found in silage is *Acetobacter*. These bacteria can sometimes initiate the aerobic deterioration of corn silage, though they usually start spoilage simultaneously with lactate-assimilating yeasts. Acetic acid bacteria produce acetic acid by oxidation of ethanol, and once ethanol is utilized, they consume other substrates such as lactic and acetic acids (Spoelstra et al., 1988).

Bacilli and Listeria

Bacilli and listeria are gram-positive bacteria that can be either obligate aerobe or facultative anaerobe. Most bacilli are spore formers and can be found during the late stages of aerobic spoilage (Lindgren et al., 1985). Spores of bacilli produced during aerobic spoilage can be transferred from feces to the udder and end up in the milk, causing deterioration of milk or even foodborne illness (Te Giffel et al., 2002). *Listeria sp.* are ubiquitous in nature, and many are able to grow under harsh conditions such as near freezing, in the presence of high salt concentrations, and over a relatively wide pH range. *L. monocytogenes* is a pathogenic species that can cause food-borne illness, but also diseases in cows and spontaneous abortions (Farber and Peterkin, 1991; Driehuis et al., 2018).

The Ensiling Process and Microbial Succession

The ensiling process is divided into four stages: aerobic, fermentation, stable, and feed-out. During the aerobic phase that lasts several hours once the silo is sealed, the plant, aerobic microorganisms, and facultative aerobic microorganisms are actively consuming the oxygen trapped between the forage material. At this phase, the forage pH is about 6.0 to 6.5, which allows the activity of plant enzymes, such as proteases and amylases (Elferink et al., 2000).

Aerobic bacteria and fungi do not contribute to silage fermentation and will die as soon as the silo is sealed, and the oxygen is consumed. The most abundant groups of

bacteria in fresh forage are LAB and enterobacteria, usually exceeding 10^6 cfu/g of fresh forage. The epiphytic LAB are abundant on crops and essential for natural fermentation of silages. The numbers of LAB increase after chopping due to the release of sugars that are used as a fermentation substrate. Enterobacteria, however, can be detrimental to silage fermentation by competing for substrates with LAB and by producing acetic acid, nitrites, and nitrogen oxide gases. Clostridia and bacilli are generally at much lower populations; however, their populations might increase if silage comes in contact with manure contaminated soil (Pahlow et al., 2003).

When anaerobic conditions are achieved in the silo, the fermentation phase starts. This stage can last from days to weeks depending on the crop and environmental conditions and is characterized by growth of LAB that should become the dominant microorganisms in the silage. Depending on the type of LAB, they produce mainly lactic acid and, in lower quantities, acetic acid, which decreases corn silage pH to approximately 3.8 (Elferink et al., 2000). The faster the pH drops and fermentation ends, the more nutrients are conserved.

The first LAB to dominate the ensiling process are the homofermentative, followed by the heterofermentative as the ensiling progress and the nutrient availability became scarcer. Zhou et al. (2016) showed that in whole-plant corn silage ensiled at 25°C the homofermentative *Lactobacillus plantarum* and *Pediococcus pentosaceus* dominated the fermentation as quickly as within one day. After one week of ensiling, there was a shift in abundance towards the heterofermentative *Lactobacillus buchneri*, which remained dominant until the end of the ensiling process.

During the stable phase, if anaerobic conditions are maintained and no drastic changes occur in the silo, the numbers of most microorganisms decrease. Some acid-tolerant microorganisms can survive ensiling by entering a dormant state (e. g. yeasts and molds) or forming spores (e. g. clostridia and bacilli), while others remain partially active (e.g. *L. buchneri*). Additionally, several proteases and carbohydrases that are tolerant to acid conditions can remain active in the silo and slowly degrade substrates (Elferink et al., 2000).

At the feed-out phase, silage is exposed to air and lactate-assimilating yeasts and acetic acid bacteria, to a lesser extent, start to multiply and to degrade organic acids, increasing silage pH and temperature. The rise in pH is the starting point for another wave of spoilage, usually caused by bacilli, molds, and enterobacteria, which leads to an even higher silage mass temperature (Elferink et al., 2000). Spoilage of silage due to air exposure decreases its nutritional value as microorganisms consume nutrients, such as sugars, VFAs, and ethanol (Muck, 2010).

Feeding spoiled silage can negatively affect animal performance and health. Whitlock et al., 2000 observed that steers fed spoiled silage had decreased DM intake, lower digestibility of DM, organic matter, crude protein, neutral detergent fiber, and acid detergent fiber, and reduced cohesion of the forage mat in the rumen compared to steers fed stable silage. Microorganisms present in the spoiled silage can cause health problems in ruminants. *Listeria monocytogenes* can cause infections in the brain and uterine tissues when the animal consumes contaminated silage and eye infection by direct contact. Molds can produce mycotoxins that might cause metabolic and hormonal imbalances and

inflammation. Additionally, microorganisms present in spoiled silage can cause food-born illnesses in humans or affect milk quality. *Listeria monocytogenes* can cause listeriosis that causes gastrointestinal or systemic illnesses. Shiga toxin-producing *E. coli* can cause from intestinal discomfort to more dangerous disease, such as hemorrhagic colitis. *Bacillus cereus* spores can reach the milk and negatively affect the organoleptic properties of milk products (Driehuis et al., 2018).

According to Wilkinson and Davies (2013), biochemical, microbiological, physical, and management practice are the main factors impacting the aerobic stability of silage. The biochemical composition of silage can affect its aerobic stability. The acetic acid content of the silage is usually correlated with high aerobic stability because this organic acid has antifungal properties and inhibits the growth of undesirable microorganisms that cause spoilage when silage is exposed to air. High DM forages, in which the water activity is not enough to fully support LAB growth, the WSC are usually not completely utilized. In this case, when silage is exposed to air, the residual WSC can be used by undesirable microorganisms and reduce the aerobic stability of the silage. Regarding microbiological factors, yeasts are the microorganisms with higher correlation with poor aerobic stability, especially if their numbers are higher than 10^5 cfu/g of fresh forage. The major physical and management factors influencing aerobic stability are silage packing density, permeability, porosity, and speed of silo filling which directly affect the amount of oxygen penetrating the silo (Wilkinson and Davies, 2013). Moreover, high ambient temperatures can reduce silage stability by stimulating the growth of undesirable microorganisms (Ashbell et al., 2002).

During each phase of the ensiling process, essential goals need to be achieved to obtain good quality silage. During the aerobic phase, it is essential to exclude air from the silo rapidly. At the fermentation phase, lactic acid must be quickly produced to guarantee a rapid drop in pH. During the stable phase and feed out, air should be prevented from entering the silo (Kung, 2010).

The Use of Chemical Additives in Silage Production

McDonald et al. (1981) classified silage additives into the following categories: fermentation stimulants (LAB, sources of carbohydrates, and enzymes), fermentation inhibitors (primarily strong acids or chemicals with strong antimicrobial properties, aerobic deterioration inhibitors (primarily weak organic acids), nutrients, and absorbents. The most common types of silage additives used today includes fermentation stimulants from bacterial cultures based on LAB followed by aerobic deterioration inhibitors chemicals that are directly added to the silage. Fermentation stimulants containing homofermentative or facultative heterofermentative LAB can produce lactic acid and reduce silage pH at a more rapid rate than epiphytic flora, preventing nutrient degradation by the inhibition of plant enzymes and undesirable microorganisms. However, since 2001, a heterofermentative LAB, *Lactobacillus buchneri* has been used to improve the aerobic stability of silages because of its ability to produce moderate amounts of acetic acid, which has antifungal characteristics. As this organism does not increase the rate of fermentation, *L. buchneri* can currently be classified as an aerobic deterioration inhibitor (Schmidt and Kung, 2010).

Classically, aerobic deterioration inhibitors are used to alter the metabolism of lactate-assimilating yeasts that are the primary initiators of aerobic spoilage and are described for their ability to improve the aerobic stability of silages (Henderson, 1993). Silages that remains fresh without spoiling for long periods of time after exposure to air is desirable because its nutritive value and intake potential remain high. Although the use of *L. buchneri* to improve aerobic stability has markedly increased since its first introduction, its efficacy is still dependent on its ability to survive, grow, and produce sufficient amounts of acetic acid to suppress the growth of yeasts in silages. In studies from our lab (unpublished data) we have conducted studies where the added *L. buchneri* was unable to produce acetic acid, but the reasons for this finding are unknown. Thus, the use of chemical additives can be advantageous because they are not dependent on growth of an added microbe and production of sufficient inhibitory end products.

Chemical additives might be useful to overcome some management problems such as inadequate filling and packing, high DM silages (challenging to pack and to ferment well), short or prolonged ensiling time, slow feed out rate, and feed out during warm days. Another advantage of chemical additives is that they can be applied to improve aerobic stability after fermentation occurred (e.g., added to a total mixed ration with silage), whereas bacterial inoculants are not effective when applied to ensiled forage (Kung, 2014). Drawbacks of chemical additives include their relatively higher costs and usually higher volume of application rates (1 to 3 L/t) when compared to microbial inoculants (Muck et al., 2018).

Weak organic acids are the most common chemical additives used to improve aerobic stability. Acids such as acetic and propionic acid, act by reducing aerobic spoilage through the inhibition of aerobic microorganisms, such as yeasts, molds, and some aerobic bacteria (Danner et al., 2003; Kung et al., 1998). The classical weak-acid theory proposes that the inhibitory effect of weak organic acids occurs by the reduction of intracellular pH caused by the passage of undissociated molecules through the membrane and their dissociation in the cytoplasm. The dissociated acid reduces intracellular pH, which can be normalized by pumping protons and anions out of the cell by intermembrane transporters. As pumping protons out of the cell is a process with a high-energy requirement, ATP depletion might occur (Holyoak et al., 1996). Also, the anion of the weak acid, which is much less membrane-permeable than the undissociated acid, might accumulate in the cell and reach toxic concentrations (Pampulha and Loureiro-Dias, 1990; Russell, 1992). Membrane disruption and enzyme inhibition are some of the possible mechanisms of anion toxicity (Krebs, 1983; Holyoak, 1999). Weak acids such as benzoic and sorbic acid disrupt the cell membranes of yeasts such as *Saccharomyces cerevisiae* causing oxidative disruption under aerobic conditions (Piper et al., 2001).

Acids salts are commonly used in lieu of straight acids because they are less corrosive to machinery and less hazardous to workers (Henderson, 1993). Potassium sorbate is the potassium salt of sorbic acid. Sorbic acid and potassium sorbate are used in the food industry in baked and dairy products, on fruits and vegetables, and on pharmaceuticals and cosmetic products. Sorbates selectively inhibit specific yeasts and molds, although they do not affect growth of desirable bacteria such as LAB (Robach and

Sofos, 1982). Sorbates are effective in fermented food systems when applied at 0.02 to 0.20% (Stopforth et al., 2005). Neves et al. (1994) evaluated the effects of sorbic acid against a hundred yeasts strains isolated from spoiled foods and beverages. They found that a concentration of 0.05% of the preservative inhibited 60% of the strains.

Sodium benzoate is a preservative used in the food, cosmetic, and pharmaceutical industries to control growth of yeasts and fungi. In the food industry, it is mainly used in acidic systems, such as beverages, fruit products, pickles, jelly, and bakery products. Sodium benzoate (pKa= 4.2) is more effective in acidic food because only the undissociated form of the acid has antimicrobial properties and dissociation increases with higher pH. Concentrations of 0.05% to 0.10% of the undissociated form of the acid are sufficient to inhibit most of yeasts and fungi. Most bacteria are resistant to low doses of this preservative, but it can inhibit spore-forming bacteria at 0.01 to 0.02% concentrations of the undissociated acid (Chipley et al., 2005).

Several environmental factors or food chemical properties can affect the effectiveness of sorbates, such as pH, water activity, microbial numbers, type, temperature and length of storage, and the interaction with other chemical compounds of the food system or other additives (Liewen and Marth, 1985).

Studies have shown that the sorbic acid mode of action is different from that of other weak acids and is more similar to that of ethanol. Sorbates can act as a membrane-active molecule (Stratford and Anslow, 1998). Whereas acetic acid ($C_2H_4O_2$) is hydrophilic, sorbic ($C_6H_8O_2$) and benzoic acids ($C_7H_6O_2$) are lipophilic and can more easily penetrate the cell membrane. The toxicity of a lipophilic organic acid is related to

the length of its carbon chain. The more carbons, the higher is the acid lipophilicity and consequently the more toxic it is (Strafford et al., 2009).

Strafford et al. (2013), compared the mode of action of acetic acid and sorbic acid on *Saccharomyces cerevisiae* and found that whereas acetic acid acted as a classical weak acid and drastically decreased intracellular pH (4.7), sorbic acid only slightly decreased the pH (6.3). Sorbic acid acted by inhibiting the plasma membrane H^{+-} ATPase proton pump (Pma1p). This intermembrane protein is responsible for transporting protons out of the cell and generating a difference in the electrical potential between the inside and outside of the cell. Membrane potential is essential for the functioning of secondary transporters, responsible for the transport of several ions and molecules, and once disrupted can cause cell death (Michelet and Boutry, 1995).

Abbott et al. (2007), compared transcriptional changes in *S. cerevisiae* in response to the more lipophilic substances benzoate and sorbate, and the less lipophilic acetate and propionate. Even though these compounds did not cause drastic differences in physiological responses, differences in transcriptional modulation were massive. Benzoate and sorbate had a more significant influence on cell wall-related transcripts, while acetate and propionate responses were more related to intermembrane transport. Although compounds with similar lipophilicity showed closer related transcriptional responses, each specific compound led to different transcriptional modifications. Therefore, due to differential transcriptional responses, different compounds could act synergistically and increase the inhibition rate of a chemical additive. Also, combining different chemicals could potentially inhibit a broader range of microorganisms.

One of the uses of nitrite in the food industry is to inhibit growth of *Clostridium botulinum*. In silage, sodium nitrite is used as a fermentation inhibitor and can reduce the incidence of undesirable fermentations, such as those carried out be clostridia. The effective concentration of sodium nitrite is 0.02% (Noel et al., 1990). The effects of nitrite are highly dependent on pH. For each unit of decrease in pH, its antimicrobial capacity increases 10 times (Van Laack, 1994). Cammack et al. (1999) proposed four bacteriostatic modes of action of nitrite and its derived compounds: 1) reaction with iron-sulfur proteins that are essential to bacterial energy metabolism; 2) modification of proteins by deamination, by reacting with compounds that contain SH, or by reacting with thiols; 3) changes in DNA and gene expression; and 4) inhibition of the production of cell wall constituents or alteration of cell wall or membrane permeability.

Effects of Sodium Benzoate, Potassium Sorbate, and Sodium Nitrite on the Fermentation and Aerobic Stability of Corn Silage and HMC

Since different additives usually have diverse modes of action, their synergistic action might be the key when trying to increase application range and efficacy of additives. Stanojevic et al. (2009) evaluated the efficacy of sodium nitrite, sodium benzoate, and potassium sorbate alone and every possible combination of pairing these compounds on inhibiting *Bacillus subtilis*, *Bacillus mycoides*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus flavus*, *Fusarium oxysporum*, *Candida albicans*, *Trichoderma harsianum*, and *Penicillium italicum*. They found that combinations of chemicals were more effective against microorganisms than each

chemicals alone. The combination of sodium nitrite and sodium benzoate had a synergistic effect against 40% of the species evaluated and the combination of sodium nitrite and potassium sorbate against 30%.

A chemical additive containing sodium benzoate, potassium sorbate, and sodium nitrite was able to reduce numbers of clostridia in forages with DM contents lower than 30% and to decrease numbers of yeasts in crops with DM content higher than 35%. As a result, the additive improved fermentation conditions and aerobic stability (Knicky and Spörndly, 2011).

Few studies have evaluated the effects of sodium benzoate, potassium sorbate, and sodium nitrite on HMC. Loučka R. (2010) showed that Safesil (active ingredients: sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Goteborg, Sweden) applied at 3 or 6 L/t of fresh matter equally improved the aerobic stability of HMC compared to control. Da Silva et al. (2015) analyzed the effects of different doses of Safesil in HMC after 21 and 90 d of ensiling. The low application level (2 L/t of fresh matter) was enough to reduce the concentration of ethanol, increase DM recovery, and greatly improve the aerobic stability of HMC. Since the additive contains sodium nitrite that is considered toxic above a certain threshold, and that can be converted to nitrate and other compounds that could be toxic, the authors measured the concentrations of nitrate-N and nitrite-N in silage treated with the higher level of the chemical additive (4 L/t of fresh matter) and ensiled for short periods of time (0, 3 and 7 d). At all times, the concentrations of these compounds were within the range considered safe for ruminant feed.

Some studies have evaluated the efficacy of the combination of sodium benzoate, potassium sorbate, and sodium nitrite on improving aerobic stability of corn silage under challenging conditions. Savage et al. (2016) showed that whole-plant corn silage treated with Safesil (3 L/t of fresh matter) had a lower concentration of ethanol, lower numbers of yeasts, and higher aerobic stability than untreated silage. Additionally, the treatment overcame a 2 h air-stress per week for 100 days. Knicky and Spörndly (2015) found that Safesil (5 L/t of fresh matter) reduced ethanol production, maintained low pH, and improved aerobic stability of whole-plant corn silage under challenging conditions, such as low packing density and two 8 h air-stresses during the ensiling period.

Weiss et al., (2016) evaluated the combination of sodium benzoate and potassium sorbate on corn silage under two challenging conditions, delayed fill (ensiling after 24 h) and air stress (24 h at 3 different days). When forage was filled with delay, the chemical additive lowered the concentration of acetate, probably due to inhibition of enterobacteria, compared to the control and in silage treated with LAB. In air-stressed silage, this response was even more substantial. Additionally, the additive reduced ethanol production in prompt and delay-filled silage, and to a lesser extent in air-stressed silage. Although the concentration of ethanol declined, the additive did not reduce numbers of yeasts, and only increased aerobic stability under non-challenging conditions. The authors concluded that it is hard to predict the results of silage fermentations since many variables and their numerous interactions may affect the outcome.

Improving aerobic stability and DM recovery are not the only the goals of researchers studying chemical additives. The reduction of the environmental impact of

silage making is also a concern. Knicky et al. (2014) showed that timothy silage treated with Safesil produced less gas, especially CO_2, compared to control and silage treated with a bacterial inoculant. Furthermore, no differences among treatments were found in NOx gas content, which is a concern when using additives rich in nitrites. Therefore, by reducing CO_2 production, this additive can not only decrease DM loss but also diminish the environmental impact of silage making.

To the best of our knowledge, there have been no studies done on the effects of chemical additives on the composition of the microbial community of corn silage or high moisture corn by HTS. Studies have shown that Safesil improves aerobic stability even without changes in numbers of yeasts (Da Silva et al., 2015; Kung et al., 2018). Therefore, the additive might be improving stability not by inhibiting yeast growth but by favoring the dominance of yeasts that have reduced spoilage capacity or by affecting the bacterial population. HTS could be a useful tool to gain a better understanding on how chemical additives affect silage microorganisms and improve aerobic stability because it allows the identification of OTUs at a higher taxonomic level, and of new and low abundance OTUs (Kumar et al., 2015).

Accessing the Composition of The Microbial Community of Silage

Before molecular tools were available, the study of silage microbiology relied on culture-based methods. Microorganisms were quantified and identified by growth and screening on selective media, biochemical analysis, or morphological analysis of stained

cultures under a microscope. Although these techniques are useful, they are laborious and time-consuming and unsuitable for identifying microorganisms that are not culturable. Moreover, these techniques may not be sufficient to classify microorganisms at higher taxa levels or when trying to analyze the entire composition of the microbial community of an environment. As the field of molecular biology developed, it became possible to sequence parts or the entire genome of bacteria and fungi. Therefore, it is now possible to more precisely identify and quantify microorganisms that play an essential role in silage fermentation.

Muck (2013) published an extensive review on the advances of silage microbiology in the first decade of the years 2000. The author discussed the importance of DNA based methods in enabling the identification and quantification of unculturable microorganisms, and in facilitating the analysis of the composition of the microbial community and dynamics. Amplification of genes by PCR followed by sequencing is a method used to identify species, whereas RT-PCR is a technique used to quantify these microorganisms. For community analysis, there are four commonly used PCR based methods: Denaturing Gradient Gel Electrophoresis (DGGE), Terminal Restriction Fragment Length Polymorphisms (T-RFLP), Length Heterogeneity PCR (LH-PCR), and Automated rRNA Intergenic Spacer Analysis (ARISA).

During the past years, researchers have been migrating from analyzing DNA amplified by PCR with electrophoretic means to analyzing it by high throughput sequencing technology (HTS). The improvements in HTS have made it possible to generate large amounts of data at low cost and to identify unculturable, new and low-

abundance microorganisms in a variety of environments (Kumar et al., 2015). Few studies have been done using HTS to analyze silage microbial communities, but the method seems to be promising to broaden the understanding of this system. HTS was successfully used to identify new species of microorganism in silages and to evaluate changes in microbial population. Variations in the composition of the microbial community due to forage type, ensiling method, and process stage were effectively analyzed by HTS in recent years (McAllister et al., 2018).

Duniere et al. (2017) were the first group to describe the core bacterial and fungal microbiome of small grains during ensiling and after aerobic exposure using Illumina sequencing. They conclude that HTS is a valid tool for microbial community analysis and that this technology could boost the development of new additives once it can help to understand the interaction between additives and silage native microbial population. Kraut-Cohen et al. (2016) evaluated by HTS the dynamics and spatial composition of the bacterial community of corn, sorghum, corn-sorghum, and wheat silages from bunker silos. They concluded that the microbiome of the silage was more influenced by its localization in the silo than by the geographic location of the silo, type of crop, or time of sampling. Silages sampled from the center and sides of the silo were better preserved than the ones from the shoulders. Well-preserved silages had a community with more than 90% LAB and showed a lower diversity and higher evenness compared to spoiled silages, which had a low LAB and high *Enterobacteriaceae* abundance.

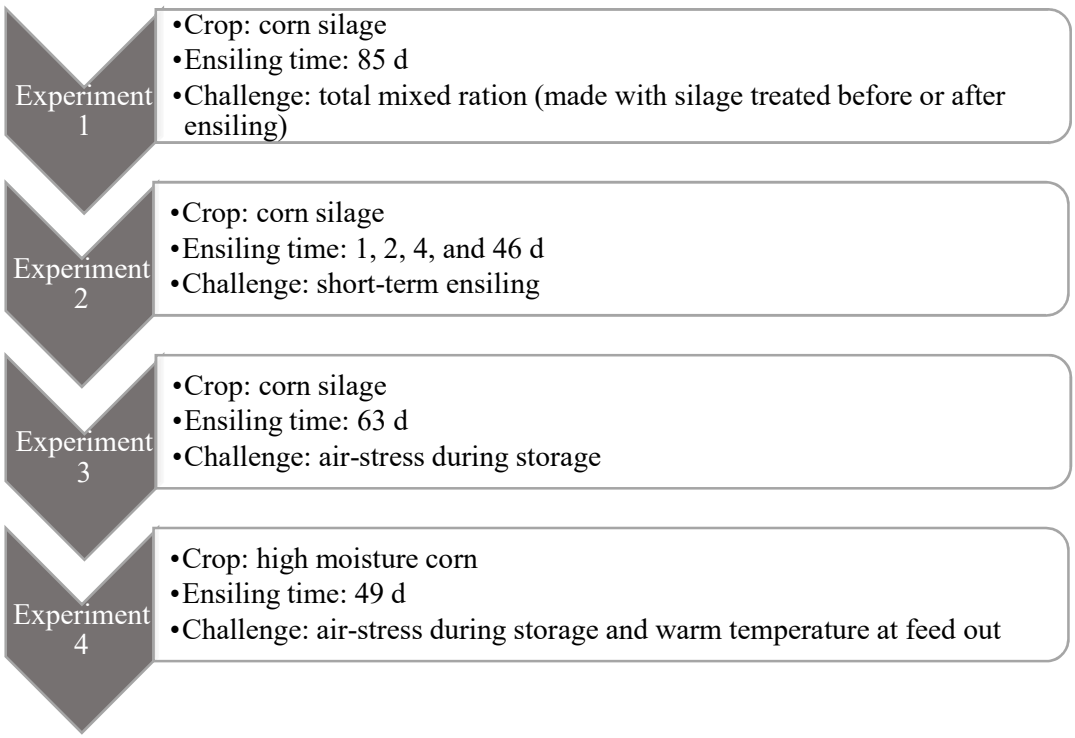
Besides all the advantages of HTS previously mentioned, this technology has some drawbacks. Some of the concerns regarding HTS are sampling, extraction, and

storage methods, PCR bias, primer type, sequencing, and bioinformatics workflow (McAllister et al., 2018). Pinloche et al. (2013), in a study evaluating the influence of probiotics in the rumen microbiome by HTS, highlighted that differences in sequencing technologies and data analysis might lead to different results regarding the microbiological composition of ruminal samples. Moreover, the choice of the primer and the specific hypervariable region of the 16s rRNA gene to be sequenced can influence the output. Jiang et al. (2017) found discrepancies between qPCR and Miseq analysis when evaluating the effect of probiotics in the rumen microbiome. Although a good correlation between the two methods was found for some species, a low correlation was observed for other species.

Objectives

The objectives of this dissertation were to evaluate the effects of a chemical additive containing sodium benzoate, potassium sorbate, and sodium nitrite on the composition of the bacterial and fungal communities of whole-plant corn silage and high moisture corn; and to assess its effectiveness on improving the fermentation and aerobic stability of silages subjected to different ensiling conditions.

The following scheme shows the crop, ensiling time, and challenging condition of each Experiment that composes this dissertation.



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Chapter 2

EFFECTS OF A CHEMICAL ADDITIVE ON THE COMPOSITION OF THE FUNGAL COMMUNITY, FERMENTATION, AND AEROBIC STABILITY OF CORN SILAGE

Abstract

The growth of yeasts during silage fermentation is undesirable because they compete with LAB for fermentable sugars, they result in poor dry matter (DM) recovery, and many species are responsible for initiating spoilage when silage is exposed to air. Organic acids can inhibit growth of yeasts thus improving fermentation and aerobic stability of silages. The objective of this study was to evaluate the effects of Safesil (SF, active ingredients: 20% sodium benzoate, 10% potassium sorbate, and 5% sodium nitrite) (Salinity, Göteborg, Sweden) on the composition of the fungal community of whole-plant corn by high throughput sequencing of the ITS1 region; and to assess its capacity to improve aerobic stability of corn silage and total mixed rations (TMR). Whole-plant corn was harvested at 39% DM, chopped, and packed into four individually replicated silos per treatment (7.5 L) at a density of about 224 kg of DM/m^3 without (CTRL) or with the addition of SF at 2 L/t of fresh forage weight. After 85 d of ensiling, portions of four replicates of CTRL silage, SF silage, or CTRL silage treated with SF at 2 L/t of fresh

forage weight after ensiling were used to make TMRs containing 40% (DM basis) of corn silage, 20% alfalfa silage, and 40% of a grain mix. SF silages had a lower pH, fewer yeasts, and lower concentrations of ethanol than CTRL. Compared to CTRL, SF silage had a lower relative abundance of *Candida tropicalis*, a yeast specie capable of producing ethanol and assimilating lactic acid. SF silage (241 h) and TMR made with silage treated before (71 h) or after ensiling (82 h) had markedly increased aerobic stability compared to CTRL silage (37 h) and TMR (41 h). The combination of sodium benzoate, potassium sorbate, and sodium nitrite not only reduced the total numbers of yeasts but also changed the fungal community of corn silage, greatly improving aerobic stability.

Introduction

Whole-plant corn silage is widely used in the United States to feed dairy cattle (Erdman et al., 2011). Corn silage is usually fed as part of a total mixed ration (TMR), as feeding TMR helps dairy cows to reach maximum performance (Lammers et al., 2003). Using good quality silages is essential to produce a stable TMR (Kung, 2010).

Yeasts are undesirable in both the anaerobic and aerobic stages of silage production. When in an anaerobic environment, some yeasts can ferment sugars and produce ethanol and CO_2, causing DM losses (McDonald et al., 1991). When exposed to air, yeasts can oxidize sugars or lactic acid, producing CO_2, H_2O, and heat.

When exposed to air, the degradation of lactic acid by lactate-assimilating yeasts causes and increase in silage pH and allows for growth of aerobic bacteria and molds that cause further spoilage (Elferink et al., 2000). Spoilage causes the loss of silage fermentation products, decreasing its nutritional value (Whitlock et al., 2000). Moreover, feeding spoiled silage to cattle can negatively affect their performance and health (Wilkinson, 1999).

When crop characteristics or management practices are not optimal, several additives can be applied to improve the aerobic stability of silages and TMR (Knick and Sporndly, 2011; Knick and Sporndly, 2015; Seppala et al., 2016). Potassium sorbate, sodium benzoate, and sodium nitrite are additives that in combination can inhibit undesirable microorganisms and delay spoilage. Potassium sorbate and sodium benzoate have antifungal properties, whereas sodium nitrite can inhibit the growth of anaerobic

bacteria that cause undesirable fermentations, such as clostridia and enterobacteria (Woolford, 1975).

The species of yeasts found in silage differ from farm to farm, and the factors that cause such variability are uncertain (Santos et al., 2017). While DNA fingerprinting methods have provided some insight into the types of yeasts responsible for aerobic spoilage (Muck, 2013), high throughput sequencing (HTS) is now the preferred method for community analyses due to its ability to generate large amounts of data at low cost and to identify unculturable, new and low-abundance microorganisms (Kumar et al., 2015). Therefore, this method was used to determine the effects of an additive on the composition of the fungal community of corn silage produced under conditions prevailing in North America

Objectives

The objectives of this study were to evaluate the effects of an additive cotaining sodium benzoate, potassium sorbate, and sodium nitrite on the composition of the fungal community of whole-plant corn silage by HTS; and to determine its effectiveness on improving the aerobic stability of whole-plant corn silage and TMR.

Materials and Methods

Crops and Silos

Whole-plant corn (TMF2H708b, Dow AgroSciences, Indianapolis, IN) was harvested at approximately 39% DM from the University of Delaware Farm (Newark, DE) and chopped to a theoretical length of 19 mm using a pull-type chopper (3975, John Deere, Moline, IL) equipped with kernel processer (roller gap set to 1.4 mm). Shortly after chopping, 300 ml of water (CTRL) or 300 ml of Safesil diluted in water at 2 L/t of fresh forage weight (SF, active ingredients: 20% sodium benzoate, 10% potassium sorbate, and 5% sodium nitrite, Salinity, Göteborg, Sweden) were applied to 4 individually replicated piles of 26 kg of forage using a hand sprayer. Forage from each pile was packed into 7.5-L laboratory silos at a packing density of approximately 224 kg of DM/m^3. Silos were sealed with plastic lids with O-ring seals and allowed to ensile for 85 d at 22 ± 2°C.

Aerobic Stability Measurements

Upon silo opening, approximately 2 kg of a representative sample from each replicate of CTRL silage and SF silage were returned to laboratory silos. Portions of CTRL silage, SF silage, or CTRL silage treated with SF at 2 L/t of fresh forage weight after ensiling were used to make TMRs. Corn silage represented 40% of the TMR (DM basis), and the remainder of the TMR was comprised of 20% alfalfa silage and 40% of a grain mix. The composition of the grain mix (Renaissance Nutrition Center Inc,

Norristown, PA, USA) is shown in Table 2.1 and Table A.1. The chemical composition of the grain mix was provided by the manufacturer, and that of the alfalfa was analyzed by Cumberland Valley Analytical Services (Waynesboro, PA, USA). A thermocouple wire was placed in the geometric center of each silage and TMR mass, and temperatures were recorded every 15 min using a data logger DataTaker DT85 (Thermo Fisher Scientific, Australia Pty, Scoresby, VIC, Australia). During aerobic exposure (22 ± 2°C) all laboratory silos were covered with 2 layers of cheesecloth which allowed the feed to be exposed to air. Aerobic stability was calculated as the number of hours before the temperature of the silage or TMR mass rose 2°C above the baseline temperature within each replicate silo.

Chemical Analysis

Fresh forage from each CTRL pile and silages after 85 d of ensiling had their DM content and pH measured. The DM content was determined after samples were placed in a 60°C forced-air oven for 48 h. Weights of full and empty silos were measured at silo opening, and with the DM content of forages and silages, was used to calculate DM recovery.

Representative 25-g portions of fresh forage, silages, concentrate, and TMR were mixed with 225 ml of sterile quarter strength Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) and homogenized for 1 min at medium speed in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). The

homogenate was filtered through 4 layers of cheesecloth. The pH was determined on homogenized samples using an Oakton pH700 Meter (Oakton Instruments, Vernon Hills, IL, USA). Fresh forage had its buffering capacity (BC) measured according to McDonald and Henderson (1962).

Portions of the water extracts that had been filtered through 4 layers of cheesecloth were filtered through a 54 filter paper (Whatman Inc., Clifton, NJ). The extracts were acidified with H_2SO_4 (50% vol/vol) and frozen. Later, they were analyzed for lactic, acetic, propionic, and butyric acids, 1,2-propanediol, and ethanol (Muck and Dickerson, 1988) using a Shimadzu LC-20AD HPLC (Shimadzu Scientific Instruments, Columbia, MD).

The nutrient analysis was performed by Cumberland Valley Analytical Services (Waynesboro, PA, USA). A portion of each dried sample was ground using an Udy Cyclone Sample Mill (Udy Corp., Fort Collins, CO) to pass through a 1-mm screen. Total N was measured by combustion of the sample following the AOAC method 990.03 (AOAC, 2010) using a Leco CNS2000 Analyzer (Leco Corporation, St. Joseph, MI), and crude protein (CP) was calculated by multiplying the resulting total N concentration by 6.25. Soluble protein (SP) was determined by the method of Krishnamoorthy et al. (1982) and expressed as % of CP. Acid detergent fiber (ADF) was quantified on dried ground samples using the AOAC method 973.18 (AOAC, 2010), using Whatman 934-AH glass micro-fiber filters with 1.5-µm particle retention instead of fritted glass crucibles. Neutral detergent fiber (NDF) was analyzed on dried samples according to Van Soest et al. (1991) with sodium sulfite and amylase, not corrected for ash. Ash was analyzed by the

AOAC method 942.05 (AOAC, 2000), with the modification of using a 1.5-g sample, 4 h ash time, and hot weighing. Another portion of the dry sample was ground to pass a 3-mm screen and analyzed for starch (Hall, 2008).

