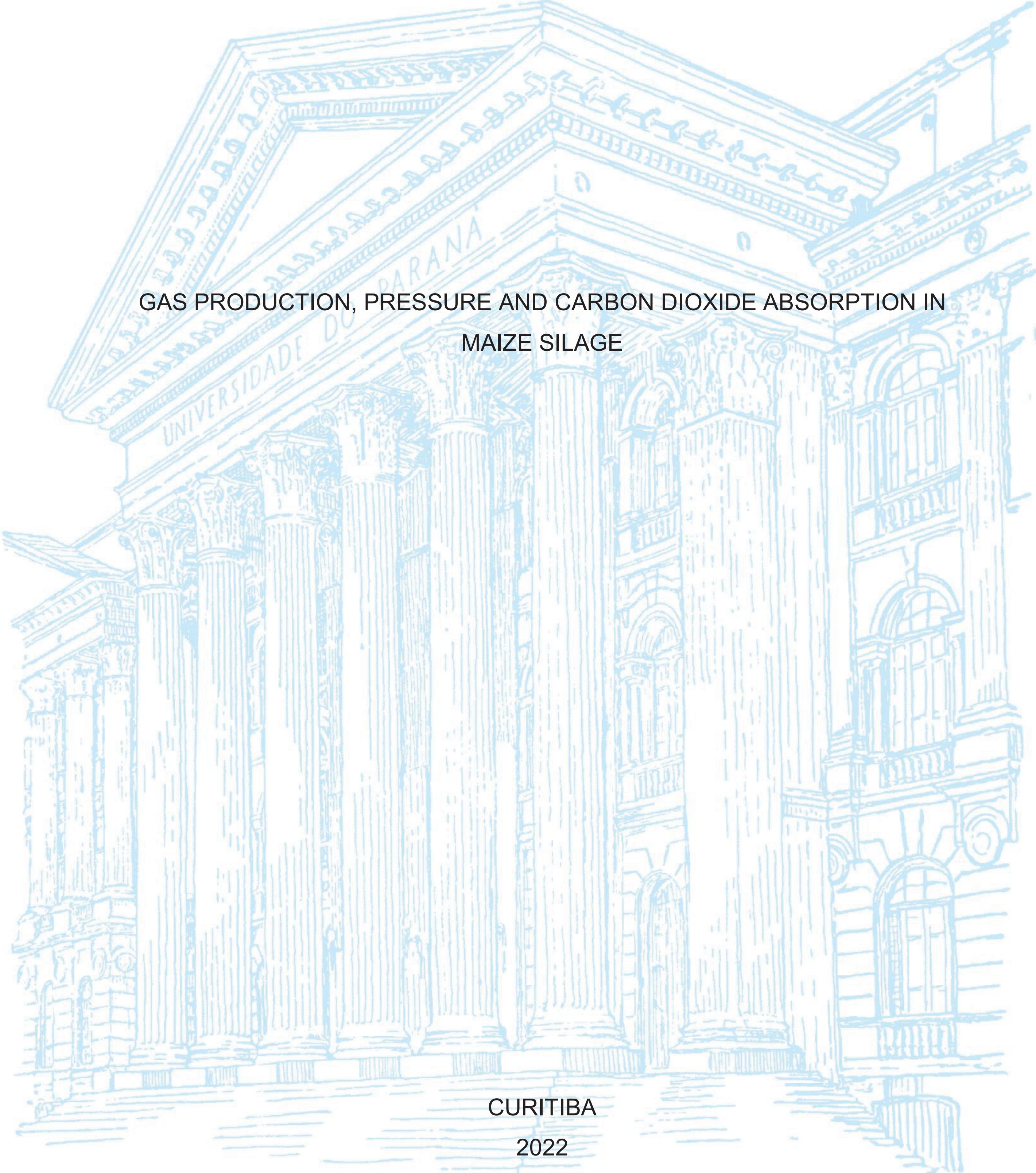
UNIVERSIDADE FEDERAL DO PARANÁ

GABRIELA LETÍCIA DELAI VIGNE

GAS PRODUCTION, PRESSURE AND CARBON DIOXIDE ABSORPTION IN MAIZE SILAGE



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GABRIELA LETÍCIA DELAI VIGNE

GAS PRODUCTION, PRESSURE AND CARBON DIOXIDE ABSORPTION IN MAIZE SILAGE

Tese apresentada ao Programa de Pós-Graduação em Zootecnia, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Zootecnia.

Orientador: Prof. Dr. Patrick Schmidt

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ZOOTECNIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **GABRIELA LETÍCIA DELAI VIGNE** intitulada: **Gas production, pressure and carbon dioxide absorption in maize silage**, sob orientação do Prof. Dr. PATRICK SCHMIDT, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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À minha família, meu noivo, amigos e colegas do CPFOR,

dedico.

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“Não há céu sem tempestades, nem caminhos sem acidentes;
Só é digno do pódio, quem usa as derrotas para alcançá-lo;
Só é digno da sabedoria, quem usa as lágrimas para irrigá-la;
Os frágeis usam a força; os fortes a inteligência.”
(Augusto Cury)

RESUMO

Uma diversidade de microrganismos está associada à ensilagem. Existe uma enorme diversidade de interações entre enzimas vegetais e atividades microbianas. Estudos recentes detectaram surpreendentemente uma nova fase de pressão negativa dentro dos silos e o aumento do teor de matéria seca após a fermentação da silagem. Essas observações justificam a realização deste estudo e afirmam a possibilidade de vias de fixação de CO_2 ocorrerem em silagens. Este ensaio teve como objetivo avaliar a produção de gás, a pressão no interior dos silos e absorção de carbono ao suplementar os silos com CO_2.

Palavras-chave: Dióxido de carbono, CO_2, Fixação de CO_2, Microbioma

ABSTRACT

A diversity of microorganisms is associated with ensilage. There is a huge diversity interaction among plant enzymes and microbial activities. Recent studies have surprisingly detected a new phase of negative pressure inside the silos and the increase in dry matter content after silage fermentation. These observations justify the conduction of this study and affirm the possibility of CO_2-fixing pathways in silages. This trial aimed to evaluate the gas production, the pressure inside the silos and the carbon absorption when supplementing the silos with CO_2.

Keywords: Carbon dioxide, CO_2, CO_2 fixation, Microbiome

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ABBREVIATIONS LIST

H_2O - Water
°C - Degrees Celsius
μl - Microliter
AAC - Acetic acid bacteria
absCO_2 - CO_2 absorption
AC - Acetic acid
ADF - Acid detergent fiber
ART-ANOVA- Aligned Rank Transform Analysis of Variance
ARTOOL - R Package for the Aligned Rank Transform for Nonparametric Factorial ANOVAs
AS - Aerobic stability
AT - Accumulated temperature
ATP - Adenosine triphosphate
C - Carbon
$C_6H_{12}O_6$ - Glucose
CFU - Colony-forming units
CH_3COOH - Acetic acid
CH_4 - Methane
CHAO - Nonparametric method for estimating the number of species in a community
CO - Carbon monoxide
CO_2 - Carbon dioxide
CODH - Carbon monoxide dehydrogenase
CP - Crude protein
CPFOR - Forage Research Center
CRD - Completely randomized design
DM - Dry matter
DMLas - Dry matter loss throughout aerobic stability test
EAC - Estimated acetic acid
Ech - Energy-converting hydrogenases
EE - Ether extract
e. g. - “Exempli gratia”, for example

Expdes.pt	- Experimental Designs Package for analysis of experimental designs in a double factorial scheme
Fat 2.dic	- Command for R Studio to analyze double factorial designs in a balanced completely randomized design
g	- Gram
g/kg	- Grams per kilogram
GHG	- Greenhouse gas
GP	- Gas production
H^+	- Ion hydrogen
H_2	- Hydrogen
H_2O	- Water
HDCR	- Hydrogen-dependent CO_2 reductase
HPLC/RI	- High performance liquid chromatography RI Knauer SmartLine 2300
HS^-	- Ion hydrosulfite
IAA	- Indole-3-acetic acid
IP	- Internal pressure
kg	- Kilogram
kg/m^3	- kilogram per cubic meter
KJ	- Kilojoule
kPa	- Kilopascal
L	- Liter
L/kg	- Liters per kilogram
L/ton	- Liters per ton
LAB	- Lactic acid bacteria
Log$_{10}$	- Base -10 logarithm
mL	- milliliter
mmHg	- Millimeters of mercury
mmWC	- millimeters of water column
mmWC/kg	- millimeters of water column per kilogram
MT	- Maximum temperature
N	- Nitrogen
Na^+	- Ion Sodium
NADH	- Nicotinamide adenine dinucleotide hydrogen coenzyme

NADP+ - Oxidized form of nicotinamide adenine dinucleotide phosphate coenzime

NAPH - Nicotinamide adenine dinucleotide phosphate coenzyme

NCBI - National Center for Biotechnology Information

NDF - Neutral detergent fiber

Nfn - NADH-dependent reduced ferredoxin: NADP+ oxidoreductase

ng - Nanogram

NO_2 - Nitrogen dioxide

OTUs - Operational taxonomic units

P - Phosphorous

PAB - Propionic acid bacteria

PAST - Paleontological Statistics (free software for scientific data analysis for ecological analysis)

PCA - Principal component analysis

PCoA - Principal coordinate Analysis

pH - Potential of Hydrogen

ppbv - Parts per billion by volume

ppmv - Parts per million by volume

PyNAST - Python Nearest Alignment Space Termination

QUIIME - Next-generation microbiome bioinformatics platform for performing microbiome analysis from raw DNA sequencing data

Rnf - Designation stems from *Rhodobacter* nitrogen fixation

RT qPCR - Real time quantitative polymerase chain reaction

SIMPER - Similarity percentage analyses

TCA - Tricarboxylic Acid Cycle

THF - Tetrahydrofolate

TMAX - Maximum temperature

ton - Tone

UCHIME - A chimera detection method to improve speed and accuracy of chimera detection

UCLUST - Algorithm for clustering unstructured data

UNIFRAC - Online tool for comparing microbial community diversity in a phylogenetic context

VOC - Volatile organic compounds

WLP - Wood–Ljungdahl pathway
WSC - Water soluble carbohydrates
HCO_3^- - Ion bicarbonate
SO_4^{-2} - Ion sulfate

SYMBOLS LIST

$\Delta G'^{\circ}$ - Gibbs energy change and equilibrium (standard free energy change)

SUMMARY

1 INTRODUCTION

Silage is currently the most used technique for conserving ruminant feed (ÁVILA; CARVALHO, 2019). Currently, 665 million tons of silage are globally consumed each year, and whole-plant corn silage accounts for more than 40% of forage in dairy cattle farms (XU *et al*., 2021).

During silage production and its use, the respiration activities proceed by plants cells and microorganisms, deamination and proteolysis, aerobic deterioration and the process of feed-out phase, can lead to significant losses of dry matter (DM) (GRANT; ADESOGAN, 2018). Most of the total DM losses is represented by loss fermentation in the form of gases (SCHMIDT *et al*., 2011). An important source of environmental impact of maize silage production and use is the emission of climate relevant gases (mainly CO_2, CH_4, N_2O) and volatile organic compounds (VOC), that can form ozone through photochemical reactions (HAFNER *et al*., 2013). So, over the last years, the interest in the environmental impacts associated with silage has strongly grown.

In order, to evaluate the greenhouse gas (GHG) emission during the fermentation of sugarcane and maize silages and the potential for GHG mitigation using additives, previous trials were developed by Forage Research Center (CPFOR) (SCHMIDT *et al*., 2011; SCHMIDT *et al*., 2012; SOUZA *et al*., 2015b). From these experiments, a system was developed using a pipeline and a graduate chamber attached to the silos for collecting and measuring all gases produced during the silage fermentation phase and storage. Souza *et al*. (2015a) observed that direct methods were more efficient than indirect methods for measuring gases losses.

In the first trials using this method for evaluating the dynamic of gas production in silages, a surprisingly pattern behavior that has never seen before in silages during the fermentation process was observed. After the gas production phase, it was observed that the internal pressure of the silos remained stable for a few days, followed by a negative pressure phase inside silos, one to two weeks after sealing. The hypothesis that it would be the accommodation of the mass inside the silo caused by the vacuum was soon discarded, after injections of atmospheric air without stabilizing the pressure (SOUZA *et al*., 2015b). With the progressive increase in the negative internal pressure inside the silos, appears as hypothesis the possibility of metabolization of the supplied air by microorganisms and the production of metabolites that would incorporate silage dry matter (SCHMIDT *et al*., 2018).

In the literature it was described the anaerobic fixation of CO_2 by acetogens bacteria (FISCHER *et al*., 1932). Acetogens utilize the Wood-Ljungdahl pathway as a terminal electron-accepting, energy-conserving, CO_2-fixing process (DRAKE; GÖßNER; DANIEL, 2008). They were competent in the formation of acetate from the H_2-dependent reduction of CO_2 (FISCHER *et al*., 1932). The first acetogenic bacteria described was *Clostridium aceticum* by Wieringa (1936). The presence of these bacteria in silages was described by MÖLLER *et al*. (1984) and the bacteria isolated was *Sporomusa ovata*. However, the autotrophic Acetyl-CoA pathway of CO_2 fixation has never been described for silages.

The existence of Wood-Ljungdahl pathway, associated with the fact of CO_2 being the main gas produced during silage fermentation at very high concentrations (about 25.000 ppmv) (SCHMIDT *et al.,* 2011) and the confirmation that acetogen bacteria have already been isolated from silage, reinforced the raised hypothesis. Therefore, in the first trial with the aim to observe exclusively the internal pressure of the silos and the possibility of CO_2 absorption, silos absorbed 5590 ± 2492 mL of CO_2 during the whole trial (SCHMIDT *et al.,* 2018).

In this sense, the analysis of the microbiome was used in order to elucidate the questions about the absorption of CO_2 and to identify the microorganism population responsible for silos internal pressure and CO_2 metabolization effects.

The review chapter is intended to carry out a compilation of information related to microorganisms involved in the ensiling process; silage DM losses, mainly focused on gas DM loss; as well to update information related to the anaerobic metabolic pathways that occur from CO_2, including the Wood-Ljungdahl pathway and the microorganisms involved.

2 LITERATURE REVIEW

2.1 THE ENSILING PROCESS

The aim of making silage is to maximize the preservation of the original nutrients in the forage crop for feeding later. So, the final quality of the silage can be close to the quality of the initial forage placed in the silo. Therefore, it is necessary that the forage harvested for ensiling must be highly digestible and contains adequate amounts of fermentable carbohydrates (KUNG Jr., 2018).

The ensiling process begins with the forage crop and ends in the moment the feed is consumed by the animal (WILKINSON; DAVIES, 2013) and it is usually divided into four phases: the aerobic phase immediately after harvest, the fermentation phase, the stable storage phase, and the feed-out phase when the silo feed is exposed to oxygen (BARNETT, 1954). The four phases have different length and intensity, and it is impossible to precisely separate one from the other.

A considerable diversity of microorganisms is associate with all ensilage phases; however, some groups have been identified with greater frequency. *Firmicutes* and *Proteobacteria* are the most abundant phyla during ensiling (OGUNADE *et al*., 2018; LIU *et al*., 2019). Among these phyla are lactic acid bacteria (LAB), propionic acid bacteria (PAB), bacteria of the *Enterobacteriaceae* family, acetic acid bacteria (AAB), spore-forming bacteria (*Bacillus* and *Clostridium*) and *Listeria* (PAHLOW *et al*., 2003). In addition to bacteria, several species of molds and yeasts are also found during ensiling (McDONALD *et al*., 1991).

Silage is conserved by anaerobiosis and environmental acidity. For acidity to occur, a fermentation process is catalyzed by microorganisms (ÁVILA; CARVALHO, 2019), that converts soluble carbohydrates from raw materials into acidic products. This process creates an acidic environment and inhibits the proliferation of harmful microorganisms, thereby preserving the nutritional content of raw materials (ZHANG *et al*., 2011). The natural microbiota of the forage chopped (epiphytic microbiota) is responsible to initiate fermentation phase in the silo (DRIEHUIS; OUDE ELFERINK, 2000; ÁVILA; CARVALHO, 2019). However, the essential epiphytic microflora for spontaneous silage fermentation is lactic acid bacteria (LAB) (PAHLOW *et al*., 2003), such as *Lactobacillus, Lacticaseibacillus, Lactilactobacillus, Lactiplantibacillus, Limosilactobacillus, Secundilactobacillus, Levilactobacillus, Lentilactobacillus, Ligilactobacillus, Lactococcus, Leuconstoc, Streptococcus, Pediococcus, Enterococcus* and *Weissella* (ZHANG *et al*., 2011; GHARECHAHI *et al*. 2017; ÁVILA; CARVALHO, 2019; ZHENG *et al.,* 2020).

Due to the recent reformulation of the nomenclature of some microorganisms of the genera *Lactobacillus,* according to Zheng *et al.* (2020), (e. g., *Lactobacillus buchneri* is now called *Lentilactobacillus buchneri*) and to facilitate our discussion, because many studies still designate the genus with the old nomenclature, in this thesis the term *Lactobacillus* will be maintained to refer to all genera like before the reformulation and including those that had their nomenclature changed (*Lactobacillus, Amylolactobacillus, Holzapfelia; Bombilactobacillus, Companilactobacillus, Lapidilactobacillus, Agrilactobacillus, Schleiferilactobacillus, Lacticaseibacillus, Paralactobacillus, Latilactobacillus, Loigolactobacillus, Dellaglioa, Liquorilactobacillus, Ligilactobacillus, Lactiplantibacillus, Furfurilactobacillus, Paucilactobacillus, Limosilactobacillus, Secundilactobacillus, Levilactobacillus, Fructilactobacillus, Aceticlactobacillus, Apilactobacillus, Lentilactobacillus*, and *Lapidilactobacillus*).

2.1.1 Epiphytic Microbiota of Silage Crops

Plants interact with various taxonomic groups of microorganisms both in the phyllosphere and rhizosphere. A number of diverse bacteria and fungi are known to inhabit leaf tissue and play pivotal roles in resistance of host plants against biotic and abiotic environmental stresses, by underappreciated metabolic pathways (TOJU; KUROKAWA; KENTA, 2019). However, the reports in the literature suggested that structural and functional diversity maize microbiome depends on multiple factors: genotype, maturity stage, geographical location, environmental factors, root proximity, nutrient availability, secondary metabolites, soil properties (pH, composition, moisture, and organic matter), agricultural practices, pathogen infection, plant species, season, mowing, field-wilting, chopping process humidity, solar radiation and plant surface structure (LIN *et al*., 1992; BAI, 2011; TOJU; KUROKAWA; KENTA, 2019; MEHTA *et al*., 2021). Although, Wu *et al*. (2021) have suggested that the diversity of epiphytic bacterial communities is more affected by plant species than by region where the plant grew. Genetic variation within and among species also affect microbiome composition in maize, because plant-associated microbiota plays a vital role in regulating various biological processes and affects a wide range of traits involved in plant growth, as well as plant responses to adverse environmental conditions (TIAN *et al*., 2020; WAGNER *et al*., 2020).

In silage technology, the epiphytic microbiota plays a key role in initiating natural silage fermentation and greatly affects the fermentation dynamic and pattern over the ensiling, impacting, in this way, in the quality of natural fermentation of silage (ÁVILA; CARVALHO, 2019; SUN *et al*., 2021a). Epiphytic microbiota on crops run throughout the whole fermentation process. These microbial communities have a succession process and compete with each other (ÁVILA; CARVALHO, 2019). So, revealing the epiphytic bacterial and fungal communities of forages can provide a scientific basis for effectively regulating and improving the fermentation process of silage (SUN *et al*., 2021a; WU *et al.,* 2021). Enterobacteria, LAB*,* acetic acid bacteria, molds and yeasts include the most observed microorganisms in fresh silage materials, varying widely in number and species composition (GUAN *et al*., 2018).

The microbial population of freshly harvested forage crops is different, both in the numbers as well as the taxonomic composition, from that found during the process of silage fermentation and in the final product (PAHLOW *et al.,* 2003). During the ensiling process, the microbiota is altered according to the characteristics of the forage crop, the ensiling techniques used and the conditions of the environment change, with a succession of genera and species. Most of the studies evaluating the succession of bacterial during silage fermentation reveal a decrease of the bacterial biodiversity in whole crop corn silage when compared to the initial and final phase (KESHRI et al., 2018; DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND, 2019; XU *et al*., 2021), and an increase in the abundance of *Lactobacillus* genera (GHARECHANI *et al*., 2017).

Drouin; Tremblay; Chaucheyras-Durand (2019) revealed that the populations of bacteria observed in maize samples prior to ensiling were mainly composed of genera belonging to *Proteobacteria* (56.4 ± 1.5%) and *Bacterioidetes* (37.4 ± 1.7%) phyla. The abundance of the *Lactobacillales* order was only 3.2 ± 0.9% of the total population, and *Leuconostocaceae* family represented between 60% and 100% of the order *Lactobacillales* found in the fresh forage.

Wu *et al*. (2021) also reported that the dominant phyla were *Proteobacteria* (70%), but followed by *Firmicutes* (13%), *Actinobacteria* (9%) and *Bacteroidetes* (7%). Enterobacteriaceae (24%) was the most predominant family and *Pseudomonas* (8%), *Acinetobacter* (4%), *Chryseobacterium* (3%), and *Hymenobacter* (1%) were the genera most identified.

Sun *et al*. (2021a) reported that the mainly genera observed in the fresh whole-corn plant was from the *Enterobacteriales* and *Flavobacteriales* order. From the *Enterobacteriales* order, *Klebsiella, Enterobacter, Rahnella, Serratia* and *Erwinia* were the most abundant genera. From the *Flavobacteriales* order, *Chryseobacterium* (a *Bacteroidetes* genera*)* was the most predominant genera.

Xu, S. *et al.* (2019) identified *Pseudomonas,* from *Pseudomonadales* order, as the most abundant genera, ranging from 10.0 to 22.9%. And Xu, D. et al. (2019) cited

Agrobacterium, *Microbacterium, Sphingobacterium*, *Chryseobacterium, Klebsiella*, *Leuconostoc* (only 2.69%), Lactobacillus (only 2.44%) and representatives of the *Enterobacterales order* and *Gammaproteobacteria class,* as the main bacteria observed in the epiphytic microbiota of fresh corn.

According to Pahlow *et al.* (2003), LAB group varies in numbers over such a wide range, depending on forage species and varieties, maturity stage of the plant and the weather. This fact may explain the difference found between the authors regarding the proportion of LAB found in epiphytic microbiome of fresh maize. Regardless of the abundance of *Lactobacillus* identified in the epiphytic microbiota, Gharechani *et al.* (2017) affirm that *Levilactobacillus brevis*, *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus* are the most identified species in fresh maize. Although, Xu, D. *et al.* (2019) observed *Lactococcus lactis* (1.58%), *Leuconostoc pseudomesenteroides* (1.13%), Companilactobacillus *paralimentarius* (1.06%), *Lactiplantilactobacillus plantarum* (0.37%) and Companilactobacillus *farciminis* (0.03%), as the mainly LAB on the fresh corn.

In some cases, representation of LAB present in epiphytic microbiota does not effectively establish the required anaerobic and acidic conditions. Attempts can be made to compensate for the low count of LAB in the epiphytic community and also to improve the fermentation efficiency, quality and aerobic stability of the silage by applying inoculant based on LAB to the ensiled plants, avoiding spoilage through the activity of other microorganisms, such as enterobacterias, yeasts, moulds, spore-forming bacterias and *Listeria* (MUCK *et al*., 2010).

Another group of microorganisms found in fresh maize is the spore-forming bacteria. The most frequent genera observed is C*lostridium* (*C. sporogenes, C. tyrobutyricum, C. butyricum and C. bifermentans*), *Bacillus* (*B. licheniformis, B. pumilus, B. coagulans, B. sphaericus, B. cereus* and *B. polymyxa*) and *Paenibacillus.* These bacteria cause several damages to the fermentation process, silage quality and animal health (PAHLOW *et al.,* 2003; ÁVILA; CARVALHO, 2019; WANG *et al*., 2021). Bacteria of these genera are usually not part of the epiphytic microbiota of crops, but may be present in fresh forage by contamination by soil or animal feces (PAHLOW *et al*., 2003; ÁVILA; CARVALHO, 2019).

Clostridium is strictly anaerobic and can produce toxins that affect animal health and decrease its productivity, because they are not degraded by fermentation and remain stable under acidic conditions (ÁVILA; CARVALHO, 2019). *Bacillus* and

Paenibacillus are aerobic or facultatively anaerobic. They can also survive the fermentation process, competing for substrates with LAB. However, some species of the genus *Bacillus* have been studied as possible inoculants (*B. subitilis, B. licheniformis and B. pumilus*), due to the production of antimicrobial substances as peptide and lipopeptide antibiotics and bacteriocins, increasing aerobic stability and the inhibition of molds (PAHLOW *et al*., 2003; ÁVILA; CARVALHO, 2019).

Another undesirable bacteria genus may be present in fresh maize is *Listeria*. However, unlike the spore-forming bacteria, it is part of the epiphytic microbiota. *Listeria* is facultatively anaerobic that requires microaerophilic conditions and can survive and even grow during silage fermentation, due to its ability to survive the acidic environment and high temperature variations. The species *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. grayi*, *L. seeligeri* and *L. Ivanovii* have been identified in different types of silage (PAHLOW *et al*., 2003; ÁVILA; CARVALHO, 2019).

Yeasts and molds also compose the epiphytic microbiota. *Tremellomycetes*, *Saccharomycetes, Dothideomycetes, Sordariomycetes* and *Microbotryomycetes* were the most classes of fungi observed in fresh samples (XU, S. *et al*. 2019). The most frequent genera identified were *Epicoccum*, *Cladosporium*, *Giberella*, *Bulleromyces*, *Neosetophoma, Sarocladium* (MIDDELHOVEN; VAN BAALEN, 1988; SANTOS *et al*., 2017; DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND, 2019), *Filobasidium, Candida*, *Mycosphaerella* (XU, D. *et al*., 2019), *Aspergillus, Fusarium, Penicillium,* and *Trichoderma* (WAMBACQ *et al.,* 2016).

2.1.2 Aerobic Phase

At the harvest time, the fresh forage harbors a range of microorganisms, the epiphytic community, but the fermentation process favors the multiplication of the LAB (McDONALD *et al*., 1991).

As much as the forage harvest occurs and it is chopped, respiration activity proceeds continuously by plants cells and epiphytic microorganisms for several hours and perhaps days if silage is poorly packed. The proteases initiate the breakdown of proteins to amino acids and carbohydrase increase the amount of carbohydrates available for fermentation (WILKINSON; DAVIES, 2013). After sealed, the respiration of aerobic microorganisms and plant cells, and the fermentation of anaerobic facultatively bacteria occur synchronously in the silo (ÁVILA; CARVALHO., 2019), until

O_2 is depleted and the pH declines (BOLSEN; ASHBELL; WEINBERG, 1996; KUNG JR., 2018). The quantity of trapped O_2 within the forage determines the respiration and enzymatic activities rate, generating heat, CO_2 and H_2O (PIT; SHAVER., 1990; PAHLOW *et al*., 2003).

The aerobic microorganisms consume soluble carbohydrates as long as there is oxygen available in the silo, decreasing the nutrient content available for LAB. Thereat, the faster the transport of the forage mass from the field to the silo, the removal of air from the forage mass and sealing the silo, the lower will be the chances of development of undesirable aerobic microorganisms, that compete with the LAB and delay the pH drop of the ensiled mass. Consequently, the lower will be the energy and the DM losses, and the effects of aerobioses are minimized, during just for a few hours (McDONALD *et al*., 1991; KUNG Jr., 2018). In the study developed by Sun *et al*. (2021a), the duration of the initial aerobic phase was 85 min, and of the identified genetic sequences in silage, 34.6% were aerobic bacteria and 28.2% were facultatively anaerobic bacteria.

The forage compaction and the silage packing density contributes to the reduction of the DM losses. The compaction processes reduce de amount of oxygen available in the initial phase of silage fermentation and improve the final quality of silage, due to the reduction of the aerobic phase during silage fermentation and decreased nutrient consumption by aerobic microorganisms (SUCU *et al*., 2016). High silage densities (≥225 kg DM/m³) promote the elimination of oxygen, increasing DM recovery (BRÜNING *et al*., 2017), the storage capacity of the silo, and reducing cost of storage.

At this stage, there is an increase in the temperature of the mass, caused by the respiration process, and a slight drop in pH, as a result of the activity of facultative aerobic or anaerobic bacteria, mainly of the family *Enterobacteriacea* and even heterofermentative LAB. The reduction in the pH (pH<4.5) limits the activity of the enzymes, shuts down the activity of enterobacteria and prevents the development of undesirable bacteria (PIT; SHAVER., 1990; McDONALD *et al*., 1991; DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND, 2019).

