University of São Paulo
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# Whole-plant corn silage treated with *Azospiriullum* sp., *Bradyrhizobium* sp. or *Bacillus* sp. compared with commercial inoculants

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Dissertation presented to obtain the degree of Master in Science. Area: Animal Science and Pastures

Piracicaba
2024

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versão revisada de acordo com a Resolução CoPGr 6018 de 2011.

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# CONTENTS

## RESUMO

### Inoculação de silagem de milho planta inteira com *Azospiriullum* sp., *Bradyrhizobium* sp. ou *Bacillus* sp. comparada com inoculação de aditivos biológicos comerciais

Investigar microorganismos não tradicionais utilizados em contextos agrícolas para diversos objetivos produtivos e relacionados à saúde, bem como sua adaptabilidade a ambientes de ensilagem, oferece uma rota para avançar as tecnologias de conservação na produção de silagem. Portanto, dois experimentos foram conduzidos para avaliar os padrões de fermentação em silagens de milho tratadas com *Bacillus* sp., *Azospirillum* sp. e *Bradyrhizobium* sp., em contraste com silagens controle ou aquelas tratadas com inoculantes comercialmente disponíveis, contendo cepas de *Lentilactobacillus* homo ou heterolácticas. Na Fase 1, a forragem colhida foi dividida em sete partes para cada um dos sete tratamentos no Experimento 1 e em oito partes para cada um dos oito tratamentos no Experimento 2. Cada parte foi subdividida em quatro pilhas e tratada separadamente com o inoculante para criar quatro replicatas para cada tratamento. De cada pilha, foram produzidos dois silos de 20 litros cada (cada um designado para um tempo específico de abertura de 45 dias ou 90 dias), e três mini bags de aproximadamente 500g cada foram preparados para estudar a dinâmica de acidificação da silagem ao longo do tempo (6, 12 e 24 horas). Os silos foram rotulados de R1 a R4, e os mini sacos de R1 a R3. Para a Fase 2, a forragem colhida foi dividida da mesma forma, mas em oito partes para cada um dos oito tratamentos. Cada parte foi subdividida em três pilhas e tratada separadamente com o inoculante para criar três replicatas para cada tratamento. De cada pilha, foram produzidos dois silos de 20 litros cada (cada um designado para um tempo específico de abertura de 45 dias ou 90 dias), e três mini bags. Os silos foram rotulados de R1 a R3, e os mini sacos de R1 a R3.A composição química, características de fermentação, cromatografia, contagem microbiana e estabilidade aeróbica foram analisadas. Os dados foram analisados usando o procedimento MIXED do SAS e o teste de significância de Tukey-Kramer ($P<0,05$) foi aplicado para Tratamento, Tempo e Interação Tratamento*Tempo. A composição química, características de fermentação e cromatografia, contagem microbiana e estabilidade aeróbica foram analisadas. Na fase 1, o experimento foi conduzido ao longo de dois dias, designados como Experimento 1 e Experimento 2. No Experimento 1, foram utilizados *Azospirillum brasiliense* (ABV5, ABV6) e *Bradyrhizobium elkanii semia* (BV36, BV27), e no Experimento 2, *Bacillus subtilis* (BV02, BV09, BV30, BV31) e *Bacillus* sp. (BV26). Em ambos os experimentos, os inoculantes foram comparados com um tratamento de controle (CON) e com as *Lactiplantibacillus plantarum* (LP) e *Lentilactobacillus buchneri* (LB). No Experimento 1, após 45 dias de armazenamento, o tratamento LB apresentou o maior pH em comparação com os outros tratamentos (3,85 vs 3,70% MS). O mesmo padrão foi observado após 90 dias de armazenamento, com ABV5, ABV6, BV36 e BV37 também exibindo concentrações de pH elevadas de forma semelhante. Entre as populações bacterianas, o tratamento LB revelou a contagem mais alta para LAB (8,20 vs. 6,10 log cfu.g-1), seguido por LP (7,29 vs. 6,25 log cfu.g-1). Os tratamentos de *Bradyrhizobium* e *Azospirillum* não diferiram do tratamento de controle para outras características de fermentação e estabilidade aeróbica. No Experimento 2, os conteúdos de ácido lático (AL) demonstraram uma interação entre aditivos e tempo de armazenamento. Ao considerar o tempo dentro dos aditivos, todos os tratamentos apresentaram um aumento nos conteúdos de AL, exceto o tratamento LB, que permaneceu inalterado. Ao examinar os aditivos dentro do tempo, aos 90 dias, as novas cepas BV09 e BV26 tiveram os maiores conteúdos, com BV02, BV30, BV31 e LP apresentando semelhanças com eles, assim como o tratamento de Controle, e o tratamento LB teve o menor conteúdo. O LEV apresentou uma queda na contagem ao considerar o impacto do tempo (4,04 para 3,44 log cfu.g-1). O tratamento LP produziu a contagem mais alta (5,03 vs 3,55 log cfu.g-1), enquanto a mais baixa foi observada no tratamento LB (<2,00 vs 3,99 log cfu.g-1). Os tratamentos restantes apresentaram semelhanças com o Controle. Em relação ao etanol, uma

interação significativa foi observada (valor de P <0,001). Ao considerar o tempo dentro dos aditivos, Controle e LP apresentaram aumento, enquanto os outros tratamentos permaneceram consistentes. Ao analisar os aditivos dentro do tempo, aos 45 dias, LP apresentou o maior conteúdo (0,68 vs 0,11 % de correção de MS), com os outros sendo mais baixos e semelhantes entre si. Aos 90 dias, LP novamente mostrou o maior conteúdo, seguido pelo Controle, enquanto BV09 e BV30 exibiram os menores conteúdos. A estabilidade aeróbica aumentou de 45 dias para 90 dias (38,5 vs 49,0 horas). Entre os aditivos, LB apresentou a maior duração (64,4 vs 40,7 horas), seguido por LP (50,8 vs 42,7 horas). BV09, BV26 e BV30 mostraram similaridade na estabilidade em relação aos tratamentos LP e Controle. No entanto, BV02 e BV31 foram apenas comparáveis ao controle, que demonstrou a menor estabilidade aeróbica. A estabilidade aeróbica das silagens não diferiu do tratamento de controle; no entanto, as cepas de Bacillus mostraram promessa no controle do metabolismo de leveduras. Na fase 2, a forragem colhida foi tratada com inoculantes de *Bacillus* sp. (BV02, BV09, BV26, BV30 e BV31) e inoculantes comerciais à base de lactobacilos (*Lentilactobacillus buchneri*, LB e *Lactiplantibacillus plantarum,* LP). Para a variável de AL, os tratamentos com aditivos mostraram diferenças apenas dentro de 12h e os maiores conteúdos foram encontrados para LP (0,29 vs 0,87% de correção de MS). Para 90 dias de armazenamento, os tratamentos BV02, BV09, BV26 e BV30 tiveram os maiores valores de AL (5,09, 4,94, 5,19, 5,05 vs 2,58% de correção de DM, respectivamente). O crescimento de BAL foi favorecido porque as contagens dos ensaios de bacilos foram superiores a 4 log cfu/g para os tratamentos. As cepas BV02 (166h) e BV30 (174h) tiveram estabilidade aeróbica semelhante ao tratamento LB (216h), e a cepa BV09 (140h) apresentou maior estabilidade aeróbica do que o Controle (70,3h). O tratamento LB, aos 45 dias de armazenamento, apresentou os maiores conteúdos de ácido acético (1,08% de correção de DM), e o tratamento de bacilos teve o mesmo comportamento que o controle, exceto BV31, igualado a LP. Para 90 dias de armazenamento, BV09 foi estatisticamente igual ao tratamento LB (0,8 vs 1,08% de correção de DM), mas os outros tratamentos de bacilos tiveram valores semelhantes ao Controle e LP. O tratamento LB teve as maiores quantidades de ácido propiônico (0,1% de correção de DM) e LP, as menores (0,009% de DM). Os outros tratamentos tiveram teores intermediários, mas os ensaios de bacilos que mostraram estabilidade aeróbica semelhante ao LB, como BV02, BV09 e BV30, demonstraram altas quantidades deste ácido (0,04, 0,06 e 0,01% de correção de DM, respectivamente). As silagens produzidas sob inoculação com cepas de *Bacillus* integraram características de fermentação de qualidade desejável e controle da deterioração aeróbica.

Palavras-chave: Silagem, Aditivo, Estabilidade aeróbica, Levedura

## ABSTRACT

### Whole-plant corn silage treated with *Azospiriullum* sp., *Bradyrhizobium* sp. or *Bacillus* sp. compared with commercial inoculants

Investigating non-traditional microorganisms utilized in agricultural contexts for diverse productive and health-related objectives, and their adaptability to a silage setting, offers a route to advance conservation technologies in silage production. Therefore, two trials were conducted to evaluate the fermentation patterns in corn silages treated *Bacillus* sp., *Azospirillum* sp., and *Bradyrhizobium* sp., in contrast to control silages or those treated with commercially available inoculants containing homo or heterolactic *Lentilactobacillus* strains. In Phase 1, the harvested forage was divided into seven parts for each of the seven treatments in Experiment 1 and eight parts for each of the eight treatments in Experiment 2. Each part was subdivided into four piles and treated separately with the inoculant to create four replicates for each treatment. From each pile, two silos of 20 liters were produced (each designated for a specific opening time of 45 days or 90 days), and 3 mini-bags of approximately 500g each were prepared to study the silage acidification dynamics over time (6,12 and 24 hours). The silos were labeled R1 to R4, and the mini-bags R1 to R3. For Phase 2, the harvest forage was divided in the same way, but into eight parts for each of the eight treatments. Each part was subdivided into three piles and treated separately with the inoculant to create three replicates for each treatment. From each pile, two silos of 20 liters were produced (each designated for a specific opening time of 45 days or 90 days), and three mini-bags. The silos were labeled R1 to R3, and the mini-bags R1 to R3. The chemical composition, fermentation characteristics, chromatography, microbial count and aerobic stability were analyzed. Data were analyzed using the MIXED procedure of SAS and the Tukey-Kramer significance test ($P<0.05$) was applied for Treatment, Time, and Treatment*Time interaction. In this experiment, in the first 6 hours, the control has the highest pH and the strains resemble LP and LB treatments, the last one with the lowest value. Chemical composition, fermentation characteristics and chromatography, microbial count, and aerobic stability were analyzed. In phase 1, the experiment was conducted over two days, designated as Experiment 1 and Experiment 2. In Experiment 1 were utilized the Azospirillum brasiliense (ABV5, ABV6) and Bradyrhizobium elkanii semia (BV36, BV27) and in Experiment 2, the *Bacillus subitilis* (BV02, BV09, BV30, BV31) and *Bacillus* sp. (BV26) and in both experiments, the inoculants were compared with a control treatment (CONT) and with *Lactiplantibacillus plantarum* (LP) and *Lentilactobacillus buchneri* (LB). In Experiment 1, after 45 days of storage, the LB treatment showed the highest pH compared to the other treatments (3.85 vs 3.70% DM). The same pattern was observed after 90 days of storage, with ABV5, ABV6, BV36, and BV37 also exhibiting similarly high pH concentrations. Among the bacterial populations, the LB treatment revealed the highest count for LAB (8.20 vs. 6.10 log cfu.$g^{-1}$), followed by LP (7.29 vs. 6.25 log cfu.g-1). The Bradyrhizobium and Azospiriullum treatments did not differ from the control treatment for other fermentation characteristics and aerobic stability. In Experiment 2, lactic acid (LA) contents demonstrated an interaction between additives and storage time. When considering time within additives, all treatments exhibited an increase in LA contents, except for LB treatment which remained unchanged. Examining additives within time, at 90 days, new strains BV09 and BV26 had the highest contents, with BV02, BV30, BV31, and LP displaying similarities to them, as well as to the Control treatment, and LB treatment had the lowest content. The LEV displayed a decline in the count when considering the impact of time (4.04 to 3.44 log cfu.$g^{-1}$). The LP treatment yielded the highest count (5.03 vs 3.55 log cfu.$g^{-1}$), while the lowest was observed in the LB treatment (<2.00 vs 3.99 log cfu.g-1). The remaining treatments exhibited similarities to the Control. Regarding ethanol, a significant interaction was observed (P value < 0.001). Considering time within additives, Control and LP exhibited an increase, while the other treatments remained consistent. Analyzing additives within time, at 45 days, LP displayed the highest content (0.68 vs.

0.11 % of DM correction), with the others being lower and similar among themselves. At 90 days, LP again showed the highest content, followed by Control, while BV09 and BV30 exhibited the lowest contents. The aerobic stability increased from 45 days to 90 days (38.5 vs. 49.0 hours). Among the additives, LB exhibited the longest duration (64.4 vs. 40.7 hours), followed by LP (50.8 vs. 42.7 hours). BV09, BV26, and BV30 showed similarity in stability to LP and Control treatments. However, BV02 and BV31 were only comparable to the control, which demonstrated the lowest aerobic stability. The aerobic stability of the silages did not differ from the control treatment; however, the Bacillus strains showed promise in controlling yeast metabolism. In phase 2, the harvested forage was treated with *Bacillus* sp. inoculants (BV02, BV09, BV26, BV30, and BV31) and commercial lactobacillus-based inoculants (*Lentilactobacillus buchneri* LB and *Lactiplantibacillus plantarum* LP). For LA variable, the additives treatments showed differences only inside 12h and the highest contents were found for LP (0.29 vs. 0.87 % of MScorr). For 90d of storage, the treatments BV02, BV09, BV26 and BV30 had the highest values for LA (5.09, 4.94, 5.19, 5.05 vs. 2.58 % of DMcorr, respectively). The growth of BAL was favored because the bacillus trial counts were greater than 4 log cfu/g for the treatments. Strains BV02 (166h) and BV30 (174h) had similar aerobic stability as LB treatment (216h), and strain BV09 (140h) presented longer aerobic stability than the Control (70.3h). LB treatment in 45 days of storage presented the highest acetic acid contents (1.08 % of DMcorr) and bacillus treatment had the same behavior as the control, except by BV31 equalized as LP. For 90 days of storage, BV09 was statistically equal to LB treatment (0.8 vs 1.08 % DM Corr) but the other bacillus treatments had similar values as the Control and LP. LB treatment had the highest quantities of propionic acid (0,1% DMcorr) and LP, the lowest (0.009% DM). The other treatments had intermediate contents but the bacillus rehearsals that showed similar aerobic stability as LB, such as BV02, BV09, and BV30 demonstrated high quantities of this acid (0.04, 0.06, and 0.01% DMcorr, respectively). The silages produced under inoculation with *Bacillus* strains integrated desirable quality fermentation characteristics and aerobic deterioration control.

