**BEATRIZ FERREIRA CARVALHO**

# SELEÇÃO DE CEPAS DE BACTÉRIAS DO ÁCIDO LÁTICO PARA ENSILAGEM DA CANA-DE-AÇÚCAR *in natura* OU ADITIVADA COM GLICERINA ACRESCIDA DE METANOL E LEVEDURA METILOTRÓFICA

**LAVRAS - MG**

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, área de concentração em Microbiologia Agrícola, para a obtenção do título de Doutor.

Orientadora

Dra. Rosane Freitas Schwan

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**Ficha Catalográfica Elaborada pela Coordenadoria de Produtos e Serviços da Biblioteca Universitária da UFLA**

Carvalho, Beatriz Ferreira.
Seleção de cepas de bactérias do ácido lático para ensilagem da cana-de-açúcar *in natura* ou aditivada com glicerina acrescida de metanol e levedura metilotrófica / Beatriz Ferreira Carvalho. – Lavras : UFLA, 2013.
203 p. : il.

Tese (doutorado) – Universidade Federal de Lavras, 2013.
Orientador: Rosane Freitas Schwan.
Bibliografia.

1. Leveduras. 2. Inoculantes microbianos em silagem. 3. *L. hilgardii*. 4. *L. plantarum*. 5. *L. brevis.* I. Universidade Federal de Lavras. II. Título.

CDD – 636.08552

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APROVADA em 03 de julho de 2013.

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| Dr. Marcos Neves Pereira | UFLA |
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**LAVRAS – MG**

**2013**

À minha filha Maria Fernanda

**DEDICO**

## AGRADECIMENTOS

A Deus, por me conceder sabedoria, força, entendimento e fé em todos os momentos;

À CAPES pela concessão de bolsa de estudo.

À Universidade Federal de Lavras e ao Departamento de Biologia.

Aos meus pais Heber e Sueli, por em nenhum momento ter medido forças para ajudar e me apoiar em tudo que foi necessário.

À minha irmã Débora, pelo companheirismo, dedicação e ajuda nos experimentos.

À professora Dra. Rosane Freitas Schwan, pela orientação, pelos ensinamentos e apoio para a concretização deste trabalho.

Aos professores Dra. Cristina, Dra Carla, Dr. Disney, Dr. Eustáquio, Dr. José Cardoso, Dr. Marcos, Dra Patrícia e Dr. Whasley pelos valiosos ensinamentos, apoio e ajuda.

Aos bolsistas de inciação científica, Willian, Milena e Cibelli, pela amizade e pela grande ajuda nos experimentos.

À Cidinha pela confiança, suporte de fé, carinho e também por todo apoio nas análises cromatográficas.

Aos pós-doutorandos Gabriela, Simone, Karina e Cíntia, pela amizade e ensinamentos.

À Rose pela paciência e amizade.

Aos colegas e ex-colegas de laboratório Gilberto, Alenir, Fernanda, Suzana, Angélica, Patrícia, Kelly, Vanessa, Mariana Dias, Igor, Karla, Claudia Auler, Claudia Puerari, Ana Luiza, Carol, Mariana Rabelo, Bárbara, Debora, Noelly, Ivani, Paulinho, Pedro, Luciana, Monique, pelos momentos de descontração.

Aos meus amigos Fabricio Alves, Olinto Lasmar, Vanessa Carvalho, Camila Meneghtti, Camila Moraes, Raquel Wolp, por estarem sempre presente mesmo distantes, pelos conselhos, pelos valiosos ensinamentos e pelo incetivo.

Muito Obrigada!

## RESUMO GERAL

A cana-de-açúcar é uma forrageira com alta produtividade por hectare. Em algumas situações a ensilagem é preferível em relação à colheita diária da forragem. A ensilagem da cana-de-açúcar resulta em altas perdas de matéria seca (MS) com consequente redução na qualidade nutricional da silagem. Os objetivos desse estudo foram selecionar cepas de bactérias do ácido lático e avaliar a adição de glicerina acrescida de metanol junto com uma levedura metilotrófica visando redução das perdas de MS, melhoria da qualidade nutricional e microbiológica e aumento na estabilidade aeróbia da silagem de cana-de-açúcar. O primeiro experimento avaliou cepas selvagens de bactérias do ácido lático (BAL) na melhoria da qualidade nutricional da silagem da cana-de-açúcar. As cepas *Lactobacillus hilgardii* UFLA SIL51 e 52 se destacaram das demais por reduzir, em média, 29,3% as perdas de MS em relação ao tratamento sem inoculante. Além de reduzir a população de leveduras e a concentração de etanol na silagem, as silagens tratadas com essas cepas apresentaram maior concentração de ácido acético e 1,2 propanodiol. A inoculação com cepas de *L. plantarum* reduziu a qualidade da silagem. O segundo experimento avaliou cepas selvagens de BAL em relação à melhoria da estabilidade aeróbia de silagem de cana-de-açúcar, as espécies de leveduras presentes na silagem após a exposição aeróbia foram identificadas. A inoculação com as cepas de BAL modificou a diversidade de espécies de leveduras após abertura dos silos. As leveduras *Candida diversa, C. ethanolica, Hanseniaspora opuntiae, Issatchenkia orientalis, Pichia fermentans, P. kudriavzevii, P. manshurica Schizosaccharomyces pombe, Debaryomyces etchellsii, Zygosaccharomyces bailii* foram identificadas nas silagens. O tratamento com as cepas *L. hilgardii* UFLA SIL51 e 52 resultaram em silagens com temperaturas mais baixas e que permaneceram mais tempo sem aquecimento. O terceiro experimento avaliou níveis de inclusão de glicerina acrescida de metanol e do *L. hilgardii* UFLA SIL52 na redução de perdas e aumento na qualidade da silagem de cana-de-açúcar, e o efeito da *P. methanolica* NCYC 1381, na redução da concentração de metanol presente na glicerina durante a ensilagem. A utilização de 4% (matéria fresca) de glicerina juntamente com *L. hilgardii* UFLA SIL52 melhorou a qualidade da silagem pela redução na concentração de fibra e etanol, redução na perda de MS, aumento na concentração final de glicerol e consequente aumento na densidade energética da silagem. Esses aditivos também aumentaram a estabilidade aeróbia. Nas condições do experimento a *P. methanolica* NCYC1381 não reduziu a concentração de metanol na silagem.



Palavras-chave: *L. hilgardii. L. plantarum. L. brevis.* Leveduras. Inoculantes microbianos em silagem. Glicerina.

**GENERAL ABSTRACT**

Sugar cane is a forage plant with high productivity per hectare. In some situations, the ensilage is preferred compared to the daily forage harvest. Sugar cane ensilage results in high dry matter (DM) losses with a consequent reduction in the nutritional quality of the silage. The objectives of this study were to select strains of lactic acid bacteria and to evaluate the addition of glycerin plus methanol along with a methylotrophic yeast aiming at reducing DM losses, improving nutritional and microbiological quality and increasing aerobic stability of sugar cane silage. The first experiment evaluated wild strains of lactic acid bacteria (LAB) in improving the nutritional quality of cane sugar silage. The *Lactobacillus hilgardii* UFLA SIL51 and 52 strains stood out from the others for reducing, in average, 29.3% of the DM losses compared to the treatment without the inoculant, in addition to reducing yeast population and the ethanol concentration in the silage. Silages treated with these strains presented the highest concentration of acetic acid and 1,2 propanediol. The inoculation with *L. plantarum* strains reduced silage quality. The second experiment evaluated wild strains of LAB regarding the improvement of the aerobic stability of sugar cane silage, identifying the yeast species present in the silage after aerobic exposure. Inoculation with LAB strains changed the diversity of yeasts species after silo opening. The yeasts *Candida diversa, C. ethanolica, Hanseniaspora opuntiae, Issatchenkia orientalis, Pichia fermentans, P. kudriavzevii, P. manshurica Schizosaccharomyces pombe, Debaryomyces etchellsii, Zygosaccharomyces bailii* were identified in the silages. The treatment with the *L. hilgardii* UFLA SIL51 and 52 strains resulted in silages with lower temperatures and which remained unheated for a longer period. The third experiment evaluated inclusion levels of glycerin plus methanol and of *L. hilgardii* UFLA SIL52 in the reduction of losses and in the quality increase of sugar cane silage, and the effect of *P. methanolica* NCYC 1381 in reducing the methanol concentration present in the glycerin during ensilage. The use of 4% (fresh weight) of glycerin along with *L. hilgardii* UFLA SIL52 improved silage quality by reducing fiber and ethanol concentration and DM loss, increasing the final glycerol concentration, resulting in an increase in the energy density of the silage. These additives also increased aerobic stability. Under the conditions of the experiment, the *P. methanolica* NCYC1381 did not reduce the methanol concentration in the silage.

Keywords: *L. hilgardii. L. plantarum. L. brevis.* Yeasts. Silage microbial inoculums. Glycerin.

**SUMÁRIO**

# PRIMEIRA PARTE

## 1 INTRODUÇÃO

A utilização da cana-de-açúcar (*Saccharum* spp.) como forrageira para alimentação de ruminantes alia o conhecimento gerado pela indústria álcool-açucareira na seleção e cultivo desta planta à produção animal. A cana é muito eficiente em sua capacidade de converter luz solar em energia para produção animal por unidade de área, o que reduz seu custo de produção por unidade de matéria seca (MS). A ensilagem da cana permite a colheita de grandes áreas em curto período, diminuindo o tempo de uso de mão-de-obra e maquinário para colheita, o que pode facilitar o manejo na fazenda e possibilitar o uso dessa forrageira durante todo ano. No entanto, a ensilagem da cana-de-açúcar resulta em altas perdas de MS em razão da fermentação alcoólica da sacarose por leveduras.

A utilização de aditivos vem sendo empregada com objetivo de melhorar características fermentativas das silagens. A inoculação com cepas de bactérias selecionadas pode modificar o padrão de fermentação no silo resultando em silagens de melhor qualidade nutricional e microbiológica. A utilização do glicerol resultante da produção de biodiesel pode compensar, de forma econômica, essa perda energética durante o processo de ensilagem dessa forrageira.

Desde a década de 80 inoculantes microbianos são utilizados como culturas iniciadoras em silagens. Os resultados da adição de inoculantes microbianos em silagens são, em geral, positivos, embora na literatura sejam encontrados trabalhos onde a adição de inoculantes não tenha efeito, ou seja, prejudicial à fermentação da silagem. Os efeitos da inoculação com espécies bacterianas são atribuídos ao maior crescimento e à produção de metabólitos

pela espécie inoculada. De acordo com Ávila et al. (2011) e Muck (2008) entre os fatores que determinam o sucesso da aplicação de microrganismos nas silagens, a compatibilidade entre o microrganismo e a planta forrageira é de suma importância. Assim microrganismos isolados do ambiente onde eles serão utilizados como culturas iniciadoras, no caso, da própria silagem, têm maior chance de apresentar resultados favoráveis quando utilizados como inoculante. Nesse cenário a seleção de cepas bacterianas isoladas da própria silagem tem grande potencial biotecnológico para o desenvolvimento de novos produtos. Apesar desse potencial, a seleção de cepas para ensilagem, utilizando critérios bem definidos, é pouco realizada.

O aumento da produção e da demanda por combustíveis alternativos aos combustíveis fósseis tem impactado não somente a produção agrícola, mas também a produção animal. No Brasil, maior produtor de bicombustíveis do mundo, com uma produção de 200 milhões de litros em fevereiro de 2013 (AGÊNCIA NACIONAL DO PETRÓLEO, GÁS NATURAL E BIOCOMBUSTÍVEIS - ANP, 2013), a grande produção de resíduos decorrente deste processo é um dos principais problemas da produção de biodiesel. Alguns desses resíduos, no entanto, têm grande potencial para serem utilizados na alimentação animal.

O glicerol é um subproduto da transesterificação de um óleo e um dos produtos secundários obtidos durante a produção de biodiesel. Pelas técnicas para produção de biodiesel, atualmente utilizadas, cada 100 kg de biodiesel produzido gera aproximadamente 10 kg do subproduto glicerina bruta, contendo quantidades variáveis de glicerol e álcool, normalmente metanol (SANTIBÁNEZ; VARNERO; BUSTAMANTE, 2011). O glicerol residual da produção de biodiesel, denominado glicerina bruta, é contaminado por resíduos dos produtos utilizados na sua extração. Dentre esses produtos, o metanol é o

contaminante mais indesejável devido a sua toxidez para animais (NIE et al., 2007).

A resposta em consumo e desempenho de ruminantes ao uso de glicerol purificado como um suplemento energético (BODARSKI et al., 2005; DEFRAIN et al., 2004; FISHER et al., 1973), ou como um substituto do milho (CARVALHO et al., 2011; DONKIN et al., 2009; PARSONS; SHELOR; DROUILLARD, 2009; SHIN et al., 2012) tem sido relatados. Entretanto, pouco é conhecido sobre o efeito da glicerina acrescida de metanol sobre o padrão de fermentação, a microbiota e as perdas fermentativas durante a ensilagem da cana-de-açúcar. Redução na concentração de FDN e aumento na concentração de MS e na estabilidade aeróbia foram observados em silagem de cana-de-açúcar e milho tratada com glicerina (DIAS JÚNIOR et al., 2010; GOMES, 2013; KREMPSER et al., 2011; OLIVEIRA et al., 2011). Em silagem da cana-de-açúcar aditivada com glicerina contendo metanol, a adição de leveduras metilotróficas, que são capazes de utilizar metanol como fonte de energia, poderia reduzir, de forma econômica, a concentração de metanol, enquanto o valor energético da glicerina compensaria as perdas energéticas que ocorrem durante a fermentação da cana-de-açúcar. *Pichia methanolica* é uma levedura capaz de metabolizar metanol (HARTNER; GLIEDER, 2006).

Diante da opção de utilizar glicerina na dieta de animais ruminantes, a adição desse aditivo na silagem facilita o manejo alimentar, uma vez que a aplicação da glicerina na silagem evita a necessidade de armazenamento desse aditivo na fazenda, evita a necessidade de incluir um aditivo liquido na dieta e possibilita a compra de lotes homogêneos de glicerina. A observação de resultados promissores da utilização de glicerina pura na ensilagem da cana-de-açúcar sugere que avaliações com glicerina acrescida de metanol devem ser conduzidas. Os efeitos desse aditivo sobre a microbiota da ensilagem devem ser

bem elucidados, bem como a presença do metanol ao longo desse processo fermentativo.

O primeiro objetivo desse estudo foi selecionar cepas selvagens de bactérias do ácido lático em relação à redução nas perdas de MS, à melhoria na qualidade nutricional e ao aumento da estabilidade aeróbia, para serem utilizadas como inoculantes em silagem de cana-de-açúcar. Em uma segunda avaliação, foi utilizada a melhor cepa selecionada nos dois experimentos iniciais juntamente com a adição de diferentes doses de glicerina acrescida de metanol, uma levedura metilotrófica foi inoculada para reduzir a concentração de metanol durante a ensilagem da cana-de-açúcar.

# 2 REFERENCIAL TEÓRICO

## 2.1 Produção de silagem de cana-de-açúcar

A ensilagem da cana-de-açúcar resulta em vantagens operacionais relativamente ao corte diário. A cana-de-açúcar pode ser considerada uma forrageira adequada para ensilagem, por possuir conteúdo relativamente alto de MS, baixa capacidade de tamponamento e teores adequados de carboidratos solúveis (CHOs) (ZOPOLLATTO; DANIEL; NUSSIO, 2009). Entretanto, uma diferença importante entre a cana-de-açúcar e outras forrageiras tradicionalmente utilizadas para ensilagem, como o milho e o sorgo, é a natureza química dos seus CHOs. A cana-de-açúcar apresenta alto teor de carboidratos não fibrosos (CNF) na forma de sacarose, um dissacarídeo constituído por glicose e frutose. Este tipo de carboidrato parece favorecer o desenvolvimento de leveduras durante a ensilagem (WOOLFORD, 1984). Leveduras convertem a sacarose em etanol, $CO_2$ e água, um exemplo típico de fermentação alcoólica, que aumenta a perda de MS e de energia na silagem (KUNG JUNIOR; STANLEY, 1982).

No Brasil, muitas pesquisas vêm sendo direcionadas na busca pela melhoria da qualidade e redução nas perdas de MS em silagem de cana-de-açúcar (ÁVILA et al., 2009; BORGATTI et al., 2012; CARVALHO et al., 2012; NOVINSKI et al., 2012). A perda de MS durante a fermentação de silagens representa o consumo de compostos fermentescíveis presentes na forragem por microrganismos. A perda de MS também pode ocorrer por lixiviação de efluentes, que ocorre principalmente quando a forrageira é colhida com baixa concentração de MS. Esse tipo de lixiviação é comumente visualizado durante a ensilagem de cana-de-açúcar. As perdas de MS da silagem de cana variam de 8,1 a 35,3% (ZOPOLLATTO; DANIEL; NUSSIO, 2009) enquanto as perdas de

MS em silagem de milho situam-se entre 5 e 14% (ARRIOLA; KIM; ADESOGAN, 2011). Esse tipo de perda durante a ensilagem representa perda de componentes nutricionais e consequentemente, perda econômica para o produtor.

## 2.2 Produção de biodiesel

A pesquisa na busca de fontes alternativas de energia derivadas de matérias primas renováveis é impulsionada por fatores econômicos, políticos, sociais e ambientais relacionados à crescente preocupação mundial com uso de combustíveis fósseis. Nesse cenário, umas das alternativas promissoras para substituir o óleo diesel derivado do petróleo é o biodiesel, um combustível produzido por fontes renováveis de energia. O biodiesel pode ser produzido a partir de gordura animal ou óleo vegetal presente em várias espécies vegetais brasileiras como mamona, dendê, girassol, babaçu, palma, algodão, coco, pinhão manso, amendoim, soja e milho. Assim, o Brasil se destaca com grande potencial para a produção de biocombustíveis. Em 2011, a produção brasileira de biodiesel foi de 2,7 bilhões de litros e como co-produto foi gerado 273 milhões de litros de glicerina (ANP, 2013).

A produção de biodiesel pode ser feita por diferentes processos, sendo a transesterificação alcoólica via catalítica a mais empregada (KRAUSE, 2008). A transesterificação consiste na reação química do óleo ou gordura com um mono-álcool de cadeia curta (metanol ou etanol), na presença de um catalisador (ácido ou básico), levando a formação de mono-ésteres (biodiesel) e glicerina (glicerol bruto) (MA; HANNA, 1999). Cada 100 kg de biodiesel produzido gera aproximadamente 10 kg do subproduto glicerina bruta, contendo quantidades variáveis de glicerol e álcool, normalmente metanol (SANTIBÁNEZ; VARNERO; BUSTAMANTE, 2011).

O crescimento na produção de biodiesel com consequente aumento na produção de glicerina bruta aumenta a disponibilidade desse co-produto, reduzindo seu valor no mercado. O excedente de glicerina não purificada representa um problema para indústria podendo causar sérios prejuízos caso seja liberado no meio ambiente. A glicerina bruta traz outros problemas como resíduos e impurezas e um processo de purificação é oneroso. Deste modo, a glicerina bruta não pode ser utilizada por indústrias que requerem um composto mais puro como, por exemplo, a alimentícia, a farmacêutica e a de cosméticos. Portanto novas formas de utilização da glicerina devem ser estudadas. Por ser fonte de energia, um possível destino para a glicerina bruta resultante da indústria do biodiesel é seu uso na composição de dietas animais e sua adição em silagens.

## 2.3 Aditivos utilizados para melhoria do processo fermentativo de silagens

Os aditivos para silagem têm sido desenvolvidos para atuar sobre o processo fermentativo, visando melhorar o valor nutritivo, reduzir a produção de etanol e as perdas de MS, controlar o crescimento de microrganismos indesejáveis e favorecer o crescimento de microrganismos dos gêneros *Lactobacillus, Enterococcus, Pediococcus e Propionibacterium*, além de aumentar a estabilidade aeróbia da silagem de cana-de-açúcar (ÁVILA et al., 2009; CARVALHO et al., 2012; FREITAS et al., 2006). Os aditivos de silagens podem ser classificados em cinco principais categorias: estimulantes de fermentação, inibidores de fermentação, inibidores de deterioração aeróbica, nutrientes e absorventes (MCDONALD; HENDERSON; HERON, 1991). Os aditivos devem ser seguros quanto ao seu manuseio, minimizar a ocorrência de patógenos e serem economicamente viáveis (HENDERSON, 1993).

A utilização de aditivos em uma fermentação não controlada como a de silagens implica em resultados de difícil previsão, uma vez que a fermentação é conduzida por uma microbiota extremamente variável. De acordo com Ávila et al. (2011), a qualidade da fermentação é influenciada pelo tipo e pela quantidade de ácidos formados, que depende das condições em que a silagem foi produzida e especialmente da população de microrganismos presentes. Por isso o tipo de aditivo mais utilizado em silagens são os aditivos microbianos (MCDONALD; HENDERSON; HERON, 1991; MUCK, 2013). A utilização de aditivos microbianos visa favorecer o crescimento de cepas específicas que são inoculadas nas silagens, fazendo com que a fermentação seja dominada por tal microrganismo inoculado. Se a cepa inoculada for criteriosamente selecionada para a forrageira em que está sendo inoculada, a inoculação provavelmente implicará em respostas positivas. Em razão das características fermentativas da silagem de cana-de-açúcar, a utilização de aditivos é fundamental para redução das perdas de MS e melhoria da qualidade fermentativa.

### 2.3.1 Aditivos microbianos

Os aditivos microbianos são classificados como estimulantes da fermentação ou inibidores da deterioração aeróbia e são empregados por meio da adição de culturas bacterianas, são os aditivos mais utilizados e estudados (MCDONALD; HENDERSON; HERON, 1991; MUCK, 2013). Os produtos comercializados, atualmente, incluem bactérias láticas homofermentativas, heterofermentativas, *Propionibacterium* ou a sua associação. Os efeitos da inoculação com espécies de bactéria do ácido lático (BAL) são atribuídos ao aumento da população desses microrganismos na silagem, a alta produção de ácidos, a aceleração do estabelecimento da anaerobiose, ao aumento na

concentração dos ácidos acético e propiônico que inibem fungos filamentosos e leveduras e a produção de bacteriocinas (DUNIÈRE et al., 2013).

Com o objetivo de promover a acidificação mais rápida da silagem são referidas as espécies *Enterococcus faecium*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus buchneri*, *Pediococcus pentosaceus*, *Enterococcus hirae*, *Propionibacterium freudenreichiic* (ADESOGAN et al., 2003; ARRIOLA; KIM; ADESOGAN, 2011; ÁVILA et al., 2009; CAI, 1999; CARVALHO et al., 2012; KUNG JUNIOR; RANJIT, 2001). Com objetivo de aumentar a estabilidade aeróbia, já foram utilizadas as espécies *Pediococcus cerevisiae*, *Propionibacterium acidipropionici*, *Pediococcus pentosaceus*, *Lactobacillus buchneri* (FILYA; SUCU, 2007; FILYA; SUCU; KARABULUT, 2004; HIGGINBOTHAM et al., 1998; REICH; KUNG JUNIOR, 2010; SCHMIDT; KUNG JUNIOR, 2010). Para reduzir o crescimento ou a atividade metabólica de microrganismos indesejáveis existem trabalhos com as espécies *Enterococcus faecium*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus casei* (MARCINAKOVA et al., 2008; STEIDLOVÁ; KALAC, 2004).

Entre os fatores que determinam o sucesso da aplicação de microrganismos nas silagens, citam-se a compatibilidade entre a planta forrageira e o microrganismo (ÁVILA et al., 2011; MUCK, 2008), a habilidade de crescimento da bactéria na massa de forragem e a inoculação de uma população suficiente em relação à epífita da forragem (ÁVILA et al., 2011; ZOPOLLATTO; DANIEL; NUSSIO, 2009). Em relação à inoculação de silagens com BAL, o uso de cepas heterofermentativas está associado à maior estabilidade aeróbia das silagens, enquanto o uso de cepas homofermentativas é relacionado a uma maior produção de ácido lático e menores valores de pH (MCDONALD; HENDERSON; HERON, 1991).

Leveduras são microrganismos indesejáveis durante a fermentação da silagem, uma vez que perdas fermentativas são atribuídas a esse grupo de microrganismo (CARVALHO et al., 2012; ZOPOLLATTO; DANIEL; NUSSIO, 2009). A falta de conhecimento relacionado às espécies e ao metabolismo das leveduras envolvidas no processo de ensilagem de diferentes forrageiras, principalmente forrageiras tropicais, muitas vezes resulta no insucesso da tentativa de se reduzir a população desse grupo de microrganismo.

Em determinadas condições, leveduras podem ser utilizadas como inoculantes em silagens com objetivos específicos. Kitamoto et al. (1999), Kitamoto, Ohmomo e Nakahara (1993) e Lowes et al. (2000) estudaram a adição de leveduras ou produtos de seu metabolismo para melhorar características de silagens. A levedura *Williopsis mrakii* produz uma substancia tóxica (HMK) a outras leveduras. Lowes et al. (2000) utilizaram o gene dessa levedura para promover uma expressão heteróloga dessa toxina em *Aspergillus niger*. O *Aspergillus niger* foi cultivado e a toxina liberada em sua forma ativa no meio de cultivo. A ação dessa toxina no aumento da estabilidade aeróbia de silagem de milho foi avaliada. Após 72 h de exposição ao ar, observou-se menor valor de pH, maior valor de MS, menor população de leveduras e bactérias do ácido acético, quando comparada com a silagem controle. Kitamoto et al. (1999) avaliaram a estabilidade aeróbia de silagem de milho inoculada com cepa de *Kluyveromyces lactis* PCK27 geneticamente modificada, a qual ficou incapacitada de utilizar o ácido lático. Em estudos laboratoriais a *K. lactis* PCK27 inibiu o crescimento de *Pichia anômala*, os autores atribuem tal fato não somente ao efeito competitivo mas também a uma proteína letal produzida. Nesse mesmo experimento, os autores obtiveram resultados divergentes da inoculação da levedura *K. lactis* PCK27, em experimentos com silos laboratoriais. Tal fato é atribuído a ampla diversidade de microrganismos

associados à deterioração aeróbia. Os autores sugerem a necessidade de novas avaliações.

Em silagem de cana-de-açúcar aditivada com glicerina bruta contendo metanol, a adição de leveduras metilotróficas, ou seja, com capacidade para utilizar metanol como fonte de energia, poderia reduzir o nível de contaminação com o metanol ao mesmo tempo em que o valor energético da glicerina compensaria a perda energética durante a fermentação da cana-de-açúcar. Nesse caso, a espécie *Pichia methanolica* NCYC1381 seria aplicada como potencial utilizadora do metanol (HARTNER; GLIEDER, 2006), que é um contaminante indesejável na glicerina bruta. Essa levedura é incapaz de utilizar a sacarose e o ácido lático como fonte energética.

#### 2.3.1.1 Seleção de microrganismos para inoculação em silagem

A seleção de microrganismos para serem utilizados como culturas iniciadoras em diversos alimentos e bebidas como cerveja, vinho, queijo, iogurte, vinagre etc., é realizada com eficiência em longa data (BOKE; ASLIM; ALP, 2010; CAMPOS et al., 2010; STEGER; LAMBRECHTS, 2000). Para o processo de ensilagem a seleção de culturas iniciadoras não é tão comum, ainda que a pesquisa por cepas especializadas tenha aumentado nos últimos anos (ÁVILA et al., 2009; CHEN et al., 2012; DOGI et al., 2013; LIU et al., 2012; SAARISSALO et al., 2007).

Embora a maioria dos resultados da inoculação de silagens com cepas microbianas sejam favoráveis, existem dados que mostram que a inoculação de determinadas cepas pioram ou não tem efeito sobre a qualidade da silagem quando comparada com a silagem sem inoculação (KLEINSCHMIT; KUNG JUNIOR, 2006; ZOPOLLATTO; DANIEL; NUSSIO, 2009). Ávila et al. (2011) ressaltam que o efeito positivo de inoculantes durante a fermentação da silagem

depende de vários fatores relacionados com a planta forrageira e com as condições de ensilagem. E ainda que, durante a seleção de cepas destinadas à ensilagem deve ser considerado: a origem do microrganismo, caracterização fisiológica e identificação, definição das características avaliadas na pré-seleção, avaliação do microrganismo em pequena e grande escala e por fim a avaliação do microrganismo em diferentes culturas. Sendo que um dos fatores que determinam o sucesso da inoculação é a compatibilidade entre planta e microrganismo (KLEINSCHMIT; KUNG JUNIOR, 2006; MUCK, 2008).

É importante ressaltar que no processo de seleção de cepas não devemos dirigir o foco da pesquisa para uma determinada espécie, uma vez que novas espécies podem apresentar resultados positivos como inoculantes em silagens. Embora microrganismos da mesma espécie apresentem características similares, diferenças entre cepas da mesma espécie são observadas (ÁVILA et al., 2010; SAARISSALO et al., 2007). Esses fatos justificam a seleção de microrganismos para cada forrageira em particular. Devendo-se considerar, no processo de seleção, as características e os problemas fermentativos de cada forragem.

Entre as características ideais requeridas em um inoculante microbiano para o processo de ensilagem, deve-se observar sua capacidade de promover uma fermentação adequada, com rápida e eficiente queda no pH, capacidade de competir com a microbiota epifítica da forrageira, ter rápido crescimento em condições de baixo pH e baixa difusão de oxigênio, não ser patogênica, não ser resistente à antibióticos, possuir a capacidade de sobreviver durante todo o processo fermentativo, melhorando a estabilidade aeróbia, inibir o crescimento de microrganismos patogênicos e deterioradores da silagem (ÁVILA et al., 2011; DOGI et al., 2013; MCDONALD; HENDERSON; HERON, 1991; SAARISSALO et al., 2007).

#### 2.3.1.2 Utilização e seleção de inoculantes microbianos em silagem de cana-de-açúcar

Como referido anteriormente, a principal dificuldade na ensilagem da cana-de-açúcar são as altas perdas de MS que ocorrem durante a fermentação dessa forrageira. Assim diversos estudos relacionados à ensilagem da cana-de-açúcar são focados em melhorar características fermentativas.

O inoculante microbiano mais utilizado em silagem de cana-de-açúcar no Brasil é composto por cepas heterofermentativas obrigatórias da espécie *L. buchneri* (ZOPOLLATTO; DANIEL; NUSSIO, 2009). A menor utilização de cepas heterofermentativas facultativas ou homofermentativas em silagem de cana-de-açúcar é resultado de um menor número de respostas favoráveis quando esse grupo de microrganismo é empregado (ÁVILA et al., 2010; VALERIANO et al., 2009; ZOPOLLATTO; DANIEL; NUSSIO, 2009). Os diferentes compostos produzidos por bactérias heterofermentativas obrigatórias podem explicar essas diferenças nos resultados. Entre os trabalhos revisados, o aumento na concentração de ácido acético é a principal diferença em relação à silagem controle, quando cepas heterofermentativas obrigatórias são utilizadas (ÁVILA et al., 2009, 2010; PEDROSO et al., 2011). O ácido acético é considerado um fungicida (DANNER, 2003; MOON, 1983) provavelmente causa uma redução na população de leveduras na silagem. As leveduras são o principal grupo de microrganismos associados à perda de MS em silagem de cana-de-açúcar (KUNG JUNIOR; STANLEY, 1982). Outros metabólitos produzidos por esses microrganismos inoculados também influenciam a fermentação. Entretanto, não foram encontrados trabalhos que avaliam outros metabolitos além dos ácidos lático, acético, propiônico e butírico e etanol em silagem de cana-de-açúcar.

Diante dos melhores resultados apresentados pela inoculação com cepas heterofementativas, Ávila et al. (2009) isolaram cepas das espécies *L. buchneri*, *L. brevis* e *L. plantarum* da própria silagem de cana-de-açúcar. A cepa *L.*

*buchneri* SIL 72 foi avaliada juntamente com uma cepa comercial da mesma espécie. A inoculação com a cepa selvagem e com a cepa comercial resultou em silagens de melhor qualidade. A silagem tratada com a cepa selvagem resultou em uma concentração, significativamente maior, de ácido propiônico, um resultado positivo e pouco comum para as condições de ensilagem. Outros trabalhos foram conduzidos avaliando as cepas selvagens isoladas por esses autores (ÁVILA et al., 2010, 2012; CARVALHO et al., 2012; VALERIANO et al., 2009), sendo os melhores resultados observados, com a inoculação da silagem de cana-de-açúcar, com cepas heterofermentativas. Nas avaliações onde a cepa selvagem SIL 72 foi comparada com cepas comerciais os resultados promovidos pela cepa selvagem foram iguais ou melhores que os das cepas comerciais, evidenciando a necessidade de pesquisas em busca de novas e melhores cepas destinadas à ensilagem da cana-de-açúcar.