Microbial Analysis

Representative 25-g portions of fresh forage, silages, concentrate, and TMR were mixed with 225 ml of sterile quarter strength Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) and homogenized for 1 min at medium speed in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). The homogenate was filtered through 4 layers of cheesecloth. The filtrate was serially diluted in Ringers solution, and the numbers of LAB were determined by pour-plating in de Man, Rogosa, and Sharpe agar (CM3651, Oxoid, Unipath Ltd., Basingstoke, UK). Plates were incubated anaerobically at 32°C for 48 to 72 h in a plastic container with anaerobic packs (Mitsubishi Gas Chemical Co., Tokyo, Japan) and an anaerobic indicator (BR0055, Oxoid, Unipath Ltd., Basingstoke, UK). The containers were vacuum-sealed in nylon-polyethylene bags (3.5-mil embossed pouches, 15.2 × 30.5 cm; Doug Care Equipment Inc., Springville, CA) using a Best Vac vacuum machine (distributed by Doug Care Equipment Inc.). Numbers of yeasts and molds were determined on malt extract agar (CM0059, Oxoid, Unipath Ltd., Basingstoke, UK) acidified with lactic acid (85%) at a rate of 0.5% vol/vol. Plates were incubated aerobically at 32°C for 48 h before enumeration. Plates with a number of colonies between 30 and 300 were used to obtain counts of colony-forming units.

Fungal Microbiome Analysis

Three replicates of 25 g of fresh forage, CTRL and SF silages were mixed with 225 ml of autoclaved Ringers solution for 2 min using a Colworth 400 stomacher (Seward, London, UK). The homogenates were filtered through four layers of cheesecloth. Next, 2 ml of the filtrates were centrifuged for 3 min at 21130.2 xg using a Centrifuge 5424 R (Eppendorf AG, Hamburg, Germany). The supernatant was discarded, and 100 microliters of autoclaved Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) was used to resuspend the pellet. Samples were kept at -80°C for further analysis.

Extraction of DNA, ITS1 amplification, and Illumina MiSeq-based sequencing were performed by the Research and Testing Laboratory (Lubbock, TX, USA). DNA extraction was done using the MoBio PowerMag Soil kit, according to manufacturer's instructions. The primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2aR (GCTGCGTTCTTCATCGATGC) (White et al., 1990; Gardes and Bruns, 1993) were used to amplify the internal transcribed spacer 1 (ITS1). Sequencing was performed with an Illumina MiSeq (San Diego, CA, USA) platform with the 2 × 250 bp paired-end method.

For sequencing processing, paired-end reads were merged and converted into FASTA files using FLASH version 1.2.11 (Magoc and Salzberg, 2011). The quality profile of the reads was checked on http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Andrews, 2010). Next, reads were filtered for quality and the primers were trimmed using QIIME 1.9.1 (Caporaso,

2010). Fungal operational taxonomic units (OTUs) were picked using open reference clustering against UNITE database at 97% similarity (Kõljalg et al., 2005). Finally, singles and double reads were filtered. The diversity analyses were performed using the phyloseq and vegan packages in R (McMurdie and Holmes, 2013; Oksanen et al. 2013). Membership and composition Venn diagrams were computed using the MetaComet web platform to determine OTU overlap among treatments (Wang et al., 2016). BLAST (Altschul et al., 1990) was used to identify sequences related to the sequences of interest with the highest Max score. Phylogenetic trees were built in MEGA 7 (Kumar et al., 2016) using the Neighbor-Joining method (Saitou and Nei, 1987) with the maximum composite likelihood substitution model after 1000 bootstrap replication.

Statistical Analysis

Data were analyzed using JMP 12 (SAS Institute, Cary, NC). Data from the silo opening were analyzed separately as a completely randomized design, with treatment as the fixed effect. Aerobic stability data from silage and TMR were analyzed separately. Differences were considered significant when $P < 0.05$, and pairwise mean comparisons were performed using Tukey's test at $P < 0.05$ (Snedecor and Cochran, 1980). Statistical analysis for the PCoA plots was done using PERMANOVA (permutational multivariate analysis of variance) by the adonis function in vegan and for CCA plot using PERMANOVA (Oksanen et al. 2013).

Results and Discussion

Chemical and Microbial Characteristics of Fresh Forage

Table 2.2 shows the DM content and chemical and microbial composition of fresh forage. The DM content of the freshly chopped forage was 39.4%. The chemical and microbial compositions were in the expected range for this feedstuff (Hafner et al., 2015; Kleinschmit et al., 2005; Kleinschmit and Kung, 2006). The grain added to the TMR had 92% DM, and 5.85 log cfu/g of fresh weight yeasts and the numbers of molds were below the lowest dilution (2 log CFU/g of fresh forage) used in this study. Alfalfa added to the TMR had 39% DM, and yeasts or molds were below the detection limit of 2 log CFU/g of fresh forage (Table 2.3).

Chemical Properties, Microbial Populations, and Aerobic Stability of Silage and TMR

Table 2.4 shows the chemical and microbial composition of untreated and treated silage after 85 d of ensiling. There were no differences in concentrations of DM, CP, SP, ADF, NDF, and starch between treatments.

After ensiling, LAB numbers were 0.6 log lower in SF than in CTRL. The lower numbers of LAB in SF compared to CTRL was probably due to the lower pH of SF, which could have caused a faster die-off of LAB, rather than the inhibition of LAB by the action of the additive. Lower numbers of LAB in silage treated with the additive containing sodium benzoate, potassium sorbate, and sodium nitrite was previously observed in high moisture corn, but in that case, it was not accompanied by a reduced

concentration of organic acids (Da Silva et al., 2015). In the present study, SF had lower concentrations of lactic and acetic acids than CTRL (4.00 *vs.* 4.82 and 0.76 *vs.* 1.23 % of DM, respectively), still it had a lower pH (3.65 *vs.* 3.74) (Table 2.5). Even though the concentration of total acids was lower in SF, it had higher lactate to acetate ratio than CTRL (5.26 *vs.* 3.92). The lower concentration of acetic acid in SF was probably caused by inhibition of enterobacteria or due to a more homofermentative fermentation. Because acetate acts as a buffer, SF possibly had a lower buffering capacity compared to CTRL, and this caused its pH to drop more. In the present study, the numbers of enterobacteria was not determined, but a reduction in its numbers by the additive containing sodium benzoate, potassium sorbate, and sodium nitrite was previously observed in alfalfa silage (da Silva et al., 2016).

SF had lower numbers of yeasts than CTRL (<2.00 *vs.* 3.96 log cfu/g of fresh weight) coupled with a decreased production of ethanol (1.27 *vs.* 2.39 % of DM) (Table 2.5). DM losses can occur when yeasts ferment sugars, producing ethanol and CO_2 (McDonald et al., 1991), however, in this study, SF did not improve DM recovery. Previous experiment had shown a reduction in the numbers of yeasts and lower ethanol production due to treatment with sodium benzoate, potassium sorbate, and sodium nitrite in silages (Knick and Sporndly, 2015), as well as an increase in DM recovery in HMC (Da Silva et al., 2015).

Treatment with sodium benzoate, potassium sorbate, and sodium nitrite markedly increased the aerobic stability of corn silage compared to CTRL (241 *vs.* 37 h) (Figure 2.2A) in silage. The reduced numbers of yeasts and lower pH of SF compared with

CTRL can explain the improvements in aerobic stability in treated silage. Knick and Sporndly (2011) previously observed low numbers of yeasts and high aerobic stability in corn silage at 39% DM treated with sodium benzoate, potassium sorbate, and sodium nitrite. TMR made with SF silage (71 h) and with CTRL silage treated with the additive after ensiling (82 h) had higher aerobic stability than TMR made with untreated silage (41 h) (Figure 2.1B). A similar finding was reported by Seppala et al. (2016) for a study evaluating TMR containing grass silage, pelleted concentrate, and brewer's grain (5:4:1). They found that TMR made with silage treated with sodium benzoate, potassium sorbate, and sodium nitrite at 3 L/t of fresh weight was stable 24 and 29 h longer than the ones made with untreated silage or with silage treated with homofermentative LAB, respectively.

When exposed to air, lactate-assimilating yeasts can degrade lactic acid and increase silage pH and temperature. The rise in pH is the starting point for another wave of spoilage that leads to an even higher increase in silage mass temperature. The first peak in temperature results from growth of yeasts, whereas the second peak from growth of aerobic bacteria and molds (Elferink et al., 2000). The heating curves and peaks for silages and TMR are shown in Figure 2.2 and Table 2.5, respectively. The heating curve of CTRL silage had two distinct peaks. The TMR-CTRL heating curve also had two peaks, but they were not as separated as on the silage. The temperature of the first peak of CTRL silage and TMR were similar (average of 32.87°C); however, the second peak in TMR-CTRL was 9.76°C above its first peak, and in CTRL silage it was approximately 3.35°C higher than its first peak. TMR-SF and TMR-RT-SF had similar heating curves,

both with only one peak. The temperature of the second peak was higher in TMR-CTRL than in CTRL (43.68 *vs.* 35.17°C). TMR-SF and TMR-RT-SF had only one peak that reached a maximum temperature of 46.31°C. TMR usually reaches higher temperatures during aerobic spoilage than silages because its components, especially grains, can have high numbers of yeasts and molds (Kung, 2010). In the present study, the high number of yeasts found in the grain added to the TMR can explain the higher temperature found in TMR compared to silages. Another explanation is that the grain added to the ration could have increased the amount of soluble sugars.

The Composition of the Fungal Community

Sequencing depth was sufficient to describe the diversity of the fungal populations in fresh forage and silages as the rarefaction curves reached a plateau (Figure B.1). Table 2.6 shows the alpha diversity indexes for fresh forage and silages ensiled for 85 d. Fresh forage had higher numbers of observed OTU and higher Chao1 and Shannon indexes than CTRL silage. Therefore, the fungal composition of fresh forage was richer and more even than CTRL silage. The higher richness in fresh forage in comparison with silage was expected. A wide range of aerobic microorganisms populate the standing corn plant, but their growth is inhibited when the silo is sealed (Pathlow et al., 2003). SF had lower numbers of observed OTU, and similar Chao1 and Shannon indexes compared with fresh forages. There were no differences between CTRL and SF after 85d of ensiling for any of the analyzed alpha diversity indexes.

The PCoA plot (Figure 2.3) shows that the replicates of a treatment clustered together and that each treatment formed clusters separated from each other. A significant Adonis test proved that the treatments did not have the same centroid, and a non-significant betadisper test showed that differences in group dispersions did not cause the results. It was concluded that ensiling and treatment affected composition of the fungal community.

Figure 2.4A shows a membership Venn diagram based on the presence or absence of an OTU in a treatment. Fresh forage had the highest (137), SF silages intermediate (105), and CTRL silages the lowest (78) number of OTUs. From a total of 189 OTUs, 46% were unique to one treatment, and fresh forage, treated silage, and untreated silage shared 25%. Fresh forage shared 6 OTUs with CTRL silages and 27 OTUs with SF silages. Untreated and treated silages shared a total of 11 OTUs. Figure 2.4B presents a composition Venn diagram constructed based on the OTUs relative abundance. Like the membership Venn diagram, fresh forage had the highest (99), SF silages intermediate (68), and CTRL silages the lowest (47) number of OTUs. 92% of the OTUs were unique to only one treatment, and 5% were shared among all treatments. Fresh forage did not share any OTU with CTRL silages but shared 3 OTUs with SF silages. CTRL and SF silages had two OTUs in common. Both the analysis of diversity indexes and the Venn diagrams showed that the composition of the fungal community of SF silage was more similar that of fresh forage than that of CTRL silage. These facts indicated that the additive helped to preserve the natural composition of the fungal community of the forage during ensiling.

The relative abundance of the genus *Candida* in fresh forage was 0.4%. The relative abundance of this genus increased after ensiling, and it was markedly higher in CTRL than in SF (52% vs. 11%). The OTU X1 and X2 at species level represented 90% of the total abundance of the genus *Candida*. Figure 2.5 shows the position of X1 and X2 in a phylogenetic tree containing the five strains with the highest Max score on BLAST. Both X1 and X2 were assigned to the species *Candida tropicalis*. Strains of *Candida tropicalis* were previously isolated from corn silage and HMC (Burmeister and Hartman, 1966; Altamirano et al., 2000;). A *Candida tropicalis* strain isolated from HMC was proved to be able to assimilate lactate (Burmeister and Hartman, 1966). Also, *Candida tropicalis* strains previously demonstrated the ability to produce ethanol (Jamai et al., 2007). The lower total numbers of yeasts and reduced relative abundance of *Candida tropicalis* in treated silage could explain why its pH and concentration of ethanol was lower compared to CTRL.

The permutation test showed that the Canonical Correspondence Analysis (CCA) plot (Figure 2.6) significantly described its predicted responses. CCA illustrated that aerobic stability was negatively correlated with numbers of yeasts, pH, and concentration of ethanol. The pH, rather than yeasts or ethanol, had a higher correlation with aerobic stability. The relative abundance of OTU 12, genus *Candida*, was positively correlated with numbers of yeasts, pH, and concentration of ethanol, and negatively correlated with aerobic stability. The OTU 12, genus *Candida*, was closely related with CTRL than with SF silages.

Conclusions

The combination of sodium benzoate, potassium sorbate, and sodium nitrite reduced the production of ethanol and markedly improved the aerobic stability of corn silage and TMR made with corn silage treated before or after ensiling. The additive capability of reducing the total numbers of yeasts and decreasing the relative abundance of *Candida tropicalis*, ethanol producer and lactate-assimilating yeasts, explained the improvements in aerobic stability.

This chemical additive can be useful to improve the aerobic stability of corn silage fed alone or as part of a TMR, minimizing heating, decreasing DM losses, and reducing risks related to feeding spoiled silage. Additionally, the additive has the potential to be used as a TMR stabilizer as its application in silage after ensiling increased TMR aerobic stability.

TABLES

Table 2.1. The composition of the grain mix added to the total mixed ration.

Item	% (as fed basis)
Corn ground	30.91
Protected soybean meal[1]	16.01
Canola meal	14.72
Citrus pulp	13.87
Distillers corn	12.43
Porcine blood	1.84
Rumen bypass fat[2]	1.37
Sodium bicarbonate	1.29
Rumen fermentation enhancer	1.21
Micronutrients premix[3]	1.03
Sugar byproduct[4]	0.97
Corn gluten meal	0.92
Calcium carbonate	0.83
Sodium chloride	0.81
Monensin[5]	0.67
Potassium carbonate[6]	0.48
Methionine Hydroxy Analog[7]	0.18
Urea	0.14
Rumen protected lysine[8]	0.13
Vitamin E[9]	0.07
Aspergillus oryzae fermentation extract[10]	0.06
Rumen protected methionine[11]	0.03
Chelated zinc[12]	0.02
Biotin[13]	0.01
Live yeast[14]	0.01

[1]Extruded and expelled soybean meal (J. L. Moyer & Sons, Inc., Turbotville, PA).
[2]MEGALAC (Church & Dwight Co., Inc, Princeton, NJ).
[3] Mil Mix 4 (Renaissance Nutrition Inc., Roaring Spring, PA). The composition described in Table A1.1
[4]Contained 92.3% sucrose (Renaissance Nutrition Inc., Roaring Spring, PA).
[5]Rumensin 90 (Elanco, Greenfield, IN).

[6]DCAD Plus (Church & Dwight Co., Inc, Princeton, NJ).
[7]HMTBa (MFP, Novus International, Inc., St. Charles, MO).
[8]AjiPro-L Generation 2 (Ajinomoto Heartland, Inc., Chicago, IL).
[9]Vitamin E, 44,100 IU/kg.
[10]Fermenten (Church & Dwight Co., Inc, Princeton, NJ)
[11]Smartamine M (Adisseo, Antony, France).
[12]MINTREX Zn (Novus International, Inc., St. Charles, MO).
[13]Microvit H Promix Biotin 2% (Adisseo, Antony, France).
[14]Levucell SC (Lallemand Animal Nutrition, Milwaukee, WI).

Table 2.2. The DM content, chemical composition (% of DM, unless stated otherwise), pH, buffering capacity, and numbers of LAB, yeasts, and molds (fresh weight basis) of freshly chopped whole corn plants.

Item	Average	Standard deviation
DM, %	39.39	0.31
CP	6.88	0.10
SP[1], % of CP	25.87	0.63
ADF	22.15	1.32
NDF	38.65	2.15
Starch	36.38	1.54
pH	5.87	0.05
BC[2], g lactic acid/kg of DM	21.00	2.37
LAB[2], log cfu/g	7.83	0.35
Yeasts, log cfu/g	6.02	0.26
Molds, log cfu/g	3.40	1.63

[1]Soluble protein.
[2]Buffering capacity
[3]Lactic acid bacteria.

Table 2.3. The DM content, chemical composition (% of DM, unless stated otherwise), pH, and numbers of LAB and yeasts (fresh weight basis) of the alfalfa silage and grain mix added to the total mixed ration.

Item	Alfalfa silage	Grain mix
DM, %	38.61	92.12
CP	17.8	25.07
SP[1], % of CP	55.7	20.1
ADF	47.9	9.94
NDF	49.40	18.56
Starch	2.30	23.36
pH	4.80	NM
LAB[2], log cfu/g	6.32	5.92
Yeasts, log cfu/g	<2.00	5.85

[1]Soluble protein.
[2]Lactic acid bacteria.
NM= not measured

Table 2.4. The DM content, chemical composition (% of DM, unless stated otherwise), lactate to acetate ratio, pH, numbers of LAB and yeasts (fresh weight basis), and DM recovery of corn silage untreated or treated with Safesil ensiled for 85 d.

Item	CTRL[1]	SF[2]	SEM	*P*-value
DM, %	38.32	38.56	0.29	0.58
CP	7.00	6.83	0.36	0.12
SP[3], % of CP	52.14	49.78	0.53	0.06
ADF	23.70	22.98	0.20	0.35
NDF	39.33	38.98	0.61	0.46
Starch	34.75	34.48	0.84	0.82
pH	3.74[a]	3.65[b]	0.01	<0.01
Lactic acid	4.82[a]	4.00[b]	0.19	0.02
Acetic acid	1.23[a]	0.76[b]	0.09	0.01
Lactate:acetate ratio	3.92[b]	5.26[a]	0.26	0.02
Ethanol	2.39[a]	1.27[b]	0.09	<0.01
LAB[4], log cfu/g	7.26[a]	6.63[b]	0.13	0.01
Yeasts, log cfu/g	3.96[a]	<2.00[b]	0.30	<0.01
DMR[5], %	96.13	97.34	0.73	0.29

[a-b] Means in rows with unlike superscripts differ ($P < 0.05$).
[1]CTRL = untreated
[2]SF = 2 L of Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden)/t of fresh matter.
[3]Soluble protein.
[4]Lactic acid bacteria.
[5]DM recovery.

Table 2.5. Average heating peaks after air exposure of corn silage untreated (CTRL) or treated with Safesil (SF) ensiled for 85 d, and of TMR made with untreated silage (TMR-CTRL), with silage treated with Safesil before ensiling (TMR-SF), or with silage treated with Safesil after ensiling (TMR-RT-SF).

	First peak		Second peak	
Treatment	Time (h)	Temperature (°C)	Time (h)	Temperature (°C)
Silage only-CTRL	74.75[b]	31.82[b]	186.50[a]	35.17[b]
Silage only-SF[1]	NA	NA	NA	NA
TMR-CTRL	75.25[b]	33.92[b]	142.25[b]	43.68[a]
TMR-SF	167.75[a]	46.31[a]	NA	NA
TMR-RT-SF	186.50[a]	45.38[a]	NA	NA
SEM	9.70	1.54	9.16	1.24
P-value	<0.01	<0.01	<0.01	<0.01

[a-c] Means in rows with unlike superscripts differ ($P < 0.05$).
[1]SF = 2 L of Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden)/t of fresh matter.
NA= No peak observed.

Table 2.6. Alpha diversity indexes of the composition of the fungal community of fresh forage and of corn silage untreated or treated with Safesil ensiled for 85 d, as analyzed by the sequencing of ITS1 using Illumina Miseq.

Index	0 d	85 d		SEM	*P*-value
	CTRL[1]	CTRL	SF[2]		
Observed	98.33[a]	40.67[b]	59.67[b]	8.84	<0.01
Chao1	103.17[a]	48.27[b]	63.67[ab]	11.61	0.04
Shannon	2.61[a]	1.67[b]	2.49[ab]	0.21	0.04
Simpson	0.87	0.66	0.83	0.06	0.08

[a-b] Means in rows with unlike superscripts differ ($P < 0.05$).
[1]CTRL = untreated.
[2]SF = 2 L of Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden)/t of fresh matter.

FIGURES

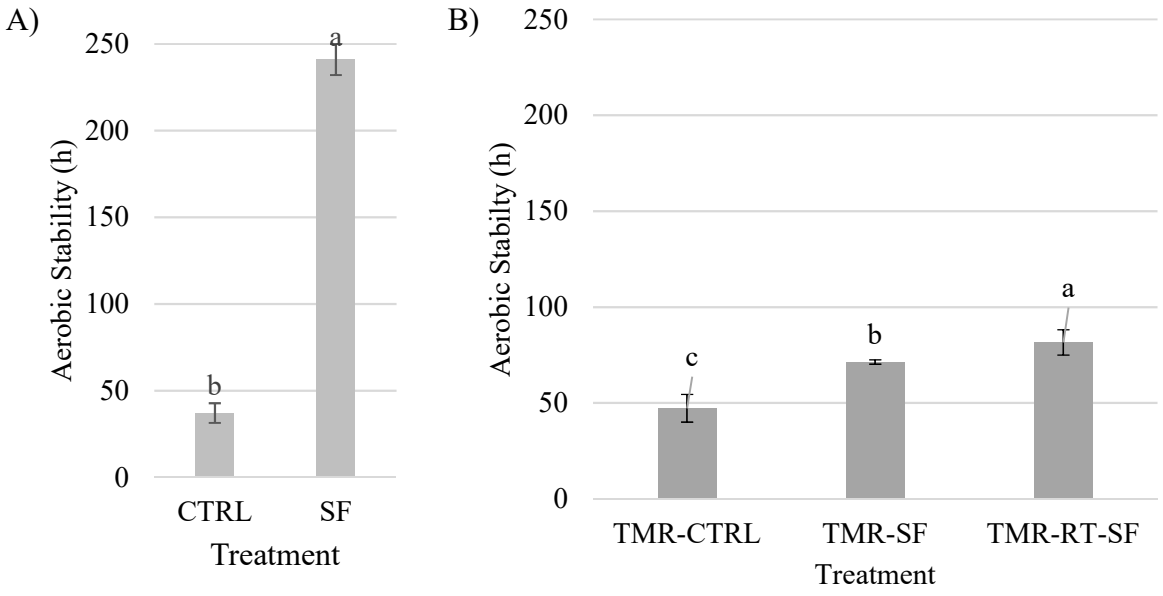

Figure 2.1. Aerobic stability of af A)untreated silage (CTRL) and silage treated with Safesil 2 L (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden)/t of fresh matter (SF) ensiled for 85 d (SEM=7.5, $P < 0.01$), and of B) TMR made of untreated silage (TMR-CTRL), of silage treated with SF before ensiling (TMR-SF), and of silage treated with SF after ensiling (TMR-RT-SF) (SEM=5.3, $P < 0.01$). $^{a\text{-}c}$ Means bars with unlike letters differ ($P < 0.01$).

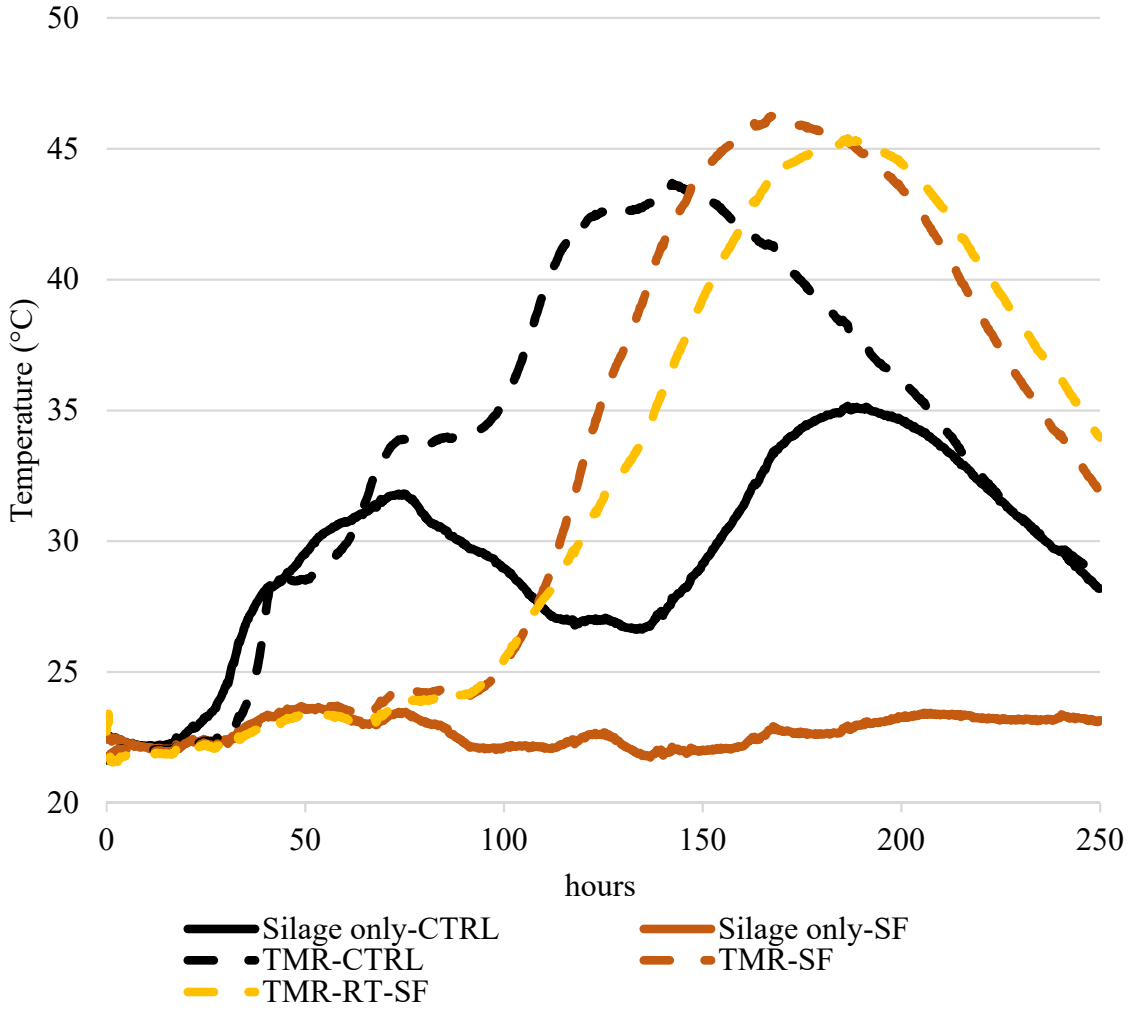

Figure 2.2. Heating curves of untreated silage (CTRL), silage treated with Safesil 2 L (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden)/t of fresh matter (SF) ensiled for 85d, and of TMR made with untreated silage (TMR-CTRL), with silage treated with SF before ensiling (TMR-SF), or with silage treated with SF after ensiling (TMR-RT-SF).

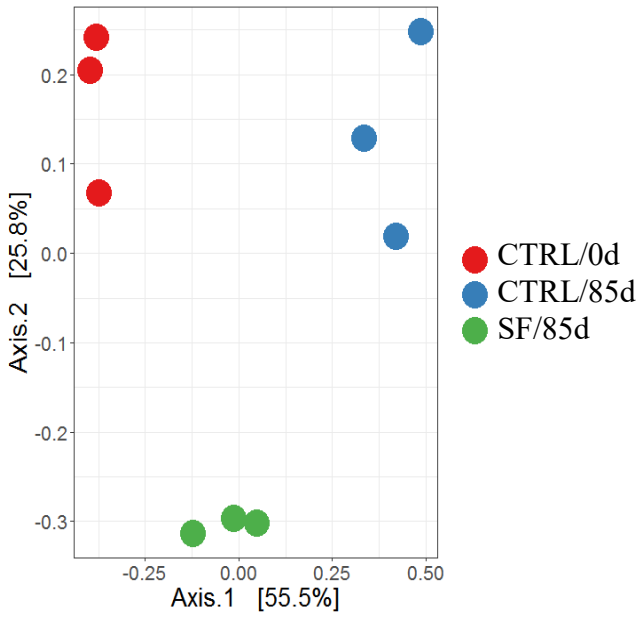

Figure 2.3. Bray-Curtis PCoA ordination based on fungi OTU from sequencing of the ITS1 using Illumina Miseq of fresh forage (CTRL/0d) and corn silage untreated (CTRL/85d) or treated with Safesil 2L (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden)/t of fresh matter (SF/85d) ensiled for 85 d.

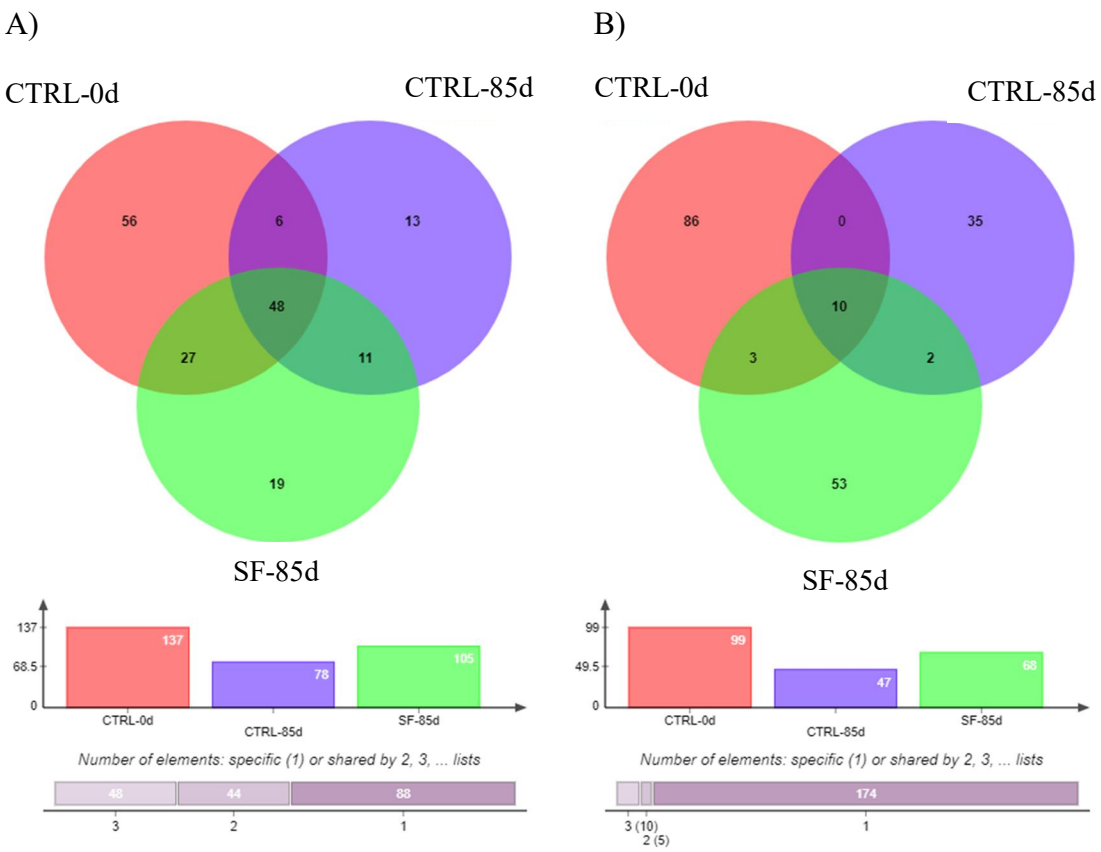

Figure 2.4. A) Membership Venn diagram build based on the presence and absence of fungi OTU and B) Composition Venn diagram based on OTU relative abundance (abundance ratio parameter of 0.8) obtained from the sequencing of the ITS1 using Illumina Miseq of four replicates of fresh forage (CTRL-0d) and corn silage untreated (CTRL-85d) or treated with Safesil 2 L (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden)/t of fresh matter (SF-85d) ensiled for 85 d. The numbers on the diagrams represent the numbers of unique or shared OTUs among treatments.

