

UNIVERSITÀ CATTOLICA DEL SACRO CUORE

Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare

Ph.D. in Agro-Food System

Cycle XXXVII

S.S.D: AGRI-09/B



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CATTOLICA
del Sacro Cuore

**From field to bunk and bales: effective decisions about
harvesting, managing, and feeding silage and hay to improve
forage quality**

Coordinator:

Ch.mo Prof. Paolo Ajmone Marsan

Candidate:

Carmelo Mastroeni

Matriculation n:

5116134

Academic Year 2023/2024



UNIONE EUROPEA
Fondo Sociale Europeo



Ministero dell'Università
e della Ricerca



PON
RICERCA
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REACT EU

La borsa di dottorato è stata cofinanziata con risorse del Programma Operativo Nazionale Ricerca e Innovazione 2014-2020, risorse FSE REACT-EU Azione IV.4 “Dottorati e contratti di ricerca su tematiche dell’innovazione” e Azione IV.5 “Dottorati su tematiche Green”

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*Alla mia famiglia
senza la quale tutto questo
non sarebbe stato possibile*

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List of abbreviations

A. alternata – *Alternaria alternata*;
A. arborescens – *Alternaria arborescens*;
A. carbonarius – *Aspergillus carbonarius*;
A. clavatus – *Aspergillus clavatus*;
A. flavus – *Aspergillus flavus*;
A. fumigatus – *Aspergillus fumigatus*;
A. niger – *Aspergillus niger*;
A. nominus – *Aspergillus nominus*;
A. ochraceus – *Aspergillus ochraceus*;
A. parasiticus – *Aspergillus parasiticus*;
A. tenuissima – *Alternaria tenuissima*;
AAB – Acetic acid bacteria
ADFom, acid-detergent fiber exclusive of residual ash;
ADICP, acid detergent insoluble CP;
ADL – acid detergent lignin;
AFB1 - aflatoxin B1;
AFB2 -aflatoxin B2;
AFG1 - aflatoxin G1;
AFG2 – aflatoxin G2;
AFM1 – aflatoxin M1;
AFs – aflatoxins;
Ammonia N, Ammonia nitrogen expressed relative to total N (TN) basis;
aNDFom, neutral-detergent fiber assayed with a heat stable amylase and exclusive of residual ash;
ANOVA – analysis of variance;
AOAC, Association of Official Analytical Chemists;
B. firmus – *Bacillus firmus*;
B. lentus – *Bacillus lentus*;
B. licheniformis – *Bacillus licheniformis*;
B. pumilus – *Bacillus pumilus*;
B. sphaericus – *Bacillus sphaericus*;
B. subtilis – *Bacillus subtilis*;
BAB – butyric acid bacteria;
BEq - benzoate equivalent;
BL - corn silage treated with *Lentilactobacillus buchneri* (DSM22501/1k20738) and *Lactococcus lactis* (DSM11037/1k2081);
bm3 – brown mid 3 mutation;
BMR – brown mid rib;
br2 – brachytic 2 mutation;
BRs – brassinosteroids;
C – cultivar;
C. butyricum – *Clostridium butyricum*;
C. tyrobutyricum – *Clostridium tyrobutyricum*;
CCH – conventional corn hybrid;
CFU - colony forming units;
CO₂ – carbon dioxide;
CP – crude protein;
CTR – control;
DM – dry matter;
dmnl - dimensionless;
dNDF - NDF digestibility evaluated after 24 h of rumen incubation;

DON – deoxynivalenol;
 EE – ether extract;
 ELP – corn silage treated with *Lactococcus lactis* (NCIMB30117/1k2083), *Lactiplantibacillus plantarum* (DSM16568/1k20720) and *Enterococcus faecium* (DSM22502/1k20602);
 EVOH – ethyl- vinyl alcohol
F. acuminatum – *Fusarium acuminatum*;
F. avenaceum – *Fusarium avenaceum*;
F. crookwellense – *Fusarium crookwellense*;
F. culmorum – *Fusarium culmorum*;
F. equiseti – *Fusarium equiseti*;
F. graminearum – *Fusarium graminearum*;
F. incarnatum – *Fusarium incarnatum*;
F. langsethiae – *Fusarium langsethiae*;
F. oxysporum – *Fusarium oxysporum*;
F. poae – *Fusarium poae*;
F. proliferatum – *Fusarium proliferatum*;
F. semitectum – *Fusarium semitectum*;
F. solani – *Fusarium solani*;
F. sporotrichioides – *Fusarium sporotrichioides*;
F. subglutinans – *Fusarium subglutinans*;
F. tricinctum – *Fusarium tricinctum*;
F. verticillioides – *Fusarium verticillioides*;
 FB1 – fumonisin B1;
 FB2 – fumonisin B2;
 FB4 – fumonisin B4;
 FB6 – fumonisin B6;
 FBA1 – fumonisin BA1;
 FBA2 – fumonisin BA2;
 FBs – fumonisins;
 FCM – fat corrected milk;
 GAs – gibberellins;
 GC - gas chromatography;
 GLM - general linear model;
 HD – corn silage compacted at high density condition;
 HDPE - high density polyethylene;
 HM – harvest moment;
 HPLC - high performance liquid chromatography;
 HSD – honestly significant difference;
 isSD 7 h – in situ starch digestibility 7 hour;
 ivNDFD – in vitro NDF digestibility;
 LA + Eth ratio, lactic acid/ acetic acid plus ethanol ratio;
 LA ratio – lactic acid/ acetic acid ratio;
 LAB - lactic acid bacteria;
 LD – corn silage compacted at low density condition;
 LD + Cl – corn silage compacted at low density condition and inoculated with *Clostridium tyrobutyricum* UC7086;
 LDPE – low-density polyethylene;
Llb. buchneri - *Lentilactobacillus buchneri*;
Llb. diolivorans - *Lentilactobacillus diolivorans*;
Llb. hilgardii - - *Lentilactobacillus hilgardii*;
Llb. kefir - - *Lentilactobacillus kefir*;

Llb. parafarraginis - *Lentilactobacillus*
Lfy - leafy allele;
 lignin(sa) - lignin determined by solubilization of cellulose with sulphuric acid;
Lim. fermentum - *Limosilactobacillus fermentum*;
Lim. mucosae - *Limosilactobacillus mucosae*;
Lim. reuteri - *Limosilactobacillus reuteri*;
 LSMEANS - least squares mean;
 MDP - mycotoxin-deactivating feed product;
 MT - mowing time;
 MTX - mycotoxin;
 NDF - neutral detergent fiber;
 NDFD - NDF digestibility;
 NDICP - neutral detergent insoluble CP;
 NIRS - near-infrared reflectance spectroscopy;
 NIV - nivalenol;
 OB - oxygen barrier;
 OM - organic matter;
 OS - WPCS treated with SiloSolve® OS (Chr. Hansen A/S, Hørsholm, Denmark) and sodium benzoate;
 OTA - ochratoxin A;
 OTC - ochratoxin C;
 OTR - oxygen transmission rate;
 OTs - ochratoxins;
P. paneum - *Penicillium paneum*;
P. roqueforti - *Penicillium roqueforti*;
 PA - polyamides;
 PAA - propionic acid bacteria;
 PCA - principal components analysis;
 PE - polyethylene;
 peNDF - physically effective NDF;
 PS - potassium sorbate;
 R1 - corn silage treated with *Lactiplantibacillus plantarum* PB (LP PB), *Lacticaseibacillus rhamnosus* LR7 (LR LR7), and *Lacticaseibacillus rhamnosus* RI (LR RI) in a ratio of 50%-25%-25%;
 R2 - corn silage treated with *Lactiplantibacillus plantarum* PB (LP PB), *Lacticaseibacillus rhamnosus* LR7 (LR LR7), and *Lacticaseibacillus rhamnosus* RI (LR RI) in a ratio of 33%-33%-33%;
 RHMC - rolled high-moisture corn;
 Rumen LM - corn silage treated with *Limosilactobacillus mucosae* UNISS B81;
 SB - sodium benzoate;
 SCH - smart corn hybrid;
 SLs - strigolactones;
 SNB - silo-NET bag;
 SP - sodium propionate;
 SPDC - corn snaplage alone or in combination with ground dry shelled corn;
 SPFH - self-propelled forage harvester;
 TLOC - theoretical length of cut;
 TMR - total mixed rations;
 TN - total N;
 TTSD - total-tract starch digestibility;
 uNDF - undigested NDF
 UV - ultra-violet;
 VFA - volatile fatty acid;