### 2.3.2 Outras classe de aditivos

Além dos aditivos microbianos diversos outros tipos de aditivos têm sido empregados na ensilagem com diferentes objetivos. Como aditivos inibidores da fermentação e inibidores da deterioração aeróbia são citados os ácidos orgânicos e a amônia. Os ácidos orgânicos são utilizados tanto como antimicrobianos quanto como acidificadores. Em estudos realizados por Moon (1983), foi verificado que os ácidos acéticos e propiônicos são melhores inibidores do crescimento das leveduras que o ácido lático e que as misturas de ácidos láticos, propiônico ou acético desempenham um efeito inibitório sinergístico. O uso de ácidos não tamponados é quase inexistente em virtude do alto custo, dos danos causados ao maquinário e do risco de acidentes durante o manuseio. A avaliação de ácido propiônico foi conduzida de forma experimental com objetivo de reduzir a população de leveduras em silagem de cana-de-açúcar.

Esse ácido foi eficiente em reduzir a população de leveduras e de clostrídios nas silagens de cana-de-açúcar (CARVALHO et al., 2012). A amônia foi aplicada em silagem de grão úmido de milho (DIAZ et al., 2013), e a uréia aplicada em silagem de trigo (BAL; BAL, 2012) e em silagem de cana-de-açúcar (BORGATTI et al., 2012) com o objetivo de reduzir leveduras e fungos filamentosos e controlar perdas fermentativas. A amônia tetraformato (amônia + ácido fórmico) foi recentemente utilizada como agente acidificador. O benzoato de sódio, propionato de sódio, nitrito de sódio e hexametilenotetramina (hexamina) foram aplicados como agentes antimicrobianos (CONAGHAN; O'KIELY; O'MARA, 2012). O cloreto de sódio também tem sido aplicado em silagens para controlar crescimento microbiano, principalmente na superfície de silos trincheira (BAL; BAL, 2012; NERES et al., 2013).

Aditivos alcalinos são utilizados para melhorar os coeficientes de digestibilidade de forrageiras, palhas e resíduos agrícolas (BAL; BAL, 2012; BORGATTI et al., 2012; CARVALHO et al., 2012). A justificativa para a utilização de bases está no fato da lignina de gramíneas ser susceptível à hidrólise provocada por álcalis em ligações covalentes do tipo éster entre a lignina e a parede celular (SOEST, 1994). Entre os aditivos alcalinos utilizados em silagens no último ano, podem ser citados: hidróxido de sódio, calcário, cal, cal hidratada, bicarbonato de sódio (BAL; BAL, 2012; BORGATTI et al., 2012, CARVALHO et al., 2012).

Existem ainda as enzimas, geralmente extraídas de microrganismos, usadas com a finalidade de aumentar a disponibilidade de CHOs e aumentar a digestibilidade da fibra (KUNG JUNIOR; STOKES; LIN, 2003). A aplicação de enzimas fibrolíticas solubiliza os carboidratos presentes na parede celular, fornecendo substratos para fermentação (DEHGHANI et al., 2012). Tal fato é principalmente vantajoso em forrageiras que contém baixa concentração de CHOs e alta concentração de proteína, com alto poder tampão. As principais

enzimas utilizadas como aditivos em silagens, no último ano, foram: xilanase, glucanase, β-glucanase, β-glucosidase, pectinase, celulase, hemicelulase e amilase, sendo estas aplicadas isoladamente ou em conjunto (DEHGHANI et al., 2012; FUGITA et al., 2012). Os resultados do uso de enzimas são dependentes da(s) enzima(s) aplicada e da forrageira. Em geral aumento na produção de ácidos, redução do pH e da concentração de nitrogênio amoniacal são observados (DEHGHANI et al., 2012).

Os aditivos classificados como nutrientes são adicionados a foragem no momento da ensilagem. Entre eles, podem ser citadas a amônia anidra, ureia, melaço, grãos de destilaria, milho moído, polpa cítrica, minerais e misturas entre eles. Esses aditivos são utilizados com a finalidade de suprir nutrientes para os microrganismos durante a fermentação ou suprir os animais que serão alimentados com essa silagem. Assim, aditivos nutrientes são mais utilizados em silagens de forrageiras que apresentam problemas de fermentação por ter conteúdo baixo de carboidratos, como é o caso de silagens de capins tropicais. Aditivos nutrientes também são utilizados em silagens nas quais ocorrem muitas perdas durante a fermentação, como a silagem da cana-de-açúcar, e em silagens de forrageiras com alto poder tampão, como de leguminosas (MCDONALD; HENDERSON; HERON, 1991). Entre os trabalhos revisados, os aditivos nutrientes que foram recentemente utilizados em silagens são: melaço e farinha de mandioca em silagem de capim Napier (*Pennisetum purpureum*) (BUREENOK et al., 2012). Os grãos de destilaria foram utilizados em silagem de festuca (*Festuca arundinacea*) e em silagem de palha de cevada (*Hordeum vulgare*) (YUAN et al., 2012). A sacarose foi utilizada em silagens de gramíneas e leguminosas tropicais (HEINRITZ et al., 2012) e em silagem de trigo (BAL; BAL, 2012). A casca de soja e grãos de milho foram adicionados em silagem de Tifton 85 (*Cynodon* spp) (NERES et al., 2013).

Como aditivo em silagem, a glicerina seria classificada como aditivo nutriente. A atuação sobre o substrato disponível para crescimento microbiano pode alterar o perfil fermentativo da silagem, reduzindo a perda de MS durante o armazenamento e aumentando o conteúdo de energia da forragem. A utilização de glicerina bruta como aditivo em silagem seria feita com o intuito de modificar a fermentação, promover o metabolismo de contaminantes dessa glicerina e utilizar o glicerol residual do processo de ensilagem como fonte de energia para animais ruminantes.

#### 2.3.2.1 Glicerina

Glicerina é o nome comercial de um líquido viscoso, incolor, inodoro, higroscópico e com sabor adocicado, quimicamente definido como glicerol ou propano-1,2,3-triol, de fórmula $C_3H_5(OH)_3$. O termo é muito utilizado na literatura como sinônimo de glicerol, apesar da glicerina ser composta por proporções variáveis de glicerol e outros compostos. O termo glicerol aplica-se somente ao composto puro, enquanto o termo glicerina aplica-se à purificação de compostos comerciais (MORRISON, 1994). O glicerol é considerado um ingrediente seguro para alimentação animal (FOOD AND DRUG ADMINISTRATION - FDA, 2006). O valor energético da glicerina bruta é proporcional à concentração de glicerol e dita seu valor comercial (LAMMERS et al., 1991). A concentração de glicerol na glicerina bruta, originária do processo de produção de biodiesel, é variável e o nível de contaminantes dessa glicerina bruta deve ser considerado. A purificação pode resultar em produtos com até 99% de glicerol, no entanto o processo de purificação é oneroso e pode tornar inviável o uso desse subproduto na alimentação animal.

A reação de transesterificação é reversível, o álcool é adicionado em excesso para deslocar a reação no sentido dos produtos. Os alcoóis utilizados

podem ser metanol, etanol, propanol ou butanol. O metanol é o principal álcool utilizado devido à redução no tempo de reação, e por sua recuperação ser mais simples e acessível economicamente que os outros alcoóis (COOPER; WEBER, 2012). O teor de metanol é particularmente importante e variável, dependendo da indústria produtora e da matéria prima empregada na produção de biodiesel (THOMPSON; HE, 2006). Variações de 0,006 a 14,98% de metanol na glicerina produzida nos EUA foram revisadas por Shurson et al. (2012). No Brasil, concentrações de 0.27 a 2,23% foram observadas em amostras de diferentes indústrias por Silva et al. (2011), Teixeira (2013) e Zacaroni (2010). Alguns países têm estabelecido um nível máximo permitido de metanol na glicerina destinada à alimentação animal: 0, 015% nos EUA e Brasil; 0,01% no Canadá; 0,2% na Alemanha e 0,5% na União Européia (HANSEN et al., 2009). As manifestações clínicas da intoxicação por metanol incluem distúrbios visuais, depressão do sistema nervoso central, com disfunção respiratória e acidose metabólica (NIE et al., 2007).

## 2.4 Glicerina na alimentação animal

O glicerol tem sido utilizado para tratamento de Cetose em bovinos desde a década de 50 (JOHNSON, 1954). Entretanto, os dados sobre inclusões de glicerol puro ou glicerina bruta em dietas de bovinos são inconsistentes. Observam-se variações de acordo com a dieta, com a fase produtiva do animal e com a qualidade da glicerina utilizada. Em uma avaliação de doses de glicerol adicionado à dieta para vacas em lactação, Donkin et al. (2009) constataram que níveis de até 15% (% da MS da dieta) de glicerina purificada (99,5) foram adequados. Aumento em produção de leite e efeito sobre balanço energético positivo foi relatado por Bodarski et al. (2005), Chung et al. (2007), Fisher et al.

(1973) e Shin et al. (2012) concluíram que teores dietéticos de até 10% de glicerol podem ser usados para vacas em lactação.

Respostas negativas também são observadas. Defrain et al. (2004) avaliaram a suplementação de glicerina bruta nos 21 dias anteriores à data prevista do parto até 21 dias pós-parto. Esses autores observaram que a glicerina foi depressora de consumo, induziu menor teor de glicose plasmática e aumentou a concentração ruminal de butirato quando comparada ao amido de milho. Wang et al. (2008) observaram que a produção diária de gordura no leite tendeu ($P$<0,10) a ser mais baixa nas vacas alimentadas com glicerol durante os primeiros 42 dias de lactação e a produção de proteína tendeu ($P$<0,09) a decrescer linearmente com o aumento da quantidade de glicerol suplementado. Zacaroni (2010) avaliou a resposta de vacas leiteiras à substituição total de milho grão moído fino por glicerina bruta; a inclusão dietética de glicerina representava 12,3% da MS. A dieta com glicerina reduziu a produção de leite ($P$=0,01). O autor ressalta que, a inclusão de 12,3% de glicerina bruta à dieta total pode ter sido alta demais. Possíveis efeitos deletérios do metanol residual da glicerina bruta podem ter induzido a resposta negativa a este tratamento. O estudo de estratégias viáveis economicamente para reduzir a concentração de metanol na glicerina bruta destinada à alimentação animal também é uma alternativa para o uso desse co-produto. Uma opção seria a metabolização do metanol por microrganismos metilotróficos durante o processo fermentativo da silagem.

O uso de glicerina como aditivo em silagem é ainda pouco estudado. Dias Júnior et al. (2010) avaliaram a adição de glicerina purificada (10% matéria fresca – MF) juntamente com os inoculantes microbianos comerciais Biomax LB (Chr Hansen, Milwaukee, EUA, composto por cepas de *Lactobacillus plantarum*, *Enterococcus faecium* e *L. buchneri*), Kera-Sil Cana (LNF Latino Americana, Bento Gonçalves, RS, composto por cepas de *L. plantarum* e

*Propionibacterium acidicipropionici*) e uma cepa de *L. buchneri* (UFLA SIL 72) isolado de silagem de cana-de-açúcar (ÁVILA et al., 2009). A inclusão de glicerol foi efetiva em reduzir o teor de fibra em detergente neutro (FDN) e aumentar o teor de MS das silagens, demonstrando ser uma estratégia para compensar a perda energética na ensilagem da cana. Os autores observaram que a inoculação da silagem com *L. buchneri* (UFLA SIL 72) foi efetiva em reduzir a perda de MS na silagem de cana de 31,2% no controle com glicerol para 15,7% no tratamento com glicerol e com esse inoculante ($P$<0,01). Foi observada redução na perda de MS não-fibrosa neste tratamento, sem afetar a perda de FDN, resultando em silagens com menor teor de FDN.

Inclusões de 0, 5, 10, 15 e 20% (MF) de glicerina semipurificada na silagem de milho e de cana-de-açúcar foram avaliadas por Gomes (2013). O autor observou aumento na concentração de MS, nutrientes digestíveis totais, matéria mineral, CNF e redução na concentração de FDN, fibra em detergente ácido, proteína bruta e extrato etéreo à medida que a inclusão da glicerina foi maior nas silagens de cana-de-açúcar e milho. Nestas silagens também foi observado aumento na digestibilidade *in vitro* da MS (DIVMS) (P<0,05) nas silagens com 15 e 20% de glicerina em relação aos níveis com 0, 5 e 10%. A glicerina melhorou a estabilidade aeróbia, mantendo o pH e temperatura baixos. Durante exposição aeróbia de silagens de milho tratadas com 5, 10 ou 15% de glicerina foi observado aumento da estabilidade aeróbia (OLIVEIRA et al., 2011) e redução nas populações de BAL, fungos filamentosos e leveduras (KREMPSER et al., 2011).

## 2.5 Metabolismo de glicerol por microrganismos

O glicerol é uma fonte de carbono assimilável por bactérias e leveduras sob condições aeróbicas e anaeróbicas (GANCEDO; GANCEDO; SOLS, 1968;

TACCARI et al., 2012). A assimilação de glicerol envolve o transporte passivo (GANCEDO; GANCEDO; SOLS, 1968) e transporte ativo (NEVES, 2004) por meio da membrana plasmática. O crescimento de microrganismos em fontes de carbono alternativas aos carboidratos, como exemplo o glicerol, requer a capacidade de sintetizar hexoses (gliconeogênese) necessárias para a produção de vários componentes celulares (MOAT; FOSTER; SPECTOR, 2002; WALKER, 1998).

A metabolização desse composto é dependente das condições de cultivo e da cepa utilizada (TACCARI et al., 2012). Entre os fatores que influenciam o metabolismo de glicerol, o fornecimento de oxigênio é provavelmente o de maior importância devido às reações de oxidação e redução que alteram os produtos finais do metabolismo (POLADYAN et al., 2013). O suprimento limitado de oxigênio durante a fermentação do glicerol favorece a produção de 1,3-propanodiol, etanol e ácido acético (XIU et al., 2007; ZHAO; CHEN; YAO, 2006). Condições adversas de temperatura e pH afetam a expressão de genes que codificam a síntese de enzimas responsáveis pelo catabolismo de fontes de carbono como o glicerol (BARBIRATO; SOUCAILLE; BORIES, 1996; GONZALEZ et al., 2008; POLADYAN et al., 2013). As vias metabólicas para utilização do glicerol são semelhantes para leveduras e bactérias resultando na produção de ácidos orgânicos, carotenóides, lipídeo microbiano e biomassa microbiana (PASTERIS; SAAD, 2009; TACCARI et al., 2012).

Até hoje foram relatadas três vias para metabolização de glicerol: a principal via envolve a enzima glicerol quinase que fosforila o glicerol a glicerol 3-P; um gliceraldeído 3-P desidrogenase oxida o gliceraldeído 3-P a dihidroxiacetona-P (GANCEDO; GANCEDO; SOLS, 1968; PASTERIS; SAAD, 2009). Uma segunda via envolve a enzima glicerol desidrogenase que oxida o glicerol a dihidroxiacetona, que é fosforilada por uma dihidroxiacetona quinase resultando em dihidroxiacetona-P. A dihidroxiacetona-P é um

importante intermediário para gliconeogênese, assim como para obtenção de vários compostos por meio de vias oxidativas. Dentre estes compostos estão os ácido cítrico, ácido succínico, ácido acético, ácido fórmico, ácido lático e etanol (BARBIRATO; SOUCAILLE; BORIES, 1996; PASTERIS; SAAD, 2009). Uma terceira via envolve a enzima glicerol desidratase. Por meio desta enzima, o glicerol é desidratado a 3-hidroxipropionaldeído (3-HPA). O 3-HPA pode ser transformado em acroleína por desidratação química sob condições ácidas ou sob altas temperaturas. O 3-HPA também pode ser reduzido pela enzima NADH dependente 1,3-propanodiol desidrogenase a 1,3-propanodiol ou pode ser oxidado a ácido 3-hidroxipropiônico (PASTERIS; SAAD, 2009).

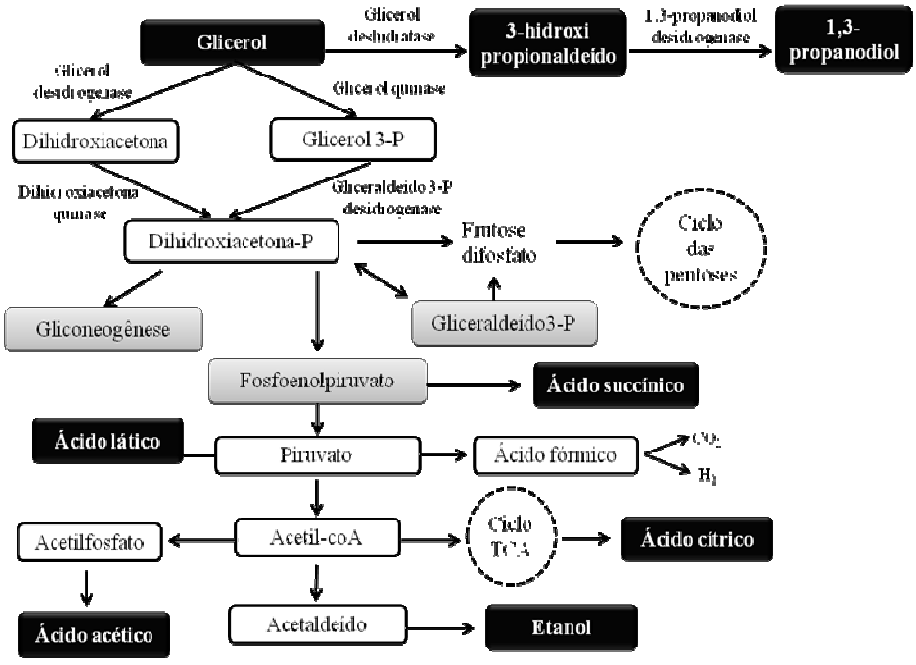


Figura 1 Vias metabólicas de assimilação de glicerol por microrganismos e seus possíveis produtos

Fonte: Modificado de Pasteris e Saad (2009) e Sun et al. (2010).

### 2.5.1 Bactérias

Cepas de BAL heterofermentativas das espécies *L. brevis, L. buchneri, L. collinoides* e *L. reuteri* podem utilizar o glicerol como um aceptor final de elétrons na co-fermentação anaeróbia com glicose (AXELSSON, 2004). Algumas cepas de *L. brevis* fermentam glicose deficientemente em condições anaeróbicas. Entretanto, outras cepas fermentam glicose se glicerol for adicionado. Os produtos da co-fermentação são lactato, acetato, $CO_2$ e 1,3-propanodiol. Nesse caso, o NADH formado durante a fermentação da glicose não é re-oxidado pela via do etanol, mas sim utilizando glicerol como aceptor final de elétrons. O glicerol é primeiramente desidratado a 3-hidroxipropionaldeído (3-HPA) e posteriormente reduzido a 1,3-propanodiol por uma $NAD^+$ 1,3-propanodiol desidrogenase (AXELSSON, 2004).

*L. reuteri* fermenta glicose tendo lactato, acetato, etanol e $CO_2$ como produtos finais, mas, mudanças na proporção para mais acetato/ menos etanol ocorrem quando glicerol é adicionado (TALARICO et al., 1990). A mesma via para redução de glicerol a 1,3-propanodiol que ocorre em *L. brevis* mostrou-se funcional em *L. reuteri* (SCHÜTZ; RADLER, 1984). Entretanto, algumas diferenças na resposta à presença de glicerol entre essas espécies podem ser notadas. Células em repouso de *L. brevis* metabolizando glicerol acumularam 1,2-propanodiol, enquanto *L. reuteri* sob essas condições acumularam e excretaram o intermediário 3-HPA (SCHÜTZ; RADLER, 1984). Esse composto é um potente antimicrobiano, denominado reuterina (PASTERIS; SAAD, 2009). Na presença de glicerol, cepas de *L. brevis e L. collinoides* também fermentam o lactato formado inicialmente a partir da glicose a acetato, etanol, $CO_2$ e 1,3-propanodiol (CUNHA; FOSTER, 1992). A formação de 1,2-propanodiol a partir da fermentação do glicerol também foi observada em enterobactérias (GONZALEZ et al., 2008). A produção de 1,3-propanodiol a partir da

fermentação anaeróbia do glicerol também foi observada em *L. diolivorans*, sendo a maior produção observada em meio contendo 0,1 mol de glicose para 1 mol de glicerol e suplementado com vitamina $B_{12}$ (PFLÜGL et al., 2012).

A adição de *L. coryniformis* 394 em silagem de palha de arroz contendo 1% de glicerol resultou em silagens com menores valores de pH, menores populações de BAL e de clostrídios e maior concentração de 3-HPA quando comparada com silagens tratadas com *L. rhamnosus* e glicerol ou *L. coryniformes* sem glicerol (TANAKA et al., 2009). Em vinho, após a fermentação alcoólica, quando os açúcares se esgotam, o glicerol pode ser utilizado por BAL para manter sua viabilidade. Dependendo de como esse glicerol é utilizado, ele pode ser responsável por modificações na qualidade do vinho. A cepa de *L. hilgardii* $X_1B$ isolada de vinho foi capaz de degradar o glicerol e produzir 3-HPA e ácido acético (PASTERIS; SAAD, 2009), ambos compostos indesejáveis durante a vinificação, porém desejáveis durante a ensilagem.

### 2.5.2 Leveduras

Em microrganismos eucarióticos, o glicerol constitui o principal composto regulador de variações de atividade de água em ambientes altamente osmofílicos (BRISSON, 2001; WANG et al., 2001). O transporte do glicerol por meio da membrana celular constitui a primeira etapa para seu metabolismo. Gancedo, Gancedo e Sols (1968) observaram que, em aerobiose, *Candida utilis* cresce mais rapidamente que *Saccharomyces cerevisae*, tendo como fonte de carbono o glicerol. Esse fato foi atribuído à maior facilidade de difusão do glicerol pela membrana plasmática de *C. utilis.* Várias espécies de leveduras dos gêneros *Candida, Pichia, Saccharomyces* e *Torulopsis* assimilam glicerol por meio da enzima glicerol-quinase na via fosforilativa. Espécies do gênero

*Hansenula* assimilam glicerol pela via fosforilativa e oxidativa (TANI; YAMADA, 1987). A expressão de enzimas dessas vias é reprimida durante o crescimento celular em substratos fermentescíveis como glicose, mas desregulado quando glicerol ou etanol é utilizado como a principal fonte de carbono (GRAUSLUND; RONNOW, 2000).

## 2.6 Metabolismo de metanol por leveduras

Leveduras metilotróficas são leveduras capazes de usar o metanol como única fonte de carbono, um número limitado de espécies de leveduras apresentam essa característica. Inicialmente, a aplicação biotecnológica dessas leveduras ficou limitada a produção de biomassa e, posteriormente observou-se que elas eram excelentes hospedeiras para produção de proteínas heterólogas (HARTNER; GLIEDER, 2006). Essas leveduras foram observadas dentro de quatro gêneros, *Hansenula*, *Pichia*, *Candida* e *Torulopsis* (HAZEU; DE BRUYN; BOS, 1972), sendo as espécies *Pichia pastoris*, *Hansenula polymorpha* (*Pichia angusta*), *Candida boidinii* e *Pichia methanolica*, os representantes mais importantes (CEREGHINO; CREGG, 2000).

Diferentes espécies de leveduras metilotróficas utilizam uma mesma via para catabolizar metanol, essa via é fortemente regulada em nível de transcrição (ELLIS et al., 1965; ROGGENKAMP et al., 1984). Como uma parte da via de utilização de metanol acontece nos peroxissomos, ocorre uma grande proliferação dessas organelas sob indução de metanol. O passo inicial no metabolismo do metanol é a oxidação desse composto a formaldeído e peróxido de hidrogênio por uma álcool oxidase. O peróxido de hidrogênio, que é tóxico para as células, é quebrado em oxigênio e água por catalases. O formaldeído pode ser tanto oxidado por duas reações subsequentes de dehidrogenases ou assimilado no metabolismo celular pela condensação com xilulose 5-P. Esta

última reação de condensação peroxisomal é catalisada por uma transquetolase chamada dihidroxiacetona sintase, que converte a xilulose 5-P e o formaldeído em compostos de 3C. Desses compostos, a dihidroxiacetona e o gliceraldeído 3-P são, posteriormente, metabolizadas no citosol. Nas reações de dehidrogenases conhecidas como via dissimilativa, o formaldeído reage com a glutationa formando S-hidroximetilglutationa que é oxidada em duas reações consecutivas (glutationa e $NAD^+$ dependente formato dehidrodrogenase) formando dióxido de carbono. Em geral, a expressão dos genes que regulam as vias de metabolismo do metanol é reprimida pela glicose e etanol e fortemente induzida pelo metanol (HARTNER; GLIEDER, 2006).

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**SEGUNDA PARTE – ARTIGOS**

**ARTIGO 1 Microbiological and chemical profile of sugar cane silage fermentation inoculated with wild strains of lactic acid bacteria**

(Artigo submetido ao periódico indexado: Journal of the Science of Food and Agriculture)

**ABSTRACT**

**BACKGROUND:** The aim of this study was to screen new strains of lactic acid bacteria (LAB) from sugar cane (*Saccharum* spp.) silage with different ensilage times. Fourteen wild LAB strains were evaluated. These strains were characterised biochemically (API 50 CHL, BioMérieux) and identified by sequencing of 16S rRNA.

**RESULTS:** The isolates were identified as *Lactobacillus plantarum*, *L. brevis* and *L. hilgardii*. Different fermentation profiles were observed among strains of the same species. The silages inoculated with *L. plantarum* specie showed the highest yeast population, ethanol concentration and dry matter loss. The silages inoculated with *L. brevis* UFLA SIL 17 and UFLA SIL 24 and *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains showed smaller dry matter (DM) loss and neutral detergent fibre (NDF) content. The silages inoculated with *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains resulted in 57% and 94% more acetic acid and 1,2-propanediol, respectively, when compared with inoculated silage.

**CONCLUSION:** Inoculation with *L. plantarum* strains was not beneficial for sugar cane silage. Obligatory heterofermentative strains showed better silage quality. *L. hilgardii* (UFLA SIL 51, UFLA SIL 52) strains are promising for use in sugar cane silage.

**Keywords:** *Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus hilgardii,* volatile fatty acids, dry matter loss.

## INTRODUCTION

Many factors contribute to the success of the natural fermentation of carbohydrate-rich foods, such as the fermentation of sugar cane (*Saccharum* spp.), traditional forage used in animal feed, mainly in Brazil. One of the main difficulties with the conservation of this forage as silage is the control of growth of yeasts. Excessive yeast growth results in high rates of dry matter (DM) loss.[1] The metabolic activity of lactic acid bacteria (LAB) plays a key role during the fermentation of the silage and is chiefly responsible for overall silage quality.[2, 3]

Each grass species has its own chemical characteristics, such as quality and quantity of carbohydrates, protein, fibre and other compounds that interfere with the fermentation process, thus favouring certain groups of microorganisms able to utilise the available substrates. For this reason, the evaluation of microorganisms destined for ensilage from different cultures should consider these characteristics.[4, 5, 6] The compatibility between the forage and the inoculant strain used is a determining factor in the success of using microbial additive.[6] In the case of sugar cane ensilage, there are studies demonstrating differences in the efficiency of inoculants containing LAB from different species or even different strains of the same species.[2]

The presence of epiphytic microorganisms also influences silage fermentation and LAB are naturally present on the surface of forage crops.[3] In most forage crops, this population is initially low but rapidly increases over the

course of the fermentation.[1, 7] However, the use of microbiological additives may promote the dominance of LAB and control the growth of undesirable microorganisms[4], in addition to promote a faster decrease in pH value. The selection of microorganisms specialised for ensilage of each forage specie has garnered interest among researchers and companies producing microbiological additives.[4, 6] Despite the great potential for LAB use as starter cultures in silage, the results of using these inoculants have been controversial depending on the substrate used and the parameters evaluated.[8, 9] Therefore, it is important the study the action of inoculants throughout the fermentation process.

The objective of the present study was to screen strains of LAB isolated from sugar cane silage, with particular interest in the species that are capable of reducing DM loss, and thus maintain the nutritive value of forage. The strains were identified at the biochemical and molecular level using 16S rRNA sequence analysis. The chemical and microbiological composition and silage fermentation characteristics were also studied.

## MATERIALS AND METHODS

### Biochemical and molecular characterisation of the utilised additives

The new strains were isolated from sugar cane silage, selected through laboratory tests (Ávila et al. unpublished data) and belong to a culture collection of the Laboratory of Microbial Physiology and Genetics at Department of

Biology / Federal University of Lavras (UFLA). Characterised strains belonging to the genus *Lactobacillus* were evaluated for the production of metabolites, and the strains with the highest production of lactic acid, acetic acid and propionic acid were selected for evaluation in PVC silos.

The strains were biochemically characterised by the use of API 50 CHL kits (BioMérieux). Gas production was evaluated in MRS (De Man Rogosa Sharpe) broth (Oxoid CM361, Basingstoke, Hampshire, England)[10]. These strains were also identified by sequencing their 16S rRNA. Each isolate was grown in MRS agar plates during 24h at 30ºC and collected with a sterile pipette tip and resuspended in 40 µl of PCR buffer. To achieve the DNA template the suspension was heated for 10 min at 95°C, and 2 µl was used in PCR experiments to amplify the full-length 16S region. An approximately 1500-bp fragment of the 16S rRNA was amplified using the forward primer 27f (5'AGAGTTTGATCCTGGCTCAG3') and the reverse primer 1512r (5'ACGGCTACCTTGTTACGACT3'). The PCR products were sequenced using an ABI3730 XL DNA Analyzer (Applied Biosystems, Foster City, California, CA). The sequences were compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

**Forage and ensilage conditions**

The silages were made in three different days, in each day one replication of each treatment (different strains and different time of silage fermentation) were made. In each day, fresh-cut sugar cane that was approximately 12 months old, was manually harvested and chopped (PP-47, Pinheiro, Itapira, SP, Brazil) to a length of 10 mm. Experimental silos (mini-silos) were used in the form of PVC tubes 10 cm diameter and 60 cm length. The tubes were sealed with tight lids containing Bunsen valves for gas release. Three silos were prepared for each evaluation day (12, 30, 61 and 126 days) with one of the 14 strains plus a control treatment. The new inoculants were pre-cultured in the laboratory and on the ensilage day were enumerated on MRS agar. The inoculation concentration was of $1.8 \times 10^6$ cfu $g^{-1}$ of fresh mater (FM). The inoculants were mixed with deionised water and sprayed in the forage, resulting in an application volume of 14.3 L $ton^{-1}$. The same volume of pure distilled water was added to the control treatment. A separate sprayer was used for each treatment to avoid cross-contamination. Each silo was packed with 3 kg of wet forage to achieve a packing density of approximately 666 kg $m^{-3}$ of FM. The weight of empty and full silos was recorded. The silos were sealed, stored at room temperature (25ºC ± 1.5ºC) and protected from sunlight and rain. After 12, 30, 61 and 126 days of ensilage, the full silos were weighed and opened. The

loss of DM was calculated using weights of the DM contents of fresh forage and silage.

**Analytical procedures**

To obtain the aqueous extract, a 25-g sample of fresh forage or sugar cane silage was blended in 225 mL of 0.1% sterile peptone water and homogenised in an orbital shaker for 20 min. The pH of each sample was then determined (DIGIMED® DM 20 Potentiometer, Digicrom Instrumentos, SP, Brazil). Aqueous extracts (2 mL) were acidified with 10 μL of 50% (vol/vol) $H_2SO_4$ and frozen prior to analysis of fermentation end products. The acidified aqueous extracts were analysed for acetic acid, butyric acid, propionic acid, lactic acid, ethanol and 1,2-propanediol by high-performance liquid chromatography according the method described by Carvalho *et al.*[7] The DM contents of each sample was determined using a forced-draft oven at 55ºC for 72 h. Dried samples were ground in a Wiley-type grinder through a 1-mm screen. DM at 105ºC was determined according AOAC (1990)[11]. Neutral detergent fibre (NDF) was analysed using the sulphite method as described by Van Soest *et al.*[12], using an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Water soluble carbohydrates (WSC) were analysed using the phenol method.[13]

**Microbiological analyses**

The other portion of aqueous extracts was used for enumeration of microorganisms. Sequential ten-fold dilutions were prepared to quantify the microbial groups. Yeasts and filamentous fungi were enumerated on Dichloran Rose Bengal Chloramphenicol Medium (DRBC, Difco; Becton Dickinson, Sparks, MD, USA). The plates were incubated at 28ºC for 72 h. Yeasts were distinguished from filamentous fungi by colony appearance and cell morphology. For enumeration of LAB, pour plating onto DeMan Rogosa Sharpe agar plus nystatin (4 mL $L^{-1}$) was used. The plates were incubated at 30ºC for 72 h. Colonies were counted on plates containing a minimum of 30 and a maximum of 300 cfu.