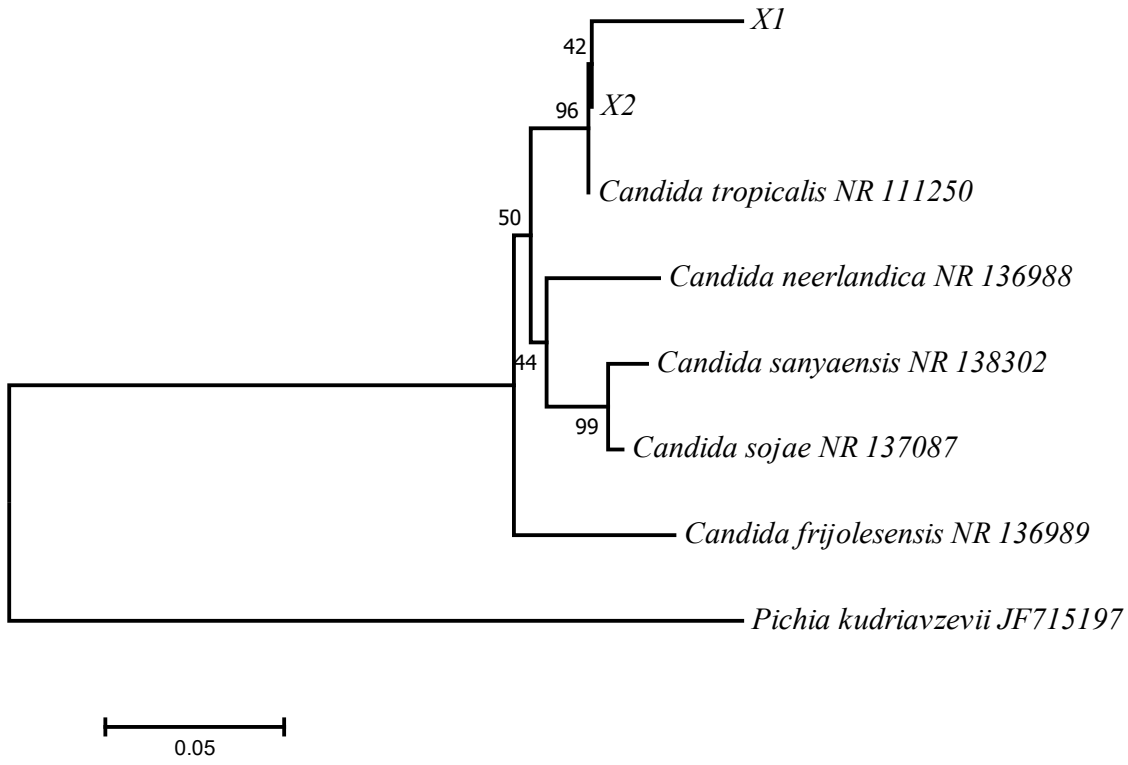

Figure 2.5. Phylogenetic tree constructed by the Neighbor-Joining method showing the position of 7 yeasts species. X1 and X2 represents the most abundant OTUs at species level for the genus Candida. The evolutionary distances were computed using the Maximum Composite Likelihood method. Bootstraps value for 1,000 replicates is shown at the nodes. *Pichia kudriavzevii* was used as outgroup. Scale bar indicates an evolutionary distance of 0.05 nucleotide substitution.

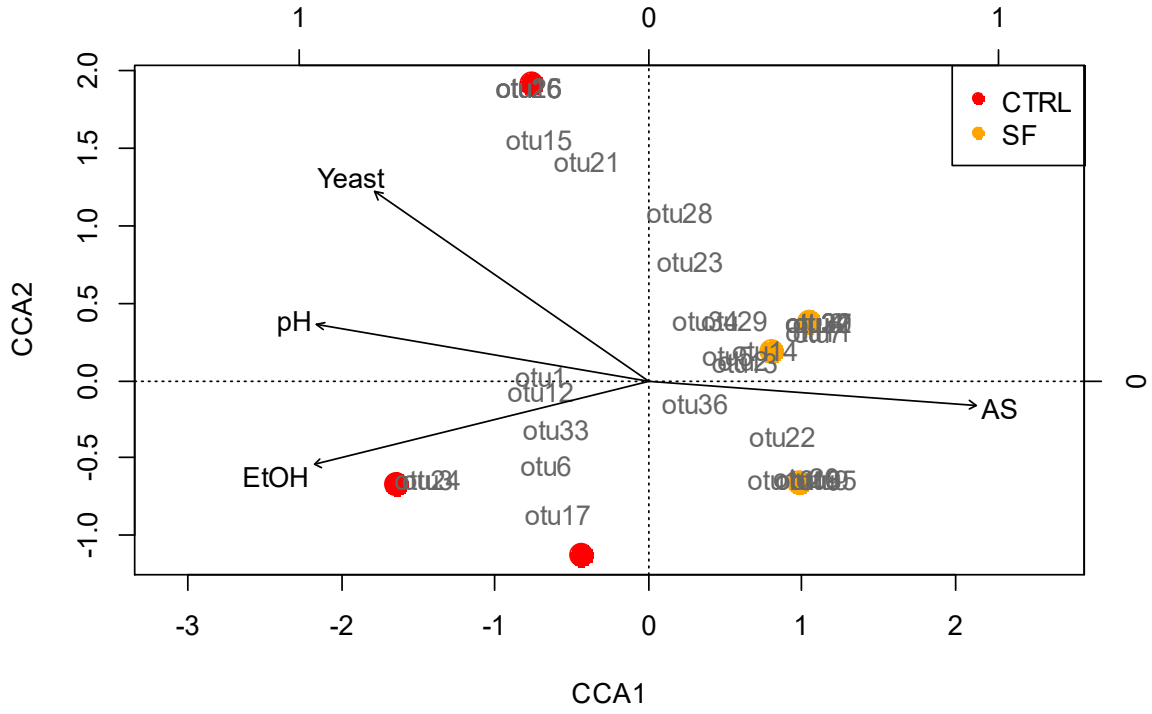

Figure 2.6. Canonical correspondence analysis (CCA) plot showing the relationships among treatments, fungi OTU at the genus level, and environmental variables. Arrows demonstrate the direction and magnitude of the environmental variables. CTRL, untreated silage ensiled for 85 d; SF, silage treated with Safesil 2 L (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden)/t of fresh matter ensiled for 85 d; AS, aerobic stability.

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Chapter 3

EFFECTS OF A CHEMICAL ADDITIVE ON THE COMPOSITION OF THE MICROBIAL COMMUNITY OF CORN SILAGE AND ITS EFFECTIVENESS IN IMPROVING THE FERMENTATION AND AEROBIC STABILITY OF CORN SILAGE AFTER SHORT PERIODS OF ENSILING

Abstract

In many circumstances, ensiled forages must be fed out almost immediately or very shortly after being packed into silos. When silages are exposed to air at feed out, yeasts and acetic acid bacteria can initiate spoilage of the feed. The objective of this study was to evaluate the effects of a chemical additive (SF, active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite, Salinity, Göteborg, Sweden) on the composition and dynamics of the microbial community of whole-plant corn silage and its effects on the fermentation and aerobic stability of corn silage ensiled for short periods of time. Whole-plant corn was harvested at 39% DM, chopped, and was untretaed (CTRL) or treated with SF at 2 (SF2) and 3 L/t (SF3) of fresh forage weight. Four individually replicated silos (7.5 L) were prepared and opened after 1, 2, 4, and 46 d of ensiling. The fermentation end-products, agar-culturable microbial population, the composition of the

microbial community (using high-throughput sequencing), and aerobic stability were measured. Samples from CTRL and SF2 silos opened after 46 d of ensiling and exposed to air for 50, 100, and 240 h were also analyzed for changes in the microbial community. Treatment with SF generally had only minor effects on the traditional fermentation end products of silage. The additive also did not affect the overall composition of the microbial community of corn silage through 46 d of ensiling. However, the composition of the microbial community changed with time of ensiling. Numbers of total yeasts were similar among treatments from 1 to 4 d, at 46 d, fewer yeasts were present in SF-treated silages than CTRL. However, treatment with SF improved the aerobic stability of silages compared to CTRL at all openings. Numbers of total yeasts were lower in SF2 compared to CTRL during aerobic exposure. The population of Lactobacillaceae in SF2 was more stable to exposure to air than in CTRL. After 50 h of exposure to air, the ribosomal gene sequence abundance of Enterobacteriaceae in CTRL silages was greater than in SF2 silages. After 240 h of aerobic exposure, sequences representing the Acetobacteraceae were the most abundant bacterial family in CTRL silages, whereas their abundance in SF2 silages was lower. The results from this study show that SF markedly improved the aerobic stability of corn silage after short periods of ensiling, with the early effects, probably due to fungistatic (rather than fungicidal) activity. Improved aerobic stability in treated silage fermented for 46 d was most likely due to lower numbers of total yeasts but also inhibition of acetic acid bacteria.

Introduction

Fermentation of whole plant corn to produce silage is a process that aims to preserve forage and to provide more uniform feeding conditions to ruminants throughout the year. The fermentation of forages occurs by the action of lactic acid bacteria (LAB) that convert water-soluble carbohydrates (WSC) to organic acids, and the active ensiling process usually takes several weeks to complete (Pahlow et al., 2003). However, it is not uncommon for farmers to feed forage before the active process of silage fermentation is complete, usually because they run out of well-fermented silage. Forages that are incompletely fermented can have decreased aerobic stability because the pH is not low enough to prevent the growth of undesirable microorganisms. When silage is exposed to air, yeasts that were dormant in the silo can start to grow and initiate spoilage. The degradation of lactic acid by lactate-assimilating yeasts increases silage pH and allows growth of aerobic bacteria and molds that cause further spoilage (Elferink et al., 2000).

The application of chemical additives before ensiling could be an option for improving the aerobic stability of partially fermented silages that have high pH. Organic acids can be applied to silage to prevent growth of fungi (Muck et al., 2018). An additive containing sodium benzoate, potassium sorbate, and sodium nitrite is known to inhibit microorganisms and reduce spoilage in silages with more than 30% DM (Knicky and Spörndly, 2011).

Due to the lack of knowledge on the effects of a chemical additive containing sodium benzoate, potassium sorbate, and sodium nitrite on the fermentation and aerobic stability of corn silage ensiled for very short periods of time, we evaluated its effects after

1, 2, 4, and 46 d of ensiling. As the impact of additives on the composition of the microbial community of silages are not well-known, we evaluated the effects of the additive on composition of corn silage bacterial and fungal communities during early fermentation stages and after exposure to air. High-throughput sequencing (HTS) was used to perform these analyses, as it is described as a promising tool to broaden the understanding of the effects of additives on silage (Duniere et al., 2017).

Objectives

The objectives of this experiment were to evaluate the effects of two application levels of an additive containing sodium benzoate, potassium sorbate, and sodium nitrite on the composition of the microbial community of corn silage and to determine its effectiveness on improving the fermentation and aerobic stability of short-term ensiled silage.

Materials and Methods

Crops and Silos

Whole plant corn (P1449AMX, DuPont Pioneer, Johnston, IA, USA) was harvested at approximately 39% DM from the University of Delaware Farm (Newark, DE) and chopped to a theoretical length of 19 mm using a pull-type chopper (3975, John Deere, Moline, IL) equipped with kernel processer (roller gap set to 1.4 mm). Shortly after chopping, 200 ml of water (CTRL) or 200 ml of Safesil diluted in water (SF, active

ingredients: 20% sodium benzoate, 10% potassium sorbate, and 5% sodium nitrite) (Salinity, Göteborg, Sweden) at 2 L/t of fresh forage weight (SF2) or at 3 L/t of fresh forage weight (SF3) were applied to forage. The treatments were applied into 4 individually replicated piles containing 31 kg of forage per treatment using a hand sprayer. Forage from each pile was packed into 7.5-L laboratory silos at a packing density of approximately 224 kg of DM/m^3. Silos were sealed with plastic lids with O-ring seals and allowed to ensile for 1, 2, 4 and 46 d.

Aerobic Stability Measurements

Upon silo opening, approximately 2 kg of a representative sample from each silo were returned to clean laboratory silos. Silos were covered with 2 layers of cheesecloth and exposed to air in the laboratory at 22 ± 2°C. A thermocouple wire was placed in the geometric center of each forage mass, and temperatures were recorded every 15 min using a data logger DataTaker DT85 (Thermo Fisher Scientific, Australia Pty, Scoresby, VIC, Australia). Aerobic stability was calculated as the number of hours before the temperature of the forage mass roses 2°C above the baseline. Samples of CTRL and SF2 ensiled for 46 d were collected after 50, 100, and 240 h of aerobic exposure.

Analytical Procedures

Fresh forage from two of the CTRL, SF2 and SF3 piles at d 0 and from each silo after ensiling was analyzed for DM and pH. The DM content was determined in after

incubation in a 60°C forced-air oven for 48 h. Weights of full and empty silos were measured at silo opening, and with the DM content of fresh and ensiled samples, were used to calculate DM recovery.

Representative 25-g portions of fresh forage and silage were mixed with 225 ml of sterile quarter-strength Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) and homogenized for 1 min in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). The homogenate was filtered through four layers of cheesecloth. The pH was determined on homogenized samples using an Oakton pH700 Meter (Oakton Instruments, Vernon Hills, IL, USA). Fresh HMC was analyzed for buffering capacity (BC) according to McDonald and Henderson (1962).

Portions of the water extracts that had been filtered through 4 layers of cheesecloth were filtered through a Whatman 54 filter paper (Whatman Inc., Clifton, NJ). The extract was acidified with H_2SO_4 (50% vol/vol) and frozen. Later, it was analyzed for lactic, acetic, propionic, and butyric acids, and for 1,2-propanediol and ethanol content (Muck and Dickerson, 1988) by a Shimadzu LC-20AD HPLC (Shimadzu Scientific Instruments, Columbia, MD). The concentration of NH_3-N was determined on the water extracts by the phenol-hypochlorite method of Weatherburn (1967). Water-soluble carbohydrates (WSC) were quantified by a colorimetric procedure (Nelson, 1944).

The nutrient analysis of fresh forage was performed by Cumberland Valley Analytical Services (Waynesboro, PA, USA). A portion of each dried sample was ground

using an Udy Cyclone Sample Mill (Udy Corp., Fort Collins, CO) to pass through a 1-mm screen. Total N was measured by combustion of the sample following the AOAC method 990.03 (AOAC, 2010) using a Leco CNS2000 Analyzer (Leco Corporation, St. Joseph, MI), and crude protein (CP) was calculated by multiplying the resulting total N concentration by 6.25. Soluble protein (SP) was determined by the method of Krishnamoorthy et al. (1982) and expressed as % of CP. Acid detergent fiber (ADF) was quantified on dried ground samples using the AOAC method 973.18 (AOAC, 2010), employing Whatman 934-AH glass micro-fiber filters with 1.5-µm particle retention instead of fritted glass crucibles. Neutral detergent fiber (NDF) was analyzed on dried samples according to Van Soest et al. (1991) with sodium sulfite and amylase, not corrected for ash. Ash was analyzed by the AOAC method 942.05 (AOAC, 2000), with the modification of using a 1.5-g sample weight, 4 h ash time, and hot weigh. Another portion of the dry sample was ground to pass a 3-mm screen and analyzed for starch (Hall, 2008).

Microbial Analysis

Representative 25-g portions of fresh forage and silage were mixed with 225 ml of sterile quarter-strength Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) and homogenized for 1 min in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). The homogenate was filtered through four layers of cheesecloth. The filtrate was serially diluted in Ringers solution, and the numbers of LAB were determined by pour-plating in de Man, Rogosa, and

Sharpe agar (CM3651, Oxoid, Unipath Ltd., Basingstoke, UK). Plates were incubated anaerobically at 32°C for 48 to 72 h in a plastic container with anaerobic packs (Mitsubishi Gas Chemical Co., Tokyo, Japan) and an anaerobic indicator (BR0055, Oxoid, Unipath Ltd., Basingstoke, UK). The container was vacuum-sealed in nylon-polyethylene bags (3.5-mil embossed pouches, 15.2 × 30.5 cm; Doug Care Equipment Inc., Springville, CA) using a Best Vac vacuum machine (distributed by Doug Care Equipment Inc.). Numbers of yeasts and molds were determined on malt extract agar (CM0059, Oxoid, Unipath Ltd., Basingstoke, UK) acidified with lactic acid (85%) at a rate of 0.5% vol/vol. These plates were incubated aerobically at 32°C for 48 h before enumeration of yeasts and molds. Plates with a number of colonies between 30 and 300 were used to obtain counts of colony-forming units.

Microbiome Analysis

Four replicates of 25 g of fresh forage, of CTRL and SF2 silages ensiled for 1, 4, and 46 d, as well as of CTRL and SF2, ensiled for 46 d, after 50, 100, and 240 h of exposure to air were mixed with 225 ml of autoclaved Ringers solution for 2 min using a Colworth 400 stomacher (Seward, London, UK). The homogenates were filtered through four layers of cheesecloth. Next, 2 ml of the filtrate was centrifuged for 3 min at 21130.2 xg using a Centrifuge 5424 R (Eppendorf AG, Hamburg, Germany). The supernatant was discarded, and 100 microliters of autoclaved Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) was used to resuspend the pellet. Samples were kept at -80°C until further analysis.

Extraction of DNA, 16S rRNA and ITS1 amplification, and Illumina MiSeq-based sequencing were performed by the Research and Testing Laboratory (Lubbock, TX, USA). DNA was extracted done using the MoBio PowerMag Soil kit, according to manufacturer's instructions. For bacterial analysis, the primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGTCAATTCMTTTRAGTTT) were used to amplify the V4 and V5 hypervariable regions of the 16S rRNA (Baker et al., 2003). For fungal analysis, the primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2aR (GCTGCGTTCTTCATCGATGC) (White et al., 1990; Gardes and Bruns, 1993) were used to amplify the internal transcribed spacer 1 (ITS1). Sequencing was conducted with an Illumina MiSeq (San Diego, CA, USA) platform with the 2 × 250 bp paired-end method.

For sequence processing, paired-end reads were merged and converted into FASTA files using FLASH version 1.2.11 (Magoc and Salzberg, 2011). The quality profile of the reads was checked on http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Andrews, 2010). Next, reads were filtered for quality and the primers were trimmed using QIIME 1.9.1 (Caporaso, 2010). Operational taxonomic units (OTUs) picking was done using open reference clustering at 97% sequence similarity against Greengenes (version 13_8) or UNITE for bacteria and fungi, respectively. Finally, singles and double reads were filtered. The diversity analyses were performed using the phyloseq and vegan packages in R (McMurdie and Holmes, 2013; Oksanen et al. 2013).

Statistical Analysis

Data were analyzed using JMP version 12 (SAS Institute, Cary, NC). Data for fresh forage at d 0 were analyzed separately as a completely randomized design. Data from the silo openings was analyzed as a 3 × 4 factorial arrangement of treatments in a completely randomized design. The model included the fixed effects of the additive, days of ensiling, and their interaction. Data from the post aerobic exposure samples were analyzed as a 2 × 3 factorial arrangement of treatments in a completely randomized design. The model included the fixed effects of the additive, time after aerobic exposure, and their interaction. Differences were considered significant when $P < 0.05$, and pairwise mean comparisons were performed using Tukey's test at $P < 0.05$ (Snedecor and Cochran, 1980). Statistical analysis for the PCoA plots was done using PERMANOVA (permutational multivariate analysis of variance) by the adonis function in vegan (Oksanen et al. 2013).

Results

Chemical and Microbial Characteristics of Fresh Forage

Table 3.1 shows the chemical composition and numbers of LAB, yeasts, and molds of the freshly chopped whole corn plant. We found no differences for any of the parameters analyzed at d 0 among treatments.

Chemical Properties, Aerobic Stability, and Composition of the Microbial Community of Silage

Table 3.2 shows the DM recovery, pH, and numbers of LAB and yeasts in silage. The recovery of DM was not affected by SF, but it was highest in silage ensiled for 1 d (99.11%) compared to silages ensiled for longer periods of time (a range of 94.48 to 96.39%). Silage pH decreased with time of ensiling (from 4.39 on d 1 to 3.64 on d 46) and was higher in SF3 (4.01) than it was to CTRL and SF3 (both 3.98). The concentration of lactic acid was unaffected by treatment with SF, but it increased with time of ensiling on d 1, 2, 4, and 46 to 1.50, 2.76, 3.42, and 5.66%, respectively. Concentrations of ethanol were similar between CTRL (0.98%) and SF3 (0.89%) and similar between SF2 (0.78%) and SF3 (0.89%). Concentrations of ethanol were similar and higher at 2 and 46 d (average of 1.02 % of DM) than at 1 and 4 d (average of 0.77 % of DM). Numbers of LAB were unaffected by SF, but they decreased with time of ensiling (9.29 to 7.79 log cfu/g on d 1 and 46, respectively). There was an interaction between d of ensiling and additive treatment because on 1, 2, and 4 d, no differences in numbers of yeasts were observed among treatments, but at d 46, treated silages had lower numbers of yeasts (1.14 cfu/g) compared with untreated silages (3.22 log cfu/g).

Treatment with SF did not affect the chemical composition of silage after 46 d of ensiling except for changes in NH3-N and WSC (Table 3.3). Specifically, silages treated with both levels of SF had a lower concentrations of NH_3-N and higher concentrations of WSC compared to untreated silage.

Silages treated with both levels of SF had markedly higher aerobic stability compared to untreated silages after all periods of ensiling (Figure 3.1). For SF2 and SF3 after 2, 4, and 46 d of ensiling stability was at least 240 h (time measurements ended), whereas CTRL was stable for 57, 81, and 117 h, respectively. There was no correlation between aerobic stability and yeasts at 1, 2, and 4 d of ensiling. However, at 46 d of ensiling, there was a negative correlation ($R^2 = 0.99$, $P < 0.01$) between aerobic stability and numbers of yeasts (Figure 3.2).

Table 3.4 shows the pH and microbial numbers of CTRL and SF2, ensiled for 46 d, after exposure to air. After 50 h of exposure to air, SF2 had lower numbers of yeasts than CTRL, but there were no differences in pH or numbers of molds. After 100 h, numbers of yeasts increased in both treatments but remained lower in SF2 compared to CTRL. The pH and numbers of molds remained at the same levels as at 50 h. After 240 h, the numbers of yeasts in CTRL increased to more than 9 log cfu/g of fresh forage, whereas in SF2 it only reached 4.7 log cfu/g of fresh forage. Additionally, CTRL had a higher pH and numbers of molds than SF2. After 50 and 100 h of aerobic exposure, there were no differences in concentrations of organic acids and ethanol between treatments. After 240 h of exposure to air, the levels of lactic and acetic acid remained stable in SF2 but decreased in CTRL. The concentration of ethanol decreased in both treatments after 240 h of exposure to air.

The Composition of the Microbial Community

Sequencing depth was sufficient to describe the diversity of the microbial population as the rarefaction curves reached a plateau (Figures B.2-B.5). Tables 3.5 and 3.6 show the alpha diversity of bacterial and fungal communities, respectively, in fresh forage, silages, and post aerobic exposure samples. Table 4 shows the alpha diversity of the bacterial community. After 240 h of aerobic exposure, CTRL had lower numbers of observed OTU, Chao 1 index, and Shannon index than SF2.

Figure 3.3A shows the PCoA plot for the composition of the bacterial community of fresh forage and silage after 1, 4, and 46 d of ensiling. A significant Adonis test proved that the treatments did not have the same centroid, and a significant betadisper test showed that differences in group dispersions caused the results. Samples from each ensiling day clustered together independent of additive treatment, indicating that the composition of the bacterial community changed over time and was not influenced by the additive. Figure 3.3B shows the PCoA plot for the composition of the bacterial community of silage, ensiled for 46 d, after exposure to air. A significant Adonis test proved that the treatments did not have the same centroid, and a significant betadisper test showed that differences in group dispersions caused the results. All treatments, independent of time of exposure to air clustered together, except for CTRL after 240 h after exposure to air.

We observed a day effect in the relative abundance of Lactobacillaceae, Leuconostocaceae, and Enterobacteriaceae. The relative abundance of Lactobacillaceae sequences was 13% at 1 d, increased to 80% at 4 d, and declined to 64% at 46 d.

Leuconostocaceae sequence abundance was 72% at 1 d, declined at 4 d to 7%, and remained low at 4% at 46 d. Enterobacteriaceae relative abundance was 4 and 6% at 1 and 4 d, respectively, and increased to 17% at 46 d (Figure 3.4A). In samples collected after exposure to air, there was an additive effect for relative abundance of Lactobacillaceae showing that SF2 had a higher relative abundance of Lactobacillaceae sequences than CTRL (77 *vs.* 52%). There was an interaction between additive and time of exposure to air for relative abundances of Acetobacteraceae, Enterobacteriaceae, and Leuconostocaceae sequences. After 50 h of aerobic exposure, CTRL had a higher relative abundance of Enterobacteriaceae sequences than SF2 (19 *vs.* 10%). After 240 h of exposure to air, CTRL had a higher relative abundance of Acetobacteraceae (68 *vs.* 4%) and a lower relative abundance of Leuconostocaceae sequences (0.1 *vs.* 4%) than SF2 (Figure 3.4B).

Figure 3.5A shows the PCoA plot for the composition of the fungal community of fresh forage and silage after 1, 4, and 46 d of ensiling. A significant Adonis test proved that the treatments did not have the same centroid, and a non-significant betadisper test showed that differences in group dispersions did not cause the results. Fresh forage samples clustered separated from ensiled samples. There was a day effect for the relative abundance of Acremonium sequences; their relative abundance was higher at 1 d (15%) and lower at 4 and 46 d (2%) (Figure 3.6). Figure 3.5B shows the PCoA plot for the composition of the fungal community of silage, ensiled for 46 d, after exposure to air. A significant Adonis test proved that the treatments did not have the same centroid, and a non-significant betadisper test showed that differences in group dispersions did not cause

the results. Clusters were not well defined, but SF2 after 50 h of exposure to air formed a cluster separated from other samples.

Discussion

The chemical composition and numbers of LAB, yeasts, and molds of the freshly chopped whole corn plant in this study was in the typical range for this feedstuff (Kleinschmit et al., 2005; Kleinschmit and Kung, 2006). During ensiling, treatment with the low dose of the additive did not affect the numbers of LAB or the production of lactic acid, which is not surprising as sorbates have selective inhibition against catalase-positive microorganisms and do not efficiently inhibit catalase-negative microorganisms such as LAB (Liewen and Marth, 1985). Sodium nitrite is more prone to inhibit LAB than potassium sorbate and sodium benzoate, but according to Woolford (1975), sodium nitrite can only inhibit LAB at a concentration of 0.02% at pH 4, and in the present study the application of the low dose of the additive resulted in a concentration of sodium nitrite of 0.01%.

Enterobacteria can be responsible for proteolytic processes in silage (Heron et al., 1993). When silage is exposed to air and its pH increases, enterobacteria can multiply. Since the numbers of enterobacteria were not determined in this study, the reason treatment reduced the concentration of NH_3-N is unclear. Even though the numbers of total culturable enterobacteria were not measured, we evaluated the relative abundance of the order Enterobacteriaceae. The relative abundance of Enterobacteriaceae after 50 h of

exposure to air was higher in CTRL than SF. Because treatments had the same pH at this time point, the additive rather than the pH was the probable cause of Enterobacteriaceae inhibition. A reduction in the numbers of enterobacteria by Safesil was previously observed in alfalfa silage (Da Silva et al., 2016). Similarly, Stanojevic et al. (2009) showed that sodium benzoate, potassium sorbate, and sodium nitrite could inhibit *Escherichia coli* at a concentration similar to those applied in the present study. They also observed a synergistic action between sodium nitrate and sodium benzoate and between sodium nitrite and potassium sorbate against this microorganism. Because yeasts produce ethanol by the fermentation of WSC (McDonald et al., 1991), their reduction in numbers after 46 d of ensiling partially explains the higher concentration of WSC and lower production of ethanol in treated compared to untreated silage.

The marked increased stability for treated corn silage after very short periods of ensiling (1, 2, and 4 d) could not be explained by changes in the composition of bacterial or fungal communities during ensiling or by a reduction in the total numbers of agar-culturable yeasts. Only after 46 d of ensiling did the additive reduce the numbers of culturable yeasts in the silage and a correlation between aerobic stability and numbers of yeasts was observed.

Environmental conditions such as pH, length of exposure, dose, and type of targeted strain will determine the effects of organic acids on microorganisms (Neal et al., 1965; Schroeder and Bullerman, 1985). For example, sorbates can have both fungistatic and(or) fungicidal activity (Sofos and Busta, 1981). Specifically, benzoates and sorbates are more effective at a low pH when these molecules are in the undissociated form, which

have antimicrobial effects (Chipley et al., 2005; Robach and Sofos, 1982). Palou et al. (2002) showed that potassium sorbate, sodium benzoate, and their combination were fungistatic but not fungicidal against strains of Penicillium on citrus fruit. In our experiment, silage pH decreased progressively with each d of ensiling (4.39, 4.07, 3.87, and 3.64 for days 1, 2, 4 and 46, respectively). When diluted in Ringers solution, yeast cells were removed from interacting with the organic acids and when grown on agar, conditions were extremely favorable for growth. Short-term exposure to antifungal acids in silage maybe enough to alter the metabolism of yeasts during aerobic exposure especially as they remain within the hostile environment. Thus, after short periods of ensiling the additive we used might have had a fungistatic effect, whereas after a long-term exposure the effect was more fungicidal. Kung et al. (2018) recently reported similar findings as there were numerically larger declines in numbers of yeasts in corn silage treated with Safesil as the days of ensiling progressed from 5 to 30. Similarly, Da Silva et al. (2015) observed that Safesil at 4 L/t of fresh weight markedly improved the aerobic stability of HMC after 21 and 90 d of ensiling. However, a reduction in numbers of yeasts by treatment was observed only after 90 d of ensiling and not at 21 d.

Alpha diversity indexes indicated that the bacterial population was more stable over time in treated silage. In contrast, the bacterial population of untreated silage lost diversity and was less even over time, which was caused by the dominance of Acetobacteraceae over other bacterial orders. After 240 h of exposure to air, SF remained stable with a high relative abundance of Lactobacillaceae. On the other hand, CTRL had higher numbers of yeasts and relative abundance of Acetobacteraceae, which consumed

lactic acid, acetic acid, and ethanol and increased silage pH. Strains belonging to the order Acetobacteraceae have been found to initiate the aerobic deterioration of corn silage, usually simultaneously with lactate-assimilating yeasts (Spoelstra et al.1988). Even though potassium sorbate is mostly used to inhibit growth of fungi, it can inhibit certain bacteria such as *Acetobacter*, *Bacillu*s, *Clostridium*, *Pseudomonas*, *Salmonella*, *Staphylococcus*, and *Escherichia coli* (Baker and Grant, 2018). Therefore, besides the fungistatic hypothesis, another possible explanation for improvements in aerobic stability by Safesil, despite its inability to reduce numbers of yeasts, is that it might have inhibited acetic acid bacteria that can also initiate spoilage.

Conclusion

We found that the additive containing sodium benzoate, potassium sorbate, and sodium nitrite markedly improved the stability of corn silage after very short periods of storage (1, 2, and 4 d), probably due to fungistatic (rather than fungicidal) effects. To our best knowledge, this is the first study showing an improvement in aerobic stability as early as only 1 d after ensiling using this mixture of organic acids and the first study to show its effects on the microbial community of corn silage. Our findings suggest that the additive might be useful for producers who cannot wait several weeks for silage to cure and that must feed silage immediately or very soon after harvest.

TABLES

Table 3.1. The DM content, chemical composition (% of DM, unless stated otherwise), pH, buffering capacity, and numbers of LAB, yeasts, and molds (fresh weight basis) of freshly chopped whole corn plants untreated or treated with two concentrations of an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1,2].

Item	CTRL	SF2	SF3	SEM	P-value
DM, %	40.00	38.30	38.90	0.28	0.05
CP	8.50	8.60	8.35	0.16	0.58
SP[3], % of CP	24.12	24.42	23.35	1.90	0.85
NH_3-N	0.02	0.02	0.02	0.00	NA
ADF	19.75	20.00	20.15	1.41	0.98
NDF	36.40	35.60	36.25	2.35	0.97
Starch	36.55	34.70	37.00	2.49	0.80
Ash	3.81	3.83	4.39	0.43	0.60
WSC[4]	7.27	8.91	7.77	0.96	0.54
pH	5.88	5.94	5.89	0.03	0.51
BC[5], g lactic acid/kg	20.58	23.71	23.80	0.74	0.09
LAB[6], log cfu/g	6.35	6.41	6.42	0.11	0.90
Yeasts, log cfu/g	5.04	4.85	5.11	0.15	0.52
Molds, log cfu/g	4.97	4.89	4.39	0.39	0.58

[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight; SF3 = 3.0 L of Safesil/t of fresh forage weight.
[3]Soluble protein.
[4]Water soluble carbohydrates.
[5]Buffering capacity.
[6]Lactic acid bacteria.
NA= not applicable.

Table 3.2. The DM recovery (%), pH, chemical composition (% of DM), and numbers of LAB and yeasts (log_{10} cfu/g of fresh weight) of corn silage treated with two concentrations of an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] after 1, 2, 4 and 46 d of ensiling.

Item[2]	DMR[3]	pH	Lactic acid	Acetic acid	ETOH[4]	LAB[5]	Yeasts
Day 1							
CTRL	100.00	4.37	1.67	0.63	0.83	9.29	5.39^{a}
SF2	98.91	4.37	1.67	0.63	0.70	9.28	5.42^{ab}
SF3	98.79	4.42	1.15	0.37	0.71	9.30	5.38^{a}
Day 2							
CTRL	94.57	4.05	3.05	0.93	1.04	9.15	4.81^{bcd}
SF2	96.00	4.06	2.63	0.78	1.08	9.14	4.80^{bcd}
SF3	96.48	4.11	2.61	0.78	0.97	9.10	4.92^{abc}
Day 4							
CTRL	99.20	3.85	3.40	0.83	0.84	9.15	4.77^{cd}
SF2	95.36	3.88	3.53	0.85	0.65	9.07	4.63^{cd}
SF3	96.04	3.90	3.33	0.82	0.87	9.09	4.31^{d}
Day 46							
CTRL	95.40	3.64	5.64	1.15	1.21	7.79	3.22^{e}
SF2	95.40	3.62	5.76	1.16	0.83	7.80	1.00^{f}
SF3	92.63	3.65	5.59	1.14	0.99	7.76	1.27^{f}
Additive means							
CTRL	97.29	3.98^{b}	3.44	0.88^{a}	0.98^{a}	8.85	4.55
SF2	96.42	3.98^{b}	3.40	0.85^{ab}	0.81^{b}	8.82	3.96
SF3	95.99	4.01^{a}	3.17	0.78^{b}	0.89^{ab}	8.81	3.97
Day means							
1	99.11^{a}	4.39^{a}	1.50^{d}	0.54^{c}	0.75^{b}	9.29^{a}	5.40
2	95.68^{b}	4.07^{b}	2.76^{c}	0.83^{b}	1.03^{a}	9.13^{b}	4.84
4	96.39^{b}	3.87^{c}	3.42^{b}	0.83^{b}	0.79^{b}	9.10^{b}	4.57
46	94.48^{b}	3.64^{d}	5.66^{a}	1.15^{a}	1.01^{a}	7.79^{c}	1.83
SEM	1.29	0.02	0.19	0.05	0.11	0.08	0.10

Cont.