VOC - volatile organic compound;
WPCS - whole plant corn silage
WSC - water soluble carbohydrates;
Y -year;
ZEN - zearalenone;
 α -ZEL - α -zearanol;
 β -ZEL - β -zearalenol;

Abstract

Improving the nutritional quality of forages is crucial to maximize the performance of dairy and beef cattle. Thus, producing high-quality forages, both silage and hay, is dependent on management decisions and practices applied among ensiling and haymaking. This Thesis was developed considering the academical course in which topic related to silage and hay were investigated. In particular: a literature review about mycotoxin occurrence, two trials (i.e., farm and laboratory conditions) testing silage additives, one trial referred to the evaluation of forage feeding value of corn hybrids for silage production, and one trial related to the management aspects of four alfalfa cultivars selected for hay were conducted.

Among regulated mycotoxins, the most studied and known for their effects are aflatoxins (AFs) AFB1, AFB2, AFG1 and AFG2, as well as the AFM1 for its high importance in dairy sector, deoxynivalenol (DON) and its metabolites 3/15 acetyl-DON and 3-glucoside DON, T-2 and HT-2 toxins, zearalenone (ZEN), fumonisins, in particular that belong to the class B, and ochratoxin A. Due to the emergence of multiple mycotoxins that are detectable in feed utilized in ruminant diets, such as ensiled forage, there is now a growing focus on investigating these compounds by the scientific community to understand their toxicity for animal health.

The first trial was carried out testing a combination of bacterial mixture and sodium benzoate (OS) in comparison to a control (CTR) group to reduce the aerobic spoilage in the top layer of an experimental bunker silo. The OS solution allowed to reduce the aerobic deterioration in the silage top layer showing a better microbiological profile and aerobic stability than the control group. In addition, the application of the combined solution allowed to reduce the DM losses from the top layer.

The second study aimed to evaluate the effects of lactic acid bacteria (LAB) inoculants on fermentative, chemical, and aflatoxin contamination in corn silage packed at different levels of density. Two commercial inoculants, two pure LAB mixtures and one animal-derived LAB were tested in comparison to a CTR group. High density conditions allowed to achieve a well-fermented silage with an optimal aerobic stability. Although the different combinations of LAB did not change the final fermentative profile, silages treated with the animal-derived LAB alone presented a higher content of acetic acid, propionic acid, ethanol, and total alcohols, and an aerobic stability on average higher by 3.7%, 1.3% and 8.1% in comparison to the commercial inoculants, the pure LAB mixtures and the CTR group respectively.

The aim of the third study was to evaluate the forage feeding value of three corn hybrids carrying the *br2* mutation (SCH) in comparison to two conventional corn hybrids (CCH) harvested at 5 different maturity stage (HM). Fiber digestibility, starch digestibility and estimated energy content (milk/ton) through Milk2006 and Milk2024 were evaluated. The SCH had higher energy content (milk/ton) than CCH hybrids. In this study, SCH had lower fibrous fractions, higher starch content and in situ starch digestibility 7 h (isSD7 h) when compared with CCH. In addition, higher neutral detergent fiber digestibility (NDFD) at 120 h and lower undigested neutral detergent fiber (uNDF) at 240 h evaluated in vitro were observed for SCH, highlighting that enhancing fiber digestibility is a key factor to improve WPCS. Further studies are warranted to elucidate the agronomical aspects of these new corn hybrids and their effects on animal performance.

The fourth study aimed to evaluate yield, nutritional value, and morpho-physiological traits of four alfalfa cultivars, cut three times, with five different mowing timings (i.e., 21, 25, 30, 35, and 40 days), in an experimental field under traditional management of the alfalfa ley. Dry matter (DM) yield increased with later mowing times. Significant differences in crude protein (CP) yield were observed across cultivars, mowing timings, and years. Later mowing times resulted in higher DM yield but lower CP concentration and increased lignification, reducing fiber digestibility. These findings underscore the importance of strategic cultivar selection and adaptive mowing practices to balance forage yield and quality.

1. Literature review on the ensiling process

Silage is a fermented product from a crop with a sufficient moisture content stored in anaerobic conditions. Ensiling is widely practiced in livestock systems allowing to minimize losses during the harvest and storage periods (Grant and Adesogan, 2018). Considering the great benefits in producing silage, this forage preservation practice is based on the conversion of simple plant sugars, such as glucose and fructose, to lactic acid by lactic acid bacteria (LAB) under anaerobic conditions (McDonald et al., 1991; Rooke and Hatfield, 2003).

The ensiling process is usually divided in 4 phases: 1) the initial aerobic phase after harvest, 2) the fermentation phase, 3) the stable or storage phase in the silo, and 4) the feed-out phase.

The initial aerobic phase starts after harvesting and chopping of the forage which will be ensiled in a silo (e.g., bunker silos, drive-over piles, tower silos, round bales, plastic tubes). Before sealing the silo, packing the forage is crucial to reduce the mass porosity, which in combination with proper sealing reduce the trapped oxygen, that is rapidly consumed by plant cells and aerobic and facultative microorganisms, as it may triggers biological and chemical processes that consume nutrients and energy producing water, carbon dioxide (CO₂), heat and free ammonia (Borreani et al., 2018). During this phase, the obligate and facultative aerobic microorganisms such as molds, yeasts, and bacteria dominate on the lactic acid bacteria (LAB) which are the essential microflora to start the fermentation. Speed-up this phase can significantly contribute to decrease nutrient losses.

The fermentation phase starts when oxygen concentration decreases. This stage can continue for about a couple of weeks until one month depending on crops and ensiling conditions. The main microorganisms involved in the fermentation phase are LAB, enterobacteria, clostridia, and yeasts (Pahlow et al., 2003). The LAB are the essential group of microorganisms that lead the fermentation pathways by fermenting sugars to organic acids ensuring the well preservation of the ensiled crop. During this phase, losses associated to fermentation may occur depending on the dominant species and their substrates (Borreani et al., 2018). The LAB are able to lead two fermentation pathways: homofermentative pathway in which LAB produce only lactic acid and no

dry matter (DM) loss occur in this pathway; whereas in heterofermentative pathway LAB ferment glucose producing lactic acid, acetic acid, CO₂, ethanol. However, when fermentation pathways are led by enterobacteria, clostridia, or yeasts the DM losses tend to drastically increase during this phase. Notably, yeasts ferment glucose producing ethanol, CO₂ and water, whereas clostridia produce butyrate, CO₂ and water from lactate or glucose, causing DM losses that approach 50% (Pahlow et al., 2003). Limiting the DM losses during the fermentation phase can be achieved by forage processing properly, forage wilting (if needed), well-compaction during silo filling, and effective sealing to allow LAB to dominate the fermentation in order to promote a fast dropping of pH, as well as the use of silage additives capable of inhibiting the growth of undesirable microorganisms and increasing nutrient recovery.

The stable or storage phase in the silo starts when the silo achieves a whole anaerobic condition and the intensity of fermentation decreases. In anaerobic conditions, some species can persist during this stage by entering a dormant phase such as yeasts and molds, or spore forming as bacilli, and the LAB population usually declines by 3 logarithmic below their numerical peak due to the inhibition from the low pH and fermentation products they produced (Pahlow et al., 2003). Maintaining proper sealing is crucial to minimizing the DM losses in the course of storage period.