According to Sun *et al*. (2021a), from 3 to 5 hours after sealing, the most dominant bacteria was *Pantoea.* When silage pH was less than 5.5, *Pantoea* abundance reduce. *Klebsiella* and *Enterobacter* also increased in the first 5 hours and then turned down. *Weissella* increased to 42.4% at 10 hours after the beginning of the aerobic phase, becoming dominant within 10 to 24 hours (pH ≤ 5.5), then decreased to 4.05% at 3 days. *Leuconostoc* went up in the first 24 hours and then turned down and *Lactobacillus* became the most prevalent after 2 days, when pH ≤ 4.25, reaching 70.5% of the OTUs at 3 days. The increase of *Lactococcus* and *Pediococcus* to 3.94% happened at 2 days and then decreased to 2.28% at 3 days.

From 1 to 3 days of fermentation, Drouin; Tremblay; Chaucheyras-Durand (2019) and Xu, S. *et al.* (2019) also reported a succession of dominance between the *Proteobacteria* phyla and the *Firmicutes* phyla. Drouin; Tremblay; Chaucheyras-Durand (2019) reported that the Firmicutes phyla was composed mainly by the families *Leuconostocaceae* and *Streptococcaceae* (mainly related to *Lactococcus* genera) and which represented 50% or more of the total population. The *Proteobacteria* was composed mainly by *Enterobacteriaceae* family and *Citrobacter*, *Brenneria*, *Enterobacter*, *Erwinia* and *Serratia* were the most abundant genera. During this time, they observed an increase in the relative abundance of *Lactobacillaceae,* however *Lactobacillus* represent a small fraction

At 3 days, Gharechani *et al.* (2017) observed the increase in the abundance of *Lactobacillaceae* family (90.3% on average), largely due to the family *Leuconostocaceae* and the reduction in *Enterobacteriaceae* family (GHARECHANI *et al.,* 2017).

According to Sun *et al*. (2021a), the *Enterobacteriaceae* family (mainly *Klebsiella, Enterobacter* and *Citrobacter* genera) and the *Erwiniaceae* family (mainly *Pantoea* and *Erwinia* genera) represent the major aerobic bacterial activity during the first days of silage fermentation. These bacteria are followed by heterofermentative LAB (mainly *Weissella*, *Leuconostoc, Lactococcus* and *Pediococcus* genera) (SUN *et al.,* 2021a), indicating that the enterobacteria and heterofermentative LAB might be the reason for reducing the pH and achieving the anaerobic environment to the development of the *Lactobacillus*. However, some heterofermentative LAB, e.g., L. buchneri, needs about 30 to 60 days to become apparent in silage (MUCK *et al*., 2018).

Regarding to fungal succession, Drouin; Tremblay; Chaucheyras-Durand (2019) reported the completely disappearance of the genera *Epicoccum* in the first day of ensiling, while *Neosetophoma* and *Peyronellaea* became the dominant genera after 1 and 2 days, respectively. *Epicoccum* and *Neosetophoma* are saprophytic molds, and several species of the genera *Peyronellaea* are plant pathogens (DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND, 2019).

2.1.3 Fermentation phase and stable storage phase

After the oxygen has been depleted, the anaerobic phase begins. There is an increase in LAB population, which produces lactic acid by the fermentation of soluble carbohydrates, promoting a faster and more efficient reduction in pH, due to the lactic acid is stronger than acetic acid. With the extinction of oxygen among the forage particles, the breathing process ceases and only the fermentation process prevails. Thus, after the increase in the mass temperature in the initial phase of the ensiling process, the temperature decreases and tends to stabilize until the next infiltration of O_2 (PIT; SHAVER ,1990).

If the production of lactic acid is not sufficient to lower the pH to values that inhibit *Enterobacteria, Clostridium, Listeria* and propionic acid bacteria, these bacteria can continue to grow, performing other secondary fermentations (ÁVILA; CARVALHO, 2019). But, in well-compacted silage with adequate soluble carbohydrates and DM content, LAB dominates the process resulting in good silage (PIT; SHAVER, 1990).

The LAB are classified into three groups according to the main metabolic routes as well as the fermentation products. The homofermentative species metabolize hexoses via the Embden–Meyerhoff pathway to pyruvate as the key metabolic intermediate and produce mainly lactic acid. The obligately heterofermentative LAB metabolize hexoses via the phosphoketolase pathway to pyruvate and acetyl-phosphate as key intermediates and produce lactic acid, as well as significant amounts of acetic acid, mannitol, ethanol and CO_2. And facultatively heterofermentative LAB are in an intermediate position, being able to perform the two metabolic routes according to the oxygen and glucose concentrations in the environment (PIT; SHAVER, 1990; McDONALD *et al.,* 1991; GÄNZLE, 2015; ÁVILA; CARVALHO, 2019; ZHENG *et al.,* 2020). In addition to these compounds, LAB can synthesize a wide variety of other compounds, such as acetate, propionic acid, formate, acetoin, 2,3-butanediol, diacetyl, 1,3-propanediol, 1,2-propanediol, 3-hydroxypropional (reuterin), bacteriocins, 3-hydroxydecanoic acid, 3-(R)-hydroxytetradecanoic acid, 4-hydroxybenzoic acid, vanillic acid, acetaldehyde and several others such as oligosaccharides, amino acids, fatty acids, vitamins, and aromatic compounds, depending on the genetic characteristics of the available microorganisms, substrates and environment conditions (McDONALD *et al.,* 1991; AXELSSON, 2004; XU, D. *et al.,* 2019)

The *Lactobacillaceae* family starts to multiply and appears in bacterial composition of day 2-4 of maize silage (DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND*,* 2019; XU *et al*., 2021; SUN *et al*., 2021a). Gharechani *et al*. (2017) and Sun *et al*. (2021a), observed the increase of *Lactobacilllus* and *Pediococcus* throughout the first 2 days of the fermentation process, and the decline of *Pediococcus* abundance after 3 days.

According to Drouin; Tremblay; Chaucheyras-Durand (2019), on day 4 of the fermentation process, there was a major increase in the relative abundance of *Lactobacillaceae* members. According to Xu *et al.* (2021), at 3 days, the abundance of *Lactobacillus* increased to 70.5% and *L. brevis* and *L. plantarum* are the major species.

However, *Enterobacteriaceae* family were still present, with *Citrobacter* and *Serratia* as the main genera. The relative abundance of OTUs related to *Leuconostocaceae* was lower during this phase of fermentation compared to the observed values at 1 and 2 days of incubation, possibly related to their poor tolerance to low pH compared to the others LAB (DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND., 2019). Sun *et al*. (2021a) observed the reduction of the abundance of *Weissella* and *Leuconostoc* at 10 days of fermentation.

Xu *et al.* (2021) and Drouin; Tremblay; Chaucheyras-Durand (2019), observed the dominance of the *Lactobacillus* genera at 7-8 days and Hu *et al.* (2018) at 5 days of fermentation with an abundance of 49.54%, followed by *Klebsiella* (9.03%). Xu *et al*. (2021) reported that *Lactobacillus* genera reached more than 60% of the relative abundance in 7 days of fermentation, and *L. parabrevis*, *L. brevis* and *L. coryniforms* were the major species at that time. However, despite the dominance of the *Lactobacillus* genera, *Weissella* (23.3%) and *Leuconostoc* (16.5%) were still present (Xu, S. *et al*. 2019).

At 10 days of fermentation, Sun *et al.* (2021a) related the abundance of *Lactobacillus,* reaching 80.5% and then reducing at 57.9% at 60 days. At 14 days, Xu *et al.* (2021) reported an increase of the species *L. farciminis* and *L. paralimentarius*.

After 16 days of fermentation, Drouin; Tremblay; Chaucheyras-Durand (2019) related that OTUs affiliated to the *Pediococcus* genera were retrieved and *Lactococcus* slowly disappeared from the community. Sun *et al.* (2021a) also observed the decrease in *Lactococcus*, reaching 0,85% at 60 days. However, they reported that *Pediococcus* went down to 0,85% just at 30 days, but then increased to 2.04% at 60 days.

From day 32 to 64, the relative abundance of *Lactobacillales* remained similar to 16 days of fermentation, however the relative population of genera *Lactobacillus* increased (DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND*,* 2019). On 30 days of fermentation, Xu *et al.* (2021) observed an increase in the population of *Companilactobacillus heilongjiangensis*. On 45 days of fermentation, *Lentilactobacillus buchneri, Levilactobacillus spicheri* and *Lacticasei pantheris* were enriched. And after 90 days, Lactobacillus species such as *Lactobacillus acetotolerans*, *Secundilactobacillus silagei*, *Lentilactobacillus parafarraginis*, *Lentilactobacillus buchneri* and *Secundilactobacillus odoratitofui* dominated the bacterial community (XU, D. *et al*., 2019; XU *et al.* 2021).

Wang *et al*. (2021), after 300 days of ensilage, observed that the main bacteria genera continued to be *Lactobacillus* and the main *Lactobacillus* species were *L. buchneri*, *Lentilactobacillus parafarraginis and Lentilactobacillus kefiri*. Guan *et al*. (2018) also observed *Lactobacillus* as the predominant microbial genus.

Most of the studies evaluating the succession of bacterial during silage fermentation reveal a decrease of the bacterial biodiversity from prolonged fermentation process in whole crop corn silage, when compared to the initial and final phase (KESHRI *et al*., 2018; DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND, 2019; XU *et al*., 2021), due to the increase of *Lactobacillus* genera.

Keshri *et al*. (2018), Xu, D. *et al*. (2019) and Sun *et al.* (2021a) also reported the increase of the abundance of *Lactobacillus* during fermentation, reaching more than 90% after 90 days (XU, D *et al*., 2019). Hu *et al.* (2018) observed the highest level of *Lactobacillus* at 40 days, reaching 64.1%, during 60 days of fermentation. Xu, S. *et al*. (2019) observed the peak of *Lactobacillus* at 30 days throughout 90 days of fermentation and Xu *et al.* (2021) observed the increase in *Lactobacillus* abundance until the opening of the silo at 60 days. Sun *et al*. (2021a), Hu *et al.* (2018) and Xu, S. *et al.* (2019) also verified the exponential growth of representatives of the *Lactobacillus* genera throughout the fermentation process; however, they reported that at 60 and 118 days, there was a little decrease in *Lactobacillus* population (80.5% to 57.9%, 64.1% to 63.2% and 66% to 55.3%, respectively).

The explanation for those findings may be related to the stabilization of the fermentation process and the storage time of silage. The duration of fermentation process or the fermentation intensity depends largely on the available substrate and the environment conditions (pH and anaerobiosis) (ÁVILA; CARVALHO, 2019). During fermentation phase, growing conditions become unsuitable for most of the microorganisms, due to the decrease of the nutrients available and the reduction of pH to values below 4. In the moment at which no or minimal changes occur, the ensiled material enters a stability stage, and the metabolism of these microorganisms became very low or did not occur, suppressing some populations (GUAN *et al*., 2020). However, even though reactions during the stable phase are minimal, the storage time affects the fermentative characteristics of a silage, such as the microbial population and fermentation products (BORREANI *et al*., 2014).

The eukaryote succession in the fermentation phase corresponds to an increase in yeast related OTUs (DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND, 2019). The main yeast genera found in this phase are *Candida*, *Dipodascus*, *Hannaella*, *Hanseniaspora*, *Kazachstania*, *Metschnikowia* (DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND, 2019) *Saccharomyces, Geotrichum* (XU, S. *et al.*, 2019) and *Pichia* (SANTOS *et al*., 2017).

According to Xu, S. *et al.* (2019), after ensiling, *Candida* increased and predominated, peaking at day 61 (75.3%), and thereafter declining by day 118 (46.7%). After 118 days, *Kazachstania, Pichia, Dipodascus, Metschnikowia*, and *Geotrichum* increased, but *Kazachstania* remained the most prevalent during all fermentation phase. While *Filobasidium, Cryptococcus, Sporobolomyces*, and *Mycosphaerella* continued to decline.

Drouin; Tremblay; Chaucheyras-Durand (2019) verified that *Kazachstania* was the dominant yeast genera after 4 and 8 days of fermentation and subsequently decreased. By day 32, it represented only a low ratio of the total population. After 64 days of ensiling, the OTU related to *Candida* clearly dominated the control treatment (DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND, 2019).

Santos *et al.* (2017) detected 15 yeast species in corn silage. *Candida ethanolica* (21.2%), *Saccharomyces bulderi* (17.6%), *Pichia anomala* (10.0%), *Kazachstania unispora* (9.6%) and *S. cerevisiae* (8.0%) were the most prevalent yeasts and represented 66.4% of the relative abundance. The remaining 33.6% was represented by 10 other species that were presented in frequencies ≤7.5%. In their trial, the yeast count varied from 5.4 to 6.3 $\log_{10}$ cfu/g of silage with 347 g/kg of DM. This value is considered low according to Middlhoven (1998), who claims that silages having a population in excess of 10^5 cfu/g of DM are particularly prone to aerobic deterioration.

May; Smiley; Schmidt (2001) revealed that *P. anomala* were the dominant yeast present 2 to 3 months after ensiling in maize silage. And Wang *et al.* (2021) reported the dominant fungal genera were *Kazachstania,* followed by *Candida, Rhizomucor, Cladosporium* and *Rhizopus* and the most identified species were *Kazachstania bulderi, Candida xylopsoci* and *Rhizopus microspores*.

Approximately 90% of the isolated fungi were molds, both in fresh forage and silage (KELLER *et al*., 2013). Keller *et al.* (2013) reported that *Aspergillus* was the most frequent genera of filamentous fungi isolated from pre- and post-fermented (90 days) maize silage. However, *Penicillium, Cladosporium, Euroticum, Fusarium, Alternaria* and *Curvularia* were the other genera found as most frequent ones. Regarding the genera *Aspergillus*, the predominant species identified in pre-fermented samples were *Aspergillus flavus* (40%) and the *A. niger* aggregate (23%), while in post-fermentation samples *Aspergillus fumigatus* (17%). Among the species of the genera *Penicillium*, the most prevalent in pre-fermentation silage were *Penicillium citrinum* (65%), *Penicillium islandicum* (25%) and *Penicillium glabrum* (10%); and in post-fermentation samples, *P. citrinum* (50%), *P. islandicum* (17%), *Penicillium griseofulvum* (15%), *Penicillium funiculosum* (13%), and *P. glabrum* (5%) were the most observed species. The *Fusarium* species most frequent isolated in pre-fermented silage were *Fusarium verticillioides* (80%) and *Fusarium graminearum* (20%), while in post-fermented silage, *F. verticillioides* (100%) was the most observed specie.

Dunière *et al.* (2015) and Borreani; Tabacco, (2014) affirmed that yeast and mold counts reduce throughout fermentation phase. According to Ferrero *et al.* (2018), the fermentation process, combined with the longer ensiling duration, greatly reduced the yeast and mold count compared to those observed at harvesting. However, their population increased after silage exposure to air, reaching a plateau between 7 and 14 days (DUNIÈRE *et al*., 2015; FERRERO *et al*., 2018).

Ferrero *et al*. (2018) reported that the number of yeasts and molds in fresh maize forage was 6.46 and 5.26 $\log_{10}$ cfu/g, respectively; and after 250 days of fermentation, they count reduced to 2.17 $\log_{10}$ cfu/g and <0.01 $\log_{10}$ cfu/g, respectively. After 14 days of aerobic stability (AS), yeasts and molds count were 6.14 and 8.49 $\log_{10}$ cfu/g, respectively. Drouin *et al*. (2020) reported counts of yeast and molds in the silos of 2.7 and 2.3 $\log_{10}$ cfu/g of fresh maize silage after 156 days of fermentation. Xu, S. *et al*. (2019) also observed a reduction in the fungi count after ensiling. In fresh maize forage a total of 7.22 $\log_{10}$ cfu/g DM of yeasts and 5.99 $\log_{10}$ cfu/g DM of total fungi was observed. In silage, after 118 days of fermentation, the count of yeasts was 5.26 $\log_{10}$ cfu/g DM and the count of total fungi was 1.61 $\log_{10}$ cfu/g DM (XU, S. *et al*., 2019). However, Keller *et al*. (2013) reported an average total fungal count of $3.34 \times 10^3 \pm 2.72 \times 10^3$ cfu/g of fresh maize forage and $5.74 \times 10^4 \pm 1.82 \times 10^4$ cfu/g of silage, after 90 days of fermentation. The average total fungal count found by Keller *et al.* (2017) exceeded the limit of total fungal counts recommended as a quality standard for animal feeds that is 1×10^4 cfu/g (GMP, 2008).

Fungi often thrive well in environments with high humidity, high temperature, and oxygen access during all stages of plant production and storage (Egal *et al.,* 2005). Keller *et al.* (2013) collected 464 samples of corn silage from the most important regions of Brazil during two sampling periods and the pH values of silage varied from 4.2 to 6.5. The elevated pH (>6.0) caused by the growth of spoilage-causing yeasts results in the active growth of molds when anaerobic environment is disturbed, particularly in poorly managed silage (SCHMIDT *et al*., 2015), justifying the high fungal counts observed by Keller *et al*. (2013).

2.1.4 Feed-out phase

This phase starts when the silo is opened and is highly influenced by removal rate and silo face handling, as well as ambient temperature and silage compaction. In this phase, the re-exposure of the mass to oxygen provides the development of those microorganisms that have survived (in the active form or in the form of spores) the fermentation process, such as fungi and aerobic bacteria that were latent in anaerobic conditions (PAHLOW *et al*., 2003). Silages exposed to air can deteriorate as a result of the aerobic microbial activity during the feed-out, and losses can reach 70% of DM in the top layer and near to the sidewalls of the bunkers, due to the catabolism of the residual digestible carbohydrates and silage fermentation products (BOLSEN *et al*., 1993; DOLCI *et al*., 2011). For this reason, the enzymes produced by each member of the microbial community (both fungi and bacteria), associated with the forage and its residual nutrients can greatly influence the rate and the extent of decomposition of the ensiled material (MAY; SMILEY; SCHMIDT, 2001).

The spoilage of silage due to exposure to air decreases the nutritive value and leads to negative effects on animal performance and health, due to the relationship with the proliferation of potentially pathogenic microorganisms, fungal degradative enzymes and secondary metabolites and mycotoxin synthesis (DEACON, 2006; RICHARD *et al.,* 2009). It is estimated that losses of DM during aerobic deterioration are more important than the losses occurring in the initial aerobic phase and the main fermentation phase, when silage management is inadequate (WILKINSON; DAVIES, 2013).

Oxygen entering the silo allows microorganisms to use aerobic biochemical pathways and dormant cells may be reactivated, leading to major biochemical changes (WILKINSON; DAVIES, 2013). It is generally accepted that yeasts play a major role in initiating aerobic spoilage of silage, because they are facultative anaerobes and acid tolerant (JONSSON; PAHLOW, 1984; ÁVILA; CARVALHO., 2019). However, in maize silages, acetic acid bacteria occasionally can start the degradation process (DOLCI *et al.*, 2011) in silages with low yeast numbers (KUNG *et al.*, 2018). Acetic acid bacteria can grow at low pH and on ethanol, producing acetic acid. Once ethanol has been exhausted, AAB can grow on acetic acid, producing CO_2, water and heat. This will raise pH and permit other aerobic microorganisms to grow (MUCK *et al.*, 2010). The increase in the biochemical activity, linked to the development of yeasts, acetic acid bacteria and *Bacillus,* triggers a rise in temperature inside the maize silage mass, which is the most noticeable sign of the process (DROUIN *et al.*, 2020).

Yeasts are able to ferment sugars anaerobically and produce ethanol and CO_2 when trace amounts of oxygen are present (WOOLFORD, 1990; McDONALD *et al.*, 1991). Although, they can oxidize sugars and lactic acid, resulting in the production of CO_2, H_2O and heat. So, the lactate-assimilating yeasts (*Saccharomyces, Candida* and *Pichia*) are generally the main initiators of the aerobic spoilage of silage (PAHLOW *et al.*, 2003). Due to its metabolism, yeasts cause high DM loss, and rise in silage pH to a level that allows the growth of molds and opportunistic bacteria and reduction in AS (aerobic stability) (McDONALD *et al.*, 1991). Drouin *et al.* (2021) reported the abundance of *Issatchenkia* genus after 10 days of AS and the increase of pH from 3.9 to 5.5, in agreement with the drastically decreased in lactic acid concentration from 34.4 to 5.2 g/kg of DM. Therefore, yeasts are considered undesirable in silages. However, some species of yeast are used as probiotics for ruminants and the inoculation of *S. cerevisiae or S. paradoxus* (10^3 cfu/g) did not alter the fermentation profiles, microbiology population, gas production, in vitro DM disappearance, microbial protein synthesis and AS of the corn silage (DUNIÈRE *et al.*, 2015). Xu, S. *et al.* (2019), studying the impact of *S. cerevisiae* in maize silages, also verified that the inoculation of S. cerevisiae did not alter the fermentation profiles (p <0,01), and fungal and bacterial community (p <0,01).

Molds are less acid-tolerant than yeasts, and usually they are involved with silage deterioration. Their metabolism significantly reduces the nutritional quality of the feed and synthesis of mycotoxins may occur simultaneously, mainly when molds are exposed to adverse conditions (ÁVILA; CARVALHO, 2019). Mycotoxins in maize silage comes mostly from the development of fungi on forage in the field, mainly by molds of the *Aspergillus, Penicillium* (KELLER *et al*., 2013; ÁVILA; CARVALHO, 2019)*, Fusarium*, *Alternaria* (KELLER *et al*., 2013), *Monascus and Byssochlamys* genera (ÁVILA; CARVALHO, 2019). These fungal genera can produce mycotoxins such as aflatoxins, ochratoxins, trichothecenes, zearalenone, deoxynivalenol, fumonisins, and several others (OGUNADE *et al*., 2018; REISINGER *et al*., 2019). However, the mycotoxins with the highest prevalence in maize silage are zearalenone and deoxynivalenol, and both are *Fusarium* toxins (SCHMIDT *et al*., 2015; REISINGER *et al*., 2019).

Although mycotoxins in corn silage are derived mainly from the field, they can be produced during ensiling (such some aflatoxins), and at the same time could be degraded or bound by silage bacteria and produced over again during aerobic deterioration. However, many common molds do not produce mycotoxins, and the presence of molds in silage does not indicate the presence of mycotoxins nor does their absence confirm mycotoxins are absent (ZAIN, 2011).

The succession of microorganisms can explain the two-phase profile in the temperature curve during exposure to air. According to Merry and Davies (1990), the first peak corresponds to an increase in yeast and acetic acid bacteria, and the second peak might correspond to the growth of molds.

Acetic acid bacteria have been identified in the aerobic stages of fermentation, mainly in the feed-out phase, associated with aerobic deterioration. Most of the species are obligate aerobes, and have high tolerance to acidic condition. *Acetobacter* and *Gluconobacter* are examples of acetic acid bacteria. They metabolize carbohydrates and fermentation products to produce acetic acid (weak acid), causing an increase in the pH and loss of DM in the form of CO_2. Besides, acetic acid may be a factor in reducing silage intake (ÁVILA; CARVALHO, 2019). According to Gerlach *et al*. (2021), in silages with acetic acid concentrations above 17 g/kg DM, an increase of 1 g acetic acid/kg DM led to a reduction of 1.2 g in DM intake per 100 kg body weight. The reason why acetic acid reduces DM intake is not yet clarified; however, the most plausible reason is because acetic acid has a pungent smell, that reduces the palatability of silage (GERLACH *et al*., 2021). Because acetate is produced in large amounts in the rumen, it seems unlikely that DM intake was metabolically controlled by dietary acetate (DANIEL *et al*., 2013).

Drouin *et al*. (2020) developed a trial to observe the bacteria succession during aerobic stability of maize silage after 159 days of fermentation and observed the dominance of *Lactobacillus* (89.1%) up to 5 days after the aerobic exposure test. According to Hu *et al*. (2018), the genera *Lactobacillus* can decrease in an average ratio of 30% in 3 days of AS.

Weissella was observed in the silage in small proportion, but it dropped until the final of the AS test. The proportion of the genera *Pediococcus* in the second day of AS increased, but until de 5th day it disappeared. On the fourth and fifth day of fermentation, few representatives of the *Acetobacter* genera began to develop, and in the sixth day, they dominated the OTUs of the relative abundance until the end of the AS test. After 6 days of AS, the abundance of *Paenibacillus* genera (*Bacillales* spore-forming) subtly increased, reaching 11.5% by day 10. Also starting from day 6, higher abundances of OTUs related to *Lanchnoclostridium* genera (*Clostidiales*) were present. However, OTUs related to *Acetobacter* (*Rhodosphiriallales*) was still the most prevalent genera until the end of the AS test (DROUIN *et al*., 2020).

Xu, S. *et al*. (2019) also observed the dominance of *Lactobacillus* in the first 3 days of AS test. However, from day 4 to day 7, *Paenibacillus* and *Bacillus* corresponded to the most OTUs.

Drouin *et al*. (2020) also evaluated the fungal succession during AS test and observed that the *Ascomycota* was the dominant phyla, with over 95% of the total abundance. In this study, they observed a higher relative abundance for the fungal microbiome when compared to prokaryotes. At the opening of the silos (plastic bags) with the maize silage, *Kazachstania*, *Saccaromyces* and *Issatchenkia* were the prevalent genera, but *Penicillium*, *Wickerhamomyces* and *Candida* were also observed. *Saccharomyces, Issatchenkia* and *Kazachstania* were dominant until day 6. From then on, fewer *Kazachstania* were detected, whereas the relative abundance of *Penicillium* increased. In the first few days of aerobic exposure, *Issatchenkia* emerged and *Kazachstania* decreased. OTUs related to *Saccharomyces* increased slightly on the first day of exposure to air and remained stable thereafter. On the fourth day, Pichia genera increased, but decreased until the final of AS test, without disappearing. On the day 7, fewer *Kazachstania* were detected, whereas the relative abundance of OTUs related to *Penicillium* increased, remaining high up to the last day of the trial. At the end of AS test, the prevalent genera were *Penicillium, Issatchenkia* and *Saccharomyces.*

However, Xu, S. *et al*. (2019) observed *Kazachstania* as the most abundant genera in the first 3 days of AS. Its abundance increased in the first 3 days to 75.5% and then declined to 40.4% after 7 days. The abundance of *Geotrichum* and *Dipodascus* increased after 3 days and, after 7 days, *Geotrichum, Dipodascus* and *Kazachstania* were the most relatively abundant genera.

Drouin *et al*. (2020) also screened the samples during AS test for mycotoxins and related fungal metabolites. A total of 13 mycotoxins were identified, including metabolites produced by *Penicillium*, *Fusarium* and *Alternaria*. Upon the opening of silo, zearalenone, fumonisin and beauvericin were observed. On day 1 of AS test, the mean concentration of beauvericin (toxin produced by *Fusarium*) was similar to or higher than the reported maximum concentration according to regulations and recommendations set by the European Commission for dairy feeds (214 µg/kg of fresh silage) (REISINGER *et al*., 2019). Brazilian legislation only regulates that the maximum tolerated limit of aflatoxin, which must be 50 µg/kg, for any raw material to be used directly or as an ingredient for animal feed, with no prediction for other mycotoxins (BRASIL, 1988).

The information presented above confirms the importance of microbial community composition in the ensilage process. There are many microorganisms, interactions and metabolites involved in silage production. According to Ávila and Carvalho (2019), the ensiling process can be considered as a batch fermentation, composed of an extremely dynamic fermentation process with high metabolites production, from non-sterile substrate with ample opportunities of contamination, in which succession of different groups and species of microorganisms occur in different ways. Therefore, it is essential to comprehend the epiphytic forage and silage microorganisms, its interaction and its dynamics in relation to silage characteristics and management. Thus, it could be possible to intervene during the silage processes, in order to minimize DM losses, as much as possible, and reduce the aerobic deterioration of the silage.

2.2 GAS DRY MATTER LOSSES AT FERMENTATION PHASE AND MEASUREMENT METHODS

The amount of DM losses during the ensilage process is totally influenced by the microbial species, their succession and substrates. DM and quality losses occur during the ensiling process, from the field through the feeding phase. However, one of the main stages where losses occur are from forage harvest to silo sealing (aerobic phase), fermentation and oxygen exposure during storage and feed-out phases (BORREANI *et al*., 2018), representing 3% to 36% of the total losses, according to McDonald *et al*. (1991).

The losses associated with the aerobic phase and the fermentation phase of ensilage are mainly from CO_2 production, and are generally large and linked primarily to microbial species, forage species, forage DM and water-soluble carbohydrates (WSC) content, particle size, the time between harvest and silo sealing, compaction, sealing effectiveness and silage temperature (McDONALD *et al*., 1991; GOESER *et al*., 2015; BORREANI *et al*., 2018; BÜNING *et al*., 2018).