Effects and interactions[6]	P – value						
A	0.49	<0.01	0.12	0.02	0.04	0.26	<0.01
D	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
A × D	0.35	0.64	0.58	0.05	0.29	0.48	<0.01

[a-f]Means in columns with unlike superscripts differ (P < 0.05).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight; SF3 = 3.0 L of Safesil/t of fresh forage weight.
[3]DM recovery.
[4]Ethanol.
[5]Lactic acid bacteria.
[6]A = effect of additive; D = effect of day of ensiling; A × D = interaction of additive by d of ensiling.

Table 3.3. The DM content (%) and chemical composition (% of DM, unless stated otherwise) of corn silage treated with two concentrations of an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] after 46 d of ensiling[2].

Item	CTRL	SF2	SF3	SEM	P-value
DM	37.80	37.73	36.59	0.49	0.10
CP	8.53	8.58	8.73	0.08	0.23
SP[3], % of CP	43.96	43.47	42.96	0.98	0.98
NH_3-N	0.06[a]	0.03[b]	0.03[b]	0.01	<0.01
ADF	22.68	21.68	23.05	0.86	0.53
NDF	38.83	36.88	38.15	1.24	0.55
Starch	31.53	33.03	30.93	1.43	0.58
Ash	4.07	3.99	4.33	0.26	0.64
WSC[4]	0.08[b]	0.15[a]	0.14[a]	0.01	<0.01

[a-b] Means in rows with unlike superscripts differ ($P < 0.05$).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight; SF3 = 3.0 L of Safesil/t of fresh forage weight.
[3]Soluble protein.
[4]Water soluble carbohydrates.

Table 3.4. The pH, and chemical composition (% of DM, unless stated otherwise), and numbers of yeasts and molds of untreated silage (CTRL) and silage treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] at 2L/t of fresh matter (SF2) ensiled for 46 d after 50, 100, and 240 h of exposure to air[2].

Item	pH	Lactic acid	Acetic acid	ETOH[3]	Yeasts	Molds
50 h of aerobic exposure						
CTRL	3.67^{b}	5.40^{a}	1.13^{a}	1.02^{a}	4.27^{cd}	<1.00^{c}
SF2	3.66^{b}	5.29^{a}	1.06^{ab}	0.74^{ab}	2.23^{e}	<1.00^{c}
100 h of aerobic exposure						
CTRL	3.72^{b}	4.88^{a}	0.96^{ab}	0.86^{ab}	5.89^{b}	<1.00^{c}
SF2	3.66^{b}	4.92^{a}	0.97^{ab}	0.65^{b}	2.96^{de}	<1.00^{c}
240 h of aerobic exposure						
CTRL	5.40^{a}	1.08^{b}	0.11^{c}	0.06^{c}	9.05^{a}	7.63^{a}
SF2	3.63^{b}	4.81^{a}	0.84^{b}	0.27^{c}	4.69^{bc}	3.23^{b}
SEM	0.07	0.25	0.05	0.07	0.32	0.15
Effects and interactions[4]	---------- P – value ----------					
T	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
A	<0.01	<0.01	<0.01	0.12	<0.01	<0.01
T × A	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a-e]Means in columns with unlike superscripts differ (P < 0.05).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight; SF3 = 3.0 L of Safesil/t of fresh forage weight.
[3]Ethanol.
[4]T = effect of time of sampling; A = effect of additive; T × A = interaction of time of sampling by additive.

Table 3.5. Alpha diversity indexes of the composition of the bacterial community, as analyzed by the sequencing of the V4-V5 hypervariable region of the 16S rRNA using Illumina Miseq, of fresh forage and corn silage treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] ensiled for 1, 4, and 46 d, and of silages after 50, 100, and 240 h of exposure to air.

Additive[2]	Time	Observed	Chao1	Shannon	Simpson
CTRL	0	296.00^{abc}	337.16^{abcd}	4.28^{a}	0.97
	1	264.75^{bc}	331.91^{bcd}	2.40^{bc}	0.78
	4	346.75^{abc}	436.64^{ab}	2.97^{bc}	0.86
	46	368.25^{ab}	427.51^{abc}	3.14^{b}	0.87
	46+50h	332.50^{abc}	356.20^{abcd}	3.19^{b}	0.88
	46+100h	308.75^{abc}	345.66a^{bcd}	3.00^{bc}	0.85
	46+240h	51.25^{d}	69.94^{e}	1.41^{d}	0.54
SF2	0	329.00^{abc}	348.29^{abcd}	4.72^{a}	0.98
	1	289.28^{abc}	348.05^{abcd}	2.65^{bc}	0.81
	4	372.75^{a}	443.33^{a}	3.12^{b}	0.88
	46	346.5^{abc}	405.87^{abcd}	3.05^{b}	0.86
	46+50h	300.50^{abc}	337.52^{abcd}	2.90^{bc}	0.84
	46+100h	281.00^{abc}	325.03^{cd}	2.45^{bc}	0.73
	46+240h	261.75^{c}	311.69^{d}	2.25^{c}	0.67
	SEM	29.11	40.00	0.21	0.05
P-value	A	0.01	0.02	0.22	0.90
	T	<0.01	<0.01	<0.01	<0.01
	A × T	<0.01	<0.01	<0.01	0.06

[a-d] Means in columns with unlike superscripts differ (P < 0.05).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight.
[3]A = effect of additive; T = time; A × T = interaction of additive by time.

Table 3.6. Alpha diversity indexes of the composition of the fungal community, as analyzed by the sequencing of the ITS1 using Illumina Miseq, of fresh forage and corn silage treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] ensiled for 1, 4, and 46 d, and of silages after 50, 100, and 240 h of exposure to air.

Additive[2]	Time	Observed	Chao1	Shannon	Simpson
	0	59.50	61.33	2.68^{ab}	0.87^{a}
	1	131.50	144.26	2.07^{abc}	0.72^{ab}
	4	84.75	91.14	2.20^{abc}	0.70^{ab}
CTRL	46	63.50	68.91	1.89^{abcd}	0.69^{ab}
	46+50h	35.50	36.21	1.55^{cd}	0.61^{ab}
	46+100h	15.25	17.88	0.16^{e}	0.05^{c}
	46+240h	6.25	9.00	0.08^{e}	0.03^{c}
	0	124.00	134.67	2.92^{a}	0.87^{a}
	1	117.50	123.09	1.83^{abcd}	0.64^{ab}
	4	90.50	99.84	1.97^{abc}	0.66^{ab}
SF2	46	51.50	58.83	2.14^{abc}	0.72^{ab}
	46+50h	38.00	38.38	2.11^{abc}	0.73^{ab}
	46+100h	16.75	17.00	1.58^{bcd}	0.68^{ab}
	46+240h	20.50	22.00	0.98^{de}	0.41^{b}
	SEM	16.37	18.15	0.25	0.09
P-value	A	0.18	0.21	<0.01	<0.01
	T	<0.01	<0.01	<0.01	<0.01
	A x T	0.18	0.15	<0.01	<0.01

[a-e] Means in columns with unlike superscripts differ ($P < 0.05$).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight.
[3]A = effect of additive; T = time; A × T = interaction of additive by time.

FIGURES

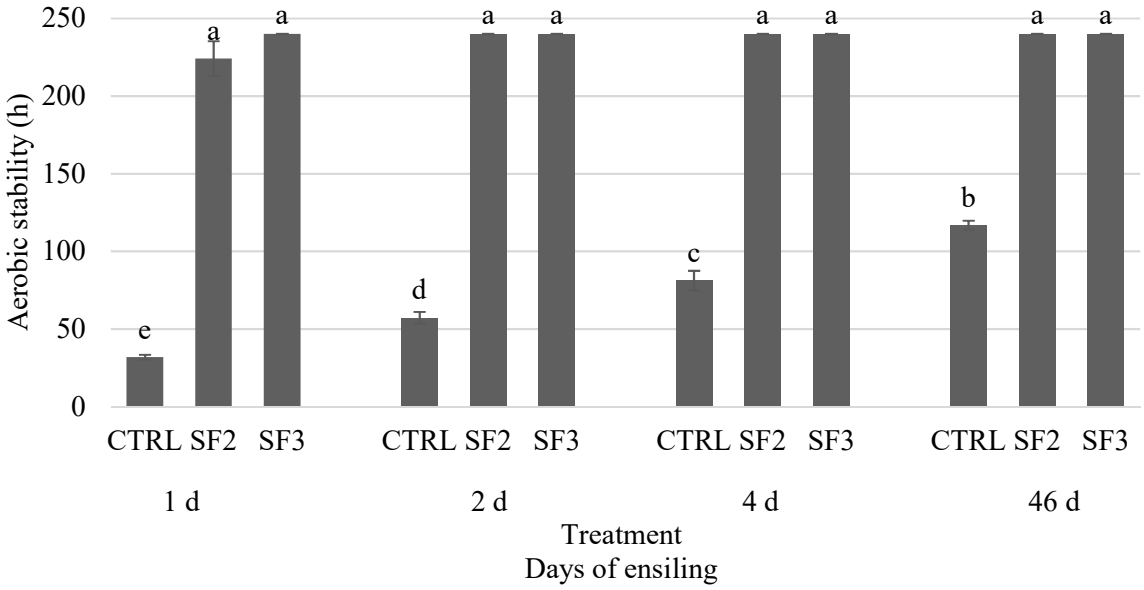

Figure 3.1. Aerobic stability of untreated silage (CTRL) and silage treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh matter (SF2) or 3 L/t of fresh matter (SF3) after 1, 2, 4, and 46 d of ensiling (SEM= 3.90, $P < 0.01$). [a-e] Means bars with unlike letters differ ($P < 0.05$).

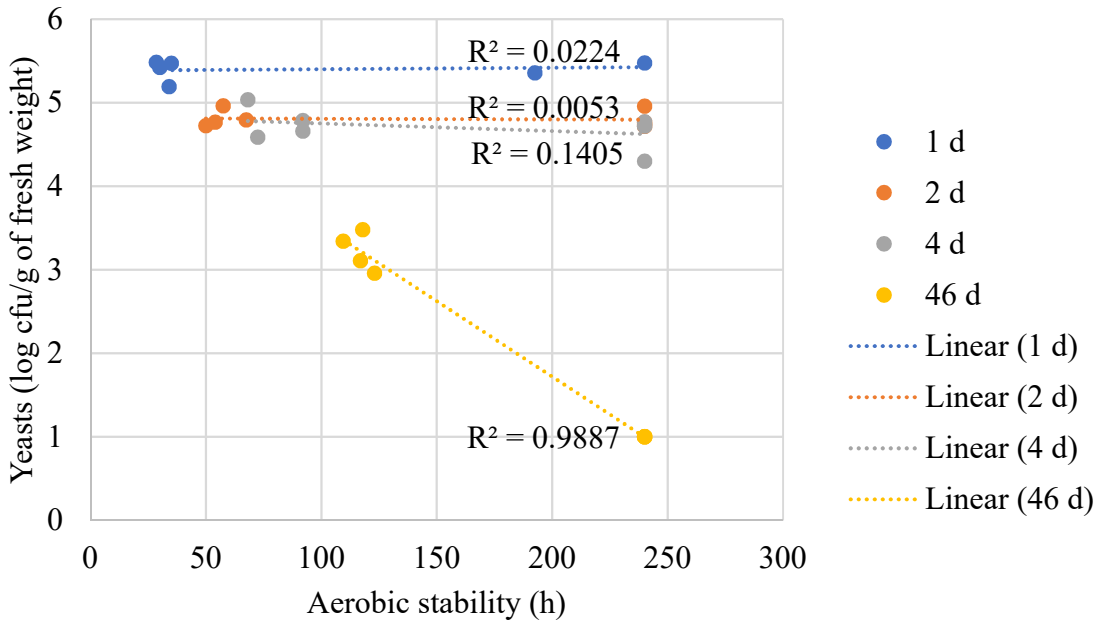

Figure 3.2. Scatter plot and linear regressions showing correlations between aerobic stability and numbers of yeasts in untreated silage and silage treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh matter after 1, 2, 4, and 46 d of ensiling.

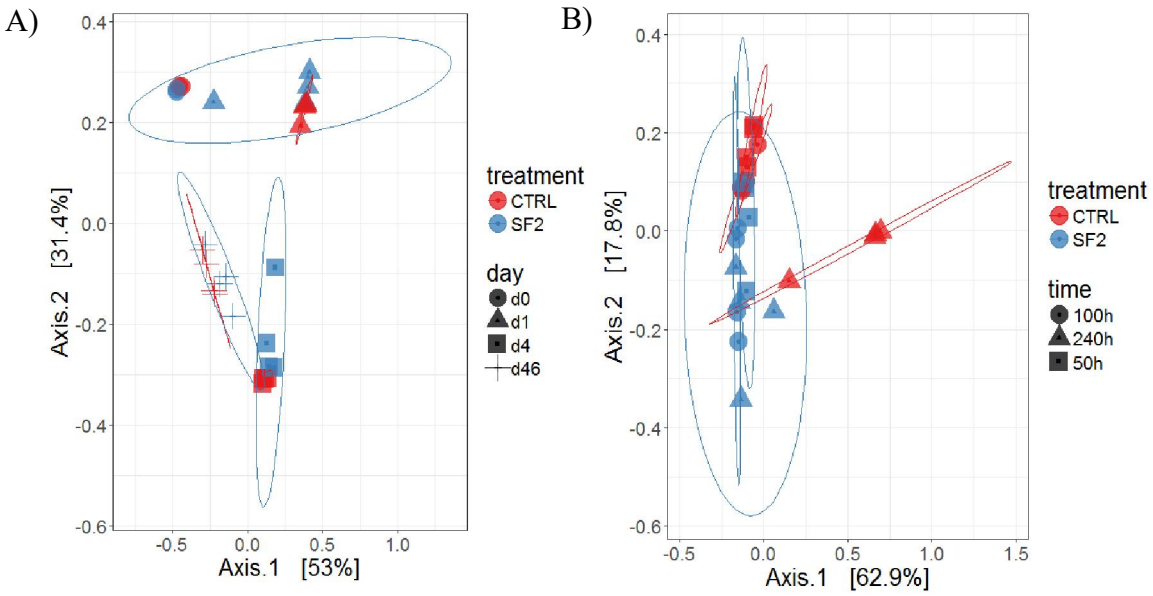

Figure 3.3. Principal coordinate analysis (PCoA) plot with weighted unifrac dissimilarity of the composition of the bacterial community, as analyzed by the sequencing of the V4-V5 region of the 16S rRNA using Illumina Miseq, of A) fresh forage (0 d) and corn silage (1, 4, and 46 d d) untreated (CTRL) or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh forage weight (SF2) ensiled for 1, 4, and 46 d and B) in silages ensiled for 46 d after 50, 100, and 240 h of exposure to air.

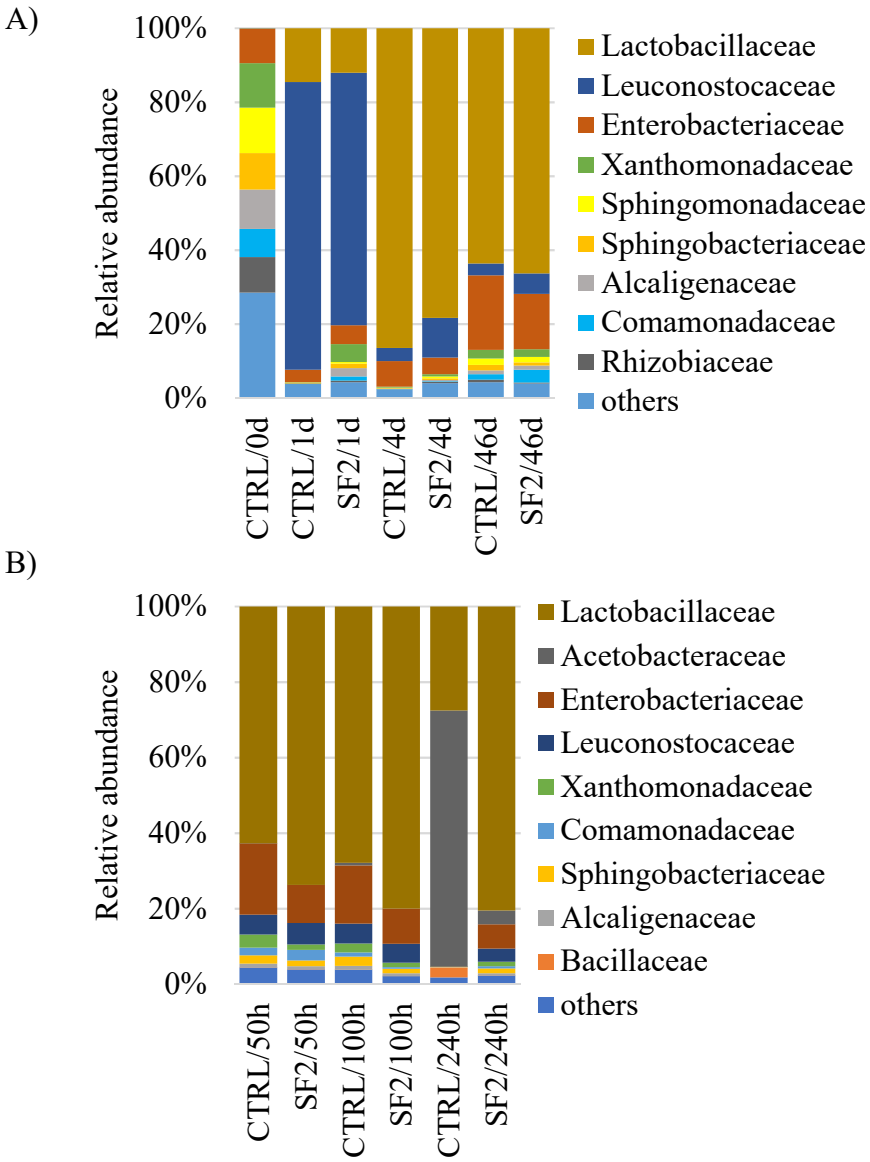

Figure 3.4. Average relative abundance of sequences from A) bacteria orders in silages untreated (CTRL) or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh forage weight (SF2) ensiled for 1, 4, and 46 d and B) in silages ensiled for 46 d after 50, 100, and 240 h of exposure to air, as analyzed by the sequencing of the V4-V5 region of the 16S rRNA using Illumina MiSeq platform.

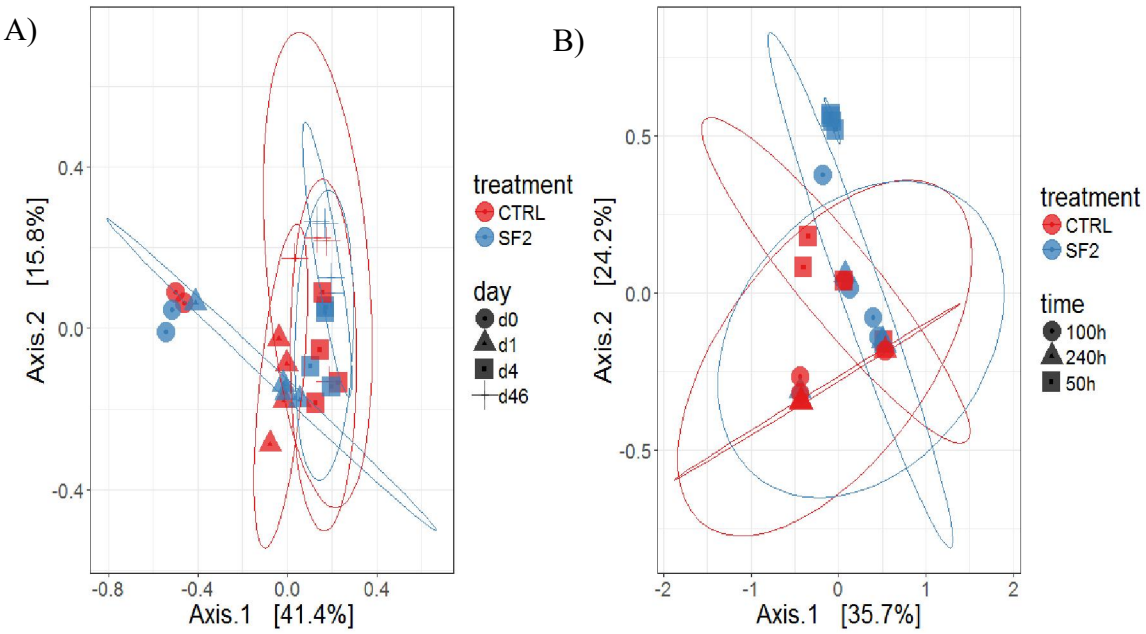

Figure 3.5. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity of the composition of the fungal community, as analyzed by the sequencing of the ITS1 using Illumina Miseq, of A) fresh forage (0 d) and corn silage (1, 4, and 46 d) untreated (CTRL) or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh forage weight (SF2) ensiled for 1, 4, and 46 d and B) in silages ensiled for 46 d after 50, 100, and 240 h of exposure to air.

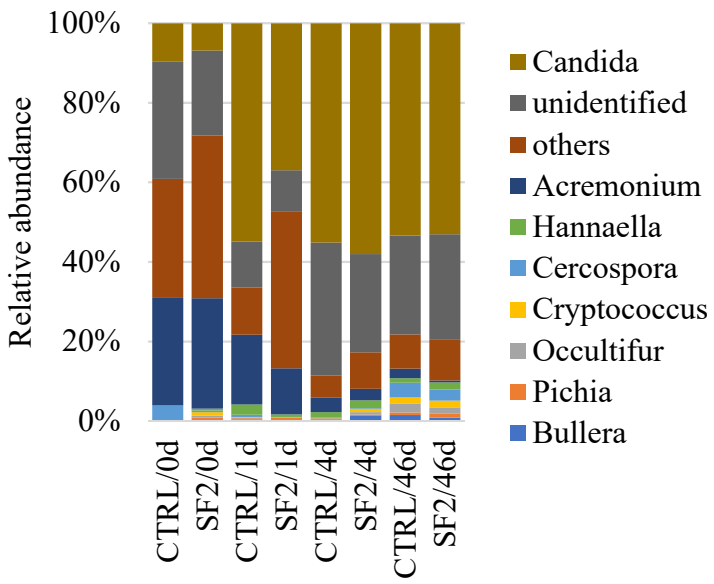

Figure 3.6. Average relative abundance of sequences from fungi genera in silages untreated (CTRL) or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh forage weight (SF2) ensiled for 1, 4, and 46 d, as analyzed by the sequencing of the ITS1 using Illumina MiSeq platform.

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Chapter 4

EFFECTS OF A CHEMICAL ADDITIVE ON THE COMPOSITION OF THE MICROBIAL COMMUNITY OF CORN SILAGE AND ITS EFFECTIVENESS IN IMPROVING THE FERMENTATION AND AEROBIC STABILITY OF CORN SILAGE STORED WITH OR WITHOUT AIR STRESS

Abstract

During storage silage can be exposed to air, which can stimulate the growth of undesirable microorganisms leading to spoilage. The objective of this study was to evaluate the effects of a chemical additive on the composition and dynamics of the microbial community of whole-plant corn silage, and its effectiveness in improving fermentation and aerobic stability and overcoming an air-stress challenge during storage. Whole-plant corn was treated with Safesil (Salinity, Göteborg, Sweden; active ingredients: 20% sodium benzoate, 10% potassium sorbate, and 5% sodium nitrite) at 2 L/t of fresh forage weight (SF2) or 3 L/t (SF3). Untreated silage served as control (CTRL). Ten individually replicated silos for each treatment were packed in 7.5-L laboratory silos. Half of the silos were not air-stressed (NS), and the other half were air-stressed (AS) for 2 h/week. After 63 d of ensiling, all silos were opened and exposed to

air. CTRL and SF2 were sampled after 115 and 260 h of exposure. After 63 d of ensiling, the pH was higher in CTRL than treated silages. In NS silages, SF2 had the same and SF3 lower numbers of yeasts than CTRL. In AS silages, neither additive level affected the numbers of yeasts. Based on high-throughput sequencing data, *Candida* abundance was the same in CTRL and SF2 in NS silages and higher in SF2-AS. CTRL-AS had the lowest abundance of *Candida* and was dominated by *Pichia*. SF2 and SF3 reduced the production of ethanol and increased DM recovery compared to CTRL, in both NS and AS silos. In NS silage after 115 h of exposure to air, CTRL and SF2 had a high abundance of Lactobacillaceae, the same pH, and concentrations of lactic and acetic acids. However, SF2 had lower numbers of yeasts and concentration of ethanol than CTRL. After 115 h of aerobic exposure, AS silages had a high abundance of Acetobacteraceae. CTRL had higher pH and numbers of yeasts and lower concentrations of lactic acid, acetic acid, and ethanol than SF2. After 260 h of aerobic exposure in NS silos, CTRL had high pH and numbers of yeasts, and low concentrations of lactic acid, acetic acid, and ethanol. In contrast, SF2 remained stable and with high abundance of Lactobacillaceae and low pH. In AS silos, CTRL and SF2 had similar pH, concentrations of lactic acid, acetic acid, and ethanol, and numbers of yeasts after 260 h. Treated silage was more stable than CTRL. The low dose of the additive did not reduce the total numbers of yeasts, but still markedly improved aerobic stability. Air stress during storage modified the composition of the microbial community of corn silage. The additive overcame the effects of air stress possibly by inhibiting specific microorganisms that were stimulated by oxygen, such as *Pichia*.

Introduction

Plants and microorganisms use air trapped in forage during harvesting or at the ensiling time to respire, which oxidizes nutrients, produces heat and reduces digestibility (Woolford, 1990). Preventing oxygen from entering the silo is essential to produce good quality silage, but inevitably, silage is exposed to air during feed-out.

When silage is exposed to air, aerobic yeasts that were dormant in the silo begin to grow and oxidize sugars and lactic acid, producing CO_2, H_2O, and heat. Acetic acid bacteria can also initiate the aerobic deterioration of corn silage, though they usually start spoilage simultaneously with lactate-assimilating yeasts (Spoelstra et al., 1988). The consumption of lactic acid by lactate-assimilating yeasts and acetic acid bacteria increases silage pH. The high pH of silage allows for growth of aerobic bacteria and molds that cause further spoilage (Elferink et al., 2000).

According to Borreani et al. (2018), harvesting, respiration and fermentation, effluent production, and exposure to air during storage and feed-out account for most of the DM losses during silage production. DM losses due to exposure to air alone if management practices are not optimum and if no appropriate silo covering is available can reach up to more than 20% and 30%, respectively.

Chemical additives can be used in silage production to overcome the risks brought by bad management practices, unwanted crop characteristics, or challenging environmental conditions such as air-stress during storage. Chemical additives can inhibit microorganisms that cause undesirable fermentations or spoilage and reduce DM losses,

improve feed quality, or improve sanitary conditions (Henderson, 1993). Sodium benzoate, potassium sorbate, and sodium nitrite are salts that in combination act synergistically to inhibit microorganisms (Stanojevic et al., 2009).

An additive containing sodium benzoate, potassium sorbate, and sodium nitrite is known to improve silage fermentation (Knicky and Spörndly, 2011; Kung et al., 2018). However, there is a need for more studies evaluating the effectiveness of this additive on improving aerobic stability of corn silage under North America conditions, especially in challenging situations, such as air stress during storage. Moreover, there is little knowledge of its effects on the composition of the bacterial and fungal communities of silage after ensiling and on the dynamic of those communities after exposure to air.

Objectives

The objectives of this experiment were to evaluate the effects of two application levels of an additive containing 20% sodium benzoate, 10% potassium sorbate, and 5% sodium nitrite on the composition and dynamics of the microbial community of whole plant corn silage; and to determine its effectiveness on overcoming an air-stress challenge during silo storage.

Materials and methods

Crops and Silos

Whole plant corn (P1449AMX, DuPont Pioneer, Johnston, IA, USA) was harvested at approximately 39% DM from the University of Delaware Farm (Newark, DE) and chopped to a theoretical length of 19 mm using a pull-type chopper (3975, John Deere, Moline, IL) equipped with kernel processer (roller gap set to 1.4 mm). Shortly after chopping, 200 ml of water (CTRL) or 200 ml of Safesil diluted in water (SF, active ingredients: 20% sodium benzoate, 10% potassium sorbate, and 5% sodium nitrite) (Salinity, Göteborg, Sweden) at 2 L/t of fresh forage weight (SF2) or 3 L/t of fresh forage weight (SF3) were applied to forage. The treatments were applied into five individually replicated piles per treatment containing 32 kg of forage using a hand sprayer. Forage from each pile was packed into 7.5-L laboratory silos at a packing density of approximately 224 kg of DM/m^3 and sealed with plastic lids with O-ring seals. Silos used for air stress had three holes of 1.60 cm diameter that were plugged with rubber stoppers and sealed with silicon glue. Two holes were located 5 cm above the bottom of the silo, 180° from each other. The third hole was on the lid of the silo. All three holes were opened for 2 h per week for 9 weeks, then resealed with the plugs and silicon glue.

After 63 d of ensiling, samples from control non-air-stressed (CTRL-NS), control air-stressed (CTRL- AS), Safesil 2 L/t non-air-stressed (SF2-NS), Safesil 2 L/t air-stressed (SF2-AS), Safesil 3 L/t non-air-stressed (SF3-NS), and Safesil 3 L/t air-stressed (SF3-AS) silos were analyzed.

Aerobic Stability Measurements

Upon silo opening, approximately 2 kg of silages from each silo were returned to clean laboratory silos. Silos were covered with 2 layers of cheesecloth and exposed to air in the laboratory at 22 ± 2°C. A thermocouple wire was placed in the geometric center of each forage mass, and temperatures were recorded every 15 min using a data logger DataTaker DT85 (Thermo Fisher Scientific, Australia Pty, Scoresby, VIC, Australia). Aerobic stability was calculated as the number of hours before the temperature of the forage mass roses 2°C above the baseline. Samples of CTRL-NS, CTRL-AS, SF2-NS, and SF2-AS were collected after 115 and 260 h of aerobic exposure for analysis.

Analytical Procedures

Fresh forage from four CTRL piles at d 0 and samples from each silo after ensiling were analyzed for DM and pH. The DM content was determined after 48-h incubation in a 60°C forced-air oven for 48 h. Weights of full and empty silos were measured at silo opening, and with the DM content of fresh and ensiled samples, were used to calculate DM recovery.

Representative 25-g portions of fresh forage and silage were mixed with 225 ml of sterile quarter-strength Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) and homogenized for 1 min in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). The homogenate was filtered through four layers of cheesecloth. The pH was determined on homogenized samples

using an Oakton pH700 Meter (Oakton Instruments, Vernon Hills, IL, USA). Fresh forage was analyzed for buffering capacity (BC) according to McDonald and Henderson (1962).

Portions of the water extracts that had been filtered through four layers of cheesecloth were filtered through Whatman 54 filter paper (Whatman Inc., Clifton, NJ). The extract was acidified with H_2SO_4 50% vol/vol and frozen. Later, it was analyzed for lactic, acetic, propionic, and butyric acids, and for 1,2-propanediol and ethanol production (Muck and Dickerson, 1988) by a Shimadzu LC-20AD HPLC (Shimadzu Scientific Instruments, Columbia, MD). The concentration of NH3-N was determined on the water extracts by the phenol-hypochlorite method of Weatherburn (1967). Water-soluble carbohydrates (WSC) were quantified by a colorimetric procedure (Nelson, 1944).

The nutrient analysis of fresh forage was performed by Cumberland Valley Analytical Services (Waynesboro, PA, USA). A portion of each dried sample was ground using an Udy Cyclone Sample Mill (Udy Corp., Fort Collins, CO) to pass through a 1-mm screen. Total N was measured by combustion of the sample following the AOAC method 990.03 (AOAC, 2010) using a Leco CNS2000 Analyzer (Leco Corporation, St. Joseph, MI), and crude protein (CP) was calculated by multiplying the resulting total N concentration by 6.25. Soluble protein (SP) was determined by the method of Krishnamoorthy et al. (1982) and expressed as % of CP. Acid detergent fiber (ADF) was quantified on dried ground samples using the AOAC method 973.18 (AOAC, 2010), using Whatman 934-AH glass micro-fiber filters with 1.5-µm particle retention instead of

fritted glass crucibles. Neutral detergent fiber (NDF) was analyzed on dried samples according to Van Soest et al. (1991) with sodium sulfite and amylase, not corrected for ash. Ash was analyzed by the AOAC method 942.05 (AOAC, 2000), with the modification of using a 1.5-g sample, 4 h ash time, and hot weighing. Another portion of the dry sample was ground to pass a 3-mm screen and analyzed for starch (Hall, 2008).

Microbial Analysis

Representative 25-g portions of fresh forage and silage were mixed with 225 ml of sterile quarter-strength Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) and homogenized for 1 min in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). The homogenate was filtered through four layers of cheesecloth. The filtrate was serially diluted in Ringers solution, and the numbers of LAB were determined by pour-plating in de Man, Rogosa, and Sharpe agar (CM3651, Oxoid, Unipath Ltd., Basingstoke, UK). Plates were incubated anaerobically at 32°C for 48 to 72 h in a plastic container with anaerobic packs (Mitsubishi Gas Chemical Co., Tokyo, Japan) and an anaerobic indicator (BR0055, Oxoid, Unipath Ltd., Basingstoke, UK). The container was vacuum-sealed in nylon-polyethylene bags (3.5-mil embossed pouches, 15.2 × 30.5 cm; Doug Care Equipment Inc., Springville, CA) using a Best Vac vacuum machine (distributed by Doug Care Equipment Inc.). Numbers of yeasts and molds were determined on malt extract agar (CM0059, Oxoid, Unipath Ltd., Basingstoke, UK) acidified with lactic acid (85%) at a rate of 0.5% vol/vol. These plates were incubated aerobically at 32°C for 48 h before

enumeration. Plates with a number of colonies between 30 and 300 were used to obtain counts of colony-forming units.

Microbiome Analysis

Four replicates of 25 g of fresh forage, CTRL and SF2 non-air-stressed, or air-stressed silages, and post aerobic exposure samples were mixed with 225 ml of autoclaved Ringers solution for 2 min using a Colworth 400 stomacher (Seward, London, UK). The homogenates were filtered through four layers of cheesecloth. Next, 2 ml of the filtrate was centrifuged for 3 min at 21130.2 xg using a Centrifuge 5424 R (Eppendorf AG, Hamburg, Germany). The supernatant was discarded, and 100 µl of autoclaved Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) was used to resuspend the pellet. Samples were kept at -80°C for further analysis.