In the fourth phase, the silo is open for feeding the herd and during this period the ensiled mass is exposed to air (Wilkinson and Davies, 2013a). During the feed-out phase oxygen penetrates through the silo face, especially in the peripheral areas of the silo due to the higher porosity in those zones which are more difficult to pack (Borreani et al., 2007), and the oxygen flow is proportional to porosity (Pitt and Muck, 1993). The effect of low compaction in these areas is associated to an increase of silage temperature compromising the preservation of silage during the storage and feed-out phases with the occurrence of mycotoxins (Brüning et al., 2018; Gallo et al., 2016a). Oxygen penetration within the ensiled mass triggers the growth of aerobic microorganisms. Generally, yeasts are known to be the initiators of the aerobic deterioration, consuming sugars and fermentation products and increasing silage temperature and pH (Pahlow et al., 2003). This deterioration process is completed by molds. However, acetic acid bacteria have been found to be the precursors of the aerobic deterioration somewhat in corn silage, the reason is not clearly understood (Dolci et al., 2011; Spoelstra et al., 1988). Despite the lower nutritive value, spoiled silages can be source of indirect losses adversely affecting animal performance and health (Kung et al., 1998). Therefore, spoiled silage can affect the metabolism and immunity of ruminants causing problems to humans due to transfer of pathogens and mycotoxins from silage to animal products (Gallo et al., 2015).

Silage fermentation and microorganisms' performance

The fermentation process is led by huge number of microorganisms under anaerobic conditions that use fermentable sugars such as glucose or fructose to produce organic acids. However, the microorganisms involved in the fermentation process can differently affect the final quality of the ensiled feed, leading somewhat to undesirable fermentations. The substrate present, environmental conditions and the oxygen availability define the quality of the silage produced, regardless of the ensiled material (Ávila and Carvalho, 2020). These characteristics are related to the microbiota present in the material being ensiled and on the conditions that can affect the development of the microbiota (Ávila and Carvalho, 2020).

The microbial population stands on the fresh crop is completely different from the ones involved during the fermentation or feed-out phases. The most significant phyla identified during ensiling belong to Firmicutes and Proteobacteria (Liu et al., 2019; Ogunade et al., 2017). The mentioned phyla present bacteria that are known to be desirable among the fermentation process such as lactic acid bacteria (LAB) and propionic acid bacteria (PAB), whereas the undesirable species during ensiling belong to the Enterobacteriaceae family, acetic acid bacteria (AAB), spore-forming bacteria (Bacillales and Clostridiales), and *Listeria* (Pahlow et al., 2003). In addition, several species belong to molds and yeasts have been found and considered undesirable as well (McDonald et al., 1991).

Lactic Acid Bacteria (LAB)

The LAB are essential microorganisms for proper silage fermentation. The bacteria belonging to this group mainly produce lactic acid or other fermentation products. Taxonomically all LAB belong to the Lactobacillales order and spread across four families: Lactobacillaceae, Leuconostocaceae, Enterococcaceae, Streptococcaceae (Zheng et al., 2020). The main genera of LAB found in silage are *Lactiplantibacillus*, *Lentilactobacillus*, *Pediococcus*, *Lacticaseibacillus*, *Enterococcus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Weissella*.

The LAB can be classified in three groups:

homofermentative LAB, which ferment hexoses producing exclusively lactic acid, but they cannot ferment pentoses due to they lack phosphoketolase;

facultative heterofermentative LAB, which ferment hexoses as homofermentative LAB, and pentoses because they have both aldolase and phosphoketolase;

obligate heterofermentative LAB, that ferment hexoses to other fermentation end-products in addition to lactic acid.

Optimum temperature for LAB growth ranges between 27 and 38°C (Yamamoto et al., 2011). Although LAB have been considered as anaerobes, most of them can grow under aerobic conditions, consuming oxygen and allowing to establish an anaerobic conditions in the forage mass (Yamamoto et al., 2011). Moreover, the presence of air may influence the metabolism of some LAB (Condon, 1987) following by a reduction in lactic acid production (Kim and Adesogan, 2006). Knowledge regarding the metabolism of LAB is used to explain how these microorganisms act in the course of fermentation. Metabolic variation occurs between strains of the same species may be associated with the environments in the strains developed, such as tolerance to high temperatures (Ávila and Carvalho, 2020).

A fast initial acidification of the ensiled mass can help to control the growth of enterobacteria and clostridia. Generally, enterobacteria and clostridia continue to growth during the first steps of the ensiling process until an inhibitory concentration of undissociated acids and a sufficiently low pH have been achieved (Pahlow et al., 2003). The optimum pH level varies among the ensiled crop, even though it is linked to the concentration of lactic acid ($pK_a = 3.86$) and buffering capacity of the crop (Kung et al., 2018a). Lactic acid is generally the main fermentation end-product in silages (Kung et al., 2018) and it contributes to drop silage pH due to its lower dissociation constant (pK_a) than acetic ($pK_a = 4.75$) and propionic acids ($pK_a = 4.87$). For instance, corn silages have a lower final pH (e.g., 3.7-4.0) compared to legume silages (e.g., 4.3-5.0) due to corn plant has a lower buffer capacity than legume crops (e.g., 200-250 and 500-550 mE/kg of DM, respectively; McDonald et al., 1991). Therefore, treating legume silages with homofermentative LAB may allow to achieve a rapid dropping in pH if crop conditions (e.g., DM and sugar concentration) are favorable (Silva et al., 2020).

Propionic acid bacteria (PAB)

The PAB are considered as Gram-positive, facultatively anaerobic bacteria. These bacteria can ferment carbohydrates, polyols and organic acids, including lactic acid and gluconic acid (Ávila and Carvalho, 2020). Throughout glycolysis or pentose phosphate pathway, the substrates are oxidized to pyruvate; afterward, pyruvate is reduced to propionic acid through the Wood-Werkman cycle or oxidized to acetic acid and CO_2 . Although propionic acid is known for its

antifungal capacity (Pahlow et al., 2003), the role of PAB in silage is not clear and the small amounts of propionic acid often found in well-fermented silages are not sufficient to explain the presence of these bacteria. Exceptionally, higher concentrations of propionic acid can be found in poorly fermented silages, mainly due to clostridial activity (McDonald et al., 1991). Furthermore, propionic acid can be found in silages where *Lb. diolivorans* is active contributing to the conversion of 1,2-propanediol to 1-propanol and propionic acid (Krooneman et al., 2002).

Enterobacteria

Enterobacteria are classified as facultatively anaerobic Gram-negative bacteria usually found in poorly preserved silage. Several species ferment glucose to acetic acid, succinic acid, ethanol, and 2,3 butanediol. Generally, the number of enterobacteria found on forage crops is higher than LAB at ensiling, due to their ability to survive under unfavorable conditions during the cold, dry winter period (Pahlow et al., 2003). The population of enterobacteria is generally high on forages at vegetative period of growth and it usually increases after the first ensiling days, reaching a peak of $10^8 - 10^{10}$ cfu/g of fresh matter (FM) in grass or legume silages; whereas in corn silages a short-term increase in the number of enterobacteria can only be observed if microbiological determinations are carried out during the first day after ensiling, due to the fast acidification, that will prevent the proliferation of enterobacteria once pH has dropped below 4.5 (Pahlow et al., 2003). Due to their facultative anaerobic nature, enterobacteria are able to compete with LAB for nutrients prior to and after ensiling. Several species belong to the enterobacteria can degrade proteins, producing ammonia and biogenic amines, which are undesirable in silage (McDonald et al., 1991; Rooke and Hatfield, 2003). Biogenic amines are known to be toxic compounds that adversely affect silage palatability in ruminants (Driehuis and Elferink, 2000). Additionally, enterobacteria can reduce nitrate (NO_3^-) to nitrite (NO_2^-), and nitrite to ammonia and nitrous oxide (N_2O) (Spoelstra, 1985). During the exposure of air, nitric oxide is oxidized to a mixture of gaseous nitrogen oxides (NO_2 , N_2O_3 , and N_2O_4), that sometimes are visible as a yellow-brown gas escaping from a silo. In conclusion, nitric oxide and nitrogen dioxide react to water realizing nitrous acid (HNO_2) and nitric acid (HNO_3) that cause respiratory irritation (Driehuis and Elferink, 2000).