During ensiling process, metabolism of microorganisms and plant cells always lead to gas generation, which includes high percentage of CO_2 (BUENO *et al*., 2020; CHEN *et al*., 2021), methane (CH_4), nitrous oxide (N_2O), in addition to volatile organic compounds (VOC), such as lactic acid, acetic acid, butyric acid, alcohols, aldehydes, esters and ketones (Daniel, 2011; LEIP *et al*., 2015; BUENO *et al*., 2020).

Homofermentative LAB convert glucose and fructose into 2 moles of lactate, 2 moles of H_2O and 2 moles of ATP, resulting in no DM and losses. However, heterofermentative LAB can produce a diversity of end products according to the hexose used as substrate. In the fermentation of glucose, for each mol of glucose reduced, 1 mol of CO_2 is formed, generating 1% gross energy loss from silage (McDONALD *et al*., 1991; GOESER *et al*., 2015; BORREANI *et al*., 2018). *Leuconostocaceae* family were considered the main sources of CO_2 production during fermentation (ZHAI; PÉREZ-DÍAZ, 2020).

However, clostridia and yeasts are the main microorganisms involved in losses during the fermentation process. Yeasts produce ethanol from glucose, resulting in 2 moles of CO_2. Clostridia produce butyrate from lactate and glucose, resulting in 2 moles of CO_2 and 2 moles of H_2. In addition, during proteolysis, clostridia produce 1 mol of CO_2 (McDONALD *et al*., 1991; BORREANI *et al*., 2018).

Enterobacterias are facultatively anaerobic. They are part of epiphytic microbiota (such as *Erwinia herbicola* and *Rahnella aquitilis*), and their presence in silage is determined by initial conditions of fermentation. During ensilage, *Escherichia coli, Hafnia alvei* and *Serratia fonticola* can be observed. They are involved in the aerobic phase of silage fermentation, contributing to the reduction of residual O_2 concentration in forage mass. The most of the gas production in silages is due to the Enterobacteria metabolism in this phase. Enterobacteria compete with LAB for nutrients during fermentation, however the most enterobacteria do not proliferate and their viability decreases at pH values lower than 4.5 to 5.0. Therefore, a rapid and sufficient decline in pH, decreases enterobacterial growth and survival in the silage. According to Sun *et al*. (2021b), packing density is positively correlated with *Lactobacillus* ($p< 0.05$), but negatively correlated with *Enterobacter* ($p< 0.05$). The higher packing density decrease the abundances of *Enterobacter* (from 47.4% to 35.4%) and increase the abundance of *Lactobacillus* (from 1.8% to 17.0%). In this way, the packaging density can influence the rate of DM recovery, as well as the emission of gas by the silage.

Enterobacterias are also associated with poorly preserved silage, since when silage with high pH is aerobically exposed, it can provide favorable conditions for growth of these bacteria (DRIEHUIS; ELFERINK, 2000). Their metabolism differs from species, however lactic, acetic and succinic acids, ethanol, 2,3 butanediol, CO_2 and water can be produced. From lactate, they produce ethanol, acetate, 1 mol of CO_2 and 1 mol of H_2 (McDONALD *et al*., 1991; ÁVILA; CARVALHO, 2019).

Another bacteria sometimes detected in silage is the propionic acid bacteria. Just as clostridia, these bacteria are responsible for the formation of propionic acid. They can ferment a variety of carbon sources, such as carbohydrates, polyols and organic acids (lactic acid and gluconic acid) and the main fermentation products are propionic, acetic and succinic acid. From lactate, 1 mol of acetate, 1 mol of CO_2 and 1 mol of H_2 are produced. They can be fastidious anaerobic or facultatively anaerobic bacteria and the only specie that has been identified in silage is *Propionibacterium acidipropionici* (McDONALD *et al*., 1991; ROSSI; DELLAGLIO, 2007; ÁVILA; CARVALHO, 2019).

Therefore, gas production during ensilage is commonly the main source of DM losses (CHEN *et al*., 2021; SCHMITHAUSEN *et al*., 2022). In addition, CO_2 production during ensiling is a source of greenhouse gases (GHG) emissions (CHEN *et al*., 2021), participating in 28% of the anthropogenic greenhouse gases emissions from livestock production in Brazil (ALBUQUERQUE *et al*., 2020). It is known that CO_2 is a primary GHG, which causes global warming by trapping heat in the atmosphere, and alone accounts for around 77% of the total GHG emissions (CHEN *et al*., 2021).

Of the total gases emitted by silage, most of them are made up of CO_2 (about 20%-90% of the gas), according to Schmidt *et al*. (2011), especially in the early stage of ensiling (greater than 60%) (McENIRY; FORRISTAL; O'KIELY, 2011). And, according to Cai *et al*. (1997), more than 6.0 L of gas per kg of fresh matter was produced throughout 60 days fermentation. Over the past 20 years, multiple efforts have been made to mitigate GHG emissions from livestock production and manure treatment. However, little attention has been paid on CO_2 emission during ensiling (CHEN *et al*., 2021).

For this reason, Schmidt *et al.* (2011) proposed a system for evaluating the GHG emission during the fermentation phase of sugarcane silages and evaluated the mitigation potential using additives. This system was lately described by Bueno *et al.* (2020), and is composed of a 1 liter graduated low-density polyethylene beaker connected to each silo by a silicone hose passing through a 3-way stop tap to measure directly the daily gas production. These authors observed a higher gas production during the first 7 days of fermentation, and a strong influence of silage temperature over the gas production. The total amount of 2.08 L/kg DM of gas was produced, which was mainly composed by CO_2 (23054 ppmv – 99.9%) with low levels of CH_4 (2 ppmv) and N_2O (937 ppbv).

Using the same system, Schmidt *et al.* (2012) evaluated the gas emission from fermentation of corn silage stored for 63 days and reported a gas production of 424 L per ton of ensiled forage, and CO_2 was also the main gas produced (19,458 ppmv – 99.9%), and low levels of CH_4 (7 ppmv) and N_2O (1 ppbv) were detected. Compared to the sugarcane silages that produced 2,080 L per ton of ensiled forage in 66 days of fermentation, the corn silage produced significantly less gases.

Due to the observations raised by Schmidt *et al*. (2011; 2012), Souza (2015) compared the conventional methodology for evaluating fermentation losses by gravimetric estimation (Jobim *et al*., 2007) with the new methodology of direct measurement. The authors stated that the new model have greater sensitivity. Souza (2015) also reported that the correction of DM content for volatile compounds according to the equation proposed by Weissbach (2009) increased the incidence of negative values for estimating losses. The negative loss (DM gain), although frequent, is usually considered experimental error and excluded from the data (SOUZA, 2015). In addition, they observed that, after the period of gas production (first 3 to 7 days after the sealing silos), there was a phase of negative pressure that could be perceived until the opening of the silos.

Souza *et al*. (2015) developed a trial to simulate infiltration of atmospheric air in maize silages through low quality polyethylene sheets comparing to hermetic sealing. In all silos, they again observed negative values for gravimetric dry matter losses and gas losses; and air injection did not affect those variables. During 66 days of fermentation, gas production happened mainly in the first 5 days and decreased until 21 days. Gas production was 4.77 L/ton DM and 2.6 L of air were injected into silos, but this volume of gas was not recovered, and the internal pressure of the silos was not stabilized. The intention of this trial was to verify if the air injections would stabilize the negative pressure inside the silos and if it would change the values of DM loss.

Novinski *et al.* (2016) evaluated the gas production and the internal silo pressure dynamic in maize silage. Silos were hermetically closed; internal pressures were measured by a water column manometer and gases by the apparatus described by Bueno *et al*. (2020). They observed that the pressure was positive in the first seven days, average 9 mmH_2O. After this period the pressure began showing negative, increasing over the days (reaching values more negative than -1,180 mmWC, since the manometer utilized had measurement limitation). They also observed that the DM increased 3.55 g/Kg DM.

Novinski (2018) also verified the period of negative pressure inside the silos and it declined until the opening day. The average internal pressure assessment was -611 mmWC, however some silos with maize silage showed negative values, closed to -1,300 mmWC. They also observed that silages stored at hot temperature (27.1°C) produced approximately 700 mL of gas per kg of DM more than the silages in cold environment (15.2°C), in the accumulated period of 95 days.

Schmidt *et al.* (2018) also evaluated the pressure inside the maize silage. Additionally, the authors have infused pure CO_2 into the silos, in order to balance the internal pressure of the silos to the atmospheric one. According to the authors, silos gas production lasted 11 days (3235 ± 388 mL/kg DM). Twelve days after, the negative pressure inside the silos was detected and silos that were kept closed showed an increase of the negative pressure until 101 days after sealing. The CO_2 was made available for the silos 28 days after sealing and a total of 19 weekly supplies of CO_2 were made during the trial. Silos absorbed 5,590 ± 2,492 mL of CO_2 during the whole trial, and continued showing negative pressure. At the opening, the average pH of the silages was 3.76 ± 0.03 in the silos that were kept closed for pressure measurement and 3.63 ± 0.02 in the silos that received the CO_2 supplies.

The chemistry reaction of CO_2 with the water present in the silage could be an explanation for the volume of air injected into the silos not being recovered. The solubility of CO_2 in water depends on pressure, temperature, pH and composition of the liquid phase (ILLERA *et al*., 2019); and is directly proportional to the pressure. However, it varies in more complex way with temperature. At pressures <100 bar (<1019.74 mmWC), CO_2 solubility decreases with increasing temperature. However, at pressures >100 bar (>1019.74 mmHG), CO_2 solubility actually increases with increasing temperature. According to Acién *et al*. (2017) and Soeswanto; Wahyuni and Trirahayu (2020), at 1 atm and 25°C, the maximum CO_2 solubility in water is 1.5 g CO_2/ kg H_2O, which corresponds to 831 mL CO_2/ L H_2O. In addition, in the presence of sugar (glucose, sucrose and fructose), ethanol and organic acids (citric and malic) in liquid medium, CO_2 solubility is lower when compared with pure water (CALIX; FERRENTINO; BALABAN, 2008; ILLERA *et al*., 2019). Thus, the incorporation of this gas seems to be related to some biochemical reactions instead of CO_2 solubility.

Schmidt *et al*. (2018) concluded that the CO_2 disappearance inside the silo seems to be incorporates into the silage mass, leading to the hypothesis that mechanisms of CO_2 and/or nitrogen fixation can be happening into the silo and the CO_2 absorption would be the probable cause for the negative pressure. They suggested that microbial population may be CO_2-fixing under anaerobic environment, possibly by the Wood-Ljungdahl pathway and producing acetate from CO_2.

2.3 CO_2-FIXING METABOLIC PATHWAYS

Carbon fixation is one of the dominant biochemical processes in the biosphere and is the biological process through which CO_2 is converted to organic compounds (SATO; ATOMI, 2010; BAR-EVEN; NOOR; MILO, 2012). Organisms that fix CO_2 provide the organic carbon necessary to support the existence of all heterotrophic life on our planet (SATO; ATOMI, 2010). Autotrophic CO_2 fixation represents the most important biosynthetic process in nature, being responsible for the net fixation of 7 x

10^{16} g carbon annually, corresponding to the conservation of 2.8 x 10^{18} kJ of energy (Berg, 2011). There are six natural pathways of autotrophic CO_2 fixation: Calvin–Benson–Bassham cycle, reductive tricarboxylic acid cycle, 3-hydroxypropionate cycle (or 3-hydroxypropionate/malyl-CoA cycle), 3-hydroxypropionate/4-hydroxybutyrate cycle, the dicarboxylate/4-hydroxybutyrate cycle and reductive acetyl-CoA pathway (Berg, 2011).

The reductive tricarboxylic acid cycle and the 3-hydroxypropionate bicycle are operate just for bacteria and the dicarboxylate-4-hydroxybutyrate cycle and the 3-hydroxypropionate-4-hydroxybutyrate cycle are used just by archaea (BAR-EVEN; NOOR; MILO, 2012).

The organisms that use the reductive tricarboxylic acid cycle and the dicarboxylate-4-hydroxybutyrate cycle are restricted to anaerobic environments. The reductive acetyl-CoA pathways, performed by both acetogenic and methanogenic microorganisms, can only tolerate oxygen at a very low level. These pathways employ oxygen-sensitive enzymes or cofactors that operate only in organisms that occupy anaerobic environments (BAR-EVEN; NOOR; MILO, 2012).

2.3.1 CALVIN–BENSON–BASSHAM CYCLE

The reductive pentose phosphate cycle (also referred as Calvin-Benson) is the predominant mechanism of autotrophic CO_2 fixation in nature and is used by many prokaryotes and all plants fix CO_2 into biomass. The key enzymes are ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and phosphoribulokinase which are not sensible to O_2, and the end product of this pathway is glyceraldehyde-3-phosphate (BERG, 2011). The RubisCO have low affinity for CO_2. The cycle requires nine ATP equivalents and six NADPHs for the synthesis of one glyceraldehyde-3-phosphate molecule (BERG, 2011). The Calvin–Benson cycle is the CO_2-fixing pathway developed by photosynthetic & chemolithotrophic microorganisms such as plants, algae, cyanobacteria (e.g., *Synechococcus, Anacystis* and *Anabaena*), and many aerobic or facultative aerobic proteobacteria (alpha, beta, and gamma subgroups) (SATO; ATOMI, 2010). It was also shown for *Sulfobacillus spp*., iron and sulfur-oxidizing members of the firmicutes, some mycobacteria, green nonsulfur bacteria of the genus *Oscillochloris* (phylum *Chloroflexi*), purple sulfur bacteria (e.g., *Chromaticum*), purple nonsulfur bacteria (e.g., *Rhodobacter, Rhodospirillum*, and *Rhodopseudomonas*), and hydrogen bacteria (e.g., *Ralstonia* and *Hydrogenovibrio*).

(SATO; ATOMI, 2010; BAR-EVEN; NOOR; MILO, 2012). This cycle has not been found in Archaea (BAR-EVEN; NOOR; MILO, 2012).

2.3.2 REDUCTIVE TRICARBOXYLIC ACID CYCLE

Reductive Tricarboxylic Acid Cycle or the reductive citric acid cycle is the pathway used by photosynthetic (*Chlorobaculum tepidum* and *Chlorobium limicola*) and chemolithotrophic microorganisms (*Hydrogenobacter thermophilus* and *Desulfobacter hydrogenophilus*), mainly distributed in anerobic organisms such as green sulphur bacteria (*Chlorobium* and *Chlorobaculum* genera), members of the thermophilic bacterial phylum Aquificae, members of the *Nitrospira phylum* and Leptospirillum), and members of the alfa, delta and epsilon subdivisions of proteobacteria (SATO; ATOMI, 2010; BERG, 2011). This cycle may in fact be widespread among anaerobic or microaerobic bacteria, but until now it had not been found in the archaeal domain. It reverses the reactions of the oxidative citric acid cycle (Krebs cycle) and forms acetyl-CoA from two CO_2 molecules. The primary CO_2 fixation product of the cycle, acetyl-CoA, must be further converted to other intermediates as pyruvate/phosphoenolpyruvate, oxaloacetate, and 2-oxoglutarate. Three reactions of the conventional Krebs cycle are considered to be irreversible and have to be substituted by other reactions and enzymes to reverse the cycle: succinate dehydrogenase is replaced by fumarate reductase, NAD-dependent 2-oxoglutarate dehydrogenase by ferredoxin-dependent 2-oxoglutarate synthase, and citrate synthase by ATP-citrate lyase. These three enzymes are the characteristic enzymes of the cycle. This cycle is well established in *Hydrogenobacter thermophilus*, *Desulfobacter hydrogenophilus*, *Chlorobaculum tepidum* and *Chlorobium limicola* (BERG, 2011).

2.3.3 3-HYDROXYPROPIONATE BI-CYCLE

The 3-Hydroxypropionate/Malyl-CoA Cycle consists of two overlapping metabolic cycles (bi-cycle) and is developed by green non-sulfur phototrophs bacteria of the Chloroflexaceae family, which grow preferentially under photoheterotrophic conditions, and the only representative of this family that can be autotrophic is *Chloroflexus aurantiacus* (SATO; ATOMI, 2010). In total, the two cycles combined result in the formation of pyruvate from conversion of bicarbonate and also result in the production of intermediates such as acetyl-CoA, glyoxylate and succinyl-CoA. The energy costs of the 3-hydroxypropionate bi-cycle are high. It requires ten ATP equivalents (BERG, 2011). The key enzyme is the biotin-dependent acetyl-CoA/propionyl-CoA carboxylase and, although the pathway involves 19 steps, only 13 enzymes were required, due to the presence of a number of bi- and multifunctional enzymes in this pathway (BERG, 2011).

2.3.4 3-HYDROXYPROPIONATE/4-HYDROXYBUTYRATE CYCLE

The 3-Hydroxypropionate/4-Hydroxybutyrate Cycle results in the formation of acetyl-CoA from two bicarbonate molecules. This cycle is developed by chemolithotrophic microorganisms such as aerobic thermophilic archaea of the order *Sulfolobales,* anaerobic *Stygiolobus azoricus,* by *Crenarchaeota*, and, possibly, by the mesophilic *Thaumarchaeota* phylum (SATO; ATOMI, 2010; BERG 2011). The enzymes of this cycle tolerate oxygen and the synthesis of one pyruvate from this pathway requires five ATP equivalents (BERG, 2011).

2.3.5 DICARBOXYLATE/4-HYDROXYBUTYRATE CYCLE

The Dicarboxylate/4-Hydroxybutyrate Cycle was identified in the anaerobic hyperthermophilic archaeas of the *Thermoproteales* and *Desulfurococcales* and results in the formation of acetyl-CoA from one molecule of CO_2 and one molecule of bicarbonate. The enzymes of this cycle are oxygen sensitive and the synthesis of one pyruvate from this pathway requires nine ATP equivalents (BERG, 2011).

2.3.6 REDUCTIVE ACETYL-COA PATHWAY

The last pathway is also named Wood–Ljungdahl pathway or the acetyl-CoA (acetyl coenzyme A) pathway (BAR-EVEN; NOOR; MILO, 2012) This pathway is characterized by the use of H_2 as an electron donor, and CO_2 as an electron acceptor to produce acetyl-CoA which is catabolized to acetate as the main final product. This pathway is reductive and it is the only linear pathway of carbon fixation that can occur exergonically in the direction of CO_2-assimilation among CO_2 assimilation pathways. It is terminal electron-accepting process, that reduces CO_2 to acetyl-CoA, and provides the cell a mechanism for the fixation of CO_2 and/or other one-carbon moieties compounds into biomass (DRAKE; KÜSEL; 2003; SATO; ATOMI, 2010; MÜLLER; FRERICHS, 2013; XAVIER; PREINER; MARTIN, 2018).

The Wood-Ljungdahl pathway was discovery in 1932 by Fischer *et al*. (1932) and the first acetogen to be isolated was *Clostridium aceticum* from soil, by Wieringa, in 1936. However, this pathway was elucidated just in 1958 by Harland G. Wood and Lars G. Lungdahl and, only in the 80's, the chemical and enzymological features of the pathway were resolved. The acetate production from H_2 and CO_2 is considered the oldest biochemical pathway and the first life-sustaining process on earth, since it is the only pathway of carbon dioxide fixation that also yields adenosine triphosphate (ATP). Nowadays, the acetyl-CoA pathway is recognized as a fundamental component of the global carbon cycle and essential to the metabolic potential of many different prokaryotes (DRAKE; GÖßNER; DANIEL, 2008). It is estimated that acetogenesis yields trillions of kg of acetate globally each year (RAGSDALE, 2008).

The organisms capable of using the reductive acetyl-CoA pathway can only tolerate oxygen at a very low level, because the pathway employs two of the most oxygen sensitive enzymes known: NADPH-dependent formate dehydrogenase and the CO-dehydrogenase-acetyl-CoA synthase complex (BAR-EVEN; NOOR; MILO, 2012). The Wood–Ljungdahl pathway entails oxygen sensitive catalysts, as its enzymes are replete with iron and nickel sulfur centers, essential for electron transfer and catalysis (XAVIER; PREINER; MARTIN, 2018).

This pathway consists of two reductive branches: the methyl and the carbonyl branches, that together reduce CO_2 and hydrogen to acetyl-CoA, and posteriorly to mainly acetate, and has a free energy change of approximately -95 kJ per mol acetate synthesized (DRAKE *et al*., 2008; MÜLLER; FRERICHS, 2013; KATSYV; MÜLLER, 2020). And the two major enzymes involved in these processes are carbon monoxide dehydrogenase and acetyl CoA synthase complex (RAGSDALE, 1991).

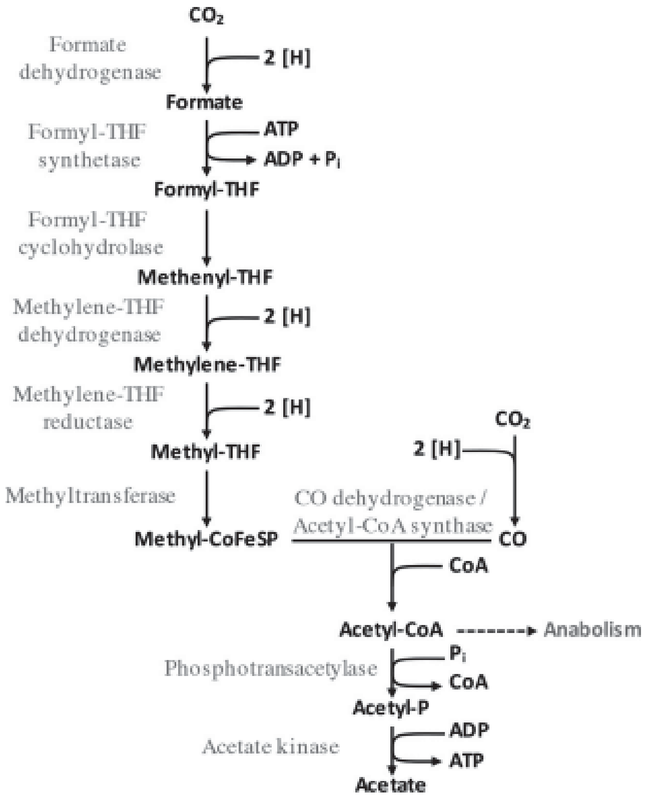
According to Ragsdale (1991), there are two parts in the acetyl-CoA pathway: (1) reduction of CO_2 to methyltetrahydrofolate (methyl-THF) and (2) synthesis of acetyl-CoA from methyl-THF and a carboxyl donor, such as CO or CO_2 and CoA. The equation that represents this pathway when acetate is the sole end product is the Equation 1. This equation represents the mechanism of autotrophic CO_2 fixation.

$$4H_2 + 2CO_2 \leftrightarrows CH_3COOH + H^+ + 2\,H_2O \qquad (1)$$

The Wood-Ljungdahl pathway is illustrated in Figure 1. The pathway begins with the eight reducing equivalents required to reduce CO_2, that are derived from hydrogen via hydrogenase (RAGSDALE, 1991). The reduction of a CO_2 to CO occurs in the carbonyl branch. The CO_2 is reduced to a carbonyl group (CO), via the carbon monoxide dehydrogenase/acetil-CoA synthase. The other CO_2 is reduced to a methyl group in the methyl branch (RAGSDALE, 1991). Regarding the first step of the methyl branch, one molecule of CO_2 is reduced to formate, catalyzed by formate dehydrogenase. Subsequently, formate is activated and bound to tetrahydrofolate (THF) to formyl-THF by formyl-THF synthase. The formation of formyl-THF is endergonic and requires the hydrolysis of ATP (adenosine triphosphate). In the next step, water is split off the formyl-THF by methenyl-THF-cyclohydrolase. The stepwise reduction of formyl-THF to methyl-THF is catalyzed by the enzyme methylene-THF dehydrogenase and methylene-THF reductase. The methyl group is transferred to a corrinoid iron–sulfur protein by a methyltransferase, and finally the enzyme complex carbon monoxide dehydrogenase/acetil-CoA synthase fuses the methyl group, the carbonyl group, and coenzyme A to form the central intermediate acetyl CoA.

Acetyl-CoA is then either incorporated into cell carbon (anabolism) or converted to acetate (by phosphotransacetylase and acetate kinase) and/or other products (catabolism) (RAGSDALE; PIERCE, 2008). The net synthesis of ATP formed via substrate-level phosphorylation is zero because one mol of ATP is gained in the acetate kinase reaction but the activation of formate, an intermediate of the pathway, requires one mole of ATP (GRAHAME, 2003; MÜLLER; FRERICHS, 2013). So, the microorganism growth by the production of acetate from H_2 + CO_2 is linked to ATP generation mechanisms for energy production (KATSYV; MÜLLER, 2020).

FIGURE 1. ACETIL-CoA PATHWAY AND THE BRANCHES



SOURCE: SCHUCHMANN and MÜLLER (2016).

LEGEND: The Acetil-CoA pathway consists of two reductive branches: the methyl and the carbonyl branches, that together reduce CO_2 to acetyl-CoA. Abbreviations: THF, tetrahydrofolate; CoFe-SP, corrinoid iron-sulfur protein; [H], reducing equivalent; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate.

The ability to utilize the acetyl-CoA pathway to form cell carbon is observed in acetogenic bacteria, methanogenic archaea and sulfate-reducing bacteria. Acetogenic bacteria generate acetyl-CoA from CO_2 and H_2, that is transformed in acetic acid as their sole or major end product of autotrophic fermentation (Equation 1). Sulfate-reducing bacteria runs the Wood-Ljungdahl pathway in reverse and generate metabolic energy by coupling the endergonic oxidation of acetate to H_2 and CO_2 (the reverse of Equation 1) to the exergonic reduction of sulfate to sulfite (Equation 2). Hydrogenotrophic methanogens grow on H_2 + CO_2 by the Wood-Ljungdahl pathway in the reductive direction (like acetogens, Equation 1) for CO_2 fixation. However, they conserve energy by the conversion of H_2 + CO_2 to CH_4 (Equation 3), since the formation of CH_4 is more favorable than acetate synthesis by interfacing the Wood-Ljungdahl pathway to the pathway of methanogenesis (RAGSDALE; PIERCE, 2008). In addition, acetoclastic methanogens (*Methanosarcina* and *Methanosaeta*) are able to utilize acetate as a carbon and energy source producing CH_4 and CO_2 as final products (DYKSMA; JANSEN; GALLERT, 2020).

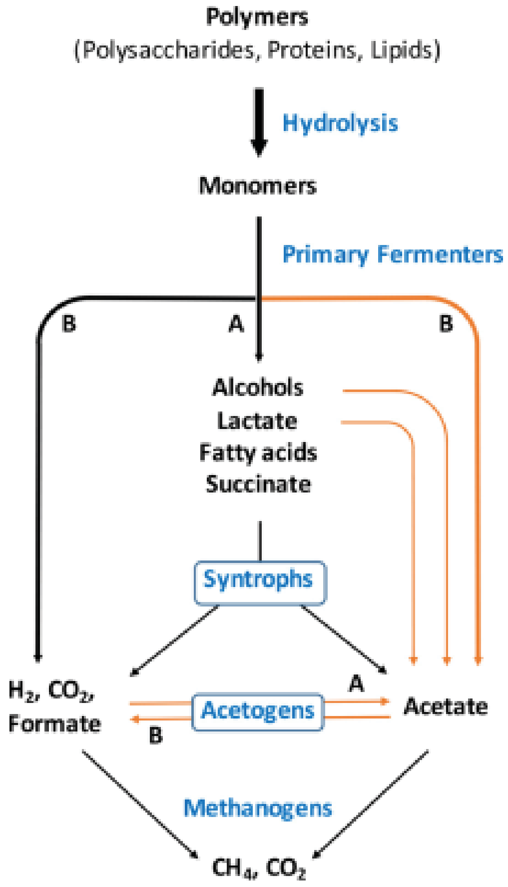
$$SO_4^{2-} + CH_3COOH + 2H^+ \leftrightarrows HS^- + 2CO_2 + 2H_2O \qquad (2)$$
$$CO_2 + 4H_2 \leftrightarrows CH_4 + 2H_2O \qquad (3)$$
$$CH_3COOH + H_2O \leftrightarrows CH_4 + HCO_3^- \qquad (4)$$

The use of acetyl-CoA synthase for a variety of processes in nonacetogens, such as methanogens, sulphate reducers, hydrogenogens, and possibly anammox bacteria, illustrates how widely various features of the acetyl-CoA pathway are distributed among evolutionarily diverse functional groups of prokaryotes. In some cases, acetogens can create a trophic relationship with nonacetogenic bacteria which are capable of rapidly consuming oxygen, but do not compete with the acetogens for the same substrate, allowing the acetogens growth under aerated conditions (DRAKE; KÜSEL; 2003). Acetogens have been found in a methanogenic mixed population. In these ecosystems, CO_2 and H_2 are utilized by acetogens and hydrogenotrophic methanogenic archaea, that compete for substrate (RASGDALE; PIERCE, 2008; MÜLLER; FRERICHS, 2013;). Thermodynamically acetogens are in disadvantageous relative to methanogens and sulfate-reducing bacteria, since the energy yield from the conversion of CO_2 and H_2 to methane is greater than that for conversion to acetate (free energy change - $\Delta G'_0$ = -131 kJ per mole methane and $\Delta G'_0$ = -95 kJ per mole acetate, respectively) (DRAKE; KÜSEL; 2003; SCHUCHMANN; MÜLLER, 2016). In addition, the larger amount of energy from the methanogenic reaction allows growth and energy conservation of methanogens at much lower substrate concentrations than necessary for acetogenesis, thus outcompeting them (SCHUCHMANN; MÜLLER, 2016). The competition is tightened when sulfate is present, since H_2 is a common substrate for sulfate reducers that can compete both with acetogens and with methanogens in the presence of sulfate. Therefore, if sulfate is present, sulfate reducers should be the dominant H_2-consuming species, and if not, methanogens should take over methanogenic reaction. However, acetogens seem to be more robust and can grow at lower temperatures, at slightly acidic pH and seem to be more resistant to oxygen comparing to methanogenic microorganisms (SCHUCHMANN; MÜLLER, 2016). Furthermore, the metabolism of acetogens is more flexible and not restricted to a few compounds, as in methanogens (MÜLLER; FRERICHS, 2013).