Keywords: Silage, Aditive, Aerobic stability, Yeast

**LIST OF TABLES**

## 1. INTRODUCTION

The ensiling dates back over 3000 years, with ancient civilizations like the Egyptians and Greeks preserving grains and entire forage crops in storage silos (Wilkinson et al., 2003). In 1984, Woolford described silage as "the product formed when grass or other material of sufficiently high moisture content, liable to spoilage by aerobic microorganisms, is stored anaerobically". This statement emphasizes that the key factor in successful silage production lies in the dynamic interactions of microorganisms during the process. Therefore, biological inoculation has become the prevailing method used to modulate the fermentation process in silage production (Wilkinson and Muck, 2019). This process can be categorized into four primary stages: the initial aerobic phase, the intense fermentation phase, the stable phase, and the aerobic feed-out phase (Weinberg and Muck, 1996; Pahlow et al., 2001).

During the initial three stages, the crucial factor determining the quality of ensiled forage is the initial drop in pH (Wang et al., 2009; Muck et al., 2018). To achieve this, homofermentative inoculants are extensively utilized, with *Lactiplantibacillus plantarum* being one of the most renowned strains (Kung et al., 2018). This acidification process arises from the fermentation of hexoses, such as glucose, by these bacteria. It predominantly yields lactic acid, which is the primary strong acid in silage (Mc Donald, 1991; Muck et al., 2018). However, for the aerobic feed-out phase, lactic acid alone does not suffice to uphold the aerobic stability of the mass, as yeasts play a pivotal role in instigating aerobic spoilage. These yeasts, which assimilate lactate, have the potential to elevate the silage pH, thereby facilitating the proliferation of other harmful microorganisms (Driehuis et. al, 1999). To regulate yeast activity, acetic acid plays a significant role. This is due to the ability of protonated acetic acid to penetrate the plasma membrane, causing intracellular acidification and triggering programmed cell death (Burtner et al., 2009). Considering this requirement, the study conducted by Oude Elferink et al. (2001) served as the foundation for heterofermentative inoculants. This research demonstrated the capability of *Lentilactobacillus buchneri* to convert moderate quantities of lactic acid into acetic acid and 1,2-propanediol. Following these findings, several studies substantiated the strain's ability to manage aerobic stability effectively (Ranjit and Kung, 2000, Mari et al., 2009; Kristensen et al., 2010; Tabacco et al., 2011).

In silage production, most commercially accessible inoculants generally contain one or both varieties of lactic acid bacteria. However, investigating novel organisms capable of thriving in silage environments, including specialized soil inoculants, represents a means to enhance silage conservation technologies. This exploration can lead to the identification of shared attributes

within a single inoculant, such as the acidification of forage and enhancement of aerobic stability. *Azospirillum brasilense* is a microorganism that thrives in both anaerobic and aerobic conditions, yet it predominantly operates in low-oxygen environments, especially when nitrogen is present in the medium. This metabolic preference is vital for reducing oxygen levels in fresh forage, shortening the aerobic phase during silage production, and promptly inhibiting the growth of molds, yeasts, and certain bacteria (Okon and Itzigsohn, 1995; Pahlow et al., 2003). Similarly, Bradyrhizobium elkanii semia, which is a nitrogen-fixing microorganism thriving in aerobic environments, effectively consumes oxygen during the aerobic phase of silage production. Moreover, when oxygen is scarce, these advantageous bacteria produce nitrite, which exhibits antimicrobial properties (Klebanoff, 1993; Polcyn and Luciński, 2003). Another advantageous trait of this microorganism is its capability to produce surface polysaccharides, as noted by Bomfeti et al. (2011). These polysaccharides can be utilized by lactic acid bacteria, enhancing their functionality in the silage environment.

Bacillus subtilis stands out as a promising inoculant for controlling spoilage microorganisms in silage (Phillip and Fellner 1992; Basso et al., 2012). These bacteria are commonly found in soil and play a significant role in the rhizosphere. Investigated for its fungicidal properties, B. subtilis demonstrates a wide-ranging antibiotic effect against both fungi and bacteria, primarily through lipopeptides cyclic compounds like surfactin, iturine, and fungicin. Additionally, under anaerobic conditions, B. subtilis induces the expression of genes responsible for lactate and acetate production(Todovora and Kozhuharova, 2009; Lanna et al., 2010). Studies by Basso et al. (2012) indicated that the inoculation of B. subtilis led to a reduction in yeast levels and increased aerobic stability in whole-plant corn silage. Similar outcomes were observed by Lara et al. (2016), where silages inoculated with B. subtilis exhibited lower yeast incidence after one day of air exposure and demonstrated improved aerobic stability.

The objective of those studies was to examine the pH decline patterns in corn silages treated with the new strains, analyze their dry matter and nutrient composition, evaluate fermentative properties, and assess aerobic stability. Additionally, establish comparisons between silages inoculated with *Bacillus* sp., *Azospirillum* sp., and *Bradyrhizobium* sp. against control silages or those treated with commercial inoculants based on homo or heterolactic *Lactobacillus*.

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## 2. WHOLE-PLANT CORN SILAGE TREATED WITH *Azospiriullum* SP., *Bradyrhizobium* SP. OR *Bacillus* SP. COMPARED WITH COMMERCIAL INOCULANTS


### Abstract

Exploring unconventional microorganisms used in agricultural crops for different productive and health-related purposes, which can thrive in a silage environment, represents a pathway to enhance silage conservation technologies. This study aimed to investigate the fermentation patterns in corn silages treated with nine newly isolated strains. Furthermore, it sought to establish comparisons between these treated silages and those inoculated with *Bacillus* sp., *Azospirillum* sp., and *Bradyrhizobium* sp., in contrast to control silages or those treated with commercially available inoculants containing homo or heterolactic *Lentilactobacillus* strains. The experiment was conducted over two days, designated as Experiment 1 and Experiment 2. In Experiment 1 were utilized the *Azospirillum brasiliense* (ABV5, ABV6) and *Bradyrhizobium elkanii semia* (BV36, BV27) and in Experiment 2, the *Bacillus subitilis* (BV02, BV09, BV30, BV31) and *Bacillus* sp. (BV26) and in both experiments, the inoculants were compared with a control treatment and with *Lentilactobacillus*. The harvested forage was divided into four separate piles, each treated with the inoculant to create four repetitions. Chemical composition, fermentation characteristics and chromatography, microbial count, and aerobic stability were analyzed. In Experiment 1, after 45 days of storage, the LB treatment showed the highest pH compared to the other treatments (3.85 vs 3.70% DM). The same pattern was observed after 90 days of storage, with ABV5, ABV6, BV36, and BV37 also exhibiting similarly high pH concentrations. Among the bacterial populations, the LB treatment revealed the highest count for LAB (8.20 *vs.* 6.10 log cfu.g$^{-1}$), followed by LP (7.29 *vs.* 6.25 log cfu.g-$^{1}$). The *Bradyrhizobium* and *Azospiriullum* treatments did not differ from the control treatment for other fermentation characteristics and aerobic stability. In Experiment 2, lactic acid (LA) contents demonstrated an interaction between additives and storage time. When considering time within additives, all treatments exhibited an increase in LA contents, except for LB treatment which remained unchanged. Examining additives within time, at 90 days, new strains BV09 and BV26 had the highest contents, with BV02, BV30, BV31, and LP displaying similarities to them, as well as to the Control treatment, and LB treatment had the lowest content. The LEV displayed a decline in the count when considering the impact of time (4.04 to 3.44 log cfu.g$^{-1}$). The LP treatment yielded the highest count (5.03 vs 3.55 log cfu.g$^{-1}$), while the lowest was observed in the LB treatment (1.99 vs 3.99 log cfu.g$^{-1}$). The remaining treatments exhibited similarities to the Control. Regarding ethanol, a significant interaction was observed (P value < 0.001). Considering time within additives, Control and LP exhibited an increase, while the other treatments remained consistent. Analyzing additives within time, at 45 days, LP displayed the highest content (0.68 vs. 0.11 % of DM correction), with the others being lower and similar among themselves. At 90 days, LP again showed the highest content, followed by Control, while BV09 and BV30 exhibited the lowest contents. The aerobic stability increased from 45 days to 90 days (38.5 vs. 49.0 hours). Among the additives, LB exhibited the longest duration (64.4 vs. 40.7 hours), followed by LP (50.8 vs. 42.7 hours). BV09, BV26, and BV30 showed similarity in stability to LP and Control treatments. However, BV02 and BV31 were only comparable to the control, which demonstrated the lowest aerobic stability. The aerobic stability of the silages did not differ from the control treatment; however, the *Bacillus* strains showed promise in controlling yeast metabolism.


Keywords: Additives, Aerobic stability, Unconventional microorganisms

## 2.1. Introduction

Ensiling is a millennial biologic method of preservation of fresh crops based on spontaneous lactic acid fermentation under anaerobic conditions. In 1980 and 1990, the researchers focused on understanding silage microbiology and bacterial inoculants' role, which evidenced the effectiveness of these additives in enhancing silage preservation and maximizing production outcomes (Wilkinson et al., 2003).

Biological products play a significant role in the vision of future agriculture. Over the past decade, farmers' perception has recognized a noticeable shift, leading to increased use of microbial bio-inputs. For silage, the majority of commercially available inoculants typically include one or both types of lactic acid bacteria (LAB) (Muck et al., 2018). Typically, inoculants containing facultative heterofermentative LAB like *Lactiplantibacillus* plantarum ferment water-soluble carbohydrates into organic acids, particularly lactic acid. This process results in a rapid reduction of silage pH and effectively suppresses the growth of undesirable bacteria (Queiroz et al., 2013). On the other hand, heterolactic inoculant bacteria like *Lentilactobacillus buchneri* metabolize water-soluble carbohydrates into antimicrobial acids, such as acetic and propionic acids, effectively inhibiting the growth of fungi responsible for spoilage (Elferink et al., 2001; Kung et al., 2018).

In Brazil, soil inoculants for biological $N^2$ fixation are widely used and contribute to the economic viability of the soybean and maize crop, making it less dependent on N-fertilizers (Garcia et al., 2021; Hungria, 2019). Exploring potential new organisms resistant to a silage environment, including specific soil inoculants, is a way to improve technologies in silage conservation and discover common effects in a single inoculant, such as forage acidification and aerobic stability.

*Azospirillum brasilense* is an anaerobic or aerobic organism but is preferentially microaerophilic in both the presence and absence of combined nitrogen in the medium (Okon and Itzigsohn, 1995). This metabolism can be essential to consume oxygen in fresh forage, minimize the aerobic phase of silage, and control molds, yeasts, and some bacteria as soon as possible (Pahlow et al., 2003). However, under oxygen-limiting conditions, these plant-growth-promoting bacteria are also responsible for producing nitrite by the dissimilatory nitrate reduction pathway in addition to nitric oxide (NO) and nitrous oxide ($N^2O$) (Hartmann and Zimmer, 1994) and this compost has antimicrobial effects (Klebanoff, 1993) what can be applied for the spoiled microorganism in silage. In a similar vein, *Bradyrhizobium elkanii semia*, a nitrogen-fixing aerobic organism, could efficiently utilize oxygen during the aerobic silage phase and under conditions where oxygen is limited, these beneficial bacteria are also accountable for the production of nitrite (Polcyn and

Luciński, 2003). Another characteristic of those rhizobial microorganisms is their ability to produce surface polysaccharides, specifically exopolysaccharides, comprised of neutral glucans or hexose residues (Bomfeti et al., 2011). Lactic acid bacteria utilize this last compound to generate lactic acid and acetic acid, which will enhance the preservation of silage (Muck et al., 2018).

*Bacillus subtilis* is another microorganism known for its remarkable resilience in adverse environments, similar to those encountered in silage (Guo et al., 2022). Significantly, the inoculation of *Bacillus subtilis* has shown the potential to improve silage fermentation. This is attributed to its broad-spectrum antibiotic activity against fungi and bacteria, and its ability to induce the expression of genes responsible for lactate and acetate production under anaerobic conditions (Yin et al., 2023; Todovora and Kozhuharova, 2009; Cruz Ramos et al., 2000).

The objective of this study was to examine the pH decline patterns in corn silages treated with 9 new strains, analyze their dry matter and nutrient composition, evaluate fermentative properties, and assess aerobic stability. Additionally, establish comparisons between silages inoculated with *Bacillus* sp., *Azospirillum* sp., and *Bradyrhizobium* sp. against control silages or those treated with commercial inoculants based on homo or heterolactic *Lactobacillus*.