**Statistical analysis**

The experiment was carried out in randomised blocks, each block corresponds to a day of silage production. The treatments were assigned in a factorial arrangement, consisting of 15 experiments (14 LAB strains and a control without inoculant) and four silage fermentation periods (12, 30, 61 and 126 days). For each inoculant, there were prepared 12 silos (three replications and four opening days), totalling 180 experimental units. The data were analysed by SISVAR ®, by a model containing the fixed effects of blocks, inoculants,

days of ensilage and the interactions between the inoculants and the days of ensilage. The means were compared using Scott-Knott test.

The principal component analyses (PCA) were performed using the software XLSTAT 7.5.2 (Addinsoft's, New York, N.Y., U.S.A.) for grouping data of the fermentation products, DM loss, DM and NDF with evaluated strains. The data utilized in the PCA analysis were related to the average results of all ensiling periods evaluated.

## RESULTS

### Biochemical characteristics and molecular identification of strains

The characteristics, identification and API 50 CHL (BioMérieux) fermentation patterns of selected strains are shown in Tables 1 and 2. The *L. brevis* UFLA SIL 33, UFLA SIL 17, UFLA SIL 24, UFLA SIL 25 and, UFLA SIL 27 and *L. hilgardii*, UFLA SIL 51 and UFLA SIL 52 strains produced gas when cultivated in MRS broth.

**Table 1.** Properties and identification of the lactic acid bacteria (LAB) strains evaluated.

| Strain | Involved acid [1] | Gas production[2] | Biochemical identification | Molecular identification[3] |
|---|---|---|---|---|
| UFLA SIL 19 | Lactic | - | *L. plantarum* 99,9% | *L. plantarum* 98% (FJ669130.1)[4] |
| UFLA SIL 32 | Lactic | - | *L. plantarum* 99,9% | *L. plantarum* 99% (HM218291.1) |
| UFLA SIL 33 | Lactic | + | *L. brevis* 96,3% | *L. brevis* 98% (FJ227316.1) |
| UFLA SIL 34 | Lactic | - | *L. plantarum* 99,9% | *L. plantarum* 98% (HM218291.1) |
| UFLA SIL 17 | Acetic | + | *L. brevis* 99,8% | *L. brevis* 97% (FJ532364.1) |
| UFLA SIL 24 | Acetic | + | *L. brevis* 99,9% | *L. brevis* 99% (FJ227316.1) |
| UFLA SIL 25 | Acetic | + | *L. brevis* 96,3% | *L. brevis* 98% (FJ227316.1) |
| UFLA SIL 27 | Acetic | + | *L. brevis* 96,3% | *L. brevis* 98% (FJ227316.1) |
| UFLA SIL 35 | Acetic | - | *L. plantarum* 99,9% | *L. plantarum* 98% (HM218291.1) |
| UFLA SIL 41 | Propionic | - | *L. plantarum* 99,9% | *L. plantarum* 99% (HM218291.1) |
| UFLA SIL 42 | Propionic | - | *L. plantarum* 99,6% | *L. plantarum* 99% (HM218291.1) |
| UFLA SIL 46 | Propionic | - | *L. plantarum* 99,6% | *L. plantarum* 99% (HM218291.1) |
| UFLA SIL 51 | Propionic | + | *L. buchneri* 99,8% | *L. hilgardii* 99% (HM217953.1) |
| UFLA SIL 52 | Propionic | + | *L. buchneri* 99,8% | *L. hilgardii* 99% (HM217953.1) |

[1] Involved acid in the screening process based on the production of metabolites in sugar cane broth (Ávila et al unpublished data);
[2] From MRS broth;
[3] Sequencing of 16S rRNA;
[4]The number in parentheses refers to the access code in Gen-Bank.

All strains of the evaluated lactic acid bacteria were able to ferment L-arabinose, D-ribose, D-glucose, D-fructose and D-maltose. None of the strains

produced acid from glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, L-sorbose, dulcitol, inositol, inulin, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-frucose, L-frucose, D-arabitol and L-arabitol. Among the strains identified as *L. plantarum*, UFLA SIL 19, UFLA SIL 32, UFLA SIL 34, UFLA SIL 35 and UFLA SIL 41 fermented a higher number of carbohydrates (Table 2). Among these carbohydrates, the sucrose, that is the main carbohydrate present in the sugar cane. The strains UFLA SIL 42 and UFLA SIL 46, also identified as *L. plantarum*, were able to ferment 21 carbohydrates, but did not presenting fermentation positive results for sucrose, methyl-αD-mannopyranoside and D-rafinose.

The strains identified as *L. brevis* also showed differences in use of carbohydrates, but none produced positive sucrose fermentation results (Table 2). The strains UFLA SIL 17 and UFLA SIL 24 were able to use, respectively, twelve and fifteen carbohydrates and were differentiated only by the ability to use potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. The remaining evaluated strains of *L. brevis* fermented eight carbohydrates and were differentiated from the other strains by the incapacity to produce acid from methyl-βD-xylopyranoside, D-mannitol, methyl-αD-glucopyranoside, N-acetylglucosamine, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate.

The strains UFLA SIL 51 and UFLA SIL 52 were biochemically identified as *L. buchneri* (99.8%) and were able to ferment eleven different carbohydrates, including sucrose (Table 2). The 14 strains were also identified by the sequencing of the 16S region of the rRNA to confirm the biochemical identification. The sequencing results identified the strains UFLA SIL 51 and UFLA SIL 52 as *L. hilgardii* (98%). In the identification of other 12 strains, the sequencing results confirmed the biochemical identification.

**Table 2.** Fermentation patterns (evaluated using API 50 CHL strips; BioMerieux) of lactic acid bacteria strains evaluated.

| Item | UFLA SIL 19♦ | UFLA SIL 32♦ | UFLA SIL 33‡ | UFLA SIL 34♦ | UFLA SIL 17‡ | UFLA SIL 24‡ | UFLA SIL 25‡ | UFLA SIL 27‡ | UFLA SIL 35♦ | UFLA SIL 41♦ | UFLA SIL 42♦ | UFLA SIL 46♦ | UFLA SIL 51* | UFLA SIL 52* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D-Galactose | + | + | + | + | + | + | + | + | + | + | + | + | - | - |
| D-Glucose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Fructose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Manose | + | + | - | + | - | - | - | - | + | + | + | + | - | - |
| L-Rhamnose | w | w | - | w | - | - | - | - | - | w | w | w | - | - |
| D-Manitol | + | + | - | + | + | + | - | - | + | + | + | + | - | - |
| D-Sorbitol | + | + | - | + | - | - | - | - | + | + | + | + | - | - |
| Methyl-αD-Mannopyranoside | + | + | - | + | - | - | - | - | + | + | - | - | - | - |
| Methyl-αD-Glucopyranoside | - | - | w | - | + | + | w | - | - | - | + | + | - | - |
| N-Acetylglucosamine | + | + | w | + | + | + | w | w | + | + | + | + | - | - |
| Amygdalin | + | + | - | + | - | - | - | - | + | + | + | + | - | - |
| Arbutin | + | + | - | + | - | - | - | - | + | + | + | + | - | - |
| Esculin ferric citrate | + | + | w | + | - | - | w | - | + | + | + | + | - | - |
| Salicin | + | + | - | + | - | - | - | - | + | + | + | + | - | - |
| D-Celobiose | + | + | - | + | - | - | - | - | + | + | + | + | - | - |
| D-Maltose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Lactose | + | + | - | + | - | - | - | - | + | + | + | + | - | - |
| D-Melibiose | + | + | + | + | + | + | + | + | + | + | + | + | - | - |
| D-Saccharose | + | + | - | + | - | - | - | - | + | + | - | - | + | + |
| D-Trehalose | + | + | - | + | - | - | - | - | + | + | + | + | - | - |
| D-Melezitose | + | + | - | + | - | - | - | - | + | + | + | + | + | + |
| D-Rafinose | + | + | - | + | - | - | - | - | + | + | - | - | + | + |
| Gentiobiose | + | w | - | + | - | - | - | - | w | + | + | + | - | - |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D-Turanose | + | + | - | + | - | - | - | - | + | + | - | - | - | - |
| Potassium Gluconate | w | w | w | w | w | + | w | w | w | w | - | - | + | + |
| Potassium 2-Ketogluconate | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| Potassium 5-Ketogluconate | - | - | w | - | w | + | w | w | - | - | - | - | + | + |

[1]+ = Positive reaction; – = negative reaction; and w = weakly positive reaction. All strains gave negative results for glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, L-sorbose, dulcitol, inositol, inulin, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-frucose, L-frucose, D-arabitol, L-arabitol.

♦ *L. plantarum* specie, ‡ *L. brevis* specie, * *L. hilgardii* specie

**Fresh forage**

The characteristics of the sugar cane prior to the ensilage are shown in Table 3. LAB, filamentous fungi and yeasts were observed in the forage at population levels of 7.51, 5.07 and 5.72 log cfu $g^{-1}$ of forage, respectively. The average density obtained after the sealing of the mini-silos was 634 kg $m^{-3}$ of fresh forage.

**Table 3.** Chemical and microbial composition of fresh whole-plant of sugar cane.

| Item | Mean / Standard deviation |
|---|---|
| Lactic acid bacteria (log cfu $g^{-1}$ fresh forage) | 7.51* ± 0.617 |
| Yeasts (log cfu $g^{-1}$ fresh forage) | 5.72 ± 0.186 |
| Filamentous fungi (log cfu $g^{-1}$ fresh forage) | 5.07 ± 0.153 |
| pH | 5.75 ± 0.046 |
| Dry matter (DM) (g $kg^{-1}$) | 282 ± 1.17 |
| Neutral detergent fibre (g $kg^{-1}$ DM) | 480 ± 1.78 |
| Water soluble carbohydrates (g $kg^{-1}$ DM) | 247 ± 0.92 |
| Density (kg $m^{-3}$) | 634.1 ± 19.9 |

*Each mean was obtained in nine replicates.

**Chemical composition of the silages and fermentative loss**

The addition of different strains influenced ($P < 0.01$) the concentrations of DM, NDF and the losses of DM ($P < 0.01$) (Table 4). The silages inoculated with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 and *L. brevis* UFLA SIL 24 and UFLA SIL 17 strains resulted in the lowest DM losses. These strains also

presented the highest concentrations of DM and the lowest concentrations of NDF. In these variables also occurred modifications during the ensilage days ($P < 0.01$). However during the ensilage period, all silages showed similar behaviour (non-significant interaction between the factors). The concentration of DM decreased, while those of NDF and the losses of DM increased from the twelfth to the sixty-first day of fermentation. On the sixty-first day, the concentrations of these variables stabilised (Fig. 1A and 1B).

No significant difference in pH values of the silages was observed between the inoculated strains ($P = 0.98$). Thus, the modifications that occurred throughout the fermentation time were similar between the silages, in which the pH was stable until the 61th day of fermentation. From the 61th to the 126th day of fermentation, an increase in pH was observed (Fig. 1C).

When WSC concentration was examined, a significant interaction between the factors strains and days of ensilage ($P < 0.01$) was observed (Table 5). The differences between the strains were observed until day 30 of ensilage. At twelve days, the lowest concentration of WSC was observed in the silages treated with the *L. hilgardii* UFLA SIL 51and UFLA SIL 52 strains and the highest concentration was observed in the silage inoculated with the strain *L. plantarum* UFLA SIL 19 strain (Table 5).

**Table 4.** Dry matter (DM), neutral detergent fibre (NDF) and DM loss (DML) (DM basis) of sugar cane silages with novel inoculants.

| Inoculants | DM | NDF | DML |
|---|---|---|---|
| | g $kg^{-1}$ DM | | % of ensiled DM |
| Control | 263 b* | 571 b | 16.4 c |
| UFLA SIL 19♦ | 250 c | 623 a | 21.3 a |
| UFLA SIL 32♦ | 250 c | 631 a | 21.2 a |
| UFLA SIL 33‡ | 270 a | 576 b | 14.6 c |
| UFLA SIL 34♦ | 248 c | 651 a | 22.9 a |
| UFLA SIL 17‡ | 270 a | 556 b | 13.1 d |
| UFLA SIL 24‡ | 275 a | 569 b | 12.5 d |
| UFLA SIL 25‡ | 267 b | 565 b | 14.2 c |
| UFLA SIL 27‡ | 271 a | 570 b | 13.9 c |
| UFLA SIL 35♦ | 251 c | 638 a | 21.4 a |
| UFLA SIL 41♦ | 250 c | 639 a | 20.5 a |
| UFLA SIL 42♦ | 255 c | 630 a | 18.4 b |
| UFLA SIL 46♦ | 253 c | 642 a | 18.6 b |
| UFLA SIL 51* | 274 a | 578 b | 11.4 d |
| UFLA SIL 52* | 269 a | 590 b | 11.8 d |
| SEM t† | 2.21 | 7.81 | 0.89 |
| Probability for the effects contained in the model. | | | |
| Inoculants (I) | <0.01 | <0.01 | <0.01 |
| Days of ensilage (DE) | <0.01 | <0.01 | <0.01 |
| I x DE | 0.37 | 0.58 | 0.77 |

*For each column, means values with different small letters are significant at P < 0.05 by Scott–Knott test. † Standard error of the means. ♦ *L. plantarum* specie, ‡ *L. brevis* specie, * *L. hilgardii* specie.

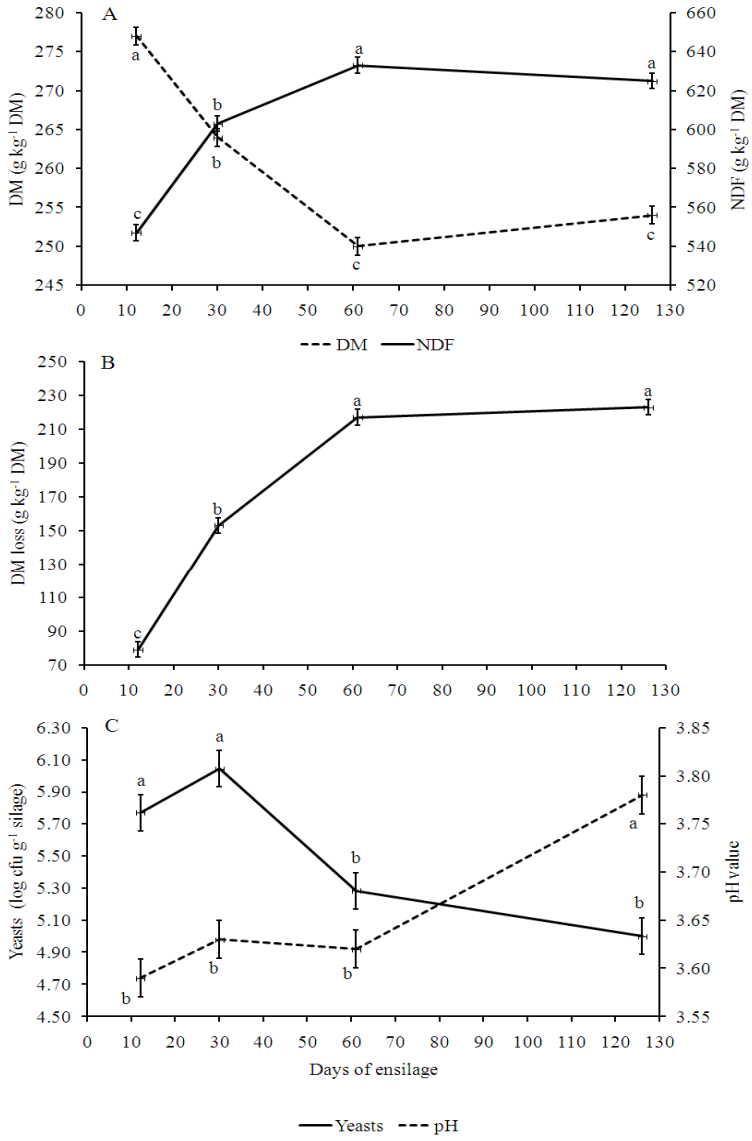


**Figure 1.** Dry matter (DM) and neutral detergent fibre (NDF) (A), DM loss (B) and number of yeasts and pH values (C) during ensilage of sugar cane. For yeast population means: $P < 0.01$ for inoculants (I); $P < 0.01$ for day of ensilage (DE): $P = 0.23$ for interaction T × DE. For pH means: $P = 0.97$ for treatment (T); $P < 0.01$ for day of ensilage (DE); $P = 0.99$ for interaction T × DE. Mean values with different lowercase letters are significant at $P < 0.05$ by Scott–Knott test. Bars represent the standard error of the means.

Throughout the ensilage, the highest average losses of WSC occurred from 12 to 30 days (52.6%). From the 61th and 126th days of fermentation, there was no difference between the WSC concentrations of the silages and the WSC disappearance were of 42% (Table 5).

There was a interaction ($P < 0.01$) between the added strains and the days of ensilage in regards to the concentrations of ethanol, 1,2-propanediol and of lactic, acetic, propionic and butyric acids. The exception was for the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains, which showed low rates of ethanol throughout the fermentation, the silages treated with the other strains and the control silage resulted in an increase in the ethanol concentration during the ensilage period. Ethanol concentration was the most outstanding differences between silages after the thirtieth day of fermentation. On the 126th day of ensilage, the strains UFLA SIL51 and UFLA SIL52 showed the lowest concentrations of ethanol.

**Table 5.** Effects of inoculation with different strains and of different days of ensilage on the concentrations of water soluble carbohydrate (WSC), ethanol and lactic acid in sugar cane silages.

| Days of ensilage | Inoculants (strains) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Ctr** | **UFLA SIL19♦** | **UFLA SIL32♦** | **UFLA SIL33‡** | **UFLA SIL34♦** | **UFLA SIL17‡** | **UFLA SIL24‡** | **UFLA SIL25‡** | **UFLA SIL27‡** | **UFLA SIL35♦** | **UFLA SIL41♦** | **UFLA SIL42♦** | **UFLA SIL46♦** | **UFLA SIL51*** | **UFLA SIL52*** |
| | **WSC (g kg$^{-1}$ of DM)** | | | | | | | | | | | | | | |
| 12 | 136.2bA* | 171.4 aA | 148.5 bA | 110.3 cA | 128.8 bA | 105.3 cA | 109.6 cA | 117.3 cA | 113.1 cA | 144.5 bA | 138.7 bA | 134.5 bA | 134.5 bA | 75.5 dA | 66.7 dA |
| 30 | 78.1 aB | 88.1 aB | 72.8 aB | 33.4 bB | 69.5 aB | 49.8 bB | 57.4 aB | 34.9 bB | 46.7 bB | 73.1 aB | 60.2 aB | 56.6 aB | 68.8 aB | 41.2 bB | 38.2 bB |
| 61 | 25.3 aC | 33.6 aC | 32.3 aC | 23.2 aB | 21.8 aC | 25.5 aC | 24.5 aC | 30.8 aB | 24.0 aC | 24.6 aC | 31.6 aC | 25.4 aC | 21.2 aC | 23.4 aB | 21.1 aB |
| 126 | 13.1 aC | 14.1 aC | 14.6 aC | 13.1 aB | 14.2 aC | 13.7 aC | 12.0 aC | 13.3 aB | 13.8 aC | 13.5 aC | 13.8 aC | 15.9 aC | 12.5 aC | 20.9 aB | 19.4 aB |
| Level of significance | Inoculants | | | | Days of ensilage | | | | | Inoculants x Days of ensilage | | | | | SEM† |
| | <0.01 | | | | <0.01 | | | | | <0.01 | | | | | 7.56 |
| | **Ethanol (g kg$^{-1}$ of DM)** | | | | | | | | | | | | | | |
| 12 | 26.7 bB | 48.9 aC | 51.4 aC | 19.3 bB | 73.8 aC | 23.2 aB | 17.0 bB | 11.2 bB | 18.1 bB | 61.5 aC | 45.2 aC | 55.2 aB | 54.3 aC | 27.9 aA | 35.7 aA |
| 30 | 46.6 dB | 90.1 cB | 113.3 bB | 73.5 cA | 164.5 aB | 35.5 dB | 46.1 dB | 85.3 cA | 70.1 cA | 131.4 bB | 148.0 aB | 85.7 cB | 117.4 bB | 38.6 dA | 38.2 dA |
| 61 | 137.1 bA | 189.3 aA | 210.1 aA | 87.2 cA | 217.8 aA | 76.2 cA | 94.4 cA | 88.9 cA | 102.7 cA | 185.8 aA | 208.3 aA | 187.5 aA | 180.4 aA | 39.9 dA | 44.4 dA |
| 126 | 104.2 cA | 174.0 aA | 171.5 aA | 72.3 cA | 207.1 aA | 85.1 cA | 80.2 cA | 77.3 cA | 77.2 cA | 200.5 aA | 137.6 bB | 180.6 aA | 144.1 bB | 32.4 dA | 36.0 dA |
| Level of significance | Inoculants | | | | Days of ensilage | | | | | Inoculants x Days of ensilage | | | | | SEM |
| | <0.01 | | | | <0.01 | | | | | <0.01 | | | | | 14.14 |
| | **Lactic acid (g kg$^{-1}$ of DM)** | | | | | | | | | | | | | | |
| 12 | 17.2 cB | 42.6 aB | 41.8 aA | 30.1 bA | 40.8 aA | 28.4 bB | 28.2 bB | 27.5 bA | 29.8 bA | 34.1 bB | 40.1 aA | 33.8 bB | 37.1 aB | 31.6 bA | 32.0 bA |
| 30 | 25.2 bB | 41.4 aB | 23.9 bB | 28.5 bA | 38.3 aA | 25.4 bB | 27.2 bB | 27.3 bA | 29.0 bA | 42.0 aA | 42.6 aA | 38.3 aB | 32.5 bB | 32.4 bA | 31.0 bA |
| 61 | 46.6 aA | 55.3 aA | 51.8 aA | 34.5 bA | 48.5 aA | 35.5 bA | 36.4 bA | 36.8 bA | 35.7 bA | 43.7 aA | 47.9 aA | 48.1 aA | 43.7 aA | 34.6 bA | 31.0 bA |
| 126 | 48.6 aA | 47.1 aB | 43.8 aA | 29.6 bA | 45.8 aA | 36.9 bA | 37.5 bA | 33.9 bA | 34.5 bA | 48.1 aA | 42.3 aA | 54.7 aA | 46.0 aA | 30.0 bA | 27.6 bA |

| Level of significance | Inoculants | Days of ensilage | Inoculants x Days of ensilage | SEM |
|---|---|---|---|---|
| | <0.01 | <0.01 | <0.01 | 3.30 |

* For each row, mean values with different lowercase letters are significant at P < 0.05 by Scott–Knott test. For each column, mean values with different capital letters are significant at P < 0.05 by Scott–Knott test. † Standard error of the means of two-way interactions (inoculants and days of ensilage). ♦ *L. plantarum* specie, ‡ *L. brevis* specie, * *L. hilgardii* specie.

The silages inoculated with *L. plantarum* strains produced higher quantities of lactic acid than the silages inoculated with *L. brevis* and *L. hilgardii* strains (Table 5). In the silages inoculated with the strains UFLA SIL 33, UFLA SIL 34, UFLA SIL 25, UFLA SIL 27 and UFLA SIL 41, at 12 days of fermentation, the concentration of lactic acid peaked and did not increase until the 120th day. However, for other silages, the concentration increased at varying rates.

With the exception of the silages treated with the *L. plantarum* UFLA SIL 34, UFLA SIL 27, UFLA SIL 35, UFLA SIL 42, UFLA SIL 46 and *L. brevis* UFLA SIL 27 strains, the presence of acetic acid in the silage increased showing the highest values observed after 126 days of ensilage (Table 6). Considering all periods evaluated, the highest average of acetic acid concentrations were observed in the silages inoculated with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains, followed by the silages inoculated with *L. brevis* UFLA SIL 33, UFLA SIL 25, UFLA SIL 24, UFLA SIL 27 and UFLA SIL 17.

**Table 6.** Effects of inoculation with different strains and of different days of ensilage on the concentrations of acetic acid, propionic acid, 1,2 propanediol and butyric acid in sugar cane silages.

| Days of ensilage | Inoculants (strains) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Ctr** | **UFLA SIL19♦** | **UFLA SIL32♦** | **UFLA SIL33‡** | **UFLA SIL34♦** | **UFLA SIL17‡** | **UFLA SIL24‡** | **UFLA SIL25‡** | **UFLA SIL27‡** | **UFLA SIL35♦** | **UFLA SIL41♦** | **UFLA SIL42♦** | **UFLA SIL46♦** | **UFLA SIL51*** | **UFLA SIL52*** |
| | **Acetic acid (g kg$^{-1}$ of DM)** | | | | | | | | | | | | | | |
| 12 | 3.3 bB* | 1.6 bB | 1.6 bB | 7.5 aB | 1.8 bA | 7.3 aB | 6.6 aB | 7.3 aB | 7.4 aA | 1.4 bA | 1.4 bB | 2.6 bA | 2.1 bA | 7.6 aB | 8.4 aB |
| 30 | 2.8 bB | 2.0 bB | 2.0 bB | 9.0 aB | 3.3 bA | 5.0 bB | 7.0 aB | 11.5 aB | 10.4 aA | 2.2 bA | 2.6 bB | 2.6 bA | 2.1 bA | 12.8 aB | 12.2 aB |
| 61 | 9.7 bA | 6.0 aB | 3.9 cB | 9.8 bB | 4.1 cA | 10. 5 bA | 10.5 bB | 10.6 bB | 9.9 bA | 3.3 cA | 3.6 cB | 8.9 bA | 3.2 cA | 19.7 aA | 22.5 aA |
| 126 | 10.9 bA | 11.1 bA | 9.2 cA | 20.3 aA | 7.3 cA | 12.9 bA | 20.2 aA | 15.7 aA | 13 bA | 5.8 cA | 13.3 bA | 5.8 cA | 6.9 cA | 21.5 aA | 20.6 aA |
| Level of significance | | | Inoculants | | | Days of ensilage | | | Inoculants x Days of ensilage | | | | SEM† | | |
| | | | <0.01 | | | <0.01 | | | <0.01 | | | | 1.92 | | |
| | **Propionic acid (g kg$^{-1}$ of DM)** | | | | | | | | | | | | | | |
| 12 | 4.0 aA | 2.4 aA | 4.3 aC | 3.5 aB | 5.1 aA | 3.7 aB | 3.4 aA | 3.1 aB | 3.4 aB | 4.3 aB | 4.4 aB | 3.2 aA | 3.8 aA | 4.2 aA | 4.5 aA |
| 30 | 1.8 cA | 2.5 cA | 5.5 bB | 6.5 aA | 6.3 aA | 4.8 bB | 4.8 bA | 6.9 aA | 6.5 aA | 6.2 aA | 7.0 aA | 2.8 cA | 4.0 cA | 4.6 bA | 4.7 bA |
| 61 | 3.1 cA | 3.4 cA | 7.6 aA | 7.4 aA | 5.4 bA | 6.5 aA | 3.4 cA | 5.2 bA | 5.2 bA | 6.4 aA | 6.6 aA | 3.3 cA | 3.5 cA | 4.0 cA | 4.1 cA |
| 126 | 3.3 aA | 2.7 aA | 1.9 aD | 1.8 aB | 1.8 aB | 1.4 aC | 2.0 aA | 1.9 aB | 1.8 aB | 1.8 aC | 3.3 aB | 2.9 aA | 2.8 aA | 2.2 aB | 2.0 aB |
| Level of significance | | | Inoculants | | | Days of ensilage | | | Inoculants x Days of ensilage | | | | SEM | | |
| | | | <0.01 | | | <0.01 | | | <0.01 | | | | 0.63 | | |
| | **1,2 propanediol (g kg$^{-1}$ of DM)** | | | | | | | | | | | | | | |
| 12 | 0.5 aA | 0.5 aA | 0.3 aA | 0.4 aA | 0.5 aA | 0.5 aA | 0.4 aA | 0.3 aA | 0.4 aA | 0.5 aA | 0.5 aA | 0.5 aA | 0.5 aA | 0.7 aD | 1.1 aD |
| 30 | 0.3 cA | 0.5 cA | ,0.5 cA | 0.5 cA | 0.5 cA | 0.4 cA | 0.4 cA | 0.5 cA | 0.5 cA | 0.5 cA | 0.6 cA | 0.6 cA | 0.5 cA | 14.5 bC | 19.3 aC |
| 61 | 1.7 cA | 0.5 cA | 0.6 cA | 0.6 cA | 0.6 cA | 0.7 cA | 0.5 cA | 0.6 cA | 0.6 cA | 0.6 cA | 0.7 cA | 0.6 cA | 0.6 cA | 32.8 bA | 39.9 aA |
| 126 | 2.6 cA | 0.5 cA | 0.5 cA | 0.6 cA | 0.6 cA | 0.7 cA | 0.5 cA | 1.0 cA | 0.5 cA | 0.6 cA | 0.7 cA | 0.7 cA | 0.7 cA | 30.3 bB | 34.7 aB |
| Level of significance | | | Inoculants | | | Days of ensilage | | | Inoculants x Days of ensilage | | | | SEM | | |
| | | | <0.01 | | | <0.01 | | | <0.01 | | | | 0.78 | | |
| | **Butyric acid (g kg$^{-1}$ of DM)** | | | | | | | | | | | | | | |
| 12 | 0.8 aA | 0.9 aA | 1.2 aB | 0.8 aB | 1.5 aB | 1.0 aB | 1.2 aB | 1.1 aB | 1.2 aB | 0.7 aC | 1.3 aB | 1.0 aA | 2.4 aB | 1.3 aB | 1.1 aA |
| 30 | 0.4 aA | 0.9 aA | 1.5 aB | 2.1 aB | 2.1 aB | 1.5 aB | 1.3 aB | 1.9 aB | 2.5 aB | 1.8 aC | 1.8 aB | 0.4 aA | 0.9 aB | 1.3 aB | 1.5 aA |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 61 | 0.0 bA | 0.0 bA | 3.3 aA | 2.5 aB | 4.9 aA | 2.3 aB | 2.6 aB | 3.6 aB | 3.6 aB | 3.5 aB | 3.2 aB | 0.4 bA | 1.9 bB | 1.2 bB | 1.3 bA |
| 126 | 0.0 dA | 0.0 dA | 5.3 bA | 7.0 bA | 5.9 bA | 5.1 bA | 5.1 bA | 6.3 bA | 6.7 bA | 9.9 aA | 7.0 bA | 0.9 dA | 5.5 bA | 5.4 bA | 3.2 cA |

| Level of significance | Inoculants | Days of ensilage | Inoculants x Days of ensilage | SEM |
|---|---|---|---|---|
| | <0.01 | <0.01 | <0.01 | 0.76 |

* For each row, mean values with different lowercase letters are significant at $P < 0.05$ by Scott–Knott test. For each column, mean values with different capital letters are significant at $P < 0.05$ by Scott–Knott test.† Standard error of the means of two-way interactions (treatments with the strains and days of ensilage). ♦ *L. plantarum* specie, ‡ *L. brevis* specie, * *L. hilgardii* specie.

The concentration of propionic acid was variable throughout the ensilage period for each strain (Table 6). Analysing the averages of all of the periods of evaluation, the silages treated with the *L. plantarum* UFLA SIL 41, UFLA SIL 32, UFLA SIL 35, UFLA SIL 34, and *L. brevis* UFLA SIL 17, UFLA SIL 33, UFLA SIL 25 and UFLA SIL 27 strains resulted in the highest concentrations of propionic acid (Table 6). In general, the presence of 1,2-propanediol in the silage was low and there was no significant difference throughout the ensilage, except in the silages treated with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains. In these silages, the concentration of the metabolite increased from the thirtieth day of ensilage and was significantly higher (Table 6).

During the ensilage period, no increase was observed in the butyric acid concentration in the control silage and in the silages treated with the UFLA SIL 19, UFLA SIL 42 and UFLA SIL 52 strains. The remaining silages showed increased butyric acid concentration. At 126 days of ensilage, butyric acid was not detected in the control silage or in silage treated with the *L. plantarum* UFLA SIL 19 strain.