Extraction of DNA, 16S rRNA and ITS1 amplification, and Illumina MiSeq-based sequencing were performed by the Research and Testing Laboratory (Lubbock, TX, USA). DNA was extracted done using the MoBio PowerMag Soil kit, according to manufacturer's instructions. For bacterial analysis, the primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGTCAATTCMTTTRAGTTT) were used to amplify the V4 and V5 hypervariable regions of the 16S rRNA (Baker et al., 2003). For fungal analysis, the primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2aR (GCTGCGTTCTTCATCGATGC) (White et al., 1990; Gardes and Bruns, 1993) were used to amplify the internal transcribed spacer 1 (ITS1). Sequencing was performed

with an Illumina MiSeq (San Diego, CA, USA) platform with the 2 × 250 bp paired-end method.

For sequence processing, paired-end reads were merged and converted into FASTA files using FLASH version 1.2.11 (Magoc and Salzberg, 2011). The quality profile of the reads was checked on http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Andrews, 2010). Next, reads were filtered for quality and the primers were trimmed using QIIME 1.9.1 (Caporaso, 2010). Operational taxonomic units (OTUs) picking was done using open reference clustering at 97% sequence similarity against Greengenes (version 13_8) or UNITE for bacteria and fungi, respectively. Finally, singles and double reads were filtered. The diversity analyses were performed using the phyloseq and vegan packages in R (McMurdie and Holmes, 2013; Oksanen et al. 2013). Membership Venn diagrams were computed using the MetaComet web platform to determine OTU overlap among treatments (Wang et al., 2016). BLAST (Altschul et al., 1990) was used to identify sequences related to the sequences of interest with the highest Max score. Phylogenetic trees were built in MEGA 7 (Kumar et al., 2016) using the Neighbor-Joining method (Saitou and Nei, 1987) with the maximum composite likelihood substitution model after 1000 bootstrap replication. Krona was used to plot microbiome data from different taxa levels (Ondov et al., 2011).

Statistical Analysis

Data for was analyzed using JMP version 12 (SAS Institute, Cary, NC). Data of fresh forage at d 0 were analyzed separately as a completely randomized design. Data from the silo opening was analyzed as a 3 × 2 factorial arrangement of treatments in a completely randomized design. The model included the fixed effects of the treatment, air stress, and their interaction. Data from the post aerobic exposure samples were analyzed as a 2 × 2 × 3 factorial arrangement of treatments in a completely randomized design. The model includes the fixed effects of the treatment, air stress, and sampling time, and their interactions. Differences were considered significant when $P < 0.05$, and pairwise mean comparisons were performed using Tukey's test at $P < 0.05$ (Snedecor and Cochran, 1980). Statistical analysis for the PCoA plots was done using PERMANOVA (permutational multivariate analysis of variance) by the adonis function in vegan (Oksanen et al. 2013).

Results

Chemical Properties and Microbial Populations of Fresh Forage and Silage, and Aerobic Stability of Silage

Table 4.1 shows the chemical and microbial composition of the freshly chopped whole corn plant.

After 63 d of ensiling (Table 4.2), silages treated with both levels of the additive had higher DM, lower concentrations of CP, SP, and NH_3-N than CTRL. SF2 had lower,

and SF3 had a numerically lower concentration of ADF and NDF compared to CTRL. There were no differences in concentrations of starch and ash among treatments. SF3 had the highest, SF2 the intermediate and CTRL the lowest concentration of WSC. Silages subjected to air stress had higher DM and lower concentration of NH_3-N than non-stressed silages.

After 63 d of ensiling (Table 4.3), CTRL had higher acetic acid concentration and higher pH than SF. Untreated silages lost approximately 13% DM during 63 d of ensiling, whereas silages treated with the lowest dose of SF lost 8% and with the highest dose lost 5% of DM. SF3 had the lowest, SF2 intermediate, and CTRL the highest pH. Air-stressed silages had higher DM recovery and pH than non-stressed silos. The CTRL and SF2 had the same response to air stress regarding numbers of LAB. SF2 and SF3 had a lower concentration of acetic acid than CTRL. There was an interaction between treatment and air stress for concentrations of lactic acid and ethanol and numbers of LAB and yeasts. In both CTRL and SF2, air-stressed silages had lower lactic acid production than non-stressed, whereas SF3 silages had similar numbers of lactic acid independent of the air stress status. In both non-stressed and air-stressed silos, SF2 and SF3 reduced the production of ethanol compared to CTRL. CTRL and SF2 air-stressed had lower numbers of LAB compared CTRL and SF2 non-stressed. SF3 had no detectable LAB on the lowest dilution evaluated (4 log cfu/g fresh forage weight). In non-stressed silos, CTRL and SF2 had the same and SF3 the lowest numbers of yeasts. In silos subjected to air stress, neither additive levels reduced the numbers of yeasts in comparison with untreated silage. Molds counts were below the detection limit of 1 log

CFU/g of fresh forage. In non-stressed silages, SF2 and SF3 markedly increased the aerobic stability compared to CTRL (>260 *vs.* 132 h). In silages submitted to air stress, CTRL was stable for 41 h, whereas SF2 was stable for 98 h and SF3 for 239 h (Figure 4.1).

After 63 d of ensiling, silages were exposed to air and collected for analysis after 115 and 260 h of exposure (Table 4.4). There were interactions among treatment, air stress, and hours of aerobic exposure for pH, concentrations of lactic acid, acetic acid, and ethanol, and numbers of yeasts and molds. In non-air stressed silos after 115 h of exposure to air, CTRL and SF2 had the same pH and concentration of lactic and acetic acids; however, SF2 had a lower concentration of ethanol and numbers of yeasts than CTRL. In non-air-stressed silos after 260 h of aerobic exposure, CTRL had higher pH and numbers of yeasts and lower concentrations of lactic acid, acetic acid, and ethanol than SF2. In air-stressed silos after 115 h of aerobic exposure, CTRL higher pH and numbers of yeasts and lower concentrations of lactic acid, acetic acids, and ethanol than SF2. In air-stressed silos after 260 h, CTRL and SF2 had the same pH, concentrations of lactic acid, acetic acid, and ethanol, and numbers of yeasts. After 115 h of exposure to air, all silages had undetectable levels of molds (< 1 log cfu/g fresh forage weight). After 260 h, SF2 had higher numbers of molds than CTRL, in both air-stressed and non-stressed silos.

The Composition of the Microbial Community

Sequencing depth was sufficient to describe the diversity of the microbial population as the rarefaction curves reached a plateau (Figure B.6-B.9). Table 4.5 shows the alpha diversity indexes for bacterial and fungal communities of fresh forage, silages, and post aerobic exposure samples. For bacteria, fresh forage and silages had a higher number of observed OTU and Chao1 index than post-aerobic exposure samples. CTRL-AS had higher Shannon index than SF2-AS after 115 and 260 h of exposure to air. Independent of air-stress status, CTRL had a higher Simpson index than SF2 after 115 and 260 h of exposure to air. For fungi, SF2-NS after 63 d of ensiling had a higher number of observed OTUs, and Chao1, Shannon, and Simpson indexes than SF2-AS.

Figure 4.2A shows a Venn diagram build based on the presence or absence of bacterial OTU in a treatment. Fresh forage had the lowest (390), SF2 silages intermediate (464), and CTRL silages the highest (483) number of OTUs. From a total of 529 OTUs, 0.2% were unique to one treatment, and 51% were shared among all treatments. CTRL-NS shared four, CTRL-AS and SF2-NS shared two, and SF2-AS did not shared OTUs with fresh forage. Figure 4.2B presents a Venn Diagram constructed based on the presence or absence of fungal OTUs. Fresh forage and SF2-NS had the highest (103), CTRL-NS and CTRL-AS intermediate (75), and SF2-AS the lowest (27) number of OTUs. From a total of 155 OTU, 35% of the OTUs were unique to only one treatment, and 12% were shared among all treatments. Both CTRL-NS and CTRL-AS shared five OTUs with fresh forage, whereas SF2-NS shared ten OTUs and SF2-AS shared none.

Figure 4.3A shows the PCoA plot for the composition of the bacterial community of fresh forage and silage after 63 d of ensiling. A significant Adonis test proved that the treatments did not have the same centroid, and a significant betadisper test showed that differences in group dispersions caused the results. Fresh forage samples formed a cluster separated from all silages, which clustered together. This result indicates that the composition of the bacterial community of fresh forage was different from the silages, but that the additive or air-stress did not affect the composition of the bacterial community as much as ensiling. Figure 4.3B shows the PCoA plot for the composition of the bacterial community of silage after exposure to air. A non-significant Adonis test proved that the treatments had the same centroid, and a non-significant betadisper test showed that differences in group dispersions did not cause the results. In this case, all treatments independent of time of exposure to air clustered together.

Based on sequence data, the main bacterial families in fresh forage were Xanthomonadaceae, Enterobacteriaceae, Sphingobacteriaceae, Leuconostocaceae, Alcaligenaceae, and Comamonadaceae. Figure 4.4A shows the relative abundance of bacteria families of silage after 63 d of ensiling. In non-stressed silos, SF2 had a lower abundance of Leuconostocaceae and Enterobacteriaceae than CTRL, but in air-stressed silos, the abundance of these microorganisms was the same in both treatments. Air stress tended to increase ($P = 0.08$) Acetobacteraceae abundance. Figure 4.4B shows the families relative abundance after exposure to air. There was an interaction between additive and air-stress for the relative abundance of Acetobacteraceae and Lactobacillaceae. In air-stressed silos, SF2 had higher abundance of Acetobacteraceae

than CTRL, and in non-stressed silos, the abundance was the same in both SF2 and CTRL. In non-stressed silos, SF had higher abundance of Lactobacillaceae than SF2. However, in air-stressed silos, the abundance was the same in both SF2 and CTRL. CTRL tended ($P = 0.06$) to have higher abundance of Enterobacteriaceae than SF2. After 260 h of aerobic exposure, CTRL had higher abundance of Bacillaceae than SF2.

Figure 4.5A shows the PCoA plot for the composition of the fungal community of fresh forage and silage after 63 d of ensiling. A significant Adonis test proved that the treatments did not have the same centroid, and a not significant betadisper test showed that differences in group dispersions did not cause the results. Fresh forage and non-stressed silages samples formed a cluster, whereas CTRL air-stressed and SF2 air-stressed formed different clusters. This result indicates that the composition of the fungal community of fresh forage was similar for CTRL and SF2 non-stressed silage, but that CTRL and SF2 air-stressed had exclusively different fungal community compositions. Figure 4.5B shows the PCoA plot for the composition of the fungal community of silage after exposure to air. A not significant Adonis test proved that the treatments had the same centroid, and a not significant betadisper test showed that differences in group dispersions did not cause the results. In this case, all treatments, independent of time of exposure to air clustered together.

Figure 4.6A shows that *Candida* and *Acremonium* were the main fungi genera in fresh forage. The relative abundance of the genus *Candida* in non-stressed silages was higher in CTRL than SF2 (83 vs. 47% of the total abundance). In air-stressed silos, CTRL had a lower abundance of *Candida* than SF2 (8 vs. 99%) and was dominated by *Pichia*

(87%). CTRL non-stressed silage had the highest abundance of *Kodamaeae* compared to CTRL air-stressed and treated silages. After exposure to air, SF2 had higher abundance of *Candida* than CTRL (Figure 4.6B).

Figure 4.7 shows the position of some of the most frequent fungal OTUs found in untreated and treated silage after 63 d of ensiling relative to species with the highest max score on BLAST. OTU X1 was assigned to *Saccharomyces cerevisiae*, X2 to *Candida humilis*, X3 to *Candida tropicalis*, X4 and X5 to *Candida quercitrusa*, X6 to *Pichia kudriavzevii*, and X7, X8, X9, X10 to *Kodamaea ohmeri*.

Figure 4.8 shows the composition of the fungal community of silages after 63 d of ensiling and the relative abundance of the primary species. The relative abundance of *Candida tropicalis* was higher in CTRL than SF2 (49.5 *vs.* 25.2%), and higher in non-stressed than air-stressed silos (63.4 *vs.* 11.2%). There was an interaction between additive and air stress for the relative abundance of *Candida humilis* and *Pichia kudriavzevii*. SF2 subjected to air-stress had a higher relative abundance of *Candida humilis* than other treatments (98.1 *vs.* 0.1%). CTRL subjected to air-stress had a higher relative abundance of *Pichia kudriavzevii* than other treatments (71.5 *vs.* 0.5%).

Discussion

The chemical and microbial compositions of fresh whole corn plant were in the expected range for this feedstuff (Hafner et al., 2015; Kleinschmit et al., 2005). After 63 d of ensiling, the additive reduced the concentration of CP, SP, and NH_3-N. Da Silva et

al. (2015) also observed lower concentrations of SP and NH_3-N in HMC treated with Safesil and explained that this reduction was due to the attenuation of proteolytic processes during ensiling. Air stress slightly decreased the concentration of NH_3-N.

Acetic acid can increase the buffering capacity of the silage. Therefore, the lower pH in treated silage could be explained by its lower concentration of acetic acid, and possible lower buffering capacity, compared to untreated silage. The additive restricted silage fermentation, which was evidenced by the reduction in numbers of LAB and by the higher concentration of WSC compared to untreated silage. The inhibition of LAB by the additive might not have been directly caused by its antimicrobial action but rather by the low pH of treated silage, which can inhibit growth and fermentation.

The increased DM recovery in silage treated with the additive compared to untreated silage could be explained by the lower production of ethanol and acetic acid in treated silage. The higher DM recovery in silages submitted to air stress compared to non-stressed silages was not expected, as substrate degradation by aerobic microorganisms was anticipated.

Some bacterial and fungal OTU from the fresh plant remained after ensiling. Ensiling was the primary factor affecting the composition of the bacterial community, but both air-stress and treatment markedly modified the composition of the fungal community of the silages. We found the main yeasts species found in untreated silage in this study were *Candida tropicalis* and *Pichia kudriavzevii*, which are known to assimilate lactate (Wang et al., 2018).

The air-stress model used in the current study stimulated the growth of yeasts and modified the composition of the microbial community resulting in reduced aerobic stability of corn silage. Air stress increased the numbers of yeasts and tended to increase the relative abundance of Acetobacteraceae, which comprises species that can consume the organic acids of silage and initiate aerobic spoilage (Spoelstra et al.1988). Air-stress reduced the relative abundance of *C. tropicalis* in both untreated and treated silages and stimulated the dominance of *P. kudriavzevii* in untreated silage and of *Candida humilis* in treated silage.

Candida humilis was previously identified in sugarcane silage and corn silage (Santos et al., 2015; Carvalho et al., 2016). Santos et al. (2017) identified 15 yeasts species in corn silage collected from nine different farms in the United States and found that *C. humilis* was the 13th most common species across samples. Dolci et al. (2011) isolated *Acetobacter* from corn silage covered with polyethylene film after 2 d of aerobic exposure but in silage covered with oxygen barrier film, these bacteria occurred only after 7 d of exposure to air. They also isolated *P. kudriavzevii* from silage covered with polyethylene film after 7 and 9 d of aerobic exposure but did not find this strain in silage covered with oxygen barrier film. The findings of Dolci et al. (2011) were similar to the ones from the current study. In both cases, oxygen seems to have stimulated *Acetobacter* and *Pichia* growth.

Because *Acetobacter* are aerobic bacteria, simulation of growth by oxygen was expected. In non-air stressed silo, the main yeast specie was *C. tropicalis* in both treated and untreated silage. In air-stressed silos, *P. kudriavzevii* (CTRL) and *C. humilis* (SF2)

dominated the silage to the detriment of *C. tropicalis*. A possible explanation is that *C. tropicalis* is more tolerant of anaerobic conditions than these other species or that the higher pH of the air-stressed silos was more favorable for the growth of *P. kudriavzevii* and *C. humilis*. In air-stressed silos, the reasons different yeasts species were selected in treated and untreated silages could be due to differences in resistance to the additive itself or a response to changes in silage characteristics caused by the additive. In air-stressed silos, CTRL had higher pH and higher concentrations of acetic acid and ethanol than SF2. De Melo Pereira et al. (2012) showed that *P. kudriavzevii* was more tolerant to ethanol than *C. humilis.* Moreover, *C. humilis* is inhibited by acetic acid (Neysens and Vuyst, 2005).

The improvement in aerobic stability by the additive appeared due to different reasons in non-stressed and air-stressed silos. The low dose of the additive did not reduce the numbers of yeasts in non-stressed silos. Therefore, the reduction in the number of total yeasts could not explain the increased stability of treated silages that were not subjected to air stress. It is possible that the cause of the increased aerobic stability of treated silage was the reduction in the relative abundance of the lactate assimilating yeasts *C. tropicalis*.

In air-stressed silage, the additive was unable to limit Acetobacteraceae development, as observed in SF2-AS by its high relative abundance and by the decline in ethanol and a small increase in acetic acid at 115 h, which progressed to the consumption of acetate and lactate after ethanol was depleted. These results are consistent with the onset of *Acetobacter* in silage as first described by Spoelstra (1988). Even though the

additive did not completely inhibit the Acetobacteraceae population, it shifted the yeasts populations and markedly improved aerobic stability of air-stressed silage compared to CTRL. Air-stressed silage treated with the additive was dominated by *C. humilis* as the additive prevented the growth of *P. kudriavzevii*, which was stimulated by the air influx in untreated silage. The greater aerobic stability in treated silage compared to untreated was probably because the first was dominated by *C. humilis,* which is unable to assimilate lactate or assimilate it slowly, whereas untreated silage was dominated by *P. kudriavzevii*, which is an efficient lactate-assimilator (Nel and Walt, 1968; Meroth et al., 2003).

Conclusions

The low dose of the additive containing sodium benzoate, potassium sorbate, and sodium nitrite did not reduce the numbers of culturable yeasts but still markedly improved aerobic stability of corn silage. In non-air-stressed silos, a reduction of the relative abundance of the lactate-assimilating yeasts *Candida tropicalis* might have explained the improvement in aerobic stability by the low dose of the additive. In air-stressed silos, the additive overcame the harmful effects of air possibly by preventing the dominance of specific microorganisms that were stimulated by oxygen, such as the lactate-assimilating yeasts *Pichia kudriavzevii*.

TABLES

Table 4.1. The DM content, chemical composition (% of DM, unless stated otherwise), pH, buffering capacity, and numbers of LAB and yeasts (fresh weight basis) of freshly chopped whole corn.

Item	Average	Standard deviation
DM, %	36.30	0.85
CP	8.80	0.42
SP[1], % of CP	32.95	4.07
NH_3-N	0.03	0.00
ADF	20.03	1.86
NDF	35.40	2.52
Starch	35.08	3.15
Ash	4.29	0.51
WSC[2]	12.48	0.11
pH	5.60	0.05
BC[3], g lactic acid /kg	21.67	3.77
LAB[4], log cfu/g	7.59	0.29
Yeasts, log cfu/g	5.63	0.34

[1]Soluble protein.
[2]Water soluble carbohydrates.
[3]Buffering capacity.
[4]Lactic acid bacteria.

Table 4.2. The DM content (%) and chemical composition (% of DM, unless stated otherwise) of corn silage treated with two concentrations of an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] subjected or not to air stress, ensiled for 63 d.

Item[2]	DM	CP	SP[3], % of CP	NH_3-N	ADF	NDF	Starch	Ash	WSC[4]
CTRL									
NS	31.42	9.38	50.53	0.09	22.16	37.92	31.94	5.03	0.00
AS	32.74	9.32	48.07	0.08	21.86	36.94	33.34	4.14	0.00
SF2									
NS	33.53	8.84	46.15	0.06	19.62	33.64	35.78	3.97	0.12
AS	33.94	8.92	45.07	0.05	20.72	35.80	33.64	4.48	0.11
SF3									
NS	34.18	9.02	46.34	0.06	20.72	35.38	31.64	4.74	0.20
AS	35.76	8.94	44.97	0.04	21.06	35.46	32.10	4.05	0.29
Additive means									
CTRL	32.08^{c}	9.35^{a}	49.30^{a}	0.09^{a}	22.01^{a}	37.43^{a}	32.64	4.59	0.00^{c}
SF2	33.74^{b}	8.98^{b}	45.66^{b}	0.06^{b}	20.17^{b}	34.72^{b}	34.72	4.23	0.12^{b}
SF3	34.97^{a}	8.88^{b}	45.61^{b}	0.05^{b}	20.89^{ab}	35.42^{ab}	31.87	4.40	0.25^{a}
Air-stress means									
NS	33.04^{b}	9.08	47.69	0.07^{a}	20.83	35.65	33.12	4.58	0.11
AS	34.15^{a}	9.06	46.03	0.06^{b}	21.21	36.07	33.03	4.22	0.13
SEM	0.45	0.08	1.05	0.00	0.61	0.90	1.48	0.28	0.03
Effects and interactions[5]	---------- *P* – value ----------								
A	<0.01	<0.01	<0.01	<0.01	0.02	0.02	0.16	0.45	<0.01
S	<0.01	0.75	0.08	<0.01	0.45	0.57	0.94	0.14	0.17
A×S	0.42	0.53	0.66	0.78	0.52	0.23	0.48	0.05	0.15

[a-b] Means in columns with unlike superscripts differ ($P < 0.05$).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight; SF3 = 3.0 L of Safesil/t of fresh forage weight; NS= no air stress; AS= 2 h air/week stress.
[3]Soluble protein.
[4]Water soluble carbohydrates.
[5]A = effect of additive; S = effect of air stress; A × S = interaction of additive by air stress.

Table 4.3. The DM recovery (%), pH, chemical composition (% of DM), and numbers of LAB and yeasts (log cfu/g of fresh weight) of corn silage treated with two concentrations of an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] subjected or not to air stress, ensiled for 63 d.

Item[2]	DMR[3]	pH	Lactic acid	Acetic acid	ETOH[4]	LAB[5]	Yeasts
CTRL							
NS	85.04	3.69	6.81^{a}	1.35	4.78^{a}	6.98^{a}	2.74^{b}
AS	88.59	3.75	4.86^{bc}	1.31	3.86^{b}	5.30^{b}	4.91^{a}
SF2							
NS	91.53	3.56	5.79^{ab}	1.03	1.44^{c}	6.61^{a}	1.86^{bc}
AS	92.48	3.60	4.64^{c}	1.01	1.50^{c}	5.22^{b}	4.71^{a}
SF3							
NS	93.45	3.53	5.17^{bc}	0.97	0.86^{d}	<4.00^{c}	<1.00^{c}
AS	97.16	3.59	4.94^{bc}	1.13	1.02^{d}	<4.00^{c}	4.54^{a}
Additive means							
CTRL	86.81^{c}	3.72^{a}	5.84	1.33^{a}	4.32	6.14	3.83
SF2	92.00^{b}	3.58^{b}	5.22	1.02^{b}	1.47	5.92	3.29
SF3	95.31^{a}	3.56^{c}	5.06	1.05^{b}	0.94	<4.00	2.77
Air-stress means							
NS	90.00^{b}	3.60^{b}	5.92	1.12	2.36	5.86	1.87
AS	92.74^{a}	3.64^{a}	4.81	1.15	2.13	4.84	4.72
SEM	1.19	0.01	0.24	0.06	0.09	6.98	0.22
Effects and interactions[6]	---------- *P* – value ----------						
A	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
S	<0.01	<0.01	<0.01	0.44	<0.01	<0.01	<0.01
A×S	0.43	0.20	<0.01	0.21	<0.01	<0.01	0.02

[a-d] Means in columns with unlike superscripts differ ($P < 0.05$).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight; SF3 = 3.0 L of Safesil/t of fresh forage weight; NS= no air stress; AS= 2 h air/week stress.
[3]DM recovery.
[4]Ethanol.
[5]Lactic acid bacteria.
[6]A = effect of additive; S = effect of air stress; A × S = interaction of additive by air stress.

Table 4.4. The pH, chemical composition (% of DM), and numbers of yeasts and molds (log cfu/g of fresh weight) of corn silage treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] subjected or not to air stress ensiled for 63 d, after 115 and 260 h of exposure to air.

Item[2]	pH	Lactic acid	Acetic acid	ETOH[3]	Yeasts	Molds
115 h						
CTRL						
NS	3.78^{c}	5.72^{a}	0.94^{a}	2.67^{a}	7.07^{b}	<1.00^{c}
AS	6.20^{b}	0.51^{c}	0.05^{b}	0.00^{d}	9.09^{a}	<1.00^{c}
SF2						
NS	3.59^{c}	5.82^{a}	1.07^{a}	1.10^{b}	3.45^{c}	<1.00^{c}
AS	3.78^{c}	4.23^{b}	1.14^{a}	0.50^{c}	6.95^{b}	<1.00^{c}
260 h						
CTRL						
NS	7.11^{a}	0.38^{c}	0.06^{b}	0.00^{d}	9.17^{a}	<1.00^{c}
AS	6.34^{ab}	0.00^{c}	0.05^{b}	0.00^{d}	8.86^{a}	<1.00^{c}
SF2						
NS	3.61^{c}	4.57^{ab}	0.90^{a}	0.71^{bc}	4.03^{c}	4.29^{b}
AS	6.39^{ab}	0.36^{c}	0.04^{b}	0.00^{d}	8.57^{a}	8.72^{a}
SEM	0.18	0.32	0.16	0.09	0.20	0.11
Effects and interactions[4]	---------- *P* – value ----------					
A	<0.01	<0.01	<0.01	0.17	<0.01	<0.01
S	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
T	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
A×S	0.09	0.82	0.81	<0.01	<0.01	<0.01
A×T	<0.01	0.42	0.38	<0.01	0.58	<0.01
S×T	0.84	0.02	0.90	<0.01	0.04	<0.01
A×S×T	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a-d] Means in columns with unlike superscripts differ ($P < 0.05$).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight; NS= no air stress; AS= 2 h air/week stress.
[3]Ethanol.

[4]A = effect of additive; S = effect of air stress; T = effect of time; A × S = interaction of additive by air stress; A × T = interaction of additive by time; S × T = interaction of air stress by time; A × S × T = interaction of additive by air stress by time.

Table 4.5. Alpha diversity indexes of the composition of bacterial and fungal communities, as analyzed by the sequencing of the V4-V5 region of the 16S rRNA and ITS1 using Illumina Miseq, of fresh forage, corn silage untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] subjected or not to a 2h/week air stress ensiled for 63 d, and of silages after 115 and 260 h of exposure to air [2].

Time	Index	Bacteria				Fungi			
		Observed	Chao1	Shannon	Simpson	Observed	Chao1	Shannon	Simpson
0 d	CTRL	321.25^{a}	357.16^{a}	4.42^{a}	0.96^{a}	51.75^{a}	54.4^{a}	1.73^{ab}	0.60^{a}
63 d	CTRL-NS	357.25^{a}	436.18^{a}	3.38^{ab}	0.87^{ab}	41.25^{ab}	43.25^{abc}	1.01^{abc}	0.37^{ab}
	CTRL-AS	309.00^{a}	381.92^{a}	2.67^{bcd}	0.81^{abc}	34.00^{abc}	41.67^{abc}	0.70^{bc}	0.31^{ab}
	SF2-NS	311.00^{a}	407.81^{a}	2.27^{bcde}	0.69^{bcd}	51.50^{a}	52.69^{ab}	2.12^{a}	0.71^{a}
	SF2-AS	312.25^{a}	371.64^{a}	3.00^{bc}	0.84^{abc}	9.00^{c}	15.50^{cd}	0.11^{c}	0.04^{b}
63 d + 115h	CTRL-NS	205.25^{b}	224.82^{b}	2.39^{bcde}	0.72^{abcd}	26.75^{abc}	28.50^{abcd}	1.08^{abc}	0.46^{ab}
	CTRL-AS	140.50^{bcd}	172.80^{bc}	1.91^{cde}	0.60^{cd}	11.00^{c}	12.00^{d}	0.97^{abc}	0.48^{ab}
	SF2-NS	188.50^{bc}	214.05^{b}	1.50^{ef}	0.49^{de}	23.75^{bc}	23.88^{bcd}	1.32^{abc}	0.50^{ab}
	SF2-AS	78.75^{d}	104.82^{c}	0.53^{f}	0.19^{f}	14.00^{c}	15.83^{cd}	0.80^{bc}	0.40^{ab}
63 d + 260h	CTRL-NS	105.50^{cd}	122.39^{c}	2.54^{bcde}	0.82^{abc}	14.75^{c}	17.00^{cd}	0.84^{abc}	0.40^{ab}
	CTRL-AS	143.25^{bcd}	177.14^{bc}	3.17^{b}	0.87^{ab}	15.25^{c}	16.38^{cd}	0.74^{bc}	0.34^{ab}
	SF2-NS	195.50^{bc}	220.00^{b}	1.61^{def}	0.48^{de}	21.75^{bc}	23.36^{cd}	1.36^{abc}	0.56^{ab}
	SF2-AS	78.00^{d}	97.56^{c}	0.72^{f}	0.26^{ef}	17.00^{bc}	22.88^{cd}	0.59^{bc}	0.25^{ab}
	SEM	18.57	18.09	0.23	0.05	5.12	5.79	0.26	0.11
	P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.02

[a-f] Means in columns with unlike superscripts differ ($P < 0.05$).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF2 = 2.0 L of Safesil/t of fresh forage weight; NS= no air stress; AS= 2 h air/week stress.

FIGURES

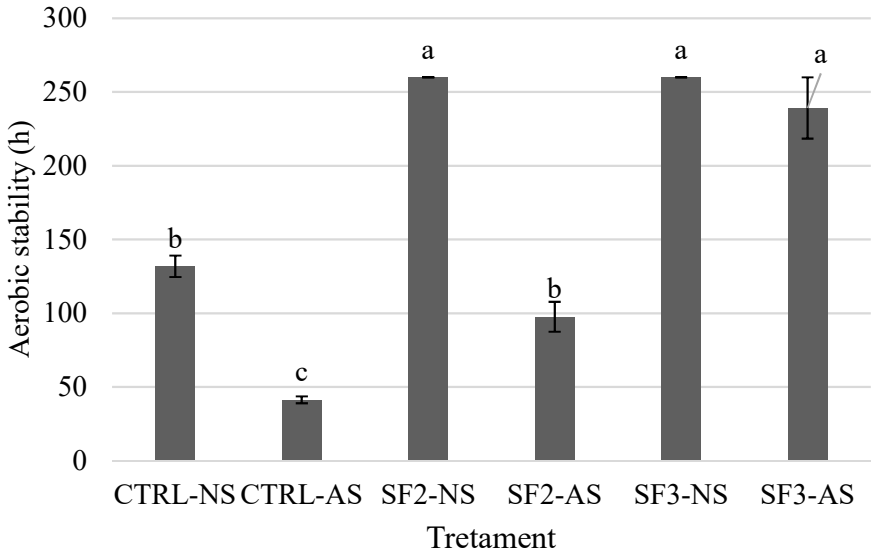


Figure 4.1. Aerobic stability of silage untreated (CTRL) and treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh matter (SF2) or at 3 L/t of fresh matter (SF3), subjected (AS) or not (NS) to a 2 h/week air stress, ensiled for 63 d. Air stress was applied 2 h/week for 9 weeks (SEM= 9.53, $P < 0.01$). [a-c] Means bars with unlike letters differ ($P < 0.05$).

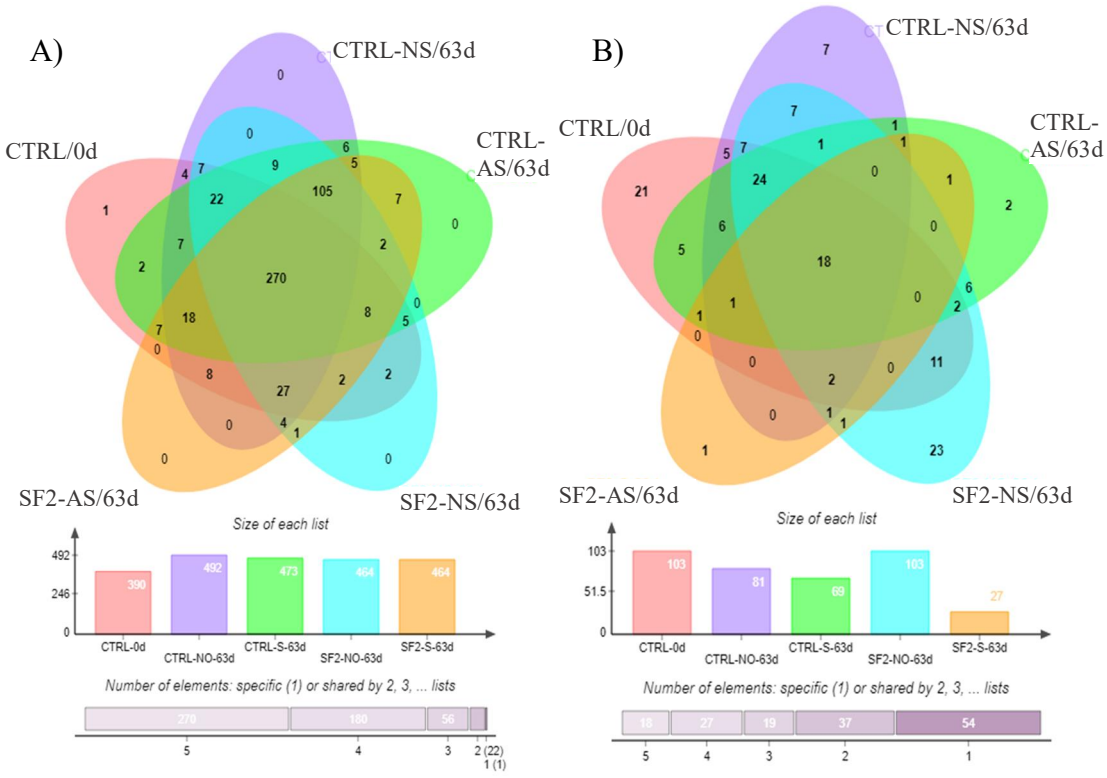


Figure 4.2. A) Venn diagram built based on the presence and absence of OTUs in fresh forage (CTRL/0d) and corn silage untreated (CTRL/63d) or treated with Safesil 2 L (active ingredients of sodium nitrite, potassium sorbate, and sodium benzoate; Salinity, Göteborg, Sweden)/t of fresh matter ensiled for 63 d (SF2/63d), subjected (AS) or not (NS) to a 2 h/week air stress of a) bacteria from the sequencing of the V4-V5 region of the 16S rRNA and B) fungi from the sequencing of ITS1, using Illumina MiSeq platform. The numbers on the diagrams show the numbers of unique or shared OTUs among treatments.