Another feature of the acetogens is the reverse acetogenesis, as indicated in Figure 2. Whereas acetogens produce acetate under high H_2 pressures, some acetogens can reverse this process if the H_2 pressure becomes very low. In this case, acetate is oxidized, presumably by reversing the Wood-Ljungdahl pathway. This adds to the remarkable flexibility that extends beyond a huge number of potential substrates to the possibility of completely reversing a metabolic pathway, depending on the environmental conditions (SCHUCHMANN; MÜLLER, 2016). However, in contrast to methanogens, which produce a gas that can be easily evaluated to assess their *in-situ* activities, the activities of acetogens are more difficult to assess under field conditions (DRAKE; KÜSEL; 2003).

The Figure 2 illustrates the relationship between the metabolism of acetogens and methanogens. Acetogens can take over functions of primary fermenters when utilizing carbohydrates or specialized substrates such as methoxylated compounds (methanol). They also can convert substrates such as alcohols or lactate. All this metabolic potential separates acetogens from methanogens, that are restricted to H_2 plus CO_2, CO, formate, methanol, methylamines, and acetate; and, to a lesser extent, from sulfate reducers that can, viewing all members of this group together, use many different substrates as well. However, most single sulfate-reducing species have a limited substrate spectrum and often only use H_2, short-chain fatty acids, and ethanol. However, acetogens and methanogens can also maintain a syntrophic relationship, such as when methanogens remove acetate in favor of the acetogen, converting it in CH_4 (SCHUCHMANN; MÜLLER, 2016).

FIGURE 2. RELATIONSHIP BETWEEN THE METABOLISM OF ACETOGENS AND METHANOGENS



SOURCE: SCHUCHMANN and MÜLLER (2016).

LEGEND: Acetogens and methanogens use the Wood-Ljungdahl pathway. Acetogens mainly produce acetate and methanogens produce methane to generate energy. Under low hydrogen pressure in a well-balanced fermentation, the major routes are labeled with B. Higher hydrogen pressures lead to an increased flux through the routes marked with an A. Conversions where acetogens can participate are shown in orange.

Their ability to reduce CO_2 to acetyl-CoA has made acetogens prime candidates for the production of biocommodities from CO_2. In addition, a number of acetogens can grow on and produce acetyl-CoA from CO or syngas, that contains a mixture of CO, H_2 and CO_2. Ethanol is already produced from syngas with *Clostridium ljungdahlii* on an industrial scale. Other products of interest are, for example, acetate, butanediol or biodiesel (MÜLLER; FRERICHS, 2013).

2.4 ACETOGENIC BACTERIA

Acetogenic bacteria, acetogens or acetate producing bacteria are anaerobic bacteria ubiquitous in nature and most of them are gram-positive. They are strictly anaerobic bacteria characterized by a reductive pathway in which two moles of CO_2 or

CO are reduced to one mol of acetyl-CoA, that can be incorporated into biomass or, in most cases, be converted to acetate as the end product by the Wood–Ljungdahl pathway (KATSYV; MÜLLER, 2020). This reaction provides only very little energy ($\Delta G°' = -95$ kJ/mol), still, acetogens evolved special adaptations to make possible their growth from this conversion (SCHUCHMANN; MÜLLER, 2016). If acetogens grow with CO as sole carbon source, one molecule enters the carbonyl branch directly, while another CO is oxidized to CO_2 by the carbon monoxide dehydrogenase (CODH) with simultaneous formation of reduced ferredoxin (KATSYV; MÜLLER, 2020). Therefore, they have come into focus for an alternative CO_2-based bioeconomy. These bacteria use the Wood–Ljungdahl pathway to CO_2 fixation and to the synthesis of ATP. Since CO_2 fixation can be driven by H_2 oxidation, these bacteria also capture and store H_2, a key process in the biohydrogen economy (MÜLLER, 2019). The key enzyme involved in their metabolism is the carbon monoxide dehydrogenase/acetyl CoA synthase complex, which differentiates acetogens from organisms that synthesize acetate by other metabolic pathways (SCHUCHMANN; MÜLLER, 2016;).

Acetogens are exclusively found in the domain Bacteria. Over 100 acetogenic species, representing 31 genera, were discovery. The most known species are *Acetobacterium* and *Clostridium*, but also other members of the Firmicutes, Spirochaetes (e.g., *Treponema primitia),* Acidobacteria (e.g., *Holophaga foetida*), Proteobacteria, Chloroflexi and Planctomycetes phylums can use this pathway. *Acetitomaculum, Acetoanaerobium, Acetobacterium, Acetohalobium, Acetonema, Alkalibaculum*, *Blautia, Butyrubacterium, Bryantella, Calderihabitans*, *Caloramator, Carboxydothermus*, *Clostridium, Desulfotignum, Desulfotomaculum*, *Fuchsiella*, *Eubacterium, Holophaga, Marvinbryantia, Moorella, Natroniella, Natronoincola, Oxobacter, Ruminococcus, Sporomusa, Syntrophococcus, Terrisporobacter*, *Thermoacetogenium, Thermoanaerobacter, Trindallia* and *Treponema* are the bacterial genera which contain species of acetogens. In some genera, e.g., *Acetobacterium* and *Sporomusa*, the members are exclusively acetogenic. However, many acetogens can be found in the genera containing both acetogenic and nonacetogenic bacteria (e.g., *Clostridium, Blautia, Eubacterium, Thermoanaerobacter* and *Treponema*) (DRAKE, 1994; DRAKE; KÜSEL; 2003; DRAKE; GÖßNER; DANIEL, 2008; RAGSDALE; PIERCE, 2008; SATO; ATOMI, 2010; NOOR; MILO, 2012; MÜLLER; FRERICHS, 2013; BAR-EVEN; BENGELSDORF *et al*., 2018).

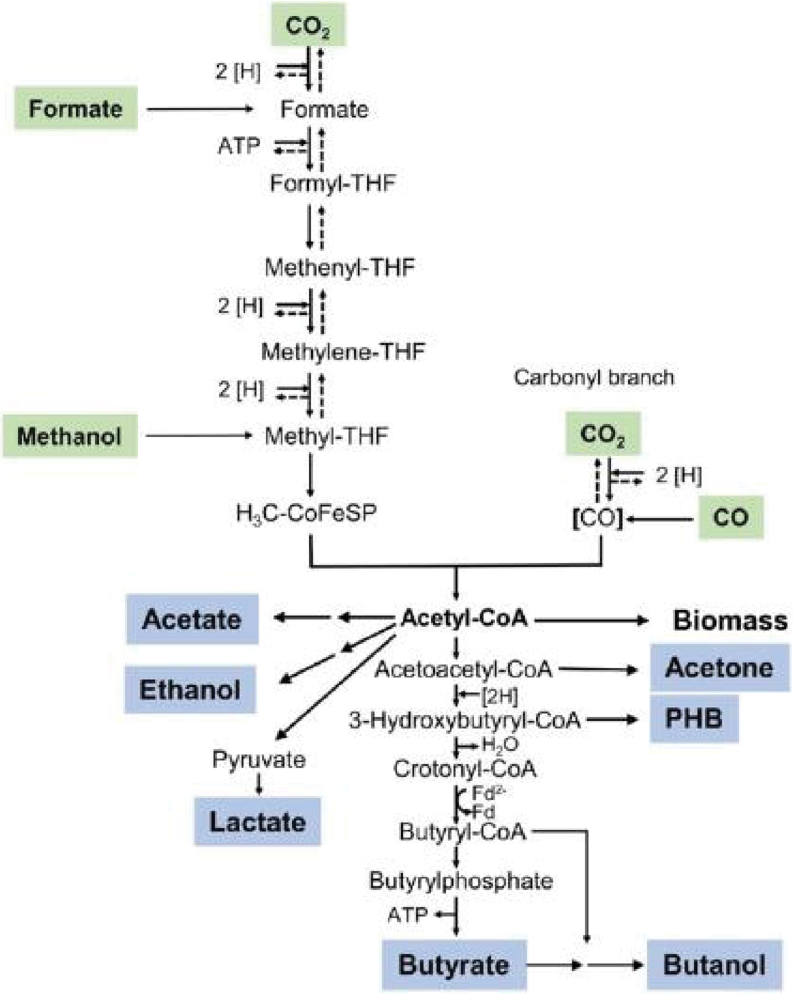
Acetogens constitute a phylogenetically diverse bacteriologic group, that can inhabit diverse habitats, ranging from different soils to termite hindgut, and also extremophiles ecosystems with respect to temperature, pH, and salinity. The optimal growth temperatures of the different strains range from 20 to 72°C and pH optima range between pH 5.4 and 9.8, but some strains grow in pH around 4.5. Thermophilic acetogens belong to the class of *Thermoanaerobacterales*, whereas mesophilic acetogens belong predominantly to the classes *Clostridiales* and *Selenomonadales* and are further classified as members of eight different families (BENGELSDORF *et al*., 2018).

Based on their 16S rRNA gene sequence similarities, acetogens are not tightly clustered phylogenetically, but are widely dispersed throughout the domain Bacteria (DRAKE; KÜSEL; 2003). Acetogens are widely distributed among a few members of many phyla, clearly indicating that acetogenesis is a metabolic, not a phylogenetic trait (RAGSDALE; PIERCE, 2008). Consequently, there are difficulties inherent in detecting, identifying, and characterizing diversity and distributions of acetogenic bacteria. Molecular biological approaches have been very useful for studies of the ecology of many functional groups of microorganisms, but acetogens present some particular challenges (LOVELL; LEAPHART, 2005).

Acetogens are among the metabolically most versatile anaerobic organisms. Their most prominent metabolic feature is autotrophic growth; however, most members also show an outstanding metabolic flexibility for utilizing a vast variety of different substrates (SCHUCHMANN; MÜLLER, 2016). The reducing power can be derived from the oxidation of inorganic, but also organic carbon sources. This group of bacteria can grow autotrophically (synthesizing organic compounds from inorganic substances) on H_2 and CO_2 or CO and/or heterotrophically (employs organic compound as an external source of energy) on numerous organic compounds (for example, carbohydrates, formate, acetoin, glycerol, lactate, pyruvate, glyoxylate, glycolate, n-propanol, n-butanol, oxalate, fumarate, short-chain fatty acids, aldehydes, substituent groups of aromatic compounds, methyl-groups (methanol), arginine and numerous other organic and halogenated substrates). Some species also can grow mixotrophically (use a mix of different sources of energy and carbon) on H_2 and an organic substrate (DRAKE; KÜSEL; 2003; LOVELL; LEAPHART, 2005; RAGSDALE; PIERCE, 2008; MÜLLER; FRERICHS, 2013; VALGEPEA *et al*., 2016; KATSYV; MÜLLER, 2020). In contrast to autotrophic growth, which is hardly competitive, metabolic flexibility is seen as a key ability of acetogens to compete in ecosystems, and might explain the almost-ubiquitous distribution of acetogenic bacteria in anoxic environments (SCHUCHMANN; MÜLLER, 2016). For this reason, although the reduction of CO_2 to acetate is the main characteristic of the metabolism of acetogenic bacteria, under certain conditions, less than 3 moles of acetate per hexose or even no acetate is produced during the growth of an acetogen, depending on the organism, growth conditions and substrate utilized (MÜLLER; FRERICHS, 2013).

The other natural metabolic products of acetogens are butyrate, ethanol, butanol, hexanoate, hexanol, acetone, 2,3-butanediol, formate, lactate, isopropanol, 3-hydroxybutyrate, 3-hydroxypropionate, isoprene, farnesene, butyrate, butanoic acid butyl ester, methylethylketone, and isobutanol (Figure 3) (BENGELSDORF *et al*., 2018). The pH control and the solubility of the substrate hydrogen have been identified as the most important rate-limiting parameters of autotrophic acetate production (SCHIEL-BENGELSDORF; DÜRRE, 2012), and high temperatures and pressures are often required for efficient catalysis (MÜLLER, 2019).

FIGURE 3. THE WOOD–LJUNGDAHL PATHWAY AND SOME OF THE NATIVE PRODUCTS



SOURCE: LITTY and MÜLLER (2021).

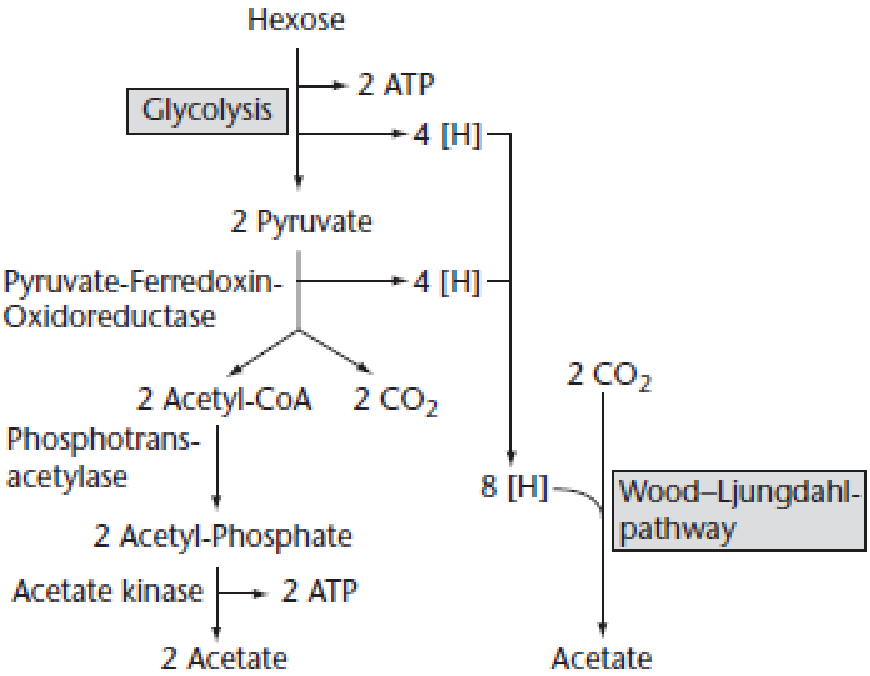
LEGEND: Acetyl-CoA is the precursor of biomass and produce a wide range of natural products (blue). The pathways leading from acetyl-CoA to products are not complete and miss intermediates, reducing equivalents and ATP input/output. Only the pathway leading to butyrate is complete. Abbreviations: CoFeSP, corrinoid/iron sulfur protein; Fe, ferredoxin; Fe^{2+}, reduced ferredoxin; THF, tetrahydrofolate; CoA, coenzyme A; [H], reducing equivalent.

During heterotrophic growth, with sugars as energy and carbon source, glycolysis is coupled to the Wood–Ljungdahl pathway via the pyruvate: ferredoxin oxidoreductase, which catalyzes the reaction from pyruvate to acetil-CoA, with concomitant production of CO_2. The resulting CO_2 is reduced in the Wood–Ljungdahl pathway, with reducing equivalents formed during glycolysis to produce additional acetyl-CoA (Equation 5) (RAGSDALE, 1991; RAGSDALE; PIERCE, 2008).

$$C_6H_{12}O_6 \leftrightarrow 3CH_3COOH \qquad (5)$$

The hexose is oxidized by way of the Embden–Meyerhof–Parnas pathway (glycolysis) to yield 2 moles of pyruvate, which are then split by the pyruvate: ferredoxin oxidoreductase to 2 moles of acetyl-CoA, reduced ferredoxin and CO_2. Acetyl-CoA is further converted to acetate by phosphotransacetylase and acetate kinase. This pathway yields 4 moles of of ATP by substrate level phosphorylation and it is the highest possible ATP-yield in a fermenting bacterium. This type of fermentation is defined as homoacetogenesis, since acetate is the sole end product formed (MÜLLER; FRERICHS, 2013).

FIGURE 4. HOMOACETIC CONVERSION OF HEXOSE TO ACETATE



SOURCE: DRAKE; GÖßNER; DANIEL (2008).

LEGEND: Fermentation of hexoses to acetate by heterotrophic acetogenic bacteria. This fermentation is referred to as homoacetogenesis. The two molecules of CO_2 that are reduced to acetate in the acetyl-CoA pathway can be derived from exogenous CO_2, rather than the CO_2 that is produced via the decarboxylation of pyruvate. Abbreviations: ATPSLP, ATP that is produced by substrate-level phosphorylation; [H], reducing equivalent.

The oxidation of various substrates, in addition to producing various products, generate different reduced electron carriers (KATSYV; MÜLLER, 2020) during heterotrophic growth. However, the electron carriers coupled to oxidation reactions may be different from electron carriers involved in the reductive branch. Therefore, a redox balancing module is essential to produce the correct electron carriers in the right amount for the Wood–Ljungdahl pathway. This is possible due to the conversion of redox carries into each other or balancing the cellular ferredoxin, H_2 and NADH pools by Rnf complex (designation stems from *Rhodobacter* nitrogen fixation) (MÜLLER, 2019; KATSYV; MÜLLER, 2020; KREMP; ROTH; MÜLLER, 2020; KUHNS *et al*., 2020).

In autotrophic growth, acetogens all employ the Wood-Ljungdahl pathway for CO_2 reduction, but they differ in how they couple it to the synthesis of ATP. The electrons for CO_2 reduction are ultimately derived from oxidation of H_2 (KREMP; ROTH; MÜLLER, 2020). From a thermodynamic perspective, the overall reduction of two molecules of CO_2 to acetate, with H_2 as the electron donor is an exergonic reaction. However, this is only the sum of the free energy changes of all intermediate reactions. Some individual reactions can be more exergonic, whereas others can be endergonic. Indeed, some reactions involve high energy barriers, and only few individual reactions are thought to be exergonic enough to enable energy conservation in the form of the generation of a chemiosmotic ion gradient (SCHUCHMANN; MÜLLER, 2014).

The largest thermodynamic barrier in the Wood–Ljungdahl pathway is the reduction of CO_2 to CO in the carbonyl branch of the pathway. This redox couple has a very low standard redox potential, which is more negative than the H^+/H_2 couple. The known electron carriers in the Wood–Ljungdahl pathway are NADH, NADPH and ferredoxin, and of these, only reduced ferredoxin can provide electrons for this reduction. Nevertheless, there is a large energetic barrier associated with the electron flow from hydrogen to ferredoxin, but all acetogens, that have been examined so far, solve this problem in a similar way, using enzymes that are capable of flavin-based electron bifurcation during this electron transport. The electron-bifurcation hydrogenases reduce ferredoxin and NAD simultaneously with H_2 (SCHUCHMANN; MÜLLER, 2014; KREMP; ROTH; MÜLLER, 2020).

The reactions that are involved in the flow of carbon in the methyl branch of the Wood–Ljungdahl pathway are highly conserved in all acetogenic bacteria. However, the electron flow in the redox reactions, that are involved in this branch, differs strongly between acetogens, regarding the electron carrier used. In the first step of this pathway, where CO_2 is reduced to formate, the oxidation of NADH is not sufficient to drive this reaction. However, ferredoxin, NADPH and H_2 all have physiological redox potentials that can possibly be used for this reaction. But the electron carrier utilized will depend on the acetogen specie (KATSYV *et al*., 2021). In the next step of the methyl branch of the Wood–Ljungdahl pathway, methenyl-THF is reduced to methylene-THF, in a slightly exergonic reaction that uses either NADH or NADPH as the reductant. Methylene-THF is then reduced to methyl-THF. This reaction has the most positive redox potential of the Wood–Ljungdahl pathway, and is highly exergonic even with NADH as the electron donor. However, the methylene-THF reductase, mostly, is not involved in the generation of a chemiosmotic gradient. In some acetogens, the reduction of methylene-tetrahydrofolate is catalyzed by an enzyme that consists of RnfC and/or Met, that couple the electron transfer from reduced ferredoxin to NAD, with the translocation of ions across the cytoplasmic membrane, generating generate ATP by ATP synthase. Therefore, none of the reactions that are involved in the flow of carbon in the Wood–Ljungdahl pathway is directly responsible for energy conservation in acetogenic bacteria. Instead, chemiosmosis is coupled to the reactions of the Wood–Ljungdahl pathway via the accumulation of reduced ferredoxin and balance of electron flow, which is then oxidized by membrane-integral protein complexes to generate a chemiosmotic gradient that is used for ATP generation (SCHUCHMANN; MÜLLER, 2014).

The low potential electron carrier ferredoxin can be reduced by only a few enzymes such as glycerol aldehyde 3-phosphatedehydrogenase or pyruvate: ferredoxin oxidoreductase, both employed during glycolysis by heterotrophic growth (KUHNS *et al*., 2020). During the autotrophic growth, ferredoxin must be reduced with H_2 as electron donor, but this reaction is highly endergonic. The energy barrier is overcome by electron-bifurcating hydrogenases. The electron bifurcation is a mode of energetic coupling of two redox reactions catalyzed by soluble enzyme complexes. The endergonic electron transfer reaction is achieved by coupling it to an exergonic electron transfer from the same electron donor to a second acceptor (SCHUCHMANN; MÜLLER, 2016; KUHNS *et al*., 2020). $NADP^+$ reduction with NADH is also endergonic and acetogens use an electron bifurcation to overcome the energetic barrier by an NADH-dependent reduced ferredoxin: $NADP^+$ oxidoreductase (Nfn). However, although the same reactions could be driven by ATP hydrolysis or an energized membrane, the electron bifurcation is the least expensive strategy (MÜLLER, 2019).

Some acetogens do not depend on cofactor such NADP or ferredoxin to catalyze the enzymes of the Wood–Ljungdahl pathway. They have hydrogen-dependent CO_2 reductase (HDCR), which uses H_2 directly for CO_2 reduction (MÜLLER, 2019; KUHNS *et al*., 2020).

In the metabolism of acetogens and all anaerobes, redox balancing is very important and not as easy to achieve, since suitable electron acceptors are limited and the low redox potentials of the electron acceptors often lead to thermodynamic barriers. In addition, NADH and reduced ferredoxin are generated in different amounts from different substrates. For acetogens, to solve the problem of redox balancing during the reduction of two CO_2 to acetate, the reduced electron carriers are balanced to achieve the right stoichiometry of the redox reactions by soluble, electron-bifurcating enzymes or membrane bound processes (SCHUCHMANN; MÜLLER, 2016).

The Wood–Ljungdahl pathway is used to reoxidize the electron carriers that are reduced during substrate oxidation. The electron carries that were not used in the Wood–Ljungdahl pathway are also oxidized. This process led to a translocation of ions across the cytoplasmic membrane (SCHUCHMANN; MÜLLER, 2016; KATSYV; MÜLLER, 2020).

The electrochemical Na^+ gradient across the cytoplasmic membrane drives the synthesis of ATP via a unique Na^+ -F_1F_O ATP synthase. So, in the autotrophic growth, the ATP left for growth and metabolism is generated via ion gradients (such as protons and sodium ions) across membranes and ATPase, because substrate level phosphorylation does not result in a net ATP generation, as only 1 ATP is formed in the acetate kinase reaction, and 1 ATP is needed for formation of formyl-THF (SCHIEL-BENGELSDORF; DÜRRE, 2012; KUHNS *et al*., 2020).

During chemiosmosis, a transmembrane electrochemical ion gradient is established by an electron-transport chain, in which exergonic electron transfer leads to the translocation of ions or protons out of the cell, across the cytoplasmic membrane (KUHNS *et al*., 2020). The established electrochemical ion potential is then the driving force for ATP synthesis, by a F_1F_O-ATP synthase (MÜLLER, 2019; SCHOELMERICH; MÜLLER, 2020). In all off this process, 0.3 mol of ATP is synthesized via the formation of one mol of acetate, with H2 + CO2 as substrate. When acetogen is able to oxidize CO as substrate, the yield is 1.5 ATP/mol of acetate (KATSYV; MÜLLER, 2020).

There are two main mechanisms of energy conserving in acetogens: the Rnf complex (ferrodoxin-NAD^+ oxidoreductase) and the energy-converting hydrogenases (Ech complex - ferrodoxin-H^+ oxidoreductase) (MÜLLER, 2019; KATSYV; MÜLLER, 2020; SCHOELMERICH; MÜLLER, 2020).

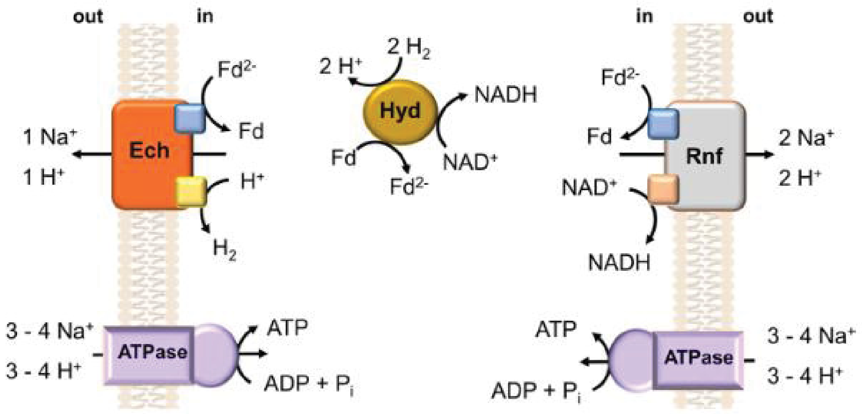
The Rnf- and the Ech-complex are membrane-bound enzymes that both use reduced ferredoxin as reductant, both resulting in the generation of a transmembrane gradient, which in turn is used for ATP synthesis. Acetogenic are generally classified as Rnf-containing or Ech-containing according to their form of energy conservation, however, some acetogenic have the cooccurrence of Rnf and Ech genes, for example: *Clostridium scatologenes* and *Sporomusa ovata* (XAVIER; PREINER; MARTIN, 2018; KATSYV; MÜLLER, 2020; SCHOELMERICH; MÜLLER, 2020).

The membrane-bound ferrodoxin: NAD^+ oxidoreductase is encoded by the *rnf* genes and also referred to as Rnf complex. The Rnf complex have six subunits (two subunits are predicted to be membrane integral, the other four are cytosolic) and is composed by flavins and iron-sulfur center as electron carriers and catalyzes ferrodoxin: NAD^+ oxidoreductase activity. The Rnf complex can export either protons or sodium ions by oxidizing reduced ferredoxin and transferring the electrons to NAD+, thus generating NADH. This gradient is then used for energy conservation by a Na^+-dependent ATP synthase, that drives a Na^+-dependent ATP synthesis, via a F_1F_O ATP synthase. This electron transport was coupled to a primary and electrogenic Na^+ transport into inverted membrane vesicles. In some acetogens species (e.g., *Clostridium ljungdahlii* and *Clostridium autoethanogenum*), Rnf complex is not Na^+, but H^+ motive (SCHIEL-BENGELSDORF; DÜRRE, 2012; MÜLLER; FRERICHS, 2013; KATSYV; BENGELSDORF *et al.*, 2018; MÜLLER, 2020).

The Ech complex generates a proton gradient by oxidizing reduced ferredoxin and transferring the electrons to protons, thus forming hydrogen. So, it couples H_2 metabolism to the electrochemical ion gradient across the membranes and leads to the establishment of a H^+ and Na^+ gradient, but just H^+ is harnessed for energy conservation by the H^+ -dependent ATP synthase. This electron transport is coupled to the export of ions across the cytoplasmic membrane and the electrochemical ion gradient established drives the synthesis of ATP, also via a F_1F_O H^+-dependent ATP synthase (KATSYV; MÜLLER, 2020; SCHOELMERICH; MÜLLER, 2020).

Continuous and fast fluctuations in energy availability can be more easily met by Ech as coupling site than Rnf. This is because redox energy is directly transformed and saved in the form of a chemiosmotic gradient by Ech, since excess electrons are simply discarded as molecular H_2. Contrarily, in Rfn complex, the redox energy used for the formation of an electrochemical ion gradient must be stoichiometrically balanced, since electrons remain in the system as NADH. Getting rid of excess redox energy requires a precise adjustment of protein abundance, which takes time and energy (SCHOELMERICH; MÜLLER, 2020).

