



Effects of natamycin and *Lactobacillus buchneri* on the fermentative process and aerobic stability of maize silage

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ABSTRACT. The present study was aimed to evaluate the reduction in fermentative losses and the improvement of aerobic stability of maize silage treated with *Lactobacillus buchneri* bacteria, antifungal natamycin and a combination of *L. buchneri* and natamycin. The study was completely randomized using four treatments with four replicates (silo) each. The treatments were as follows: C – control (forage without additives), NA – forage with low dose of natamycin (8 g/t) addition, LB – forage inoculated with low dose of *L. buchneri* (5×10^4 cfu/g) and NLB – forage treated with both natamycin (8 g/t) and *L. buchneri* (5×10^4 cfu/g). The losses of dry matter (DM) and gas, effluent production, chemical composition, yeast count and aerobic stability were calculated for all treatments. During fermentation, NLB produced more propionic and lactic acids and caused less DM and gas losses than other treatments ($P < 0.01$). The positive effect of NLB on yeast inhibition improved the aerobic stability of maize silage ($P < 0.05$). Thus, the combination of low doses of natamycin and heterolactic bacteria *L. buchneri* can reduce fermentative losses and improve the aerobic stability of maize silage after exposure to air.

Introduction

Maize silage is used all over the world, and the whole maize is the number one forage for silage production in Brazil (Schmidt et al., 2015). Its quality varies depending on a variety of factors, such as the type of hybrid, maturity at the time of harvest and ensiling technique and duration (Lim et al., 2015; Schmidt et al., 2015; Kung et al., 2018), as well as the environmental conditions, such as temperature during ensiling, storage, silage density and feed out (Bernardes et al., 2018; Borreani et al., 2018). Moreover, the quality also depends on additives such as inoculants (e.g. lactic acid bacteria – LAB), chemicals and enzymes (Muck et al., 2018; Fabiszewska et al., 2019) that improve the fermentation process

and dry matter (DM) recovery of the silages (Kung et al., 2018). The high content of lactic acid produced during anaerobic fermentation together with the residue of soluble carbohydrates makes maize silage vulnerable when exposed to air (McDonald et al., 1991). According to Muck et al. (2018), the facultative heterofermentative LAB which ferment hexoses, i.e., glucose, are the same as obligate homofermenters which produce almost exclusively lactic acid. In contrast, the obligate heterofermenters produce other compounds from hexoses in addition to lactic acid, including acetic acid (Muck et al., 2018; Fabiszewska et al., 2019). After opening the silo, the present yeast metabolizes lactate and degrades silage, which raises the mass pH reducing its nutritional value (Ranjit et al., 2002) and

allowing the growth of spoilage microorganisms (Weiss et al., 2016). In order to avoid the growth of yeast and other spoilage microorganisms, maize silage can be supplemented with additives with different levels of effectiveness (Muck et al., 2018).

Lactobacillus buchneri and some chemical additives may effectively improve the aerobic stability of silage because moderate increases in acetic acid potentially inhibit yeasts responsible for initiating aerobic spoilage (Weiss et al., 2016; Muck et al., 2018). A decrease in yeast counts and an increase in aerobic stability were observed in sugarcane silage treated with chemicals (sodium benzoate, potassium sorbate) or bacterial additives (Pedroso et al., 2008). Natamycin (pimaricin) has not yet been fully evaluated as an inhibitor of the growth of specific microorganisms during fermentation and aerobic exposure of silage, despite its potential to improve silage aerobic stability (Woolford et al., 1980). Natamycin is a bacteriocin obtained from *Streptomyces natalensis* culture. It binds to an ergosterol molecule present in the cell wall of mould and yeast but not bacteria (te Welscher et al., 2008). There are several different silage additives that help to preserve forage. Based on the effect of additives on silage preservation, four categories have been established: (1) fermentation stimulators, (2) fermentation inhibitors, (3) aerobic deterioration inhibitors and (4) nutrients and absorbents (McDonald et al., 1991; Kung et al., 2018). Natamycin is an inexpensive bacteriocin that is widely used as a food additive for the preservation of cheese (Var et al., 2006), salamis, juices and wine (Medina et al., 2007), but not feed. In the European Union, natamycin is used for the surface treatment of dairy products (European Parliament and Council Directive No 95/2/EC, 1995). The mammalian digestive system poorly absorbs natamycin, which is almost entirely excreted in faeces, making it safe for humans and farm animals (European Agency for the Evaluation of Medicinal Products, Committee for Veterinary Medicinal Products, 1998).