Wieringa (1958) investigated the inhibition of butyric acid fermentation by nitrite. In this study, he observed that the nitrite, as a reduction product of plant nitrate, suppressed butyric acid fermentation in grass silages. The antimicrobial effect of nitrite is based on the released nitric acid and the emerging nitrogen oxides. Notably, nitric oxide penetrates the bacteria cell wall inhibiting the glycolysis catalyzing enzymes.

Clostridia

The genus *Clostridium* comprises Gram-positive, endospore-forming bacteria. Most species are obligate anaerobes, whereas some species may be aerotolerant (Ávila and Carvalho, 2020). Bacteria belong to the genus *Clostridium* are not part of the epiphytic microbiota of crops and occur through soil and manure contamination (Pahlow et al., 2003). Depending on the stocking conditions and animal feed contamination, the BAB spore concentration in slurries and manures ranges between 2.30 to 4.70 log₁₀ most probably number(MPN)/g (Östling and Lindgren, 1991). The clostridia often develop when the pH is high in silages with a high moisture content (Pahlow et al., 2003). Clostridia usually take their energy through the fermentation of carbohydrates, organic acids, and proteins. On the other hand, Clostridia can grow at lower pH values than enterobacteria and bacilli, making them more difficult to control. Therefore, it is crucial to understand the interaction of pH, DM content and crop (Muck, 2010).

Pahlow et al. (2003) stated that the most common clostridia found in silage can be classified within three groups:

Proteolytic clostridia, which ferment amino acids. When pH is close to 5, these clostridia can proliferate and plant proteolytic enzymes start to hydrolyze the plant protein releasing free amino acids that are readily available for metabolism by clostridia. The main pathways of amino acid catabolism include deamination and coupled oxidation and reduction of pairs of amino acids (Stickland reaction) leading to a production of NH₃ and a mixture of organic acids (e.g., acetic, propionic, and butyric acids) and other products (e.g., alcohols).

Clostridium butyricum group, capable of proliferate at a level of pH somewhat similar to the proteolytic group, and they are able to ferment a huge range of carbohydrates. The main fermentation products are butyric and acetic acids.

Clostridium tyrobutyricum, which has been widely studied in silage fermentation due to its adverse effects on the quality of hard cheese. This bacterium is able to ferment lactic acid producing butyric acid, hydrogen, and CO₂ resulting in energy and DM losses.

Beyond their occurrence in poorly fermented silages, aerobic deterioration can trigger clostridial development. Incorrect ensiling practices may favor air penetration within the ensiled mass adversely affecting the quality of the silage and enhancing the increase of spore-forming bacteria such as clostridia (Gallo et al., 2016a; Zucali et al., 2015). Usually, the peripheral areas (e.g., upper and lateral sides of the bunker silos) are more prone to spoilage due to greater risk of air influx (G. Borreani and Tabacco, 2010; Gallo et al., 2016b). Feeding large dairy herds with a total mixed ratio (TMR), obtained by mixing forages (e.g., hay and silage), concentrates, by-products, minerals, vitamins, and additives, is widely applied in Italy (Mosconi et al., 2023). Focusing on silage composition, highlighting that the microbial contamination by butyric acid bacteria (BAB),

especially *C. tyrobutyricum*, may change among farms because the raw materials within the TMR define the microbial load level (Driehuis, 2013). Mosconi et al. (2023) estimated the contamination levels of *C. tyrobutyricum* in silage and TMR implementing a prediction model by using a real-time PCR approach including the residues of TMR and silage. Notably, they stated that the residues of TMR and silage represent a reservoir of microbial contamination among the following TMR preparation. Therefore, it is crucial to lead a proper management practice of the bunker silo among the disposal of the de-ensiled material in addition to a recurring cleaning of the mixing wagon in order to limit *C. tyrobutyricum* contaminations.

Bacilli

Bacilli are endospore-forming bacteria belonging to the family *Bacillaceae*. These bacteria differ from clostridia due to their ability to grow aerobically, whereas clostridia are obligate anaerobes (Pahlow et al., 2003). However, all bacilli are obligate aerobes some of them are considered as facultative anaerobes that can ferment a wide range of carbohydrates to organic acids (e.g., acetic, lactic, and butyric acids) or ethanol, 2,3-butanediol, and glycerol.

Bacillus spp. seem to have no role in starting aerobic deterioration of silage (Holden, 1989), even if they can affect the later stages of aerobic deterioration after deterioration started by yeasts (Spoelstra, 1985). Several species such as *B. lentus*, *B. firmus*, and *B. sphaericus* (McDonald et al., 1991) have been found during the aerobic deterioration.

Bacillus spores can contaminate raw milk during the milking time due to contamination with cow feces (Pahlow et al., 2003). Thus, not only clostridial spores but also *Bacillus* spores from silage can pass through the rumen and gut contaminating cow feces. *Bacillus cereus* is considered the most important spoilage organism of pasteurized milk for the ability of some strains to produce three types of enterotoxins such as haemolytic toxin, nonhaemolytic toxin and enterotoxin (Phelps and McKillip, 2002).

In the last few decades, studies have revealed that many organisms previously classified in the *Bacillus* genus actually represent different genera of the *Bacilli* class (Durak et al., 2006). Members of the *Paenibacillus* genus are facultative anaerobic organisms that produce spores in swollen sporangia and some of these organisms excrete diverse assortments of extracellular polysaccharide-hydrolyzing enzymes (Shida et al., 1997). The occurrence of *Bacillus* and *Paenibacillus* spores in silage is of great concern to dairy producers (Giffel et al., 2002), due to they can survive pasteurization in spore form and are able to grow under refrigeration, resulting in product spoilage and limiting the shelf life of pasteurized fluid milk (Ivy et al., 2012). Among the *Paenibacillus* spp., the most frequently reported are *Paenibacillus odorifer*, *Paenibacillus graminis*,

Paenibacillus amylolyticus, *Paenibacillus c.f. peoriae*, and *Paenibacillus polymyxa* in milk (Ivy et al., 2012; Driehuis et al., 2016), and *Paenibacillus polymyxa*, *Paenibacillus pabuli*, and *Paenibacillus macerans* in silages (Borreani et al., 2013; Driehuis, 2013; Driehuis et al., 2016).

Nevertheless, the presence of *Bacillus* spp. is undesirable in silage, some species have been used as possible inoculants. Several species of *Bacillus* can produce antimicrobial substance as peptide and lipopeptide antibiotics and as bacteriocins; indeed, the bacteriocins of *Bacillus* have a larger action spectrum than those produced by LAB (Abriouel et al., 2011). Notably, positive results concern increasing in aerobic stability with the inoculation of *B. subtilis* (Lara et al., 2015) and the inhibition of molds with *B. subtilis*, *B. licheniformis* and *B. pumilus* (Pahlow et al., 2003).

Listeria

Listeria is genus of bacteria with a facultatively anaerobic metabolism that needs microaerophilic environments and can synthesize acids from glucose without gas production (Ávila and Carvalho, 2020). *Listeria monocytogenes* is a microorganism that causes listeriosis a type of feed-borne infection that affects both animals and humans (Coblentz and Akins, 2018). This microorganism has been observed mainly in raw milk, stating that its presence may occur through fecal contamination of teats ends during milking (Visser et al., 2007). This bacterium is able to survive in acidic environment and high temperatures (Abbas and Jaber, 2012). Historically, listeriosis in livestock system has been associated with poor-quality silages preserved in round bales (McDonald et al., 1991). Notably, the incidence of listeriosis might be correlated with the development of baled-silage techniques, and that early use of pre-formed bags to create anaerobiosis might have facilitated the proliferation of *Listeria* by allowing low levels of oxygen to enter the sealed bale, particularly through the tied end (Coblentz and Akins, 2018). The presence of oxygen may affect the survival during the storage period and feed-out acting in the phase of aerobic deterioration of silage (Nucera et al., 2016). It is possible to avoid the growth and contamination of these bacteria through proper silage management, enabling a rapid pH drop and the establishment of an anaerobic environment.