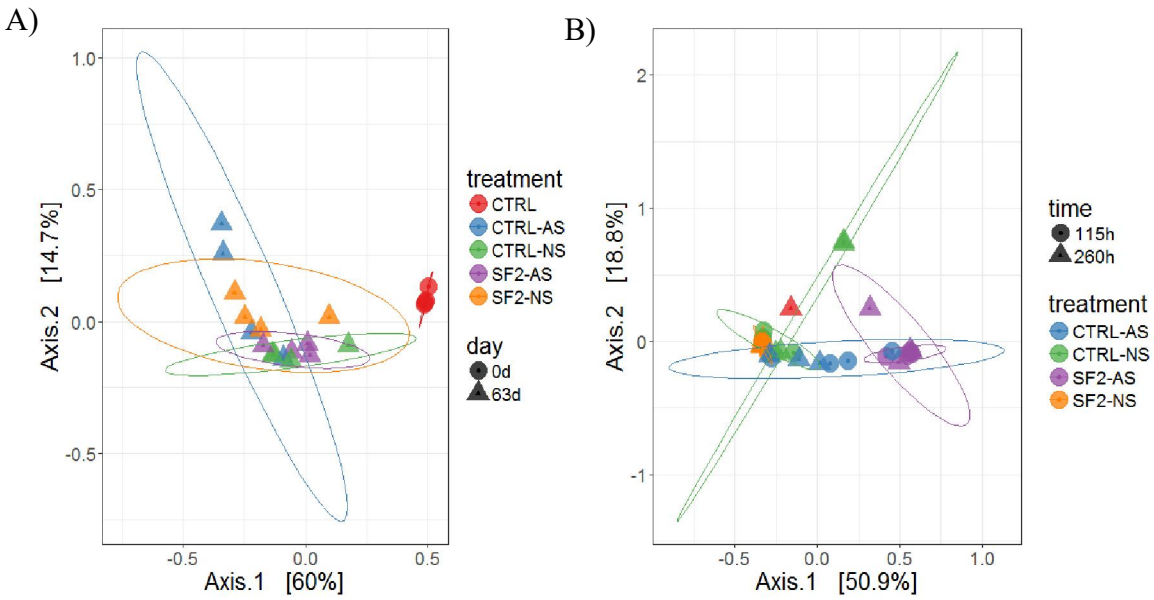


Figure 4.3. Principal coordinate analysis (PCoA) plot with weighted unifrac dissimilarity of the composition of the bacterial community, as analyzed by the sequencing of the V4-V5 region of the 16S rRNA using Illumina Miseq, of four replicates of A) fresh forage (0 d) and corn silage (63 d) untreated (CTRL) or treated with Safesil 2L (active ingredients of sodium nitrite, potassium sorbate, and sodium benzoate; Salinity, Göteborg, Sweden)/t of fresh matter (SF2) not subjected (NS) or subjected (AS) to a 2 h/week air stress and from B) silages after 115 and 260 h of exposure to air.

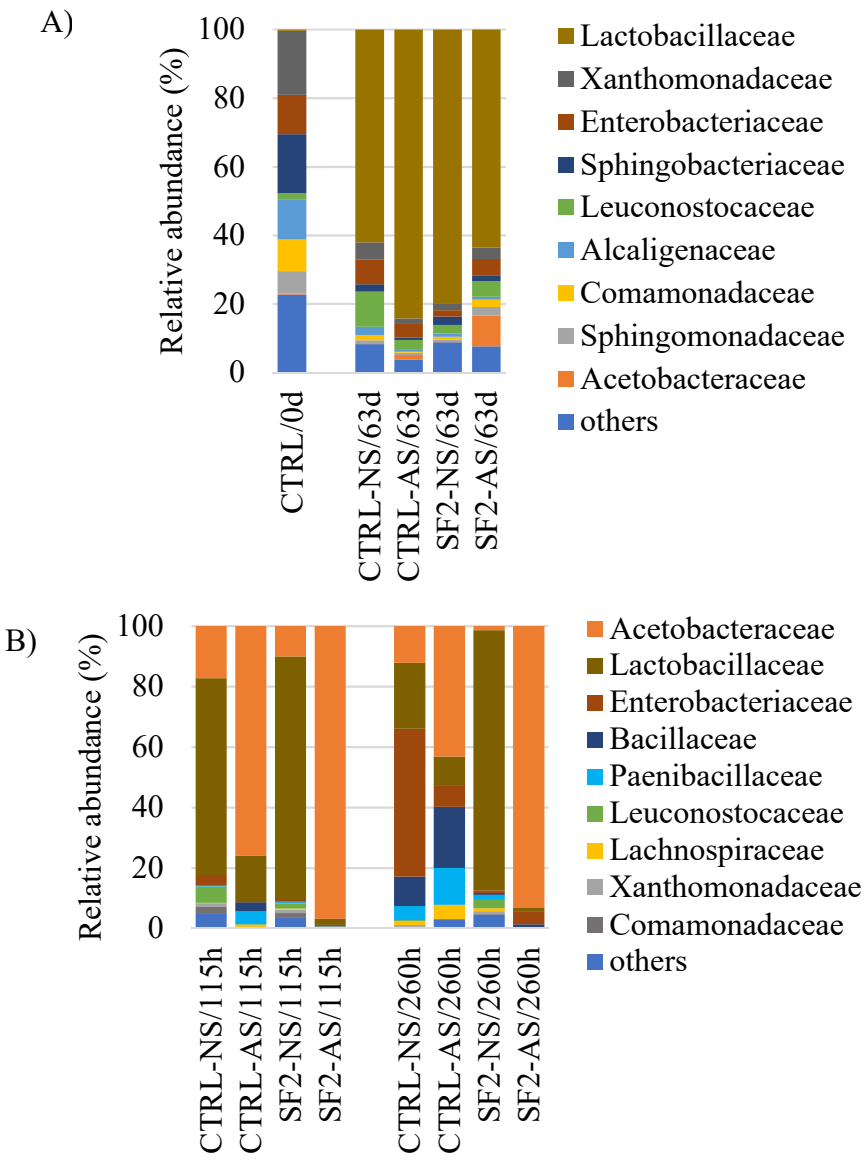


Figure 4.4. Relative abundance from sequences of A) bacteria families in fresh forage and silages untreated (CTRL) or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh forage weight (SF2) subjected (AS) or not (NS) to a 2 h/week air stress ensiled for 63 d and B) in silages after 115 and 260 h of exposure to air, as analyzed by the sequencing of the V4-V5 region of the 16S rRNA using Illumina MiSeq platform.

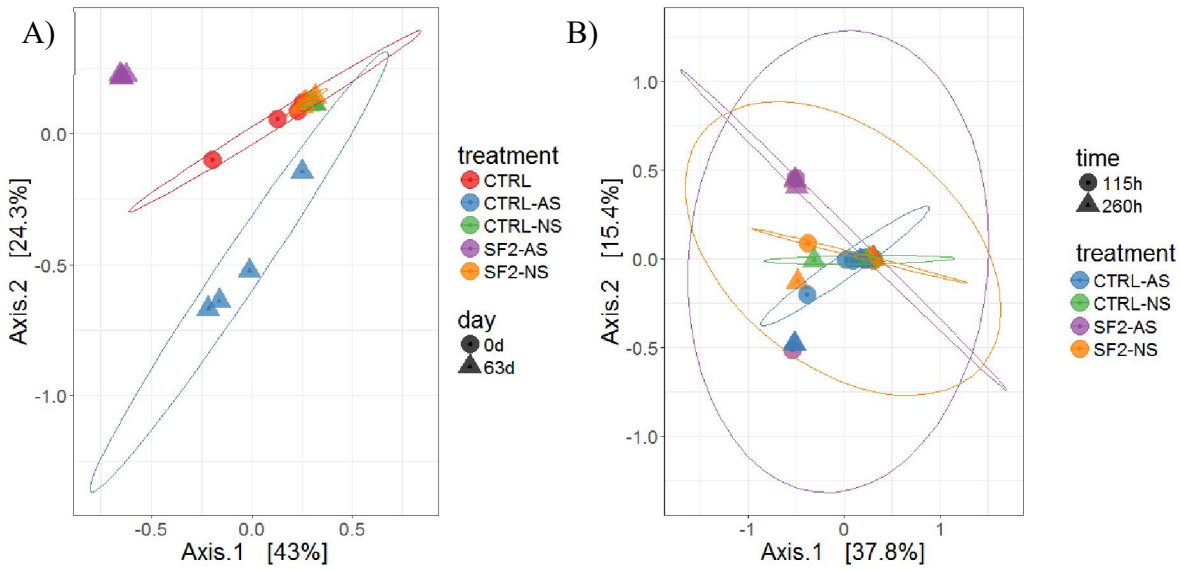


Figure 4.5. Principal coordinate analysis (PCoA) plot with Bray-Curtis dissimilarity of the composition of the fungal community, as analyzed by the sequencing of the ITS1 using Illumina Miseq, of A) fresh forage (0 d) and corn silage (63 d) untreated (CTRL) or treated with Safesil 2L (active ingredients of sodium nitrite, potassium sorbate, and sodium benzoate; Salinity, Göteborg, Sweden)/t of fresh matter (SF2) subjected (AS) or not (NS) to a 2 h/week air stress and from B) silages after 115 and 260 h of exposure to air.

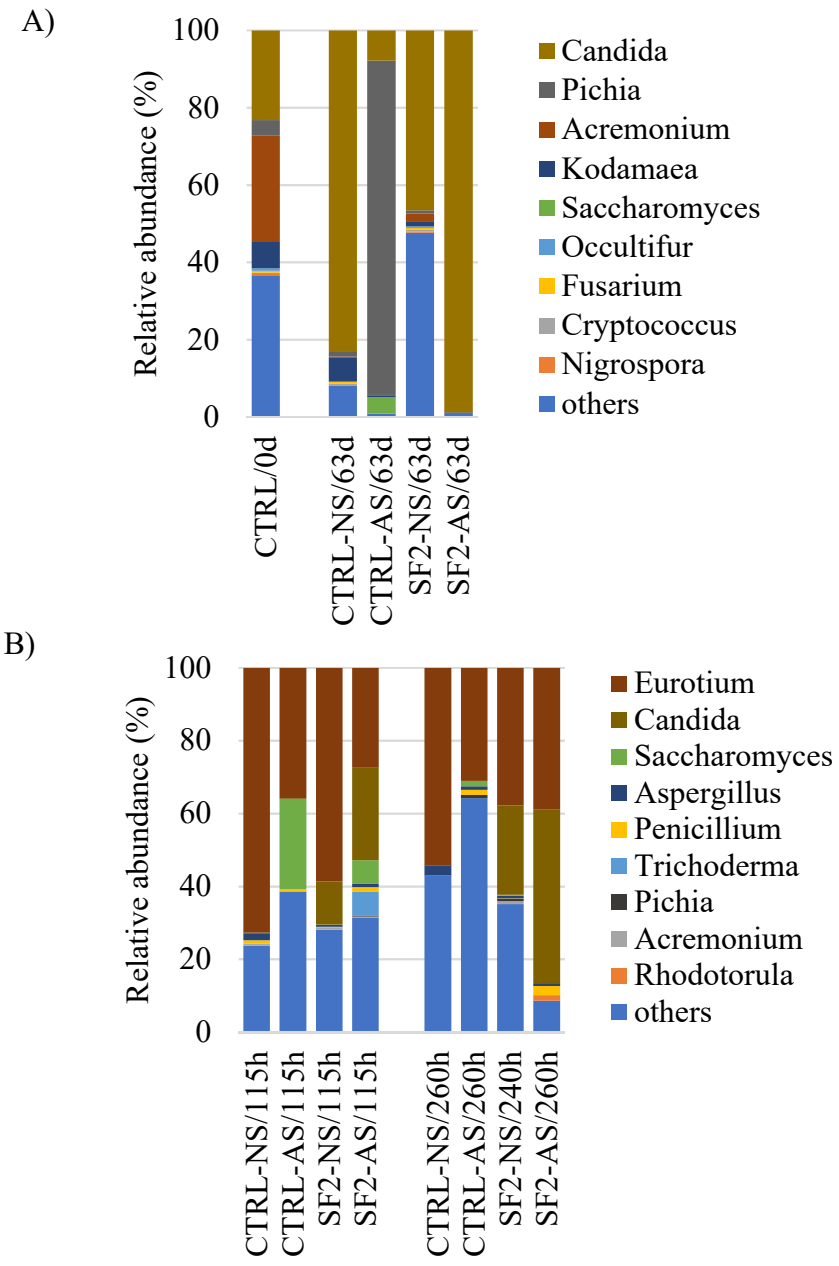


Figure 4.6. Relative abundance from sequences of A) fungi genera in fresh forage and in silages untreated (CTRL) or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh forage weight (SF2) subjected (AS) or not (NS) to a 2 h/week air stress ensiled for 63 d and B) in silages after 115 and 260 h of exposure to air, as analyzed by the sequencing of ITS1 using Illumina MiSeq platform.

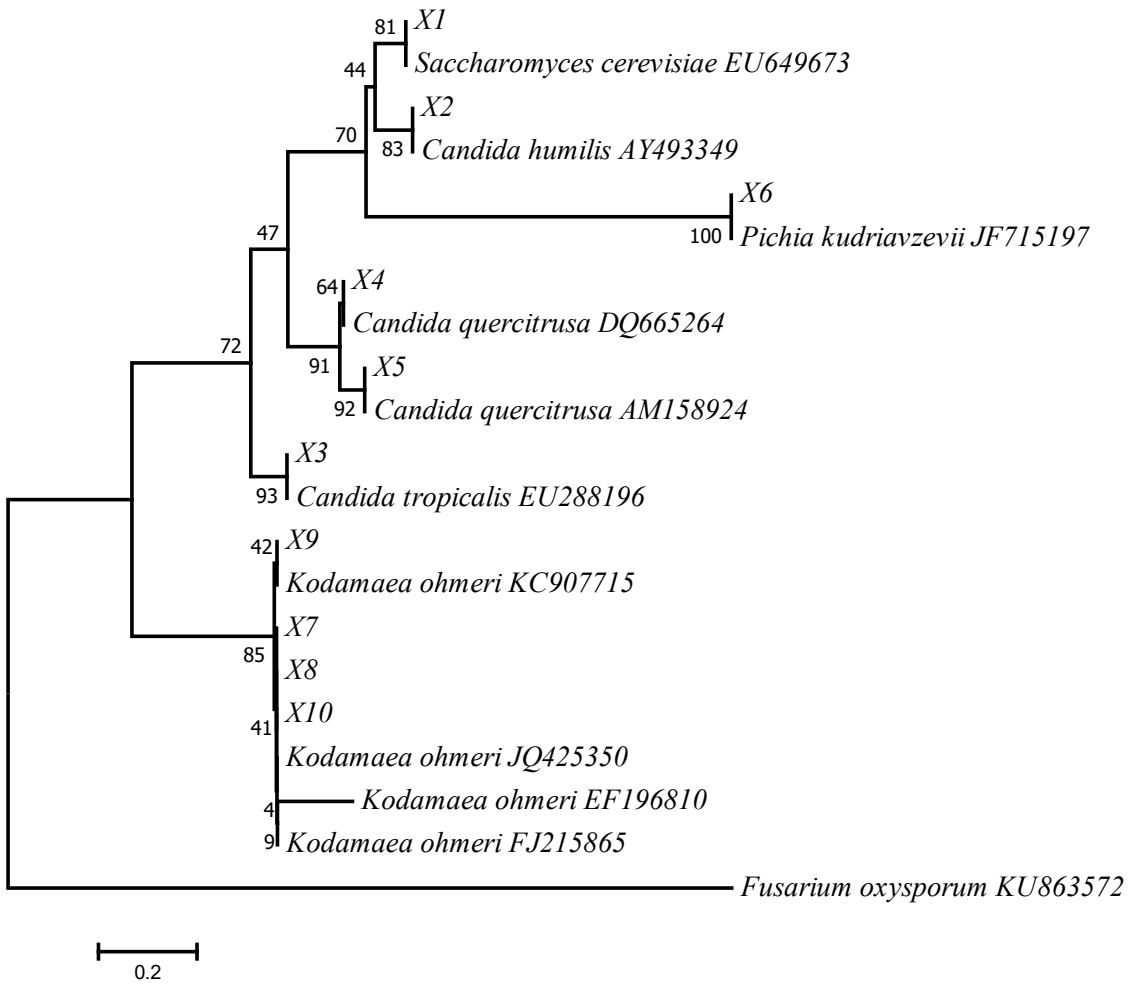
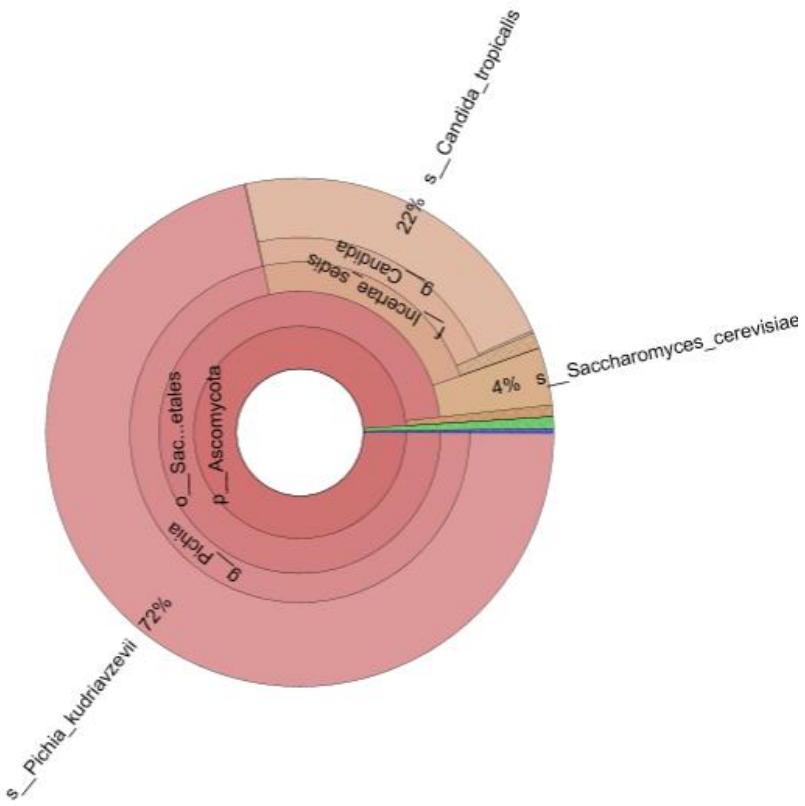
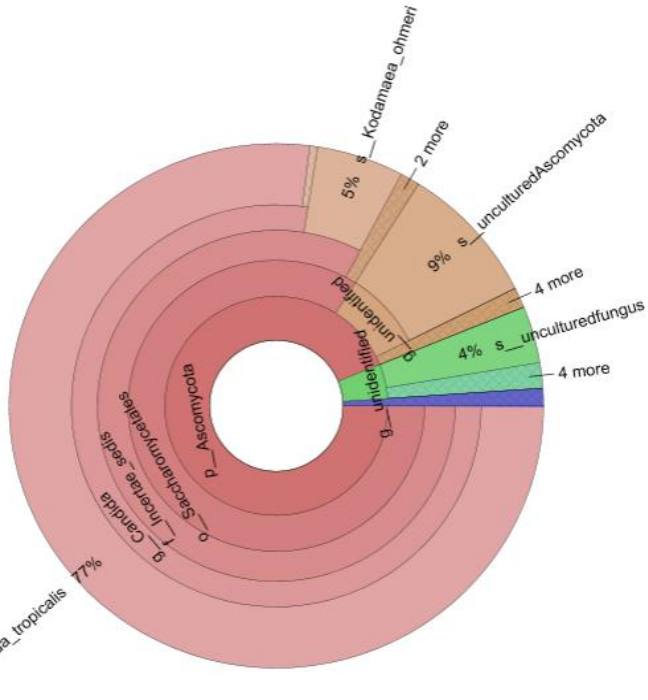


Figure 4.7. Phylogenetic tree constructed by the Neighbor-Joining method showing the position of yeasts species based on ITS1 region sequences. The evolutionary distances were computed using the Maximum Composite Likelihood method. Bootstraps value for 1,000 replicates is shown at the nodes. X1-X10 represents OTUs at species level. *Fusarium oxysporum* was used as outgroup. Scale bar indicates an evolutionary distance of 0.2 nucleotide substitution.

CTRL-AS



CTRL-NS



Cont.

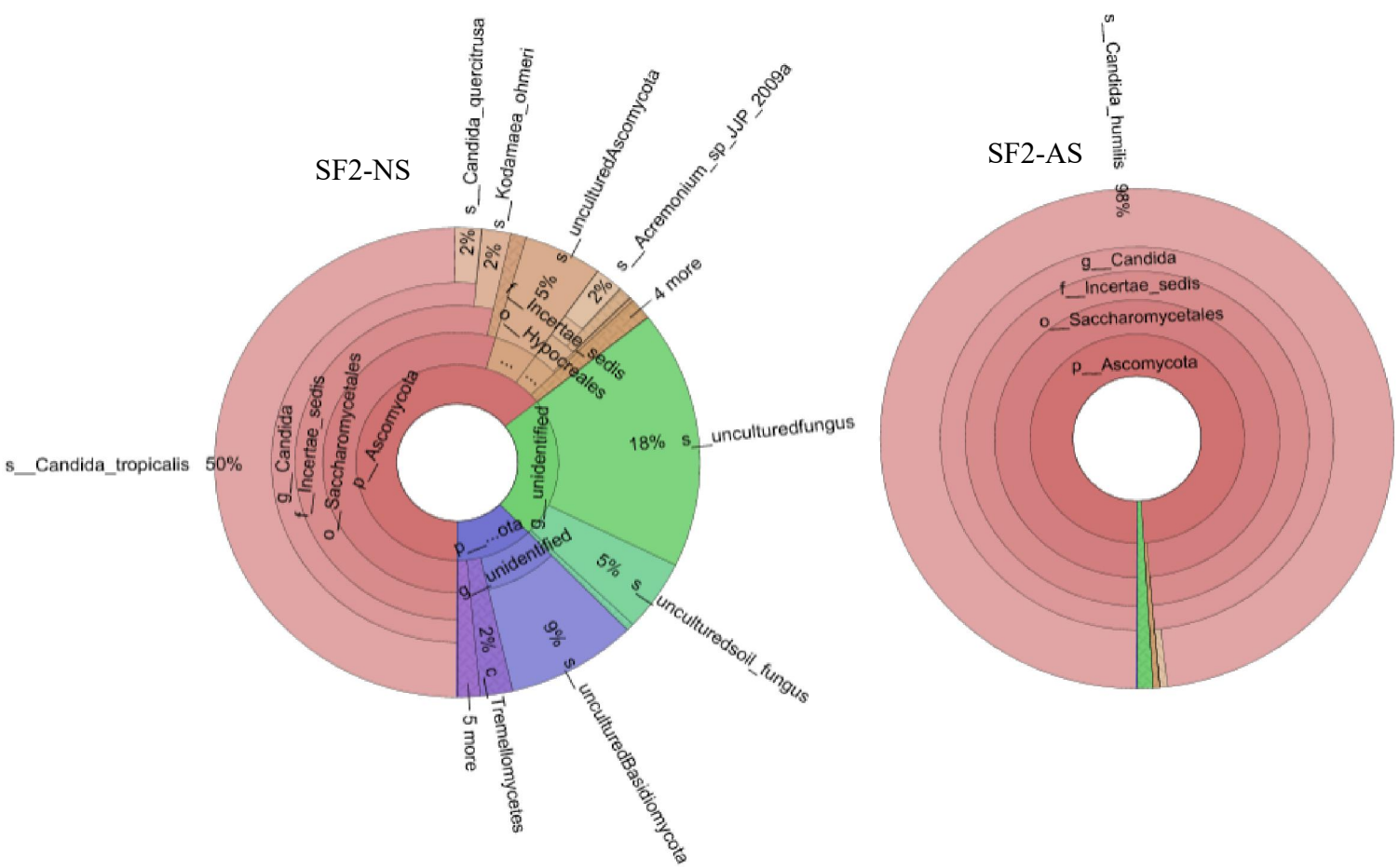

SF2-NS
s__Candida_quercitrusa
s__Kodamaea_ohmeri
s__unculturedAscomycota
s__Acremonium_sp._JJP_2009a
4 more
2%
2%
5%
2%
f__Incertae_sedis
o__Hypocreales
...
...
18%
s__unculturedfungus
g__unidentified
5%
s__unculturedsoil_fungus
9%
s__unculturedBasidiomycota
2%
c__Tremellomycetes
5 more
p__...ota
g__unidentified
s__Candida_tropicalis 50%
g__Candida
f__Incertae_sedis
o__Saccharomycetales
p__Ascomycota
SF2-AS
s__Candida_humilis 98%
g__Candida
f__Incertae_sedis
o__Saccharomycetales
p__Ascomycota

Figure 4.8. Krona plot illustrating the composition of the fungal community, based on High-Throughput Paired-end Illumina Sequencing of the ITS1, of silages untreated (CTRL) or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2 L/t of fresh forage weight (SF2) subjected (AS) or not (NS) a 2 h/week air stress ensiled for 63 d. Rings represent different taxonomic ranks (phylum, class, order, family, genus, and species, respectively) and numbers represent the relative abundance of each species.

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Chapter 5

EFFECTS OF A CHEMICAL ADDITIVE ON THE COMPOSITION OF THE BACTERIAL COMMUNITY OF HIGH MOISTURE CORN AND ITS EFFECTIVENESS ON IMPROVING FERMENTATION AND AEROBIC STABILITY OF AIR-STRESSED HIGH MOISTURE CORN SUBMITTED TO AEROBIC SPOILAGE AT ROOM AND WARM TEMPERATURES

Abstract

The objective of this experiment was to determine the effectiveness of a chemical additive containing sodium benzoate, potassium sorbate, and sodium nitrite (Safesil, active ingredients: 20% sodium benzoate, 10% potassium sorbate, and 5% sodium nitrite) (Salinity, Göteborg, Sweden) at 3 L/t (SF3) of fresh weigh on improving the fermentation characteristics and aerobic stability of high moisture corn (HMC). To better understand the impact of the additive on the fermentation and aerobic stability of HMC, the composition of its bacterial community was analyzed by high throughput sequencing. HMC (32% moisture) was untreated (CTRL) or treated with SF (3 L/t). Twenty replicated silos (7.5 L) per treatment were packed at a density of 658 kg of DM/m^3. Half of the silos were not air stressed, whereas the other half were submitted to a 12-h per

week air stress. After 49 d, half of the silos were subjected to aerobic spoilage at 22°± 2°C and the other half at 34°C°± 2°C. Samples were analyzed for microbial populations, fermentation profile, and aerobic stability. The additive reduced the relative abundance of Enterobacteriales and decreased proteolysis. Additionally, it reduced the numbers of yeasts and ethanol production, increasing DM recovery. The additive markedly improved the aerobic stability of HMC and overcame the harmful effects of air-stress and high temperatures.

Introduction

High moisture corn (HMC) is an alternative to feeding dry corn to dairy cows. HMC is produced by harvesting the corn before the kernels dry, usually at more than 24% moisture content, followed by ensiling (Lardy, 2016). Some of the advantages of HMC compared to dry corn are the elimination of drying costs, reduction of field losses, and prolongation of the harvesting season, since it can be harvested after corn silage and before dry corn (Swanson, 2009). Additionally, HMC can have a higher starch digestibility compared to dry corn, because it is harvested in an early stage of maturity and because it undergoes the ensiling process (Hoffman et al., 2011). Despite these benefits, the low moisture content and low concentration of fermentable sugars of HMC can limit the extent of the fermentation, and its high starch content can stimulate the growth of yeasts. Therefore, it can have high numbers of yeasts and low aerobic stability (Bucholtz, 2012; Kung et al., 2007).

As HMC is exposed to air at feed-out, aerobic microorganisms that were dormant in the silo start to grow. Aerobic yeasts oxidize sugars or lactic acid, producing CO_2, H_2O, and heat. The consumption of lactic acid by lactate-assimilating yeasts increases pH, which allows for the growth of aerobic bacteria and molds that cause further spoilage. Spoilage of silage due to exposure to air causes not only DM loss but also the loss of fermentation products (Whitlock et al., 2000).

Air penetration can be detrimental to HMC nutritional value, DM recovery, and aerobic stability. Management factors, physical properties, such as packing density, permeability, and porosity affect directly the amount of oxygen penetrating the silo

(Wilkinson and Davies, 2012). Even when anaerobiosis is quickly achieved during harvesting and silo filling operations, one is not exempt from the detrimental effects of air, which can enter the silo during storage. Birds, rodents, and even farmers themselves can produce holes in silos, allowing air to penetrate (Woolford, 1990). Air stress in the silo can have substantial economic consequences since aerobic spoilage during storage can cause DM losses as high as 30 to 40% (Kung, 2010).

Not only air stress during storage but also warm temperatures at feed-out can decrease aerobic stability. Producers in warm climate areas face several problems during the silage making process. Warm temperatures can negatively affect corn yield and quality and reduce the aerobic stability of silages (Bernardes et al., 2018). Ashbell et al. (2002) found that the largest increase in numbers of yeasts and the most considerable reduction in aerobic stability in corn silage occurred at 30 °C rather than at 10 °C, 20 °C, and 40 °C. Warm temperatures stimulate yeast growth. However, when the heat is extreme, it could cause a reduction in numbers of yeasts and an increase in aerobic stability, but those temperatures are rare and usually not observed for prolonged periods in most of the areas that produce corn silage.

Sodium benzoate, potassium sorbate, and sodium nitrite are additives that in combination can reduce aerobic spoilage of HMC (Da Silva et al., 2015). Even though it is known that this additive has antimicrobial properties, there is little knowledge of its effects on the composition of the bacterial community of HMC. Moreover, there is a need for more studies evaluating the effectiveness of this additive on improving HMC aerobic

stability under challenging conditions, such as air stress during storage and warm temperatures at feed-out.

Objectives

The objectives of this experiment were to determine the effects of an additive contaiing sodium benzoate, potassium sorbate, and sodium nitrite on the composition of the bacterial community of HMC; and to evaluate its effectiveness on improving aerobic stability under challenging conditions, such as air stress during storage and warm temperatures at feed out.

Materials and methods

Crops and Silos

Shelled corn (unknown hybrid) from Chesapeake Gold Farms (Rising Sun, Maryland) was harvested with a John Deere 9400 combine (Moline, Il) at about 32% moisture and ground through a Gehl hammer mill (Model # 170, Manitou Americas, Inc., West Bend, WI). Freshly ground HMC was transported to the University of Delaware where it was treated within 3 h of harvest. Treatment with 300 ml of water (CTRL) or with 300 ml of Safesil diluted in water (SF, active ingredients: 20% sodium benzoate, 10% potassium sorbate, and 5% sodium nitrite) (Salinity, Göteborg, Sweden) at 3 L/t of fresh forage weight were applied to the forage. The treatments were applied to five individually replicated piles per treatment containing 41 kg of HMC using a hand

sprayer. Samples from each pile were packed into four 7.5-L laboratory silos at a packing density of approximately 658 kg of DM/m^3 that were sealed with plastic lids with O-ring seals. Half of the silos were packed into air stress silos that had three holes of 1.60 cm diameter that were plugged with rubber stoppers and sealed with silicon glue. Two holes were located 5 cm above the bottom of the silo 180° from each other. The third hole was on the lid of the silo. All three holes were opened for 12 h per week for 7 weeks, then resealed with the plugs and silicon glue. After 49 d of ensiling, half of the silos were subjected to aerobic spoilage at 22 ± 2°C and the other half at 34 ± 2°C.

Aerobic Stability Measurements

Upon silo opening, approximately 4 kg of HMC from each silo were returned to clean laboratory silos. Silos were covered with two layers of cheesecloth and exposed to air at 22 ± 2°C or 34 ± 2°C. A thermocouple wire was placed in the geometric center of each forage mass, and temperatures were recorded every 15 min using a data logger DataTaker DT85 (Thermo Fisher Scientific, Australia Pty, Scoresby, VIC, Australia). Aerobic stability was calculated as the number of hours before the temperature of the HMC mass rose 2°C above the baseline.

Analytical Procedures

Fresh HMC from five CTRL piles and HMC from each silo after ensiling were analyzed for DM and pH. The DM content was determined after 48-h incubation in a

60°C forced-air oven. Weights of full and empty silos were measured at silo opening, and with the DM content of fresh and ensiled samples, were used to calculate DM recovery.

Representative 25-g portions of fresh and ensiled HMC were mixed with 225 ml of sterile quarter strength Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) and homogenized for 1 min in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). The homogenate was filtered through four layers of cheesecloth. The pH was determined on homogenized samples using an Oakton pH700 Meter (Oakton Instruments, Vernon Hills, IL, USA). Fresh HMC was analyzed for buffering capacity (BC) according to McDonald and Henderson (1962).

Portions of the water extracts that had been filtered through four layers of cheesecloth were filtered through Whatman 54 filter paper (Whatman Inc., Clifton, NJ). The extract was acidified with H_2SO_4 (50% vol/vol) and frozen. Later, it was analyzed for lactic, acetic, propionic, and butyric acids, and for 1,2-propanediol and ethanol content (Muck and Dickerson, 1988) by a Shimadzu LC-20AD HPLC (Shimadzu Scientific Instruments, Columbia, MD). The concentration of NH_3-N was determined in water extracts by the phenol-hypochlorite method of Weatherburn (1967). Water-soluble carbohydrates (WSC) were quantified by a colorimetric procedure (Nelson, 1944).