Various chemical substances may inhibit the growth of undesirable microorganisms and enhance dry matter (DM) recovery of silage. Until now, the aerobic stability of silage was investigated after the forage treatment with only bacterial inoculant or only a chemical additive, there is no research examining the combination of these two types of silage additives (Knicky and Spörndly, 2011). Woolford et al. (1980) observed a small decrease in the number of yeast and mould during the aerobic exposure of grass and maize silage with the addition of natamycin. New additives reducing fermentation losses while improving the

aerobic stability of silage remain an important subject in forage conservation research (Muck et al., 2018). Based on current knowledge (Woolford et al., 1980; Knicky and Spörndly, 2011; Muck et al., 2018), studies on the association of *L. buchneri* with bacteriocins such as natamycin (Woolford et al., 1980, D'Urso et al., 1990), especially in low doses, still remain missing in the forage preservation literature. The current study combines *L. buchneri* and natamycin and assesses the effect of their mixture on the fermentative process and aerobic stability of maize silage to fill this gap. Therefore, the objective of this study was to evaluate the reduction of fermentative losses and the improvement of aerobic stability of maize silage treated with *L. buchneri* bacteria, antifungal natamycin, as well as a combination of *L. buchneri* and natamycin.

Material and methods

Crop and ensiling

Hybrid maize (32R22H, Pioneer® Seeds, Santa Cruz do Sul, Brazil) was harvested at 126 day of maturity with a self-propelled machine regulated for the theoretical particle length of 12 mm. The forage was weighed separately to compose each of the four treatments (wet basis): C – untreated control silage, LB – forage inoculated with *L. buchneri* NCIMB 40788 (5×10^4 cfu/g), NA – forage with natamycin (8 g/t), NLB – forage with natamycin (8 g/t) and *L. buchneri* (5×10^4 cfu/g). All additives were prepared in distilled water, sprayed over the chopped forage and then mixed with a 70% ethanol sterilized pitchfork.

The forage was ensiled into 16 experimental silos (four replicates for each treatment) made of 20-liter plastic buckets (290 mm in diameter and 340 mm in height), equipped with a Bunsen valve at the lid to release the fermentation gas. Each silo contained 12.5 kg of silage compacted by slight trampling to achieve a bulk density of 625 kg/m³ (wet basis). A 2 cm height plastic platform was placed on the bottom of each silo to estimate and collect the production of effluents. Once weighed and sealed, all silos were stored at 22 ± 5 °C.

During ensiling and after applying the additives, representative samples of each treatment were duplicated to assess their DM content and chemical composition. After 90 days of storage, all silos were individually weighed once again for assessing gravimetric losses as gas, effluent or total DM losses, according to Jobim et al. (2007). When opened, silage samples were taken from each silo, and the pH was immediately determined (Kung et al., 1984).

All silos were completely emptied of their silage content and the obtained silages were thoroughly mixed in 60-liter plastic bags. Representative samples (500 g) of each experimental silo unit were taken and pressed with a hydraulic press to obtain silage juice. The juice was stored in Eppendorf® tubes (5 ml), acidified with 0.8 ml of formic acid (98%; Merck®, Darmstadt, Germany) and immediately frozen (-20°C) for later analysis of fermentation end-products. Next samples (350 g) were dried up in a forced air circulation oven (55°C for 72 h), ground using a Willey mill (1-mm sieve) and used for chemical analysis.

The silage aerobic stability was assessed as described by Kung et al. (2000) on 4 kg silage samples, obtained from each replicate. Those samples were placed in plastic buckets and stored at $23 \pm 1^{\circ}\text{C}$, with the temperature being taken from the center of the matter for each sample, every 3 h for an assessment period of five days. Aerobic stability (AS) was defined as the time in hours for the temperature to rise by 2°C from the storage temperature. The accumulated temperature (the sum of all the temperature measurements) and the maximum temperature obtained during air exposure were also recorded.

Chemical and yeast analysis

Nitrogen content for crude protein (CP) estimation was calculated using the Dumas method (Wiles et al., 1998). Neutral detergent fibre (aNDFom) content calculated using α -amylase and sodium sulphite and acid detergent fibre (ADF) content (Van Soest et al., 1991) were adapted from the ANKOM200 fibre analyser (ANKOM Technology, Fairport, NY, USA) according to ANKOM Technology procedures. The hemicellulose (HEM) was determined using the difference between aNDFom and ADF contents. Concentrations of ether extracts (EE), DM and ashes were measured according to the methods of the AOAC International (2012). The concentration of lactic acid in silages was quantified using the colorimetric method according to Pryce (1969), whereas concentrations of acetic, propionic and butyric acids were determined with use of gas chromatography according to Erwin et al. (1961).