## 2.2. Material and Methods

### Silage

Whole-plant corn silage was harvested at the University of São Paulo, Brazil, with a DM content of 35%. The treatments were categorized into Experiment 1 and Experiment 2, with the same harvested forage employed for both sets of experiments. Therefore, for each replication of every treatment, a clean plastic canvas was employed to apply the additive to the forage. After the application, two experimental silos of 20-liter-bucket were produced (each designated for a specific opening time of 45 days or 90 days), and 3 mini-bags of approximately 500g each were prepared to study the silage acidification dynamics over time (6,12 and 24 hours). For each subsequent replication, a new clean plastic canvas was utilized. In Experiment 1, inoculants based on *Azospirillum* sp. and *Bradyrhizobium* sp. were applied at the same dose of 4.5 x 107 g/kg of fresh forage, utilizing the following strains: ABV5 (*Azospirillum brasiliense*), ABV6 (*Azospirillum brasiliense*), BV37 (*Bradyrhizobium elkanii semia 5019*), and BV36 (*Bradyrhizobium elkanii semia 587*). In Experiment 2, inoculants based on Bacillus sp. were used at the same dose as Experiment 1, incorporating the following strains: BV02 (*Bacillus subtilis*), BV09 (*Bacillus subtilis*), BV30 (*Bacillus subtilis*), BV31 (Bacillus subtilis), and BV26 (*Bacillus* sp.). In both experiments, aside from these treatments, silages without any additives (control treatment) were produced, as well as silages

treated with commercial inoculants LB (*Lentilactobacillus buchneri*), and LP (*Lactiplantibacillus plantarum*). The applied dose was an experimental quantity chosen to facilitate the observation of microorganism fermentation. In both experiments, four replications were conducted for each treatment in the silo buckets, and three replications were performed for the bags.

The buckets were unsealed at two different time points, 45 and 90 d after ensiling, while the vacuum bags were opened after 6, 12, and 24 h of storage. Each bucket was unsealed on a tarp, which was subsequently disposed of after sample collection. The contents of the bucket and vacuum bags were thoroughly mixed, and samples were randomly collected.

**Laboratory analysis**

Silage samples were dried in a forced ventilation oven for 72h at 55°C. Then, ground through a 1-mm screen (Wiley Mill, Arthur H. Thomas). Sub-samples were analyzed for DM (AOAC, 1990; methods 934.01). Sub-samples were analyzed for DM, ash, and ether extract (EE) (AOAC, 1990; methods 934.01, 942.05 and 920.39, respectively). The ash-free neutral detergent fiber (NDF) was analyzed by filtration in a porous crucible with sodium sulfite and heat-stable amylase (Mertens, 2002). The ash-free acid detergent fiber (ADF) was analyzed by filtration in the porous crucible (Mertens, 2002). Starch content was determined by an enzymatic method according to (Hall, 2009). The crude protein (CP) was measured by the Dumas method (Leco® FP-2000A nitrogen analyzer; Leco Corp., St. Joseph, MI). Soluble protein (% of CP) was estimated by submitting the samples to a borate phosphate buffer bath at 39°C for 1 h and filtered on Whatman™ N° 541 filter paper (Krishnamoorthy et al., 1982). Soluble protein content was estimated from the difference between the silage sample CP content and the residual CP content of the filtered sample. The bromatological characteristics of the fresh corn-whole plant forage of both Experiments are present in Table 1.

Table 1. Chemical characteristics of whole crop corn fresh forage

| | DM, % | NDF, % | OM, % | CP, % | ASH, % |
|---|---|---|---|---|---|
| Experiment 1 | 37,1 ± 1,6 | 49,9 ± 1,6 | 95,5 ± 0,5 | 7,1 ± 0,3 | 4,5 ± 0,5 |
| Experiment 2 | 38,0 ± 1,8 | 45,3 ± 1,7 | 95,7 ± 0,5 | 7,0 ± 0,10 | 4,3 ± 0,5 |

**Fermentation profile and chromatography**

Silage samples were collected in the opening of the buckets and vacuum bags and an aqueous extract was prepared (25 g + 225 g of deionized water). The pH was measured (DM 20 pH meter, Digimed Analitica, São Paulo, Brazil), and fermentation end products were determined by gas chromatograph with a mass detector (GCMS QP2010 Plus, Shimadzu, Kyoto, Japan),

using a capillary column (Stabilwax, Restek, Bellefonte, PA, 60 m, 0.25-mm, i.d., 0.25, 09 m), lactic acid was measured by colorimetry (Pryce, 1969). The dry matter content, corrected for volatiles, was obtained as follows (Weissbach, 2009): DMcorr (g/kg as fed) = DMoven (g/kg as fed) + acetone (g/kg as fed) + n-alcohols (g/kg as fed) + 2-butanol (g/kg as fed) + 2,3-butanediol (g/kg as fed) + 0.95 × volatile fatty acids (g/kg as fed) + 0.77 × 1,2-propanediol (g/kg as fed) + 0.08 × lactic acid (g/kg as fed). The alcohols included methanol, ethanol, and propanol, and the volatile fatty acids included acetic acid, propionic acid, iso-butyric acid, and n-butyric acid.

**Microbial count**

Microbial profile analyses used the aqueous extract supernatant described before that was mixed into decimal sequential dilutions ($10^1$ to $10^6$) and plating was in triplicate. Lactic acid bacteria count (LAB) was performed in an MRS culture medium with antifungal Natamicine (0.25 g/L). Yeast count (LEV) was made from plating in a malt agar plus lactic acid at 0,5% concentration. The plates were incubated at 32 °C for 48h and the colonies were counted.

**Aerobic stability**

Aerobic stability was defined as the time elapsed until silage temperature increases 2°C above room temperature (O'Kiely, 1993). Samples (3kg) were weighed into plastic buckets and exposed to air for 10 days in a room with a controlled temperature. The temperature of samples and room was recorded every 15 minutes using dataloggers (iMini, Impac, São Paulo, Brazil).

**Statistical analysis**

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC), as a completely randomized design with a factorial arrangement of treatments, 8(1 control and 7 inoculants) x 2 lenght of storage, and 4 replicates per treatment. The model was following: $Yij = \mu + Ai + Tj + ATij + eij$, where $\mu$ = overall mean, Ai = fixed effect of additive (i = BV02, BV09, BV30, BV23, BV26, LB, LP or CONT), Tj = fixed effect of storage length (j = 45 or 90 days), ATij = interaction between additive and length of storage, and eij = error. The 20-liter buckets were represented as the experimental unit. For bags, the factorial arrangement of

treatments was 8 inoculants x 3 times of storage (6,12 and 24 hours), and 3 replicates per treatment. The covariance structure for the repeated measurements was chosen based on Akaike's information criterion among variance components (VC), compound symmetry (CS), first-order autoregressive (AR(1)), or unstructured covariance structure (UN). All means were compared using Tukey's test at 5% probability. Statistical significance was considered at $P \leq 0.05$.

## 2.3. Results and Discussion

### Experiment 1

Lactic acid, produced by lactic acid bacteria, is a common and abundant acid in corn silage, with a strong acidification power (Kung et al., 2018). Therefore, as the concentration of lactic acid increases over time after sealing, the pH of the silage decreases, controlling spoilage microorganisms (Pahlow et al., 2003, Ellis et al., 2016). Table 2 presents the pH and lactic acid data at 6, 12, and 24 hours after the ensiling on day 1. Notably, strains of the same species (the *Azospirillum* group and the *Bradyrhizobium* pair) showed similar pH values during the initial six hours of fermentation, indicating a homogeneous application of the inoculant in the mass. In addition, LB showed untypical 6-hour low pH for a heterolactic bacteria (4.25), which converts lactic acid to acetic acid, a weak acid (Elferink et al., 2001).In this case, LP exhibited the highest pH at 6 hours compared to all other treatments (4.96). However, the microorganisms gradually move towards similar and suitable pH values over time for the first day of a closed silo. The inclusion of homofermentative LAB during the ensiling of forage can improve the fermentation process by generating elevated levels of lactic acid and quickly lowering the pH of the silage (Ranjit and Kung, 2000), as observed in the LP treatment of Experiment 1 where the highest concentrations of lactic acid were found (3.64% DM), indicating that lactic acid performed as expected. In a similar vein, the lowest content of lactic acid was observed in the LB treatment in Experiment 1 (2.31% DM), which aligns with expectations due anaerobic conversion of moderate amounts of lactic acid to acetic acid and 1,2-propanediol (Oude Elferink et al., 2001). During the initial 24 hours of fermentation, it was observed that *Azospirillum* and *Bradyrhizobium* did not exhibit any significant effect in modulating the fermentation process. Wood et al. (1998) demonstrated that at a pH of 4.5, the membrane potential of Azospirillum was depolarized, which could account for the underdevelopment of this bacteria in silage. Strains of Bradyrhizobium exhibit a broad range

of pH tolerance (Zahran et al., 1999), yet in this experiment, they do not appear to alter the fermentation profile.

The nutrient profile presented in Table 3 reveals silages with appropriate bromatological composition and similar variations among the new strains. Overall, the strains contributed to the preservation of silage nutrients.

Understanding the fermentation patterns in silage is essential to provide insight into the metabolic activity of the new strains. In the case of *Azospirillum* and *Bradyrhizobium*, there was a pH increase over the storage time, whereas this trend was not observed in the Control, LP, and LB treatments. After 45 days of storage, the LB treatment showed the highest pH compared to the other treatments (3.85% DM). The unconventional strains exhibited low values at 45 days, comparable to those of LP and the Control. However, by 90 days, their values were similar to those of LB. However, the new strains exhibit diverse and non-homolactic metabolisms, while still ensuring effective acidification of the mass. Conversely, the non-Lactobacillus strains exhibited an upward trend in pH values over time, from 45 to 90 days, highlighting their heterofermentative metabolism.

The pH drop is linked to the growth of lactic acid bacteria (LAB) and the lactic acid produced by them. It is noteworthy that lactic acid bacteria constituted the predominant bacterial group across all categories of corn silages (Kalúzová et al., 2022). As indicated in Table 5 for Experiment 1, the new strains did not disrupt the development of epiphytic LAB, as their values were comparable to the control. The acid lactic production was consistent with LAB account and pH drop results, and the strains of *Azospirillum* and *Bradyrhizobium* contributed to efficient acidification of the silages, but this positioning, concerning traditional inoculants, makes these strains less promising as they were similar to the control. However, the unexpected increase in lactic acid over time contradicts the typical pattern observed in corn silages, where there is usually an increase in heterolactic bacteria (Okoye et al., 2023). This discrepancy may be attributed to the fact that tropical grasses tend to have higher populations of heterofermentative lactic acid bacteria (LAB) compared to temperate grasses (Li et al., 2019).

Homolactic fermentation enhances silage quality by expediting the initial stage of ensiling through the rapid fermentation of water-soluble carbohydrates (WSC) into lactic acid,

leading to a swift decrease in pH. On the other hand, heterolactic fermentation generates a significant amount of acetic acid, effectively preventing aerobic deterioration during the ensiling process (Borreani et al., 2018). In line with the low efficiency in yeast control in Experiment 1, the acetic acid levels of the new strains were comparable to those of the control silage and consistently lower than LB and LP.

The ethanol contents also indicate poor yeast control, as ethanol is a product of yeast fermentation (Weiss et al., 2016). In Experiment 1, the LP treatment demonstrated elevated ethanol contents (0.67 at 45d and 1.28 at 90 d), as anticipated since lactic acid is not as effective in inhibiting yeast growth compared to acetic and propionic acid (Moon, 1983; Elferink et al., 2001). The ethanol levels of the new strains were similar to those of *L. buchneri*, but also comparable to the control, asserting the observation of lower aerobic stability for the new strains when compared to LB. Therefore, this positioning, concerning traditional inoculants, makes these strains less promising as they were similar to the control.

Upon exposure to air during silo opening or after removal, fermentation acids and other compounds in silage undergo oxidation by aerobic bacteria, yeasts, and molds. The silage's aerobic stability is crucial to ensure it delivers well-preserved nutrients to animals with minimal mold spores and toxins (Wikinson and Davies, 2013). As demonstrated earlier, there is a correlation between acetic acid levels and yeast control, which subsequently affects aerobic stability. This is because protonated acetic acid can permeate the plasma membrane, leading to intracellular acidification and programmed cell death (Burtner et al., 2009). In Experiment 1, as mentioned earlier, confirming the low efficiency in yeast control, the acetic acid levels of the new strains were similar to those of the control silage and consistently lower than LB. This corroborates the finding of lower aerobic stability for the new strains compared to LB (Table 5).

It is well-established that propionic acid acts as an inhibitor of yeasts and molds, thus enhancing silage preservation (Kung et al., 2003; Morais et al., 2017). Furthermore, it plays a crucial role in maintaining aerobic stability during feed-out (Coblentz and Akins, 2021). Nevertheless, in both Experiment 1 and 2, there was no consistent pattern of increased production of this compound by the tested strains. Butyric acid indicates clostridial fermentation, which can lead to poorly preserved silage (Gibson, 1965; Zheng et al., 2018). In those experiments, overall, the butyric acid concentrations were minimal, indicating good fermentation.