Acetic acid bacteria

Acetic acid bacteria (AAB) are Gram-negative with a high tolerance to acidic conditions, with the ability to cause an incomplete oxidation of alcohols, sugars, and lactic acid (Ávila and Carvalho, 2020). Most of these bacteria are obligate aerobic and have been identified as potential initiator of the aerobic deterioration in silage (Spoelstra et al., 1988). The AAB belong to the family of *Acetobacteraceae*, which includes two subgenera, *Acetobacter* and *Gluconoacetobacter*. The species belonging to *Acetobacter* can oxidize acetate and lactate to CO₂ and water, whereas

Gluconoacetobacter cannot (Pahlow et al., 2003). The presence of these bacteria has been reported only in corn silage, indicating that AAB alone can initiate aerobic deterioration (Spoelstra et al., 1988).

Yeasts

Yeasts are involved in the aerobic deterioration of silage. Due to their ability to tolerate acidic conditions and remain viable in absence of oxygen, these microorganisms can utilize lactic acid as carbon source to grow rapidly when silage is exposed to air mainly during feed-out (Ávila and Carvalho, 2020), but also during storage. The main fermentation products produced by yeasts are ethanol and CO₂, by other fermentation products such as alcohols, volatile fatty acids, and lactate can be produced in anaerobic conditions (Pahlow et al., 2003). The presence of yeasts in silage is responsible for high DM losses and low aerobic stability, as they can assimilate lactic acid upon air exposure.

In silages, yeast growth may be favored by several factors such as the increased availability of water-soluble carbohydrates, temperatures close to 20°C and air infiltration (Weiss et al., 2016). Nevertheless, anaerobic conditions and short-chain fatty acids (e.g., acetic and propionic acids) may inhibit yeast growth. The molecules of these undissociated short-chain fatty acids can diffuse into the cell dropping the intracellular pH realizing H⁺ ions (Pahlow et al., 2003).

Molds

Molds belonging to the genus of *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma* and others have been isolated from corn and grass before ensiling (Wambacq et al., 2016). Forage contamination occurs in the field, and molds may be detected in silage during all stages of silage fermentation. Their growth causes a loss of nutrients, contamination with mycotoxins and possible fungal diseases through the colonization of animal and human tissues, such as aspergillosis (Puntenney et al., 2003).

The incidence of molds in silage is linked to the presence of oxygen and increase in pH levels. Several molds isolated from silage do not tolerate low concentrations of oxygen and low pH values; although, molds species such as *Penicillium roqueforti* and *Penicillium paneum* may survive or grow under these conditions (Wambacq et al., 2016). The presence of *Aspergillus fumigatus* in silage has been associated with the hemorrhagic bowel syndrome (HBS), a disorder that affects dairy cattle. Notably, HBS is characterized by a drop in milk production, abdominal pain due to obstructed bowel, anemia, and death (Elhanafy et al., 2013).

Mycotoxins

Mycotoxins are a group of secondary metabolites of molds. Their production is related to certain conditions such as exposure to adverse conditions (e.g., nutrient availability, pH, water activity, temperature). If consumed by animals, mycotoxins may cause severe diseases such as intoxication, suppression of the immune system, reductions in production and feed conversion, increased infections and hormonal imbalances (Bennett and Klich, 2003; Vila-Donat et al., 2018). Additionally, mycotoxins present in livestock ration can be transferred to the milk and meat; for instance aflatoxins in cattle feed can be metabolized into aflatoxin M1 by cows (Gallo et al., 2015). Mycotoxins that are generally present in silage include trichothecenes, fumonisins, aflatoxins, zearalenone (ZEA), mycophenolic acid, and roquefortine C (Ogunade et al., 2018).

Preventing mycotoxin contamination among the ensiling process can be led adopting several expedients. In the field, mycotoxin contamination can be mitigated by minimizing environmental stress to the plant adopting proper agronomic practices such as using varieties or hybrids tolerant to biotic conditions of a specific area, management of crop residues and weeds, scheduling of irrigation to avoid drought stress, crop rotation, and accurate fertilization (Ogunade et al., 2018). The harvest period should be carefully led considering the DM content of crop, the cutting height of the standing crop avoiding to minimize soil contamination due to a possible occurrence of *Fusarium* spores in soil (Jouany, 2007). Low pH and anaerobic conditions during ensiling largely inhibit toxigenic fungi (e.g., *Fusarium*, *Aspergillus*, *Monascus*, *Rhizopus*, and *Geotrichum*), and mitigate the production of mycotoxins in silage (Pahlow et al., 2003). Molds occurrence can be observed in the peripheral areas of the bunker silo such as the top layer and the sidewalls that are more prone to oxygen penetration in comparison to the core of the bunker, due to it is difficult to achieve a well-packing condition (Gallo et al., 2016a). Reducing mycotoxin contamination during feed-out can be achieved measuring the aerobic stability of silage, unloading the silo face daily referring to the environmental conditions (e.g., 15 cm in the winter and 25 cm in the summer) followed by a proper disposal practice of the spoiled ensiled material in the top layer of the bunker (Bernardes et al., 2021).

Volatile organic compounds production in silage

The production of volatile organic compounds (VOC) in silage is mainly led by microorganisms during fermentation and storage phases. It is widely known that during the pre-harvest stage aerobic microflora prevails on crops without contributing to silage preservation due to it belongs to the obligate aerobic bacteria (Pahlow et al., 2003). Using LAB is a broad practice to enhance the number of desirable bacteria at ensiling and drive the fermentation pathways. For

instance, inoculating silage with obligate heterofermentative LAB, such as *Lentilactobacillus buchneri*, may promote acetic acid production enhancing the aerobic stability of silage (Muck et al., 2018). The major part of VOC is generated by heterofermentative LAB or undesirable microorganisms such as enterobacteria, clostridia, and yeasts (Hafner et al., 2013). Brüning et al. (2018) observed that delayed sealing increased ethyl ester concentrations (i.e., ethyl lactate and ethyl acetate) at silo opening due to yeasts growth which could support ethyl esters production or provide the prominent role of ethanol in the esterification process suggested by Weiss et al. (2016).

Acids

Acetic acid is often the second highest concentrated compound found in silage covering a range between the 5 to 40 g/kg DM. Indeed, moderate acetic acid concentrations in silage may be advantageous for inhibiting yeasts activity and enhancing aerobic stability during the air exposure, because this acid has a strong antifungal propriety (Kung et al., 2018a). This acid is mainly produced by facultative heterofermentative and obligate heterofermentative LAB, enterobacteria, and clostridia. Considering the fermentation pathways led by the above-mentioned microflora, it is possible to detect high concentrations of acetic acid (>40-60 g/kg DM) in wet silages (McDonald et al., 1991). Wet silage inoculated with *Llb. buchneri* often has high concentrations of acetic acid. Consequently, a high concentration of acetic acid is not always synonym of poor fermentation. Anyway, very high concentrations of acetic acid (>35-40 g/kg DM) are undesirable as it depresses feed intake depending on the silage inclusion in the TMR (Bandla et al., 2024).

Propionic acid is usually present in low concentrations in well-fermented silages (<10 g/kg of DM). Propionibacteria are involved in converting glucose and lactic acid to propionic acid as the main end fermentation product, but they are able to produce acetic acid. However, the benefits of applying these bacteria as silage inoculant is not consistent and it is unlikely that they can compete with the epiphytic microbiota during silage fermentation. (Pahlow et al., 2003; Kung et al., 2018). Propionic acid can be also produced by clostridia or by *Llb diolivorans* (Hafner et al., 2018). Silages high in butyric acid, due to clostridial activity, may have significant levels of propionic acid (McDonald et al., 1991). On the other hand, Krooneman et al. (2002) reported that silages treated with *L. buchneri* can have high concentrations of propionic acid because it can be synthesized by *L. diolivorans* from 1,2 propanediol.