Yeasts were counted in the samples collected at the silo opening day (D0) and after three (D3) and five (D5) days of aerobic exposure of the silages. The yeast analyses were adapted from Ávila et al. (2009). All samples underwent previous 1/10 dilutions (25 g of silage in 225 ml of sterile 8.5 g/l NaCl solution). The solution was stirred and filtered, and 2 ml of the extract was used to prepare further dilu-

tions, ranging from 10^{-1} to 10^{-7} . The plating technique was performed in triplicate, in a pour plate agar culture Sabouraud medium (Himedia®, Kennet Square, PA, USA) with 10% tartaric acid (1 ml of acid/100 ml of agar) to obtain a pH of 4.5. All plates were incubated in a bio-oxygen demand (BOD) incubator at 26°C for 144 h. The colony-forming units (cfu) per gram of silage (cfu/g) expressed the measured amount of yeast. These units considered the mean amount of yeast in three replicates of each sample.

Statistical analysis

The experimental design was completely randomized in four treatments, with four replicate (silo) arrangements. The Shapiro-Wilk's and Bartlett's tests were used to test data normality and homogeneity distribution, respectively. The data of the adequacy of the normally distributed residuals and homogeneous variance ($P < 0.05$) was submitted to a treatment effect test. The yeast count was transformed into \log_{10} to obtain the normal distribution and presented on a wet weight basis. The fixed factor in the model of the yeast count was the day of sample collection following the opening of the silo (D0, D3 and D5). The interaction between the day of sample collection and treatment for the yeast count was also included. The chemical composition, fermentation assessment, yeast counts and aerobic stability parameters of all the silages were analysed for their statistical significance using one-way analysis of variance (ANOVA); the differences between the means were considered significant at $P < 0.05$. With significant values of F, all means were compared using the Tukey's test at 0.05 of the probability level ($P < 0.05$). All analyses were performed using the JMP 13.1.0 (SAS Institute Inc., Cary, NC, USA).

Results

The composition of the fresh maize whole-plant during the ensiling was: 349 ± 28 g/kg (mean \pm SD) of DM, 69 ± 9.0 g/kg CP, 568 ± 31 g/kg of aNDFom, 222 ± 10 g/kg of ADF, 346 ± 28 g/kg of HEM, 27 ± 1.0 g/kg of EE and 27 ± 3.0 g/kg of ash.

The chemical composition of the tested maize silages differed between treatments, except for the EE and acetic acid content (Table 1). There was a higher content of aNDFom and hemicellulose in the NLB treatment compared to the other treatments ($P < 0.01$). The control silage had higher ADF content when compared to the LB treatment ($P < 0.05$), while the ash concentration was lower in the NA treatment compared to the control one ($P < 0.01$).

Table 1. Chemical composition of maize silages treated with natamycin and/or *Lactobacillus buchneri* after 90 days of storage, g/kg DM

Indices ¹	Treatments ²				SEM ³	P-value
	C	NA	LB	NLB		
DM, g/kg FM	369.2 ^a	311.0 ^c	316.7 ^c	331.0 ^b	0.59	0.01
CP	62.6 ^c	87.7 ^a	84.8 ^a	79.2 ^b	0.63	0.01
aNDFom	462.7 ^b	436.5 ^b	454.2 ^b	495.8 ^a	0.64	0.01
HEM	241.5 ^{bc}	217.4 ^c	246.7 ^b	278.2 ^a	0.63	0.01
ADF	221.1 ^a	218.9 ^{ab}	207.5 ^b	217.6 ^{ab}	0.19	0.03
EE	38.5	36.6	36.8	37.5	0.05	0.5
Ash	26.3 ^b	32.3 ^a	30.3 ^{ab}	30.7 ^{ab}	0.07	0.01
pH	3.85 ^a	3.85 ^a	3.83 ^{ab}	3.76 ^b	0.01	0.01
Lactic acid	38.5 ^b	48.1 ^{ab}	37.4 ^b	54.4 ^a	0.48	0.01
Acetic acid	11.5	14.8	14.3	12.7	0.12	0.06
Propionic acid	0.8 ^b	0.8 ^b	0.9 ^b	1.1 ^a	3.50	0.01

¹DM – dry matter, FM – fresh matter; CP – crude protein, aNDFom – neutral detergent fibre after amylase treatment an organic matter basis, HEM – hemicellulose, ADF – acid detergent fibre, EE – ether extract; ²treatments: C – control, NA – natamycin, LB – *Lactobacillus buchneri*, NLB – natamycin + *L. buchneri*; ³SEM – standard error of mean; ^{a,b} – means followed by different letters within each row differ statistically according to Tuckey's test ($P < 0.05$)

The NLB silage had a lower pH (3.76) in comparison to the other treatments, especially the control silage and NA with a pH of 3.85. The fermentation process with use of NLB treatment showed efficient lactic acid production when compared to the LB or control treatment. There were no differences in the acetic acid concentration between the treatments, even when *L. buchneri* was used. Concentration of silage propionic acid was higher in the NLB treatment whereas, the lactic acid content in this treatment was similar to the one in the NA treatment. However, no butyric acid was detected in any silage.