**Experiment 2**

In forage preservation, the anaerobic fermentation process involves converting water-soluble carbohydrates (WSC) into organic acids, primarily lactic acid. This conversion leads to a decrease in pH and inhibits the growth of undesirable microorganisms, facilitated by lactic acid bacteria (LAB) (Dunière et al., 2013). The pH of freshly harvested whole-plant corn typically falls within the range of approximately 5.5 to 6. These initial pH values decrease during the ensiling process due to lactic acid production by lactic acid bacteria (LAB) (Kung et al., 2018). It was revealed that the Bacillus strains exhibited very similar pH values at the three measurement points (6, 12, and 24 hours), indicating that they had already achieved a higher degree of acidification compared to the control silage before the 6-hour mark. The control silage gradually reduced its pH and became similar to the other treatments after 12 hours. Bai et al. (2022) demonstrated that *Bacillus subtilis* inoculations of silage led to higher lactic acid concentrations in whole-plant corn silages on day 1 of ensiled compared to the control group. This increase in lactic acid can be attributed to the fermentative pathways within the Bacillus cells, where pyruvate is converted into lactic acid (Seo et al., 2021). In control, the pH values can be explained by the lactic acid concentrations starting at only 0.7% and ending at 2.5%. The pH changes in the other treatments were consistent with the pH variations observed.

Table 10 presents the pH values for Experiment 2, and it can be observed that, except for *Lentilactobacillus buchneri*, which had a pH of 4, the other treatments resulted in pH values below 3.71. This efficient acidification is a benefit of *Bacillus* inoculation. Although the pH difference between 45 and 90 days of ensiling was only around 0.06 pH units, it was statistically significant. This observation, combined with the results from the bags, confirms that the *Bacillus* strains facilitate the pH reduction within the silo (Lara et al., 2015).

In well-preserved silage, the rapid dominance of lactic acid bacteria (LAB) during the initial fermentation process ensures a stable and low pH in the forage. This dominance prevents spoilage microorganisms from surviving, contributing to the effective preservation of the silage. (Yang ate al., 2019). For Experiment 2, the LAB count in the LB silage was higher than in the other treatments. The BV02 strain of Bacillus subtilis stood out for modifying the fermentation environment while ensuring high LAB counts coexist. In general, LAB counts exceeding 5 log $cfu.g^{-1}$ indicated satisfactory silage preservation. Another essential characteristic of these silages

was the high production of lactic acid, especially at 90 days. Bai et al. (2020) demonstrated the silage treated with *B. subtilis* had the highest concentration of lactic acid. This can be attributed to the accelerated growth of homofermentative lactic acid bacteria (LAB) facilitated by the antimicrobial peptide-producing bacillus during the fermentation process. As observed in Experiment 1, the LAB count was higher than the count at 90 days of evaluation

Acetic acid is an important yeast control. Due to its pKa of 4.76 and the intracellular pH being generally higher than that of common media, acetic acid dissociates upon cell entry (Chaves et al., 2021). This accumulation, based on the pH differential, leads to yeast inactivation or death. The higher the presence of yeasts capable of assimilating lactate, the faster the silage begins to spoil, leading to increased deterioration (Auerbach et al., 2021). In Experiment 2, the new strains exhibited promising acetic acid production for 90d, surpassing that of the control, except for BV26. Acetic acid serves various biological functions, including acting as a metabolic signal within bacteria. In the case of *B. subtilis*, its formation occurs when glycerol is utilized as a carbon source, undergoing a sequential conversion process from glycerol to pyruvate, then acetyl-CoA, followed by acetyl-phosphate, ultimately resulting in acetate production (Chen et al., 2015). In the case of LEV control, the new strains exhibited lower counts compared to the LP treatment, which may be attributed to acetic acid production.

During the feed-out phase, the reintroduction of oxygen into the silage can lead to temperature spikes in unstable silages. This occurs because spoilage microorganisms, like yeasts, oxidize fermentation by-products, such as lactic acid, to fuel their growth (Pahlow et al., 2001; Wikinson and Davies, 2013). In Experiment 2, the responses in aerobic stability correlated with yeast counts, with *Bacillus* silages displaying similar times to the Control for stability breakdown. A better understanding of stability responses can be obtained by examining the acetic acid concentrations in the silages (Table 10). However, the acetic acid production by these strains was not significant enough to affect yeast counts and silage heating. Nevertheless, it does make these strains more promising for further research endeavors.

## 2.4. Conclusion

In conclusion, Experiment 1 revealed that the *Azospirillum* sp. and *Bradyrhizobium* sp. strains exhibited efficient fermentation profile but did not demonstrate distinctive qualities that would warrant their use as silage inoculants. In Experiment 2, the aerobic stability of the treated silages were similar to the control treatment. Nevertheless, the *Bacillus* strains displayed potential in controlling yeast metabolism, based on their enhanced acetic acid concentration. This supported the hypothesis of combining effective acidification with potential improvements in aerobic stability.

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# 3. AEROBIC SPOILAGE CONTROL IN WHOLE PLANT CORN SILAGE TREATED WITH *Bacillus subtilis* COMPARED TO *Lactiplantibacillus plantarum* AND *Lentilactobacillus buchneri* INOCULANTS


## Abstract

Modulating the microbiological environment in silages through additives is a pathway to enhance the beneficial organic acids profile and control spoilage microorganisms. Therefore, a microorganism additive that acidifies the mass and promotes aerobic stability is essential to ensure silage with minimal losses. In this study, the harvested forage was divided into four piles, each treated separately with *Bacillus* sp. inoculants (BV02, BV09, BV26, BV30, and BV31) and commercial lactobacillus-based inoculants (*Lentilactobacillus buchneri* LB and *Lactiplantibacillus plantarum* LP). the harvested forage was divided into eight parts for each of the eight. Each part was subdivided into three piles and treated separately with the inoculant to create three replicates for each treatment. From each pile, two silos of 20 liters were produced (each designated for a specific opening time of 45 days or 90 days), and 3 mini-bags of approximately 500g each were prepared to study the silage acidification dynamics over time (6,12 and 24 hours). The silos were labeled R1 to R3, and the mini-bags R1 to R3. The chemical composition, fermentation characteristics, chromatography, microbial count and aerobic stability were analyzed. Data were analyzed using the SAS MIXED procedure, and the Tukey-Kramer significance test ($P<0.05$) was applied for Treatment, Time, and Treatment*Time interaction. In this experiment, in the first 6 hours, the control has the highest pH and the strains resemble LP and LB treatments, the last one with the lowest value. For LA variable, the additives treatments showed differences only inside 12h and the highest contents were found for LP (0.29 vs. 0.87 % of MScorr). For 90d of storage, the treatments BV02, BV09, BV26 and BV30 had the highest values for LA (5.09, 4.94, 5.19, 5.05 vs. 2.58 % of DMcorr, respectively). The growth of BAL was favored because the bacillus trial counts were greater than 4 log cfu.g$^{-1}$ for the treatments. Strains BV02 (166h) and BV30 (174h) had similar aerobic stability as LB treatment (216h), and strain BV09 (140h) presented longer aerobic stability than the Control (70.3h). LB treatment in 45 days of storage presented the highest acetic acid contents (1.08 % of DMcorr) and bacillus treatment had the same behavior as the control, except by BV31 equalized as LP. For 90 days of storage, BV09 was statistically equal to LB treatment (0.8 vs 1.08 % DM Corr) but the other bacillus treatments had similar values as the Control and LP. LB treatment had the highest quantities of propionic acid (0,1% DMcorr) and LP, the lowest (0.009% DM). The other treatments had intermediate contents but the bacillus rehearsals that showed similar aerobic stability as LB, such as BV02, BV09, and BV30 demonstrated high quantities of this acid (0.04, 0.06, and 0.01% DMcorr, respectively). The silages produced under inoculation with bacillus strains integrated desirable quality fermentation characteristics and aerobic deterioration control.


Keywords: Silage, Aditive, Aerobic stability, Acetic acid, Lactic acid

## 3.1. Introduction

Brazil represents the third world's largest producer of maize with 22.7 million hectares of area harvested in 2022/23 (USDA, 2022). In addition, an increase in housed systems in dairy farms and beef cattle is observed in the country (ANUALPEC, 2022) and these systems require conserved forage all year which may improve the production of silages. In tropical areas, maize silage is the most energy-dense forage source used for ruminants (Daniel et al., 2019). This forage

plant also has desirable fermentable characteristics including the possibility of harvest with suitable dry matter (DM) content, low buffering capacity, and adequate water-soluble carbohydrates (WSC) level (McDonald et al., 1991). Even with previous features, the insertion of additives in the silage production process is a pathway to modulate the microbiological communities (Xu et al., 2020; Sun et al., 2021) and to increase the beneficial organic acids profile in silage (Muck et al., 2018; Kung et al., 2018), mitigating losses to have a competitive final product.

Biological additives are an important technology for affording quality corn silage and approximately 25% of Brazilian farmers use inoculants (Daniel et al., 2019; Bernardes & Do Rêgo, 2014). They are safe and easy to handle, noncorrosive to machinery, and less polluting than chemical additives (Henderson, 1993; Fylai, 2003). Besides that, these additives add beneficial microorganisms to dominate fermentation which inhibits the growth of undesirable anaerobic organisms (e.g., enterobacteria and clostridia) and the growth of aerobic microorganisms responsible for panel heating, indicating oxidation losses (Kung et al., 2003). Homofermentative lactic acid bacterias (LAB) such as *Lactiplantibacillus plantarum* has been one of the first bacterial inoculants used and they are still applied to help pH drop in silage (Muck and Kung, 1997, Wilkinson et al., 2003). However, high lactic acid content decreases aerobic stability in the mass because some microorganisms like yeast degrade this acid in oxygen presence (Keshri et al., 2018). Aerobic stability is a term used to determine how long silage will stay cool and not spoil after exposure to air. After fermentation is complete and the silage has been exposed to air during feeding or storage, yeasts usually initiate heating and dropping the pH, opening the opportunity for another spoilage microorganism (Woolford, 1990). These microorganisms produce end products that reduce animal performance and can affect immune function (Kung et al., 2010). Ensuring aerobic stability using heterofermentative additives to avoid spoilage silage in the feed-out phase is sustainable management. An example is *Lentilactobacillus buchneri* which converts lactic acid to acetic acid upgrading the aerobic stability of the silage by controlling yeasts (Elferink et al., 2001). Acetic acid is found undissociated at a low pH silage environment and in this form is capable of diffusing across the yeast cell membrane, causing changes in the pH environment of the cytosol and inhibiting many metabolic functions (Piper et al., 2001). In this context, efforts to study a microorganism additive that acidifies the mass and promotes aerobic stability are essential to ensure silage with minimal losses.

*Bacillus subtilis* is a promising inoculant to control spoilage microorganisms from silage (Phillip and Fellner 1992; Basso et al., 2012). These bacteria are habitual in soil and they are an important population of the rhizosphere (Lanna et al., 2010). Studied as a fungicide, *B. subtilis* exhibits

broad-spectrum antibiotic activity against fungi and bacteria (Todovora and Kozhuharova, 2009), the main ones are lipopeptides cyclic compounds belonging to the surfactin, iturine, and fungicin family (Lanna et al., 2010). Moreover, under anaerobic conditions, the expression of genes that produce lactate and acetate is induced (Cruz Ramos et al., 2000). Basso et al. (2012) found that doses of *B. subtilis* inoculation decreased the yeast account and increased the aerobic stability in whole-plant corn silage. Similar results were found by Lara et al. (2016), when silages inoculated had a lower incidence of yeasts with one day of air exposure and presented better aerobic stability. Therefore, the objective of this study was to detail aerobic spoilage control in whole-plant corn silage treated with a new strain of Bacillus-based inoculants compared to Lactobacillus sp. inoculants.

## 3.2. Materials and Methods

### Silage

Whole-plant corn silage was harvested at the University of São Paulo, Brazil, and treated with inoculants containing strains of BV02 (*Bacillus subtilis*), BV09 (*Bacillus subtilis*), BV30 (*Bacillus subtilis*), BV31 (*Bacillus subtilis*), BV26 (*Bacillus sp.*), LB (*Lentilactobacillus buchneri*), and LP (*Lactiplantibacillus plantarum*), each at a dose of 4.5 x $10^7$ g/kg of fresh forage. Additionally, a control treatment without any additives was prepared (CONT).

the harvested forage was divided into eight parts for each of the eight. Each part was subdivided into three piles and treated separately with the inoculant to create three replicates for each treatment. From each pile, two silos of 20 liters were produced (each designated for a specific opening time of 45 days or 90 days), and 3 mini-bags of approximately 500g each were prepared to study the silage acidification dynamics over time (6,12 and 24 hours). The silos were labeled R1 to R3, and the mini-bags R1 to R3.

### Chemical composition

Silage samples were dried in a forced ventilation oven for 72h at 55°C. Then, ground through a 1-mm screen (Wiley Mill, Arthur H. Thomas). Sub-samples were analyzed for DM (AOAC, 1990; methods 934.01). For the characterization of fresh forage and all silages treatments, the contents of starch, crude protein (CP), soluble protein (SP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were measured using near-infrared spectroscopy (Foss NIRsystems 5000-M, n° série 6349). Each sample was packed into a cylindrical sample holder equipped with a quartz window (Part number 60013287; Foss-NIR System) and scanned,

between 400 and 2,498 nm, as described by Mentink et al. (2006). Prediction equations for determining nutrient composition were obtained from the NIRS Forage and Feed Test Consortium website (http://nirsconsortium. org/). These equations were calibrated for CP (methods 984.13, 988.05, and 990.03; AOAC International, 2012), NDF assayed with α-amylase and sodium sulfite (method 2002.04; AOAC International, 2012) and expressed inclusive of residual ash (aNDF), and starch (Bach Knudsen, 1997) using 754 (R2 = 0.88), 1,193 (R2 = 0.93), and 320 (R2 = 0.97) fermented WPCS samples, respectively, from participating laboratories from all over the United States and collected over more than 20 yr. The bromatological characteristics of the fresh forage are present in Table 12.