**Microbial population of the silages**

There was a significant interaction between the factors inoculants and ensilage days ($P < 0.01$) in regards to LAB population (Fig. 2). At 30 and 61

days of ensilage, the greatest populations were observed in the silages treated with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains, followed by the control silage. At 126 days of ensilage, a change was observed. The UFLA SIL 51 and 52 strains showed a great decrease in LAB population when compared to the remaining silage. The silages treated with the UFLA SIL 32, UFLA SIL 27 and UFLA SIL 46 strains resulted in an increase in the populations of LAB at 126 days, while the silages with the remaining strains resulted in population decrease from the twelfth day of fermentation.

There was no interaction between days of ensilage and added strains on the yeasts population ($P = 0.23$). The addition of the new strains significantly modified the populations of yeasts in the silages ($P < 0.01$). The silages inoculated with the *L. hilgardii* and *L. brevis* strains showed the lowest yeast population, while silages treated with *L. plantarum* strains showed the highest yeast population (Fig. 3). During the fermentation period, the population of yeasts decreased from the thirtieth day ($P < 0.01$) (Fig. 1C). The presence of filamentous fungi was not observed in the silages.

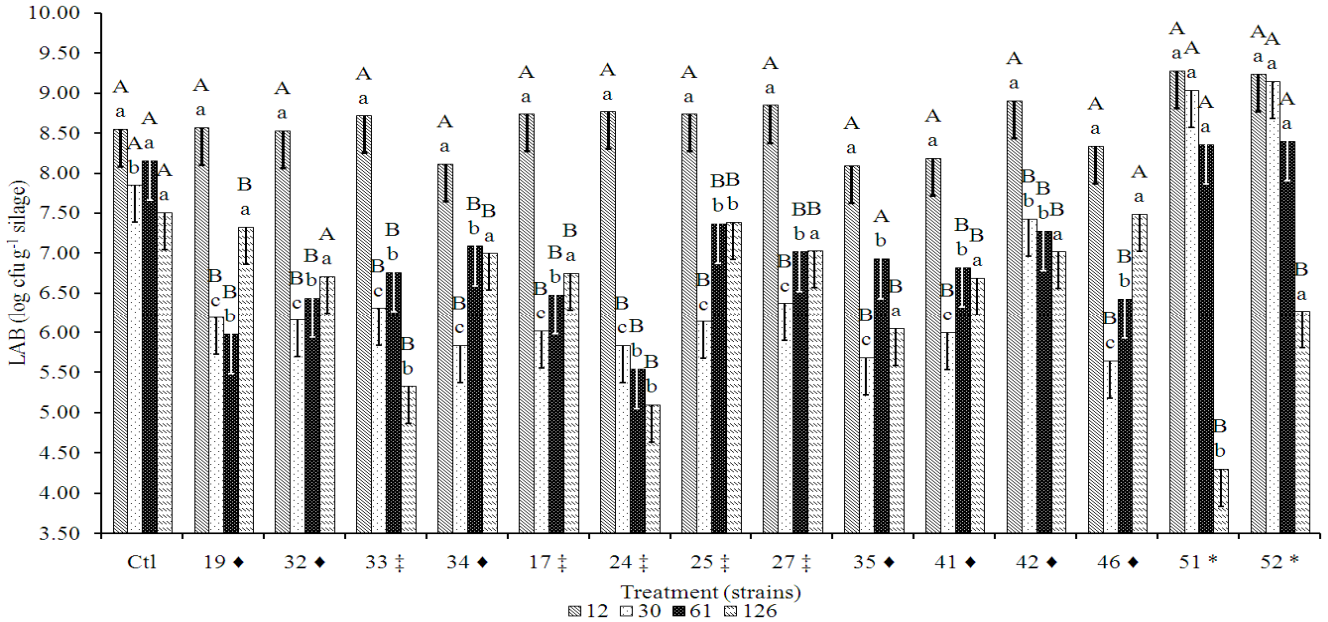


**Figure 2.** Lactic acid bacteria (LAB) populations in sugar cane silages inoculated with different strains at different days of ensilage. $P < 0.01$ for inoculants (I), $P < 0.01$ for days of ensilage (DE), $P < 0.01$ for interaction T × DE. For each evaluation day, means followed by the same lower case letter do not differ at $P < 0.05$ by Scott–Knott test. Inoculants means followed by the same capital letter do not differ at $P < 0.05$ by Scott–Knott test. Bars represent the standard error of the means. ♦ *L. plantarum* specie, ‡ *L. brevis* specie, * *L. hilgardii* specie.

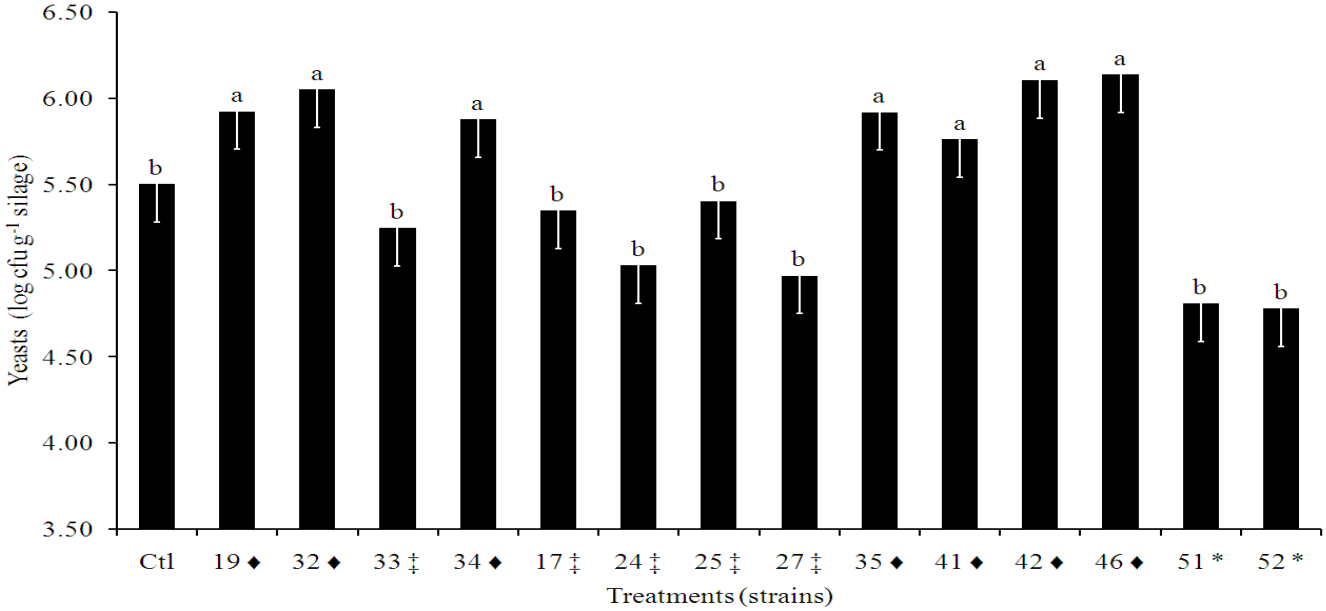


**Figure 3.** Yeast populations in sugar cane silages at different days of ensilage. $P < 0.01$ for inoculants (I); $P < 0.01$ for day of ensilage (DE): $P = 0.23$ for interaction T × DE. For each strain evaluated, sampled time means followed by the same lowercase letter do not differ at $P < 0.05$ by Scott–Knott test. Bars represent the standard error of the means. ♦ *L. plantarum* specie, ‡ *L. brevis* specie, * *L. hilgardii* specie.

**Multivariate analyses**

In order to obtain a better view of the relationship between the DM loss, NDF, DM, fermentation products and the strain inoculated in silage, the average of data from Tables 4, 5 and 6 were subjected to principal component analysis (PCA). Figure 4 shows the biplot of PCA. The first (PC 1) and second (PC 2) principal components explain 58.6% and 19.9%, respectively, of the total variance.

A plot of the results (Fig. 4) shows the formation of two groups. One of the groups is located on the negative part of the first factor, and includes the silages inoculated with obligatory heterofermentative of *L. brevis* and *L. hilgardii* strains; the other group is closely related to the positive part of the axis, and includes the silages treated with facultative heterofermentative *L. plantarum* strains.

Component 2 allowed for the differentiation of the silages inoculated with *L. brevis* strains from the silages inoculated with *L. hilgardii* strains. The obligatory heterofermentative strains were correlated with high concentration of acetic acid, 1,2-propanediol, DM and LAB population, within this group the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 showed the highest 1,2-propanediol production. High concentration of ethanol, NDF, lactic acid and yeasts population were correlated with high DM loss, and *L. plantarum* strains were correlated with these variables (Fig. 4).

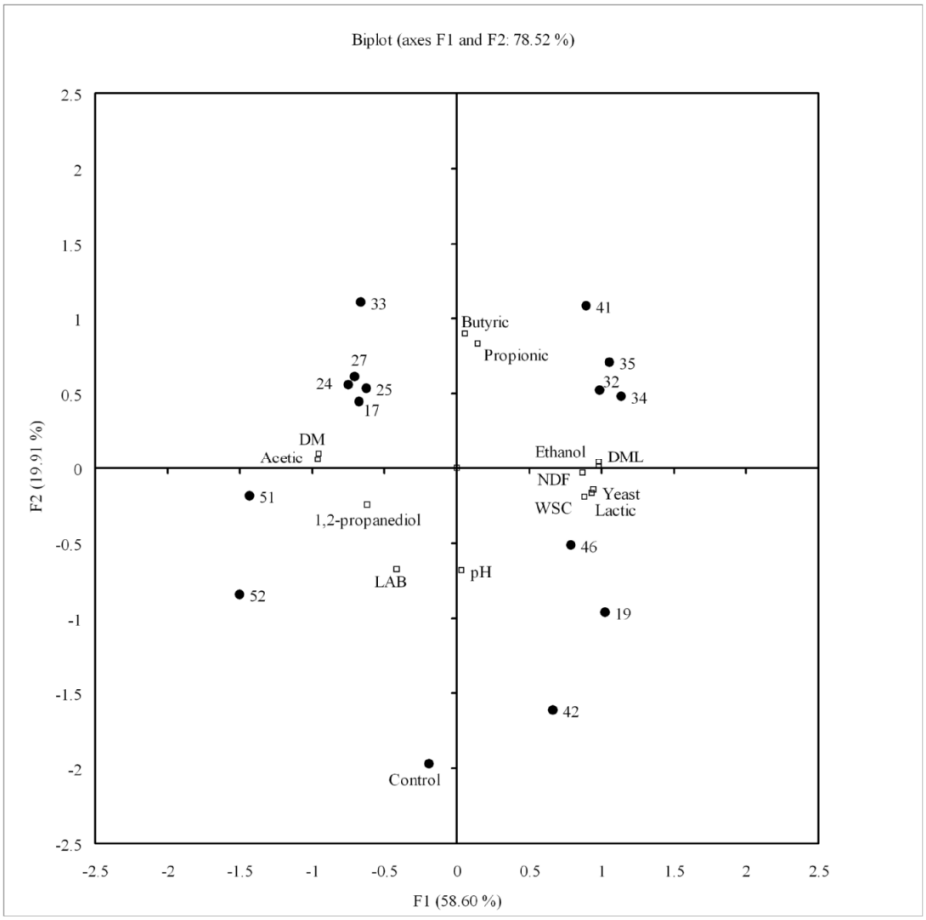


**Figure 4.** Principal component analysis (PCA) of the fermentation products, dry matter (DM), DM loss (DML), neutral detergent fibre (NDF), water soluble carbohydrates (WSC) population of lactic acid bacteria (LAB) and yeasts in sugar cane silage treated with wild strains of LAB.

## DISCUSSION

Among the studied strains, UFLA SIL 19, UFLA SIL 32, UFLA SIL 34, UFLA SIL 35, UFLA SIL 41, UFLA SIL 42 and UFLA SIL 46 were identified as *L. plantarum*. UFLA SIL 33, UFLA SIL 17, UFLA SIL 24, UFLA SIL 25 and

UFLA SIL 27 were identified as *L. brevis*. These species are commonly found in silages and have been previously identified and used as inoculants in sugar cane silages[2, 5] and other forages.[14, 4, 15] Two strains (UFLA SIL 51 and UFLA SIL 52) were identified as *L. hilgardii*. There were no data found in the literature about the isolation, identification or use of these species as a starter culture in forage silages. There are reports on the occurrence of *L. hilgardii* in lactic fermentations under acidic and anoxic conditions closely resembling those that prevail in silage and where several other species of *Lactobacillus* are present. *L. hilgardii* has been found during vinification and wine storage[16, 17] in sugary kefir grains[18] and during ricotta cheese natural fermentation.[19] Heinl *et al.*[20] verified that the metabolism of both *L. hilgardii* and *L. buchneri* are similar and possess the ability to degrade lactic acid and form acetic acid and 1,2-propanediol in anaerobic conditions.[20]

The initial grouping of strains, based on the type of acid produced, did neither relate to further strain identification nor to the carbohydrate fermentation standards. As shown in Tables 1 and 2, strains of the same species may produce different types of acid, as main products of the metabolism, as well as different fermentation standards. These inconsistencies can be attributed to the fact that physiological properties are based on phenotypic expression possibly influenced by regulation, whereas the PCR methods are strictly based on DNA sequence.[17] Thus, bacteria of the same species can produce a range of different enzymes that

influence its ability to use different substrates. This also explains the divergence in the relationship between the biochemical identification and the identification through the 16S rRNA sequencing of two evaluated strains. The strains UFLA SIL 51 and UFLA SIL 52 were biochemically identified as *L. buchneri*, however, the results of the sequencing indicated they were strains of the species *L. hilgardii*. The fact that these strains are physiologically and genetically correlated[16, 20, 22] can also explain the ambiguity in the identification. The fermentative variety of the studied strains confirms the need to select inoculants not only by species but also by strain, as observed by Ávila *et al.*[5]

The DM, WSC and NDF content in sugar cane prior to ensilage found in this study was low but within the range of observations reported previously in the literature.[2, 5, 7] These characteristics are appropriate to provide an adequate fermentation, mainly due to the high concentration of soluble carbohydrates. The LAB count found in fresh sugar cane (7.51 cfu $g^{-1}$ of forage) of the present study was above that observed by Ávila *et al.*[2, 5] and Carvalho *et al.*[7] This epiphytic microbiota of LAB, naturally present in forage crops is responsible for silage fermentation and also influences silage quality. The population of yeasts and filamentous fungi found was within the variation described in the literature for this type of forage.[2, 5, 7]

DM losses during the fermentation of sugar cane constitute the main problem involved in the ensilage of this forage. Thus, this should be the main

characteristic evaluated in the selection of inoculant strains for the culture. The *L. brevis* UFLA SIL 17 and UFLA SIL 24 and *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains showed, simultaneously the best results, i.e. lower DM losses, higher DM concentrations and lower NDF concentrations. These strains presented obligatory heterofermentative metabolisms, wherein they were able to produce gas during the growth in MRS broth (Table 1). Heterofermentative LAB can use both pentoses and hexoses to obtain energy.[23] In contrast to homofermentative metabolism these LAB produce in addition to lactic acid, ethanol and acetic acid.[23] Weak organic acids, such as acetic acid, act to inhibit the growth of yeasts,[24, 25] which are main responsible for the DM losses in sugar cane silages. As a consequence of the inoculation with these strains, the silages resulted in lower DM losses (Table 5). Increase in DM losses and NDF and decrease in DM concentration throughout sugar cane fermentation are common characteristics during ensilage.[1] In the present study, the increase in the DM losses and in the NDF concentrations and the decrease in the DM concentrations were more intense until the sixty-first day of ensilage. At this point, a stabilisation was observed as evaluated and confirmed at 126 days of ensilage (Fig. 1).

The decrease in the concentration of carbohydrates during the ensilage in all silages occurred quickly (Table 5). By the 12th day of fermentation, 50% of the WSC were consumed and by the 126th day, consumption reached 94%.

Pedroso *et al*.[1] also observed a decrease in WSC concentration in the beginning of the fermentation of this forage. In the silages inoculated with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains, the utilization of carbohydrates present in the forage occurred more swiftly than in the silages inoculated with the remaining LAB strains and in the control silage. As a consequence, at 12 days of fermentation, a lower concentration of carbohydrates (Table 5) and a higher LAB population (Fig. 2) was recorded in the silages treated with these strains. Despite the swifter initial use of WSC in the beginning, its concentration in the silages treated with these strains was not different from the control silage and the silages treated with the other strains at the end of ensilage process. These results are different from the results obtained by Ávila *et al*.[2] who observed different concentrations of residual WSC in sugar cane silages with or without inoculants. In their study, the WSC concentrations of the silages inoculated with *L. buchneri*, at 90 days of fermentation, was higher (18.5 g $kg^{-1}$) than in the non-inoculated silages (12.7 g $kg^{-1}$).

As a consequence of the lower population of yeasts present in the silages inoculated with the UFLA SIL 51 and UFLA SIL 52 strains, a lower concentration of ethanol was also observed in these silages (Table 5). During the ensilage, the concentration of ethanol increased in all silages, with the exception of those with the UFLA SIL 51 and UFLA SIL 52 strains. The presence of ethanol in the silages is undesirable; despite this compound being a source of

energy for ruminants[26] it also indicates the growth of the yeasts and DM loss. Although the heterofermentative LAB produce ethanol, yeasts are mainly associated with the presence of this metabolite in sugar cane silages.[1, 2] The higher concentration of ethanol, observed in the silages inoculated with *L. plantarum* strains, possibly resulted from the fermentative activity of yeasts in those silages. The yeast population in those silages was higher than in the control silage (Fig. 3).

Silages inoculated with *L. plantarum* showed lower concentrations of acetic acid compared to silages inoculated with heterofermentative strains (Table 6). This fact may have favoured the yeasts growth. Interaction between yeasts and *Lactobacillus* in silages has not been previously described. However, interactions of this type wherein LAB and yeasts interact beneficially have been observed in other types of fermentation.[18, 27, 28, 29] The lack of competition for the main carbon source appears to be one of the prerequisites for the stability of LAB/yeast associations in food fermentations.[27] Dead cell lysis also guarantees a supply of amino acids. The interaction between yeasts and bacteria involves stimulation or inhibition and the specific mode of interaction is dependent on the type of yeasts as well as bacteria.[30]

The presence of lactic acid in the silage is considered an indicator of fermentation, but not necessarily an indicator of successful fermentation. For a successful fermentation, values above 30 g $kg^{-1}$ DM are typically desired.[31] In

the silages treated with the *L. plantarum*, *L. brevis* UFLA SIL 33 and *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains this concentration was achieved by day 12 of fermentation, while the control silage resulted in only 17.2 g $kg^{-1}$ DM by that day (Table 5). At the 126th day of ensilage, the highest concentrations of lactic acid were observed in the silage inoculated with the strains identified as *L. plantarum* (UFLA SIL 19, UFLA SIL 32, UFLA SIL 34, UFLA SIL 35, UFLA SIL 41, UFLA SIL 42 and UFLA SIL 46). The specie *L. plantarum* is classified as having a facultative heterofermentative metabolism. The species is able to ferment hexoses and pentoses, as it constitutively expresses aldolase and phosphoketolase enzymes.[23] Lactic acid is the main product formed when the glycolytic pathway is used by LAB to obtain energy from hexoses.[23] The *L. brevis* and *L. hilgardii* strains have an obligate heterofermentative metabolism, that is, they make use of the pentose phosphate pathway and, thus, proportionally produce smaller quantities of lactic acid than *L. plantarum*.

Weak organic acids such as, propionic, acetic and butyric acids, inhibit the growth of yeasts and filamentous fungi.[24, 25] However, the presence of butyric acid in silage is undesirable because it reduces the acceptability of the feed, and the consequent decrease of the intake, in addition to its association with fermentations by pathogenic bacteria of the *Clostridium* genus.[32] The increase in the butyric acid concentration as a result of the inoculation with LAB

strains was previously observed by Carvalho *et al.*.[7] Corsetti *et al.*[33] observed that LAB species produce butyric acid during the fermentation of carbohydrates. The decarboxylation of 2-oxo acids and deamination of amino acids are the two possible routes for producing butyric, n-valeric and propionic acids among these genera.[34] Concentrations of butyric acid lower than 25 g $kg^{-1}$ of DM in silages are tolerable.[32] Thus, the concentrations of butyric acid observed in the present study may be considered low (Table 6). The silages treated with the obligatory heterofermentative strains showed the highest concentrations of acetic acid. In particular, those silages treated with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains resulted in 55% more acetic acid than the silages treated with the remaining strains and control experiment. Some obligatory heterofermentative LAB and other facultative heterofermentative strains do not express or express in a constitutive way acetaldehyde dehydrogenase, which is one of enzymes responsible for the reduction of acetyl-CoA into ethanol. Thus, the production of ethanol is practically null in this group of LAB[21] and, consequently, there is an increase in the concentration of acetic acid as a final fermentation product.

The increase in acetic acid concentration throughout the fermentation can be also resulted from the anaerobic conversion of the lactic acid into acetic acid and 1,2-propanediol, generally related to the species *L. buchneri*.[21] According to the proportions of the lactic and acetic acids, and the 1,2-propanediol observed in the silages treated with the strains UFLA SIL 51 and

UFLA SIL 52 (Tables 5 and 6), this conversion can be inferred. The LAB degradation of the lactic acid may be associated with the preservation of cell viability[21], and also explains why the concentration of lactic acid was lower and the concentration of 1,2-propanediol and acetic acid was higher in the silages inoculated with the strains UFLA SIL 51 and UFLA SIL 52 than that of the control silage.

With the exception of the silages treated with the strains *L. hilgardii* UFLA SIL 51 and UFLA SIL 52, the concentration of 1,2-propanediol in the silages was low and similar to levels observed in the control silage (Table 6). The formation of the 1,2-propanediol is initiated by the conversion of lactate into lactaldehyde by a lactaldehyde dehydrogenase. The second step of 1,2-propanediol formation is the conversion of lactaldehyde into 1,2-propanediol. This step is catalysed by a putative lactaldehyde reductase.[21] This metabolic pathway has been described for the *L. buchneri* and *L. hilgardii* species, suggesting that the inoculated microorganisms must have acted in an efficient manner during the fermentation.

The concentration of propionic acid in the different silages did not showed large variation, even in the silages treated with the *L. plantarum*, *L. brevis* and *L. hilgardii* strains that showed a greater production of this acid during pre-selection screening. In the case of silages not inoculated with *Propionibacterium*, the presence of propionic acid may also be associated with

the epiphytic presence of *L. diolivorans*[37], which is able to use 1,2-propanediol to produce propionic acid.

The silages inoculated with UFLA SIL 51 and 52 resulted in higher LAB populations until the sixty-first day of fermentation. The WSC concentrations in the silages treated with this strains also decreased more rapidly in the beginning of the fermentation, which coincided with the high LAB population observed in the same period. As these strains were able to ferment the sucrose (Table 2), it is likely that they used this carbohydrate to sustain their growth, without requiring previous metabolic processing by other microorganisms. The filamentous fungi populations were lower than the detectable minimum in all the silages (< 2 log cfu $g^{-1}$). This is agreement with the results of a study by Ávila *et al.*[2, 5] who did not observe the presence of filamentous fungi after the sugar cane ensilage. The inhibition of fungal growth can occur due to low oxygen and evaluated concentrations of lactic and acetic acid or via through the actions of bacteriocins produced by LAB present in the silages.[38]

The correlation among high ethanol and NDF concentration with the highest DM loss in sugar cane silage has been observed in others studies.[1] This study was the first to observe that in sugar cane silage, lower DM loss were correlated with the highest concentrations of 1,2-propanediol and acetic acid. Unlike observed in this study (Fig.4), in corn[39] and grass[40, 41] silages with high

concentration of 1,2-propanediol and acetic acid were associated with high DM loss.

## CONCLUSION

Inoculation with the new wild strains exerted influence over the microbiological and chemical compositions of the silages. The different behaviours of the strains during carbohydrate fermentation and throughout the ensilage period reinforce the need to use specific strains selected for each forage plant. The clustering of the strains according to the main acid produced was not related with the best results. Therefore, strains that produce the same acid did not always present the same characteristics during the ensilage. The use of facultative heterofermentative LAB, particularly the species *L. plantarum*, was not beneficial for the sugar cane ensilage. In these treatments, the highest DM losses, ethanol concentrations and yeast populations were observed. The presence of ethanol, latic acid and yeasts population were more associated with higher DM loss among all variables evaluated. Silage inoculation with obligatory heterofermentative strains produced the best results. In particular, the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains showed superior results, reducing 29% the DM losses compared to uninoculated silage. This strains are promising for use as microbial inoculant in sugar cane silages.

**ACKNOWLEDGEMENTS**

The authors thank CNPQ (Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil), FAPEMIG (Fundação de Amparo a Pesquisa de Minas Gerais), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), and Lallemand Animal Nutrition SAS for financial support and scholarships.

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**ARTIGO 2** **Yeasts associated with the aerobic deterioration of sugar cane silage inoculated with new tropical strains of lactic acid bacteri**

(Artigo submetido ao periodico indexado: Grass and Forage Science)

**Abstract**

The aims of this study were to investigate the changes that occur in sugar cane (*Saccharum* spp.) silage after aerobic exposure, to identify the major species of yeasts associated with the aerobic deterioration process and to select lactic acid bacteria (LAB) strains that can improve the aerobic stability of this silage after silo opening. Fourteen wild LAB strains belonging to *L. plantarum*, *L. brevis* and *L. hilgardii* were evaluated. Samples of sugar cane silage were collected 0, 96 and 216 h after aerobic exposure. The chemical data and temperatures were evaluated. The yeast species associated with the aerobic deterioration of silage have been identified. The strains tested were able to modify the fermentative, chemical and microbiological parameters and the diversity of yeasts species of silage after aerobic exposure. It was not possible to observe any association between the facultative or obligatory heterofermentative fermentation patterns and the increased aerobic stability of silage. The aerobic stability were more correlated with high acetic acid and 1,2-propanediol concentration. The *Lactobacillus hilgardii* UFLA SIL 51 and UFLA SIL 52 strains, which are obligatory heterofermentative, promote an increase in the aerobic stability of silage.

**Key Words**: Yeasts, *Lactobacillus hilgardii, L. brevis, L. plantarum*, fermentation products.

**Introduction**

There are a number of features required for an inoculant strain in the ensiling process; the inoculant strain should be able to promote a rapid decline in the pH value, reduce the dry matter (DM) loss, survive throughout the fermentation process, and improve the aerobic stability by inhibiting the growth of spoilage microorganisms in silage after the silos are opened (Saarisalo *et al*., 2007). Homofermentative lactic acid bacteria (LAB) have been selected to increase lactic acid concentration in the silo, but aerobic stability may be impaired because lactic acid can be easily oxidized by yeasts when the silage is exposed to air (Pahlow *et al*., 2003). Heterofermentative LAB has attracted attention as an alternative additive to inhibit aerobic deterioration (Danner *et al*., 2003; Driehuis *et al*., 1999; Ávila *et al*., 2009). The heterofermentative species *Lactobacillus hilgardii* recently isolated from sugar cane (*Saccharum* spp.) silage (Ávila *et al*., 2013) may be a novel option to improve the aerobic stability in the silage of this forage. In environments rich in organic acids and under aerobic conditions yeasts are more resistant than other microorganisms. Yeasts can use a wide range of organic substances as carbon sources, including lactate, acetate, citrate, malate, succinate, propionate and ethanol (McDonald et al., 2001; Pahlow *et al*., 2003). When silage deteriorates, its nutritional value is reduced because of the loss of fermentation products that are potentially digestible substrates (Wilkinson and Davies, 2012; Daniel *et al*., 2013). Animal

health and the quality of milk may be adversely affected as a result of the development of undesirable microbial spores (Pahlow *et al*., 2003) and toxins (Borreani and Tabacco, 2010). Deteriorated silage has reduced palatability and may be refused by animals. The yeast species involved in aerobic deterioration in sugar cane silage are not known. Because there is a correlation between the yeast population in silage and the loss of aerobic stability (McDonald *et al*., 1991; Pahlow *et al*., 2003; Wilkinson and Davies, 2012), it is necessary to distinguish the major yeast species present during the exposure of silage to air.

This study aimed to investigate the changes that occur in sugar cane silage with or without microbial inoculants after aerobic exposure, to identify the major yeasts species associated with the aerobic deterioration process of these silages and to select bacterial strains that can improve the aerobic stability after silo opening.

## Materials and Methods

### Forage and ensiling conditions

The wild LAB strains isolated from sugar cane silage were identified according to Ávila *et al*., (2013). The strains termed UFLA SIL 19, UFLA SIL 32, UFLA SIL 34, UFLA SIL 35, UFLA SIL 41, UFLA SIL 42 and UFLA SIL 46 of the *L. plantarum* species; UFLA SIL 33, UFLA SIL 17, UFLA SIL 24, UFLA SIL 25 and UFLA SIL 27 of the *L. brevis* species; UFLA SIL 51 and

UFLA SIL 52 of the *L. hilgardii* species; and a control treatment without inoculants were evaluated. The silages were made with fresh-cut sugar cane that was approximately 12 months old. The silages were made in three different days, in each day one replication of each treatment with strains were made. In each day, the sugar cane was manually harvested and chopped (PP-47, Pinheiro, Itapira, SP, Brazil) to a length of 10 mm. Experimental silos (mini-silos) were used in the form of PVC tubes 10 cm diameter and 60 cm length. The tubes were sealed with tight lids containing Bunsen valves for gas release. Three silos were prepared for each of the fourteen strains plus the control treatment. The new inoculants were pre-cultured in the laboratory and, on the ensiling day, were enumerated on De Man Rogosa Sharpe agar (Oxoid CM361, Basingstoke, Hampshire, England). The inoculation concentration was of $1.8 \times 10^6$ cfu $g^{-1}$ fresh matter (FM). The inoculants were mixed with deionized water and sprayed onto the forage, resulting in an application volume of 14.3 L $ton^{-1}$. An identical volume of pure distilled water was added to the control treatment. A separate sprayer was used for each treatment to avoid cross-contamination. Each silo was packed with 3 kg of wet forage to achieve a packing density of approximately 666 kg $m^{-3}$ FM. The silos were sealed, stored for 126 days at room temperature (average of 25ºC) and protected from sunlight and rain.

**Evaluation of aerobic stability**

To evaluate the aerobic stability, 2 kg of silage was placed in plastic buckets and kept at a controlled temperature of 24ºC (±0.5ºC). In the center of each bucket, a data logger (MI-IN-D-2-L Escort) programmed to measure the temperature every 30 minutes was placed. The silage temperature was evaluated for 216 h. During aerobic exposure, silage samples were collected at 0, 96 and 216 h and frozen for later evaluation of the pH value, water soluble carbohydrates (WSC), DM and products of fermentation. The data from time to the loss of aerobic stability [the number of hours required for the silage to exceed the ambient temperature by 2ºC (Moran et al., 1996)], the maximum temperature, and the time to reach maximum temperature were analyzed.

**Analytical procedures**

To obtain the aqueous extract, 25 g sample of fresh forage or sugar cane silage was blended in 225 mL of 0.1% sterile peptone water and homogenized in an orbital shaker for 20 min. The pH of each sample was determined (DIGIMED® DM 20 Potentiometer, Digicrom Instrumentos, SP, Brazil). The aqueous extracts (2 mL) were acidified with 10 μL of 50% (vol/vol) $H_2SO_4$ and frozen prior to the analysis of the fermentation end products. The fermentation end products were evaluated in the control silage and in the two samples of silages treated with the obligatory heterofermentative strains that showed the

best and the worst results in the temperature evaluation. The acidified aqueous extracts were analyzed for citric acid, tartaric acid, malic acid, succinic acid, lactic acid, acetic acid, propionic acid, butyric acid, ethanol and 1,2-propanediol by high-performance liquid chromatography according to the method described by Carvalho *et al*. (2012).

The DM contents of each sample were determined using a forced-draft oven at 55ºC for 72 h. Dried samples were ground in a Wiley-type grinder through a 1-mm screen. DM at 105ºC was determined according AOAC (1990). The water soluble carbohydrates were analyzed using the phenol method (Dubois *et al*., 1956).

**Identification of yeasts**

The remaining portion of the aqueous extracts was used for the enumeration and identification of the yeasts using one repetition for each treatment. Sequential ten-fold dilutions were prepared. The yeast were enumerated by surface inoculation on Dichloran Rose Bengal Chloramphenicol Medium agar (DRBC, Difco; Becton Dickinson, Sparks, MD, USA), and the plates were incubated at 28°C for 96 h.