Significantly lower dry matter losses (DML) and gas losses were observed in the NLB treatment after 90 days of storage (Table 2). The different treatments, however, did not significantly affect the effluent production which wet basis average was 17.6 kg/t.

Table 2. Fermentation loss and effluent production of maize silages treated with natamycin and/or *Lactobacillus buchneri* after 90 days of storage

Indices ¹	Treatments ²				SEM ³	P-value
	C	NA	LB	NLB		
DML, g/kg DM	68.9 ^b	70.6 ^b	88.8 ^b	21.0 ^a	0.70	0.01
Gases, g/kg DM	67.5 ^b	68.5 ^b	87.1 ^b	19.5 ^a	0.69	0.01
Effluent, kg/t FM	14.3	22.0	18.7	15.4	0.13	0.12

¹DML – dry matter losses, DM – dry matter, FM – fresh matter; ²treatments: C – control, NA – natamycin, LB – *Lactobacillus buchneri*, NLB – natamycin + *L. buchneri*; ³SEM – standard error of mean; ^{a,b} – means followed by different letters within each row differ statistically according to Tuckey's test ($P < 0.05$)

Table 3. Least-square means of development of the counts of yeast in maize silages treated with natamycin and/or *Lactobacillus buchneri* and exposed to air for different number of days (day 0, 3 and 5), log₁₀ cfu/g of silage

Indices	Treatments ¹				SEM ²	P-value
	C	NA	LB	NLB		
Day 0	2.95 ^{Aa}	2.23 ^{Aa}	1.92 ^{Ab}	0.26 ^{Ab}	0.31	0.01
Day 3	7.67 ^{Ca}	6.63 ^{Bab}	6.47 ^{Bab}	5.99 ^{Bb}	0.23	0.04
Day 5	6.85 ^{Bb}	8.74 ^{Ca}	7.78 ^{Cab}	6.32 ^{Cb}	0.29	0.01
P-value	0.01	0.01	0.01	0.01		

¹treatments: C – control, NA – natamycin, LB – *Lactobacillus buchneri*, NLB – natamycin + *L. buchneri*; ²SEM – standard error of mean; ^{A,B,C} – (days comparison for each treatment separately) means followed by different capital letters within each column differ statistically according to Tuckey's test ($P < 0.05$); ^{a,b} – (treatments comparison for each day separately) means followed by different letters within each row differ statistically according to Tuckey's test ($P < 0.05$)

There was a significant difference ($P < 0.05$) in yeast growth between the treatments during five days of aerobic exposure (Table 3). The NLB treatment showed lower yeast count at the time of silo opening in comparison to the control and NA treatment ($P < 0.01$). A significant effect of the day on the yeast count was observed ($P < 0.01$). Yeast growth increased on the third day from silo opening regardless used treatment. Similar values of yeast counts between the control, NA and LB treatments on D0 and D3 were observed. There was a significant interaction between treatment and the day of sample collection following the opening of the silo ($P < 0.001$). Yeast count was lower in the NLB treatment than in the control one on the D3 of air exposure. No significant difference was found between the NLB, LB and control silages on D5 of exposure to air, whereas the highest yeast number was in NA treatment on D5 ($P < 0.01$).

Among stability parameters of silages, there were no differences between treatments for accumulated and maximum temperatures (Table 4). The NLB treatment improved the aerobic stability of silage ($P < 0.05$) when compared to the control one. Similar aerobic stability was found in the NA and LB silages.

Table 4. Stability parameters of maize silages treated with natamycin and/or *Lactobacillus buchneri* and exposed to air for 5 days

Indices	Treatments ¹				SEM ²	P-value
	C	NA	LB	NLB		
Accumulated temp., °C	1215	1215	1205	1159	9.42	0.10
Maximum temp., °C	36	41	40	42	1.29	0.36
Aerobic stability, h	51 ^b	54 ^{ab}	52 ^b	64 ^a	1.86	0.02

¹treatments: C – control, NA – natamycin, LB – *Lactobacillus buchneri*, NLB – natamycin + *L. buchneri*; ²SEM – standard error of mean; ^{a,b} – means followed by different letters within each row differ statistically according to Tuckey's test ($P < 0.05$)

Discussion

This study aimed to assess the reduction in fermentative losses and the improvement in aerobic stability of maize silage treated with *L. buchneri* bacteria, antifungal natamycin and the mixture of *L. buchneri* and natamycin. The contents of aNDFom and hemicellulose in the NLB treated silage were higher than for the other treatments, with the ADF content remaining constant. Maize silages inoculated with *L. buchneri* do not usually show significant differences in NDF content (Ranjit et al., 2002; Reich and Kung, 2010); even though, an increase in the NDF digestibility of maize silage can be observed in some cases (Filya and Sucu, 2010). The NLB treatment provided greater solubility of hemicelluloses from aNDFom (56%) when compared to silage with a single additive (LB = 54%, control = 52% and NA = 50%). This probably occurred due to bacterial enzymatic activity and acid hydrolysis of the NDF fraction (Muck and Kung, 1997).