Butyric acid is the main product of clostridial fermentations led by three groups of clostridia detected in silages: proteolytic clostridia that are able to ferment amino acids producing the common volatile fatty acids found in silage such as acetic, propionic, butyric, iso-acids (e.g., iso-butyric, iso-valerianic, and valerianic acid); *Clostridium butyricum* that typically ferments a huge amount of carbohydrates to butyric and acetic acids; and *Clostridium tyrobutyricum* which is able to

ferment lactic acid to butyric. In well-fermented silages this acid should not be detected at high concentrations, because it leads to a huge DM loss and poor energy recovery (Pahlow et al., 2003). Clostridial silages are characterized by high levels of pH and high concentrations in acetic acid, ammonia nitrogen, biogenic amines, and soluble crude protein (Kung et al., 2018).

Alcohols

Ethanol is the most common alcohol found in silages produced by different microorganisms, mainly obligate heterofermentative LAB and yeasts, through the fermentation of sugars. Heterofermentative LAB ferment one mole of glucose to one mole of lactic acid, one of ethanol, and one mole of CO₂; whereas yeasts generate 2 mol of ethanol and 2 mol of CO₂ (McDonald et al., 1991). Ethanol production in silage is not desirable due to DM losses in terms of CO₂ without producing acid during fermentation. Silages contained high levels of ethanol, such as more than 30-40 g/kg of DM, are often related to yeasts activity that are able to assimilate lactic acid when silage is exposed to air increasing silage pH and temperature and allowing the increase of aerobic microflora (Kung et al., 2018).

Some strains of LAB may produce minor forms of alcohols through amino acid catabolism (Hafner et al., 2013). Referring to this fermentation pathway, Edwards and Peterson (1994) reported that *Lactiplantibacillus plantarum* and other *Lactobacillus* species produced some alcohols, such as butanol, hexanol, 3-methyl butanol, and 2-phenylethanol, that are presented at low concentrations in silages. The concentrations of 1-propanol are apparently low in the majority of silages, even if they can be high in corn silage treated with *L. buchneri* with values equal to 10.2 g/kg of DM (Hafner et al., 2014) reaching a peak of 21.7 g/kg of DM in grass silages (Kristensen et al., 2010a). 1-propanol is known to be a secondary end product of yeasts fermentation (McDonald et al., 1991); however, this alcohol is produced by the activity of *L. diolivorans* which can naturally occur metabolizing 1,2 propanediol (Krooneman et al., 2002).

Methanol is likely produced from pectin demethylation during silage fermentation (Fall and Benson, 1996). Then, it is likely that damage to plant cells during harvest and chop in crops for silage may trigger the action of pectinesterase and polygalacturonase facilitating the degradation of pectins and the formation of methanol (Pedrolli et al., 2009).

Aldehydes and ketones

Aldehydes and ketones can be considered as silage volatile compounds that seemingly influence feeding behavior, palatability, and ingestion (Chmelová, 2010). Aldehydes occurrence in silage may be related to the activity of several LAB and yeast which use sugar and amino acids as substrates to synthesize these volatile compounds (Liu and Pilone, 2000; Østlie et al., 2003).

Acetaldehyde is a product synthesized in the middle step among the carbohydrate's fermentation led by heterofermentative LAB and it can be produced in huge amounts by several species lacking in the dehydrogenase enzyme needed to reduce it to ethanol (Simpson et al., 2012). Nevertheless, it is an intermediate in those pathways and it seldomly accumulates due to this route. Even though, ascertaining the occurrence of the single aldehydes in silage might be difficult due to the fact that some of them, such as a part of aliphatic aldehydes in silage may be originated from cut forage and produced after plant damage through the lipoxygenase pathway of unsaturated fatty acids oxidation (Kalač, 2011).

Referring to ketones, data on these volatile compound concentrations in silage are lacking. Several bacteria can be involved in the formation of ketones during the fermentation process. Acetone seems to be produced through the metabolic pathways of clostridia (Daniel et al., 2013). Langin et al. (1989) found that the average of acetone concentration in corn and grass silages ranged between 7 mg/kg DM, and butanone among 10.5 to 13.3 mg/kg; whereas levels of ketones in red clover silage were lower than green red clover or its hay (Figueiredo et al., 2007).

Esters

The production of esters in silage may be related to the LAB activity among the fermentation of water-soluble carbohydrates producing as end-fermentation products acids which decrease the pH of silage allowing the formation of an acidic environment, or by the abiotic esterification processes with the alcohols (Weiss et al., 2016). Recently, it has been observed that the formation of esters is a process that involves certain yeast species which are able to produce these volatile compounds through a reaction between ethanol and acetyl co-enzyme A (CoA) mediated by acetyl transferases (Piškur and Compagno, 2014). Weiss et al. (2016) observed that the production of ethyl esters of lactic and, to a lesser amount, acetic acid was linked to the ethanol concentration emphasizing the role of the alcohol in the formation of esters in silage. Particularly, during the early fermentation phases ethyl acetate can be produced directly by biochemical pathways; on the other hand, ethyl lactate is formed by chemical reaction of ethanol and lactic acid (Weiß et al., 2020). However, a fraction of these esters may derive from the metabolic pathways of certain ester-producing yeasts (Fredlund et al., 2004; Saerens et al., 2010), both assimilators (e.g., *Pichia*, *Issatchenkia*) and non-assimilators (e.g., *Saccharomyces*) of lactic acid have been found in silage (Pahlow et al., 2003; Santos et al., 2017). Considering this scenario, Auerbach et al. (2020) stated that ethyl ester-producing yeasts had no role in the production of ethyl ester and that ethanol might be considered as the main driver for the ethyl acetate accumulation.

Silage additives

Using silage additives has been widely practiced by farmers to enhance silage quality. Generally, silage additives can be grouped in 4 categories considering their effects on silage preservation: i) fermentation stimulants, ii) inhibitors of fermentation, iii) aerobic deterioration inhibitors, and iv) nutrients and absorbents (Kung et al., 2003; McDonald et al., 1991). A new categorization of silage additives was recently proposed because silage additives can have more than one effects based on the above classification. Notably, this new classification divides silage additives into 6 groups: i) homofermentative LAB, ii) obligate heterofermentative LAB, iii) combination of inoculants including obligate heterofermentative LAB plus homofermentative or facultative heterofermentative LAB, iv) other inoculants (different from LAB species), v) chemicals additives [e.g., formic acid (improve fermentation), salts with anti-clostridial effect (improve fermentation), and salts with anti-fungal effect (improve aerobic stability)], and vi) enzymes (Muck et al., 2018). Considering the new categorization, in this section will be focused on the application of microbial inoculants (e.g., homo and heterofermentative LAB) and chemical additives.

Microbial inoculants

The LAB inoculants are generally employed to ensure silage quality by enhancing the fermentation process and aerobic stability of the ensiled mass. Usually, LAB inoculants are selected referring to their ability to rapidly drop pH through the fermentation of water soluble carbohydrates (WSC) to lactic acid, thus preserving nutrients (Guo et al., 2023). According to the end-products of glucose fermentation, the LAB are classified in two groups: i) homofermentative LAB, that produce two moles of lactic acid from one mole of glucose; ii) heterofermentative LAB, that produce one mole of lactic acid, one mole of carbon dioxide (CO₂), and either one mole of ethanol or one mole of acetic acid from one mole of glucose (Rabelo et al., 2016). On the other hand, it has been considered a new classification for LAB, as follows: i) obligate homofermentative LAB, that are unable to ferment pentoses due to the lack enzyme of phosphoketolase; ii) facultative heterofermentative LAB, that differ from the obligate homofermentative group because they are able to ferment pentoses; and iii) obligate heterofermentative LAB, that ferment hexoses to other products (Muck et al., 2018).