FIGURE 5. THE RFN AND ECH ENERGY CONSERVING MECANISMS IN ACETOGENS



SOURCE: ROSENBAUM and MÜLLER (2021).

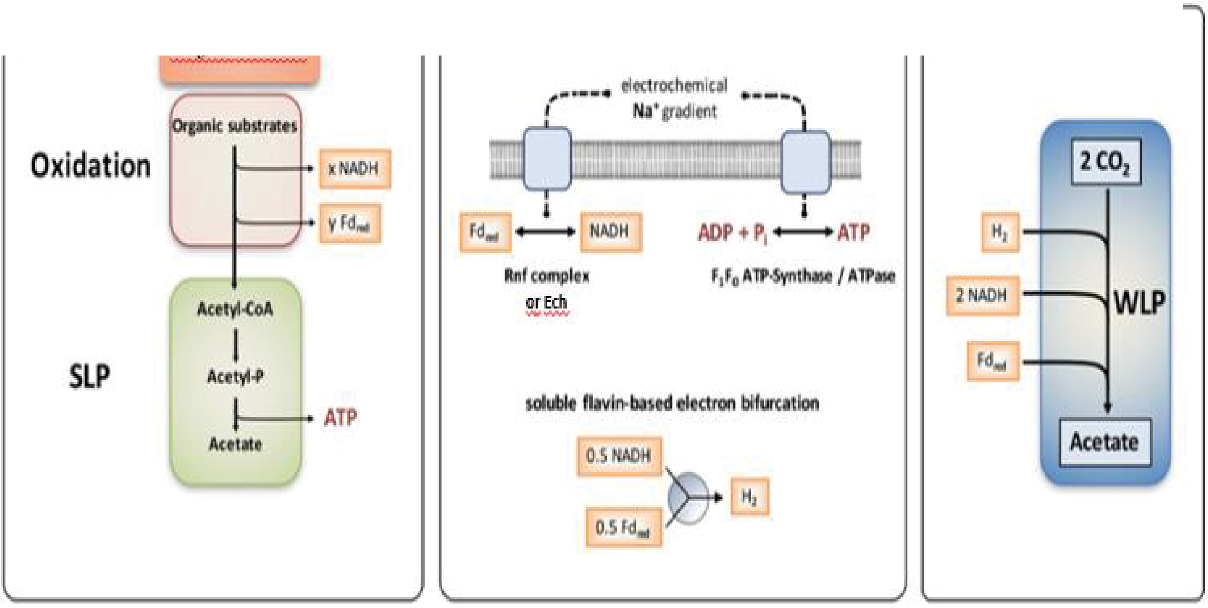
LEGEND: Energy conservation by a chemiosmotic mechanism in acetogenic bacteria. Depicted is an Ech- (left) and a Rnf-containing respiratory chain (right). An electron-bifurcating, ferredoxin and NAD-reducing hydrogenase provides reduced ferredoxin as fuel for both electron transport chains.

The arginine deiminase pathway (also referred as arginine dihydrolase pathway) represents another way of energy conservation in some acetogens. It enables the conversion of arginine to ornithine, carbon dioxide, and ammonia with the concomitant formation of 1mol of ATP. L-Arginine is converted to L-citrulline and ammonia by an arginine deiminase. Afterward, the functional carbamoyl group of L-citrulline is coupled to phosphate by an ornithine yielding L-ornithine and carbamoyl-phosphate. Carbamoyl-phosphate phosphorylates ADP (adenosine diphosphate) by the action of a carbamate kinase and yields ATP, carbon dioxide, and ammonia. The key enzyme of this pathway is the L-arginine/L-ornithine transporter, that allows stoichiometric exchange of substrate L-arginine and end product L-ornithine (BENGELSDORF *et al*., 2018).

Some acetogens contain cytochromes in their membranes. When cytochromes were discovered, they were thought to be linked to the respiratory chain involved in acetogenesis. Unfortunately, there is no evidence for that. Indeed, it may well be that cytochromes are not involved in acetogenesis (CO_2 reduction) but in the reduction of alternative electron acceptors such as nitrate (VISSER *et al*., 2016; KATSYV; MÜLLER, 2020).

In summary, the catabolism of the acetogens can be separated into three modules (Figure 6). Firstly, electros of the oxidized substrates are transferred to NAD^+ and ferredoxin (Figure 6A). Either a soluble hydrogenase using electron bifurcation or a membrane-bound (Rfn or Ech) can promote the redox balancing between modules A and C. The Rnf or Ech complex is coupled to ATP synthesis via an electrochemical sodium ion gradient and a Na^+-dependent F_1F_O ATP synthase/ATPase (Figure 6B). So, the Wood–Ljungdahl pathway can be regarded as an energy-neutral, cytoplasmic enzyme system for reoxidation of NADH and reduced ferredoxin (Figure 6C) (SCHUCHMANN; MÜLLER, 2016).

FIGURE 6. STEPS OF ACETOGEN CATABOLISM

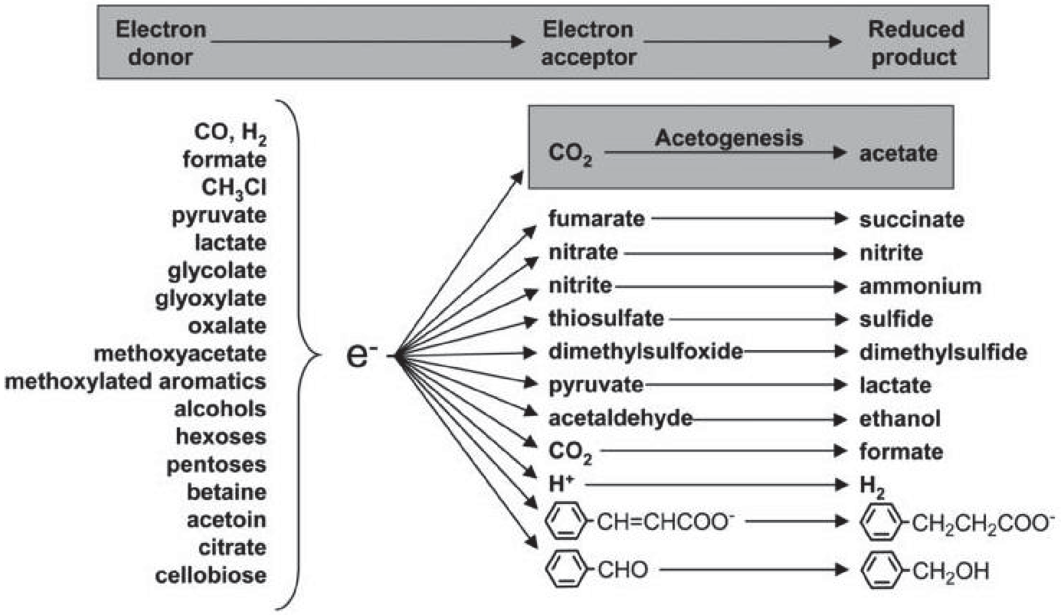


SOURCE: SCHUCHMANN and MÜLLER (2016).

LEGEND: The acetogen metabolism is composed of well-defined modules. Abbreviations: FDred, reduced ferredoxin; ADP, adenosine diphosphate; ATP, adenosine triphosphate; WLP, Wood–Ljungdahl pathway.

In addition to being able to use various electron donor substrates to grow, the acetogens can use a diverse number of terminal electron acceptors substrates, associated with another metabolic pathways, resulting in different reduced end products (Figure 7). Besides CO_2, electron acceptors include nitrate, nitrite, thiosulfate, and dimethylsulfoxide. Each acetogen displays a different propensity relative to the use of alternative electron acceptors, and it depends on the condition of growth and the environmental, presence of substrates, availability of CO_2, presence of O_2 and the ability of the acetogens to accommodate a wide range of unstable redox conditions. *Morella thermoacetica* and *Moorela thermoautotrophica* preferentially use nitrate as the terminal electron acceptor, which is reduced to nitrite and ammonium. Nitrate provides more energy to the cell, because it has higher redox potential than CO_2 as electron-accepting, thus it is not surprising that nitrate is preferred over CO_2. However, nitrate blocks carbon assimilation by the Wood-Ljungdahl pathway and no acetate is produced. The pathway blocking occurs due to the transcriptional regulation of genes encoding Wood-Ljungdahl enzymes (DRAKE; KÜSEL; 2003; RAGSDALE; PIERCE, 2008; MÜLLER; FRERICHS, 2013).

FIGURE 7. THE MANY POSSIBLES OF ELETRON DONORS AND ACCEPTORS AND THE RESULTING REDUCED END PRODUCTS IN ACETOGENESIS



SOURCE: DRAKE; GÖßNER; DANIEL (2008).

Although the basic process of CO_2 reduction to acetate is the same in every acetogenic species, the enzymes and redox factors involved are dissimilar, which opens a multitude of options to connect the CO_2 reduction pathway to many different oxidative modules. This is an exciting opportunity for biotechnology, since it allows the use of many different feedstocks and combinations thereof (MÜLLER, 2019).

In the last few years, the industrial interest in autotrophic production of bulk chemicals, as well as biofuels has risen intensively. The biotechnological production of chemicals and biofuels using anaerobic gaseous fermentation has important advantages compared to the corresponding chemical production. The biotechnological production is far more insensitive against variations or contaminations in the composition of the gaseous substrate and leads to a higher product specificity. Additional benefits are the possibility to use non-food feedstocks and the reduction of greenhouse gas emissions (DÜRRE, 2017).

The acetogens are already used on industrial scale to convert waste gas (syngas) into ethanol in Asia and Europe; and many more products (e.g., butanol, acetone and isopropanol) are currently under investigation, as a renewable energy source that do not emit CO_2 or, better still, capture CO_2 and convert it into valuable compounds, that generally come from crude oil based chemical manufacturing processes, that are non-renewable sources (DÜRRE, 2017).

A challenging task for the future is clearly to improve the energetic status of acetogens, that could be achieved by implementing additional ATP-yielding pathways through molecular biology techniques (MÜLLER, 2019).

The publication of genome sequences and the development of further molecular biological techniques, especially transformation protocols, pushed the use of acetogenic organisms as a production platform based on gases as sole carbon and energy source. Recombinant DNA approaches now opened the door to construct acetogens, synthesizing important industrial bulk chemicals and biofuels (SCHIEL-BENGELSDORF; DÜRRE, 2012; KATSYV; MÜLLER, 2020).

The presence of acetogenic bacteria in silages was described by Möller *et al*. (1984) and the isolated bacteria was Sporomusa ovata (DRAKE; GÖßNER; DANIEL, 2008). However, the Wood-Ljungdahl pathway has never been described for silages. The silage environment (anaerobiosis, pH and temperature) is prone for the development of these worldwide spread microorganisms. In the future, silos can be used as a bioreactor fixing pollutant gases from farm activities (CO, CO_2, N_2O) into high quality nutritive compounds of feed (acetate) (SCHMIDT; NOVINSKI; ZOPOLLATTO, 2018).

2.5 FINAL CONSIDERATIONS

There are many microorganisms and interactions among plant enzymes and the activities of microbial species. Numerous metabolites are produced during ensiling, and there are important interactions between the metabolites and the microbes.

However, it is important to note that most of the studies only report the identification of the microorganisms, metabolites and the pathways most abundant during the ensilage process, sometimes just confirming concepts already established in the silage universe. Maybe, there are some species whose impact on the community is large and disproportionately large to its relative abundance (POWER *et al.*, 1996), that are being overlooked. Furthermore, most diversity studies have focused on the LAB group, and thus there is a lack of information on other groups of microorganisms and particularly on the interactions between them (ÁVILA; CARVALHO, 2020).

In recent years, the knowledge of new species and the metabolism of different species and strains have been increased, which have improved the understanding of their interactions, however, this knowledge needs to be developed further, due to the diversity of and the interaction between microorganisms (ÁVILA; CARVALHO, 2019).

The observations found in the trials developed by the Forage Research Center explain the reason to conducted this work and affirm the possibility of a new metabolic pathway occurence in silages that, to the best of our knowledge, has never been described before: the Wood–Ljungdahl pathway. This pathway is used by some microorganisms to fix CO_2 as an energy source. The acetogens are anaerobic bacteria characterized to convert two moles of $CO_{2,}$ in the presence of H_2 to one mol of acetyl-CoA and then further to acetate or other products, which could explain the negative pressure inside the silos and the negative losses after silage fermentation.

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4 CHAPTER I - INTERNAL PRESSURE AND CARBON DIOXIDE ABSORPTION DYNAMICS IN SILAGES: A NOVEL APPROACH IN MAIZE SILAGE MICROBIOLOGY

Abstract

The aim was evaluating the dynamics of gas production, the internal pressure and carbon dioxide absorption throughout the fermentation of whole-plant corn silage (271 g/kg DM). Twenty-four PVC silos were filled (145 or 180 kg/m³ DM). Once the silos stopped the gas production, 12 silos were kept closed to pressure measurements and another 12 silos were weekly supplied with CO_2. Maize silage microbiota incorporate CO_2 and increase silage quality and dry matter content. Gas production was higher for the 180 kg DM/m^3 bulk density. All silos showed negative pressure. *Lactobacillus, Paenibacillus, Coprococcus* and *Erwinia* were responsible for the differences among treatments.

Key-word: Carbon dioxide, CO_2, CO_2 fixation, Microbiome

4.1 INTRODUCTION

This study came as a sequence of previous trials developed by the Forage Research Center. The first assays were initiated with the purpose of evaluating the greenhouse gas (GHG) emission during the fermentation of sugarcane and maize silages; and the potential for mitigation GHG using additives (SCHMIDT *et al*., 2011; 2012; SOUZA *et al*., 2015). At that time, the pollutant potential of silage production was poorly understood in comparison with the quantitative importance of this food in animal production system.

For accessing directly, the gas production and to collect the gases to evaluate its composition, it was developed a system that consists of an experimental PVC silo attached to a graduated chamber immersed in water, coupled to a 3-way valve (SCHMIDT *et al*., 2012; BUENO *et al*., 2020).

In the first trials using this method, it was surprisingly observed a pattern behavior never described before in silages during the fermentation process: the formation of negative pressure inside the silos. The hypothesis that it would be the accommodation of the mass inside the silo that caused the vacuum was soon discarded, after injections of atmospheric air without stabilizing the pressure (SOUZA *et al*., 2015). In addition, forced air injection at limited volume into lab silos do not increase dry matter losses nor gas production, and the volume of air applied into the silo was not recovered. Moreover, laboratory-scale silos often present negative values for gravimetric dry matter losses. And, although it is a usual finding in silage trials, negative values for DM losses are commonly considered as experimental errors and rarely discussed (SOUZA *et al*., 2015).

The progressive increase in the negative internal pressure of the silos, detected in many trials that applied the same methods, led to the hypothesis of a metabolization of the supplied air by microorganisms and the production of metabolites that would be incorporated into the silage dry matter (SCHMIDT; NOVINSKI; ZOPOLLATO, 2018).

In a pilot assay, SCHMIDT, NOVINSKI, and ZOPOLLATO (2018) tried to balance the negative internal pressure using as “inert gas” 99% pure carbon dioxide (CO_2). The authors made the gas available through a closed chamber, without forcing it inside the silos. The volume of absorbed gas was taken. After a week, the silos were surprisingly showing negative pressure again, and a new supply took place. The CO_2 continued being absorbed, and a total of 19 weekly CO_2 supplies were done along the 5 months of the trial. The silos absorbed 5590 ± 2492 mL of CO_2/silo during the whole trial.

Studies prove that several species, from the genera *Clostridium, Pseudomonas, Stenotrophomonas, Acetobacterium*, and others could convert CO_2 into organic products through microbial electrosynthesis for anaerobic-autotrophic growth (CHEN *et al*., 2021). Besides that, the anaerobic fixation of CO_2 by acetogenic bacteria is well reported (FUCHS, 1986) and the presence of these bacteria in silages was already described by Möller *et al*. (1984). However, the autotrophic Acetyl-CoA pathway of CO_2 fixation (the “Wood-Ljungdahl pathway”) has never been described for silages.

On the basis of the above, and assuming that silage packing density affect DM recovery and gas production of the silages (SUN *et al*., 2021b), the aim of this work was to evaluate and to measure gas production, pressure inside silos and carbon dioxide absorption throughout the maize silage fermentation phase in two packing densities. The microbial community and the products resulting from the silage fermentation process was also analyzed, in order to identify the microorganism population related to the negative pressure and CO_2 absorption.

4.2 MATERIAL AND METHODS

4.2.1 Experimental settings and ensiling

The procedures were conducted at the Forage Research Center (CPFOR) of Federal University of Paraná, in Curitiba, PR, Brazil. No herbicides or pesticides were used during planting.

Whole plant maize (hybrid PH285VyHR Pionner®) was harvested at 20 cm from the ground. The forage was processed in a stationary forage chopper adjusted to a theoretical length of cut of 10 mm. Twenty-four experimental PVC silos (8.8 L) were manually filled up in layers with the homogenized chopped forage. The maximum time interval between chopping and closing the silos was six hours. Samples of the fresh forage (1,000 g) were taken for chemical characterization. The DM content of whole corn plant at harvest was 271 g/kg DM and water-soluble carbohydrates (WSC) contents were 71.03 g/kg dry matter (DM) (Table 1). Other chemical compounds to characterize the maize forage before ensiling are presented in Table 1.

TABLE 1. CHEMICAL COMPOSITION OF THE HARVESTED MAIZE FORAGE AT ENSILAGE DAY

Item	Maize Forage
pH	6,02
Chemical composition (g/kg DM)	
Dry matter	271
Crude protein	52.9
NDF	547.8
ADF	263.2
IVDMD	762.92
EE	11.89
Ash	27.77
Water-soluble carbohydrates (g/kg DM)	
Raffinose	ND
Sucrose	1.10
Maltose	4.88
Xylose	ND
Glucose	19.55
Galactose	ND
Arabinose	ND
Fructose	38.83
Mannitol	3.69
Inulin	2.98
WSC total	71.03

pH: hydrogen potential; NDF: neutral detergent fiber assayed with heat-stable amylase; ADF: acid detergent fiber; IVDMD: In vitro dry matter digestibility; EE: ether extract; WSC total: total water-soluble carbohydrates; ND: not detected (below detection limit); each n = 1 (based on at least two analytical replicates).

Maize forage were either compacted to 145 kg DM/m^3 (n=12) or 180 kg DM/m^3 (n=12) and silos were sealed with an adhesive plastic polymer (Selabond®) applied around the cap, trying to get hermetically sealed silos. To achieve the target bulk density, the chopped forage was individually weighed and 4.5 or 5.6 kg were compacted according to the respective treatment. The treatments were composed of 6 replicates and they were: (-) 145 kg DM/m^3 (bulk density of 145 kg DM/m^3 and no CO_2-supply); (-) 180 kg DM/m^3 (bulk density of 180 kg DM/m^3 and no CO_2-supply), (+) 145 (bulk density of 145 kg DM/m^3 and CO_2-supply) and (+) 180 (bulk density of 145 kg DM/m^3 and CO_2-supply).

Silos were equipped with an apparatus to measure and recover the volume of gas produced (n=24), according to Bueno et al. (2020). In the lid of the cylinder-shaped silos, a 2-mm hole was made and a hollow metal pin inserted to connect a silicone hose, that passed through a three-way stopcock for gas measurement. Each silo was connected to a 1-L graduated low-density polypropylene cylinder (Ø-6.5 cm; 43 cm long). Those cylinders were placed with the mouth facing down and immersed in water to avoid any gas leakage. The lid and all the connections were sealed airtight (Figure 8).

FIGURE 8. PVC silos filled with chopped maize forage, closed with a proper lid, sealed adhesive plastic polymer and equipped with an apparatus to measure gas production.

During the first phase of silage fermentation, gas production (GP) measurements were directly taken several times per day in all silos by recording the gas trapped in the cylinder, and releasing it by the stopcock. The total GP was the sum of all measurements of each silo during the fermentation period. GP was tested just for the two bulk densities (145 kg DM/m^3 or 180 kg DM/m^3), since at this moment no CO_2 supply had been done.

Once the silos stopped the gas production and started showing negative pressure (day 19th after sealing), twelve silos were submitted to a weekly supply of gaseous dioxide carbon (CO_2 - RhomaGas®, purity degree ≥ 99,92%) (treatments (+) 145 and (+) 180, 6 replicates each) for 31 weeks. CO_2-supplies were made by the three-way stopcock. The access to the silo was closed and the cylinder immerse in water was filled up with CO_2 and the initial volume was recorded. Then, the valve was opened in order to balance the pressure inside the silo. After 30 seconds, the valve was closed again, and the absorbed volume (absCO_2) was taken as the difference between the initial and final volume of CO_2 inside the cylinder. Subsequently, the remaining CO_2 was discarded from inside the cylinder. In this way, CO_2 was not forced inside the silo (Figure 9).

FIGURE 9. Chambers filled with CO_2 available for silage absorption.

The other 12 silos (treatments: (-) 145 and (-) 180, 6 replicates each) remained closed, and were weekly submitted to the evaluation of internal pressure (IP) (the sum of all measurements) and the maximum internal pressure (MP) (the maximum pressure observed in the weekly measurements), using a mercury column manometer (Figure 10). The silo was coupled to the manometer and the valve was directed to allow access to the inside of the silo. After the measurement, the access to the silo was closed.

FIGURE 10. Assessment of the internal pressure of the silos.

Silos remained stored for 233 days in a controlled-temperature room (24±1 ºC), to avoid variations of the gas measurements due to the temperature. At opening time, silos were weighed again and the DM losses (DML) were estimated as the difference between the initial and final DM weight of each silo as described by JOBIM *et al*. (2007). Each replicate was taken and thoroughly mixed in a sterile plastic bag. Two samples (1,000 g) were collected from each replicate.

4.2.2 Laboratory and Chemical analyzes

The pre-ensiled forage and the silages, both in duplicate, were dehydrated in a forced ventilation oven at 65°C until reaching constant weight, for assessing the DM content (AOAC, 1990). Dried samples were grounded through a 1-mm screen in a Wiley mill.

The absolute dry matter (105°C) content was determined (AOAC, 1990) and corrected for the loss of volatiles during drying using the equation proposed by WEISSBACH and STRUBELT (2008). Hence, all presented data are based on DM corrected for loss of volatile compounds.

Silage pH was determined in water (25 g silage and 225 mL distilled water, homogenized by one minute), according to KUNG *et al*. (2000).

The dried pre-ensiled forage and silage of each replicate samples were analyzed for crude protein content (CP) (AOAC, 1990), ether extract (EE) by Soxhlet (AOAC, 1990), ash (AOAC, 1990), neutral detergent fiber (NDF - assayed with a heat stable amylase and sodium sulfite) (MERTENS, 2002), and acid detergent fiber (ADF) (VAN SOEST, 1973). In vitro DM digestibility (IVDMD) was determined using a Daisy II incubator (ANKOM Technology, Macedon, USA) (HOLDEN, 1999). The solutions were prepared according to TILLEY and TERRY (1963).

4.2.3 Chemical and fermentation profile analyses

A cold-water extract was prepared by gently shaking the fresh silage samples (50g) with 200 mL distilled water and 1 mL toluene and refrigerated overnight (4°C). The extracts were then filtered using MN 615 filter paper and frozen (-4°C) (WEISS; KAISER, 1995). For the WSC analyses, cooled samples (100 g) and 250 mL of distilled water were ground and mixed in a food processor for five minutes. Samples of the mixture (30 g) were freeze-dried. The filtered extracts and the freeze-dried samples, properly stored (4-8°C), were conducted for analysis to Faculty of Life Science of Humboldt Universität zu Berlin, Germany.

Lactic acid was analyzed using methodology describe by WEISS and KAISER (1995). The volatile fatty acids (acetic, propionic, butyric, valerian and caproic) and alcohols (methanol, ethanol, propanol, butanol, 1,2-propanediol, 2-butanol and 2,3-butanediol) were determined by WEISS (2001) and the detection limit for each variable was 0.001% of DM. Acetone, ethyl lactate, ethyl acetate, propyl acetate, were also determined (WEISS; SOMMER, 2012) and the detection limit for each variable was 0.0001% of DM. The ammonia-N (N-NH_3) was analyzed colorimetrically based on the Berthelot reaction, using a continuous flow analyzer (CFA, San++, Skalar Analytical, Breda, Netherlands). The WSC concentration (raffinose, sucrose, maltose, xylose, glucose, galactose, arabinose, fructose, mannitol and inulin) was determined according to VON LENGERKEN and ZIMMERMANN (1991) by HPLC/RI detection. All fermentation product variables were determined in duplicate.

In order to correlate the potential acetogenic reaction to the verified data, the estimated acetic acid production (EAC) was calculated based on the hypothetical assumption that all CO_2 supplied for the silos would be converted to acetic acid through the acetogenic reaction ($2\,CO_2 + 4\,H_2 \leftrightarrow C\,H_3COOH + 2\,H_2O$) described by Wieringa (1936).

4.2.4 Aerobic Stability analyses

After opening the silos, homogenization and sampling, the remaining silages were exposed to air at controlled temperature room (22.0 ± 0.75 °C) for 10 days. For aerobic stability assessment, 3 kg of silage were placed in 20-L plastic buckets on platforms 5 cm from the ground. The silage temperature of each replicate was recorded every five minutes by data loggers inserted at the geometric center of the mass inside the buckets. Ambient temperature was also recorded by two data loggers distributed across the room. Aerobic stability (AS) was defined as the number of hours that the silage remained stable before reaching 2°C above room temperature (MORAN *et al.*, 1996). The other variables calculated to determine the post-opening silage preservation time were: accumulated temperature (AT = defined as the sum of the temperature above room temperature) (O'KIELY, 1993), the maximum temperature recorded (MT) and hours to reach maximum temperature (TMT). Dry matter losses during aerobic exposure (DMLas) were also estimated (JOBIM *et al*., 2007).

4.2.5 Microbial population analyses

4.2.5.1 Total genomic DNA extraction

Approximately 50 g of the silage was ground in liquid nitrogen and 500 mg of each sample was subjected to DNA extraction using the ZR Fecal DNA MiniPrep® kit (Zymo Research, Irvine, CA), following the manufacturer's instructions. The integrity of the extracted DNA was determined by 1.0% (m/v) agarose gel electrophoresis, prepared in TAE buffer (400 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA). The concentration of DNA extracted from the samples was analyzed using NanoDrop® 2000 (Thermo Fisher Scientific, Waltham, MA).

4.2.5.2 Sequencing of the total 16S rRNA gene using Illumina MiSeq

To produce the amplicon of the V4 hypervariable region of the 16S rRNA gene, the extracted DNA was subjected to amplification using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and barcoded 806R (5'-GGACTACHVGGGTWTCTAAT-3') (CAPORASO *et al*., 2011). Amplification conditions were determined for final volume reactions of 25 µL with GoTaq®G2 DNA Polymerase system (Promega, Madison, WI) according to manufacturer instructions, and with 20ng of DNA. Amplification cycling was performed on a Veriti thermal cycler (Applied Biosystems, Waltham, MA) programmed to do the following thermal process: 94°C for 3 min, 18 cycles of 94°C for 15 s, 50°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. The reaction products were quantified with QubitTM HS DNA (Thermo Fisher Scientific, Waltham, MA), pooled and sequenced in a MiSeq System with MiSeq Reagent 500v2 kit (Illumina, Inc., San Diego, CA).

4.2.5.3 Sequence analysis

The analysis of the *fastq* file containing the raw sequences were performed using Quantitative Insights into Microbial Ecology (QIIME) (CAPORASO *et al*., 2010a). Briefly, the reads (R1 and R2) were joined using the command join_paired_ends.py.

The raw reads were pre-processed for quality control, adapters trimming and filter. The main criteria for trimming were sequencing oligonucleotides and barcodes length, and low-quality bases at 3’ extremes (quality score < 20). The valid sequences were grouped into OTUs using the UCLUST method at a similarity of 97% and aligned with PyNAST (CAPORASO *et al*., 2010b). The taxonomic affiliation of each representative sequence of OTUs was performed by comparison with the sequences available in the SILVA database (QUAST *et al*., 2013). Subsequently, the UCHIME method (EDGAR *et al*., 2011) was applied for chimeric sequences removal. Singletons and doubletons were also removed from the data set. After these steps, a sample normalization was carried, which consisted in re-sampling the data, generating a new normalized file, where all the samples were analyzed with the same number of sequences, based on the minimum sequencing effort. The sequence data were submitted to the NCBI database under biosample ID SAMN19011649.