The higher concentration of lactic and propionic acids during fermentation in the NLB treatment likely came from the yeast inhibition, retaining soluble carbohydrates for bacterial growth. The NLB treatment showed efficient homolactic fermentation. The found lactic acid concentration (54.4 g/kg DM) was similar to the one found by Da Silva et al. (2014) in maize silages (54.7 g/kg DM) treated with commercially available *L. buchneri* strains (CNCM I-4323). Silages inoculated with *L. buchneri* are known to lower lactic acid, because of a moderate conversion in favour of acetic acid (Kung et al., 2018). On the other hand the higher concentrations of propionic acid are stated due to the addition of *L. diolivorans* which ferment 1,2-propanediol (Krooneman et al., 2002; Kung et al., 2018). In the present study, the association of *L. buchneri* with natamycin appears not to increase the effect of bacteria action, since the acetic acid concentration showed no changes. The acid level found in the present study shows that natamycin has no negative effect on lactic acid bacteria. D'Urso et al. (1990) observed the adequate average values of lactic acid (44.1 g/kg DM) and acetic acid (18.7 g/kg DM) in a triticale silage, both treated and untreated with natamycin. These values are respectively lower and higher than those of the NA treatment in the present study, but D'Urso et al. (1990) used more than 100 g of natamycin per t of fresh matter in the combination with inoculants. The differences in used doses and ensiled material make the

comparison between studies unreliable. Moreover, the silages in the present study, except NLB one, demonstrated the concentrations of propionic acid similar to the recommended concentration by Kung et al. (2018), with <1 g/kg DM in maize silages. The concentration of propionic acid in the NLB treatment (1.1 g/kg DM) was slightly higher than in the other treatments, probably due to the lower yeast competitive metabolism during fermentation, along with a synergistic effect between the *L. buchneri* and other heterofermentative *Lactobacilli* (Krooneman et al., 2002).

Dry matter losses during fermentation reduce silage quality (Borreani et al., 2018) and they can be prevented by using additives (Muck et al., 2018). The usage of mixture of additives with different modes of action may be a strategy to reduce the cost of microbial inoculants as the same effect can be found using lower dose of bacteria. In the present trial, the combination of natamycin and *L. buchneri* (5×10^4 cfu/g) promoted a lower DM and gas losses during fermentation when compared to other silage treatments. Although the limitation of gravimetric estimates of losses based on oven-dried samples, Restelatto et al. (2019) demonstrated a significant DM and gas losses in silages inoculated with *L. buchneri* (4×10^5 cfu/g). The inoculation of *L. buchneri* solely in maize silages has not produced an efficient reduction of DML (Ranjit et al., 2002). In contrast, Reich and Kung (2010) tested a higher dose of *L. buchneri* (4×10^5 cfu/g) in maize silage and observed lower DML as compared to silage without additive. Through the production of acetic acid, the addition of *L. buchneri* improves the aerobic stability of maize silages (Kung et al., 2018). The lack of effect of LB treatment in the present study was likely related to a lower applying rate in comparison to other studies (Reich and Kung, 2010; Muck et al., 2018). Furthermore, Schmidt et al. (2012) did not find a decrease in fermentative losses in natamycin treated maize silage. However, Woolford et al. (1980) found a decrease of aerobic deterioration of maize silage after natamycin treatment but only at a high application rate (270 g/t). Therefore, the combination of additives may reduce the amount of used additives simultaneously increasing the recovery of DM from the final product.

The NLB treatment demonstrated the beneficial effects of associating the two additives. It lowered the yeast growth on the silo opening day (D0) in comparison to the control and NA treatments; no changes were observed in the single-additive treatments (natamycin or *L. buchneri*). This was

probably related to the synergistic effect of the different modes of action over the yeast metabolism. According to Brik (1981), the pKa of natamycin is 4.6, which is higher than the pH found in silages. Thus, natamycin may inhibit the growth of yeast in the first aerobic phase of the ensiling while improving the environmental conditions for LAB and for *L. buchneri* growth.

Efficient fermentation raises the amount of acids in silage (Santos et al., 2016). *L. buchneri* produces high levels of acetic acid that inhibit yeast growth (Muck and Kung, 1997), an effect is ten times more effective than leaving silage untreated (Kleinschmit and Kung, 2006). Although no acetic acid concentration differences were observed between the treatments in the present study, *L. buchneri* might have also contributed to those combined effects. The low yeast count at D0 in the NLB treated silage was the result of combined action of natamycin plus acetic acid and the higher amount of propionic acid (11 g/kg).