The nutrient analysis of fresh HMC was performed by Cumberland Valley Analytical Services (Waynesboro, PA, USA). A portion of each dried sample was ground using an Udy Cyclone Sample Mill (Udy Corp., Fort Collins, CO) to pass through a 1-mm screen. Total N was measured by combustion of the sample following the AOAC

method 990.03 (AOAC, 2010) using a Leco CNS2000 Analyzer (Leco Corporation, St. Joseph, MI), and crude protein (CP) was calculated by multiplying the resulting total N concentration by 6.25. Soluble protein (SP) was determined by the method of Krishnamoorthy et al. (1982) and expressed as % of CP. Acid detergent fiber (ADF) was quantified on dried ground samples using the AOAC method 973.18 (AOAC, 2010), using Whatman 934-AH glass micro-fiber filters with 1.5-µm particle retention instead of fritted glass crucibles. Neutral detergent fiber (NDF) was analyzed on dried samples according to Van Soest et al. (1991) with sodium sulfite and amylase, not corrected for ash. Ash was analyzed by the AOAC method 942.05 (AOAC, 2000), with the modification of using 1.5 g sample, 4 h ash time, and hot weighing. Another portion of the dry sample was ground to pass a 3-mm screen and analyzed for starch (Hall, 2008).

Microbial Analysis

Representative 25-g portions of fresh and ensiled HMC were mixed with 225 ml of sterile quarter strength Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) and homogenized for 1 min in a Proctor-Silex 57171 blender (Hamilton Beach/Proctor-Silex Inc., Washington, NC). The homogenate was filtered through four layers of cheesecloth. The filtrate was serially diluted in Ringers solution, and the numbers of LAB were determined by pour-plating in de Man, Rogosa, and Sharpe agar (CM3651, Oxoid, Unipath Ltd., Basingstoke, UK). Plates were incubated anaerobically at 32°C for 48 to 72 h in a plastic container with anaerobic packs (Mitsubishi Gas Chemical Co., Tokyo, Japan) and an anaerobic indicator (BR0055,

Oxoid, Unipath Ltd., Basingstoke, UK). The container was vacuum-sealed in nylon-polyethylene bags (3.5-mil embossed pouches, 15.2 × 30.5 cm; Doug Care Equipment Inc., Springville, CA) using a Best Vac vacuum machine (distributed by Doug Care Equipment Inc.). Numbers of yeasts and molds were determined on malt extract agar (CM0059, Oxoid, Unipath Ltd., Basingstoke, UK) acidified with lactic acid (85%) at a rate of 0.5% vol/vol. These plates were incubated aerobically at 32°C for 48 h before enumeration. Plates with a number of colonies between 30 and 300 were used to obtain counts of colony-forming units.

Bacterial Community Analysis

Four replicates of 25 g of fresh HMC, and CTRL and SF HMC after ensiling were homogenized with 225 ml of autoclaved Ringers solution for 2 min using a Colworth 400 stomacher (Seward, London, UK). The homogenate was filtered through four layers of cheesecloth. Next, 2 ml of the filtrates were centrifuged for 3 min at 21130.2 xg using a Centrifuge 5424 R (Eppendorf AG, Hamburg, Germany). The supernatant was discarded, and 100 µl of autoclaved Ringers solution (Oxoid BR0052G, Oxoid, Unipath Ltd., Basingstoke, UK) was used to resuspend the pellet. Samples were kept at -80°C for further analysis.

Extraction of DNA, 16S rRNA amplification, and Illumina MiSeq-based sequencing were performed by the Research and Testing Laboratory (Lubbock, TX, USA). DNA was extracted using the MoBio PowerMag Soil kit, according to

manufacturer's instructions. The primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGTCAATTCMTTTRAGTTT) were used to amplify the V4 and V5 hypervariable regions of the 16S rRNA (Baker et al., 2003). Sequencing was done with an Illumina MiSeq (San Diego, CA, USA) platform with the 2 × 250 bp paired-end method.

For sequencing processing, paired-end reads were merged and converted into FASTA files using FLASH version 1.2.11 (Magoc and Salzberg, 2011). The quality profile of the reads was checked on http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Andrews, 2010). Next, reads were filtered for quality and the primers were trimmed using QIIME 1.9.1 (Caporaso, 2010). Operational taxonomic units (OTUs) picking was done using open reference clustering against Greengenes (version 13_8) at 97% sequence similarity. Finally, singles and double reads were filtered. Diversity analyses were performed using the phyloseq and vegan packages in R (McMurdie and Holmes, 2013; Oksanen et al. 2013). Membership Venn diagram was computed using the MetaComet web platform to determine OTU overlap among treatments (Wang et al., 2016).

Statistical Analysis

Data were analyzed using JMP version 12 (SAS Institute, Cary, NC). Data of fresh HMC was analyzed separately as a completely randomized design. Data from the silo opening was analyzed as a 2 × 2 factorial arrangement of treatments in a completely

randomized design. The model included the fixed effects of the treatment, air stress, and their interaction. For aerobic stability measurements, data were analyzed as a 2 × 2 × 2 factorial arrangement of treatments in a completely randomized design. The model included the fixed effects of the treatment, air stress, temperature, and their interactions. Differences were considered significant when $P < 0.05$, and pairwise mean comparisons were performed using Tukey's test at $P < 0.05$ (Snedecor and Cochran, 1980). Statistical analysis for the PCoA plots was done using PERMANOVA (permutational multivariate analysis of variance) by the adonis function in vegan (Oksanen et al. 2013).

Results

Chemical Properties, Microbial Populations, and Aerobic Stability of Fresh and Ensiled HMC

Tables 5.1 and 5.2 show the chemical and microbial composition of fresh HMC harvested at about 68% DM and HMC after 49 d of ensiling, respectively. There was an air stress effect for CP, SP, ADF, and NDF. HMC submitted to air stress had higher concentrations of CP and NDF and lower concentrations of SP and ADF compared to non-stressed silages. Treatment with additive reduced the concentration of SP, NH_3-N, ADF, and starch compared to CTRL. In non-stressed silos, there was no difference in concentration of WSC between treatments. However, in air-stressed silos, CTRL had lower WSC content than SF.

After 49 d of ensiling (Table 5.3), there were no differences in pH, concentrations of lactic and acetic acid, numbers of LAB and molds among treatments. There was an interaction between additive and air stress for numbers of yeasts. CTRL subjected to air stress had higher numbers of yeasts than non-air-stressed, whereas SF had equally lower numbers of yeasts independent of the air stress status. SF had a lower concentration of ethanol and higher DM recovery compared to CTRL.

Figure 5.1 shows the aerobic stability of HMC after 49 d of ensiling at 22°C and 34°C. There was an interaction between treatment, air stress, and temperature. All SF, regardless of air stress during storage or temperature during spoilage, were stable for at least 250 h (time measurements ended). At 22°C, CTRL non-stressed spoiled after 56 h and CTRL air-stressed spoiled after 11 h. At 34°C, CTRL non-stressed spoiled after 26 h and CTRL air-stressed spoiled immediately after exposure to air.

The Composition of the Bacterial Community

Sequencing depth was sufficient to describe the alpha diversity of the bacterial population as the rarefaction curves reached a plateau for all but two samples, which were discarded from this analysis (Figure B.10). Table 5.4 shows the alpha diversity indexes of fresh and ensiled HMC. There were no differences in Shannon or Simpson indexes. Fresh HMC had lower numbers of observed OTU and lower Chao1 index than HMC after ensiling. There were no differences due to additive treatment.

Figure 5.2 shows a membership Venn diagram based on the presence or absence of OTUs. From a total of 307 OTUs, 2% were unique to one treatment, and 31% were shared among fresh and ensiled HMC. Ensiled HMC of all treatments shared 46% of OTUs. CTRL air-stressed and non-stressed shared exclusively seven OTUs, while SF air-stressed and non-stressed did not share any exclusive OTU. CTRL non-stressed and SF non-stressed, and CTRL air-stressed and SF air-stressed shared one OTU. CTRL non-stressed and SF air-stressed and CTRL air-stressed and SF non-stressed did not share any OTU.

The PCoA plot (Figure 5.3) shows that fresh HMC samples clustered separated from the ensiled samples. Ensiled HMC formed a unique cluster independent of treatment or air-stress status. A significant Adonis test proved that the treatments did not have the same centroid, and a non-significant betadisper test showed that differences in group dispersions did not cause the results.

Figure 5.4 shows the relative abundance of bacterial orders in HMC. Fresh HMC had higher abundance of Enterobacteriales. After ensiling, the relative abundance of Enterobacteriales decreased, and it was higher in CTRL than in SF (13 vs. 5%). The opposite happened with the relative abundance of Lactobacillales, which increased with ensiling and was lower in CTRL than in SF (87 vs. 94%).

Discussion

The chemical and microbiological properties of fresh HMC found in the present study were in the expected range for this feedstuff (Taylor and Kung, 2002; Kung et al., 2007). During ensiling, the higher levels of CP and NDF and the lower levels of SP and ADF in air-stressed silos than in non-stressed silos showed that air stress diminished proteolytic and fibrolytic processes. Da Silva et al. (2015) found that treatment with the same additive used in the present study reduced the concentrations of SP and NH_3-N in HMC, but they did not find any changes in ADF and starch. In summary, both air stress and treatment with the additive diminished substrate degradation. Enterobacteria can be responsible for proteolysis and ammonia production during ensiling (Heron et al., 1993). The reduction in relative abundance of Enterobacteriales by the additive supported the theory that enterobacteria were responsible for the lower concentrations of SP and NH_3-N found in SF.

Abound 30% of the OTUs present in the fresh HMC were also found after ensiling, still, the composition of the bacterial community was more affected by ensiling than by additive or air-stress. During ensiling, the anaerobic conditions select anaerobic bacteria relative to aerobic bacteria that naturally occur on the plant. One of the main microorganisms that naturally occur in the corn plant is enterobacteria. A reduction in pH by LAB during fermentation inhibits growth of enterobacteria. The presence of enterobacteria in HMC is undesirable because it competes with LAB for nutrients, causes proteolysis, and can increase the degree of spoilage after exposure to air (Driehuis and Elferink, 2000). In the present study, the additive reduced the relative abundance of

Enterobacteriales in favor of Lactobacillales. It was previously reported that the combination of sodium benzoate, potassium sorbate, and sodium nitrite reduced the numbers of enterobacteria in alfalfa silage (Da Silva et al., 2016). Stanojevic et al. (2009) showed that sodium benzoate, potassium sorbate, and sodium nitrite inhibited *Escherichia coli*. The author also showed that sodium benzoate and potassium sorbate had the same and sodium nitrate had the lowest minimal inhibitory concentration against *E. coli* and that there was a synergistic action between sodium nitrate and sodium benzoate and between sodium nitrite and potassium sorbate against this microorganism.

LAB consume WSC during the fermentation process, and the residual WSC that is not used by LAB can be consumed by yeasts and molds (Wilkinson and Davies, 2013). Therefore, the higher the numbers of yeasts, the lower is the expected residual concentration of WSC. Additive and air stress did not affect the numbers of LAB or the concentrations of lactic, acetic acids; because of this, variances in the concentration of WSC were most likely caused by differences in numbers of yeasts. In non-stressed silo, there were no differences in concentration of WSC between CTRL and SF, but in air-stressed silo, CTRL had a lower concentration of WSC than SF. In non-stressed silos, the difference in numbers of yeasts between untreated and treated silage was only 1.50 log cfu/g of fresh weight, and this might not have been enough of a difference to influence the concentration of WSC. In air-stressed silo, untreated silage had 3.96 log cfu/g of fresh weight more yeasts than treated silage, which resulted in higher consumption of WSC and the observed lower concentration of residual WSC. Da Silva et al. (2015) observed

an increase in the concentration of residual WSC due to treatment with Safesil and explained that it was caused by a reduction in numbers of yeasts.

In anaerobic conditions, yeasts can ferment sugars and produce ethanol and CO_2 as the main products (Pathlow et a., 2003). With the dissipation of ethanol and CO_2 to the atmosphere, there is a loss of DM. The combination of sodium benzoate, potassium sorbate, and sodium nitrite was demonstrated previously to be effective against the growth of yeasts in forages (Knicky and Spörndly, 2011). In the present study, the decreased production of ethanol and increased DM recovery in both non-stressed and air-stressed silos treated with the additive followed a reduction in numbers of yeasts. Da Silva et al. (2015) also observed a decreased production of ethanol and increased DM recovery in HMC due to treatment with the same additive in air-tight silos.

Sporndly and Persson (2015) demonstrated that air stressing silos regularly during storage is an effective way to study aerobic stability since air leakage stimulates yeasts growth and decreases the aerobic stability of silage. In the present study, air-stress stimulated yeast growth and reduced the aerobic stability of untreated HMC. The additive, however, overcame the effects of air stress by inhibiting the excessive growth of yeasts due to air stimulation. The additive reduced the numbers of yeasts equally in non-stressed and air-stressed silos. Consequently, SF independent of air-stress status had markedly higher aerobic stability than CTRL. Improvements in aerobic stability by this additive under air stress conditions were previously observed in corn silage (Knicky and Spörndly, 2015; Savage et al., 2016). However, the present study was the first study to

show that this additive can improve the aerobic stability of HMC under air stress conditions.

Not only air penetration in the silo but also high temperatures at feed-out can stimulate the growth of yeasts and reduce aerobic stability. Ashbell et al. (2002) showed that corn silage exposed to air at 30°C had higher numbers of yeasts, CO_2 production, and pH, and lower aerobic stability than silage exposed to air at 10, 20, and 40°C. In the present study, untreated HMC had lower aerobic stability when exposed to 34°C than to 22°C after silo opening. The decline in aerobic stability was even more dramatic in untreated silos when a combination of high temperature after silo opening and air stress during storage occurred. On the other hand, the additive efficiently overcame the effects of air stress, high temperature, and their combination.

Conclusions

Treatment with a combination of sodium benzoate, potassium sorbate, and sodium nitrite reduced the relative abundance of Enterobacteriales and the numbers of yeasts in HMC, improving fermentation and aerobic stability. The additive was able to overcome the detrimental effects of air stress during storage and of warm temperatures at feed out on aerobic stability.

TABLES

Table 5.1. The DM content, chemical composition (% of DM, unless stated otherwise), pH, buffering capacity, and numbers of LAB, yeasts, and molds (fresh weight basis) of fresh high moisture corn before ensiling.

Item	Average	Standard deviation
DM, %	67.99	0.14
CP	7.44	0.11
SP[1], % of CP	15.05	0.88
NH_3-N	0.01	0.00
ADF	3.86	0.40
NDF	9.80	0.74
Starch	75.22	0.99
Ash	1.08	0.09
WSC[2]	1.16	0.13
pH	6.19	0.03
BC[3], g lactic acid /kg	5.01	0.49
LAB[4]	6.02	0.08
Yeasts	4.71	0.20
Molds	4.27	0.20

[1]Soluble protein.
[2]Water soluble carbohydrates.
[3]Buffering capacity.
[4]Lactic acid bacteria.

Table 5.2. The DM content (%) and chemical composition (% of DM, unless stated otherwise) of high moisture corn treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] subjected or not to air stress, ensiled for 49 d.

Item[2]	DM, %	CP	SP[3], % of CP	NH_3-N	ADF	NDF	Starch	Ash	WSC[4]
CTRL									
NS	66.87	7.53	41.17	0.02	3.23	7.35	77.98	1.22	0.68[a]
AS	67.41	7.62	36.22	0.03	2.94	9.04	77.30	0.98	0.27[b]
SF									
NS	68.01	7.54	37.14	0.02	2.96	8.04	76.74	1.08	0.53[a]
AS	68.08	7.70	34.29	0.02	2.20	8.54	75.92	1.12	0.75[a]
Additive means									
CTRL	67.14	7.58	38.65[a]	0.03[a]	3.08[a]	8.20	77.64[a]	1.10	0.48
SF	68.05	7.62	35.70[b]	0.02[b]	2.58[b]	8.29	76.33[b]	1.10	0.64
Air-stress means									
NS	67.44	7.53[b]	39.18[a]	0.02	3.09[a]	7.70[b]	77.36	1.15	0.61
AS	67.75	7.66[a]	35.25[b]	0.03	2.57[b]	8.79[a]	76.61	1.05	0.51
SEM	0.49	0.50	0.60	0.00	0.19	0.44	0.55	0.08	0.06
Effects and interactions[5]	---------- *P* – value ----------								
A	0.08	0.32	<0.01	0.04	0.01	0.82	0.02	0.99	0.01
S	0.54	0.01	<0.01	0.42	0.01	0.02	0.16	0.20	0.11
A×S	0.64	0.49	0.10	0.11	0.20	0.16	0.89	0.08	<0.01

[a-b]Means in columns with unlike superscripts differ ($P < 0.05$).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF = 3.0 L of Safesil/t of fresh weight; NS= no air stress; AS= 12 h/week.
[3]Soluble protein.
[4]Water soluble carbohydrates.
[5]A = effect of additive; S = effect of air stress; A × S = interaction of additive by air stress.

Table 5.3. The DM recovery (%), pH, chemical composition (% of DM), and numbers of LAB, yeasts, and molds (log_{10} cfu/g of wet weight) of high moisture corn treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1] subjected or not to air stress, ensiled for 49 d.

Item[2]	DMR[3]	pH	Lactic acid	Acetic acid	ETOH[4]	LAB[5]	Yeasts	Mold
CTRL								
NS	97.93	4.14	1.06	0.43	0.58	7.06	4.44[b]	2.86
AS	98.58	4.16	0.93	0.49	0.59	7.61	7.01[a]	2.70
SF								
NS	99.74	4.15	0.91	0.44	0.17	7.07	2.94[c]	2.91
AS	99.75	4.18	0.80	0.51	0.13	7.22	3.05[c]	1.79
Additive means								
CTRL	98.26[b]	4.15	1.00	0.46	0.59[a]	7.34	5.73	2.78
SF	99.75[a]	4.17	0.86	0.48	0.15[b]	7.15	3.00	2.35
Air-stress means								
NS	98.84	4.15	0.99	0.44	0.38	7.07	3.69	2.89
AS	99.17	4.17	0.87	0.50	0.36	7.42	5.03	2.25
SEM	0.69	0.30	0.08	0.10	0.03	0.17	0.19	0.72
Effects and interactions[6]	----------	----------	----------	----------	*P* – value	----------	----------	----------
A	0.04	0.52	0.11	0.86	<0.01	0.31	<0.01	0.56
S	0.64	0.37	0.17	0.51	0.56	0.06	<0.01	0.38
A×S	0.65	0.95	0.94	1.00	0.37	0.26	<0.01	0.51

[a-c] Means in columns with unlike superscripts differ ($P < 0.05$).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF = 3.0 L of Safesil/t of fresh weight; NS= no air stress; AS= 12 h air stress/week.
[3]DM recovery.
[4]Ethanol.
[5]Lactic acid bacteria.
[6]A = effect of additive; S = effect of air stress; A × S = interaction of additive by air stress.

Table 5.4. Alpha diversity indexes of fresh and ensiled high moisture corn untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite[1], submitted or not to air stress, and ensiled for 49 d[2].

Item	CTRL/0d	CTRL-NS/49d	CTRL-AS/49d	SF-NS/49d	SF-AS/49d	SEM	*P*-value
Observed	99.75[b]	231.25[a]	217.33[a]	210.67[a]	208.25[a]	14.14	<0.01
Chao1	116.50[b]	254.43[a]	261.74[a]	231.76[a]	245.42[a]	14.91	<0.01
Shannon	2.95	2.93	2.70	2.86	2.37	0.23	0.26
Simpson	0.86	0.87	0.85	0.87	0.72	0.05	0.10

[a-b] Means in rows with unlike superscripts differ ($P < 0.05$).
[1]Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden).
[2]CTRL = untreated; SF = 3.0 L of Safesil/t of fresh weight; NS= no air stress; AS= 12 h/week.

FIGURES

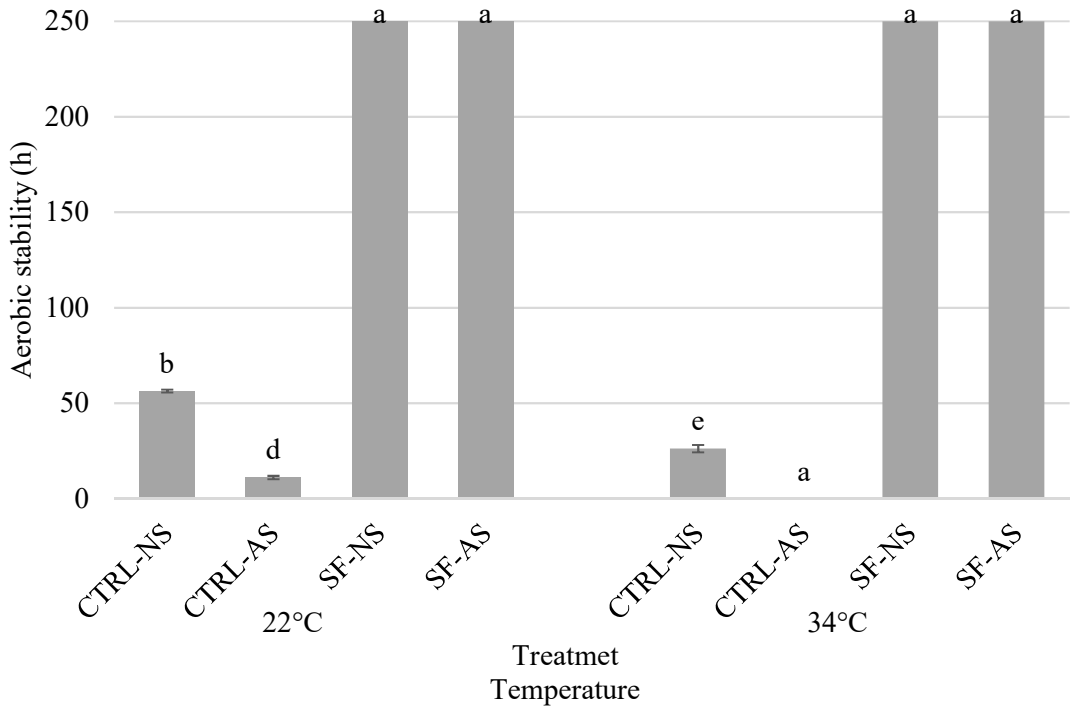

Figure 5.1. Aerobic stability of high moisture corn untreated non-air-stressed (CTRL-NS) or air-stressed (CTRL- AS) and treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite (Salinity, Göteborg, Sweden) at 3L/t of fresh weight (SF) non-air-stressed (SF-NS) or air-stressed (SF-AS) ensiled for 49 d. Air stress was applied for 12 h/week for 7 weeks (SEM= 26.21, $P < 0.01$). $^{a\text{-}e}$ Means bars with unlike letters differ ($P < 0.05$).

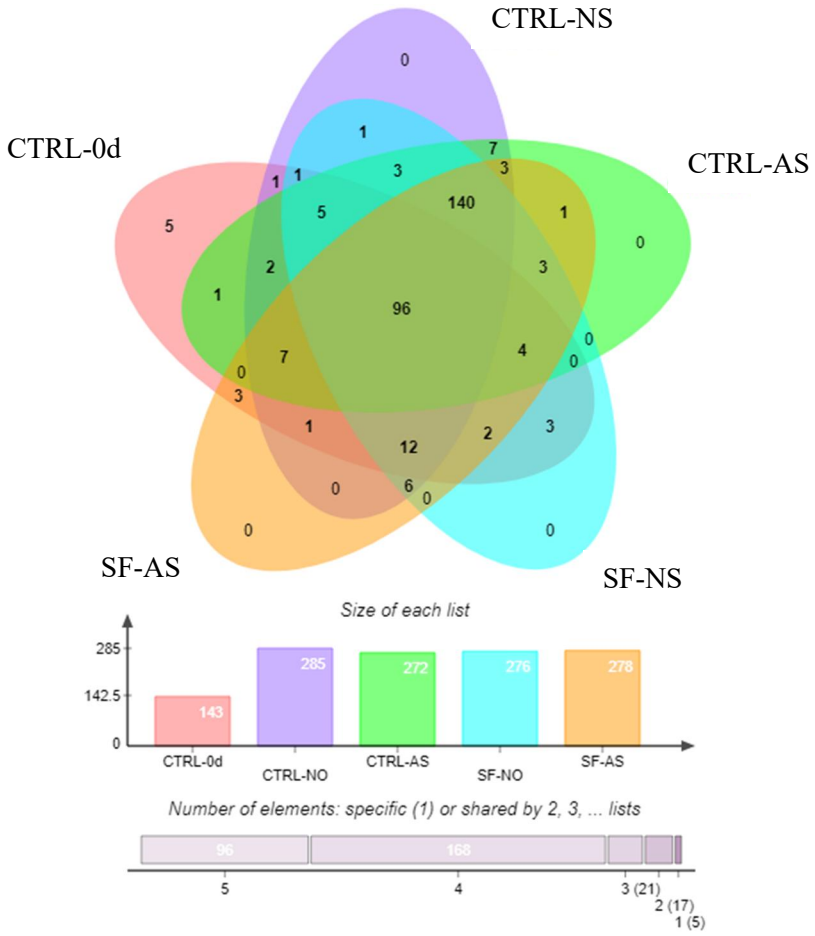

Figure 5.2. Venn diagram built based on the presence and absence of bacteria OTU, from sequencing of the V4-V5 region of the 16S rRNA using Illumina Miseq, in fresh high moisture corn (CTRL-0d) and high moisture corn untreated (CTRL) or treated with Safesil 3 L (active ingredients of sodium nitrite, potassium sorbate, and sodium benzoate; Salinity, Göteborg, Sweden)/t of fresh weight ensiled for 49 d (SF), not subjected (NS) or subjected (AS) to a 12 h/week air stress. The numbers on the diagrams show the numbers of unique or shared OTU among treatments.

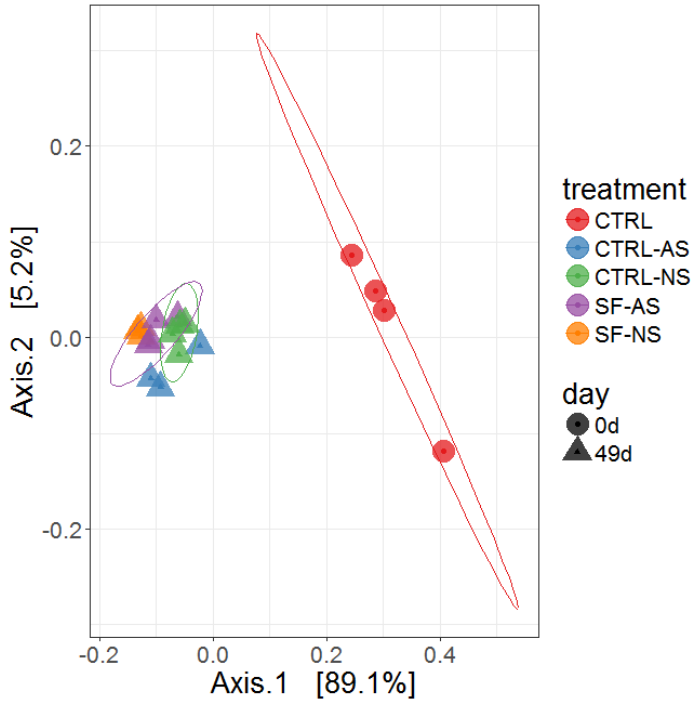

Figure 5.3. Principal coordinate analysis (PCoA) plot with weighted unifrac dissimilarity of the composition of the bacterial community, as analyzed by the sequencing of the V4-V5 region of the 16S rRNA using Illumina Miseq, of fresh high moisture corn (0 d) and high moisture corn untreated (CTRL) or treated with Safesil 3 L (active ingredients of sodium nitrite, potassium sorbate, and sodium benzoate; Salinity, Göteborg, Sweden)/t of fresh weight (SF) ensiled for 49 d, not subjected (NS) or subjected (AS) to a 12 h/week air stress.

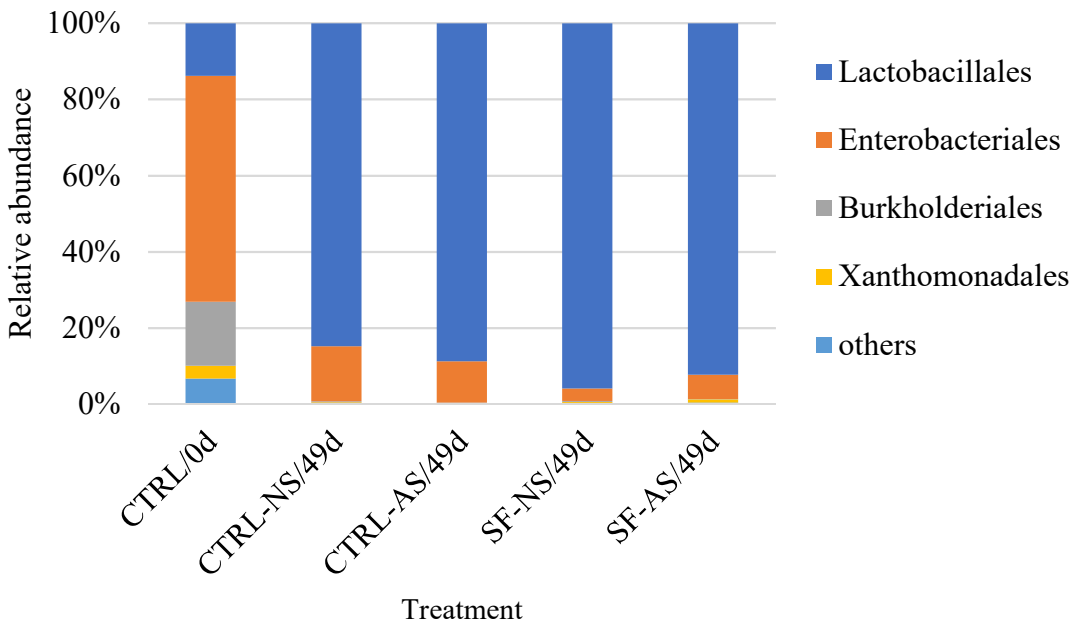

Figure 5.4. Relative abundance of bacteria orders as analyzed by the sequencing of the V4-V5 region of the 16S rRNA using Illumina MiSeq platform of fresh high moisture corn and high moisture corn untreated (CTRL) or treated with Safesil 3 L (active ingredients of sodium nitrite, potassium sorbate, and sodium benzoate; Salinity, Göteborg, Sweden)/t of fresh weight (SF) ensiled for 49 d, not subjected (NS) or subjected (AS) to a 12 h/week air stress.

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SUMMARY

Several insights can be taken regarding the efficacy of an additive containing sodium benzoate, potassium sorbate, and sodium nitrite when analyzing the Experiments 1, 2, and 3 performed in corn silage, treated with sodium nitrite, potassium sorbate, and sodium benzoate at 2 L/t of fresh weight, and Experiment 4 in high moisture corn (HMC), treated at 3 L/t of fresh weight,

The additive reduced the concentration of NH_3-N by about 30 to 50% in all the Experiments. The additive did not affect the numbers of lactic acid bacteria in corn silage ensiled for 46 and 63, or HMC ensiled for 49 d. However, it reduced the numbers of lactic acid bacteria after 85 d.

In all Experiments, silage treated with the additive had increased concentration of residual water-soluble carbohydrates, which was coupled with a reduction in the concentration of ethanol. The concentration of ethanol decreased 17, 47, 70, 61, and 75 % in corn silage ensiled for 46 d, 85 d, and 63 d in non-stressed silos and in air-stressed silos, and in HMC ensiled for 49 d, respectively. Improvements in DM recovery (6 and 2%) were only observed when the ethanol production declined more than 60%.

The additive at least doubled the numbers of hours that silage or HMC were stable after exposure to air in all experiments. Even in challenging conditions such as short-ensiling time, air stress during storage, and high temperatures at silo opening the additive markedly increased aerobic stability.

The additive reduced the numbers of yeasts after 46, 49, and 85 d of ensiling, in Experiments 2, 4, and 1, respectively. The additive did not reduce the numbers of yeasts in Experiment 2 in corn silage ensiled for 1, 2, and 4 d or in Experiment 3 after 63 d. There was a significant negative correlation between numbers of yeasts and aerobic stability in Experiments 1, 2, and 4 and a tendency to a correlation in Experiment 3, when considering non-stressed silos untreated or treated with the additive. Correlations of -0.99, -0.33, and -0.83 were found for corn silage ensiled for 46, 63, and 85 d, respectively, and a correlation of -0.86 for HMC ensiled for 49 d. However, no correlation was observed for corn silage ensiled for short-periods (1, 2, and 4 d), possibly because the additive had fungistatic and not fungicidal activity. In Experiment 3, the additive caused a shift in yeasts populations, decreasing the relative abundance of lactate-assimilating yeasts species, which could explain the improvements in aerobic stability even when numbers of yeasts were not reduced.

CONCLUSIONS

Treatment with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite improved the fermentation of corn silage and high moisture corn by reducing the concentrations of NH_3-N and ethanol compared to untreated feed. Additionally, the additive markedly improved the aerobic stability of total mixed rations made with corn silage treated before or after ensiling, of corn silage ensiled for short periods of time, of corn silage subjected to air-stress during storage, and of high moisture corn challenged with air-stress during storage and with high temperatures at feed-out. High-throughput sequencing facilitated the understanding of how the additive improved fermentation and aerobic stability, based on its effects on the composition of the microbial community of silage.

Appendices

Appendix A

MICRONUTRIENTS PREMIX COMPOSITION

Table A.1 Composition of the micronutrients premix from the grain mix added to the total mixed ration.