4.2.6 Statistical analyses

Statistical analyses were performed with R Studio, version 1.4.1106 (R CORE TEAM, 2020). Initially, the outliers were removed. The residuals were tested for normal distribution by Shapiro-Wilk test and for variance homogeneity by Bartlett test. In the instance of normal distribution, data were analyzed as a completely randomized design following a 2x2 factorial scheme with two silo densities (145 e 180 kg DM/m^3) and two conditions (with or without CO_2 supplies) with six replicates. Two-way ANOVA was performed using fat2.dic from ExpDes.pt package. When significant interaction, effects were detected by the global F test, pairwise comparisons were performed using Tukey test. In the instance of non-normal distribution, firstly data were log10-transformed (internal pressure and xylose), prior to analysis to obtain normal distribution. When data transformation was not efficient to normalize the distribution (AS; TMAX, TMT, CP, raffinose, galactose, inulin, methanol, 2-3 butanediol and ethyl acetate), data were analyzed with ART-ANOVA (aligned ranks ANOVA), which is used to process nonparametric factorial analyses) (WOBBROCK *et al*., 2011). Data were transformed into aligned ranks and ANOVA was performed by ARTool package and pairwise comparisons among aligned rank means were processed by art.con (WOBBROCK *et al*., 2011).

GP were tested for all silos (CO_2-supplied and no CO_2-supplied) by a completely randomized design with two treatments (145 e 180 kg DM/ m^3 bulk densities). IP and MP were analyzed just for the silos that kept closed by a completely randomized design with two treatments (145 e 180 kg DM/m^3 bulk densities). The total volume of CO_2 absorbed per silo (absCO_2) and the EAC were analyzed for CO_2-supplied silos by a completely randomized design with two treatments (145 e 180 kg DM/m^3 bulk densities). GP, IP, MP, absCO_2 and EAC were tested using dic function from ExpDes.pt. package. Pearson correlation test was applied for VOC composition and absCO_2 to evaluate the associations between individual silage fermentation characteristics at silo opening and total CO_2 absorption. For all statistical analysis, P values ≤0.05 were considered significant.

For bacterial community structure and composition, three replicates of each treatment were considered outliers (data of the silos that showed sealing problems were disregarded). The tested samples were: treatment (-) 145 kg DM/m^3: 2, 3 and 5; treatment (+) 145 kg DM/m^3: 2, 4 and 5; treatment (-) 180 kg DM/m^3: 1, 3 and 4; and treatment (+) 180 kg DM/m^3: 3, 4 and 6.

The changes in the bacterial community structure and composition of the whole-plant corn silage were examined through alpha-diversity and beta-diversity analysis. Alpha diversity metrics (Chao 1, Observed OTUs and PD whole tree) were calculated using QIIME (CAPORASO *et al*., 2010a), and this data were analyzed by a one-way analysis of variance (one-way ANOVA) using R software (R CORE TEAM, 2020) followed by a Tukey test at 5% significant level and plotted as a boxplot.

To evaluate the differences in the bacterial community structure among treatments, a pairwise dissimilarity matrix between samples was calculated using weighted UniFrac (LOZUPONE; KNIGHT, 2005), and the results of the PCoA analysis were plotted on a two-dimensional plot using the software PAST 3.03 (HAMMER *et al*., 2001). Relative abundance (RA) of the main genera (RA > 1%) was calculated using QIIME (CAPORASO *et al*., 2010a), and this data was used to build the taxonomic graphs at genus level. SIMPER analysis based on Bray-Curtis index was applied to assess which bacterial OTUs were primarily responsible for the observed differences using PAST software. A one-way ANOVA using R software, followed by a Tukey's test at 5% significant level was used to determine significant different taxa among treatments.

The bacterial taxa abundances reported from SIMPER analysis and contributing to ≥ 0.1% to dissimilarities in bacterial community structures among treatments were used to create another matrix with the variables DML, GP (L/kg DM), AS, CP, NDF, ADF and fermentation end-products. To establish the correlation between the variables mentioned above, a principal component analysis (PCA) with a biplot was adopted and was generated using the software PAST 3.03 (HAMMER *et al*., 2001).

4.3 RESULTS

4.3.1 Gas production, pressure and CO_2 absorption

At silo opening, in the hollow metal pin inserted to connect the silicone hose at the bottom of four silos (treatment (-) 180 kg DM/m^3: replicates 2 and 5; treatment (+) 145 kg DM/m^3: replicate 1; and treatment (+) 180 kg DM/m^3: replicate 1) was observed microbial growth reddish in color. The colonies observation under an optical microscope proved to be fungal colonies. In the replicates 1 and 4 of the treatment (-) 145 kg DM/m^3 and replicates 2 and 5 of the treatment (+) 180 kg DM/m^3, were observed few white colonies of molds in the top surface (5 cm) of the silage. These replicates were considered outliers for bacterial community structure and composition analyses, as this proves the airflow due to poor silo sealing and for corresponding to the silos that performed less vacuum formation and that had lower CO_2 absorption.

An undesirable variation inside treatments in both bulk densities for internal pressure and CO_2 absorption was detected. These variability among replicates are probably due to some silo sealing failure, and impacted the observation of some treatment effects (Table 2).

TABLE 2. GAS PRODUCTION, PRESSURE AND CO_2 ABSORPTION THROUGHOUT 233 DAYS

Item	**All silos**		**Mean**	**SEM**	**CV (%)**	***p-value***
	145 kg/m^3	**180 kg/m^3**				
GP, L	8.47	9.04	8.75	0.18	10.19	0.12
GP, L/kg DM	5.92^b	6.29^a	6.10	0.06	4.54	<0.01**

Item	**No CO_2 supplies**		**Mean**	**SEM**	**CV (%)**	***p-value***
	145 kgm^3	**180 kg/m^3**				
IP, mmWC/kg DM	-19394.81^a	-6906.22^b	-13150.52	0.11	9.49	0.04*
MP, mmWC	-1266.67	-840.00	-1053.33	145.54	47.86	0.15

Item	**CO_2 supplies**		**Mean**	**SEM**	**CV (%)**	***p-value***
	145 kg/m^3	**180 kg/m^3**				
absCO_2, L/kg DM	10.7^a	3.38^b	7.03	1.32	65.22	<0.01**
EAC, g/kg DM	49.29^a	12.74^b	31.01	6.41	71.56	<0.01**

GP: gas production; IP: internal pressure; MP: maximum pressure; absCO_2: total CO_2 absorbed; EAC: estimated production of acetic acid; SEM: Standard error of the means; CV: coefficient of variation; L: liters; L/kg DM: liters per kilogram of dry matter; mmWC: millimeters of water column; mmWC/kg DM: millimeters of water column per kilogram of dry matter; g/kg DM: grams per kilogram of dry matter; p-value: based on F Test; * ($p < 0.05$) and ** ($p < 0.01$); Internal pressure (log10-transformed before analyze); n = 6 per treatment.

The GP lasted nine days (Chart 1). The measurement intervals were determined according to the speed of gas production: every hour for the first three days and gradually reducing until 9th day. From the 9th to the 19th day, the silos were constantly monitored. After the 9th day, no silo produced gas. Only after the 12th day, in some silos were possible to measure a slight negative internal pressure. However, only on the 19th day, the beginning of vacuum formation was verified in all silos.

The GP (L/kg DM) was different for both bulk densities ($p<0.01$), however, GP (L) was the same for both bulk densities ($p>0.05$) (Chart 1 and Table 2).

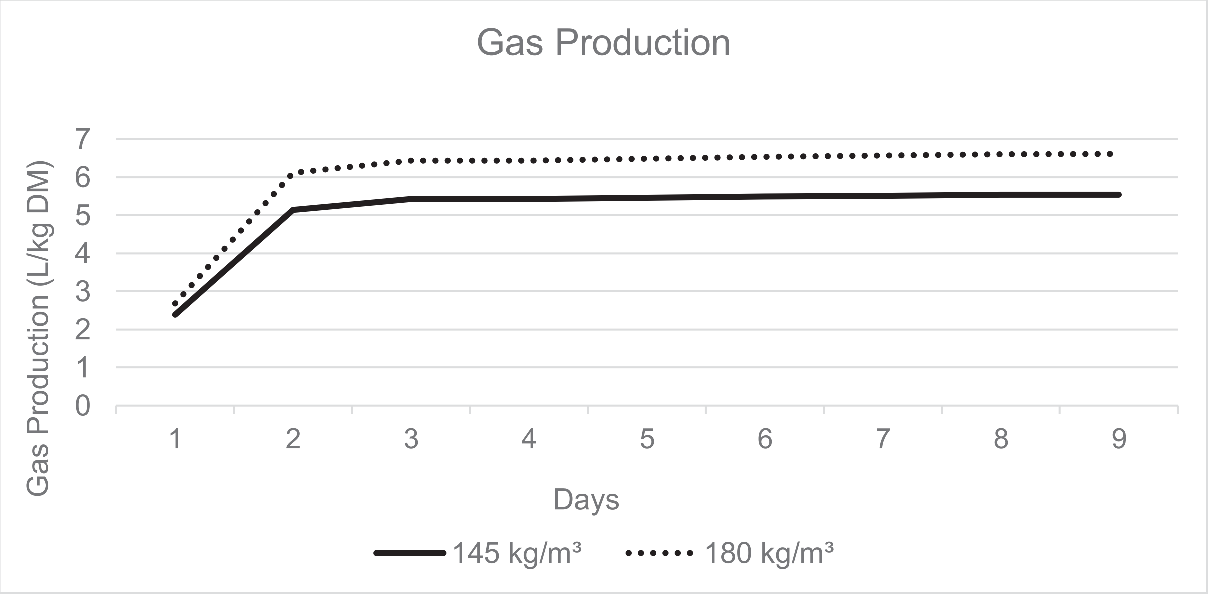


CHART 1. Gas production (L/kg DM) throughout the first phase of fermentation for the two bulk densities (n=6 per treatment).

The IP was more negative for the silos with 145 kg DM/m³, comparing with the silos 180 kg DM/m³ ($p<0.05$). However, MP was the same for both bulk densities ($p>0.05$). Even after the removal of outliers, the coefficient of variation remained high, which explain the lack of effect of bulk densities on MP (Chart 2 and Table 2).

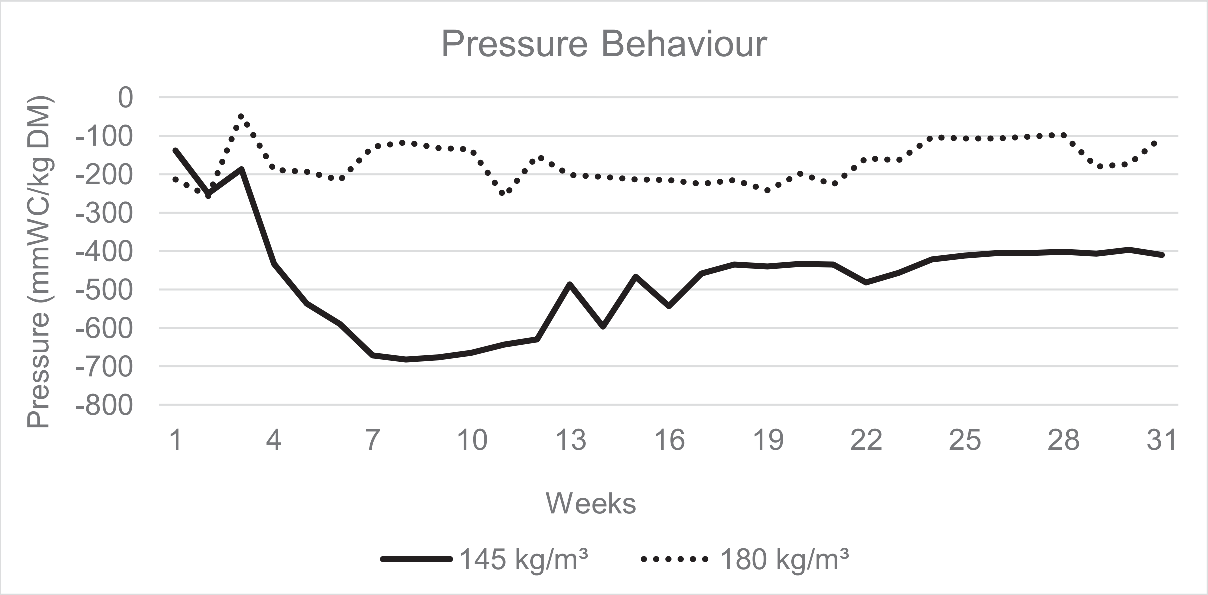


CHART 2. Negative pressure (mmWC/kg DM) behavior inside the silos (n=6 per treatment) throughout the trial for the two bulk densities.

The $absCO_2$ and EAC were higher for the lower bulk density ($p<0,01$) (Table 2). The lower bulk density treatment presented an increase in CO_2 absorption throughout the trial, while the 180 kg DM/m^3 treatment seemed to reach a plateau at the fifth months (Chart 3).

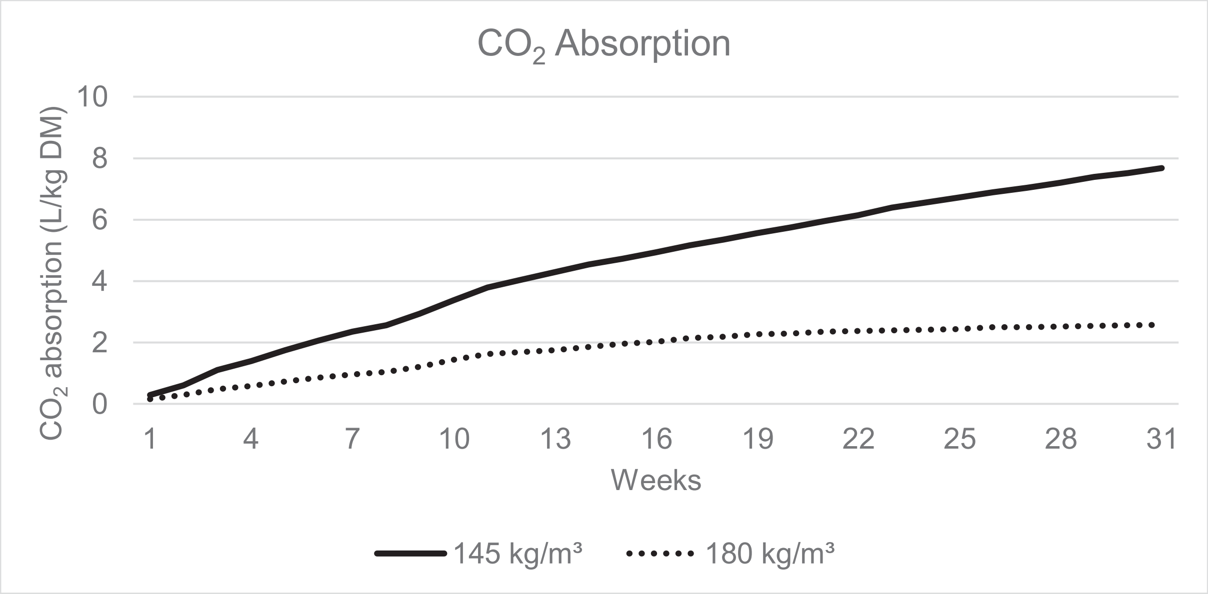


CHART 2. Accumulated CO_2 absorption pattern for the two bulk densities (n=6 per treatment) tested throughout the experiment.

4.3.1 Fermentation profile and volatile organic compounds at silo opening

The fermentation profile of the silage at silo opening is presented in Table 3. The DM of all treatments were subtly higher for silages when compared to fresh forage. There was a decrease in pH (6.02 to 3.68) throughout fermentation phase. The WSC content reduce during the fermentation up to 47%, compared to the maize forage. In addition to the reduction in WSC content, changes in the composition of the WSC were also observed. Sucrose, maltose and glucose, present in maize forage, were not found in maize silage. Raffinose, xylose and galactose were not observed in maize forage, but were detected in maize silage. Arabinose was detected neither in forage or silage. And there was a decrease in fructose (97.93%) and an increase in mannitol (86.75%) throughout fermentation phase (Table 3). In addition, butyric acid, propionic, valerian and caproic acids, propanol, butanol, 2-butanol, acetone and propyl acetate contents were below the detection limit.

4.3.2 Influence of condition and bulk densities in chemical composition and fermentation profile

The CO_2 supplies did not influence silage pH, nevertheless pH was higher for silos with bulk density of 145 kg DM/m^3 ($p<0.01$). Bulk density affected the content of EE and 1,2-propanediol. The EE content was lower and the 1,2-propanediol content was higher for the density of 145 kg DM/m^3 ($p<0,01$). The CO_2 supplies slightly increased the content of DM (2.1%), 2,3-butanediol (14.6%) and ethyl lactate (50%) ($p<0.05$). Acetic acid tended to be higher for the CO_2-supplied silages ($p=0.097$). The content of ethanol was higher for silos that did not receive CO_2 supplies ($p<0.05$). And a trend ($p=0.08$) of moderate positive correlation ($r=0.53$) between CO_2 absorption and the content of acetic acid was detected. The WSC total content was not influenced by main effects or their interaction. Raffinose, xylose and galactose were affected by interaction between the condition and the bulk densities ($p<0.05$) (Table 3). No other effect of treatment was detected.

TABLE 3. EFFECT OF CONDITION (C), BULK DENSITIES (D) AND CONDITION X BULK DENSITIES (C X D) ON CHEMICAL COMPOSITION, WATER-SOLUBLE CARBOHYDRATES AND ON FERMENTATION PROFILE OF THE SILAGES AT SILO OPENING

Item	Bulk densities		Condition		Mean or Median	SEM Or IQR	Effects		
	145 kg/ m^3	180 kg/m^3	No CO_2	CO_2			C	D	C x D
pH	$3,70^a$	$3,65^b$	3,68	3,67	3,68	0,01	0,41	0,002*	0,700
Chemical composition (g/kg DM)									
Dry matter	284.5	281.7	280.0^b	286.0^a	283.1	0.14	0.01	0.25	0.28
Crude protein	54.5	54.4	54.0	54.7	54.5	0.04	0.13	0.96	0.96
NDF	421.0	423.7	428.7	416.1	422.4	0.44	0.17	0.76	0.65
ADF	230.9	234.0	237.2	22.77	232.4	0.25	0.07	0.54	0.72
IVDMD	780.2	779.0	775.3	783.9	779.6	0.32	0.21	0.85	0.58
EE	21.3^b	26.0^a	22.0^b	25.3^a	23.7	0.09	0.03	0.00**	0.44
Ash	24.3	23.6	24.9	23.1	24.0	0.05	0.09	0.50	0.89
Water-soluble carbohydrates (g/kg DM)									
Raffinose	1.17	0.00	1.26	0.00	1.04	0.20	0.00	0.07	0.01*
Xylose	3.60	3.74	3.99	3.36	3.67	0.13	0.00	0.34	0.02*
Galactose	2.04	2.49	2.26	2.26	2.26	0.21	0.95	0.19	0.00**
Fructose	0.69	0.92	0.98	0.62	0.80	0.14	0.23	0.44	0.87
Mannitol	27.49	28.21	27.63	28.44	27.85	0.54	0.29	0.51	0.35
Inulin	1.63	1.43	1.08	1.85	1.63	0.11	0.15	0.91	0.05
WSC total	36.82	37.54	37.55	36.81	37.18	0.51	0.49	0.51	0.80
Volatile Organic Compounds (g/kg DM)									
Lactic acid	56.22	59.99	57.07	59.11	58.01	0.25	0.70	0.49	0.46
Acetic acid	17.10	15.69	15.44	17.35	16.40	0.06	0.09	0.21	0.37
N-NH_3	0.87	0.85	0.84	0.88	0.87	0.00	0.57	0.71	0.56
Ethanol	7.05	7.34	8.12^a	6.29^b	7.20	0.04	0.02	0.70	0.08
Methanol	0.33	0.31	0.32	0.31	0.33	0.00	0.35	0.24	0.60
1,2 Propanediol	0.75^a	0.33^b	0.47	0.61	0.54	0.01	0.32	0.00**	0.68
2,3 Butanediol	0.46	0.43	0.41^b	0.48^a	0.46	0.00	0.02	0.05	0.20
Ethyl Acetate	0.00	0.00	0.00	0.01	0.00**	0.00	0.74	0.12	0.48
Ethyl Lactate	0.01	0.01	0.01^b	0.02^a	0.01*	0.00	0.00	0.43	0.43

NDF: neutral detergent fiber assayed with heat-stable amylase; ADF: acid detergent fiber; IVDMD: In vitro dry matter digestibility; EE: ether extract; NH_3-N: ammonia-N; WSC total: total water-soluble carbohydrates; SEM: Standard error of the means; IQR: interquartile range; D – effect of bulk density; C – effect of condition (closed or CO_2 supply); C x D – interaction effect; g/kg DM: grams per kilogram of dry matter; n = 6 per treatment; * ($p < 0.05$) and ** ($p < 0.01$); Based on Tukey's test for variables: DM, NDF, ADF, IVDMD, EE, crude ash, xylose (log10-transformed before analysis), fructose, mannitol, lactic acid, acetic acid, ammonia, ethanol, 1,2 propanediol and ethyl lactate; Based on global rank test for variables: CP, raffinose, galactose, inulin, methanol, 2,3 butanediol, ethyl acetate.

The unfolding of condition and bulk density interaction for the raffinose, xylose and galactose contents are described in Table 4. The raffinose content in the bulk density of 180 kg DM/m^3 was higher in silage that did not receive CO_2-supplies. In CO_2-supplied silages, raffinose content was higher in the density of 145 kg DM/m^3 than in the density of 180 kg DM/m^3. Comparing the CO_2-supplied silages, it was possible to observe that xylose content was 16,89% higher in the density of 180 kg DM/m^3 compared with the density of 145 kg DM/m^3. In the lower bulk density, no CO_2-supplied silages showed the higher xylose content when compared with CO_2-supplied silages. CO_2-supplied silages had the content of galactose 56,5% higher in bulk density of 180 kg DM/m^3 compared with the density of 145 kg DM/m^3. In the silages with the density of 145 kg DM/m^3, galactose content was 1,93 times greater in no CO_2-supplied silages than the 180 kg DM/m^3 silages (Table 4).

TABLE 4. THE UNFOLDING OF CONDITION AND BULK DENSITY INTERACTION OF RAFFINOSE, XYLOSE AND GALACTOSE

Bulk densities	Treatments		SEM or IQR
	No CO_2	CO_2	
	Raffinose (g/kg DM)		
145 kg DM/m^3	1.32^{Aa}	1.08^{Aa}	0.28
180 kg DM/m^3	1.17^{Aa}	0.00^{Bb}	0.28
	Xylose (g/kg DM)		
145 kg DM/m^3	4.16^{Aa}	3.05^{Ab}	0.20
180 kg DM/m^3	3.82^{Aa}	3.67^{Ba}	0.12
	Galactose (g/kg DM)		
145 kg DM/m^3	2.45^{Aa}	1.27^{Ab}	1.18
180 kg DM/m^3	1.62^{Aa}	2.92^{Ba}	1.39

SEM: Standard error of the means; IQR: interquartile range; g/kg DM: grams per kilogram of dry matter; Means followed by lowercase letters in rows and uppercase letters in columns differ statistically by Tukey's test or global rank test ($p<0.05$); Based on Tukey's test for variables: xylose (log10-transformed before analyze); Based on global rank test for variables: raffinose and galactose; n=6 per treatment;

4.3.1 Dry-matter loss and aerobic stability

The effect of condition, bulk densities and their interaction (C x D) on dry-matter losses (DML) and variables assessed throughout aerobic stability test are presented in Table 5. The DML during the silage fermentation phase was 1.5 times smaller in the CO_2-supplied silages. The DMLas throughout AS test was influenced by the bulk densities, being 71% higher in in the density of 145 kg DM/m^3 compared to the density of 180 kg DM/m^3.

TABLE 5. EFFECT OF CONDITION (C), DENSITIES (D) AND CONDITION X DENSITIES (C X D) ON DRY-MATTER LOSSES (DML) THROUGHOUT 233 DAYS AND VARIABLES ASSESSED THROUGHOUT AEROBIC STABILITY TEST (AS), AFTER SILO OPENING

Item	Bulk densities		Condition		Mean or Median	SEM or IQR	Effects		
	145 kg/m^3	180 kg/m^3	No CO$_2$	CO$_2$			C	D	C x D
DML, % DM	3.35	3.43	4.07^a	2.72^b	3.39	0.33	0.05*	0.89	0.83
AS, h	190	235	182	235	216.75	10.68	0.52	0.52	0.21
MT, °C	24.8	23.2	24.2	23.8	24.25	1.26	0.55	0.21	0.47
TMT, °C	138	100	147	78.2	119.25	15.62	1.00	1.00	0.83
DMLas, %	5.70^a	1.37^b	4.31	2.75	3.53	0.95	0.38	0.02*	0.54

DML: dry matter losses; AS: aerobic stability; MT: maximum temperature reached throughout aerobic stability test ; TMT: time to reach maximum temperature throughout aerobic stability test; DMLas: dry matter losses throughout aerobic stability test**;** %: percentage; h: hours; °C: Celsius degrees; SEM: Standard error of the means; IQR: interquartile range; D – effect of bulk density; C – effect of condition (closed or CO$_2$ supply); C x D – interaction effect; n = 6 per treatment; * ($p < 0.05$) and ** ($p < 0.01$); Based on Tukey's test for variables: DML; DMLas; Based on global rank test for variables: GP; AS; MT; TMT.

The other variables analyzed during the stability test did not show differences among treatments ($p>0.05$).

4.3.2 Bacterial community structure and composition of silages

4.3.2.1 Alpha diversity index estimations

Alpha diversity is a measure of within-treatment diversity. Alpha diversity summarizes both the species richness (total number of species) and/or evenness (abundance distribution across species) within a sample (CHONG *et al*., 2020). The alpha diversity was measured by observed OTUs, Chao1 index and PD (Phylogenetic diversity) whole tree (Figure 11).

The Tukey's test for the bacterial alpha diversity is shown as a boxplot in Figure 11. There was no significant difference among treatments for the diversity indexes except for the treatment (+) 145 kg DM/m^3 for Chao 1 ($p<0.05$) and observed OTUs ($p<0.05$). The Chao1 and the observed OTUs show that the treatment (+) 145 kg DM/m^3 had more abundance and diversity of species than the other treatments. Treatment (+) 180 kg DM/m^3 present high variation among replicates.

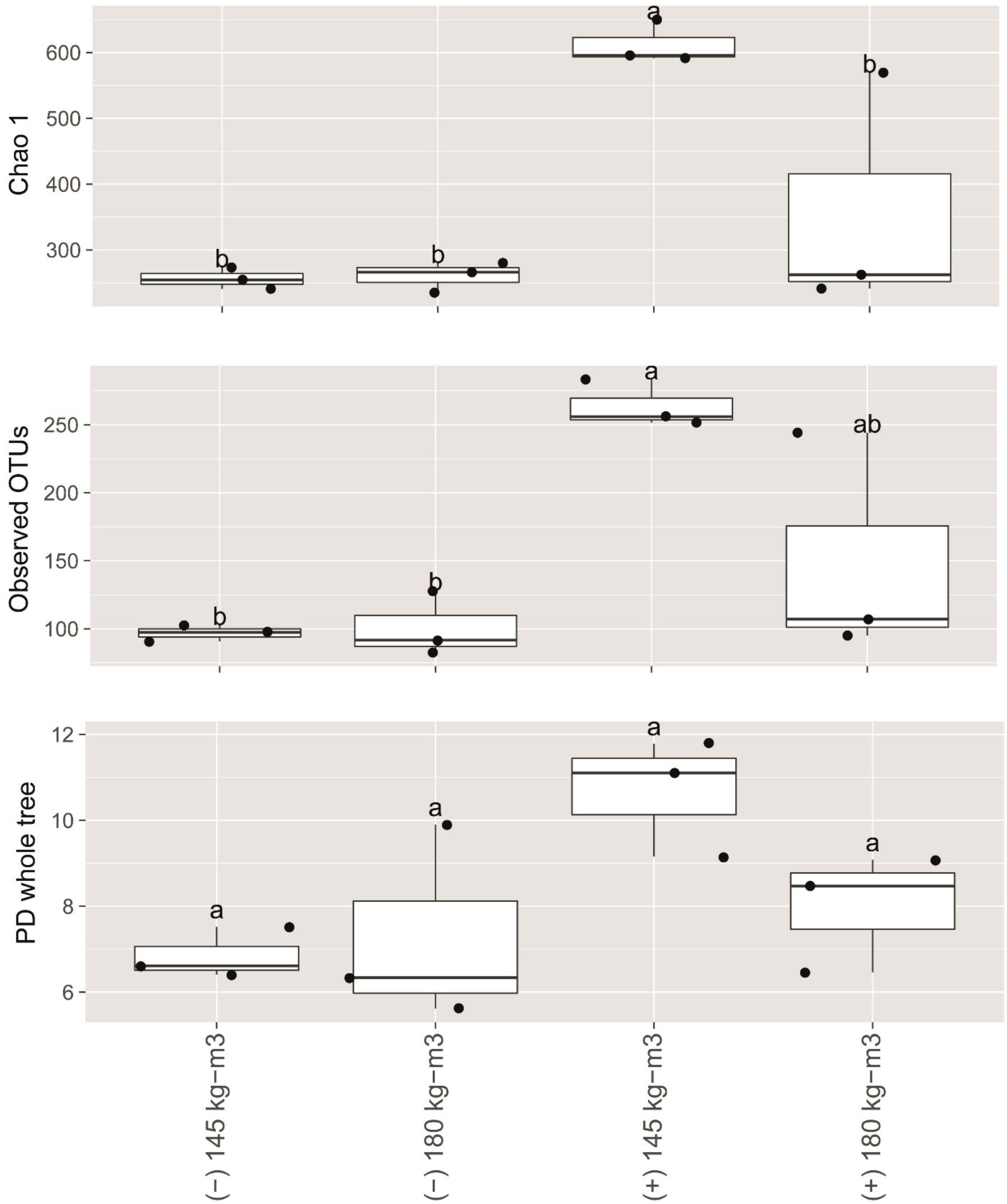


FIGURE 11. Boxplot of the bacterial alpha diversity for Chao 1, observed OTUs and PD whole tree indexes according to the treatments: (-) 145 kg DM/m^3, silo with bulk density of 145 kg DM/m^3 undergoing internal pressure measurement; (-) 180 kg DM/m^3, silo with bulk density of 180 kg DM/m^3 undergoing internal pressure measurement; (+) 145 kg DM/m^3, silo with bulk density of 145 kg DM/m^3 undergoing CO_2 availability; (+) 180 kg DM/m3, silo with bulk density of 180 kg DM/m^3 undergoing CO_2 availability. The black line represents the average, and the boxes represent the upper and lower quartile of the data. Different letters indicate significant differences ($p<0.05$).