A positive factor to consider is probably the lower dose of *L. buchneri* used in the present study (5×10^4 cfu/g) when compared to other studies. In Driehuis et al. (2001) study, the maize silage inoculated with *L. buchneri* (3×10^5 cfu/g) increased acetic acid content and reduced the yeast number. Muck et al. (2018) emphasized that *L. buchneri* effects on the aerobic stability are dose-dependent and the positive effect is stated at dose above 1×10^5 cfu/g. The effectiveness of *L. buchneri* is probably due to the capacity of these bacteria to turn lactic acid into acetic acid. Furthermore, *L. buchneri* is stated to rapidly lower the pH of ensiled material during fermentation (Filya, 2003; Filya and Sucu, 2010; Reich and Kung, 2010) and maintain a low pH for the silages during air exposure due to the greater levels of acetic acid (Filya, 2003).

In the present study no silage treatment showed efficient control of yeast growth after five days of aerobic exposure. Woolford et al. (1980) found 34.6 and 3.8% of natamycin recovery rates after 7 and 100 days of fermentation, respectively. It suggests that natamycin acts only during the first stage of the forage fermentation. Thus, its remarkable effect as silage additive is the decrease of the initial yeast population.

Mold and yeast activity during silage feed out is responsible for both lactate metabolism and an increase in silage temperature (Borreani et al., 2018). Thus, mould and yeast are considered aerobic deterioration starters. The present trial showed

no effective increase of aerobic stability with single *L. buchneri* treatment, probably related to the applied dose (Muck et al., 2018). The treatment with natamycin alone presented a non-significant increase of the aerobic stability when compared to the control one. However, the NLB treatment increased aerobic stability by 13 h.

Woolford et al. (1980) found lower accumulated temperature for maize silage with added 270 g/t natamycin (137 °C) as compared to untreated silage (207 °C). Another study with triticale and natamycin used at dose of 200 g/t presented no changes in the temperature of the silage, even after 10 days of air exposure (D'Urso et al., 1990). The present study used lower natamycin dose (8 g/t) than other former experiments in order to attend the economic viability of the data.

The combination of natamycin and *L. buchneri* inhibited the growth of yeast during fermentation, thus improved the aerobic stability of the silage for a longer period. The NLB treatment increased aerobic stability by about 20% (up to 64 h) in comparison to control silage (51 h). The combination of natamycin and *L. buchneri* led to a synergistic effect allowing for a reduction in the dosage of both additives and ensuring benefits when compared to the usage of a single additive. It is an important result concerning the costs of suitable additives to reduce aerobic deterioration.

Conclusions

The combination of low doses of natamycin and *Lactobacillus buchneri* may lead to positive effects on the fermentation of maize silages by decreasing the yeast count, reducing losses and improving aerobic stability.

References

- AOAC International, 2012. Official Methods of Analysis of AOAC International. 19th Edition. Gaithersburg, MD (USA)
- Ávila C.L.S., Pinto J.C., Figueiredo H.C.P., Schwan R.F., 2009. Effects of an indigenous and a commercial *Lactobacillus buchneri* strain on quality of sugar cane silage. *Grass Forage Sci.* 64, 384–394, <https://doi.org/10.1111/j.1365-2494.2009.00703.x>
- Bernardes T.F., Daniel J.L.P., Adesogan A.T., McAllister T.A., Drouin P., Nussio L.G., Huhtanen P., Tremblay G.F., Bélanger G., Cai Y., 2018. Silage review: Unique challenges of silages made in hot and cold regions. *J. Dairy Sci.* 101, 4001–4019, <https://doi.org/10.3168/jds.2017-13703>
- Borreani G., Tabacco E., Schmidt R.J., Holmes B.J., Muck R.E., 2018. Silage review: Factors affecting dry matter and quality losses in silages. *J. Dairy Sci.* 101, 3952–3979, <https://doi.org/10.3168/jds.2017-13837>