Item	Concentration
Vitamin A, IU/kg	888889.3
Vitamin D, IU/kg	22224.2
Vitamin E, IU/kg	5333.4
Calcium, %	14.84
Phosphorus, %	0.01
Potassium, %	0.02
Sulfur, %	0.81
Magnesium, %	34.01
Zinc, ppm	4213.2
Iron, ppm	105.31
Copper, ppm	817.5
Manganese, ppm	4200.95
Cobalt, ppm	140.7
Iodine, ppm	190.5
Selenium, ppm	65.1
Sodium chloride, %	0.02
Crude fat, %	0.20
Methionine, %	2.50

Appendix B

ALPHA DIVERSITY RAREFACTION CURVES

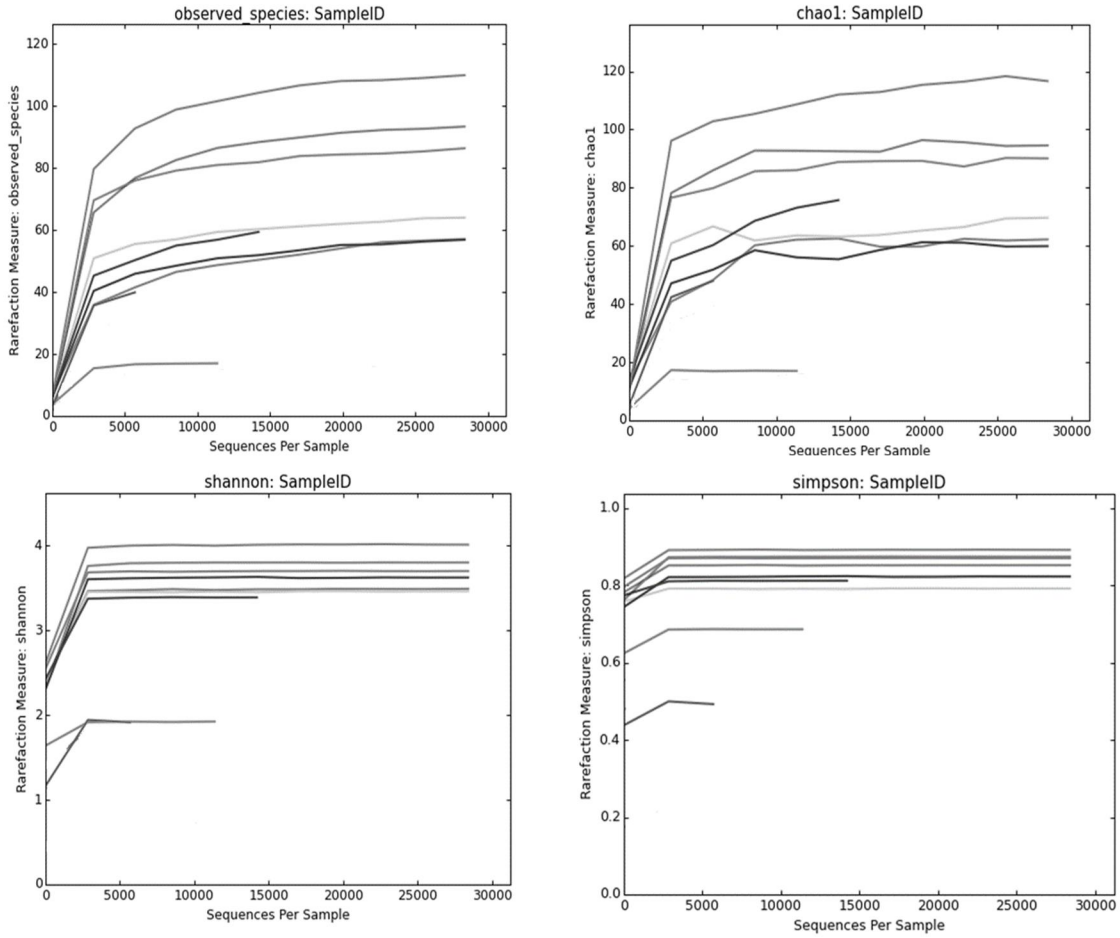

Figure B.1. Alpha diversity rarefaction curves of fungal OTU of fresh forage and silage untreated or treated with 2 L of Safesil (active ingredients of sodium benzoate, potassium sorbate, and sodium nitrite; Salinity, Göteborg, Sweden)/t of fresh matter ensiled for 85 d.

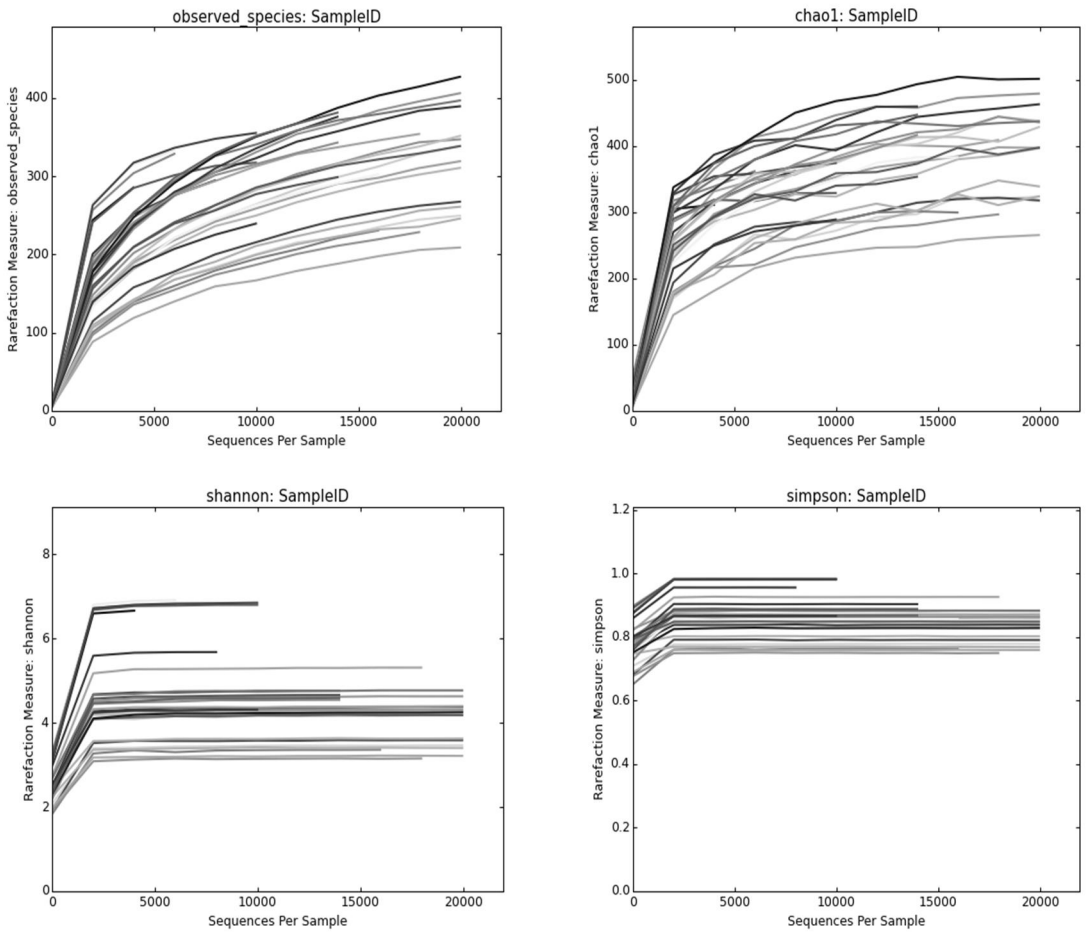

Figure B.2. Alpha diversity rarefaction curves of bacterial OTUs of fresh forage and silage untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2L/t of fresh matter ensiled for 1, 4, and 46 d.

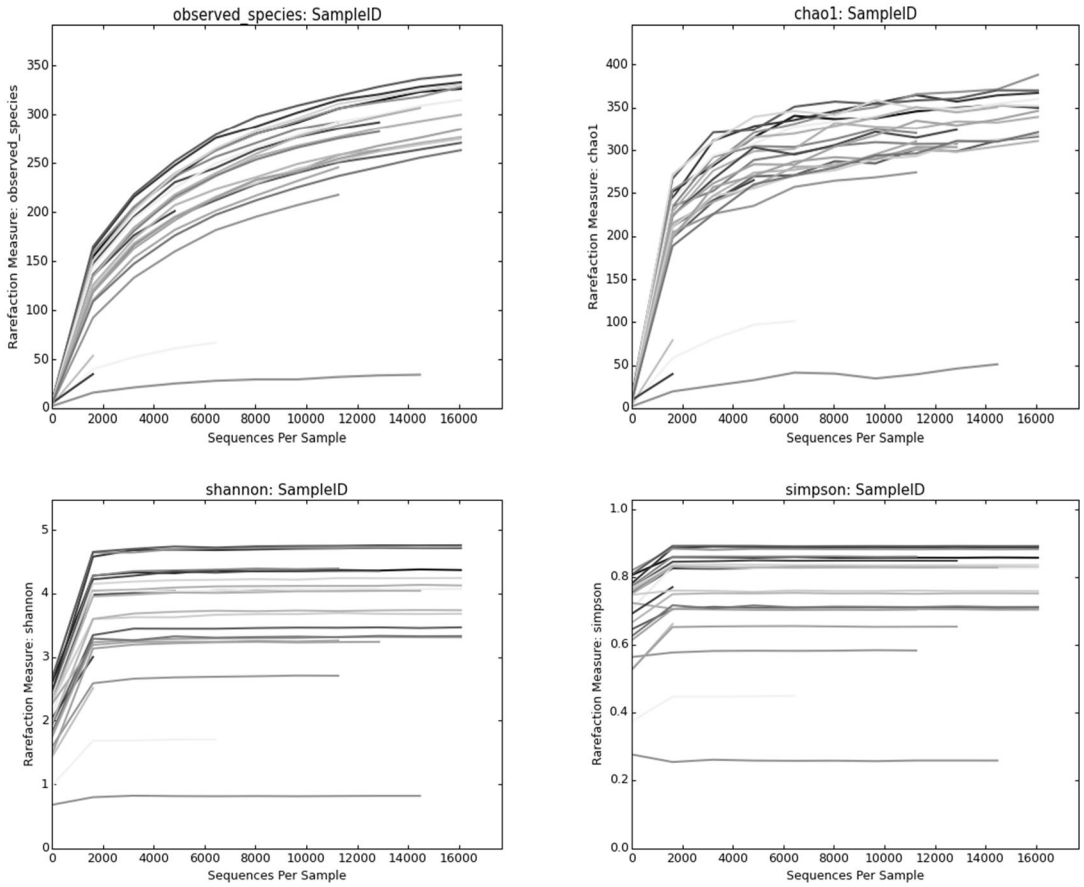

Figure B.3. Alpha diversity rarefaction curves of bacterial OTUs of silage untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2L/t of fresh matter ensiled for 46 d after 50, 100, and 240 h of exposure to air.

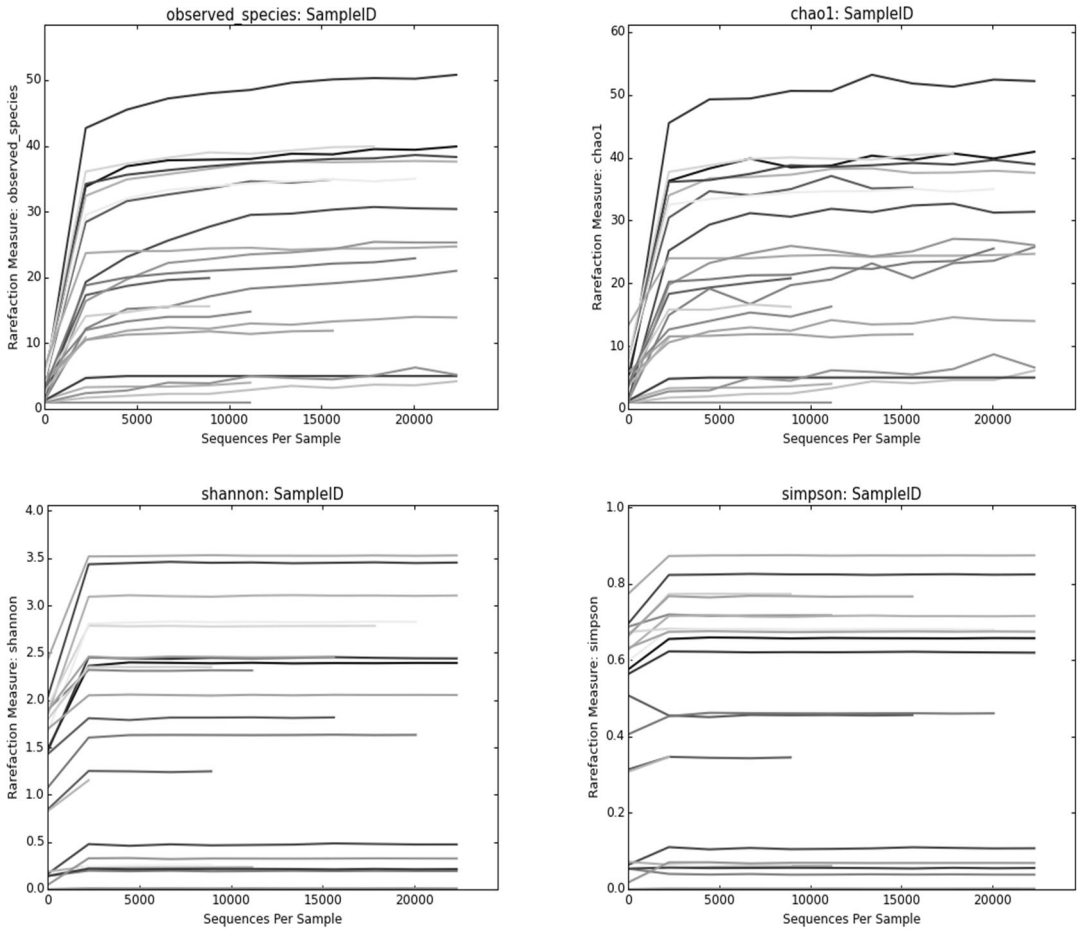

Figure B.4. Alpha diversity rarefaction curves of fungal OTUs of fresh forage and silage untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2L/t of fresh matter ensiled for 1, 4, and 46 d.

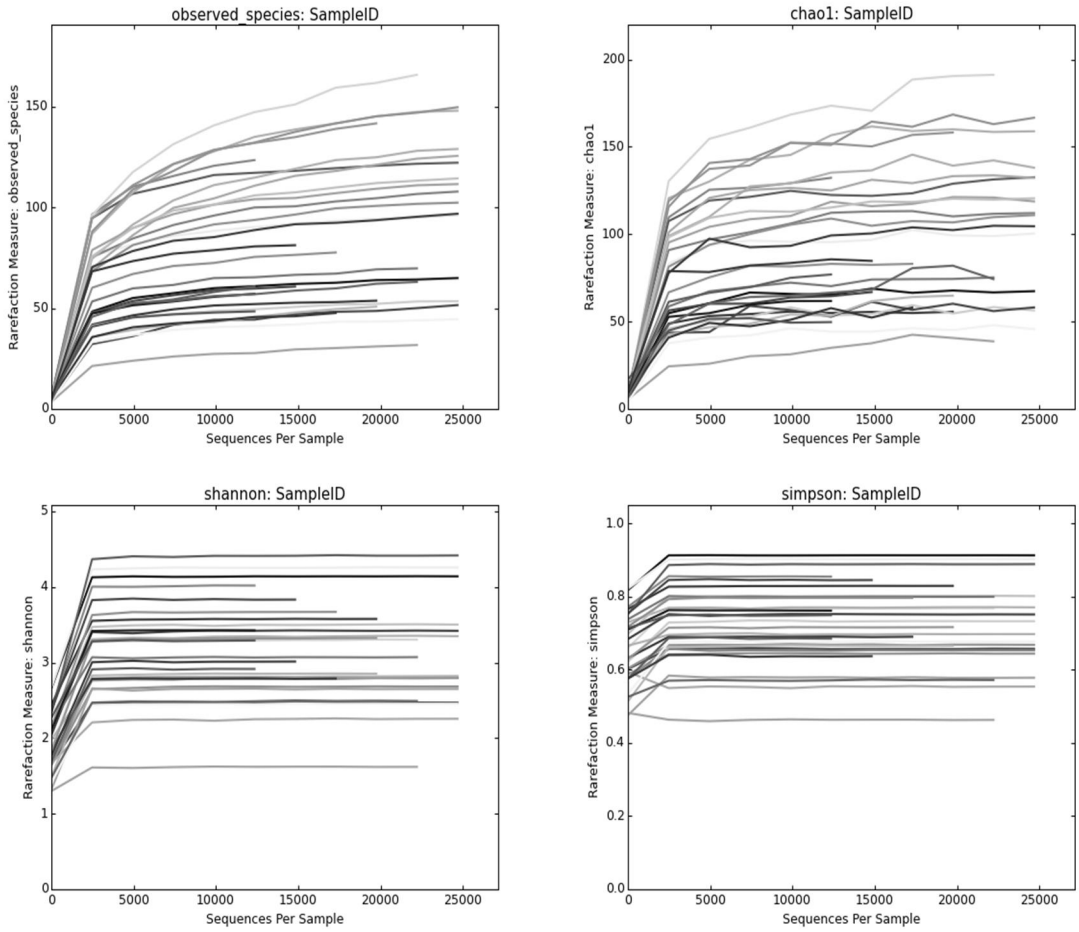

Figure B.5. Alpha diversity rarefaction curves of fungal OTUs of silage untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2L/t of fresh matter ensiled for 46 d after 50, 100, and 240 h of exposure to air.

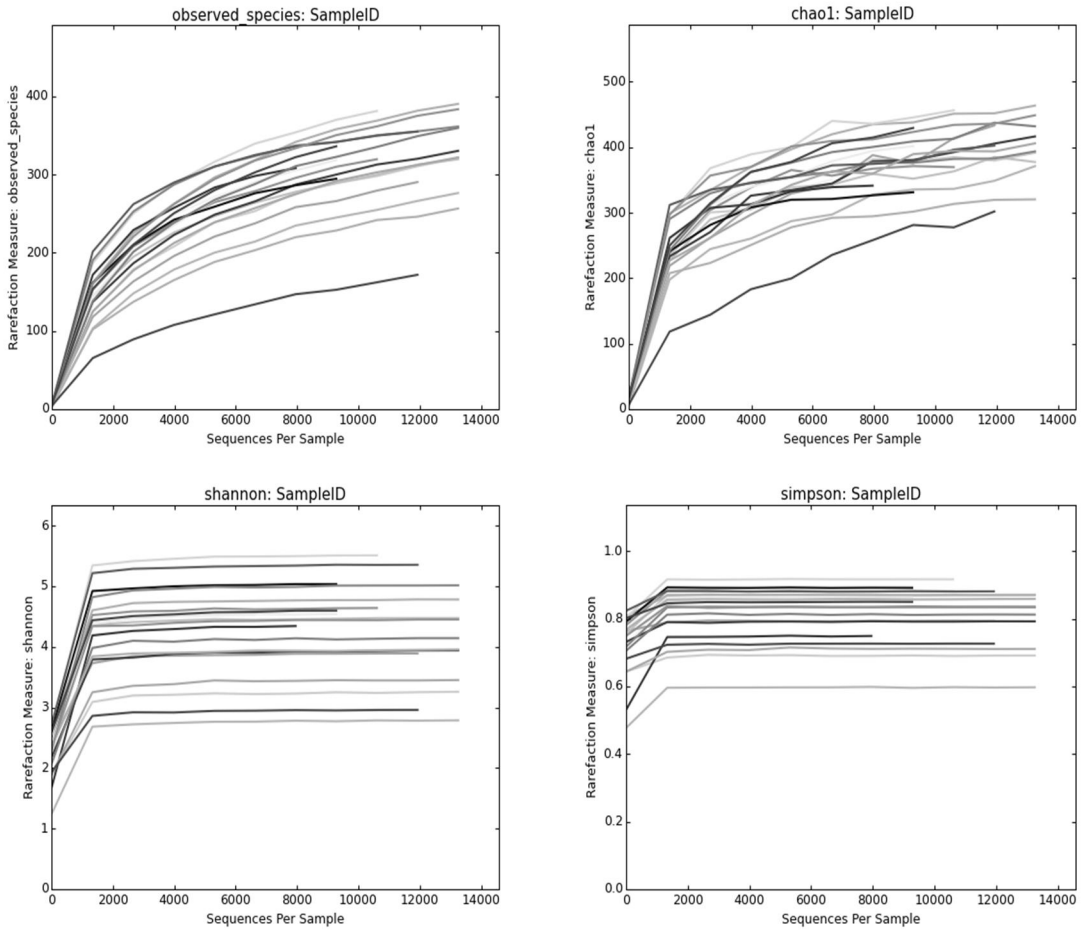

Figure B.6. Alpha diversity rarefaction curves of bacterial OTUs of fresh forage and silage untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2L/t of fresh matter ensiled for 63 d, not subjected (NS) or subjected (AS) to a 2 h/week air stress.

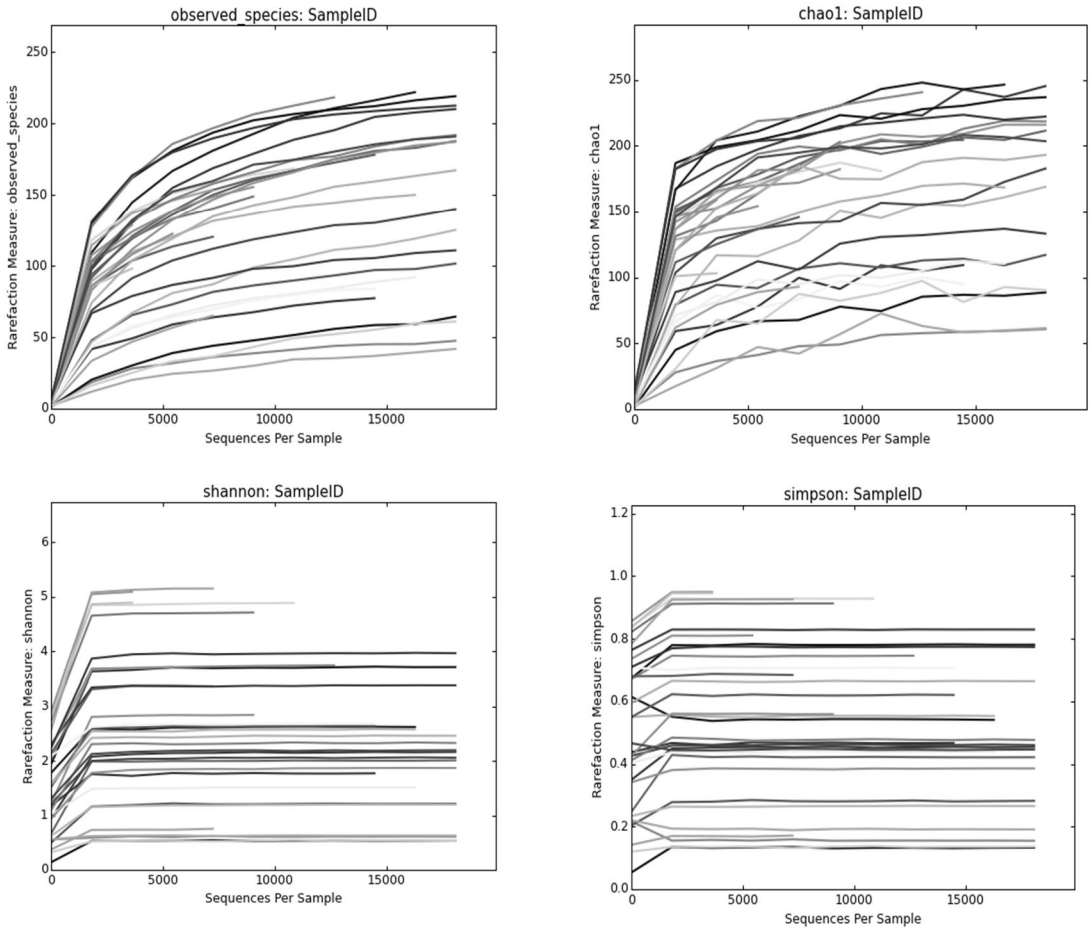

Figure B.7. Alpha diversity rarefaction curves of bacterial OTUs of silage untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2L/t of fresh matter ensiled for 63 d, not subjected (NS) or subjected (AS) to a 2 h/week air stress after 115 and 260 h of exposure to air.

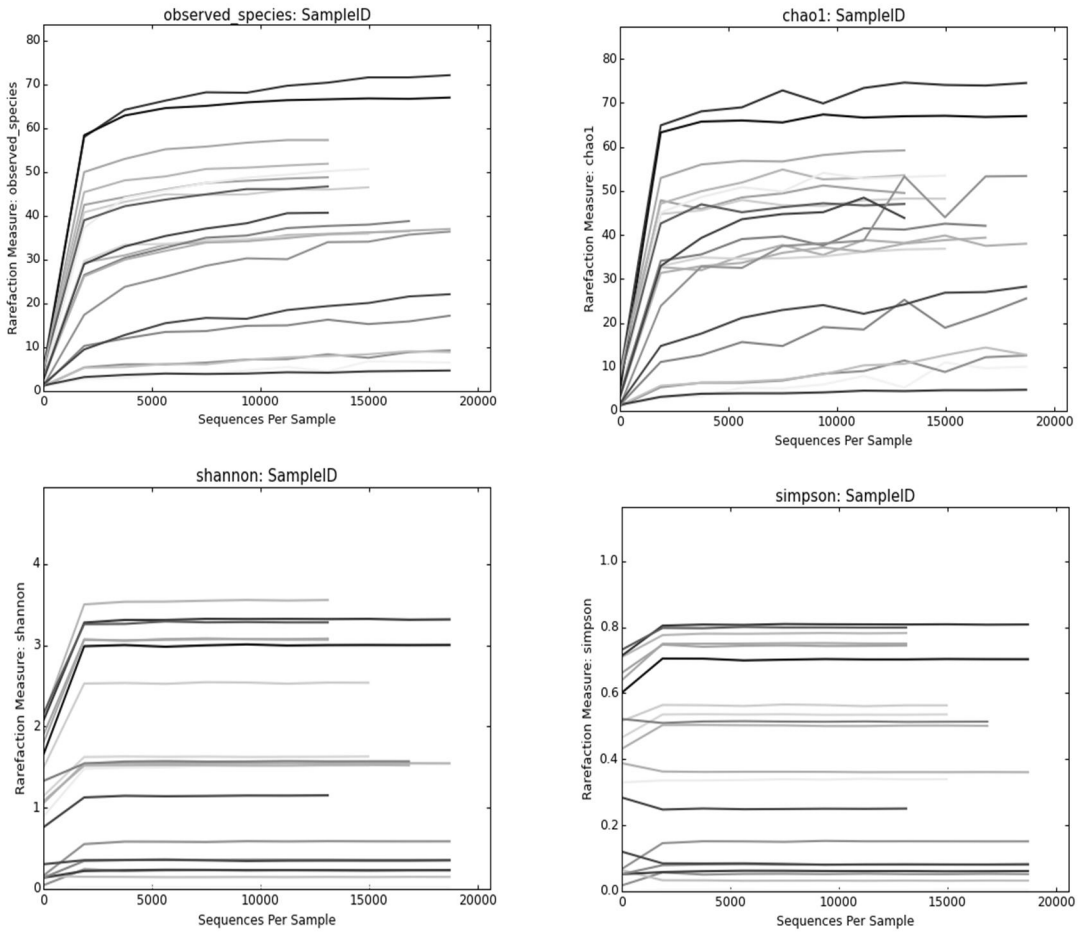

Figure B.8. Alpha diversity rarefaction curves of fungal OTUs of fresh forage and silage untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2L/t of fresh matter ensiled for 63 d, not subjected (NS) or subjected (AS) to a 2 h/week air stress.

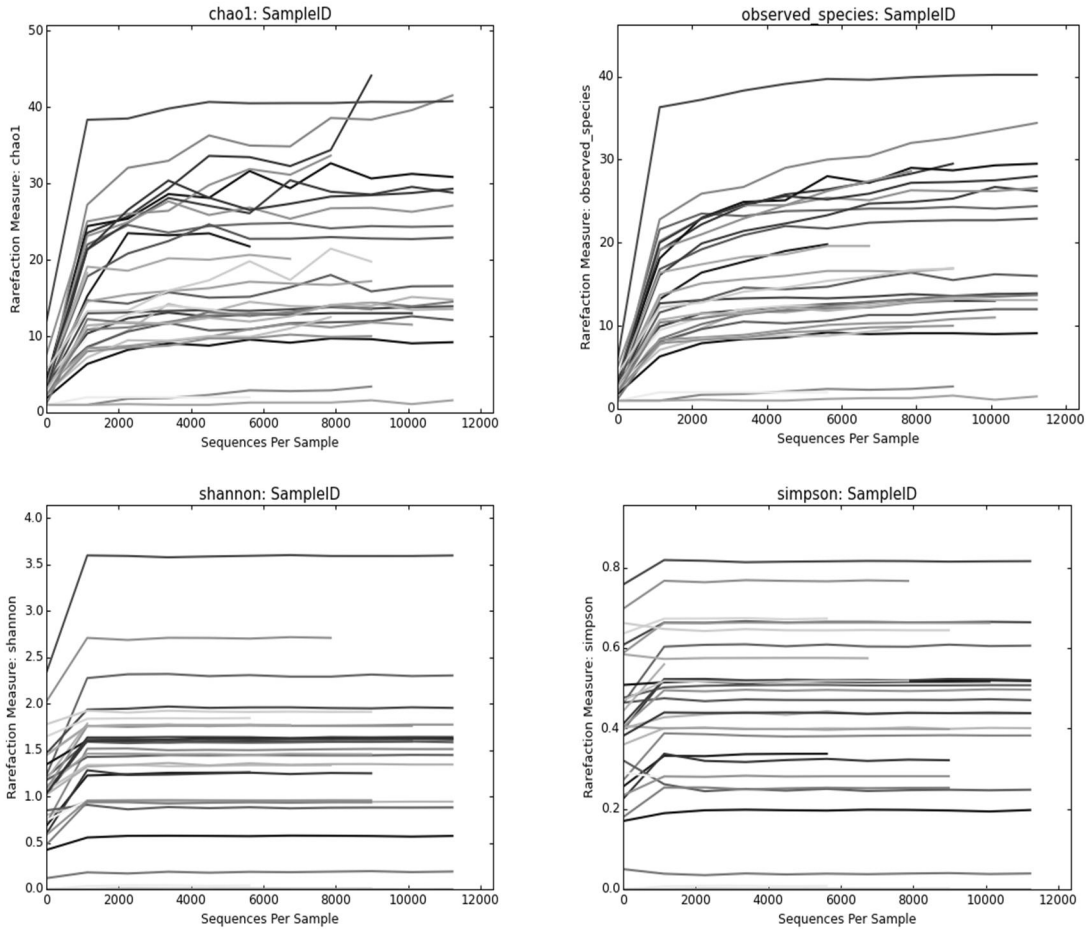

Figure B.9. Alpha diversity rarefaction curves of fungal OTUs of silage untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 2L/t of fresh matter ensiled for 63 d, not subjected (NS) or subjected (AS) to a 2 h/week air stress after 115 and 260 h of exposure to air.

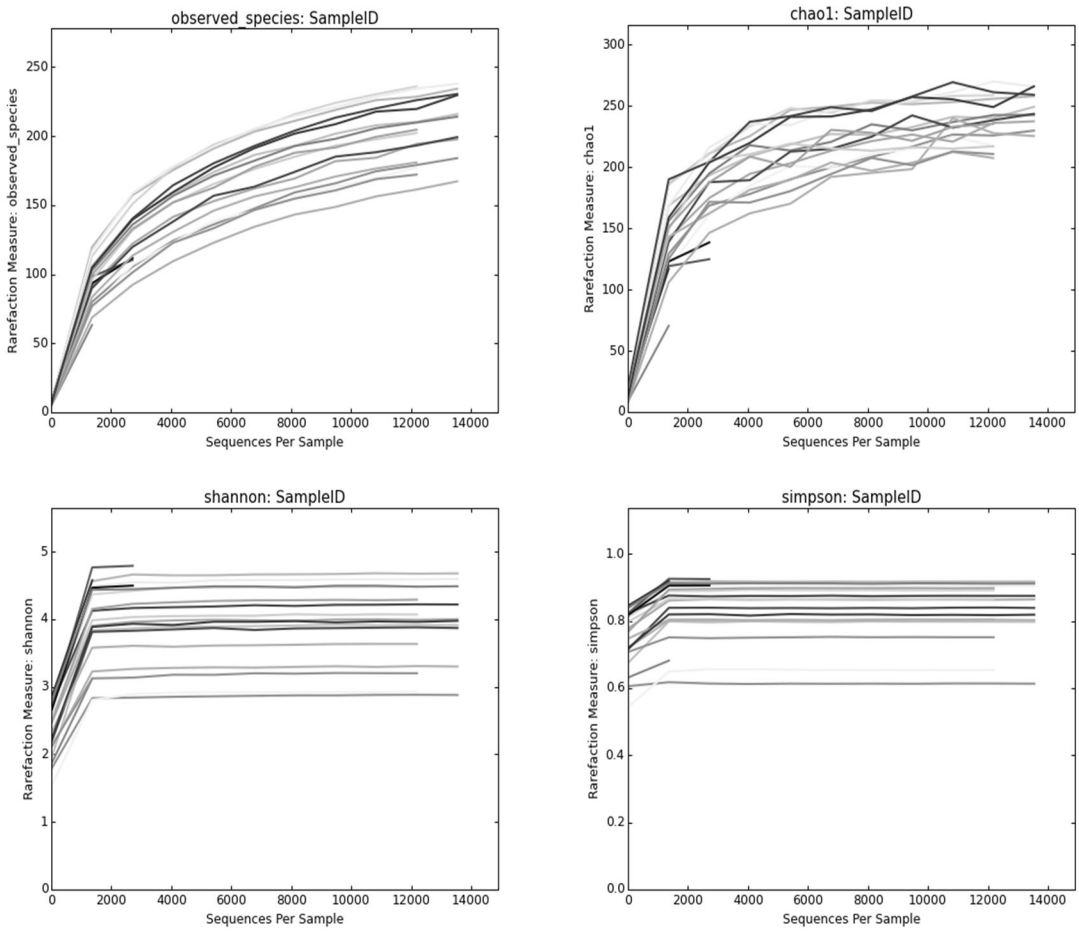

Figure B.10. Alpha diversity rarefaction curves of bacterial OTUs of fresh high moisture corn and of high moisture corn untreated or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 3 L/t of fresh weight ensiled for 49 d not subjected or subjected to a 12 h/week air stress.