4.3.2.2 Bacterial community structure and composition of whole-plant corn silages

The beta diversity is a measure of between-treatment diversity. It evaluates differences in the community composition between treatments (CHONG *et al*., 2020). The beta diversity is presented in PCoA (Figure 12), relative abundance (RA) (Figure 13), heat map (Figure 14) and PCA (Figure 15).

The PCoA combines beta diversity to a distance matrix to visualize patterns and compare sample distribution between the treatments (CHEN *et al*., 2020; CHONG *et al*., 2020). PCoA results demonstrated the bacterial community structure of silages. No dissimilarity was observed among treatments regarding the bulk density of the silos (Figure 12). However, the internal pressure and CO_2 absorption throughout the fermentation of silage influenced the bacterial community structure, since the CO_2-supplied silos clustered together over PC2 (Figure 12).

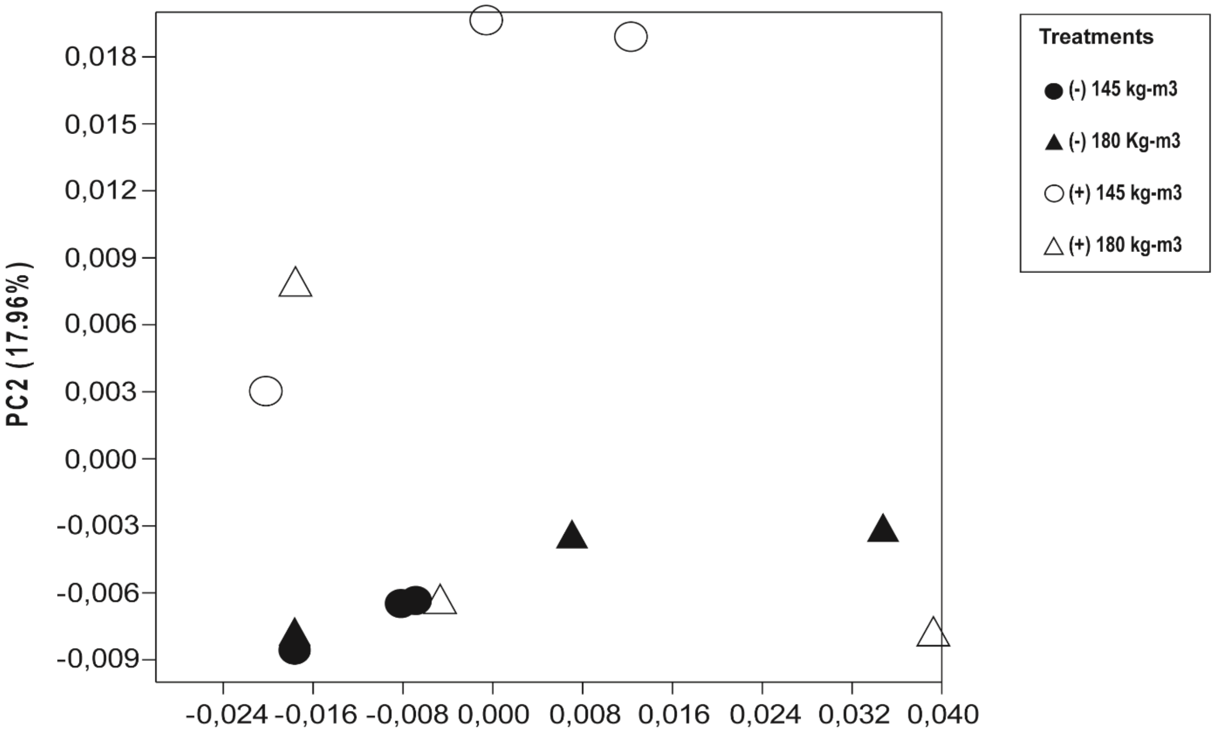


FIGURE 12. Weighted UniFrac principal coordinate analysis (PCoA) underlying the bacterial community structure of silages. (-) 145 kg DM/m^3, silo with bulk density of 145 kg DM/m^3 undergoing internal pressure measurement; (-) 180 kg DM/m^3, silo with bulk density of 180 kg DM/m^3 undergoing internal pressure measurement; (+) 145 kg DM/m^3, silo with bulk density of 145 kg DM/m^3 undergoing CO_2 availability; (+) 180 kg DM/m^3, silo with bulk density of 180 kg DM/m^3 undergoing CO_2 availability.

The relative abundance of silage bacterial community is shown in Figure 13. Relative abundance indicates the percentage of the microbiome that corresponds to a particular microbial taxonomy (AZARBAD *et al*., 2022). The valid sequences were classified into different genera, and the predominant ones were *Lactobacillus* (the number of classified sequences in this genus ranged from 81.8% in (+) 180 kg DM/m^3 to 93.7% in the (-) 145 kg DM/m^3), and *Paenibacillus* (the number of classified sequences in this genus ranged from 2.8% in the (-) 180 kg DM/m^3 to 8.4% in the (+) 180 kg DM/m^3). Other genera such as *Clostridium*, *Coprococcus, Bifidobacterium, Turicibacter, Prevotella, Azospirillum, Blautia, Ruminococcus* and *Lactococcus* also appeared, but in smaller proportions. Comparing the four treatments, there were no marked differences between them regarding bacterial community composition with exception to the genera *Erwinia* which were found in higher percentage (76%) in fresh maize samples, as expected (Figure 13).

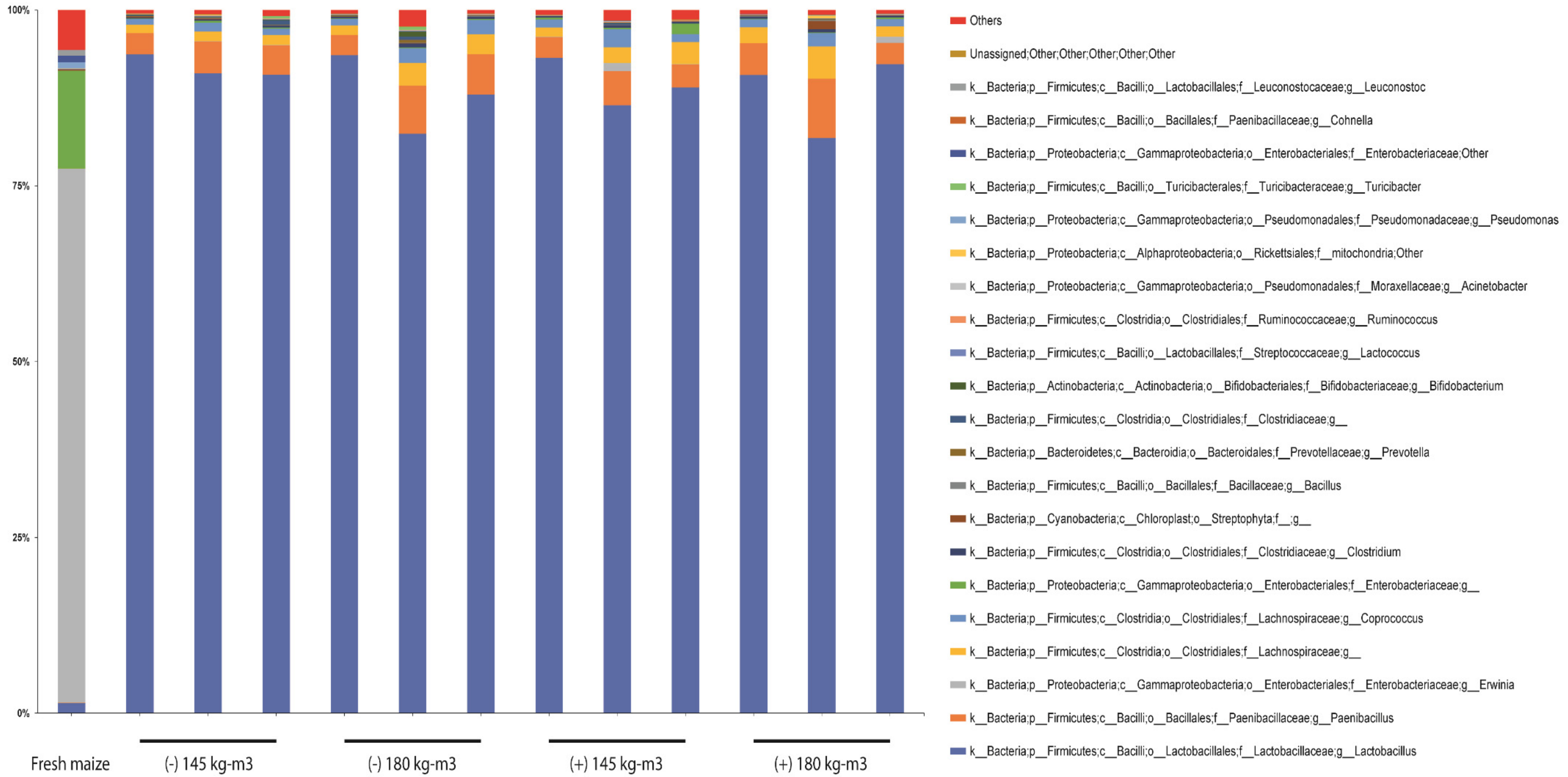
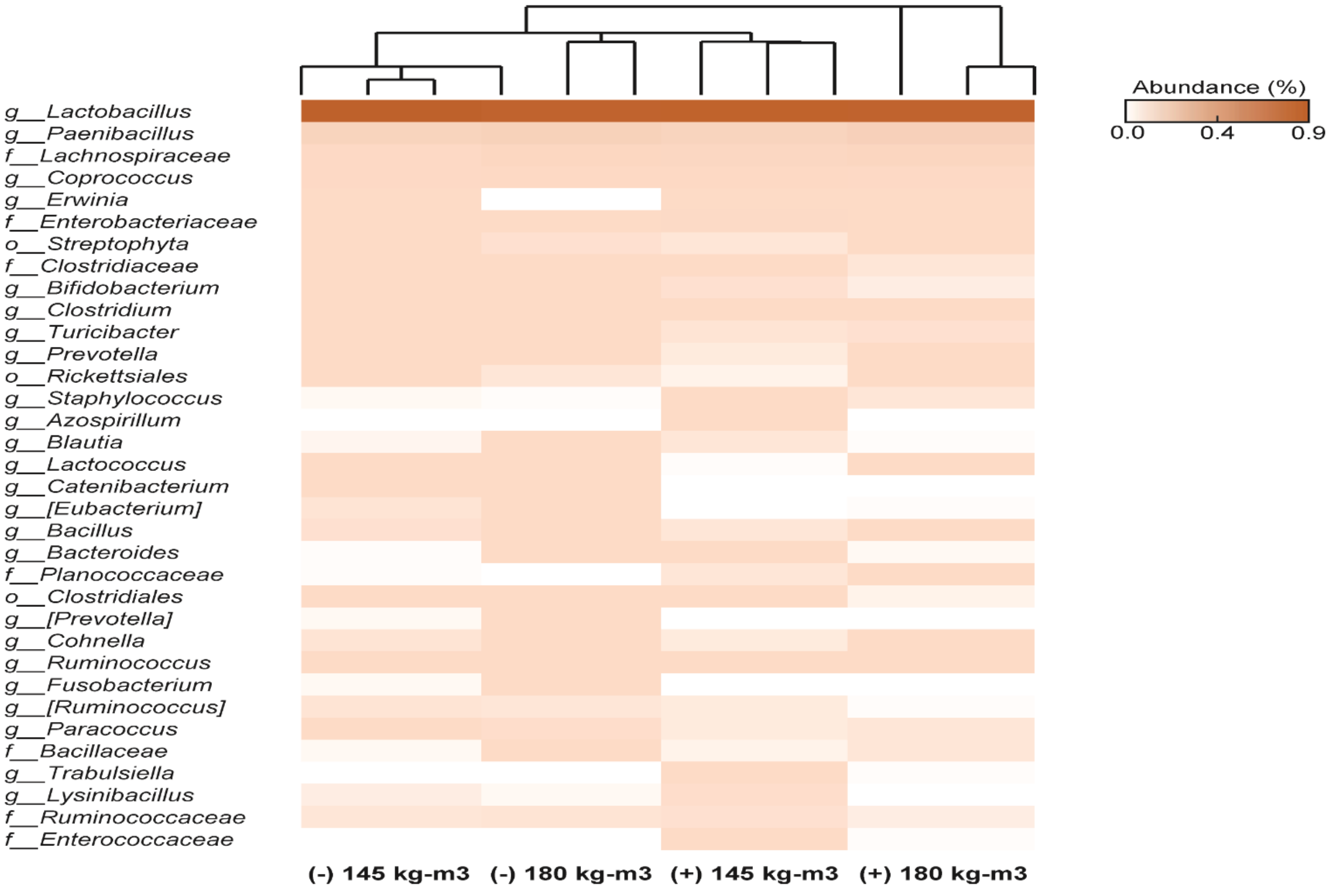


FIGURE 13. Relative abundance of bacterial genera found in silages according to the treatments: (-) 145 kg DM/m^3, silo with bulk density of 145 kg DM/m^3 undergoing internal pressure measurement; (-) 180 kg DM/m^3, silo with bulk density of 180 kg DM/m^3 undergoing internal pressure measurement; (+) 145 kg DM/m^3, silo with bulk density of 145 kg DM/m^3 undergoing CO_2 availability; (+) 180 kg DM/m^3, silo with bulk density of 180 kg DM/m^3 undergoing CO_2 availability. The graph plots the bacterial groups with abundances higher than 1%.

4.3.2.3 The most responsive OTUs to the main change in the bacterial community structure

SIMPER (similarity percentage) analysis is presented as a heat map. The SIMPER analysis calculates the contribution of each species (%) to the dissimilarity among treatments. Heat map is usually displayed in a grid wherein each row represents a genus while each column represents a treatment. The presence and the relative abundance of a gene are represented by the differences in color intensity of the boxes (PEETERS *et al*., 2021). The SIMPER evidenced that the differences among treatments were linked with few genera, with the top list 4 comprising at least 65.8% of the dissimilarity among treatments. The main genera responsible for the differences among treatments were *Lactobacillus, Paenibacillus, Coprococcus* and *Erwinia* (Figure 14). ANOVA results showed significant differences among treatments at family level when considering CO_2 availability (Figure 14). *Enterobacteriaceae* and *Staphylococcaceae* were more predominant in (+) CO_2 treatment while *Streptococcaceae* prevailed in (-) CO_2 treatments (Figure 14).



	Av. dissim	Contrib. %	Cumulative %
g__*Lactobacillus*	2.258	40.070	40.070
g__*Paenibacillus*	0.953	16.910	56.980
f__*Lachnospiraceae*	0.588	10.440	67.410
g__*Coprococcus*	0.321	5.694	73.100
g__*Erwinia*	0.162	2.878	75.980
f__*Enterobacteriaceae*	0.153	2.715	78.700
o__*Streptophyta*	0.114	2.026	80.720
f__*Clostridiaceae*	0.095	1.694	82.420
g__*Bifidobacterium*	0.074	1.319	83.740
g__*Clostridium*	0.065	1.148	84.880
g__*Turicibacter*	0.054	0.965	85.850
g__*Prevotella*	0.046	0.817	86.670
o__*Rickettsiales*	0.037	0.661	87.330
g__*Staphylococcus*	0.035	0.628	87.960
g__*Azospirillum*	0.030	0.533	88.490
g__*Blautia*	0.025	0.449	88.940
g__*Lactococcus*	0.025	0.437	89.370
g__*Catenibacterium*	0.024	0.421	89.800
g__*[Eubacterium]*	0.022	0.393	90.190
g__*Bacillus*	0.021	0.382	90.570
g__*Bacteroides*	0.020	0.352	90.920
f__*Planococcaceae*	0.019	0.336	91.260
o__*Clostridiales*	0.019	0.332	91.590
g__*[Prevotella]*	0.016	0.289	91.880
g__*Cohnella*	0.015	0.265	92.140
g__*Ruminococcus*	0.013	0.237	92.380
g__*Fusobacterium*	0.013	0.230	92.610
g__*[Ruminococcus]*	0.012	0.217	92.830
g__*Paracoccus*	0.012	0.207	93.040
f__*Bacillaceae*	0.011	0.201	93.240
g__*Trabulsiella*	0.011	0.197	93.430
g__*Lysinibacillus*	0.011	0.196	93.630
f__*Ruminococcaceae*	0.010	0.186	93.810
f__*Enterococcaceae*	0.010	0.183	94.000

FIGURE 14. Heat map showing the relative abundance of the most abundant bacterial genera contributing to differences (SIMPER analysis) in community structures among treatments: (-) 145 kg DM/m^3, silo with bulk density of 145 kg DM/m^3 undergoing internal pressure measurement; (-) 180 kg DM/m^3, silo with bulk density of 180 kg DM/m^3 undergoing internal pressure measurement; (+) 145 kg DM/m^3, silo with bulk density of 145 kg DM/m^3 undergoing CO_2 availability; (+) 180 kg DM/m^3, silo with bulk density of 180 kg DM/m^3 undergoing CO_2 availability. Only genus contributing ≥0.1% of dissimilarity among samples were selected for heat map representation and they were represented in decreased order of dissimilarity contribution. Percentages to the right indicates the average dissimilarity for each genus (Av. dissim), the percent dissimilarity contributed by each genus (Contribution %) and the cumulative percent dissimilarity among samples (Cumulative %). Color legend and scale (%) are provided in the figure.

4.3.2.4 Correlation analysis of the bacterial community and the chemical profile of silages

The PCA plot is presented in Figure 15 and it is another form to present beta diversity correlated to silage variables (CHONG *et al*., 2020). The position over the 2 dimensions on the graph indicates how variables clustered. The angle included between the arrows pointing at 2 variables determines the correlation between those variables: (very) sharp angles define (strong) positive correlations, squared angles define a null correlation, and (very) obtuse angles define (strong) negative correlations (CARVALHO-ESTRADA *et al*., 2020). In general, silos undergoing negative pressure are spread over the upper half of the graphic and those under CO_2 availability are spread over the bottom half (Figure 15). Total gas production was positively correlated with aerobic stability, *Bifidobacterium, Turicibacter* and *Prevotella*. *Lactobacillus* was positively correlated with lactic acid, ethanol, protein and NH_3-N. Together, those variables defined most of the samples belonging to the (-) 145 kg DM/m^3 and (-) 180 kg DM/m^3 sample group. On the other hand, *Erwinia* was strongly and positively correlated with 2,3-butanediol, 1,2-propanediol and acetic acid. *Paenibacillus*, *Clostridium* and *Coprococcus* were strongly and positively correlated among each other. However, they were negatively correlated with *Lactobacillus*. Together, those variables defined most of the samples belonging to the CO_2 availability group (+) 145 kg DM/m^3 and (+) 180 kg DM/m^3 samples (Figure 15).

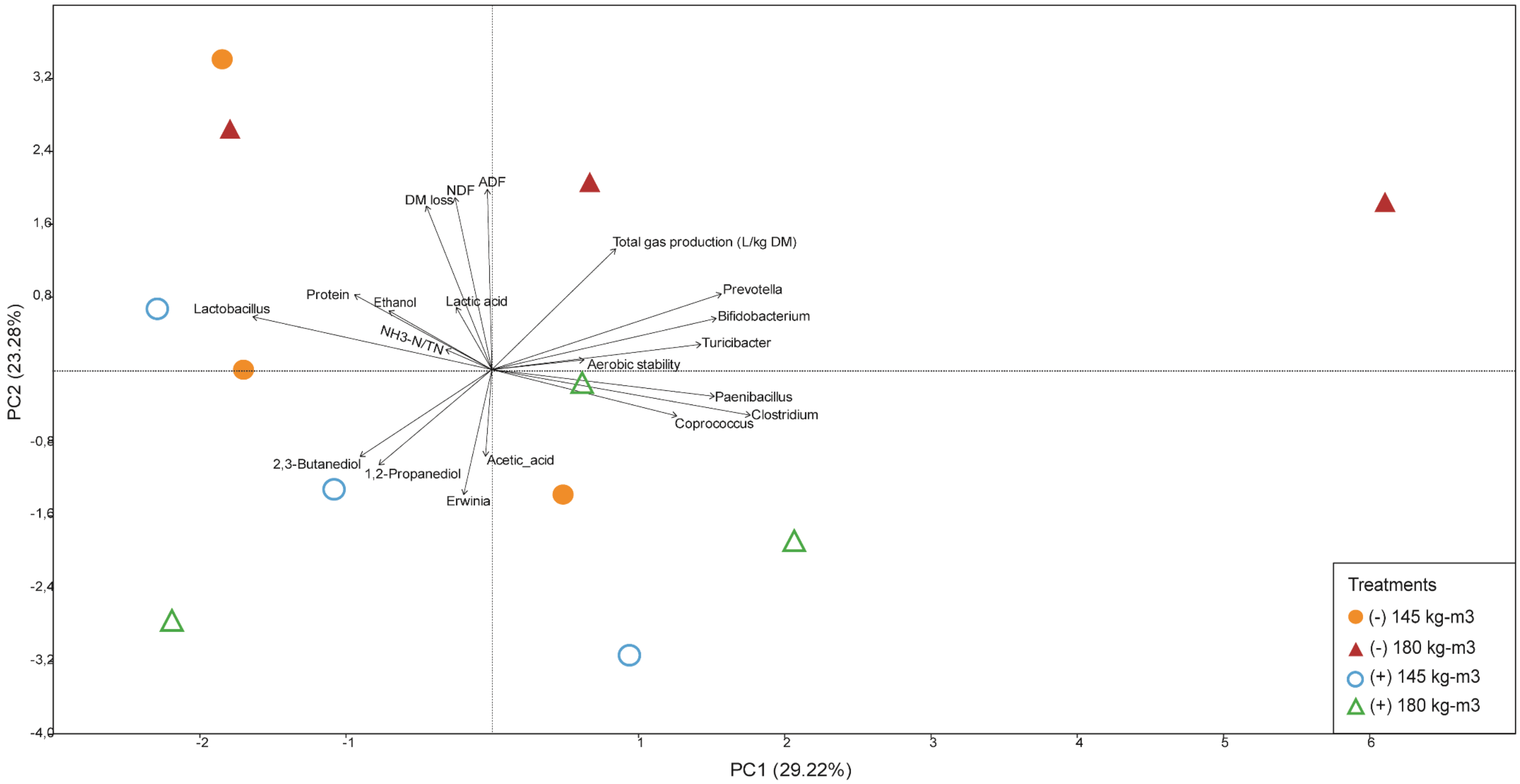


FIGURE 15. Principal component analysis (PCA) plot correlating taxonomic profile and silage chemical variables. (-) 145 kg/m^3, silo with bulk density of 145 kg/m^3 undergoing internal pressure measurement; (-) 180 kg/m^3, silo with bulk density of 180 kg/m^3 undergoing internal pressure measurement; (+) 145 kg/m^3, silo with bulk density of 145 kg/m^3 undergoing CO_2 availability; (+) 180 kg/m^3, silo with bulk density of 180 kg/m^3 undergoing CO_2 availability. Treatment (-) 145 kg DM/m^3 (replicates 2, 3 and 5, treatment (-) 180 DM/m^3: replicates 1, 3 and 4; treatment (+) 145 DM/m^3: 2, 4 and 5; treatment (+) 180 DM/m^3: 3, 4 and 6.

4.4 DISCUSSION

Despite our efforts to guarantee hermetic sealing of the silos, we have detected a huge variation inside the treatments. During all the trial, the major challenge was to provide and sustain the hermeticity of the system. Immediately after sealing the silos, gas production started very quickly. It difficulted the sealing process of the silos, due to the formation of bubbles in the adhesive plastic until it dries. Those issues showed the need to improve the methodology. Some progresses have already been done to reduce the problems with the airtight, such as the use of silicone rings specifically developed for the silo lids and for acidity conditions. These rings are attached inside of the lid, increasing the sealing between the lid and the silo. The rings, tested in subsequently experiments, collaborated to reduce the bubbles caused by the gas production at the time of silo sealing, reduced the use of the sealing adhesive and the variability among replicates. Schmidt *et al*. (in press) repeated the same trial in 2019 and detected only a very small variation inside treatments, using eight replicates.

Making silage involves a complicated biochemical process where oxygen (O_2) is rapidly consumed within the sealed environment, leading to fermentation and stable storage of the plant biomass (SUN *et al*., 2015). Silage production is intrinsically dependent on the microbial activities under aerobic and anaerobic phases. Throughout these phases, CO_2 is the major biogenic waste product of microbial metabolism in silages (PAHLOW *et al*., 2003; SCHMIDT *et al*., 2011). Therefore, the formation of CO_2 can be considered as a measure of ensiling efficiency or ensiling losses (KNICKY *et al*., 2014; CHEN *et al*., 2021; SCHMITHAUSEN *et al*., 2022).

In this experiment, all maize silages displayed a similar pattern in development of gas over time. The gas production was higher in the first two days after sealing (93% of total gas production) and gradually reduce after the third day, until it stops on the ninth day. The same pattern of gas production was found by Knicky *et al*. (2014), Novinski (2018), Melo (2020) and Chen *et al*. (2021), during the fermentation of grass ley, maize, stylo and rice silage. CO_2, CH_4 and N_2O concentration in grass and lucerne silages showed a similar trend and increased sharply at the first 2 days of ensiling (Schmithausen *et al*., 2022). In natural fermented silages, CO_2 concentration achieved the maximum at 48 hours, CH_4 in 1 day and N_2O in 2 days. However, a second peak of CH_4 was noticed to occur after 20 days of ensiling in poor fermented silages, due to the clostridial activity and/or archaea, with concomitant gas production after 20 days of ensilage (SCHMITHAUSEN *et al*., 2022). Archaea were noted in silage; however, their content was relatively low, and their impact on silage quality remains unclear (WU *et al*., 2020). For further studies evaluating the CO_2 absorption, it is recommended to evaluate fungi and archaea communities, in addition to the evaluation of the bacterial community.

In this study, the average production of gases was 6.10 L/kg DM, corroborating with the findings of Melo (2020) for maize silage (5.70 L/kg DM). However, compared to the study of Novinski (2018), who tested similar forage, DM and bulk densities, in this trial the gas production was 3 times greater and the peak was reached approximately between 4-12 hours after ensiling (Novinski (2018) observed the peak after 12 hours). It corresponds to the period of the most intensive fermentation (PAHLOW *et al*., 2003). Bueno *et al*. (2020) evaluated gas production during sugarcane ensiling and report 7.28 L/kg DM. Of this total, 129 g/ton DM was CO_2, 0,005 g/ton DM was CH_4 and 0.002 was N_2O (BUENO *et al*., 2020).

The gas production expressed in L/kg of DM differ between the bulk densities ($p<0.01$); however, gas production expressed in litters was similar. It is probably related to the huge variation due to sealing failure and the small difference between the tested bulk densities. The silages with 180 kg DM/m³ of bulk density produced 5.88% more gas than the 145 kg DM/m³. More compacted silages have a relatively greater amount of biomass or plant-cells carrying out respiration in the silo (SUN, *et al*., 2015; SHAN *et al*., 2016).