Following incubation, the number of colony-forming units (CFU) was recorded. In the samples with 96 and 216 h of aerobic exposure, each colony morphotype was counted and morphologically characterized (cell size, cell

shape, edge, color and brightness). The colonies were re-streaked and purified using YEPG agar [1% yeast extract (Merck), 2% peptone (Himedia), 2% glucose (Merck) at pH 5.6]. The purified isolates were stored at -80°C in YEPG broth containing 20% glycerol (w/w). The yeast colonies (231 isolates) were physiologically characterized by the determination of their microscopic characteristics, the fermentation of carbohydrates (sucrose, fructose and glucose) and the assimilative capacity of the lactic acid (Merck) in a concentration of 3, 6 or 9%, as described by Kurtzman et al. (2011).

The yeasts were separated according to the type of reproduction (fission or budding) and, according to the physiological results, were grouped by the Statistica program for Windows version 6.0 (Stat Soft Inc., Tulsa, OK, USA). The binary matrix was constructed with the positive or negative results of the tests described above. According to the result of the grouping, 71 strains were selected for characterization of their molecular profile by the Rep-PCR technique.

**Rep–PCR technique**

Seventy-one yeast cultures selected physiologically were grown in YEPG at 30ºC for 48 h. The isolated colonies were picked up and resuspended in 40 µl of PCR buffer. The suspension was heated for 10 min at 95°C and used as a DNA template in Rep-PCR experiments. The Rep-PCR was carried out in 2

µl of DNA added to 12.5 µl of Taq PCR Master Mix (Qiagen, SP, Brazil), 8 µl of $H_2O$, 0.25 µl of bovine serum albumin (BSA), 0.25 µl of formamide and 2 µl of the primer GTG5 (5'-GTG GTG GTG GTG GTG-3'). The Rep-PCR was performed on a Mastercycler (Eppendorf, Hamburg, Germany) under the following thermocycling program: initial denaturation of 94°C for 5 min, 30 cycles of 95°C for 30 s, 45°C for 60 s, and 60°C for 5 min, followed by a final elongation step of 60°C for 16 min. The PCR products were separated by electrophoresis on a 2.0% agarose gel in 1×TAE (4 h, 70 V) using a BenchTop 1 kb DNA ladder as a reference (Promega, Madison WI, EUA). Following electrophoresis, the gels were stained with SYBR-Green I solution (Invitrogen, Foster City, CA, USA) and documented using a transluminator Lpix Image (LTB 20x20 HE, LPix®, Brazil). The Rep-PCR profiles were normalized, and the cluster analysis was performed using Bionumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) separately according to the different types of reproduction (fission or budding). The dendrograms were calculated on the basis of the Dice coefficient and on the coefficient of similarity using the unweighted pair group method with the arithmetic averages clustering algorithm (UPGMA).

**Sequencing**

Representative yeasts strains of each Rep-PCR profile were identified by sequence analysis of the full-length of the ITS region. The yeast cultures were

grown under appropriate conditions, and the isolated colonies were resuspended in 40 µl of PCR buffer. The suspension was heated for 10 min at 95 °C, and 2 µl was added to 30 µl of Taq PCR Master Mix (Qiagen, SP, Brazil), 26 µl of $H_2O$, and 1 µl of each primer ITS 1 and ITS 4 (Nielsen et al. 2007). The PCR was performed as follows: initial denaturation at 95°C for 5 min; 30 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and a final elongation at 72°C for 10 min. The amplified products were confirmed by electrophoresis on a 1% agarose gel in 1x TAE buffer at 70 V for 30 min, stained with SYBR-Green I (Invitrogen, Foster City, CA, USA) and visualized under a transilluminator Lpix Image (LTB 20x20 HE, LPix®, Brazil). The sequencing of the amplicons was performed using an ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, California, CA), and the sequences were compared with the GenBank database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

**Statistical analysis**

The experiment was carried out in randomized blocks; each block corresponded to a day of silage. The treatments were assigned in a factorial arrangement, consisting of 14 LAB inoculants and the control (14 LAB strains and a control without inoculant) and three times of aerobic exposure (0, 96 and 216 h). The data on the pH, DM, WSC and products of fermentation were analyzed under model: $Y_{ijk} = \mu + B_i + I_j + BI_{ij} + H_k + (I \times H)_{jk} + e_{ijk}$, where: $\mu$ =

overall mean; $B_i$ = block effect (i = 1 to 3); $I_j$ = inoculant effect (j = without inoculant or with one of the 14 strains); $BI_{ij}$ = experimental error (considering treatment in each block) used for tested the inoculants effect, assumed independently and identically distributed in a normal distribution with average zero and variance $\sigma^2$; $H_k$ = hours of aerobic exposure effect (k = 0, 96 or 216 h); $(I \times H)_{jk}$ = interaction between inoculant and hour of aerobic s exposure effect; $e_{ijk}$ = experimental error, assumed independently and identically distributed in a normal distribution with average zero and variance $\sigma^2$. The temperature data were analyzed by a model containing the fixed effects of block and silage. The silage means were compared using the Scott-Knott test with the software SISVAR®.

The principal component analyses (PCA) were performed using the software XLSTAT 7.5.2 (Addinsoft's, New York, N.Y., U.S.A.) for grouping the data of the fermentation end products in silages, yeast population and hours of aerobic stability. Average of the all evaluations during aerobic exposure was evaluated using PCA analyses.

## Results

### pH value, DM and WSC

The pH values increased during the aerobic phase of silage, but this increase occurred differently between the treatments ($P < 0.01$ for the interaction

between the day of aerobic exposure and silage). In the silages treated with the obligatory heterofermentative *L. brevis* UFLA SIL 33, UFLA SIL 17, UFLA SIL 24, UFLA SIL 25 and UFLA SIL 27, *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains and in silages treated with the facultative heterofermentative *L. plantarum* UFLA SIL 32 and UFLA SIL 41 strains and in the control silage, an increase in the pH values occurred in the first 96 h of aerobic exposure (Table 1). In the silages inoculated with the others *L. plantarum* strains, this increase in pH value was observed in 216 h.

**Table 1** Values of pH, dry matter and water soluble carbohydrates in sugarcane silages treated with different strains and at different hours of aerobic exposure.

| Aerobic exposure (hours) | Ctr | UFLA SIL 19 ♦ | UFLA SIL 32 ♦ | UFLA SIL 34 ♦ | UFLA SIL 35 ♦ | UFLA SIL 41 ♦ | UFLA SIL 42 ♦ | UFLA SIL 46 ♦ | UFLA SIL 33 ‡ | UFLA SIL 17 ‡ | UFLA SIL 24 ‡ | UFLA SIL 25 ‡ | UFLA SIL 27 ‡ | UFLA SIL 51 * | UFLA SIL 52 * | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **pH** | | | | | | | | | | | | | | | |
| 0 | 3.75 B | 3.73 B | 3.76 C | 3.78 B | 3.83 B | 3.73 C | 3.82 B | 3.86 B | 3.76 B | 3.75 B | 3.77 B | 3.70 B | 3.75 B | 3.80 B | 3.84 B | 3.77 |
| 96 | 5.53 A | 4.14 B | 4.35 B | 4.11 B | 3.86 B | 4.40 B | 4.24 B | 4.28 B | 5.02 A | 4.88 A | 4.94 A | 5.22 A | 5.06 A | 4.51 A | 4.95 A | 4.63 |
| 216 | 5.06 A | 5.33 A | 5.17 A | 5.30 A | 5.19 A | 5.11 A | 4.89 A | 5.14 A | 4.87 A | 4.78 A | 4.98 A | 4.89 A | 4.68 A | 5.00 A | 4.74 A | 5.01 |
| Mean | 4.78 | 4.40 | 4.43 | 4.40 | 4.29 | 4.41 | 4.32 | 4.43 | 4.55 | 4.50 | 4.56 | 4.60 | 4.50 | 4.43 | 4.51 | |

| Level of significance | Inoculants | Days of aerobic exposure | Inoculants x Days of aerobic exposure | SEM† |
|---|---|---|---|---|
| | 0.74 | <0.01 | <0.01 | 0.20 |

| | **Dry Matter (g kg$^{-1}$ DM)** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 251.3 | 239.6 | 234.0 | 242.5 | 238.6 | 239.8 | 247.6 | 248.6 | 266.5 | 264.6 | 268.7 | 263.6 | 269.1 | 269.9 | 259.6 | 253.6 C |
| 96 | 283.3 | 243.6 | 258.8 | 254.8 | 273.9 | 260.5 | 270.1 | 259.4 | 273.8 | 301.7 | 269.3 | 287.5 | 276.3 | 267.5 | 267.9 | 269.9 B |
| 216 | 344.2 | 340.9 | 355.4 | 334.5 | 309.8 | 365.8 | 371.8 | 319.9 | 363.2 | 370.0 | 370.8 | 369.1 | 377.0 | 359.7 | 385.5 | 355.8 A |
| Mean | 292.9 b | 274.7 b | 282.7 b | 277.3 b | 274.1 b | 288.7 b | 296.5 a | 276.0 b | 301.2 a | 312.1 a | 302.9 a | 306.7 a | 307.5 a | 299.0 a | 304.3 a | |

| Level of significance | Inoculants | Days of aerobic exposure | Inoculants x Days of aerobic exposure | SEM |
|---|---|---|---|---|
| | <0.01 | <0.01 | 0.72 | 11.97 |

| | **Water Soluble Carbohydrates (g kg$^{-1}$ DM)** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0 | 13.1 B | 14.1 A | 14.6 A | 14.2 A | 13.5 A | 13.8 A | 15.9 A | 12.5 A | 13.1 B | 13.7 B | 12.0 B | 13.3 B | 13.8 B | 21.0 A | 19.4 A | 14,5 |
| 96 | 25.0 A | 16.9 A | 16.6 A | 19.3 A | 17.3 A | 16.0 A | 17.2 A | 15.1 A | 29.4 A | 24.9 A | 23.4 A | 21.8 A | 29.5 A | 18.8 A | 24.5 A | 21,0 |
| 216 | 19.8 A | 14.7 A | 13.5 A | 14.1 A | 13.1 A | 13.4 A | 15.0 A | 13.4 A | 26.7 A | 26.4 A | 18.3 A | 19.5 A | 22.5 A | 16.9 A | 20.0 A | 17,8 |
| Mean | 22.4 | 15.8 | 15.1 | 16.7 | 15.2 | 14,7 | 16,1 | 13,7 | 28,1 | 25,7 | 20,9 | 20,7 | 26,0 | 17,9 | 22,3 | |

| Level of significance | Inoculants | Days of aerobic exposure | Inoculants x Days of aerobic exposure | SEM |
|---|---|---|---|---|
| | <0.01 | <0.01 | 0.04 | 2.13 |

For each row, the mean values with different lowercase letters are significant at $P < 0.05$ according to the Scott–Knott test. For each column, the mean values with different capital letters are significant at $P < 0.05$ according to the Scott–Knott test. † Standard error of the means of two-way interactions. ♦ *L. plantarum* specie, ‡ *L.brevis* specie, ***** *L. hilgardii* specie.

The DM concentration was affected by aerobic exposure time ($P < 0.01$) and by treatment with inoculants ($P < 0.01$) (Table 1). The DM content increased during aerobic exposure time, with the mean values observed in the treatments with the obligatory heterofermentative *L. brevis* and *L. hilgardii* strains being 7.2% higher than those observed in the silages treated with the *L. plantarum* strains and in the control silage (Table 1).

There was an interaction ($P = 0.04$) between the day of aerobic exposure and inoculants on the concentration of WSC (Table 1). In the silages inoculated with *L. plantarum* UFLA SIL 19, UFLA SIL 32, UFLA SIL 34, UFLA SIL 35, UFLA SIL 41, UFLA SIL 42, and UFLA SIL 46 and *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains, no significant difference was observed in the concentrations of the WSC during the aerobic exposure. The values of the WSC increased in the silages inoculated with the *L. brevis* strains and in the control silage from 96 h of aerobic exposure.

**Temperature**

The highest maximum temperatures were observed in the treatment inoculated with *L. brevis* strains (Table 2). The maximum temperatures were lower and similar to each other in all of the other evaluated silages.

**Table 2** Dynamics of temperature in aerobic exposure of sugarcane silages and identification of strains.

| Treatment | Maximum temperature (ºC) | Time to reach maximum temperature (h) | Aerobic stability (h) | Molecular identification[2] |
|---|---|---|---|---|
| Ctr | 47.0 A[1] | 22.0 B | 14.2 | |
| UFLA SIL 19 | 45.2 A | 26.0 B | 17.0 | *L. plantarum* 98% (FJ669130.1)[3] |
| UFLA SIL 32 | 43.5 B | 42.2 A | 20.2 | *L. plantarum* 99% (HM218291.1) |
| UFLA SIL 34 | 43.0 B | 48.7 A | 21.0 | *L. plantarum* 98% (HM218291.1) |
| UFLA SIL 35 | 41.2 B | 35.7 B | 22.0 | *L. plantarum* 98% (HM218291.1) |
| UFLA SIL 41 | 43.8 B | 30.3 B | 19.8 | *L. plantarum* 99% (HM218291.1) |
| UFLA SIL 42 | 44.2 B | 28.5 B | 17.5 | *L. plantarum* 99% (HM218291.1) |
| UFLA SIL 46 | 43.2 B | 27.5 B | 17.8 | *L. plantarum* 99% (HM218291.1) |
| UFLA SIL 33 | 46.0 A | 27.2 B | 17.8 | *L. brevis* 98% (FJ227316.1) |
| UFLA SIL 17 | 46.3 A | 28.8 B | 18.2 | *L. brevis* 97% (FJ532364.1) |
| UFLA SIL 24 | 45.0 A | 33.3 B | 18.2 | *L. brevis* 99% (FJ227316.1) |
| UFLA SIL 25 | 43.8 B | 27.5 B | 16.0 | *L. brevis* 98% (FJ227316.1) |
| UFLA SIL 27 | 47.2 A | 28.0 B | 18.3 | *L. brevis* 98% (FJ227316.1) |
| UFLA SIL 51 | 41.8 B | 58.0 A | 21.5 | *L. hilgardii* 98% (HM217953.1) |
| UFLA SIL 52 | 43.8 B | 54.0 A | 30.3 | *L. hilgardii* 98% (HM217953.1) |
| *P* | 0.03 | 0.02 | 0.05 | |
| SEM† | 1.22 | 6.70 | 2.03 | |

[1] The mean values with different capital letters are significant at $P < 0.05$ according to the Scott–Knott test. [2] Sequencing of 16S rRNA (Ávila *et al*., 2013).[3] The number in parentheses refers to the access code in Gen-Bank. † Standard error of the means.

Regarding the time required to reach the maximum temperature, the silages treated with *L. plantarum* UFLA SIL 32, *L. brevis* UFLA SIL 34, *L. hilgardii* UFLA SIL 51 and *L. hilgardii* UFLA SIL 52 showed the best results (Table 2). The silages treated with these strains took, on average, 50.7 h to reach the maximum temperature, while the control silage took 22.0 h.

Although the comparison test of means did not show any differences among the treatments in relation to the time of aerobic stability, the values of a number of strains should be highlighted (Table 2). The silage treated with the UFLA SIL 52 (*L. hilgardii*) strain remained stable for 30.3 h, while the control silage lost stability in 14.2 h. The silages inoculated with *L. plantarum* strains (for example, the UFLA SIL 35 strain) were more stable than certain silages treated with the obligatory heterofermentative strains (Table 2).

In the evaluated periods of aerobic exposition the temperature and yeast population in the silage are shown in Figure 1. At the opening of the silos (0 h) the temperature was lower and similar among silages (Figure 1). In this evaluation the yeast population was lower in silages inoculated with *L. brevis* UFLA SIL 27, *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains (average 3.87 log of yeasts cfu $g^{-1}$ silage).

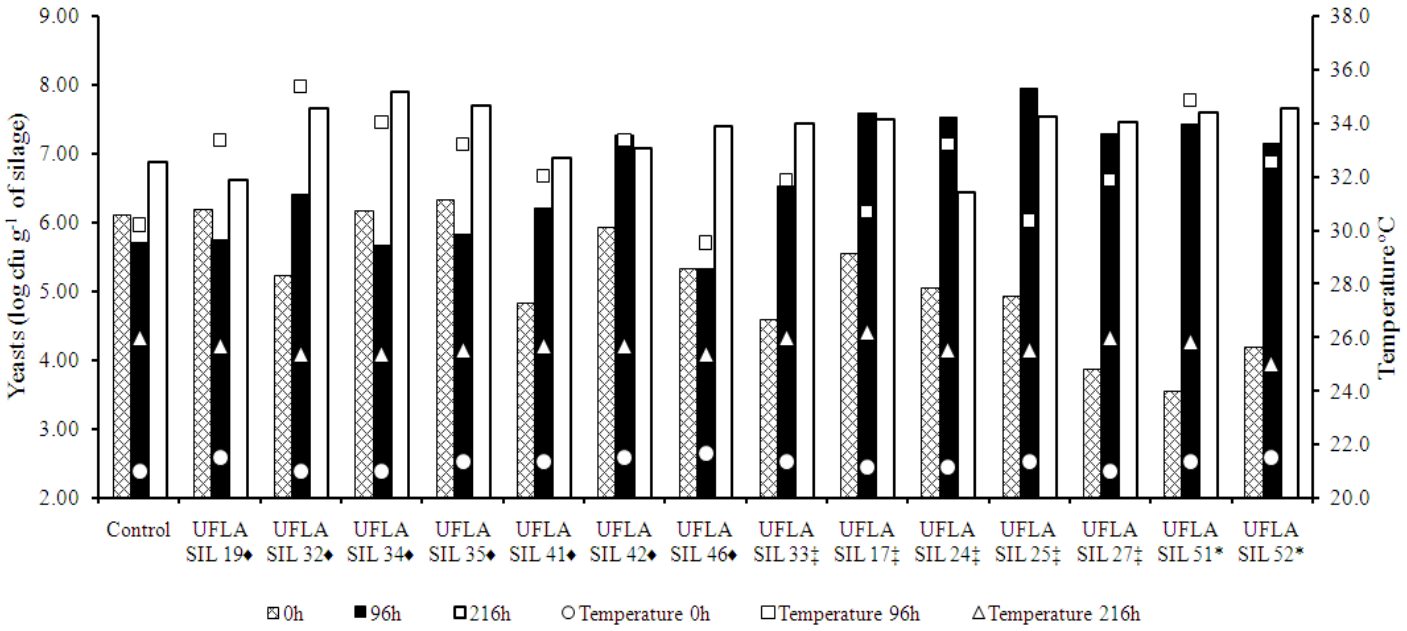


**Figure 1** Yeast population and temperature of the sugarcane silage after 0, 96 and 216 h of aerobic exposure. ♦ *L. plantarum* specie, ‡ *L.brevis* specie, ***** *L. hilgardii* specie.

At 96 h of aerobic exposure, there were marked differences between the temperatures of silage. The highest temperature was observed in the silage treated with the UFLA SIL 32 strain (35.3ºC), the lowest yeasts population (5.32 log cfu of yeasts $g^{-1}$ silage) was detected in the silage treated with the *L. plantarum* UFLA SIL 46 strain (Figure 1). In the evaluation at 216 h the silage temperature was lower and the yeast population was higher than the evaluation at 96 h, the differences among the silages temperatures and yeasts populations were lower (Figure 1).

**Fermentation end products**

The *L. hilgardii* UFLA SIL 51, UFLA SIL 52 and *L. brevis* UFLA SIL 17, UFLA SIL 24 strains are among the obligately heterofermentative strains that showed the best and worst results in relation to the temperature data compared with the control silage (Table 2). They were selected for analysis of fermentation products produced during aerobic exposure.

The concentrations of tartaric, malic, succinic and lactic acids were significantly modified only by the aerobic exposure time (Table 3). The concentrations of tartaric and malic acids remained stable from the moment of opening the silos (zero time of aerobic exposure) up to 96 h of aerobic exposure (Figure 2A). From this evaluation time, there was an increase of 99.8 and 90.9% to 216 hours of aerobic exposure for tartaric and malic acids, respectively. From

the aerobic exposure of silage, the concentration of succinic acid increased by 57.8% and the concentration of lactic acid reduced by 94.1% with up to 96 h of aerobic exposition (Figure 2B). The concentrations of these acids remained stable up to 216 h (Figure 2B).

**Table 3** Probability (*P*) for the effects contained in the model for metabolite analyses.

| **Item** | ***P* value** | | |
|---|---|---|---|
| | **Inoculants (I)** | **Hours of Aerobic stability (H)** | **Interaction (I X H)** |
| Tartaric acid | 0.29 | <0.01 | 0.15 |
| Malic acid | 0.19 | <0.01 | 0.44 |
| Succinic acid | 0.36 | <0.01 | 0.06 |
| Lactic acid | 0.16 | <0.01 | 0.07 |
| Acetic acid | 0.04 | <0.01 | <0.01 |
| Propionic acid | 0.10 | <0.01 | <0.01 |
| Butyric acid | 0.90 | <0.01 | 0.01 |
| 1,2-propanediol | <0.01 | <0.01 | <0.01 |
| Ethanol | <0.01 | <0.01 | <0.01 |

There was an interaction ($P < 0.03$) among the factors inoculants and hours of aerobic exposure on concentrations of citric acid, acetic acid, propionic acid, butyric acid, 1,2-propanediol and ethanol (Table 3). The silages treated with the *L. brevis* UFLA SIL 17 and *L. hilgardii* UFLA SIL 51, UFLA SIL 52 strains showed similar and the highest concentrations of citric acid in the evaluation with 96 h of aerobic exposure (Table 4). No changes were observed

in the citric acid concentrations in the silages inoculated with *L. brevis* UFLA SIL 24 and in the control. The highest concentrations of acetic acid were observed at the opening time of the silos (0 h of aerobic exposure) and in the treatments with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains (Table 4). After 96 h of aerobic exposure, the concentration of acetic acid reduced in all of the silages and showed similar values between them.

In the control silage and in the silage treated with the *L. brevis* UFLA SIL 17 strain no significant changes were observed in the propionic acid concentrations during the aerobic exposure (Table 4). The concentration of propionic acid was higher in the evaluation with 96 h of aerobic exposure and decreased until the assessment with 216 h in the silages treated with the *L. brevis* UFLA SIL 24, *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains.

The presence of butyric acid was observed in the inoculated silages at the opening of the silos (Table 4). The concentrations of butyric acid in the silages treated with the *L. brevis* UFLA SIL 17, UFLA SIL 24 and *L. hilgardii* UFLA SIL 51 strains were higher than the concentration in the silage treated with the *L. hilgardii* UFLA SIL 52 strain, with average values of 5.80 and 3.18 g of butyric acid per kg of DM.

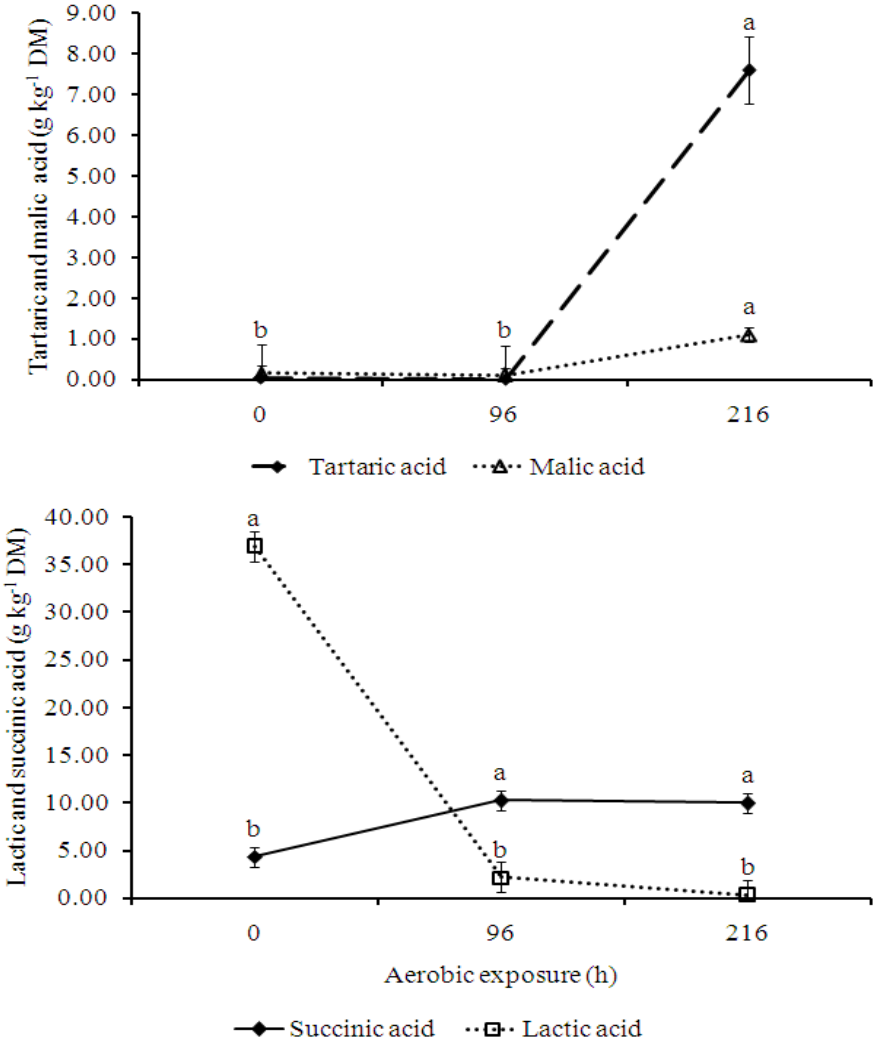


**Figure 2** Tartaric and malic acids (A) and succinic and lactic acids (B) concentrations during aerobic exposure of sugarcane silages. The mean values with different lowercase letters are significant at $P < 0.05$ according to the Scott–Knott test. For each variable bars represent the standard error of the means.

**Table 4** The effects of inoculation on the concentrations of oxalic, citric, acetic, propionic, and butyric acids, 1,2-propanediol and ethanol in sugarcane silages treated with different strains and at different hours of aerobic exposure.

| Aerobic exposure (h) | Ctr | UFLA SIL 17‡ | UFLA SIL 24‡ | UFLA SIL 51* | UFLA SIL 52* | SEM† |
|---|---|---|---|---|---|---|
| **Citric acid (g kg$^{-1}$ DM)** | | | | | | |
| 0 | 0.25 Aa | 0.79 Ba | 0.16 Aa | 0.79 Ba | 0.77 Ba | 0.375 |
| 96 | 0.48 Ab | 5.11 Aa | 0.16 Ab | 3.96 Aa | 3.99 Aa | |
| 216 | 1.26 Aa | 1.85 Ba | 1.00 Aa | 2.13 Ba | 1.78 Ba | |
| **Acetic acid (g kg$^{-1}$ DM)** | | | | | | |
| 0 | 10.85 Ab | 13.71 Ab | 9.23 Ab | 21.50 Aa | 20.61 Aa | 0.897 |
| 96 | 0.38 Ba | 0.61 Ba | 3.00 Ba | 2.01 Ba | 0.21 Ba | |
| 216 | 0.48 Ba | 0.32 Ba | 0.58 Ba | 0.53 Ba | 0.35 Ba | |
| **Propionic acid (g kg$^{-1}$ DM)** | | | | | | |
| 0 | 3.23 Aa | 1.77 Aa | 1.92 Ba | 2.18 Ba | 2.02 Ba | 0.499 |
| 96 | 5.41 Ab | 3.90 Ab | 11.06 Aa | 7.18 Ab | 5.72 Ab | |
| 216 | 3.09 Aa | 2.61 Aa | 3.05 Ba | 2.99 Ba | 2.89 Ba | |
| **Butyric acid (g kg$^{-1}$ DM)** | | | | | | |
| 0 | 0.00 Ac | 6.74 Aa | 5.29 Aa | 5.37 Aa | 3.18 Ab | 0.457 |
| 96 | 1.04 Aa | 0.00 Ba | 0.00 Ba | 0.00 Ba | 0.00 Aa | |
| 216 | 0.00 Aa | 0.00 Ba | 0.00 Ba | 0.00 Ba | 0.00 Aa | |
| **1,2-propanediol (g kg$^{-1}$ DM)** | | | | | | |
| 0 | 2.58 Ab | 0.48 Ab | 0.54 Ab | 30.32 Aa | 34.71 Aa | 0.418 |
| 96 | 1.73 Ab | 0.65 Ab | 0.00 Ab | 19.12 Ba | 12.11 Ba | |
| 216 | 0.95 Aa | 0.25 Aa | 0.00 Aa | 2.27 Ca | 4.07 Ba | |
| **Ethanol (g kg$^{-1}$ DM)** | | | | | | |
| 0 | 104.18 Ab | 77.23 aC | 171.49 Aa | 32.44 Ad | 35.99 Ad | 5.174 |
| 96 | 0.00 Ba | 0.00 Ba | 0.00 Ba | 0.00 Ba | 0.00 Ba | |
| 216 | 0.00 Ba | 0.00 Ba | 0.00 Ba | 0.00 Ba | 0.00 Ba | |

For each row, the mean values with different lowercase letters are significant at $P < 0.05$ according to the Scott-Knott test. For each column, the mean values with different capital letters are significant according to the $P < 0.05$ by Scott-Knot test. † Standard error of the means of two-way interactions. ‡ *L.brevis* specie, ***** *L. hilgardii* specie.

In the evaluation with 96 hours of aerobic exposure, the presence of butyric acid was observed only in the control treatment. With 216 h, the presence of this acid was not observed in any silage.

The silages inoculated with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains showed the highest 1,2-propanediol concentrations and lower ethanol concentrations at the opening of the silos (0 h aerobic exposure). The 1,2-propanediol concentration decreased on average by 51.9% in the silages treated with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains after 96 h of aerobic exposure. To 96 h from 216 h of aerobic exposure, the presence of 1,2-propanediol in the silage treated with the UFLA SIL 52 strain was not altered, and in the silage treated with the UFLA SIL 51 strain, it was reduced by 88.1%. The ethanol presence was observed in silages at opening (0 h aerobic stability) and was not observed throughout the aerobic exposition.

**Multivariate analyses of products of fermentation**

The average of results obtained in the evaluation with 0, 96 and 216 h of aerobic exposure of silage for yeasts population, hours of aerobic stability and products of fermentation in the silages treated with the *L. brevis* UFLA SIL 17, UFLA SIL 24 and *L. hilgardii* UFLA SIL 51, *L. hilgardii* UFLA SIL 52 strains were submitted to principal component analysis (PCA) to identify the variables that may be directly related to a specific strain (Figure 3). The first (F1) and

second (F2) principal components explained 53.92% and 29.80%, respectively, of the total variance. According to the characteristics evaluated the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains showed similar characteristics concerning the concentrations of 1,2-propanediol, acetic acid and succinic acid and were more related to the aerobic stability (Figure 3). The silages treated with the UFLA SIL 24 strain showed features nearest to the control silage (lower left quadrant), which were associated with the presence of lactic acid, malic acid, propionic acid and ethanol. In the upper right, it was possible to observe an association between treatment with the *L. brevis* UFLA SIL 17 strain and the presence of yeast, tartaric acid and butyric acid in the silages.

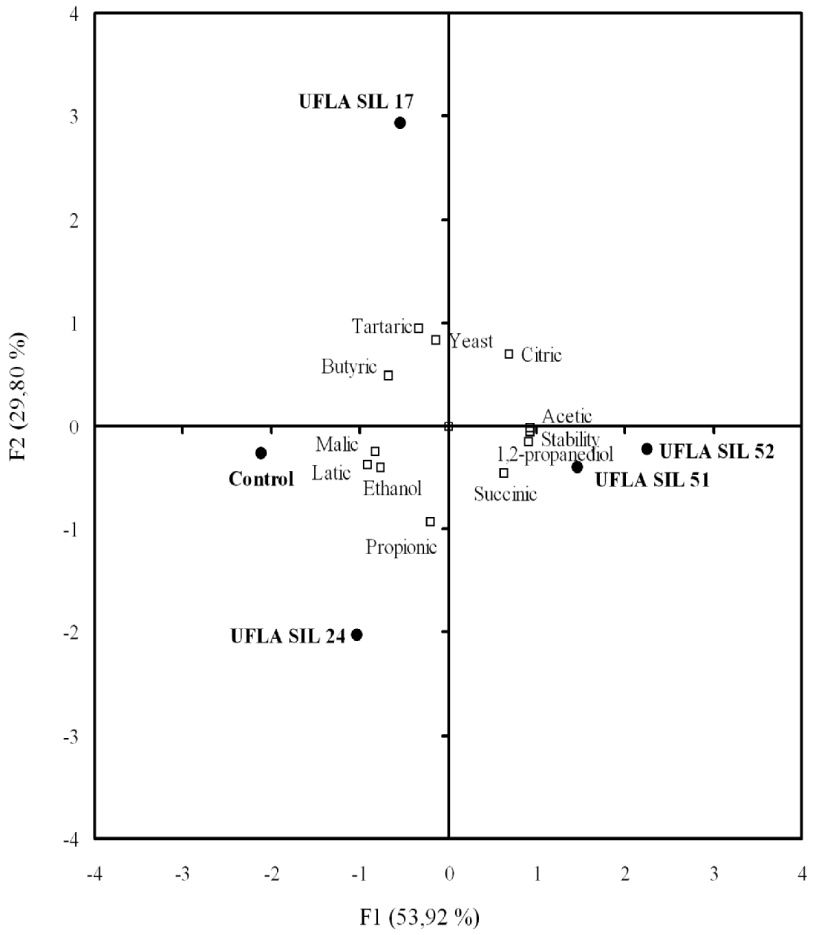


**Figure 3** Principal component analysis (PCA) of the fermentation end products during the aerobic exposure of sugarcane silages.