The obligate homofermentative LAB are able utilize sugars in forage allowing to produce lactic acid, achieve a rapid dropping of silage pH, and inhibit the growth of antagonist microorganisms and plant enzyme activities that can lead to undesirable fermentations. Nowadays, most of the bacteria in this group are taxonomically identified as facultative heterofermentative LAB (Pahlow et al., 2003). As mentioned above, the facultative

heterofermentative LAB can ferment pentoses due to phosphoketolase enzyme. These bacteria can be identified as *Lactiplantobacillus plantarum*, *Lacticaeibacillus casei*, *Enterococcus faecium*, and species belonging to the *Pediococcus* genus. The fermentation pathway led by homofermentative LAB is more efficient than heterofermentative LAB due to it allows to achieve a DM recovery close to 100% (McDonald et al., 1991). However, the effect of these inoculants varied by crop (Oliveira et al., 2017). Notably, the inoculation with these bacteria exhibits an important reduction for silage pH in tropical grasses, alfalfa, and other legume crops, but not in corn and sorghum. In sugarcane silage, homofermentative LAB inoculants decrease DM recovery (Oliveira et al., 2017). Moreover, inoculation with homofermentative or facultative heterofermentative LAB does not improve aerobic stability of silage due to lactic acid has an insufficient ability to inhibit yeasts, and likely because acetic acid, which in these circumstances is produced in low quantity, is the one with a good antifungal propriety.

The obligate heterofermentative LAB are mainly recognized to the group of *Lentilactobacillus buchneri* which includes, *Llb. buchneri*, *Llb. diolivorans*, *Llb. hilgardii*, *Llb. kefir*, *Llb. parafarraginis*, and *Levilactobacillus brevis* (Muck et al., 2018). The first recommendations about *L. buchneri* as potential silage additives reported that this type of inoculation improved the aerobic stability of silage due to an increase in acetic acid concentration, which had the ability to inhibit yeasts activity (Muck, 1996). Lately, it has been stated *L. buchneri* was able to convert in anoxic conditions moderate concentrations of lactic acid to acetic acid and 1,2 propanediol (Oude Elferink et al., 2001); although, the effects are strain-specific (Kleinschmit et al., 2005; Muck, 2004) and dose-dependent (Filya et al., 2006; Ranjit and Kung, 2000). In addition, the conversion of lactic acid to acetic acid does not occur immediately but it needs somewhat 30 or 60 days (Muck et al., 2018). Other obligate heterofermentative bacteria were identified as potential inoculants in improving aerobic stability. For instance, the addition of *Levilactobacillus brevis* may improve the aerobic stability even though the effect is dose-dependent (Danner et al., 2003). Interesting improvements in aerobic stability have been observed adding *L. hilgardii*. Notably, the inoculation with two strains of *L. hilgardii* in sugarcane silage showed high concentrations in acetic acid and 1,2 propanediol, and low DM losses, ethanol, and butyric acid concentrations (Ávila et al., 2014).

Chemical additives

Chemical additives are applied to inhibit the fermentation of undesirable bacteria, reducing losses, and preventing the growth of yeasts that can initiate aerobic deterioration in silage (Muck et al., 2018). In comparison to inoculants, chemical additives have the ability to act quickly due to their action is direct and not dependent on a microorganism to outcompete epiphytic microorganisms and to produce antifungal end products (Da Silva and Kung, 2022). The active

ingredients in chemical additives are acids (e.g., formic, sorbic, benzoic, propionic, and acetic acids) and salts (e.g., sodium benzoate, potassium sorbate, sodium nitrite).

Formic acid can be sold as silage additives alone or in combination with other chemicals. Basing on application rate and buffer capacity, formic acid can cause direct acidification and suppression of undesirable spoilage microorganisms improving silage preservation (Muck et al., 2018). When sold as a sole ingredient, formic acid concentration for the commercial additive is close to 80-85%, and it should be added with a recommended rates of 2 to 4 L/t of fresh weight forage (Kung et al., 2003). The pH dropping is linked to the dosage of formic acid applied, water concentration, and buffering capacity of the forage crops (Kung et al., 2003). For crops that are hard-to ensile, a moderate dose of formic acid will increase the lactic acid fermentation, reduce the fermentation to acetic, propionic and butyric acids, reduce proteolysis and reduce silage pH (Randby, 2000). For a long time, formic acid has been widely used, but its corrosivity on metal, skin and eye damages machinery and poses serious health risks to workers, leading to a decline in popularity. However, selling formic acid buffered with ammonia or sodium has become very popular, and usually it is blended with other chemicals, such as propionic and benzoic acids (Auerbach and Nadeau, 2019). Considering formic acid concentration at 85% of a commercial additive, the amount required to drop the pH of fresh forage to 4 was 5 g/kg for alfalfa, 3 g/kg for grass, and 2 g/kg for sorghum (Takano et al., 1973). Application of formic acid in combination with ammonium formate at 3 to 6 L/t to direct cut or wilted grass silages has been observed to cause a drop in the pH by direct acidification, restrict fermentation of WSC, and reduce acetic acid concentration and proteolysis (Conaghan et al., 2010; Saarisalo et al., 2006). However, the improvement in handling properties takes place at the expense of effectiveness, so higher application rates are required in comparison to pure formic acid (Randby, 2000).

On the other hand sorbic, benzoic, propionic, and acetic acids improve aerobic stability of silage among feed-out with a direct inhibition of yeasts and molds (Auerbach et al., 2012). The inhibitory action of the undissociated form of the acids to these microorganisms, due to this form is able to pass through the cell membranes releasing H^+ , hence acidifying the cytoplasm. Among this process, protons and anions are produced, and protons must be pumped outside the cell in order to maintain an optimum pH level within the cell. Propionic acid has been used as silage additive to inhibit yeasts and molds growth and improve aerobic stability. Due to its odor and corrosiveness as other organic acids, propionic acid is mainly used as salt (e.g., calcium, sodium, and ammonium propionate) in several commercial products (Kung et al., 2003). Applying high levels of propionic acid (10 g/kg of fresh weight forage) has been reported an improve in aerobic stability in high moisture corn grain (Sebastian et al., 1996). Sorbic acid has a stronger inhibitory

action to yeasts, molds, and other spoilage bacteria rather than benzoic acid, due to its higher acid dissociation constant (4.75) than benzoic acid (4.20) (Muck et al., 2018). Zhao et al. (2021) tested the effect of sorbic acid in comparison to a combination of inoculants on fermentation and aerobic stability of rice straw silage. The rice straw silage inoculated with sorbic acid had the lowest ethanol and ammonia nitrogen contents, and in addition it showed a better performance in terms of fermentation quality and aerobic stability than the silage with the inoculant's solution.

Sodium nitrite and hexamethylene tetramine (hexamine) are able to improve silage fermentation suppressing spoilage microorganisms. Gomes et al. (2021) investigated the effect of sodium nitrite alone or in combination with hexamine in guinea grass silage. The authors observed that both solution were efficient in controlling clostridial fermentations, reducing the fermentation losses, improving the nutritive value and securing the hygienic quality of silage. Moraes et al. (2023) examined different application rates of sodium nitrite and hexamine [e.g., low dose (0.5 g/kg + 0.325 g/kg) and high dose (1.0 g/kg + 0.65 g/kg)] on guinea grass silage harvested at different stage of maturity (e.g., 5 and 10 weeks of regrowth). Notably, the additive solution was able to restrict *Clostridium* growth and DM losses during the fermentation of guinea grass silage at both stage of maturity. Da Silva and Kung (2022) evaluated the effect of a chemical additive containing sodium benzoate (SB), potassium sorbate (PS) and sodium nitrite on fermentation and aerobic stability of whole-plant corn silage. In this study the applied salts reduced the numbers of yeasts when the DM content was higher than 320 g/kg as fed and the ethanol concentration, enhanced the DM recovery, and improved the aerobic stability of whole-plant corn silage. Auerbach and Nadeau (2013) introduced the concept of benzoate equivalent (BEq) to compare products that differ in active ingredients and application rates. Among chemical additives, potassium sorbate (PS) and sodium benzoate (SB) are present in several silage additives as part of mixtures that can include propionates (Borreani et al., 2018). Potassium sorbate has a stronger inhibitory action against yeasts than sodium benzoate (Auerbach and Nadeau, 2013; Bernardes et al., 2014). Daniel et al. (2022) defined BEq coefficients for PS, and sodium propionate (SP) in comparison to SB to establish an application rate according to BEq in order to extend the aerobic stability in rehydrated corn grain silage. They observed that PS, SB, and SP, alone or mixed, linearly improved the aerobic stability with an increase in the application rate. In addition, the BEq coefficient for PS (BEq = 1.99) was more effective than SB (BEq = 1), whereas SB was more effective than SP (BEq = 0.35) in improving the aerobic stability. Additives containing sorbate, benzoate, and other mixtures applied at sufficient dosages decrease ethanol concentrations in corn silage, mitigating the formation of ethyl esters (Weiss et al., 2016). Da Silva et al. (2015) compared different level of application of these salts at laboratory scale reporting that the DM recovery was