- Brik H., 1981. Natamycin. *Anal. Profiles Drug Subst.* 10, 513–561, [https://doi.org/10.1016/S0099-5428\(08\)60650-4](https://doi.org/10.1016/S0099-5428(08)60650-4)
- Da Silva N.C., Dos Santos J.P., Ávila C.L.S., Evangelista A.R., Casagrande D.R., Bernardes T.F., 2014. Evaluation of the effects of two *Lactobacillus buchneri* strains and sodium benzoate on the characteristics of corn silage in a hot-climate environment. *Grassl. Sci.* 60, 169–177, <https://doi.org/10.1111/grs.12053>
- Driehuis F., Oude Elferink S.J.W.H., Van Wikselaar P.G., 2001. Fermentation characteristics and aerobic stability of grass silage inoculated with *Lactobacillus buchneri*, with or without homofermentative lactic acid bacteria. *Grass Forage Sci.* 56, 330–343, <https://doi.org/10.1046/j.1365-2494.2001.00282.x>
- D'Urso G., Avondo M., Licitra G., Sinatra M., 1990. Effects of adding Na-bentonite, formic acid and pimaricin on the fermentation characteristics and aerobic deterioration of triticale silage. *Zootec. Nutr. Anim.* 16, 99–106
- Erwin E.S., Marco G.J., Emery E.M., 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci.* 44, 1768–1771, [https://doi.org/10.3168/jds.S0022-0302\(61\)89956-6](https://doi.org/10.3168/jds.S0022-0302(61)89956-6)
- European Agency for the Evaluation of Medicinal Products, Committee for Veterinary Medicinal Products, 1998. Natamycin. EMEA/MRL/342/98-FINAL. Available at <https://www.ema.europa.eu>
- European Parliament and Council Directive No 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners. *Off. J. L61*, 18.3.1995, 1–40
- Fabiszewska A.U., Zielińska K.J., Wróbel B., 2019. Trends in designing microbial silage quality by biotechnological methods using lactic acid bacteria inoculants: a minireview. *World J. Microbiol. Biotechnol.* 35, 76, <https://doi.org/10.1007/s11274-019-2649-2>
- Filya I., 2003. The effect of *Lactobacillus buchneri* and *Lactobacillus plantarum* on the fermentation, aerobic stability, and ruminal degradability of low dry matter corn and sorghum silages. *J. Dairy Sci.* 86, 3575–3581, [https://doi.org/10.3168/jds.S0022-0302\(03\)73963-0](https://doi.org/10.3168/jds.S0022-0302(03)73963-0)
- Filya I., Sucu E., 2010. The effects of lactic acid bacteria on the fermentation, aerobic stability and nutritive value of maize silage. *Grass Forage Sci.* 65, 446–455, <https://doi.org/10.1111/j.1365-2494.2010.00763.x>
- Jobim C.C., Nussio L.G., Reis R.A., Schmidt P., 2007. Methodological advances in evaluation of preserved forage quality (in Portuguese: Avanços metodológicos na avaliação da qualidade da forragem conservada). *Rev. Bras. Zootec.* 36, 101–119, <https://doi.org/10.1590/S1516-35982007001000013>
- Kleinschmit D.H., Kung L., 2006. A meta-analysis of the effects of *Lactobacillus buchneri* on the fermentation and aerobic stability of corn and grass and small-grain silages. *J. Dairy Sci.* 89, 4005–4013, [https://doi.org/10.3168/jds.S0022-0302\(06\)72444-4](https://doi.org/10.3168/jds.S0022-0302(06)72444-4)
- Knicky M., Spörndly R., 2011. The ensiling capability of a mixture of sodium benzoate, potassium sorbate, and sodium nitrite. *J. Dairy Sci.* 94, 824–831, <https://doi.org/10.3168/jds.2010-3364>
- Krooneman J., Faber F., Alderkamp A.C., Oude Elferink S.J.H.W., Driehuis F., Cleenwerck I., Swings J., Gottschal J.C., Vancanneyt M., 2002. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. *Int. J. Syst. Evol. Microbiol.* 52, 639–646, <https://doi.org/10.1099/00207713-52-2-639>
- Kung L., Grieve D.B., Thomas J.W., Huber J.T., 1984. Added ammonia or microbial inocula for fermentation and nitrogenous compounds of alfalfa ensiled at various percents of dry matter. *J. Dairy Sci.* 67, 299–306, [https://doi.org/10.3168/jds.S0022-0302\(84\)81302-8](https://doi.org/10.3168/jds.S0022-0302(84)81302-8)
- Kung L., Robinson J.R., Ranjit N.K., Chen J.H., Golt C.M., Pesek J.D., 2000. Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. *J. Dairy Sci.* 83, 1479–1486, [https://doi.org/10.3168/jds.S0022-0302\(00\)75020-X](https://doi.org/10.3168/jds.S0022-0302(00)75020-X)
- Kung L., Shaver R.D., Grant R.J., Schmidt R.J., 2018. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *J. Dairy Sci.* 101, 4020–4033, <https://doi.org/10.3168/jds.2017-13909>
- Lim J.M., Nestor K.E., Kung L., 2015. The effect of hybrid type and dietary proportions of corn silage on the lactation performance of high-producing dairy cows. *J. Dairy Sci.* 98, 1195–1203, <https://doi.org/10.3168/jds.2014-8725>
- McDonald P., Henderson N., Heron S., 1991. *The Biochemistry of Silage*. Chalcombe. Marlow, UK
- Medina Á., Jiménez M., Mateo R., Magan N., 2007. Efficacy of natamycin for control of growth and ochratoxin A production by *Aspergillus carbonarius* strains under different environmental conditions. *J. Appl. Microbiol.* 103, 2234–2239, <https://doi.org/10.1111/j.1365-2672.2007.03462.x>
- Muck R.E., Kung L., 1997. Effects of silage additives on ensiling. In: *Silage: Field to Feedbunk*. NRAES-99. Ithaca, NY (USA), pp. 187–199
- Muck R.E., Nadeau E.M.G., McAllister T.A., Contreras-Govea F.E., Santos M.C., Kung L., 2018. Silage review: Recent advances and future uses of silage additives. *J. Dairy Sci.* 101, 3980–4000, <https://doi.org/10.3168/jds.2017-13839>
- Pedroso A., Nussio L.G., Loures D.R.S., Paziani S., Ribeiro J.L., Mari L.J., Zopollatto M., Schmidt P., Mattos W.R.S., Horii J., 2008. Fermentation, losses, and aerobic stability of sugarcane silages treated with chemical or bacterial additives. *Sci. Agric.* 65, 589–594, <https://doi.org/10.1590/S0103-90162008000600004>
- Pryce J.D., 1969. A modification of Barker-Summerson method for determination of lactic acid. *Analyst* 94, 1151–1152, <https://doi.org/10.1039/an9699401151>
- Ranjit N.K., Taylor C.C., Kung L., 2002. Effect of *Lactobacillus buchneri* 40788 on the fermentation, aerobic stability and nutritive value of maize silage. *Grass Forage Sci.* 57, 73–81, <https://doi.org/10.1046/j.1365-2494.2002.00304.x>
- Reich L.J., Kung L., 2010. Effects of combining *Lactobacillus buchneri* 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. *Anim. Feed Sci. Technol.* 159, 105–109, <https://doi.org/10.1016/j.anifeedsci.2010.06.002>
- Restelatto R., Novinski C.O., Pereira L.M., Silva E.P.A., Volpi D., Zopollatto M., Schmidt P., Faciola A.P., 2019. Chemical composition, fermentative losses, and microbial counts of total mixed ration silages inoculated with different *Lactobacillus species*. *J. Anim. Sci.* 97, 1634–1644, <https://doi.org/10.1093/jas/skz030>
- Santos A.O., Ávila C.L.S., Pinto J.C., Carvalho B.F., Dias D.R., Schwan R.F., 2016. Fermentative profile and bacterial diversity of corn silages inoculated with new tropical lactic acid bacteria. *J. Appl. Microbiol.* 120, 266–279, <https://doi.org/10.1111/jam.12980>
- Schmidt P., Novinski C.O., Carneiro E.W., Bayer C., 2012. Greenhouse gas emissions from fermentation of corn silage. In: K. Kuoppala, M. Rinne, A. Vanhat-alo (Editors). *Proceedings of the XVI International Silage Conference*. Hämeenlinna, Finland, pp. 448–449
- Schmidt P., Novinski C.O., Junges D., Almeida R., de Souza C.M., 2015. Concentration of mycotoxins and chemical composition of corn silage: A farm survey using infrared thermography. *J. Dairy Sci.* 98, 6609–6619, <https://doi.org/10.3168/jds.2014-8617>