**Identification of yeasts**

A total of 231 isolates belonging to twenty-eight different morphotype yeast colonies were characterized in terms of cell morphology and physiological features. Ten species were identified, including *Candida diversa* (FR819717.1), *C. ethanolica* (FJ662418.1), *Hanseniaspora opuntiae* (FM199955.1), *Issatchenkia orientalis* (FM199958.1), *Pichia fermentans* (AY235810.1), *P. kudriavzevii* (JQ726607.1), *P. manshurica* (HE965029.1), *Schizosaccharomyces pombe* (AB054041.1), *Debaryomyces etchellsii* (AJ586528.1) and *Zygosaccharomyces bailii* (GU237058.1) (Table 5). The homology of the sequences reported in the GenBank was within 98–100%. The strains genetically identified as the same species presented different physiological characteristics (Figures 4 and 5).

The most frequently observed yeast strains in the silage during the aerobic phase were *C. ethanolica, C. diversa, Z. bailii, S. pombe, Pichia sp* and *Schizosaccharomyces sp* (Table 5). The occurrence of species of the genera *Candida* and *Schizosaccharomyces* occurred evenly between the treatments. The genus *Pichia* was not observed in the treatment with the *L. plantarum* UFLA SIL 35 strain, and *Zygosaccharomyces sp* was not observed in the treatment with the *L. brevis* UFLA SIL 25 strain. The species *D. etchellsii* was found in a lower number of treatments (Table 5).

**Table 5** The yeast species isolated and identified in sugarcane silages during aerobic exposure.

| Species | Treatments where the species was identified (nº of strain) | % of total number of isolates | Percentage of isolates capable of using 3, 6 or 9% of lactic acid | | | % isolated at each time of exposure aerobic | |
|---|---|---|---|---|---|---|---|
| | | | 3% | 6% | 9% | 96h | 216h |
| *Candida diversa* | All treatments | 15.6% | 50.0% | 40.0% | 10.0% | 60.0% | 40.0% |
| *Candida ethanolica* | 17, 19, 24, 27, 33, 34, 35, 41, 42, 46, 51, 52, Ctr | 16.7% | 43.7% | 37.5% | 18.8% | 43.6% | 56.4% |
| *Hanseniaspora opuntiae* | 19, 33, 35, 41, 51, 52, Ctr | 2.1% | 0.0% | 0.0% | 0.0% | 31.2% | 68.8% |
| *Issatchenkia orientalis* | 17, 19, 24, 27, 33, 41, 46, Ctr | 2.1% | 33.3% | 33.3% | 33.4% | 47.8% | 52.2% |
| *Pichia fermentans* | 27, 34, 41, 42 | 2.1% | 50.0% | 50.0% | 0.0% | 50.0% | 50.0% |
| *Pichia kudriavzevii* | 19, 33, Crt | 2.1% | 33.3% | 33.3% | 33.4% | 0.0% | 100.0% |
| *Pichia manshurica* | 17, 19, 27, 32, 34, 41, 42, 46, 52 | 6.3% | 66.7% | 33.3% | 0.0% | 65.0% | 35.0% |
| *Schizosaccharomyces pombe* | 17, 19, 27, 32, 33, 34, 35, 41, 42, 46, 51, 52, Ctr | 12.5% | 50.0% | 50.0% | 0.0% | 41.0% | 59.0% |
| *Debaryomyces etchellsii* | 24, 32, 33 | 2.1% | 0.0% | 0.0% | 0.0% | 76.0% | 24.0% |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Zygosaccharomyces bailii* | 17, 19, 24, 27, 32, 33, 34, 35, 41, 42, 46, 51, 52, Ctr | 15.6% | 100.0% | 0.0% | 0.0% | 52.1% | 47.9% |
| *Pichia sp* | 17, 19, 24, 25, 27, 33, 34, 41, 42, 46, 51, 52, Ctr | 12.5% | 57.1% | 42.9% | 0.0% | 45.3% | 54.7% |
| *Schizosaccharomyces sp* | 17, 19, 24, 25, 27, 32, 33, 34, 41, 42, 46, 51, 52, Ctr | 10.4% | 0.0% | 0.0% | 0.0% | 47.4% | 52.6% |

The species *H. opuntiae* and *I. orientalis* were found in 50% of the treatments (Table 5). *P. kudriavzevii* was isolated at the end of fermentation. The species *H. opuntiae, P. manshurica* and *D. etchellsii* were isolated in a higher percentage at the beginning of the aerobic exposure of the silage, and other species were isolated during the entire aerobic exposure period (Table 5). The species of *C. diversa, C. ethanolica, I. orientalis* and *P. kudriavzevii* were able to grow in a medium with up to 9% lactic acid as the sole carbon source (Table 5, Figure 4).

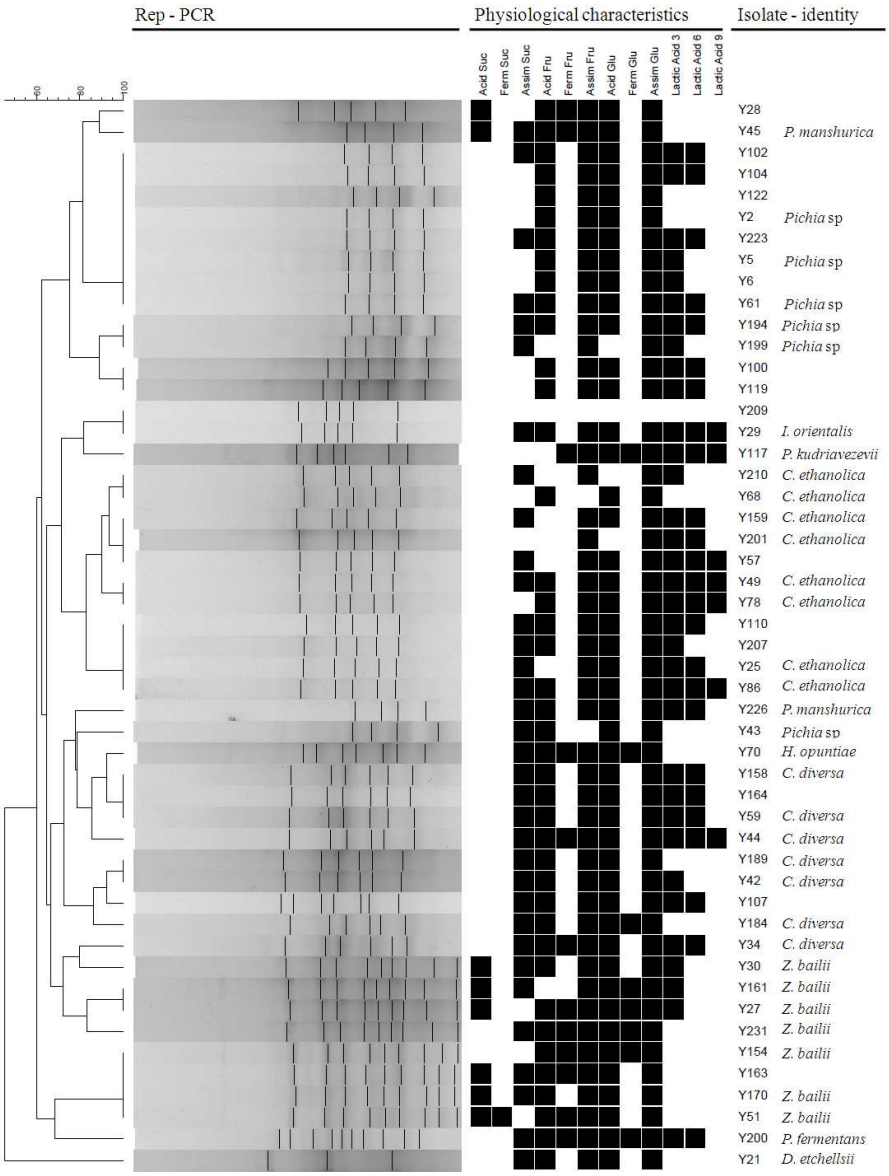


**Figure 4** Similarity analysis between the bands profiles (Rep-PCR) of budding yeast isolates during aerobic exposure of sugarcane silages. Legend: Acid – acid production, Ferm – fermentation, Assim – assimilation, Suc – sucrose, Fru – fructose, Glu – glucose, Lactic Acid 3, 6 and 9 – lactic acid assimilation at 3, 6 and 9%, respectively.

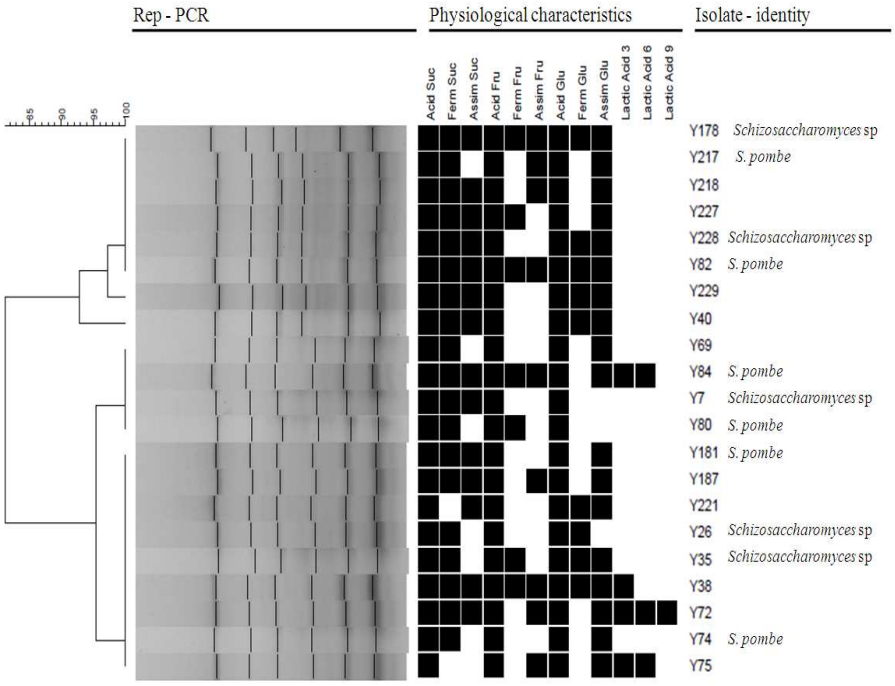


**Figure 5** Similarity analysis between the bands profiles (Rep-PCR) of fission yeast isolates during aerobic exposure of sugarcane silages. Legend: Acid – acid production, Ferm – fermentation, Assim – assimilation, Suc – sucrose, Fru – fructose, Glu – glucose, Lactic Acid 3, 6 and 9 – lactic acid assimilation at 3, 6 and 9%, respectively.

## Discussion

### Changes in chemical composition and temperature of the silage during aerobic exposure

An increase in pH value after silo opening has been observed in previous research (McDonald *et al*., 1991; Ávila *et al*., 2012; Wilkinson and Davies, 2012). The pH remained stable longer in silages treated with facultative heterofermentative *L. plantarum* strains than in silages treated with obligatory heterofermentative *L. brevis* and *L. hilgardii* strains. This finding was unexpected because, in the majority of the studies conducted (Driehuis *et al*., 1999; Wilkinson and Davies, 2012), the silages inoculated with facultative heterofermentative strains showed higher concentrations of lactic acid and lower concentrations of acetic acid, which lead to more rapid deterioration of silage. In the silages treated with *L. brevis* UFLA SIL 17 and UFLA SIL 24, *L. hilgardii* UFLA SIL 51 and UFLA SIL 52, in which the products of fermentation was measured, the increase in the pH values coincided with a reduction in the concentrations of lactic, acetic and butyric acids (Figure 2B, Table 4) and can be associated with the consumption of these acids produced during fermentation (Danner *et al*., 2003).

The increase in the DM and WSC concentrations during the aerobic exposure is the result of the dehydration of silage and the consequent concentrations of these components. The difference in the average content of

DM among the obligatory heterofermentative and facultative heterofermentative strains, with higher values in the silages treated with the obligatory heterofermentative strains, may be because of differences in the production of metabolites and in the microbial population. The highest DM concentration in the silages treated with the obligatory heterofermentative strains at silos opening (0 h) may be responsible for this event. The increased in the DM concentration during aerobic exposition was similar between the silages (Table 1). It is known that heterofermentative strains can promote an increase the aerobic stability of silage and can reduce the temperature because of the increase in acetic acid concentration (Danner *et al*., 2003; Hu *et. al*, 2009; Li and Nishino, 2011), and it has been also reported that homofermentative strains could reduce the aerobic stability of silage (Driehuis *et al*., 1999; Wilkinson and Davies, 2012). In this study, we did not observe an association between the facultative or obligatory heterofermentative strains and aerobic stability (Table 2). Although the best results were observed in the silages inoculated with the obligatory heterofermentative *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains, the facultative heterofermentative *L. plantarum* UFLA SIL 32 and UFLA SIL 34 strains showed better results than the control and the obligatory heterofermentative *L. brevis* strains (Table 2). These facultative heterofermentative strains showed a lower maximum temperature and longer time to reach the maximum temperature than the treatments with the obligatory

heterofermentative UFLA SIL 33, UFLA SIL 17, UFLA SIL 24 and UFLA SIL 27 strains. This finding shows that the wild strain selection should be at the level of strain and not species.

The obligatory heterofermentative *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains showed best results from silage temperature evaluation (Table 2). It is known that inoculation with heterofermentative strains resulted in high silage aerobic stability (Driehuis *et al.*, 1999; Wilkinson and Davies, 2012). However, not all LAB strains which have obligatory heterofermentative metabolism were positive in preventing the temperature increase of the silage (Table 2). In the evaluation of samples analysed here there were no relationship between high yeast population and high temperature of the silage (Figure 1). After silo opening the temperature increasing in the silage is correlated with an increase in yeast population (McDonald *et al.*, 1991; Pahlow *et al.*, 2003; Wilkinson and Davies, 2012). From the results observed in Figure 1 it can be concluded that for the association between yeast population and silage temperature is done sampling should be performed with smaller time intervals from the beginning of aerobic exposure.

The most important organic acids associated with silage fermentation are lactic, acetic, propionic and butyric acids. However a great number of acids can be present in silage. Organic acids can be used in LAB metabolism as a carbon source or as electron acceptors (Axelsson, 2004). To the best of our

knowledge, there are no studies in the literature reporting the quantification of citric, tartaric, malic and succinic acids in sugar cane silages. In the treatments with the *L. brevis* UFLA SIL 17 and *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains, the increase in the concentration of citric acid in the silage during 0 to 96 h of aerobic exposure (Table 4) may be due to the production of the metabolite by yeasts (Vandenberghe *et al*., 1999). According to these authors, the yeasts species of the *Candida, Saccahromicopsis* and *Hansenula* genera produce citric acid from their metabolism. In this work, it was found *C. diversa* and *C. ethanolica* species (Table 5), which may be responsible for the production of citric acid in the measured silage.

The concentration of malic acid found during aerobic exposure of sugar cane silage may be associated with the presence of yeast, especially the genera *Zygosaccharomyces* (Zelle *et al*., 2008; Taing and Taing, 2007). The *Z. bailii* species was isolated and identified in all the silages except in the silage treated with the UFLA SIL 25 strain (Table 5). Since 1924, malic acid has been identified as a product of yeast fermentation (Dakin, 1924). Malic acid production from glucose has been described in *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* species (Zelle *et al*., 2008; Taing and Taing, 2007). The presence of this acid in the silage is desirable because *in vitro* studies have shown the positive effects of malic acid on ruminal fermentation increased the concentrations of propionate and total volatile fatty acids, increased pH,

decreased methane production, decreased lactate concentration, and increased digestibility (Carro *et al*., 1999). Malic acid has been suggested as a substitute for monensin in beef cattle diets because this acid has effects on ruminal fermentation analogous to ionophores (Castillo *et al*., 2004).

Despite the addition of *Lactobacillus* strains, there was no difference among the treated silages and the control, regard to tartaric acid concentration. The concentration of tartaric acid in the silage increased over the aerobic exposure period. The presence of this acid is undesirable in food because it is a compound analogous to malic acid, which is key in the Krebs cycle and the energy supply. It is hypothesized that tartaric acid inhibits the action of malic acid in the Krebs cycle, reducing energy production in humans (Shaw et al., 1995). The enantiomer L(+)-tartaric acid can be produced by various microorganisms, including the *Acetobacter*, *Acinetobacter* (Li and Nishino, 2011) and *Pseudomonas* (Dutkiewicz *et al*., 1989) genera that were found in the silages.

Yeasts and most likely LAB species were present in silage, and the presence of succinic acid can be related to their metabolism. As the population of LAB has not been quantified, it is not known whether the increase in the concentration of succinic acid from 0 to 96 h was associated with an increase in the population of these bacteria or with an increase in yeasts. Succinic acid is a common intermediate in the metabolic pathway of a number of anaerobic and

facultative microorganisms (Zeikus *et al*., 1999). It is a common by-product by the alcoholic fermentation of yeast (Coulter *et al*., 2004). Succinate is formed from sugars or amino acids by *Propionibacterium* species, gastrointestinal bacteria such as *Escherichia coli*, *Pectinatus* sp., *Bacteroides* sp., rumen bacteria such as *Ruminococcus flavefaciens*, *Actinobacillus succinogenes*, *Bacteroides amylophilus*, *Prevotella ruminicola*, *Succinimonas amylolytica*, *Succinivibrio dextrinisolvens*, *Wolinella succinogenes*, and *Cytophaga succinicans* (Bryant and Small, 1956). A number of *Lactobacillus* strains have been reported to produce succinic acid (Kaneuchi *et al*., 1988). The majority of the succinate-producing microorganisms have been isolated from the rumen because, in this ecosystem, succinate serves as an important precursor for propionate, which is absorbed through the rumen wall for subsequent oxidation to provide energy and biosynthetic precursors for the animal.

The reduction in the concentrations of acetic, propionic and butyric acids and in the concentrations of 1,2-propanediol and ethanol may be caused by their volatilization during the aerobic exposure of silage. Lactic acid can be metabolized under aerobic conditions by yeasts (McDonald *et al*., 1991) and under aerobic and anaerobic conditions by LAB. The specie *L. buchneri* degrades lactic acid to acetic acid and 1,2-propanediol (Oude Elferink *et al*., 2001). As an increase in the concentration of these metabolites was not observed, it is probable that yeasts identified in the silage were predominantly

responsible for this process since, most of the isolated yeasts during silage aerobic exposure were able to use lactic acid as a carbon source (Table 5).

Acetic acid can be produced anaerobically or aerobically by heterofermentative LAB and aerobically by acetic bacteria. Acetic bacteria promote the oxidation of ethanol, initially produced by the yeast, to acetic acid in a reaction that is highly exothermic, thus raising the temperature of the fermentation substrate (Spoelstra *et al*., 1988). The reduction in the concentration of acetic acid could be due to volatilization or to the use of the acetic acid by acetic bacteria. According to Spoelstra *et al*. (1988), after the use of ethanol, these bacteria can completely metabolize the acetic acid, as well as the lactic acid in $CO_2$ and water, causing a marked increase in the pH.

The presence of butyric acid in high concentrations in the silages treated with the evaluated strains could be due to the metabolite production by the inoculated LAB. This microbial group can produces butyric acid during the fermentation of carbohydrates (Corsetti *et al*., 1998). The decarboxylation of 2-oxo acids and the deamination of amino acids are the two possible routes for producing butyric, n-valeric and propionic acids among these genera (Galal *et al*., 1978).

Inoculation of the sugar cane silages with the *L. brevis* UFLA SIL 24 strain allowed the highest concentration of propionic acid and acetic acid after 96 h of aerobic exposure. The production of these acids can be correlated

because the formation of propionic acid is accompanied by the formation of acetate for stoichiometric reasons and to maintain hydrogen and redox balances (Boyaval and Corre, 1995). The production of propionic acid can be from glucose or lactic acid, the latter being typically used by anaerobic or aerotolerant *Propionibacteria* (Madigan *et al.,* 2010).

Ethanol was rapidly lost after the aerobic exposition of the silages (Table 4). Decreases in the concentrations of ethanol and acetic acid from two days of aerobic exposure of sugar cane were observed by Ávila *et al*. (2012). The complete disappearance of ethanol after the aerobic exposure of silage is caused by volatilization or by the use of this compound by the acetic bacteria (Spoelstra *et al*., 1988), and because the acetic acid concentration was not increased during the evaluation, we could assume that the reduction in the concentration of ethanol was due to volatilization.

The presence of 1,2-propanediol in silage is associated with the degradation of lactic acid (Oude Elferink *et al.,* 2001). Heinl *et al*. (2012) verified that the metabolism of *L. hilgardii* and *L. buchneri* are similar and that these species possess the ability to metabolize lactic acid and to form acetic acid and 1,2-propanediol under anaerobic conditions. Under anoxic conditions, *L. diolivorans* can degrade 1,2-propanediol to 1-propanol and propionic acid (Krooneman *et al*., 2002). Because this conversion is anaerobic, the reduction in the concentration of 1,2-propanediol in the silage during the aerobic stability

assessment is possibly caused by volatilization. Concepts regarding the variations in the concentrations of fermentation end products during the aerobic exposure of silage are complex because these metabolites are associated with different groups of microorganisms. The microbiology of sugar cane silage is not completely known, despite the dominance of *Lactobacillus* (Ávila *et al*., 2009). A number of genera may be present and could participate in the fermentation process. The ideal fermentation of silage would be dominated by LAB; this premise is utopic in the uncontrolled and spontaneous fermentation process that occurs in silos.

**Multivariate analyses**

The *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains were more related to the aerobic stability than the other obligatory heterofermentative *L. brevis* strains evaluated and were highlighted by high 1,2-propanediol and acetic acid concentrations. The changes associated with the aerobic stability of the silage were more closely associated with these strains. Other studies have observed an increase in aerobic stability because of the presence of acetic acid (Danner *et al*., 2003; Wilkinson and Davies, 2012) and the presence of 1,2-propanediol (Nishino *et al*., 2003). It is possible to infer that not all heterofermentative LAB are beneficial for improving the aerobic stability of silage.

**Yeast identification**

This study is the first to identify the yeast species present during the aerobic exposure of sugar cane silage. It was not possible to confirm the presence of the following species in other silages: *C. diversa, C. ethanolica, H. opuntiae, P. manshurica, S. pombe, D. etchellsii* and *Z. bailii.* Other species, such as *I. orientalis* (O'Brien *et al*., 2007; Mansfield and Kuldau, 2007), *P. fermentans* (Ávila *et al*., 2010; O'Brien *et al*., 2007; Mansfield and Kuldau, 2007, Rossi and Dellaglio, 2007; Woolford, 1990), and *P. kudriavzevii* (Dolci *et al*., 2011; Li and Nishino, 2011), have been identified in silages. Woolford (1990) classified the yeasts associated with the deterioration of silage into two groups: the acid-users, which comprise species of *Candida, Endomycopsis, Hansenula* and *Pichia,* and the sugar users, which are species of *Torulopsis.* Yeasts of the *Torulopsis* genera were not identified in this study, possibly because of the low concentration of WSC in the silage remaining after the silo opening.

It is hypothesized that silages containing a yeast population larger than 5 log cfu $g^{-1}$ present inadequate microbial quality in the silos and are more susceptible to aerobic deterioration (McDonald *et al*., 1991). The 5 log cfu $g^{-1}$ population quoted is valid only if it contains yeasts that utilize lactate, and a high population of yeasts does not necessarily indicate that the silage will deteriorate (Jonsson and Pahlow, 1984). The silages treated with the *L. plantarum* UFLA

SIL 41, *L. brevis* UFLA SIL 33, UFLA SIL 25, UFLA SIL 27, *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains showed yeast populations lower than 5 log cfu $g^{-1}$ at the opening of the silos (Figure 1), and after aerobic exposure, the silages showed different behaviors relative to the yeast growth.

A total of 231 yeast isolates, 67.9% were able to grow in the medium containing 3% lactic acid, and 27.7% grew in the medium with 9% lactic acid. Yeasts of identical species showed different responses in relation to fermentation and assimilation of the tested carbon sources (Figures 4 and 5). Likewise our findings, Schwan et al. (2007) observed differences in the yeasts metabolisms belonging to same species isolated from fermented beverage. The differences in the metabolisms of microorganisms of identical species reinforce the need to study in detail the fermentation of different types of forage under different conditions. The data from the initial yeast population and from yeast species associated with each treatment may justify the reduced aerobic stability of these silages. The best results during aerobic exposure were observed in the silages treated with the *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains. These results might be caused by the lowest initial population of yeast and the highest concentrations of acetic acid and 1,2-propanediol since. In these silages, the occurrence of yeast species capable of utilizing lactic acid was observed (Figure 1, Table 5).

## Conclusion

The LAB strains tested as starter cultures were able to modify the fermentative, chemical and microbiological parameters of silage after aerobic exposure. The growth and metabolism of the LAB strains modified the diversity of the yeast species in the silages during aerobic exposure. The diverse behavior of the LAB strains of the identical group and species during evaluation reinforces the need to use specific strains selected for each forage plant. Silages showing highest aerobic stability were more correlated with high acetic acid and 1,2-propanediol concentration. Among the evaluated LAB strains, the obligatory heterofermentative *L. hilgardii* UFLA SIL 51 and UFLA SIL 52 strains provided the silages with lower temperatures and additional unheated time and are suitable for use in sugar cane silage.

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**ARTIGO 3 Methylotrophic yeast, lactic acid bacteria and crude glycerin as additives for sugar cane silage**

(Artigo submetido ao periodico indexado: Journal of Dairy Science)

## ABSTRACT

The ensilage of sugar cane results in high DM loss due to alcoholic fermentation, but the addition to the silage of methanol rich-glycerin from biodiesel may compensate for the energy loss during ensilage. Methanol is the most undesirable contaminant in crude glycerin destined for animal feeding. The aim of this study was to evaluate the effects of glycerin added with 1.1% of methanol and *Lactobacillus hilgardii* UFLA SIL52 in loss reduction and in increased in nutritional quality of sugar cane silage and to evaluate the effects of *Pichia methanolica* NCYC 1381 in reduction the methanol concentration of glycerin added to the silage. A randomised design with a 4 × 3 × 3 factorial arrangement of treatments was used to analyse the results. We assessed four concentrations of glycerin inclusion (0, 4, 8, 12% of fresh forage), three periods of silage fermentation (11, 34, 68 days) and three combinations of microbial additives (*L. hilgardii* UFLA SIL52 [LH], UFLA SIL52 plus *P. methanolica* NCYC1381 [LH+PM] and without any microbial additive [WI]). The glycerin addition increased the DM concentration and reduced the NDF concentration, increasing the energy density of the silage. The increase in the glycerin concentrations reduced the contents of citric, malic, lactic, acetic and propionic acids, 1,2-propanediol, methanol and ethanol, and increased the concentrations of glycerol and the aerobic stability. The LH treatment increased the concentrations of the succinic, acetic and propionic acids and 1,2-propanediol

and reduced the pH values, the yeast population, and the concentration of lactic acid and glycerol in silage. The PM treatment increased DM loss, glycerol concentration and aerobic stability. The treatment with 4% glycerin dosages and with the *L. hilgardii* UFLA SIL52 was the better treatment for improve the silage quality, therefore they are indicated as additives in sugar cane ensilage. Under the conditions of the experiment the *P. methanolica* NCYC 1381 treatment did not reduce the methanol concentration in silage.

Key Words: *Pichia methanolica*, *Lactobacillus hilgardii*, organic acids in silage, dry matter loss

## INTRODUCTION

The use of sugar cane (*Saccharum* spp.) as forage for cattle feeding is widespread in Brazil. However, sugar cane ensilage results in high dry matter (DM) loss due to fermentation of sucrose by yeasts (Kung Jr. & Stanley, 1982). The addition of glycerin resulting from biodiesel production may compensate for this energy loss during ensilage of this forage and reduce losses due to changes in the fermentation pattern (Carvalho et al., unpublished data).

Each 100 kg of biodiesel generates approximately 10 kg of glycerin as co-product , which contains variable amounts of glycerol and methanol (Santibánez et al, 2011). The methanol content is particularly important and variable. Concentrations from 0.006 to 14.98% of methanol in the glycerin produced in the U.S.A. were reviewed by Shurson et al., (2012). The toxicity of methanol to animals has been described by Nie et al. (2007). Some countries have established the maximum permitted levels for methanol in crude glycerol for animal feed: 0.015% in USA and Brazil, 0.1% in Canada, 0.2% in Germany and 0.5% in the European Union as a whole (Hansen et al., 2009; MAPA, Brasil, 2010).

The response in consumption and performance of ruminants to the use of purified glycerol as an energy supplement (DeFrain et al., 2004) or as a substitute for corn (Donkin et al., 2009; Carvalho et al., 2011) have been

reported. Although little is known about the effects of glycerin added with methanol to the fermentation pattern, microbiota, and fermentative losses of sugar cane silage.

The addition of methylotrophic yeasts that is able to use methanol as an energy source, could reduce the levels of methanol while the energy value of the glycerin compensates for the energy loss during sugar cane fermentation. *Pichia methanolica* is a yeast able to metabolize methanol (Hartner and Glieder, 2006). This yeast is unable to utilize sucrose and lactic acid as an energy source. The heterofermentative *Lactobacillus hilgardii* UFLA SIL52 isolated from sugar cane silage (Ávila et al., unpublished data) is a new option to improve the quality of sugar cane silage. Moreover, it reduces the dry matter loss of this silage plus glycerin (Carvalho et al., unpublished data). The aim of this study was to evaluate the effects of glycerin added with 1.1% of methanol and *Lactobacillus hilgardii* UFLA SIL52 in loss reduction, and increase in nutritional quality of sugar cane silage and to evaluate the ability of *Pichia methanolica* NCYC 1381 to reduce the methanol concentration of glycerin added to the silage.

## MATERIAL AND METHODS

### *Forage and ensilage conditions*

Fresh-cut sugar cane that was approximately 12 months old was manually harvested and chopped (PP-47, Pinheiro, Itapira, SP, Brazil). Experimental silos (mini-silos) were used in the form of 10 cm diameter and 60 cm long PVC tubes. The tubes were sealed with tight lids containing Bunsen valves for gas release. Each silo was packed with 3 kg of fresh matter (FM) resulting in a density of 666 kg $m^{-3}$. Glycerin doses were added at 0, 4, 8 and 12% of FM. This glycerin was added with methanol to achieve a concentration of 1.1%. To ensure the concentration of glycerin added to silage and also the amount of methanol present in this glycerin, purified glycerin was used (99.7% of glycerol) (Vetec, Duque de Caxias, RJ, Brazil), which was added with methanol (Merck, Dasmstadt, Germany) at application to the silage. Nine mini silos were prepared for each treatment, three were opened after 11 days, another three after 34 days, and the last three after 68 days. The weights of empty and full silos were recorded. Silos were sealed, and stored at room temperature (25ºC on average) and protected from sunlight and rain. After ensilage and before opening, the full silos were weighed. Dry matter (DM) loss was calculated using weights and DM contents and FM silage.