The quantity of CO_2 produced during ensiling depends on the extent and the pattern of fermentation, and is particularly influenced by microorganism activities (McGECHAN; WILLIAMS, 1994). In the initial phase of silage fermentation, gas production results from aerobic activity which uses the residual O_2 for metabolism, at the same time as produces CO_2, reaching levels up to 80% of total gas produced during this phase (WEINBERG; ASHBELL, 1994; KNICKY *et al*., 2014; CHEN *et al*., 2021). According to Driehuis and Elferink (2000), most of the gas production in silages is due to the enterobacteria metabolism, which is particularly active (HERON; WILKINSON; DUFFUS, 1993), contributing to the reduction of residual O_2 in forage mass. Melo (2020) share the same idea and affirm that the increase in time between chopping and sealing increases gas production, as a result of the increase in the aerobic microorganism population present in epiphytic microbiota. The increase in the temperature of the environment in which the silos are stored, also increases gas production, but reduces the extent of production (NOVINSKI, 2018).

During gas formation, the pressure inside the silos increases (LI *et al*., 2017; SCHMITHAUSEN *et al*., 2022), which could reach +45.4 kPa for maize silage and +19.1 kPa for ryegrass silage (LI *et al*., 2017), accentuating the difficulty to maintain the airtight and could lead to the escape of gases. Under sealing failure, high CO_2 concentrations, denser than the atmospheric, tends to drop out and to be replaced by air. In airtight silos, CO_2 dissolves in water, but rapidly exceeded the capacity for CO_2 dissolution during the early stages of ensilage (LI *et al*., 2017). Therefore, it would be expected that the internal pressure of the silos would remain positive until the end of the fermentation process. However, the opposite was observed.

When the oxygen was exhausted and the environment was acidulated, the plant cell respiration and gas producing aerobic bacteria are inhibited (CHEN *et al*., 2021). After gas production phase, a short period of stabilization of the internal pressure, that precede the formation of negative pressure inside the silos, was observed, as the same way reported by Souza (2015b), Li *et al*. (2017), Novinski (2018) and Schmidt *et al*. (2018). In this trial, this phase was observed between the 9th and 12th days for some silos, while for others silos it continued until the 19th day.

After the 19th day all silos exhibited negative internal pressure. All silos showed negative pressure throughout the trial. From the moment the negative pressure was first detected, it gradually increased until the opening. The maximum negative pressure observed was -1266.7 and -840.0 mmWC for the treatments (-) 145 kg DM/m^3 and (-) 180 kg DM/m^3, respectively. It corresponds to -12.4 and -8.2 kPa, respectively. The MP were not influenced by the bulk densities. However, the IP was 64% higher for the treatment (-) 145 kg DM/m^3, comparing to the treatment (-) 180 kg DM/m^3 ($p<0.05$). It is probably related to the higher residual gas in the porous space. The negative pressure detected can possibly be interpreted as gas disappearance from the porous of the silage, probably due to its transformation into a liquid or solid, which volume is significantly smaller.

The negative pressure inside silos was also observed by Schmithausen *et al*. (2022). They reported had observed signs of negative pressure when realizing the lids were slightly curved inwards, however they did not evaluate pressure inside the silos. Li *et al.* (2017) detected maximum negative pressure inside the silo of -7.5 kPa for maize silage and -3.1 kPa for ryegrass silage, however they analyzed the negative pressure just in 100 hours. Novinski (2018) observed negative pressure around -12.74 kPa during 93 days of maize ensiling and Schmidt *et al*. (2018) reported a mean of -5.87 kPa during 150 days of fermentation.

Novinski (2018) observed an inverse correlation between negative pressure and gas production. In addition, Li *et al*. (2017) found a positive and linearly correlation between CO_2 production and pH, assuming the reduction of pH and gas production reflects the suppression of CO_2 producing pathways and further acid production (LI *et al*., 2017). Meeske and Basson (1998) showed that in the first 10 days of ensilage, at the same time as WSC and pH declines, lactic acid increases. In the first 4 days there is a major consumption of WSC and a major increase of lactic acid concentration, and after 10 days this process stabilizes (MEESKE; BASSON, 1998).

According to Li *et al*. (2017), two phases of negative pressure occur during the ensilage. The first phase initiate after sealing the silos and preceded the gas production phase, has a quite short period (less than 5 hours) and could be the result either from loss of mass and/or from decline in silage temperature. However, this phase was not detected in this experiment, since the time elapsed between harvesting and chopping the maize forage, filling, compacting and sealing the 24 silos was around 6 hours (steps occur simultaneously), due to the amount of labor available. The negative phase observed in this study corresponds to the second phase of negative pressure described by Li *et al.* (2017) and reflect a decline in gas phase mass inside the silos, suggesting sequestration of CO_2 (LI *et al*. 2017).

Some published studies had been evaluated ensiling and the availability of CO_2. However, they investigated the potential of CO_2 in conserving forage and inhibiting the development of yeasts in silages, or traced the diffusion of gases between the silage particles (MUCK; SPOELSTRA; WIKSELAAT, 1992; McGECHAN; WILLIAMS; 1994; TRIANDAFILOV *et al*., 2017). In the best of our knowledge, it is the second study to provide CO_2 to maize silages in order to investigate the sequestration of CO_2. The first study was conducted by Schmidt *et al*. (2018).

CO_2 gas was made available to the silos without being forced inside them and each silo absorbed according to its capacity. Maize silages absorbed on average 7.03 L CO_2/kg DM. However, it was observed replicates that absorbed more than 14.0 L CO_2/kg DM. According to Acién *et al*. (2017) and Soeswanto; Wahyuni and Trirahayu (2020), at 1 atm and 25°C, the maximum CO_2 solubility in water is 1.5 g CO_2/kg H_2O, which corresponds to 831 mL CO_2/L H_2O. Even considering all water present in the silo as pure and free, silages absorbed twice more CO_2 than the theoretical maximum capacity of CO_2 solubilization in water. It proves that great part of the CO_2 absorbed by the silage was not solubilized into water silage, revealing the sequestration of CO_2 by some unidentified biochemical process. The silage pH supports this finding, since it was not influenced by the CO_2-supplies. Additionally, the DML was reduced by 66% in CO_2-supplied silos.

Silages DM content were 28.3% and pH levels range from 3.70 to 3.65, which is typically found for corn silages with 30% DM (KUNG *et al.*, 2018). CO_2-supplied silage exhibited DM content higher than the regular silos (p=0.01), showing that CO_2-supply improved DM content. Furthermore, acetic acid content tended to be higher in CO_2-supplied silages (p=0.097). In addition, a trend (p=0.08) of moderate positive correlation (r=0.53) between CO_2 absorption and the content of acetic acid was detected and the estimated acetic acid production (EAC) supports these findings.

High CO_2 concentrations have a severe lethal effect on microorganisms (>3000 ppm) (YU; CHEN, 2019). According to Muck, Spoelstra and Wikselaar (1992), high levels of CO_2 (100% of the atmosphere) decrease yeast population and improve recovery of DM. However, although high CO_2 concentrations are considered a promising sterilization technology because their effect of inactivate aerobic bacterial, some studies showed that anaerobes and facultative anaerobes were capable of growing and catalyzing reactions in environments with a high CO_2 concentration (YU; CHEN, 2019). According to Xiong *et al*. (2015), raising environment CO_2 concentration (550ppm) had significant effects on the functional structure and metabolic potential of microbial communities and many key functional genes involved in carbon, nitrogen and phosphorous cycling were stimulated. In addition, genes linked to CO dehydrogenase/acetyl-CoA synthase for the reductive acetyl-CoA pathway and Propionyl-CoA carboxylase/acetyl-CoA carboxylase for the 3-hydroxypropinate/malyl-CoA cycle are increased. Elevating CO_2 concentrations reduce the diversity of bacterial communities and shift their structure by eliminating the sensitive species and lead to the survival of only a few insensitive ones (YU; CHEN, 2019). However, our study observed an increase in specie richness and abundance for the treatment (+) 145 kg DM/m^3. The treatment (+) 180 kg DM/m^3 presented high variation, justifying the lack of effect.

Erwinia (76%), *Enterobacteriaceae* family-related OTUs (1.14%) and *Lactobacillus* (0.1%) were the OTUs most identified in fresh maize forage. These results are in agreement with the findings reported by Wu *et al*. (2021), that observed the dominance of the phyla *Proteobacteria* followed by the phyla *Firmicutes*, and also with the findings of Sun *et al*. (2021a), that observed that *Erwinia* was one of the mainly genera observed in fresh maize. *Erwinia* is a genus commonly observed in epiphytic microbiota of maize and in the first days of fermentation phase (SUN *et al*., 2021a).

CO_2-supplies did not influence the development of *Lactobacillus* during the ensilage. After 233 days of fermentation, *Lactobacillus* was the genera most found in silages, ranging from 81.8% to 93.7%, which indicate that both CO_2-supplied silages and the 145 kg DM/m^3 silages underwent a good fermentation process as well as the other treatments (the minimum packing density recommendation for maize silage is ≥225 kg/m^3 DM) (BRÜNING *et al*., 2018). The lactic acid concentrations ranged from 5.6% to 6.0% of DM and was the acid found in the highest concentration in all treatments. The lactic acid concentration find in this trial is considered normal in maize silages with DM concentrations below 30% (KUNG *et al*., 2018). Acetic acid was the second highest concentration acid found (1.63% of DM) and propionic acid and butyric acid were undetectable in all treatments, which is acceptable for maize silages (KUNG *et al*., 2018).

Paenibacillus was the second most prevalent genera in silages, ranging from 8% to 6.8%. Many *Paenibacillus* species are facultative anaerobes and can promote crop growth directly via biological nitrogen fixation, phosphate solubilization, production of the phytohormone indole-3-acetic acid (IAA), and release of siderophores that enable iron acquisition (GRADY *et al*., 2016). It also produces antimicrobials substances which include peptides, enzymes and VOC (benzenes, aldehydes, ketones, alcohols, benzothiazole, benzaldehyde, undecanal, dodecanal, hexadecanal, 2-tridecanone and phenol, being the main antifungal compounds). Hydrolytic enzymes of *Paenibacillus* can attack the cell walls of fungal and oomycete competitors. Other enzyme produced by *Paenibacillus* are amylases, cellulases and pectinases which were used to improve the nutritional quality of animal feeds (GRADY *et al*., 2016).

The internal pressure and the CO_2-supplies influenced the bacterial community structure. CO_2-supplied silages clustered together in PCA. However, there were no marked differences between treatments regarding bacterial community composition. Other genera that appeared in bacterial community of silages, but in smaller proportions, were *Clostridium, Coprococcus, Bifidobacterium, Turicibacter, Prevotella, Azospirillum, Blautia, Ruminococcus* and *Lactococcus.*

Clostridium, *Blautia* and *Ruminococcus* are genera composed of acetogenic bacteria. Acetogenic bacteria are anaerobic and can grow autotrophically using the Wood–Ljungdahl pathway (WLP), also called reductive acetyl-CoA pathway, producing, from CO_2 and H_2, the acetate as the main final product. This pathway is considered a CO_2-fixing pathway (KATSYV; MÜLLER, 2020). The presence of these bacteria, even though it did not show greater abundance in the CO_2-supplied silages, reflects the development of CO_2-fixing bacteria in silage. The literature supports this finding, since *Sporomusa ovata* has already been identified in silages (MÖLLER *et al*., 1984).

Coprococcus are obligately anaerobic bacteria generally found in rumen community. *Coprococcus* species are involved in the formation of acetate, butyrate, propionate and lactate in anaerobiosis (NOGAL *et al*., 2021). In alfalfa silage, *Coprococcus* were observed by Kang *et al*. (2021) and displayed a positive correlation with crude protein and gross energy, but negatively correlated with NH_3-N (KANG *et al*., 2021).

Bifidobacterium are anaerobic and their typical habitat is the human and animal intestinal tract (VLKOVÁ *et al*., 2009). In silages, *Bifidobacterium* was observed in the end of the fermentation, after 60 and 90 days of ensilage (ZHENG *et al*., 2017; LI *et al*., 2019; MITIKU *et al*., 2020; KANG *et al*., 2021). It is characterized by the conversion of free conjugate linoleic acid into conjugated linoleic acid, and it is an important intestinal probiotic for humans and animals (MITIKU *et al*., 2020). Some species of *Bifidobacterium* produce succinic acid through CO_2 fixation reaction. The production of succinic acid occurred through the conversion of phosphoenolpyruvate into oxaloacetate with the incorporation of CO_2, due to the action of a phosphoenolpyruvate carboxylase. Other species also produce lactic acid, acetic acid, ethanol (in lower concentrations) and mannitol during fermentation of carbohydrates (VAN DER MUELEN *et al*., 2006), and their mainly substrates used are lactose, glucose, maltose and raffinose (RUIZ-ACEINTUNO *et al*., 2020).

Prevotella belongs to the phylum *Bacteroidetes* and was described in silages by Li *et al*. (2022). The major pathways are based on glycolysis and succinate production from fumarate via phosphoenolpyruvate carboxylation and malate reduction (FRANK; DEPPENMEIER, 2018). Some species, such as *Prevotella copri*, incorporate CO_2 for biomass formation by the conversion of phosphoenolpyruvate to oxalacetate (via carboxylation) and finally to succinate by TCA cycle (5.6 mmol CO_2/g DM) (FRANK; DEPPENMEIER, 2018; FERNÁNDEZ-VELEDO; VENDRELL, 2019), another CO_2-fixing cycle (BERG, 2011). The CO_2-absorbing rate corresponds to 13,7 L of CO_2/kg DM (1 atm and 25°C), value close to that absorbed by treatment (+) 145 DM kg/m^3. In the further researches, it might be interesting to evaluate the content of succinic acid in fresh forage and silages.

Azospirillum sp. is a nitrogen-fixing bacteria associated with several silage crops, such as maize, rice, wheat and sugar cane (SOUZA *et al*., 2014). Studies recently conducted by Forage Research Center showed the incorporation of labeled 15 N_2 by silage, that was incorporated into 51 proteins including elongation factors of many bacteria common in silages, such as *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Clostridium* (Schmidt *et al*., *in press*).

Lactococcus is a homofermentative lactic acid bacteria related to *Streptococacеae*, often associated with plants and silages (GOLOMB; MARCO, 2015; DROIN *et al*., 2021). The metabolism of glucose and fructose was homolactic (PASSERINE *et al*, 2013). However, recent study shows the ability of *Lactococcus* to fix nitrogen (HIGDON *et al*., 2020). Lactococcus is also known to be bacteriocins producers that inhibited *Listeria monocytogenes* and *Staphylococcus aureus* (MORENO *et al*., 2000).

The chemical composition of the silages was considered as normal range for well-fermented silages (KUNG *et al*., 2018). In all treatments, silages showed normal color (light green to green/brown) and normal mild smell (KAISER; PILTZ, 2009), although these parameters were not evaluated in this experiment.

The pH was influenced by the bulk densities. Silages with the bulk density of 145 kg DM/m^3 exhibited the highest pH ($p<0.01$), but even so it is considered normal for well-fermented silages (KUNG *et al*., 2018). It could be explained by the higher residual O_2 among the particles in lower densities. During the aerobic exposure test (AS), DMLas was 71% higher for the density of 145 kg DM/m^3, corroborating with the pH results observed in those silages. Although, the difference between the bulk densities and CO_2-supplies, all silages presented AS above 180 hours and the maximum temperature reached was 24.8°C, indicating a good stability after aerobic exposure. In fact, those values of AS are greater than the regular values found in most trials for non-inoculated silages, and can be related to the long period of fermentation we have used in this trial.

Sugars are an important source of nutrients for microbial growth; however, this subject is little discussed on silage studies. In fresh maize, fructose, glucose and maltose were the most abundant WSC. In silage, mannitol, galactose and xylose were the higher. According to Gänzle and Follador (2012), maltose and maltodextrins are the most abundant oligosaccharides in cereal fermentations. Maltose is fermented by *Lactobacillus* (such as *L. casei, L. acidophilus, L. sanfranciensis, L. reuteri, L. fermentum, L. buchneri, L. brevis* and *L. reuteri*) in glucose and beta glucose-1-phosphate. A diversity of microorganisms can utilize sucrose as a substrate. Among the saccharolytic organisms found in maize epiphytic community, are mentioned the species from the genera *Staphylococcus, Streptococcus, Pediococcus, Lactobacillus, Lactococcus, Klebsiella, Escherichia, Erwinia, Leuconostoc*, and *Pseudomonas* (REID; ABRATT, 2005).

According to Gao *et al*. (2021) fructose additions in alfalfa silage results in desirable fermentation profiles and chemical compositions throughout the ensiling period due to the decreases in pH by its conversion in lactic acid by LAB fermentation. Several heterofermentative LAB (*Leuconostoc pseudomesenteroides, Leuconostoc mesenteroides, Levilactobacillus brevis, Limosilactobacillus reuteri*) produce mannitol in large amounts, using a mixture of fructose and glucose as an electron acceptor. *Lactobacillus sp*. and *Leuconostoc sp*. fermentation can result in mannitol formation, being able to produce concentrations up to 150 g/L (WISSELINK *et al*., 2002).

LAB also were involved in the production of galactose by lactose metabolization by beta galactosidase. Most of the LAB are efficiently capable of using the glucose portion of lactose and releasing galactose (BARROS *et al*., 2019). In addition to *Lactobacillus*, *Bifidobacterium* are widely used as sources of beta galactosidase. Some LAB species involved in this metabolism are *Bifidobacterium longum, Lactobacillus delbrueckii, Streptococcus thermophiles* and *Leuconostoc citrovorum* (SAQIB *et al*, 2017). However, not can be inferred about lactose in the trial, since the lactose analysis was not performed.

Raffinose is an oligosaccharide and is found in plants. It consists of 3 monomers, namely alfa-D-galactose, alfa-D-glucose, and alfa-D-fructose. Raffinose can increase the growth of LAB and *Bifidobacterium* (MIRANDA; CARVALHO; NERO, 2014; PASQUALE *et al*., 2020; ANGGRAENI, 2022). Reports of accumulation of raffinose in the unicellular organisms are not obtained. Thus, the role of raffinose in a unicellular organism is unknown (SENGUPTA *et al*., 2015).

According to ZARDIN *et al*. (2017), the mean of EE content found in maize silage produced by scientific studies in Brazil is 28.4 g/kg of DM. However, the EE content can vary with the content of grain in silage. The 1.2 propanediol amounts in silages can vary from 0 g/kg DM, in no treated silages, to 50 g/kg of DM, in silages treated with *L. buchneri* and stored for long period. Although the 1.2 propanediol contents in no treated silages were usually less than 10 g/kg DM (NISHINO *et al*., 2003; WEISS, 2017). The EE and the 1,2 propanediol contents were influenced by the bulk densities. The EE content was higher for 180 kg/m^3 silage, but the content observed was close to the content usually observed in maize silages found in Brazil. The 1,2 propanediol was lower for 180 kg/m^3 silage, when comparing to the 145 kg/m^3 silage ($p<0.05$). However, the 1,2 propanediol content of all treatments were below 1% of DM. The 1,2 propanediol is associated with the metabolism of *Lentilactobacillus buchneri, Limosilactobacillus reuteri* and *Lentilactobacillus diolivorans*. They are heterofermentative LAB, which slowly converts lactic acid to acetic acid and 1.2 propanediol during silo storage, improving aerobic stability, while having no effect on animal productivity (MUCK *et al*., 2018).

CO_2-supplies affected the EE, ethanol, 2,3 butanediol and ethyl-lactate contents ($p<0.05$), however the contents were found in very low concentrations. In silages, 2,3 butanediol is associated with the metabolism of facultative anaerobic bacilli. It uses different sugars and convert to organic acids, ethanol, 2,3 butanediol and glycerol (KUNG *et al*., 2018). However, some *Clostridium* also can produce 2,3 butanediol (KÖPKE *et al*., 2011). Also, enterobacteria (HÖHN-BENTZ; RADLER, 1978), lactic acid bacteria (*Leuconostoc and Lactobacillus*) and some yeasts can produce 2,3 butanediol (HEROLD; PFEIFFER; RADLER, 1995).

Esterification processes were stimulated by low pH (WEISS; AUERBACH, 2013). Ethanol that can be formed from heterolytic *Lactobacillus* and/or epiphytic yeast was involved in the esterification processes occurring in these silages (BRÜNING *et al*., 2018).

The CO_2-supplied treatments and no CO_2-supplied silages clustered separated in PCoA, showing that the condition influenced the bacterial community structure. The *Streptococaceae* family prevailed in no CO_2-supplied silage and *Enterobacteriaceae* and *Staphylococcaceae* were more predominant in no CO_2-

supplied silage. The *Streptococaceae* family are generally related with Lactococcus and *Streptococcus,* both carrying out homolactic acid fermentation. It generally exists in naturally fermented silages. As well as *Lactobacillus*, *Lactococcus* is a homofermentative LAB, important in pH decline and lactic acid accumulation, and in the whole process of silage it is generally present (KHOTA *et al*., 2016; LU *et al*., 2021).

Enterobacteraceae family is composed by various genera and is associated with dry matter loss in silage (McDonald *et al*., 1991). *Erwinia, Citrobacter, Klebsiella, Serratia* and *Salmonella* are some of the genera belonging to this family. In this family, the specie *Citrobacter amalonaticus* is known to be a CO_2-fixating bacteria and produce succinic acid by TCA cycle, and for providing electron donors (AMULYA; MOHAN, 2019). In *Staphylococcaceae* family, the only genera described in silages is the *Staphylococcus* (NI *et al*., 2016). *Sthaphylococcus aureus* and S*. xylosus* grow in anaerobiosis doing CO_2-fixation in the synthesis of aspartic acid in CO_2 enriched environments (HANCOCK; McMANUS, 1960; QUINTIERI *et al.,* 2018). However, *Sthaphylococcus* can adapt their metabolism, depending on the substrate and medium, alternating between glycolysis and the TCA cycle (QUINTIERI *et al*., 2018), what happens to most of the microorganisms.

In SIMPER analysis, *Lactobacillus*, lactic acid, ethanol, protein and N-NH_3 were positively correlated each other and define no CO_2-supplied silages. Lactobacillus produce lactic acid exclusively or associated with others compounds, such as ethanol (McDONALD *et al.,* 1991). According to Daniel *et al*. (2013), ethanol and lactic acid were positively related, due most yeast species are resistant to low pH, and actually use lactic acid as a nutrient source. However, ethanol contents were very low in all silages. The higher content of ethanol and ethyl lactate in no CO_2-supplied silages could be involved with the reaction of ethanol and lactic acid which product is the ethyl lactate (WEISS; KROSCHEWSKI; AUERBACH, 2022). *Lactobacillus* is also involved in the degradation of macromolecular proteins by hydrolyze to their amino acid catabolism, explaining the positive relation among it, protein and N-NH_3.

SIMPER analysis also correlated positively gas production, AS, *Bifidobacterium, Turicibacter* and *Prevotella* in no CO_2-supplied silages*.* Traditional fermented products are the primary sources of LAB and bifidobacteria. They produce lactic acid as a major metabolic end-product of carbohydrate fermentation and exhibit an increased tolerance to acidity (SAEZ-LARA *et al*., 2015). Some LAB strains can utilize the heterolactic acid pathway, which the major end product is ethanol and CO_2, in addition to lactic acid and acetic acid (KIM; DONG; CHOI, 2021). Acetic acid improves AS since it inhibits yeasts, because their molecules diffuse through the cell membrane and dissociate in the cytoplasm acidifying the yeast cytoplasm (NARENDRANATH; THOMAS; INGLEDEW, 2001; KUNG et al., 2018).

Prevotella produces succinate, acetate and formate as a result of starch fermentation (WITZIG *et al*., 2010). *Prevotella* was described in silages by Li *et al.* (2022) and the major pathways are based on glycolysis and succinate production, from fumarate via phosphoenolpyruvate carboxylation and malate reduction (FRANK; DEPPENMEIER, 2018). *Turicibacter* in silages was described by Wang *et al.* (2020) and Gallo *et al*. (2021), and it is a strictly anaerobic bacteria associated with butyric acid. However, their metabolism and its interaction with other microorganisms are still unclear (ZHOU *et al*. 2019), which make any inference about this microorganism impossible.

The CO_2-supplied silages were defined by the positive correlation among *Erwinia*, 2,3-butanediol, 1,2-propanediol and acetic acid and the correlation among *Paenibacillus*, *Clostridium* and *Coprococcus*. *Erwinia* often dominates fresh crops (QUEIROZ *et al.,* 2018). It is found in the epiphytic microbiota and in the early stage of fermentation phase in maize silages (DROUIN; TREMBLAY; CHAUCHEYRAS-DURAND., 2019; SUN *et al*., 2021a). Some species of *Erwinia* contains 2,3 butanediol dehydrogenase and can produce 2,3 butanediol (HÖHN-BENTZ; RADLER, 1978) and acetic acid (SPINELLI *et al*., 2012), justifying their relation with 2,3 butanediol and acetic acid.

Paenibacillus, *Clostridium* and *Coprococcus* were strongly and positively correlated among each other. *Coprococcus* species present fermentative pathways involved in the acetate formation from pyruvate, acetoin biosynthesis, butanediol biosynthesis and butyrate formation from acetyl-CoA. Some *Clostridium* species are acetogenic bacteria. Acetogens are among the metabolically most versatile anaerobic organisms. Their most prominent metabolic feature is autotrophic growth; however, they can use a metabolic flexibility for utilizing a vast variety of different substrates. *Paenibacillus* species are N-fixing bacteria that can promote *Coprococcus* and *Clostridium* metabolism. For the CO_2-fixing microorganisms, the available energy supply is the most important factor that limits the growth. Energy sources for CO_2-fixation by chemoautotrophic microorganisms include many types of inorganic compounds (namely available electron donors), such as hydrogen, nitrogen, sulfur, phosphite, and metal species (HU *et al.*, 2016).

The acetogens are already used on an industrial scale to convert waste gas (syngas) in chemicals and biofuels (DÜRRE, 2017). The CO_2 production during ensiling is a source of greenhouse gases (GHG) emissions. CO_2 causes global warming by trapping heat in the atmosphere and alone accounts for around 77% of the total GHG emissions (CHEN *et al*., 2021). In worldwide, 665 million tons of fresh silage is consumed per year (XU *et al*., 2021). According to Chen *et al*. (2021), the use of lactic acid bacteria inoculants is an effective approach to mitigate climate change caused by greenhouse gases emissions from silage, due to the reduction in 66 mL of CO_2 to each 100 g of fresh silage. If no inoculants were used, 791 tons of CO_2 could be utilized by chemolithoautotrophic microorganism to produce biofuel (DÜRRE, 2017).

In the future, the importance of efficient production of high-quality silage (for feeding animals or supplying biogas plants) will increase, especially considering the climatic changes, political regulations and the current concerns with the environmental consequences of gas emissions (KROMMWEH *et al*., 2020; SCHMITHAUSEN *et al*., 2022).

In this study, we prove that CO_2 is absorbed by the silage microbiota by the metabolization of inorganic sources in organic compounds, improving the DM content and nutritional quality of silages. In the future, silages may be a source of CO_2 mitigation at the same time as will have their DM and quality improved, due to the increase in the acetate content. Acetate is the primary product of ruminal fermentation and it is used for fatty acid synthesis in adipose and mammary tissue and as an energy source by various tissues (GUALDRÓN-DUARTE; ALLEN, 2016).

Further studies are necessary to elucidate the microorganisms and the pathways involved in this CO_2 fixation, the rate of acetate and other metabolites production, the implications in other types of silage (DM, density, forage) and the consequences in DM intake and animal productivity.

4.5 FINAL CONSIDERATIONS

This is the first study to confirm CO_2 absorption by maize silage microbiota and to prove the subsequently increase of silage quality and dry matter content. Although, we still had not been able to state which microorganisms are involved with CO_2 fixation, the prediction of the functional profiling of the bacterial community are being analyzed and could help to better understand the microorganisms involved.

CO_2-supplies did not influence the developed of *Lactobacillus* during the ensilage and *Lactobacillus* and *Paenibacillus* were the most prevalent genera in silages. Although, *Clostridium, Coprococcus, Bifidobacterium, Turicibacter, Prevotella, Azospirillum, Blautia, Ruminococcus* and *Lactococcus* also appeared, but in smaller proportions. Among them, Clostridium, Blautia and Ruminococcus are composed by acetogenic bacteria and some species of the genera Bifidobacterium and Prevotella can produce succinic acid through CO_2 fixation reaction.

To the further researches is recommended improvements in the silo sealing system and the evaluation of succinic acid content in fresh forage and silage. In addition to the evaluation of bacteria community, the evaluation of fungi and archaeas, and the use of techniques of enrichment, isolation and identification of acetogenic bacteria would be important to assess some questions that have not yet been clarified.

In the future, the importance of efficient production of high-quality silage will increase, especially considering the climatic changes, making possible the use of silage as bioreactors fixing pollutant gases from farm activities into high quality nutritive compounds of feed. For that, further studies are necessary to better elucidate the microorganisms and the pathways involved in silage CO_2-fixation.

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