- te Welscher Y.M., ten Napel H.H., Balagué M.M., Souza C.M., Riezman H., de Kruijff B., Breukink E., 2008. Natamycin blocks fungal growth by binding specifically to ergosterol without permeabilizing the membrane. *J. Biol. Chem.* 283, 6393–6401, <https://doi.org/10.1074/jbc.M707821200>
- Van Soest P.J., Robertson J.B., Lewis B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74, 3583–3597, [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)
- Var I., Erginkaya Z., Güven M., Kabak B., 2006. Effects of antifungal agent and packaging material on microflora of Kashar cheese during storage period. *Food Control* 17, 132–136, <https://doi.org/10.1016/j.foodcont.2004.09.012>
- Weiss K., Kroschewski B., Auerbach H., 2016. Effects of air exposure, temperature and additives on fermentation characteristics, yeast count, aerobic stability and volatile organic compounds in corn silage. *J. Dairy Sci.* 99, 8053–8069, <https://doi.org/10.3168/jds.2015-10323>
- Wiles P.G., Gray I.K., Kissling R.C., 1998. Routine analysis of proteins by Kjeldahl and Dumas methods: review and interlaboratory study using dairy products. *J. AOAC Int.* 81, 620–632
- Woolford M.K., Cook J.E., Hall D.M., Bonis A., 1980. The use of pimaricin as an additive to improve the aerobic stability of silage. *J. Sci. Food Agric.* 31, 558–566, <https://doi.org/10.1002/jsfa.2740310607>