The inoculants were pre-cultured in the laboratory according to Ávila et al. (2009) and enumerated on the ensilage day. The *L. hilgardii* UFLA SIL52 was enumerated on De Man Rogosa Sharpe agar (Oxoid CM361, Basingstoke, Hampshire, England) and the *P. methanolica* NCYC1381 on YEPD agar [10 g $L^{-1}$ yeast extract (Merck, Darmstadt, Germany) 10 g $L^{-1}$ soy peptone (Himedia, Mumbai, India) , 20 g $L^{-1}$ glucose (Merck, Darmstadt, Germany), 20 g $L^{-1}$ agar (Merck, Darmstadt, Germany) containing 100 mg $L^{-1}$ chloramphenicol (Sigma, St. Louis, USA) and 50 mg $L^{-1}$ chlortetracycline (Sigma, St. Louis, USA)]. The inoculants were mixed with deionised water and sprayed onto the forage, resulting in an application volume of 13.3 L $ton^{-1}$. The final concentration of microbial inoculum added to silage was 6.1 log cfu of *L. hilgardii* (UFLA SIL52) $g^{-1}$ of FM and 5.0 log cfu of *P. methanolica* (NCYC1381) $g^{-1}$ of FM. The same volume of pure distilled water was added to the control treatment. A separate sprayer was used for each treatment to avoid cross-contamination.

***Analytical procedures***

To make the aqueous extract, a sample of 25 g of fresh forage or sugar cane silage was blended in 225 mL of 0.1% sterile peptone water and homogenized in an orbital shaker for 20 min. The pH of each sample was then determined. A portion of this aqueous extract (2 ml) was acidified with 10μL of 50% (vol/vol) $H_2SO_4$ and frozen prior to analysis for fermentation end products.

Aqueous extracts were analysed concerning to the content of citric acid, malic acid, succinic acid, lactic acid, acetic acid, propionic acid, butyric acid, glycerol, methanol, 1,2-propanediol and ethanol by high-performance liquid chromatography. The apparatus (Shimadzu, Corp., Tokyo, Japan) was equipped with a dual detection system consisting of an ultraviolet detector (UV-Vis SPD-10Ai) and a refractive index detector (RID 10A). An ion exclusion column from Shimadzu (Shim-pack SCR-101H; 7.9 mm X 30 cm) operated at 50°C was used for the chromatographic separation of the acids. The same column was used for the chromatographic separation of the alcohols but it was operated at 30ºC. The mobile phase consisted of a 100 mM perchloric acid solution (pH 2.2) with a flow rate of 0.6 mL/min. The acids were detected by UV absorbance (210 nm) and the alcohols were identified using the refractive index detector.

Samples were dried at 55ºC in a forced air oven for 72 h and ground through a 1-mm mesh (Wiley mill, Thomas Scientific, Philadelphia, USA). Neutral detergent fiber (NDF) was analysed using the sulfite method described by Van Soest et al. (1991), using an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Water soluble carbohydrates (WSC) were analysed by the method of phenol (Dubois et al. 1956).

***Microbiological analysis***

Another portion of aqueous extracts was used for enumeration of microorganisms. Subsequent tenfold dilutions were prepared to quantify the microbial groups. Yeasts and filamentous fungi were enumerated (spread plating) on Dichloran Rose Bengal Chloramphenicol Medium (DRBC, Difco, Detroit, MI, USA). Plates were incubated at 28ºC for 72 h. Lactic acid bacteria (LAB) were enumerated (pour plating) using MRS agar (De Man Rogosa Sharpe, Difco, Detroit, MI, USA) containing 0.1% cysteine HCl (Merck, Dasmstadt, Germany) and 0.4% cycloheximide (0.4%) (Sigma, St. Louis, MO, USA). Plates for bacterial enumeration were kept under anaerobic incubation (AnaeroGen, Oxoid Basingstoke, UK) at 30ºC for 72 h.

***PCR-DGGE analysis***

The total DNA was extracted from samples before ensilage (fresh sugar cane) and at 68 days of fermentation using the following Protocol: DNA Purification from Tissues [(QIAamp DNA Mini Kit (Qiagen, Hilden, Germany)], in accordance with the instructions of the manufacturer. The DNA from the evaluation of bacterial community was amplified with 338fGC and 518r primers, which span the V3 region of the 16S rRNA gene (Ovreas et al., 1997). A fragment of the D1-region of the 26S rRNA gene was amplified using the eukaryotic universal primers NL1GC and LS2 (Cocolin et al., 2000).

The PCR products from the microbial communities were analysed by PCR–DGGE using a BioRad DCode Universal Mutation Detection System (BioRad, Richmond, CA, USA) according to the procedures previously described by Ramos et al. (2010). Denaturation gradients varied from 30-50% denaturant [100% denaturant corresponds to 7 M urea and 40% (v $v^{-1}$) formamide] for the bacterial community and 25-50% for the eukaryotic community. Electrophoresis was conducted at 60ºC for 5 h at a constant voltage of 160 V. After electrophoresis, the gels were stained with SYBR-Green I solution (Invitrogen, Foster City, CA, USA) (1:10.000 v/v) for 30 min and photographed with UV transillumination (302 nm; Lpix Image, Loccus Biotechnology). Bands from the PCR–DGGE gels were excised with a sterile blade and placed in 50 μL of sterile Milli-Q water at 4 °C overnight to allow the DNA to diffuse out of the polyacrylamide matrix. The DNA was subsequently re-amplified using the same primers described above. The PCR products were purified and sequenced using an ABI 3730 XL DNA Analyser (Applied Biosystems, Foster City, California, CA),and the sequences were compared with those available in the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, Maryland, USA).

***Evaluation of aerobic stability***

For evaluation of the aerobic stability, at 68 days of ensilage 2 kg of silage were placed in plastic buckets and were kept in a room with controlled temperature at 24 ºC (±0.5 ºC). In the center of each bucket a data logger (MI-IN-D-2-L Escort) was placed, programmed to measure the temperature every 0.5 h. Silage temperature was evaluated until 265 h. Data from time to loss of aerobic stability (number of hours required to silage exceed in 2 ºC the ambient temperature, Moran et al., 1996), maximum temperature, and time to reach maximum temperature were analyzed.

***Statistical analyses***

The experiment was conducted in randomized design. A 4 × 3 × 3 factorial arrangement of treatments was used to analyze the results. We assessed four concentrations of glycerin inclusion (0, 4, 8, 12% of fresh forage), three periods of silage fermentation (11, 34, 68 days) and three combinations of microbial additives [*L. hilgardii* UFLA SIL52 (LH), UFLA SIL 52 plus *P. methanolica* NCYC1381 (LH+PM) and without any microbial additive (WI)]. Data were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC 1998), under model: $Y_{ijkl} = \mu + Gly_j + Day_k + Ma_l + Gly \times Day_{jk} + Gly \times Ma_{jl} + Day \times Ma_{kl} + e_{jkl}$, where: $\mu$ = global mean; $Gly_j$ = glycerin effect (j = 0, 4, 8, 12); $Day_k$ = days of ensilage effect (k = 11, 34, 68); $Ma_l$ = microbial additive

effect (l = LH, LH+PM, WI); Gly × $Day_{jk}$ = effect of interaction between glycerin and days of ensilage; Gly × $Ma_{jl}$ = effect of interaction between glycerin and microbial additive; Day × $Ma_{kl}$ = effect of interaction between day and microbial additive and $e_{ijkl}$ = experimental error, assumed independently and identically distributed in a normal distribution with average zero and variance $\sigma^2$. Means were compared with pre-planned contrasts. The averages of dose and days of ensilage were compared with polynomial contrasts (linear, quadratic and cubic). The averages of microbial additive were compared by orthogonal contrasts: WI *vs* LH and LH *vs* LH+PM. The data of acids and alcohols were compared only at evaluation with 68 days of ensilage, thus the effect of days of ensilage was removed from the model above. The temperature data were analyzed using the same model described above without the effect of days of ensilage. Significance was defined at $P \leq 0.05$.

## RESULTS

### *Chemical composition of freshly treated sugar cane before ensilage*

The increase in glycerin dosage added in sugar cane prior to ensilage increased the concentration of DM, methanol and glycerol (Table 1). The concentrations of NDF, WSC, citric, malic, succinic and propionic acids reduced with the increase of glycerin dosage in sugar cane prior to ensilage (Table 1).

The concentration of ethanol, 1,2-propanediol, lactic, butyric and acetic acids were below to detection limits.

The succinic acid concentration, the pH value and the microorganisms population were not modified by glycerin addition. The mean population of LAB, yeasts and filamentous fungi were 6.83, 5.44 e 4.35 log of cfu $g^{-1}$ of fresh sugar cane, respectively (Table 1).

**Table 1** Chemical and microbiological composition of sugar cane aditived with 0, 4, 8 or 12% of fresh matter of glycerin prior to ensilage.

| | Glycerin dosage (% of fresh matter) | | | | SEM[1] | *P* value |
|---|---|---|---|---|---|---|
| | 0 | 4 | 8 | 12 | | |
| pH | 5.71 | 5.66 | 5.82 | 5.85 | 0.12 | 0.62 |
| Dry matter % | 27.5 | 30.0 | 33.0 | 36.7 | 0.41 | <0.01 |
| Concentration % of dry matter | | | | | | |
| Neutral detergent fibre | 52.4 | 45.2 | 38.1 | 33.0 | 0.31 | <0.01 |
| Water soluble carbohydrates | 26.6 | 23.7 | 21.1 | 18.9 | 1.04 | <0.01 |
| Citric acid | 0.78 | 0.43 | 0.43 | 0.14 | 0.118 | <0.01 |
| Malic acid | 0.41 | 0.25 | 0.17 | 0.13 | 0.027 | <0.01 |
| Succinic acid | 0.80 | 0.71 | 0.66 | 0.66 | 0.066 | 0.40 |
| Propionic acid | 0.31 | 0.23 | 0.18 | 0.17 | 0.022 | <0.01 |
| Methanol | 0.00 | 0.00 | 0.16 | 0.19 | 0.045 | <0.01 |
| Glycerol | 0.54 | 13.88 | 24.60 | 37.62 | 1.613 | <0.01 |
| Population cfu[3] $g^{-1}$ of fresh matter | | | | | | |
| Lactic acid bateria | 7.03 | 6.91 | 6.74 | 6.64 | 0.234 | 0.65 |
| Yeast | 5.47 | 5.55 | 5.38 | 5.37 | 0.075 | 0.32 |
| Filamentous fungi | 4.24 | 4.28 | 4.41 | 4.45 | 0.160 | 0.74 |

[1] SEM - standard error of the means, [2] each mean was obtained in nine replicates, [3] cfu - colony former unit

***Fermentative loss and chemical characteristics***

The DM content in silage was modified by interaction between glycerin dosage and days of ensilage ($P < 0.01$) and by interaction between glycerin dosage and microbial inoculum ($P = 0.03$) (Table 2). The DM content increased with the increase in glycerin dosage (Figures 1A and 1B), in the treatment without glycerin the DM reduced during the ensilage period but in the treatment with glycerin this reduction was not observed (Figure 1A).

**Table 2** Means of main effect and *P* value of the influence of glycerin dosage, microbial inoculums and day of ensilage and its interactions on dry matter (DM), DM loss, neutral detergent fibre (NDF), water soluble carbohydrates (WSC), pH and lactic acid bacteria (LAB) and yeast population in sugar cane silage.

| | DM | DM loss | NDF | WSC | pH | LAB | Yeast |
|---|---|---|---|---|---|---|---|
| Glicerin dosage (% of fresh matter) | % | % of ensiled DM | % DM | | | Log cfu[1] g[-1] silage | |
| 0 | 26.3 | 13.8 | 62.3 | 5.66 | 3.83 | 8.36 | 4.52 |
| 4 | 29.9 | 11.7 | 53.2 | 5.13 | 3.83 | 8.32 | 4.55 |
| 8 | 33.4 | 12.4 | 46.2 | 5.15 | 3.86 | 8.28 | 4.68 |
| 12 | 37.3 | 13.9 | 40.1 | 4.80 | 3.86 | 8.37 | 4.18 |
| SEM[2] | 0.12 | 0.56 | 5.98 | 0.358 | 0.011 | 0.058 | 0.149 |
| Microbial inoculum | | | | | | | |
| WI[3] | 31.5 | 13.1 | 50.3 | 5.61 | 3.91 | 8.29 | 4.98 |
| LH[4] | 31.8 | 12.2 | 50.6 | 4.96 | 3.81 | 8.27 | 4.29 |
| LH+PM[5] | 31.8 | 13.5 | 50.5 | 4.99 | 3.82 | 8.44 | 4.18 |
| SEM | 0.10 | 0.89 | 0.19 | 0.305 | 0.009 | 0.050 | 0.129 |
| Days of ensilage | | | | | | | |
| 11 | 31.6 | 11.6 | 48.5 | 9.36 | 3.99 | 8.23 | 4.98 |
| 34 | 32.4 | 11.8 | 50.0 | 3.91 | 3.78 | 8.18 | 5.06 |
| 68 | 31.1 | 15.4 | 52.9 | 2.28 | 3.77 | 8.58 | 3.40 |
| SEM | 0.10 | 0.48 | 0.19 | 0.308 | 0.009 | 0.050 | 0.129 |

| *P* value | | | | | | | |
|---|---|---|---|---|---|---|---|
| Glycerin dosage (GLY) | <0.01 | 0.02 | <0.01 | 0.39 | 0.03 | 0.76 | 0.10 |
| Days of ensilage (DAY) | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Microbial inoculum (INO) | 0.05 | 0.15 | 0.63 | 0.24 | <0.01 | 0.10 | <0.01 |
| GLY x DAY | <0.01 | <0.01 | <0.01 | 0.08 | <0.01 | 0.11 | 0.03 |
| GLY x INO | 0.03 | 0.02 | 0.19 | 0.67 | 0.55 | 0.85 | 0.30 |
| INO x DAY | 0.22 | 0.13 | <0.01 | 0.95 | <0.01 | 0.03 | <0.01 |
| GLY x DAY x INO | 0.23 | 0.95 | 0.03 | 0.94 | 0.02 | 0.85 | 0.27 |
| Contrasts for glycerin dosage | | | | | | | |
| Linear | <0.01 | 0.01 | <0.01 | 0.44 | 0.25 | 0.32 | 0.2 |
| Quadratic | <0.01 | 0.59 | <0.01 | 0.21 | 0.79 | 0.72 | 0.11 |
| Cubic | <0.01 | 0.04 | <0.01 | 0.35 | <0.01 | 0.84 | 0.14 |
| Contrasts for days of ensilage | | | | | | | |
| Linear | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Quadratic | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| Contrasts for microbial inoculum | | | | | | | |
| WI *vs* LH | 0.02 | 0.18 | 0.35 | 0.14 | <0.01 | 0.81 | <0.01 |
| LH *vs* LH+PM | 0.80 | 0.06 | 0.77 | 0.96 | 0.64 | 0.05 | 0.55 |

[1] cfu - colony former unit,
[2] SEM - standard error of the means,
[3] WI - without microbial inoculum,
[4] LH - *Lactobacillus hilgardii* UFLA SIL52,
[5] PM - *Pichia methanolica* NCYC 1381.

When the microbial inoculums were added in silages treated without or with 12% glycerin an increase in DM content occurred (Figure 1B). The DM content of silages treated with 4 or 8% glycerin was not modified by microbial inoculums addition (Figure 1B).

The interaction between glycerin dosage and days of ensilage ($P < 0.01$) and the interaction between glycerin dosage and microbial inoculum ($P = 0.02$)

altered the DM loss in silage (Table 2) (Figures 2A and 2B). As general average, the dry matter loss increased during the ensilage period. However, this increase was small in silages treated with glycerin (Table 2). At 11 days of ensilage, the addition of 8 or 12% glycerin increased the DM loss. In other times of fermentation, the DM losses in the treatment with glycerin increased less expressively when compared with the losses observed in the treatment without glycerin (Figure 2A). The LH treatment reduced the DM loss when added in silage without glycerin or in silage treated with 8% glycerin.

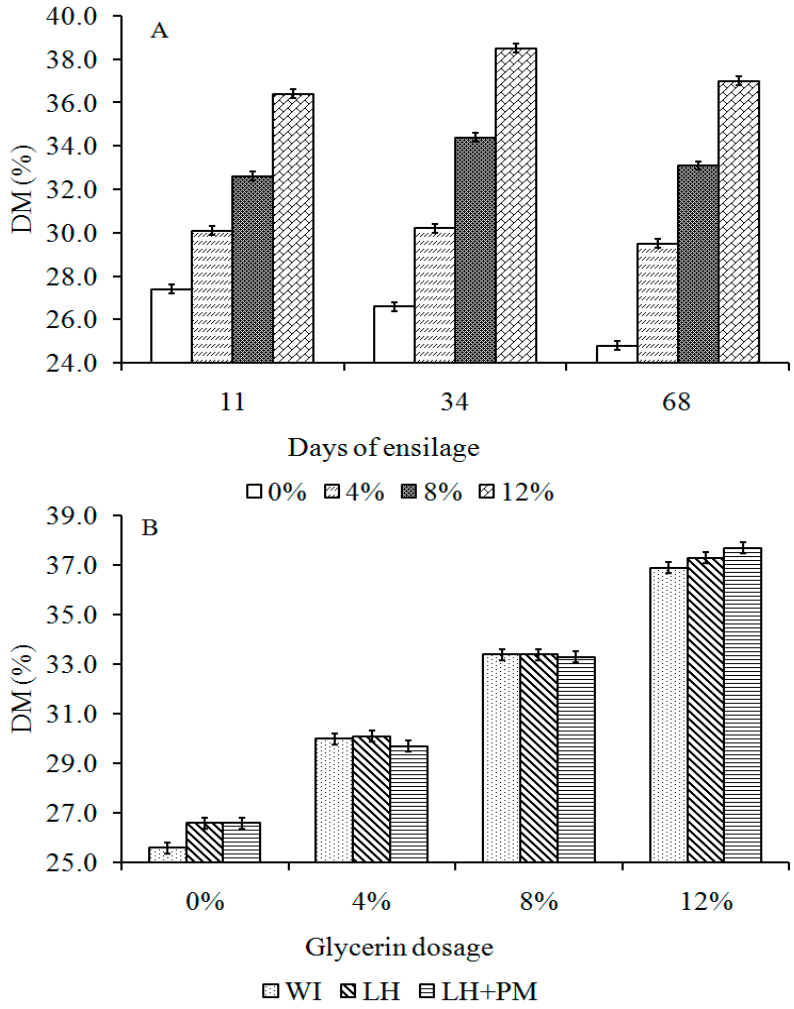


Figure 1 Dry matter (DM) in sugar cane silage treated with 0, 4, 8 or 12% (fresh matter) glycerin ensiled for 11, 34 and 68 days (A) and DM without microbial inoculums, with *Lactobacillus hilgardii* UFLA SIL52 (LH) or with *Lactobacillus hilgardii* UFLA SIL52 plus *Pichia methanolica* NCYC 1381 (LH+PM) ensiled with 0, 4, 8 or 12% (fresh matter) glycerin (B). The bars represent the standard error of the means.

In silages treated with 4 or 12% glycerin the LH treatment showed DM loss similar to the treatment without glycerin (Figure 2B). The PM treatment increased the DM loss when added to glycerin treated silage (Figure 2B).

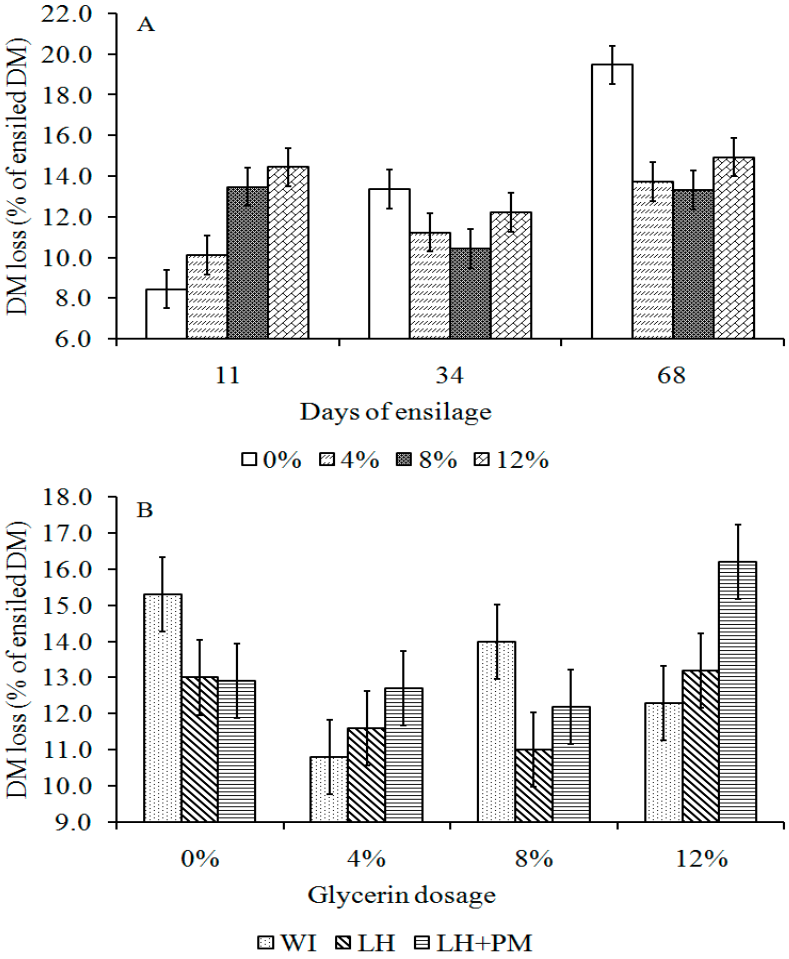


Figure 2 Dry matter (DM) loss in sugar cane silage treated with 0, 4, 8 or 12% (fresh matter) glycerin ensiled for 11, 34 and 68 days (A) and DM loss without microbial inoculums, with *Lactobacillus hilgardii* UFLA SIL52 (LH) or with *Lactobacillus hilgardii* UFLA SIL52 plus *Pichia methanolica* NCYC 1381 (LH+PM) ensiled with 0, 4, 8 or 12% (fresh matter) glycerin (B). The bars represent the standard error of the means.

The NDF content was modified by interaction between glycerin dosage and days of ensilage ($P < 0.01$) and by interaction between microbial inoculum and days of ensilage ($P < 0.01$). The increase in glycerin dosage reduced the NDF content in silage (Table 2), during ensilage period the NDF content increased. However, this increase was smaller in silages treated with glycerin (Figure 3A). The increase in NDF content during the ensilage period in the LH treatment was observed only in the evaluation with 68 days of ensilage while in the WI treatment and in the LH+PM treatment this increase occurred during all ensilage period assessed. (Figure 3B).

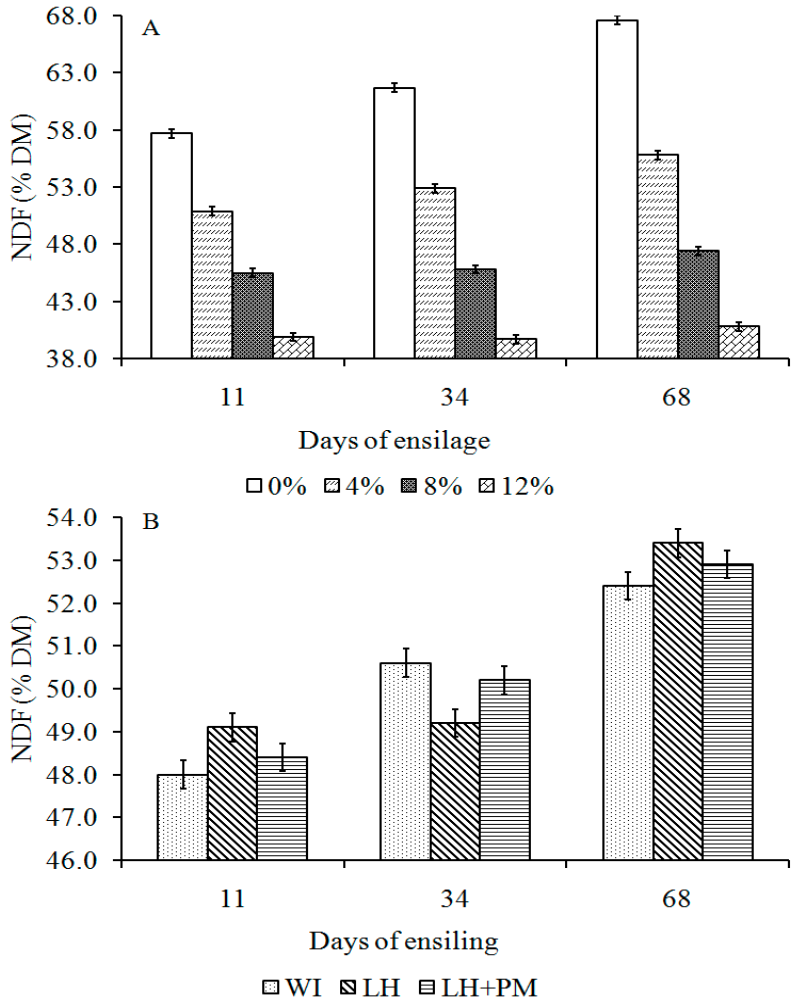


Figure 3 NDF concentration of sugar cane silage treated with 0, 4, 8 or 12% (fresh matter) glycerin ensiled for 11, 34 and 68 days (A) and NDF concentration without microbial inoculums, with *Lactobacillus hilgardii* UFLA SIL52 (LH) or with *Lactobacillus hilgardii* UFLA SIL52 plus *Pichia methanolica* NCYC 1381 (LH+PM) ensiled for 11, 34 and 68 days (B). The bars represent the standard error of the means.

The carbohydrates present in the silage reduced during the ensilage (quadratic effect) (Table 2). There was a more accentuated decrease in the WSC concentration in the beginning of ensilage when the WSC is higher (Tables 1 and 2).

The pH of the silage was modified by interaction between glycerin dosage and days of ensilage ($P < 0.01$) and by interaction between microbial inoculum and days of ensilage ($P < 0.01$) (Table 2). At 11 days of ensilage the silages treated with the highest glycerin dosage (8 and 12%) showed an average of pH value 0.1 unities greater than the other silages (Figure 4A). There was no difference in pH values of silages treated with different glycerin dosages after 34 and 68 days of ensilage (Figure 4A). The LH treatment reduced pH values in the silage (Figure 4B). In the evaluations with 11 and 34 days, the pH value in the silage treated with LH or with LH+PM was lower than WI treatment (Figure 4B). At 68 days of ensilage no differences were observed in pH values of silages.

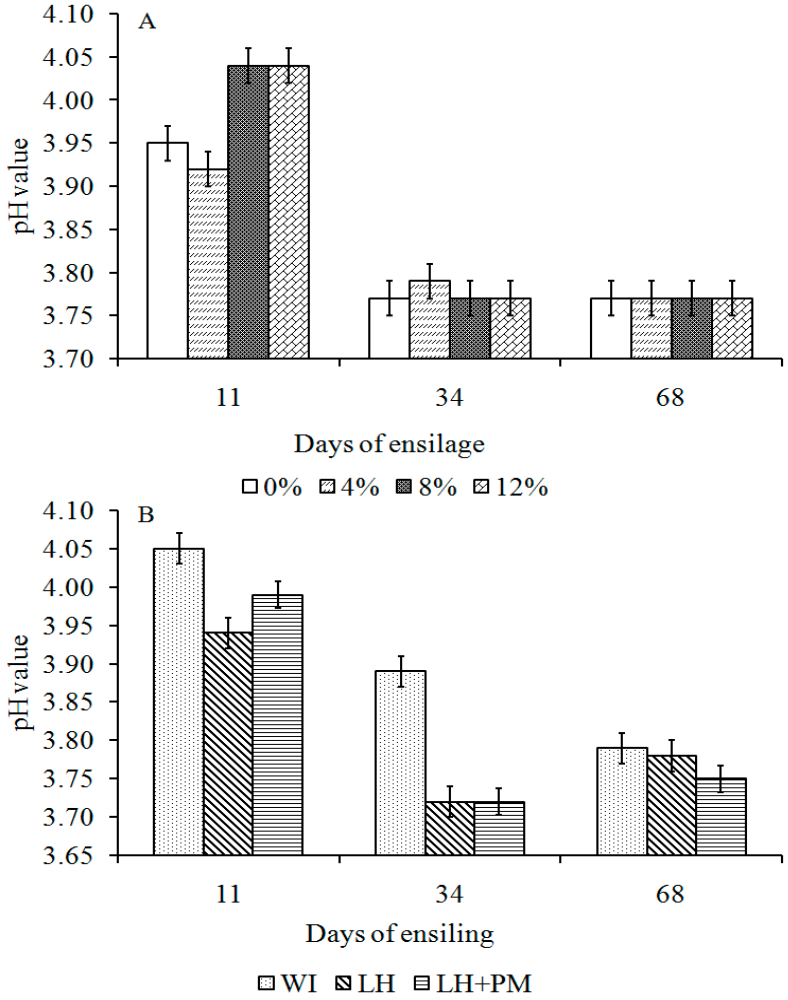


Figure 4 pH values of sugar cane silage treated with 0, 4, 8 or 12% (fresh matter) glycerin ensiled for 11, 34 and 68 days (A) and pH values without microbial inoculums, with *Lactobacillus hilgardii* UFLA SIL52 (LH) or with *Lactobacillus hilgardii* UFLA SIL52 plus *Pichia methanolica* NCYC 1381 (LH+PM) ensiled for 11, 34 and 68 days (B). The bars represent the standard error of the means.

The butyric acid concentration was below the detection limit of the technique employed (0.04g $kg^{-1}$). The amounts of citric, malic, lactic, acetic and

propionic acid, 1,2-propanediol and ethanol were reduced with the increase in glycerin dosage (cubic effect) (Table 3). The increase in glycerin dosage results in a linear reduction in methanol concentration in silage (Table 3). The glycerol concentration increased with the increase in glycerin dosage (cubic effect). The LH treatment reduced the glycerol concentration but when PM was added together with LH, the glycerol concentration was the same as the WI treatment (Table 3).

The succinic acid concentration in silage was not modified by the glycerin treatment. The LH treatment increased in 54.1% the concentration of this acid in sugar cane silage (Table 3). The LH treatment increased the concentration of acetic and propionic acids, and 1,2-propanediol, and reduced the lactic acid concentration in silage (Table 3).