Table 12. Chemical characteristics of whole crop corn fresh forage:

| DM,% | NDF,% | ADF,% | CP,% | EE,% | ASH,% |
|---|---|---|---|---|---|
| 35.8 ± 0.0 | 47,9 ± 2.5 | 28.2 ± 2.1 | 10.0 ± 0.0 | 3.95 ± 0.6 | 5.35 ± 0.8 |

**Fermentation analyses and chromatography**

Silage samples were collected in the opening of the buckets and vacuum bags and an aqueous extract was prepared (25 g + 225 g of deionized water). The pH was measured (DM 20 pH meter, Digimed Analitica, São Paulo, Brazil), and fermentation end products were determined by gas chromatograph with a mass detector (GCMS QP2010 Plus, Shimadzu, Kyoto, Japan), using a capillary column (Stabilwax, Restek, Bellefonte, PA, 60 m, 0.25-mm, i.d., 0.25, 09 m), lactic acid was measured by colorimetry (Prynce, 1969). The dry mater content, corrected for volatiles, was obtained as follows (Weissbach, 2009): DMcorr (g/kg as fed) = DMoven (g/kg as fed) + acetone (g/kg as fed) + n-alcohols (g/kg as fed) + 2-butanol (g/kg as fed) + 2,3-butanediol (g/kg as fed) + 0.95 × volatile fatty acids (g/kg as fed) + 0.77 × 1,2-propanediol (g/kg as fed) + 0.08 × lactic acid (g/kg as fed). The alcohols included methanol, ethanol, and propanol, and the volatile fatty acids included acetic acid, propionic acid, iso-butyric acid, and n-butyric acid.

**Microbial count**

Microbial profile analyses used the aqueous extract supernatant described before that was mixed into decimal sequential dilutions ($10^1$ to $10^6$) and plating was in triplicate. Lactic acid bacteria count (LAB) was performed in an MRS culture medium with antifungal Natamicine (0.25 g. $L^{-1}$). Yeast count (LEV) was made from plating in a malt agar plus lactic acid at 0,5% concentration. The plates were incubated at 32 °C for 48h and the colonies were counted.

**Aerobic stability**

Aerobic stability was defined as the time elapsed until silage temperature increases 2°C above room temperature (O'Kiely, 1993). Samples (3kg) were weighed into plastic buckets and exposed to air for 10 days in a room with a controlled temperature. The temperature of samples and room was recorded every 15 minutes using dataloggers (iMini, Impac, São Paulo, Brazil).

**Statistical analysis**

Data were analyzed using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC), as a completely randomized design with a factorial arrangement of treatments, 8 additives x 2 times of storage, and 3 replicates per treatment. The model was following: $Yij = \mu + Ai + Tj + ATij + eij$, where $\mu$ = overall mean, Ai = fixed effect of additive (i = BV02, BV09, BV30, BV23, BV26, LB, LP or CON), Tj = fixed effect of storage length (j = 45 or 90 days), ATij = interaction between additive and length of storage, and eij = error. The 20-liter buckets were represented as the experimental unit. For bags, the factorial arrangement of treatments was 8 additives x 3 times of storage (6,12 and 24 hours), and 3 replicates per treatment. The covariance structure for the repeated measurements was chosen based on Akaike's information criterion among variance components (VC), compound symmetry (CS), first-order autoregressive (AR(1)), or unstructured covariance structure (UN). The Tukey-Kramer significance test ($P<0.05$) was applied for Treatment, Time, and Treatment*Time interaction.

## 3.3. Results and Discussion

The exploration of the initial pH drop is essential to identify the extent of silage fermentation and the quality of ensiled forage (Wang et al., 2009; Denek et al., 2011). In Table 13, the variable pH and LA present an interaction between time and treatments. In the first 6 hours, the control has the highest pH and the strains resemble LP and LB treatments, the last one with the lowest value. All additives and control behaved similarly in the treatment at 12h and 24h, and in this last one, they have satisfactory pH for a successful ensile (Kung et al., 2018). Regarding the length of storage, pH dropped over time for all treatments. For LA variable, the additives treatments showed differences only inside 12h. The highest contents were found for LP, which corroborates with the homofermentative characteristics of this bacteria.

Effects of inoculation on silage nutrients were not expected. The nutrient composition in Table 14 evidence silage with total dry matter contents close to 30%, a percentage that is in the range of 28% to 40% to reach a satisfactory fermentation capacity (Weissback e Honing, 1996). The CP presented the interaction between treatment and time, but in this experiment, this is not

explored and the differences could be explained by secondary compounds produced in the fermentation of both strains and commercial inoculants.

In silage, acidification is synonymous with controlling spoilage organisms (Pahlow et al., 2003). However, the stabilization of the forage in the feed-out phase is a commercial requirement for a successful silage additive. Tables 15 and 16 showed positive fermentable aspects for all treatments and, *bacillus* presents a higher acid lactic production than the others ones. In Table 15, this variable presents an interaction between treatments and length of storage ($p<0.001$). In 45d, these bacteria had a similar result to control (2.14 vs. 2.51% of MScorr) and LP (2.14 vs. 2.26% of MScorr) but were superior to LB treatment (2.14 vs. 1.11% of MScorr). In 90d, a different scenario was exhibited with BV02 (5.09 % of MScorr) and BV26 (5.19 % of MScorr) showing the highest contents of lactic acid, followed by BV09 (4.94% of MScorr) and BV30 (5.05% of MScorr). Glycolytic enzymes like L-lactate dehydrogenase are induced in anaerobiosis and especially under conditions that favor fermentation (Smith and Neidhardt, 1983), and in *Bacillus*, this enzyme is an intermediate in the reduction of pyruvate for lactic acid formation (Ramos et al., 2000). These bacteria are potential producers of organic acids owing to their genetic information and well-characterized metabolic pathways that metabolize a high diversity of substrates and grow fast, better than Lactobacillus (Nakano and Zuber, 1998; Park et al., 2021). Nevertheless, the bacillus favored the growth of BAL, always present in counts greater than 4 log cfu/g for the treatments (Table 5). This can be explained by the Biological Oxigen-Capturing Theory which is related to the oxygen consumed by the germination and sporulation of bacillus, increasing the environment anaerobiosis (Tam et al., 2006). The production of bioactive molecules as hydrolytic enzymes (amylase and protease), antioxidative enzymes (catalase and superoxide dismutase) and surface proteins by bacillus also can enhance the development of lactobacillus, because they are sensitive to reactive oxygen species (ROS) (Yu et al., 2019).

The acid lactic is stronger than any of the other major silage acids and contributes the most to low pH (Mc Donald, 1991). Table 16 showed that the commercial inoculates work as expected for this variable, thus LP treatment with the lowest pH and LB with the highest demonstrated homolactic and heterotactic fermentation respectively (Muck et al, 2018; Oude Elferink et al., 2001). The bacillus treatments presented lower values than the LB treatment, responding to the expected impact of lactic acid production in the pH drop (Table 15 and 16).

Lactic acid alone is insufficient to maintain the aerobic stability of the mass, as yeasts play a crucial role in initiating aerobic spoilage. Yeasts, being lactate-assimilating organisms, can elevate the silage pH, facilitating the proliferation of other detrimental microorganisms. (Pahlow et al., 2003; Driehuis et. al, 1999; Kung et al., 2018). Lara et al. (2016) showed that silage treated with *B.*

*subtilis* presented 3 times longer aerobic stability when compared with untreated silage. In the same way, Bonaldi et al (2021) found better aerobic stability in the silage inoculated with *B. subtilis* above 8 log10 CFU per kg forage compared with untreated. In this experiment, the yeast count in the bacillus treatment was similar but numerically lower than that in the control (2.48 vs 3.30 log $cfu.g^{-1}$) and LP (2.48 vs 3.76 log $cfu.g^{-1}$) treatments. The LB treatment exhibited a yeast count comparable to that of BV09 (<2.00 log $cfu.g^{-1}$). This finding underscores the impact of inoculation on the modified fungal population. It is crucial to note that inoculation not only influences the bacterial population but also modulates the fungal community, potentially resulting in distinct fermentation profiles for each silage. A study by Xiao et al. (2022) demonstrated that post-ensiling, the LB treatment exhibited a concentrated presence of the *Apiotrichum* genus, whereas the LP treatment displayed a predominant presence of the *Ustilago* genus. The latter is responsible for promoting a lactic acid profile akin to LP. Ethanol is generated through yeast fermentation of the plant material during the ensiling process (Malkina et al., 2011). This alcohol stands out as the predominant volatile organic compound (VOC) emitted from maize silage (Hafner et al, 2010; Montes et al, 2010). According to Kung et al. (2018), the standard ethanol content in silage typically falls within 1 to 3% of DM. Still, in this study, the ethanol content observed in the treatments was more than ten times lower than the typical range reported. Table 15 showed that Bacillus treatments were similar contents of this alcohol as LB treatment and lower than LP. Another observed outcome was the unexpected increase in ethanol over storage time (0.05 vs 0.11 % of DMcorr for 45d and 90d, respectively), despite the lower count of LEV and higher acetic acid content in silage.

The primary volatile compound to control yeasts is acetic acid and presents the highest concentration for LB treatment in 45 days of storage which was expected by heterolactic metabolism (Oude Elferink et al., 2001; Kung et al., 2018) and *bacillus* treatment had the same behavior as the control, excepted by BV31 that equalized as LP. For 90 days of storage, BV09 was statistically equal to LB treatment (0.8 vs 1.08 % DM Corr) but the other bacillus treatments had similar values as the Control and LP (Table 4). Bathia et al. (2018) showed that *B. subtilis* ferments glucose and fructose into acetic and propionic acids. Propionic acid also inhibits yeasts and molds and improves the aerobic stability of the mass at the feed-out phase (Auerbach et al., 2012). Previously, Kleinschmit et al. (2005) demonstrated that the addition of 0.1% propionic acid to fresh matter did not enhance aerobic stability. In our study, the LB treatment exhibited the highest quantities of propionic acid (0,1% DM), lower than the concentration studied by Kleinschmit et al.

Acetic and yeast count were associated with aerobic stability, a crucial characteristic during the silage feed-out phase, closely linked to the control of spoilage microorganisms. In the current experiment, strains BV02 (166h) and BV30 (174h) had similar aerobic stability as LB treatment (216h), and strain BV09 (140h) presented longer aerobic stability than the Control (70.3h). As previously mentioned, the yeast count and acetic acid were concatenated in this analysis, but these factors alone did not fully account for the observed differences in the results. Additionally, *B. subtilis* also produces various antifungal and antibacterial metabolites, including peptides, which likely contribute to the enhancement of aerobic stability (Tavora and Kozhuhavora, 2009; Lara et al., 2016; Bai et al., 2020).

Volatile organic acids (VOC) are compounds that can explain some secondary fermentation pathways. Bacillus metabolizes starch, cellulose, proteins and oils and this pathway originates compounds like 2,3 butanediol, butanol and acetone that were not exploited before in this text (Seo et al., 2021). In Table 6, the highest content of acetone was found in CON 45d, which could be explained by clostridia metabolites (Rooke and Hatfield, 2003). Ethyl acetate was higher in LB treatment because it results from the reaction between acetic acid and ethanol (Tang et al., 2005). This showed that bacillus doesn't base its metabolism on acetic acid production. The same reaction occurs with lactic acid and ethanol but the result is ethyl lactate (Delgado et al., 2010), shown in Table 17 with the highest content in LP treatment in 90d and lowest in LB 90d, and bacillus had intermediate values, indicating this acid is not the main metabolite too.

### 3.4. Conclusion

The silages produced under inoculation with Bacillus strains integrated desirable quality fermentation characteristics and aerobic deterioration control. BV02 and BV30 had similar aerobic stability as LB commercial additive BV02 and BV30 had similar aerobic stability as LB commercial. These trains are relevant candidates for the development of a new additive.

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# 4. TABLES

Phase 1
Experiment 1

Table 2. The acidification rate (%.h$^{-1}$) of corn silages treated with *Azospirillum* sp. and *Bradyrhizobium* sp strains or with commercial inoculants, evaluated in the first 24 hours of fermentation

| Item | Treatments | | | | | | | | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | | ABV5 | | | ABV6 | | | BV36 | | | BV37 | | | LB | | | LP | | | | Trat | Time | Trat*Time |
| | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | | | | |
| pH | 4.68ab | 4.15b | 4.07b | 4.89ab | 4.19b | 4.09b | 4.83ab | 4.17b | 4.10b | 4.42ab | 4.27b | 4.14b | 4.39ab | 4.34b | 4.15b | 4.25b | 4.14b | 4.09b | 4.96a | 4.12b | 3.86b | 0.125 | 0.411 | <0.001 | 0.007 |
| Lactic acid, %DM | 1.84b | 2.99ab | 3.49a | 0.86b | 2.12b | 3.13ab | 0.50c | 2.08b | 3.11ab | 1.46b | 1.50b | 2.48ab | 1.12b | 1.78b | 2.84ab | 1.56b | 3.19ab | 2.31ab | 0.64b | 2.45ab | 3.64a | 0.291 | <0.001 | <0.001 | 0.001 |

ABV5 and ABV6 – *Azospirillum* strains. BV36 and BV37 – *Bradyrhizobium* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum.* [a-c] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 3. Total dry matter and nutrient profile of corn silages treated with *Azospirillum* sp. and *Bradyrhizobium* sp strains or with commercial inoculants, stored for 45 or 90 days.

| Item | Treatments | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | ABV5 | | ABV6 | | BV36 | | BV37 | | LB | | LP | | | Trat | Time | Trat*Time |
| | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | | | | |
| DM, % | 29.5a | 27.9b | 29.6a | 28.4ab | 29.2ab | 28.2ab | 28.3ab | 27.7b | 28.4ab | 27.9b | 27.4bc | 25.4c | 25.9c | 23.7Bd | 0.323 | <0.001 | <0.001 | 0.017 |
| NDF, % | 45.1 | 39.9 | 42.1 | 40.1 | 40 | 38 | 44.6 | 39.1 | 40.8 | 36.9 | 43.5 | 39.7 | 47.5 | 46.2 | 1,157 | <0.001 | <0.001 | 0.449 |
| CP, % | 7.27b | 6.46c | 7.92ab | 6.83bc | 7.62ab | 6.94bc | 7.73ab | 7.16bc | 7.41ab | 6.83bc | 7.12bc | 6.91bc | 7.97ab | 8.08a | 0.152 | <0.001 | <0.001 | 0.018 |
| ASH, % | 3.89b | 3.80b | 4.26ab | 3.85b | 3.96b | 3.66b | 4.53ab | 3.96b | 3.84b | 3.93b | 3.88b | 4.59ab | 4.82a | 4.64ab | 0.159 | <0.001 | 0.19 | 0.014 |

ABV5 and ABV6 – *Azospirillum* strains. BV36 and BV37 – *Bradyrhizobium* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum.* [a-c] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 4. Dry matter corrected for volatile and main fermentation profile of corn silages treated with *Azospirillum* sp. and *Bradyrhizobium* sp. strains or with commercial inoculants, stored for 45 or 90 days.

| Item | Treatments | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | ABV5 | | ABV6 | | BV36 | | BV37 | | LB | | LP | | | Trat | Time | Trat*Time |
| | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | | | | |
| DM corr, % | 30.2 | 28.8 | 30.2 | 29.4 | 29.9 | 29.2 | 29 | 28.7 | 29.1 | 28.9 | 28.4 | 26.7 | 27.3 | 25.9 | 0.317 | <0.001 | <0.001 | 0.097 |
| Lactic acid, %DMcorr | 4.26 | 6.03 | 3.76 | 5.94 | 4.47 | 5.86 | 4.54 | 5.94 | 4.64 | 6.37 | 3.05 | 4.62 | 5.58 | 6.97 | 0.173 | <0.001 | <0.001 | 0.102 |
| Acetc acid, %DMcorr | 0.19d | 0.20d | 0.17d | 0.24cd | 0.22d | 0.25cd | 0.21d | 0.30cd | 0.24cd | 0.26cd | 0.44b | 0.61a | 0.30c | 0.37bc | 0.018 | <0.001 | <0.001 | 0.001 |
| Propionic acid, mg.kg -1 | 12.0b | 25.9ab | 19.1ab | 16.7b | 46.5ab | 35.9ab | 58.3a | 15.7b | 21.8ab | 9.41b | 18.0ab | 6.86b | 16.7ab | 13.4b | 9.00 | <0.001 | 0.002 | 0.004 |
| Butiric acid, %DMcorr | 1.66 | 0.92 | 1.95 | 1.15 | 4.21 | 1.4 | 3.39 | 1.53 | 3.02 | 1.39 | 2.6 | 0.15 | 1.63 | 1.11 | 0.464 | 0.005 | <0.001 | 0.086 |
| Ethanol, %DMcorr | 0.16bc | 0.24c | 0.14b | 0.26c | 0.14b | 0.25c | 0.16b | 0.23c | 0.15b | 0.24c | 0.10a | 0.16bc | 0.67d | 1.28d | 0.069 | <0.001 | <0.001 | 0.022 |

ABV5 and ABV6 – *Azospirillum* strains. BV36 and BV37 – *Bradyrhizobium* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum.* [a-c] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 5. pH, microbial count and aerobic stability of corn silages treated with *Azospirillum* sp. and *Bradyrhizobium* sp. strains or with commercial inoculants, stored for 45 or 90 days

| Item | Treatments | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | ABV5 | | ABV6 | | BV36 | | BV37 | | LB | | LP | | | Trat | Time | Trat*Time |
| | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | | | | |
| pH | 3.68c | 3.69c | 3.69c | 3.76b | 3.71bc | 3.80ab | 3.73b | 3.79ab | 3.72bc | 3.78ab | 3.85a | 3.82ab | 3.69c | 3.72bc | 0.016 | <0.001 | <0.001 | 0.007 |
| LAB, (cfu.g-1) | 5.22 | 6.08 | 5.14 | 6.06 | 5.28 | 6.64 | 5.52 | 6.53 | 5.5 | 6.73 | 8.22 | 8.18 | 6.72 | 7.85 | 0.251 | <0.001 | <0.001 | 0.212 |
| Yeast, (cfu.g-1) | 4.96b | 4.42b | 4.81b | 6.09a | 4.94b | 5.92ab | 4.29b | 5.09b | 5.20ab | 5.78ab | <2.00c | 2.49c | 5.99ab | 4.74b | 0.222 | <0.001 | 0.003 | <0.001 |
| AE, h | 29.8 | 39.5 | 33 | 44.3 | 33 | 45.7 | 36.2 | 41.8 | 32.8 | 40.8 | 49.2 | 59 | 43.2 | 50.5 | 3.74 | <0.001 | <0.001 | 0.953 |

ABV5 and ABV6 – *Azospirillum* strains. BV36 and BV37 – *Bradyrhizobium* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. a-c Rows with unlike letters differ by Tukey's test ($P \leq 0.05$).

Table 6. Volatile fatty acids profile of corn silages treated with Azospirillum sp. and Bradyrhizobium sp. strains or with commercial inoculants, stored for 45 or 90 days

| Item | Time | Treatments | | | | | | | Mean | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Controle | ABV5 | ABV6 | BV36 | BV37 | LB | LP | | | Trat | Time | Trat*Time |
| Acetone, mg.kg$^{-1}$ DMcorr | 45 d | 2.66Ab | 3.30Aab | 5.36Aa | 4.43Aab | 3.14Aab | 4.18Aab | 3.44Aab | 3.79 | 0.656 | 0.085 | <0.001 | 0.016 |
| | 90 d | 1.89Aab | 2.07Aab | 2.27Bab | 1.47Bb | 2.93Aab | 2.60Aab | 3.96Aa | 2.46 | | | | |
| | **Mean** | 2.27 | 2.69 | 3.81 | 2.95 | 3.03 | 3.39 | 3.70 | | | | | |
| Ethyl acetate, | 45 d | 8.73Bbcd | 6.58Bd | 7.38Bcd | 10.6Bbc | 8.36Bcd | 13.3Bb | 32.3Ba | 12.4 | 2.807 | <0.001 | <0.001 | 0.033 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mg.kg$^{-1}$ DMcorr | 90 d | 23.4Ac | 28.9Abc | 27.5Abc | 32.7Ab | 28.5Abc | 40.9Ab | 124Aa | 43.7 | | | | |
| | **Mean** | 16.1 | 17.8 | 17.5 | 21.6 | 18.4 | 27.1 | 78.0 | | | | | |
| Isopropyl alcohol, mg.kg$^{-1}$ DMcorr | 45 d | 0.27 | 0.24 | 0.46 | 0.35 | 0.36 | 0.21 | 0.37 | 0.33 | 0.082 | 0.764 | 0.898 | 0.073 |
| | 90 d | 0.47 | 0.34 | 0.29 | 0.28 | 0.30 | 0.38 | 0.18 | 0.32 | | | | |
| | **Mean** | 0.37 | 0.29 | 0.37 | 0.32 | 0.33 | 0.30 | 0.27 | | | | | |
| Propyl Acetate, mg.kg$^{-1}$ DMcorr | 45 d | 0.41 | 0.33 | 0.38 | 0.38 | 0.41 | 0.23 | 0.46 | 0.38 | 0.112 | 0.681 | 0.587 | 0.989 |
| | 90 d | 0.34 | 0.34 | 0.39 | 0.40 | 0.50 | 0.38 | 0.51 | 0.41 | | | | |
| | **Mean** | 0.38 | 0.34 | 0.39 | 0.39 | 0.46 | 0.34 | 0.49 | | | | | |
| 1 Propanol, mg.kg$^{-1}$ DMcorr | 45 d | 0.85 | 0.79 | 1.10 | 1.18 | 1.35 | 0.96 | 1.14 | 1.05 | 0.317 | 0.927 | 0.596 | 0.701 |
| | 90 d | 1.00 | 1.12 | 0.76 | 0.82 | 1.10 | 1.17 | 1.19 | 1.02 | | | | |
| | **Mean** | 0.92 | 0.96 | 0.93 | 1.00 | 1.23 | 1.06 | 1.17 | | | | | |
| Ethyl Lactate, mg.kg$^{-1}$ DMcorr | 45 d | 19.2Bb | 18.7Bb | 19.5Bb | 23.5Bb | 21.0Bb | 11.7Bc | 46.7Ba | 22.9 | 4.338 | <0.001 | <0.001 | 0.044 |
| | 90 d | 35.5Ab | 40.5Ab | 34.7Ab | 37.2Ab | 35.3Ab | 17.1Ac | 111Aa | 44.5 | | | | |
| | **Mean** | 27.3 | 29.6 | 27.1 | 30.4 | 28.1 | 14.4 | 78.9 | | | | | |
| 2.3 Butanediol, mg.kg$^{-1}$ DMcorr | 45 d | 6.21 | 7.37 | 9.08 | 11.5 | 10.9 | 40.2 | 35.1 | 17.2B | 6.027 | <0.001 | <0.001 | 0.534 |
| | 90 d | 12.8 | 16.3 | 15.1 | 12.9 | 14.6 | 83.5 | 52.3 | 29.6A | | | | |
| | **Mean** | 9.50b | 11.8b | 12.1b | 12.2b | 12.7b | 61.8a | 43.7a | | | | | |
| Isobutyric Acid, mg.kg$^{-1}$ DMcorr | 45 d | 2.81 | 2.69 | 2.86 | 2.50 | 2.14 | 2.95 | 2.25 | 2.60 | 0.577 | 0.505 | 0.011 | 0.998 |
| | 90 d | 1.86 | 1.95 | 2.12 | 1.73 | 1.17 | 2.55 | 1.74 | 1.87 | | | | |
| | **Mean** | 2.33 | 2.32 | 2.49 | 2.11 | 1.65 | 2.75 | 2.00 | | | | | |
| Isovaleric, mg.kg$^{-1}$ DMcorr | 45 d | 1.08 | 1.39 | 1.78 | 3.06 | 1.92 | 1.64 | 1.00 | 1.70A | 0.494 | 0.383 | 0.003 | 0.067 |
| | 90 d | 1.32 | 0.81 | 1.25 | 0.77 | 0.63 | 0.91 | 1.04 | 0.96B | | | | |
| | **Mean** | 1.20 | 1.10 | 1.52 | 1.92 | 1.28 | 1.28 | 1.02 | | | | | |
| Propilenoglicol, mg.kg$^{-1}$ DMcorr | 45 d | 34.2Abc | 46.0Abc | 17.6Bc | 27.5Bc | 148Abc | 2638Aa | 267Ab | 454 | 76.71 | <0.001 | 0.290 | 0.017 |
| | 90 d | 58.1Abc | 58.0Abc | 79.3Abc | 91.4Abc | 14.2Bc | 4468Aa | 254Ab | 717 | | | | |
| | **Mean** | 46.1 | 52.0 | 48.4 | 59.5 | 81.3 | 3553 | 260 | | | | | |
| Valeric, mg.kg$^{-1}$ DMcorr | 45 d | 0.49 | 0.35 | 1.15 | 1.19 | 0.88 | 0.55 | 0.42 | 0.72A | 0.208 | 0.015 | 0.012 | 0.224 |
| | 90 d | 0.55 | 0.32 | 0.60 | 0.58 | 0.37 | 0.32 | 0.47 | 0.46B | | | | |
| | **Mean** | 0.52ab | 0.33b | 0.87a | 0.88a | 0.62ab | 0.43ab | 0.44ab | | | | | |

ABV5 and ABV6 – *Azospirum* strains. BV36 and BV37 – *Bradyrhizobium* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. a-c Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Experiment 2

Table 7. The acidification rate (%.h$^{-1}$) of corn silages treated with *Bacillus* strains or with commercial inoculants, evaluated in the first 24 hours of fermentation

| Item | Treatments | | | | | | | | | | | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | | BV02 | | | BV09 | | | BV26 | | | BV30 | | | BV31 | | | LB | | | LP | | | | Trat | Time | Trat*Time |
| | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | | | | |
| pH | 5.19a | 4.5b | 4.10bc | 4.37bc | 4.32bc | 3.95c | 4.45b | 4.29bc | 4.18bc | 4.35bc | 4.22bc | 4.17bc | 4.28bc | 4.18bc | 4.07bc | 4.42bc | 4.29bc | 4.15bc | 4.36bc | 4.28bc | 4.23bc | 4.49b | 4.31bc | 4.26bc | 0.099 | <0.001 | <0.001 | 0.001 |
| Lactic acid, %DM | 0.74c | 1.89bc | 2.46bc | 1.42c | 1.47c | 3.32ab | 1.52bc | 1.96bc | 2.80ab | 1.90bc | 1.86bc | 2.89ab | 2.36bc | 3.11ab | 3.77a | 1.94bc | 1.65bc | 2.55b | 1.72bc | 2.20bc | 2.64ab | 1.84bc | 2.24bc | 2.38bc | 0.21 | <0.001 | <0.001 | 0.002 |