**Table 3** Means of main effect and *P* value of the influence of glycerin dosage, microbial inoculums and day of ensilage and its interactions on fermentative parameters and in temperature evaluation in sugar cane silage.

| Glicerin dosage (% FM[1]) | Citric acid | Malic acid | Succinic acid | Lactic acid | Acetic acid | Propionic acid | 1,2-propanediol | Methanol | Ethanol | Glycerol | A.S.[6] | Max. temp.[7] | Time to reach max. temp. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | % dry matter | | | | | | | | | | hours | ºC | hours |
| 0 | 0.25 | 0.97 | 0.84 | 4.92 | 3.24 | 1.73 | 2.34 | 1.18 | 5.33 | 3.33 | 13.9 | 44.1 | 28.3 |
| 4 | 0.17 | 0.36 | 0.74 | 3.36 | 2.56 | 0.74 | 2.30 | 0.64 | 2.71 | 15.46 | 16.2 | 44.4 | 41.1 |
| 8 | 0.12 | 0.17 | 0.68 | 2.70 | 2.05 | 0.39 | 1.59 | 0.55 | 1.91 | 24.28 | 17.5 | 42.3 | 56.7 |
| 12 | 0.07 | 0.14 | 0.65 | 2.34 | 1.68 | 0.28 | 0.97 | 0.92 | 2.19 | 32.12 | 33.8 | 37.2 | 75.3 |
| SEM[2] | 0.028 | 0.054 | 0.075 | 0.239 | 0.243 | 0.001 | 0.249 | 0.136 | 0.313 | 1.405 | 2.28 | 0.55 | 10.86 |
| Microbial inoculum | | | | | | | | | | | | | |
| WI[3] | 0.12 | 0.38 | 0.39 | 4.46 | 1.67 | 0.59 | 0.63 | 0.81 | 3.34 | 20.14 | 16.3 | 44.5 | 29.9 |
| LH[4] | 0.15 | 0.41 | 0.85 | 2.67 | 2.56 | 0.84 | 2.19 | 0.88 | 2.71 | 16.11 | 18.7 | 41.4 | 46.0 |
| LH+PM[5] | 0.19 | 0.45 | 0.94 | 2.85 | 2.91 | 0.93 | 2.57 | 0.77 | 3.06 | 20.14 | 26.0 | 40.0 | 75.1 |
| SEM | 0.025 | 0.047 | 0.065 | 0.207 | 0.210 | 0.096 | 0.216 | 0.118 | 0.27 | 1.22 | 1.97 | 0.47 | 9.4 |
| *P* value | | | | | | | | | | | | | |
| Glycerin dosage (GLY) | <0.01 | <0.01 | 0.33 | <0.01 | <0.01 | <0.01 | <0.01 | 0.01 | <0.01 | <0.01 | <0.01 | <0.01 | 0.04 |
| Microbial inoculum (INO) | 0.17 | 0.55 | <0.01 | <0.01 | <0.01 | 0.05 | <0.01 | 0.80 | 0.29 | 0.04 | <0.01 | <0.01 | 0.01 |
| GLY x INO | 0.45 | 0.77 | 0.75 | 0.40 | 0.14 | 0.92 | 0.26 | 0.74 | 0.07 | 0.21 | <0.01 | <0.01 | 0.01 |
| Contrasts for glycerin dosage | | | | | | | | | | | | | |
| Linear | 0.03 | <0.01 | 0.28 | <0.01 | 0.02 | <0.01 | 0.40 | <0.01 | <0.01 | <0.01 | 0.52 | 0.45 | 0.33 |
| Quadratic | 0.03 | <0.01 | 0.39 | <0.01 | 0.04 | <0.01 | 0.19 | 0.55 | <0.01 | <0.01 | <0.01 | <0.01 | 0.18 |
| Cubic | <0.01 | <0.01 | 0.22 | <0.01 | <0.01 | <0.01 | <0.01 | 0.73 | <0.01 | <0.01 | <0.01 | <0.01 | 0.02 |
| Contrasts for microbial inoculum | | | | | | | | | | | | | |
| WI *vs* LH | 0.37 | 0.68 | <0.01 | <0.01 | <0.01 | 0.08 | <0.01 | 0.65 | 0.12 | 0.03 | 0.42 | <0.01 | 0.26 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LH *vs* LH + PM | 0.31 | 0.51 | 0.34 | 0.55 | 0.25 | 0.51 | 0.23 | 0.52 | 0.38 | 0.03 | 0.02 | 0.04 | 0.05 |

[1] FM - fresh matter,
[2] SEM - standard error of means,
[3]WI - without microbial inoculum,
[4] LH - *Lactobacillus hilgardii* UFLA SIL52,
[5] PM - *Pichia methanolica* NCYC 1381.
[6] AS - Aerobic Stability
[7] Max. temp. - Maximum temperaure

***Population of LAB and yeasts and diversity of eukaryotes and prokaryotes***

There was no growth of filamentous fungi after the fermentation process. The LAB population was altered by interaction between days of ensilage and microbial inoculum ($P$ = 0.03). During the ensilage period assessed, the LAB population increased (Figure 5). At 11 days of ensilage, the population of this microorganism was the same between microbial inoculums treatment and WI treatment (Figure 5). At 34 days of ensilage, the highest LAB population were in the treatment with microbial inoculums. At 68 days of ensilage, the LH treatment showed the smallest LAB number (Figure 5).

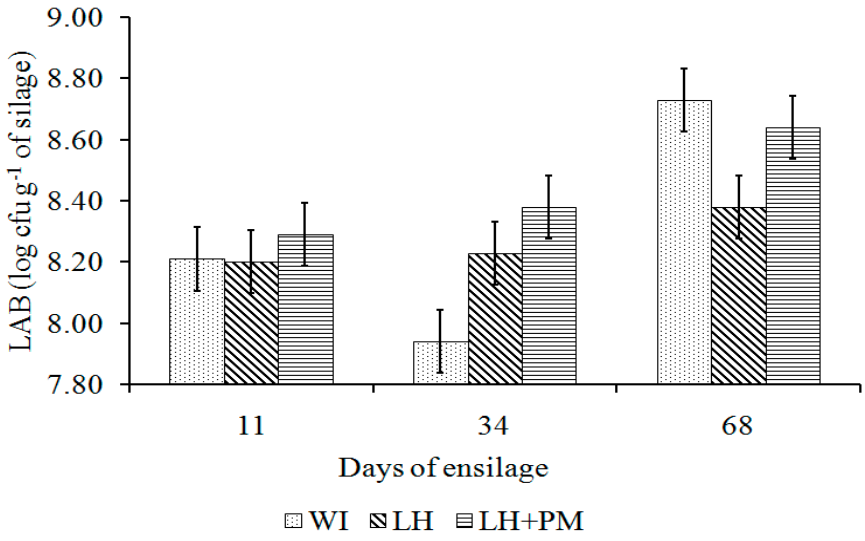


Figure 5 Lactic acid bacteria (LAB) population without microbial inoculums, with *Lactobacillus hilgardii* UFLA SIL52 (LH) or with *Lactobacillus hilgardii* UFLA SIL52 plus *Pichia methanolica* NCYC 1381 (LH+PM) ensiled for 11, 34 and 68 days. The bars represent the standard error of the means.

The interaction between glycerin dosage and days of ensilage ($P = 0.03$) and the interaction between microbial inoculum and days of ensilage ($P < 0.01$) modified the yeasts population in sugar cane silage (Table 2). The yeasts number reduced from the evaluation at 34 days of ensilage. At 68 days, this reduction was more intense in silages treated with 12% glycerin (Figure 6A). The LH treatment reduced the yeasts population and this reduction was more intense with 68 days of ensilage (Figure 6B).

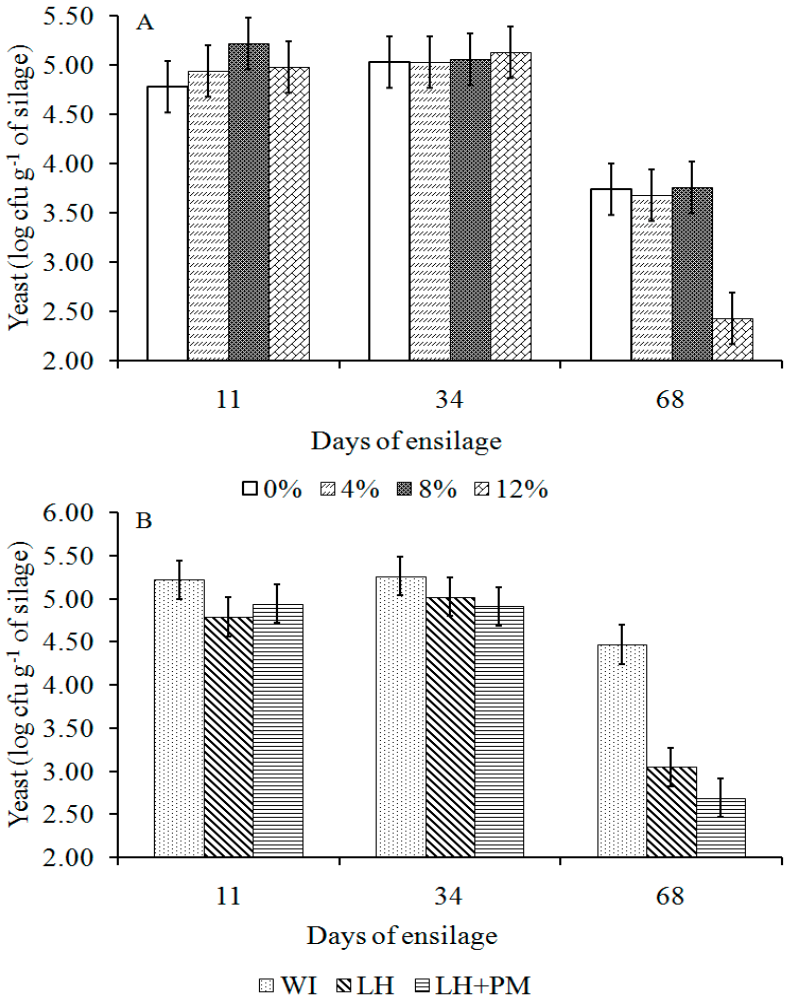


Figure 6 Yeasts population in sugar cane silage treated with 0, 4, 8 or 12% (fresh matter) glycerin ensiled for 11, 34 and 68 days (A) and yeast population without microbial inoculums, with *Lactobacillus hilgardii* UFLA SIL52 (LH) or with *Lactobacillus hilgardii* UFLA SIL52 plus *Pichia methanolica* NCYC 1381 (LH+PM) ensiled for 11, 34 and 68 days (B). The bars represent the standard error of the means.

By PCR-DGGE analysis, the community of prokaryotes and eukaryotes changed after silage fermentation (Figures 7 and 8). It can be observed that the inoculated *L. hilgardii* UFLA SIL52 strain persists during all ensilage process (bands 1, 2, 13, 15, 22, 24). The diversity of prokaryotes was slightly modified by treatment with glycerin and LH, but it was not modified by treatment with PM (Figure 7).

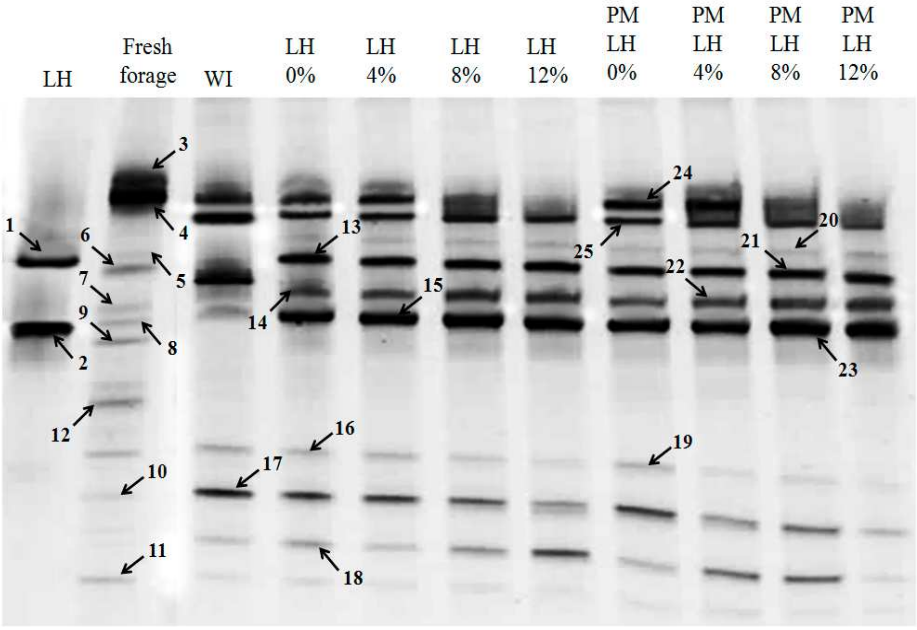


Figure 7 Prokaryote community in sugar cane treated with 0, 4, 8 and 12% glycerin and with microbial inoculums after 68 days of ensilage. Abbreviations: LH – *Lactobacillus hilgardii* UFLA SIL52, WI – without microbial inoculums, PM – *Pichia methanolica* NCYC1381. Bands: 1,2, 13, 15, 21, 23- *L. hilgardii*, 7- *Erwinia* sp., 3- *Weissella* sp., 4,24- *Weissella cibaria*, 5, 6, 8, 10, 11, 12- Uncultured bacterium, 9- *Enterobacter* sp., 14, 22, 20- *Lactobacillus diolivorans*, 16, 19- *Acetobacter pasteurianus*, 17- *A. lovaniensis*, 18- *Gluconacetobacter* sp., 25- *Lactobacillus* sp.

Many uncultured bacteria and contaminants species such as *Erwinia* sp. and *Enterobacter* sp. were observed before ensilage. *Weissela* was the only lactic acid bacteria genera found in sugar cane before and after fermentation. However, this species was not observed in silage treated with 8 or 12% glycerin (band 25, Figure 7). After ensilage, the most predominant species was *L. diolivorans* and *L. hilgardii*. Species of acetic acid bacteria *Acetobacter pasteurianus, A. lovaniensis* and *Gluconacetobacter* sp. were also observed in silages. The inoculated *P. methanolica* was not identified in the assessment of the diversity of eukaryotes (Figure 8), suggesting no establishment of this strain during fermentation. The yeasts species identified were *Candida humilis, Hanseniaspora osmophila, Hanseniaspora vineae* and *Issatchenkia orientalis* (Figure 8). By the intensity of the band, the species *C. humilis* and *I. orientalis* were in lower population in treatment with 12% glycerin compared to the other glycerin dosages. No alteration was observed in eukaryotic diversity caused by LH treatment. The *Glomus intraradices* and *Tremella globispora* fungi were identified in sugar cane just before ensilage (Figure 8).

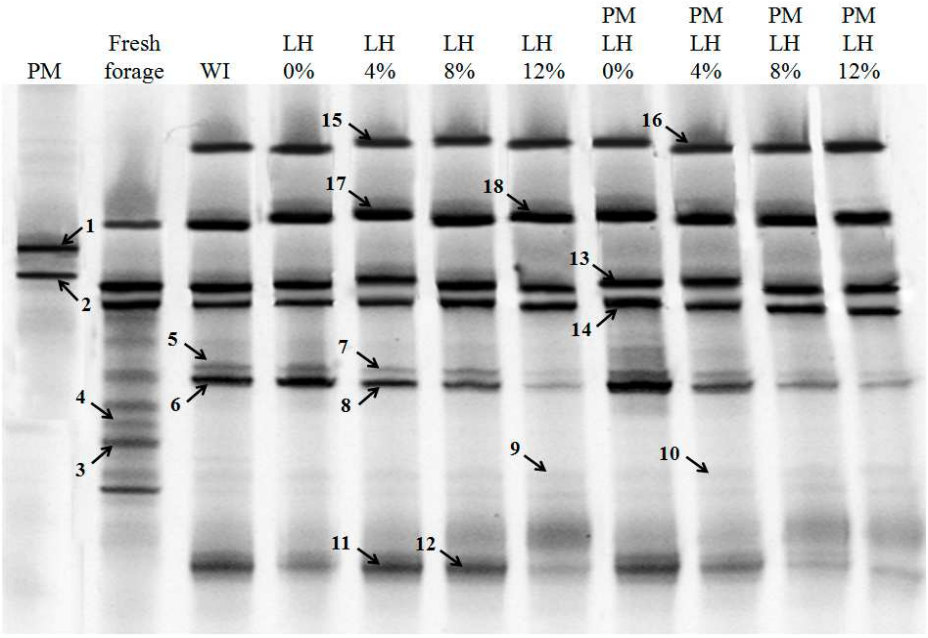


Figure 8 Eukaryote community in sugar cane treated with 0, 4, 8 and 12% glycerin and with microbial inoculums after 68 days of ensilage. Abbreviations: LH – *Lactobacillus hilgardii* UFLA SIL52, WI – without microbial inoculums, PM – *Pichia methanolica* NCYC1381. Bands: 1,2- *P. methanolica*, 3- *Tremella globispora*, 4- *Glomus intraradices*, 5, 6, 7, 8- *Candida humilis*, 9,10, 15,16- *Hanseniaspora osmophila*, 11, 12- *Issatchenkia orientalis*, 13, 14- *Torulaspora* sp., 17,18- *Hanseniaspora vineae*.

***Aerobic Stability***

An interaction was observed between treatments with glycerin and microbial inoculum ($P < 0.01$), over evaluated temperature (Table 3). Treatment with 12% glycerin increased the aerobic stability of the silage when applied together with PM treatment (Figure 9A).

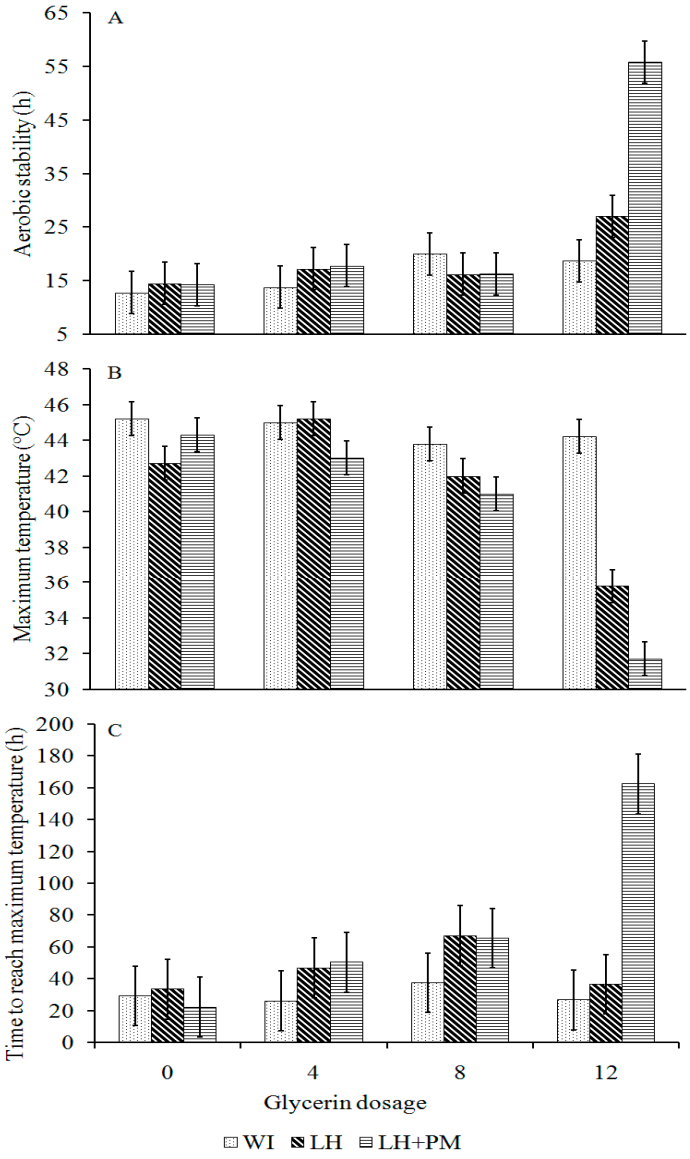


Figure 9 Aerobic stability (A), maximum temperature (B) and time to reach the maximum temperature (C) in sugar cane silage treated without microbial inoculums, with *Lactobacillus hilgardii* UFLA SIL52 (LH) or with *Lactobacillus hilgardii* UFLA SIL52 plus *Pichia methanolica* NCYC 1381 (LH+PM) combined with 0, 4, 8 or 12% (fresh matter) glycerin. The bars represent the standard error of the means.

In the WI treatment, the application of the LH treatment reduced the maximum temperature of silage (Figure 10B). In the treatment with 4% glycerin, the addition of LH+PM treatment reduced the maximum temperature of silage. In the silage with 8 or 12% glycerin, the addition of LH treatment reduced the maximum temperature of silage. This reduction was more pronounced when the PM treatment was added (Figure 9B).

In the treatment WI or with 4 and 8% glycerin, there were no differences between the applications of microbial inoculums at the time to reach the maximum temperature (Figure 9C). In silages treated with 12% glycerin the application of PM treatment increased the time to reach maximum temperature (Figure 10C).

## DISCUSSION

The chemical and microbiological characteristics of sugar cane prior to ensilage (Table 1) are within the values observed in the literature for this forage (Pedroso et al., 2005; Carvalho et al. unpublished data). The effects of glycerin addition in sugar cane about NDF, WSC, citric, malic and propionic acids concentration prior to ensilage are dilution effects caused by this additive.

The increase in DM concentration caused by glycerin addition (Table 1 and 2) was previously observed (Carvalho et al. unpublished data) and is due to increase in DM caused by the additive application. The DM concentration

reduction observed during ensilage only in the treatment without glycerin (Figure 1A) can be an indication that the added glycerin was not consumed by microorganisms during ensilage resulting in higher DM concentration in the treatments in which the glycerin was added. The LH treatment increased the DM concentration in the silage without glycerin (Figure 1B). This result can be explained by changes in the fermentation.

High DM loss is a major problem associated with sugar cane ensilage. In this study, the treatment with either 4 or 8% glycerin, and the LH treatment were able to reduce these losses (Figures 2A and 2B). The higher initial percentage of loss in the glycerin treatments (Figure 2A) is probably caused by a high effluent volume in the beginning of fermentation. In the other assessments, during the ensilage this loss is compensated by increase in DM caused by glycerin addition. As can be noted in Figure 2B, the LH treatment reduced the loss when applied with or without glycerin. In another study, evaluating different LAB strains, Carvalho et al. (unpublished data) observes reduction of 28% in the DM loss when the *L. hilgardii* UFLA SIL52 strain was added to sugar cane silage. Reduction in the DM loss resulted by the addition of this strain (*L. hilgardii* UFLA SIL52) together with 10% glycerin (% of FM) was observed in the sugar cane silage by Carvalho et al. (unpublished data). However, these authors did not observe reduction in loss when glycerin was added without the microbial inoculum. Data from this experiment and of Carvalho et al. (unpublished data)

suggest that application of doses higher than 8% of glycerin does not reduce the DM loss of sugar cane silage. As previously noted, this fact is probably due to the higher effluent volume observed when high doses of glycerin are added to silage.

The increase in concentration of fibrous components during sugar cane ensilage observed in this study (Figure 3) has been previously observed (Pedroso et al., 2005), and it is due to the concentration of this fraction after the silage consumption of fermentable components. The reduction in NDF caused by glycerin inclusion was probably due to a dilution effect (Figure 3A). As discussed for the DM concentration, the effect of glycerin addition during fermentation about the smallest increase in fiber fraction of the silage is indicative that glycerol was not consumed during fermentation of silage from sugar cane. The increase in NDF in the silage that received the LH treatment observed only after 34 days of ensilage (Figure 3B) may be due to lower initial intake of carbohydrates when this strain was added to the silage. Although in this study this fate has not been statistically significant (Table 2), Carvalho et al. (unpublished data) observed lower WSC consumption until 30 days of ensilage when this strain was added to sugar cane silage. The reduction in the WSC concentration observed (Table 2) is expected during the ensilage. The WSC is an energy source for the growth of microorganisms that act on the forage fermentation. The reduction of WSC is higher in the peak of fermentation when

microbial metabolic activity is more intense (Pedroso et al., 2005; Ávila et al., 2009).

At 11 days of ensilage, the pH values of the silages treated with 8 and 12% glycerin were slightly higher than the pH values observed in the silage treated with 4% glycerin or in the WI treatment (Figure 4A). This result can be associated to the high pH of this additive (6.5 – 7.5), or a possible buffer effect. The reduction in pH caused by the addition of LH treatment was not expected, since heterofermentative strains such as *L. hilgardii* UFLA SIL52 produce, besides lactic acid, high amounts of acetic acid (Axelsson, 2004) which was not as efficient in lowering the pH. In this study, the LH treatment resulted in silages with less lactic acid concentration but, with larger succinic, acetic and propinic acids concentration which may explain the lower pH observed (Table 3).

The fermentation products found in high concentrations in silages are lactic, acetic, propionic and butyric acids and ethanol (Kalac et al., 2011) and so are the major chemical compounds assessed in studies with silages. However, other compounds are also involved in the fermentation of forage plants, but are found in small proportions and so are rarely evaluated. The study of these compounds, found in small proportions, is important since the use of additives can alter their concentration in the silage, as observed in this study and by Carvalho et al. (unpublished data). Changes in the concentration of these compounds can explain the best or worst performance of an additive.

The reduction in the concentration of citric, malic, lactic, acetic and propionic acids and 1,2-propanediol, methanol and ethanol caused by increase in glycerol dosage (Table 3) is probably due to a dilution effect resulting from the application of this additive. Unlike observed in this study, Garai-Ibabe et al. (2008) and Pasteris and Strasser de Saad (2009) observed an increase in the acetic and lactic acids concentration when glycerol was added to the culture medium of *L. collinoides* and *L. hilgardii* $X_1B$.

Citric and malic acids are intermediate metabolites in the tricarboxylic acid cycle of living cells. The citric acid concentration did not increase with ensilage (Tables 1 and 3) indicating that this metabolite was not produced during ensilage. The malic acid concentration increased after ensilage only in silage without glycerin (Tables 1 and 3) this acid can be produced by microbial fermentation (Zelle et al, 2008). The glycerin treatment modified the microbial diversity in the silage (Figures 7 and 8) and can also have modified the metabolism of this microorganisms changing the production of these acids.

The increase of 53% in the succinic acid concentration and the reduction of 40% in the lactic acid concentration caused by the LH treatment was probably a result of the characteristic metabolism of this strain. Kaneuchi et al. (1988) observed that 35% of the LAB strains tested were able to produce succinic acid. However, these authors observed that the *L. hilgardii* JCM 1155 strain did not produce this acid. Carvalho et al. (unpublished data) observed that inoculation

of sugar cane silages with *L. hilgardii* UFLA SIL52 resulted in lower lactic acid concentrations and higher acetic acid concentration when compared to the silage without microbial inoculum or silage inoculated with *L. plantarum* strains. The *L. hilgardii* strains have an obligatory heterofermentative metabolism, that is, they use the pentose phosphate pathway and, thus, proportionally produce smaller quantities of lactic acid than *L. plantarum* (Axelsson, 2004). Some heterofermentative LAB and other facultative heterofermentative strains do not express or express in a constitutive way acetaldehyde dehydrogenase, which is one of enzymes responsible for the reduction of acetyl-CoA into ethanol. Thus, the production of ethanol is practically null in this group of LAB (Oude Elferink et al. 2001) and, consequently, there is an increase in the concentration of acetic acid as a final fermentation product. The inoculated strain probably follows this pattern, where the acetaldehyde dehydrogenase is little or not produced.

The propionic acid concentration in sugar cane silage is very variable depending on the type of additive used, and the conditions and the period of ensilage (Ávila et al., 2009; Carvalho et al., 2012; Carvalho et al., unpublished data). In the case of silages not inoculated with *Propionibacterium*, the presence of propionic acid may also be associated with the epiphytic presence of *L. diolivorans*, which is able to use 1,2-propanediol to produce propionic acid (Krooneman et al. 2002). The inoculated *L. hilgardii* UFLA SIL52 increased the 1,2-propanediol concentration in silage and the *L. diolivorans* species was

observed in the diversity evaluation (Figure 7) which explains the higher concentration of propionic acid in the LH treatment. Heinl et al. (2012) verified that the metabolism of both *L. hilgardii* and *L. buchneri* are similar and possess the ability to degrade lactic acid and form acetic acid and 1,2-propanediol in anaerobic conditions (Oude Elferink et al. 2001).

The inoculation with the *P. methanolica* NCYC 1381 strain did not reduce the methanol concentration in the silage (Table 3). Three possible explanations are i) no adaptation of the strain under the ensilage conditions resulting in a slow growth (Figure 8), ii) short period of ensilage (68d), and iii) low methanol concentration in silage. The application of methylotrophic yeasts are linked to the use of strong methanol-inducible promoters derived from genes of the methanol utilization pathway. These promoters are tightly regulated, highly repressed in the presence of non-limiting concentrations of glucose in the medium and strongly induced if methanol is used as carbon source (Hartner and Glieder, 2006).

During sugar cane fermentation, an increase in methanol concentration (Tables 1 and 3) was observed. Methanol is formed from the enzymatic hydrolysis of the methoxy groups of pectin, and its content depends on the extent that the forage is macerated to (Peinado et al., 2004). Data on low-molecular weight alcohols in silage, except for ethanol is limited. The methanol average concentrations in maize silage with dry matter below 250 g $kg^{-1}$ were

0.3 g $kg^{-1}$ DM. However, wide variations occurred (Kalac, 2011). In alfalfa (*Medicago sativa*) silage, the methanol concentration could vary from 2.1 to 3.1 g $kg^{-1}$ DM (Robinson et al, 1988). These values of corn and alfalfa silages are smaller than those observed in sugar cane silages evaluated in this study (average 0.8%). The methanol concentration in sugar cane silage is well above the allowed values for the concentration of methanol in crude glycerin in this country. According to the MAPA (Brazil 2010), glycerin for animal feeding should contain a maximum of 0.015% of methanol in Brazil. The increase in the methanol concentration resulting from the increase in the dosage of glycerin was due to the presence of methanol in this additive.

The glycerol concentration increased 83.8 and 10.2% in the silage without glycerin and in the silage treated with 4% glycerin after ensilage, respectively. In the silage treated with 8 and 12% glycerin, glycerol concentration after ensilage was reduced to 1.3 and 14.6%, respectively, possibly due to effluent loss (Tables 1 and 3). The glycerol produced by yeasts justifies the increase in the concentration of this compound in the silage inoculated with the *P. methanolica* NCYC 1381 strain. Yeasts produce glycerol during alcoholic fermentation (Smidt et al., 2012) and also osmotic changes induced the significant storage of glycerol in yeasts (Sánchez-Fresneda et al., 2013). The reduction in the yeast number observed in the treatment with *L. hilgardii* UFLA SIL52 strain may be responsible for the decrease in glycerol

concentration in the silage in which this strain was inoculated. The energy value of crude glycerin is proportional to the level of glycerol. Considering the crude energy of glycerol as 4320 kcal/kg (Lammers et al., 2008), the silages treated with 4, 8 and 12% glycerin had 78.5, 86.3 and 89.6% more energy (from glycerol) than the control, respectively.

The filamentous fungi population was below the limit of detection (< log 2). This result is commonly found in sugar cane silages (Ávila et al., 2009; Carvalho et al., unpublished data). The inhibition of fungal growth can occur due to low oxygen level and high concentrations of acetic acid or through the actions of bacteriocins produced by LAB present in the silages (Strom et al., 2002).

The results of the application of the LH treatment on the reduction in LAB population after 68 days of ensilage (Figure 5) were also observed by Carvalho et al. (unpublished data). The smaller LAB population observed in the LH treatment in assessment with 68 days of ensilage can be resulted from production of inhibitory compounds (bacteriocins). Associations of yeasts and LAB are often encountered in the production of beverages and fermented foods. Interaction between yeasts and *Lactobacillus* in silages has not been previously described. This interaction may be the response towards increase in LAB population caused by PM treatment (Figure 5). Carvalho et al. (unpublished data) observed an increase in the yeast population when *L. plantarum* wild

strains were inoculated in sugar cane silage. The lack of competition for the main carbon source appears to be one of the prerequisites for the stability of LAB/yeast associations in food fermentations, and the lysis of dead microbial cells also guarantees a supply of amino acids (Gobbetti, 1998).

After 68 days of ensilage, the reduction in yeast population was more significant in the treatment with 12% glycerin (Figure 6A). There are no reports on the toxicity of glycerol to yeasts. However, the metabolism of glycerol by LAB results in the production of 3-hydroxypropionaldehyde (3-HPA), which is an antimicrobial compound known as reuterin (Pasteris and Strasser de Saad, 2009; Tanaka et al., 2009). According to eukaryotic diversity analysis (Figure 8), the likely inhibitory effect of these compounds acted selectively with respect to yeast species, since a small population of *Candida humilis* and *Issatchenkia orientalis* were observed in 12% glycerin treatment (Figure 8).The reduction in the yeast population by LH treatment was previously observed (Carvalho et al. unpublished data). The more intense reduction at the 68 days evaluation can be a result of the accumulation of acid and other inhibitory compounds.

The increased glycerin concentrations and the use of microbial inoculum were favorable to enhance the aerobic stability, to reduce the maximum temperature, and to prolong the time to reach the maximum temperature during the aerobic silage exposition. In other studies the inoculation of sugar cane silage with the *L. hilgardii* UFLA SIL52 strain resulted in an increase in aerobic

stability (Carvalho et al., upublished data). The authors associated this result to high 1,2-propanediol and acetic acid concentrations (Carvalho et al., unpublished data; Nishino et al., 2003). The effects of *Pichia methanolica* NCYC 1381 inoculation were mainly observed after opening the silos. It can be associated with a higher growth of this strain with the greater oxygen availability.

## CONCLUSION

The sugar cane silage quality decreases with the increase in the fermentation time. The treatment of sugar cane with 4% glycerin and *L. hilgardii* UFLA SIL52 (6.1 log cfu $g^{-1}$ of FM) was able to improve the silage quality by reducing fiber and ethanol concentration, providing high glycerol availability and consequently more energy at the end of fermentation. The addition of doses higher than 4% glycerin increased the percentage loss. Glycerin and *L. hilgardii* UFLA SIL52 also increased the aerobic stability of the silage. Therefore, they are indicated as additives in sugar cane ensilage. New assessments using glycerin with higher methanol concentrations, and inoculation with a higher population, are needed to ensure the capacity of *P. methanolica* NCYC 1381 in reducing the concentration of this compound in the silage. The results of this study provide information about the influence of purified glycerin plus methanol in the ensilage process of sugar cane. In this study, we used the purified glycerin

to prevent the probable effects of other chemical contaminants except methanol contained in the residual glycerin from biodiesel production. Further studies should be conducted using crude glycerin to confirm the positive effects of the addition of glycerin in sugar cane silage.

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