Lactic acid expressed as %DM. BV02 to BV31 – *Bacillus* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. [a-c] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 8. Total dry matter and nutrient profile of corn silages treated with *Bacillus* strains or with commercial inoculants, stored for 45 or 90 days.

| Item | Treatments | | | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | BV02 | | BV09 | | BV26 | | BV30 | | BV31 | | LB | | LP | | | Trat | Time | Trat*Time |
| | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | | | | |
| DM, % | 30 | 28.9 | 27.3 | 26.7 | 26.9 | 26.6 | 27 | 26.7 | 26.8 | 26.4 | 27.4 | 26.3 | 29.2 | 28.4 | 28.3 | 27.3 | 0.407 | <0.001 | <0.001 | 0.888 |
| NDF, % | 43.1 | 40.6 | 43.8 | 39.4 | 43.8 | 42.5 | 43.1 | 42.2 | 44.6 | 42.4 | 44.6 | 41.6 | 41.4 | 34.2 | 45.2 | 44.4 | 1.03 | <0.001 | <0.001 | 0.067 |
| CP, % | 7.04ab | 6.63b | 7.75a | 6.67b | 7.48ab | 6.71b | 7.65ab | 6.84ab | 6.95ab | 6.74b | 7.43ab | 6.87ab | 7.41ab | 6.96ab | 7.36ab | 7.65ab | 0.193 | 0.018 | <0.001 | 0.022 |
| Ash, % | 3.56 | 3.93 | 4.19 | 3.88 | 4.40 | 4.15 | 4.20 | 4.40 | 4.15 | 4.34 | 4.03 | 4.07 | 3.58 | 3.84 | 3.78 | 4.00 | 0.118 | <0.001 | 0.128 | 0.057 |

BV02 to BV31 – *Bacillus* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. [a-b] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 9. Dry matter corrected for volatile and main fermentation profile of corn silages treated with *Bacillus* strains or with commercial inoculants, stored for 45 or 90 days

| Item | Treatments | | | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | BV02 | | BV09 | | BV26 | | BV30 | | BV31 | | LB | | LP | | | Trat | Time | Trat*Time |
| | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | | | | |
| DM corr, % | 30.6 | 29.9 | 27.9 | 27.7 | 27.6 | 27.7 | 27.7 | 27.8 | 27.6 | 27.4 | 28.1 | 27.4 | 30.3 | 30 | 29.6 | 29.3 | 0.401 | <0.001 | 0.162 | 0.92 |
| Lactic acid, %DMcorr | 4.18cd | 6.58b | 4.67c | 7.43ab | 4.66c | 7.91a | 4.39cd | 8.05a | 5.00c | 6.96ab | 4.82c | 7.63ab | 2.56d | 2.77d | 4.19cd | 7.52ab | 0.301 | <0.001 | <0.001 | <0.001 |
| Acetc acid, %DMcorr | 0.17d | 0.25d | 0.21d | 0.38c | 0.23d | 0.39bc | 0.23d | 0.32cd | 0.26d | 0.36c | 0.21d | 0.35cd | 0.51b | 0.76a | 0.19d | 0.31cd | 0.023 | <0.001 | <0.001 | 0.007 |
| Propionic acid, mg.kg -1 | 17.0 | 12.9 | 15.5 | 16.4 | 16.9 | 12.0 | 13.8 | 13.7 | 15.8 | 15.5 | 15.4 | 13.0 | 9.6 | 9.2 | 17.0 | 14.0 | 2.09 | 0.132 | 0.063 | 0.677 |
| Butiric acid, %DMcorr | 1.66 | 1.51 | 0.41 | 1.32 | 1.53 | 0.81 | 0.99 | 0.97 | 1.17 | 1.27 | 1.1 | 1.21 | 0.43 | 0.54 | 1.18 | 1.44 | 0.302 | 0.105 | 0.631 | 0.286 |
| Ethanol, %DMcorr | 0.13d | 0.23c | 0.06d | 0.12d | 0.06d | 0.11d | 0.10d | 0.18cd | 0.06d | 0.09d | 0.08d | 0.15cd | 0.13d | 0.23cd | 0.68b | 1.12a | 0.044 | <0.001 | <0.001 | 0.002 |

DM corr: dry matter corrected for volatile fatty acids (%); all variables are expressed in **% DMcorr**. BV02 to BV31 – *Bacillus* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. [a-c] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 10. pH, microbial count and aerobic stability of corn silages treated with *Bacillus* strains or with commercial inoculants, stored for 45 or 90 days

| Item | Treatments | | | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | BV02 | | BV09 | | BV26 | | BV30 | | BV31 | | LB | | LP | | | Trat | Time | Trat*Time |
| | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | | | | |
| pH | 3.63 | 3.7 | 3.66 | 3.77 | 3.66 | 3.72 | 3.62 | 3.68 | 3.66 | 3.73 | 3.64 | 3.7 | 3.99 | 4.07 | 3.64 | 3.66 | 0.025 | <0.001 | <0.001 | 0.667 |
| LAB, (cfu.g-1) | 5.65 | 6.13 | 6.60 | 7.34 | 5.71 | 6.45 | 5.68 | 5.58 | 5.36 | 6.21 | 5.60 | 6.78 | 8.73 | 8.34 | 6.40 | 7.60 | 0.328 | <0.001 | <0.001 | 0.142 |
| Yeast, (cfu.g-1) | 4.28 | 3.47 | 3.98 | 4.03 | 4.22 | 3.50 | 4.01 | 3.32 | 4.10 | 3.00 | 4.49 | 3.38 | <2.00 | <2.00 | 5.25 | 4.80 | 0.345 | <0.001 | <0.001 | 0.525 |
| AE, h | 32.0 | 40.7 | 30.2 | 38.7 | 36.3 | 44.7 | 37.5 | 48.2 | 37.7 | 46.5 | 34.2 | 42.7 | 54.7 | 74.0 | 45.0 | 56.7 | 3.616 | <0.001 | <0.001 | 0.844 |

BV02 to BV31 – *Bacillus* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. a-c Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 11. Volatile organic compounds profile of corn silages treated with *Bacillus* strains or with commercial inoculants, stored for 45 or 90 days

| **Item** | **Time** | **Treatments** | | | | | | | | **Mean** | **SEM** | ***P*-value** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **CON** | **BV02** | **BV09** | **BV26** | **BV30** | **BV31** | **LB** | **LP** | | | **Trat** | **Time** | **Trat*Time** |
| Acetone, mg.kg-1 DMcorr | 45 d | 3.73 | 3.87 | 3.71 | 4.53 | 3.64 | 2.21 | 4.60 | 5.82 | 4.01A | 0.784 | 0.047 | 0.050 | 0.560 |
| | 90 d | 3.78 | 3.70 | 3.51 | 2.85 | 2.68 | 2.75 | 3.22 | 4.07 | 3.32B | | | | |
| | **Mean** | 3.75ab | 3.78ab | 3.61ab | 3.69ab | 3.16ab | 2.48b | 3.91ab | 4.94a | | | | | |
| Ethyl Acetate, mg.kg-1 DMcorr | 45 d | 6.81Bc | 6.40Bc | 6.35Bc | 8.35Bbc | 6.93Bc | 8.26Bbc | 21.4Bab | 29.9Ba | 11.8 | 4.366 | <0.001 | <0.001 | 0.036 |
| | 90 d | 23.3Ab | 19.1Ab | 16.3Ab | 25.9Ab | 17.6Ab | 22.5Ab | 58.9Aa | 84.5Aa | 33.5 | | | | |
| | **Mean** | 15.1 | 12.7 | 11.3 | 17.1 | 12.3 | 15.4 | 40.1 | 57.2 | | | | | |
| Methanol, mg.kg-1 DMcorr | 45 d | 17.4 | 14.5 | 16.2 | 16.0 | 17.3 | 15.2 | 18.0 | 18.1 | 16.6B | 1.764 | 0.915 | <0.001 | 0.712 |
| | 90 d | 30.2 | 30.1 | 29.0 | 28.2 | 18.9 | 29.6 | 29.6 | 28.4 | 29.3A | | | | |
| | **Mean** | 23.8 | 22.3 | 22.6 | 22.1 | 23.1 | 22.4 | 23.8 | 23.3 | | | | | |
| Isopropyl Alcohol, mg.kg-1 DMcorr | 45 d | 0.33 | 0.66 | 0.45 | 0.38 | 0.45 | 0.46 | 0.68 | 0.37 | 0.47 | 0.387 | 0.937 | 0.181 | 0.435 |
| | 90 d | 0.53 | 0.45 | 0.22 | 0.41 | 0.67 | 0.38 | 2.30 | 1.02 | 0.75 | | | | |
| | **Mean** | 0.43 | 0.56 | 0.34 | 0.39 | 0.56 | 0.42 | 1.49 | 0.70 | | | | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Propyl Acetate, mg.kg$^{-1}$ DMcorr | 45 d | 0.34 | 0.42 | 0.43 | 0.40 | 0.62 | 0.47 | 0.62 | 0.49 | 0.47 | 0.105 | 0.785 | 0.229 | 0.154 |
| | 90 d | 0.55 | 0.37 | 0.45 | 0.53 | 0.39 | 0.25 | 0.40 | 0.38 | 0.42 | | | | |
| | **Mean** | 0.45 | 0.40 | 0.44 | 0.46 | 0.50 | 0.36 | 0.51 | 0.43 | | | | | |
| 1 Propanol mg.kg$^{-1}$ DMcorr | 45 d | 0.62Ba | 1.00Aa | 0.97Aa | 0.95Aa | 0.72Aa | 1.53Aa | 1.07Aa | 0.77Ba | 0.95 | 0.282 | 0.123 | 0.029 | 0.019 |
| | 90 d | 1.32Aab | 0.79Ab | 1.52Aab | 1.11Aab | 0.74Ab | 1.01Aab | 1.39Aab | 1.99Aa | 1.23 | | | | |
| | **Mean** | 0.97 | 0.90 | 1.24 | 1.03 | 0.73 | 1.27 | 1.23 | 1.38 | | | | | |
| Ethyl Lactate, mg.kg$^{-1}$ DMcorr | 45 d | 20.1 | 11.7 | 11.1 | 18.9 | 11.5 | 17.1 | 15.3 | 72.0 | 22.2B | 6.809 | <0.001 | <0.001 | 0.847 |
| | 90 d | 41.3 | 21.3 | 21.3 | 36.1 | 18.6 | 32.8 | 20.1 | 163 | 44.3A | | | | |
| | **Mean** | 30.7b | 16.5c | 16.2c | 27.5b | 15.1c | 25.0bc | 17.7bc | 117a | | | | | |
| 2.3 Butanodiol, mg.kg$^{-1}$ DMcorr | 45 d | 9.95 | 11.4 | 12.1 | 25.3 | 23.8 | 13.8 | 8.76 | 10.5 | 14.4B | 10.18 | <0.001 | <0.001 | 0.161 |
| | 90 d | 14.9 | 46.9 | 33.6 | 85.9 | 50.8 | 58.2 | 26.9 | 16.7 | 41.8A | | | | |
| | **Mean** | 12.4b | 29.1ab | 22.9b | 55.6a | 37.3ab | 36.0ab | 17.8b | 13.6b | | | | | |
| Isobutyric Acid, mg.kg$^{-1}$ DMcorr | 45 d | 2.49 | 2.04 | 2.38 | 2.68 | 2.34 | 3.21 | 1.41 | 2.47 | 2.38 | 0.607 | 0.019 | 0.171 | 0.109 |
| | 90 d | 2.40 | 2.56 | 2.21 | 2.40 | 4.53 | 3.91 | 2.48 | 1.49 | 2.75 | | | | |
| | **Mean** | 2.45 | 2.30 | 2.30 | 2.54 | 3.43 | 3.56 | 1.94 | 1.98 | | | | | |
| Isovaleric, mg.kg$^{-1}$ DMcorr | 45 d | 0.92 | 1.80 | 3.71 | 1.03 | 5.76 | 4.41 | 1.10 | 0.94 | 2.46 | 1.018 | <0.001 | 0.240 | 0.342 |
| | 90 d | 1.01 | 1.96 | 3.21 | 1.55 | 8.88 | 7.20 | 0.81 | 2.30 | 3.37 | | | | |
| | **Mean** | 0.97c | 1.88bc | 3.46b | 1.29bc | 7.32a | 5.81ab | 0.95c | 1.62bc | | | | | |
| Valeric, mg.kg$^{-1}$ DMcorr | 45 d | 0.24 | 0.35 | 0.42 | 0.55 | 0.49 | 0.44 | 0.45 | 0.69 | 0.45 | 0.108 | 0.061 | 0.948 | 0.052 |
| | 90 d | 0.43 | 0.55 | 0.55 | 0.64 | 0.54 | 0.41 | 0.11 | 0.37 | 0.45 | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Mean** | 0.34 | 0.45 | 0.48 | 0.59 | 0.52 | 0.43 | 0.28 | 0.53 |

BV02 to BV31 – *Bacillus* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. [a-d] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

## Phase 2

Table 13. Acidification rate (unidade %.h$^{-1}$) of corn silages treated with *Bacillus* strains or with commercial inoculants, evaluated in the first 24 hours of fermentation

| | Treatments | | | | | | | | | | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | Cont | | | BV02 | | | BV09 | | | BV26 | | | BV30 | | | BV31 | | | LB | | | LP | | | | Trat | Time | Trat*Time |
| | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | 6 h | 12 h | 24 h | | | | |
| pH | 5.29a | 4.68b | 3.89c | 4.83b | 4.52b | 3.87c | 4.75b | 4.55b | 3.86c | 4.72b | 4.57b | 3.83c | 4.82b | 4.50b | 3.87c | 4.78b | 4.61b | 3.89c | 4.63b | 4.52b | 3.77c | 4.96ab | 4.48b | 3.72c | 0.07 | <0.001 | <0.001 | 0.004 |
| Lactic acid, %DM | 0.32b | 0.34b | 0.73ab | 0.26b | 0.44b | 0.67ab | 0.32b | 0.42b | 0.93ab | 0.30b | 0.48b | 0.99a | 0.28b | 0.35b | 0.92ab | 0.27b | 0.42b | 0.97ab | 0.31b | 0.31b | 1.01a | 0.28b | 0.68ab | 0.87ab | 0.08 | 0.11 | <0.001 | 0.04 |

BV02 to BV31 – *Bacillus* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. [a-c] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 14. Nutrient composition of corn silages treated with *Bacillus* strains or with commercial inoculants, stored for 45 or 90 days

| Item | Treatments | | | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | BV02 | | BV09 | | BV26 | | BV30 | | BV31 | | LB | | LP | | | Trat | Time | Trat*Time |
| | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | | | | |
| DM, % | 30.7 | 29.9 | 32.0 | 29.3 | 32.5 | 30.3 | 33.1 | 31.7 | 30.7 | 31.5 | 30.8 | 32.9 | 28.8 | 27.4 | 30.0 | 29.3 | 1.03 | 0.008 | 0.14 | 0.37 |
| NDF, % | 39.6 | 40.5 | 42.9 | 41.9 | 38.8 | 37.6 | 41.2 | 41.4 | 42.4 | 41.3 | 40.4 | 40.4 | 41.5 | 41.4 | 40.8 | 42.4 | 2.02 | 0.55 | 0.92 | 0.99 |
| CP, % | 9.60a | 9.33ab | 9.43ab | 9.67a | 9.90a | 10.0a | 9.73a | 9.80a | 9.47a | 9.47a | 9.53a | 9.07ab | 8.53b | 9.33ab | 9.83a | 9.27ab | 0.17 | <0.001 | 0.89 | 0.01 |
| Ash, % | 3.93 | 4.00 | 3.70 | 4.50 | 3.65 | 4.05 | 3.87 | 4.27 | 3.73 | 3.77 | 3.83 | 3.83 | 4.27 | 4.30 | 4.27 | 3.80 | 0.23 | 0.28 | 0.15 | 0.18 |

BV02 to BV31 – *Bacillus* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. [a-b] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 15. Dry matter corrected content and fermentation profile of corn silages treated with *Bacillus* strains or with commercial inoculants, stored for 45 or 90 days.

| Item | Treatments | | | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | BV02 | | BV09 | | BV26 | | BV30 | | BV31 | | LB | | LP | | | Trat | Time | Trat*Time |
| | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | | | | |
| DM corr, % | 31 | 30.6 | 32.5 | 30.7 | 32.9 | 31.8 | 33.5 | 32.7 | 31.2 | 32.8 | 31.1 | 33.7 | 29.5 | 29.1 | 30.4 | 30.2 | 1.13 | 0.016 | 0.941 | 0.463 |
| Lactic acid, %DMcorr | 2.51bc | 3.19b | 2.34bc | 5.09a | 1.93bc | 4.94a | 2.10bc | 5.19a | 2.31bc | 5.05a | 2.08bc | 2.41bc | 1.11c | 0.99c | 2.26bc | 3.75ab | 0.32 | <0.001 | <0.001 | <0.001 |
| Acetc acid, %DMcorr | 0.13ab | 0.30c | 0.21bc | 0.71d | 0.17b | 0.80cd | 0.17b | 0.45cd | 0.22bc | 0.59cd | 0.12a | 0.40cd | 0.36cd | 1.08d | 0.12a | 0.37cd | 0.08 | <0.001 | <0.001 | <0.001 |
| Propionic acid, mg.kg -1 | 4,73 | 34,0 | 3,61 | 730 | 62,2 | 1191 | 2,61 | 32,1 | 47,0 | 190 | 3,73 | 28,9 | 341 | 1606 | 1,21 | 17,0 | 249.00 | <0.001 | <0.001 | 0.152 |
| Ethanol, %DMcorr | 0.05 | 0.1 | 0.04 | 0.08 | 0.04 | 0.09 | 0.04 | 0.09 | 0.04 | 0.09 | 0.03 | 0.09 | 0.06 | 0.13 | 0.1 | 0.24 | 0.02 | <0.001 | <0.001 | 0.341 |

DM corr: dry matter corrected for volatile fatty acids (%); all variables are expressed in **% DMcorr**. BV02 to BV31 – Bacillus strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. [a-c] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 16. pH. microbial count and aerobic stability of corn silages treated with *Bacillus* strains or with commercial inoculants. stored for 45 or 90 days

| Item | Treatments | | | | | | | | | | | | | | | | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cont | | BV02 | | BV09 | | BV26 | | BV30 | | BV31 | | LB | | LP | | | Trat | Time | Trat*Time |
| | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | 45 | 90 | | | | |
| pH | 3.66 | 3.56 | 3.69 | 3.81 | 3.73 | 3.78 | 3.64 | 3.61 | 3.67 | 3.65 | 3.61 | 3.64 | 4.09 | 4.09 | 3.55 | 3.41 | 0.06 | <0.001 | 0.791 | 0.47 |
| LAB, (cfu.g-1) | 2.99 | 4.29 | 4.94 | 5.47 | 4.95 | 5.43 | 3.66 | 4.98 | 5.29 | 4.55 | 4.52 | 3.59 | 6 | 5.77 | 3.86 | 4.16 | 0.69 | 0.019 | 0.434 | 0.515 |
| Yeast, (cfu.g-1) | 3.86 | 2.74 | 2.27 | 1.99 | <2.00 | <2.00 | 2.78 | 3.1 | 2.26 | 1.99 | 3.94 | 2.5 | <2.00 | <2.00 | 4.84 | 2.67 | 0.57 | 0.006 | 0.024 | 0.239 |
| AE, h | 47.3 | 93.3 | 142 | 190 | 117 | 164 | 58 | 132 | 132 | 215 | 63.3 | 182 | 216 | 216 | 42 | 63.7 | 20.5 | <0.001 | <0.001 | 0.098 |

BV02 to BV31 – *Bacillus* strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum*. [a-c] Rows with unlike letters differ by Tukey's test (P ≤ 0.05).

Table 17. Profile of volatile organic compounds of corn silages treated with *Bacillus* strains or with commercial inoculants. stored for 45 or 90 days

| Item | Time | Treatments | | | | | | | | Mean | SEM | *P*-valor | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CON | BV02 | BV09 | BV26 | BV30 | BV31 | LB | LP | | | Treat | Time | Treat*Time |
| Acetone. | 45 d | 6.70Aa | 4.79Aab | 0.66Bb | 3.38Aab | 4.16Aab | 2.82Aab | 2.10Aab | 1.71Ab | 3.29 | 1.19 | 0.002 | 0.506 | 0.002 |
| | 90 d | 1.86Ba | 5.66Aa | 4.86Aa | 2.39Aa | 2.82Ba | 4.94Aa | 0.88Aa | 1.18Aa | 2.90 | | | | |
| | **Mean** | 4.28 | 5.22 | 2.76 | 2.88 | 2.81 | 3.88 | 1.49 | 1.44 | | | | | |
| Ethyl acetate. | 45 d | 2.35 | 4.02 | 4.45 | 3.00 | 2.84 | 2.62 | 6.64 | 4.39 | 3.79B | 3.11 | <0.001 | <0.001 | 0.887 |
| | 90 d | 12.7 | 15.6 | 20.7 | 14.4 | 13.3 | 13.3 | 31.2 | 29.2 | 18.9A | | | | |
| | **Mean** | 7.52b | 9.80ab | 12.6ab | 8.68b | 8.09b | 7.94b | 18.9a | 16.7ab | | | | | |
| Methanol | 45 d | 12.9 | 13.1 | 9.17 | 10.2 | 12.4 | 7.60 | 7.79 | 7.84 | 10.1B | 2.71 | 0.452 | <0.001 | 0.413 |
| | 90 d | 23.2 | 27.8 | 31.8 | 12.4 | 28.5 | 26.0 | 27.4 | 25.0 | 27.4A | | | | |
| | **Mean** | 18.0 | 20.4 | 20.5 | 20.1 | 20.5 | 16.8 | 17.6 | 16.4 | | | | | |
| Isopropyl alcohol | 45 d | 0.30Aa | 0.69Ba | 0.50Ba | 0.37Ba | 0.24Ba | 0.44Aa | 0.38Ba | 0.43Aa | 0.42 | 1.34 | <0.001 | <0.001 | <0.001 |
| | 90 d | 1.10Ab | 7.46Aa | 6.90Aa | 5.87Aa | 4.85Aa | 1.42Ab | 11.2Aa | 0.42Ab | 4.90 | | | | |
| | **Mean** | 0.70 | 4.07 | 3.70 | 3.12 | 2.55 | 0.93 | 5.79 | 0.43 | | | | | |
| Propyl acetate | 45 d | 0.49 | 0.38 | 0.32 | 0.23 | 0.49 | 0.36 | 6.08 | 0.26 | 1.07B | 6.74 | 0.001 | 0.042 | 0.069 |
| | 90 d | 0.56 | 17.7 | 36.4 | 0.70 | 5.00 | 0.29 | 45.0 | 0.50 | 13.3A | | | | |
| | **Mean** | 0.53b | 9.07ab | 18.4ab | 0.47b | 2.74ab | 0.33b | 25.5a | 0.38b | | | | | |
| 2-Butanol | 45 d | 0.21Bab | 0.28Bab | 0.46Aab | 0.14Bb | 0.46Ba | 0.32Aa | 0.30Bab | 0.34Aa | 0.32 | 6.08 | 0.059 | <0.001 | 0.042 |
| | 90 d | 0.52Aa | 20.9Aa | 3.08Aa | 1.70Aa | 5.27Aa | 0.88Aa | 35.7Aa | 0.54Aa | 8.58 | | | | |
| | **Mean** | 0.36 | 10.6 | 1.77 | 0.92 | 2.86 | 0.60 | 18.0 | 0.44 | | | | | |
| Ethyl lactate | 45 d | 7.55Bab | 7.22Bab | 6.77Bab | 7.95Bab | 6.95Bab | 6.71Bab | 4.21Ab | 13.6Ba | 7.62 | 4.38 | <0.001 | <0.001 | 0.001 |
| | 90 d | 27.7Ab | 14.4Acd | 14.7Abcd | 20.9Abc | 20.2Abc | 22.1Abc | 6.79Ad | 80.8Aa | 25.9 | | | | |
| | **Mean** | 17.6 | 10.8 | 10.7 | 14.4 | 13.6 | 14.4 | 5.50 | 47.2 | | | | | |
| 2.3 Butanediol | 45 d | 1.63Bd | 9.59Bbc | 13.9Bbc | 12.3Bbc | 21.7Bb | 2.50Bd | 103Ba | 3.32Bcd | 21.0 | 108 | <0.001 | <0.001 | <0.001 |
| | 90 d | 182Ad | 951Aab | 1428Aa | 662Aabc | 1097Aab | 854Aab | 367Abcd | 239Acd | 722 | | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Isobutyric acid | **Mean** | 91.9 | 480 | 721 | 337 | 560 | 428 | 235 | 121 | | | | | |
| | 45 d | 2.22Aa | 1.23Ba | 1.82Aa | 2.38Ba | 2.02Ba | 2.48Ba | 2.99Aa | 2.42Aa | 2.19 | 1.42 | 0.020 | <0.001 | 0.001 |
| | 90 d | 3.30Abcd | 9.81Aabc | 5.31Aabcd | 12.5Aa | 12.0Aab | 13.0Aa | 1.98Ad | 3.04Acd | 7.63 | | | | |
| Isovaleric | **Mean** | 2.76 | 5.52 | 3.57 | 7.46 | 7.01 | 7.75 | 2.49 | 2.73 | | | | | |
| | 45 d | 3.57Aa | 2.40Aab | 0.71Ac | 1.31Abc | 1.89Aabc | 3.55Ba | 0.60Ac | 1.86Aabc | 1.99 | 0.41 | <0.001 | 0.222 | 0.002 |
| | 90 d | 1.37Bbc | 3.36Ab | 1.31Abc | 1.49Abc | 1.83Abc | 6.79Aa | 0.87Ac | 1.31Ac | 2.29 | | | | |
| Valeric | **Mean** | 2.47 | 2.88 | 1.01 | 1.40 | 1.86 | 5.17 | 0.73 | 1.58 | | | | | |
| | 45 d | 0.40 | 0.35 | 0.26 | 0.31 | 0.19 | 0.42 | 0.17 | 0.25 | 0.29B | 0.17 | 0.215 | <0.001 | 0.256 |
| | 90 d | 1.03 | 0.38 | 0.51 | 1.05 | 0.71 | 0.56 | 0.69 | 0.68 | 0.70A | | | | |
| | **Mean** | 0.71 | 0.37 | 0.39 | 0.68 | 0.45 | 0.49 | 0.43 | 0.46 | | | | | |

All variables are expressed as mg.kg-1 DM corr. BV02 to BV31 – Bacillus strains; LB - commercial *Lentilactobacillus buchneri*; LP – commercial *Lactiplantibacillus plantarum.* [a-d] Rows with unlike letters differ by Tukey's test ($P \leq 0.05$).