improved among the opening period. Improvement in aerobic were observed at farm level when sodium benzoate was added in the top layer of the bunker showing the same nutrients composition of the bunker core (Da Silva et al., 2014) due to the ability of sodium benzoate to release among the ensiling process sorbic acid which has inhibitory action on yeasts and fungi (Santos et al., 2019).

Silage storage

The aim of silage storage is to provide a proper environment for the fermentation of fresh forage, minimizing losses in feed value and hygienic quality, and to contribute to an efficient and safe feeding system within the farm (Savoie and Jofriet, 2003). Considering the environmental scenario, changes in climatic events such as temperature and rainfall are adversely affecting the yield of crop (Malhi et al., 2021). The conservation of forage as silage depends on particular conditions, such as the amount of sufficient WSC to lactic acid production and low buffering capacity to promote a rapid lowering of pH, maintaining the nutritional value of forage (Ramos et al., 2016). Particularly, climate represents an uncontrollable aspect that affects crop and animal production, and consequently forage conservation is a strategy to increase stocking rate and animal performance during the dry season (Daniel et al., 2019). During the dry season the lack and the low nutritive value of forage may compromise the animal production, following by a decreased productivity (Ramos et al., 2016). On the other hand, ensiling agro-industrial by-products with other forage or feed sources has been considered as a sustainable alternative to handle wet byproducts in ruminants diets, mitigating the environmental impact of feed production and transport, and the feed-food competition (Bueno et al., 2020; Musati et al., 2023). Restricting the focus on the Italian silage production, in this section will be discussed the storage systems referred to bunker silos and round bales.

Bunker silos

A bunker silo is a paved area bordered on two or three sides by almost vertical enclosure walls, usually less than 6 m high. Once harvested, the crop is unloaded inside the walled area, compacted by a tracked vehicle, and sealed with a plastic film. Bunker-silo walls are usually prefabricated or cast-in-place concrete. The most common type of precast wall consists of a rectangular panel cast on a pre-existing floor and lifted into an upright position by a crane after an appropriate curing period (Savoie and Jofriet, 2003).

The location of bunker silos should be pinpointed to optimize all operations correlated to filling, emptying, and feeding. The planning decisions should consider the traffic patterns of heavy

vehicles involved in transport and from field to silo, forage packing, and silage unloading for feeding. The drainage structures require a careful consideration as it follows: i) silage juice should flow traversing drains and acid-resistant pipes to a holding pit or treatment pond; ii) rainwater and snowmelt should be drained away from the silo to not obstruct daily unloading; iii) silo orientation should be chosen to minimize the effects of prevailing winds, blowing rainwater, and snow accumulation nearby the open face and the unloading area (Savoie and Jofriet, 2003).

Sizing bunker silos should take in consideration several factors base on machinery size, average silage density, face removal rate, justified DM losses, storage period, and the required amount for feeding the herd (Holmes, 1998). Considering these preliminary conditions, a bunker silo should be at least 5 m width to allow the unloading, packing operations and feeding operations (Borreani and Tabacco, 2007). Taking into account that the advance speed is related to the sequence of the season, it is generally suggested a feed-out rate of 15 cm for the winter and 25 cm for the summer in order to control the aerobic deterioration (Bernardes et al., 2021). The height dimensions should be at least 1.7-2 m, even if it depends on the removal rate; it is crucial to not fill over the top of the bunker walls to avoid tractor fatalities during the unloading and packing operations (Bolsen, 2018). The length of the bunker does not influence the feed-out rate, even though it is linked to the amount of forage that will be stored.

The density of silage mass is a factor that influences silage quality (Ruppel et al., 1995). During the packing operations, it is crucial to minimize the level of porosity avoiding the oxygen penetration within the silo. The packing process should be done continuously among the filling time (Hutnik and Kobiela, 2012). The optimum packing rate should be 1 to 4 minutes per tons of forage (McAllister and Hristov, 2000). Packing density is maximized adopting packing tractors with tires applying the greatest weight per unit of surface area (Hutnik and Kobiela, 2012). Regularly, filling and packing machines for bunker silos are insufficient in numbers and weight to get the forage mass packed at the desired density. According to Holmes and Muck (2007), with the assumptions of 4.3 m sidewall, 5.5 m peak height, 35% dry matter, 0.15 m packing layer thickness, and a harvest rate of 54.4 t as fed/h; a tractor of 14.5 t is needed to push and pack the forage to obtain a bulk density close to 705 kg/m³. It is crucial that the bunker must be of sufficient width to accommodate the numbers of tractors operating safely or more than one bunker must be filled simultaneously to allow the tractors to operate. Additionally, spreading the forage in thin layers (<20-30 cm) is also recommended to improve packing. Porosity level has been widely investigated in past developing several equations (Pitt and Muck, 1993; Richard et al., 2004) in order to contain the oxygen flow within the silo. The optimum bulk density rate suggested to limit porosity should be 705 kg/m³ (Holmes and Muck, 2007).

Once the unloading and packing operations have been completed, the forage should be sealed using a plastic film. Sealing operation is required to prevent air infiltration (i.e., keep anaerobiosis) and exclude rainfall due to precipitation which may wash organic acids and other soluble nutrients (Bernardes, 2016; Bernardes et al., 2012). The plastic film applied to cover bunker silos are generally made in polyethylene (PE) metallocene, that was firstly introduced as material to improve the properties of the conventional plastic films. Nowadays, a new generation of films have been launched on the market. These new generation films are labeled as “barrier film” due to their low permeability to the oxygen transmission (Borreani et al., 2007). Covering the sidewalls of the bunker should be done before the filling operations to ensure the exclusion of oxygen during the ensiling and storage (Bernardes, 2016). The airtight seal is usually done in two steps: i) covering the silo surface to create a first seal effect avoiding the creation of air bubbles between the forage mass and the covering film, and ii) a protection film which should be UV stabilized and ensure anaerobic conditions. In order to maintain the mechanical properties of the protection film among the storage and feed-out periods, it is crucial to stage a security net which has the function to prevent damages caused by climatic events such as rainfall, hail, or wild animal attacks.

At the completion of the sealing, it is essential to arrange a weighting on the whole bunker surface preventing a return effect of oxygen and ensuring the bond between film and forage mass. The weighting of the bunker surface can be done using different materials such as tires, soil, gravel, or tiles. These materials can have a different weighting effect on silage. Borreani and Tabacco (2007) compared two different weighting systems on a farm bunker silo covered with a single black-on-white film. The bunker was divided in two sides lengthwise: half was burdened with tires (25 kg/m²), and the other half using gravel (200 kg/m²). The different sealing systems affected the temperature in the peripheral areas of the silo. Notably, temperatures close to 40°C were reached in the side burdened with tires, whereas the temperatures of the side burned with gravel system never exceeded 30°C. Another weighting system, known as “German” system, is applied putting ballistic bags filled with gravel or send (60-70 kg/m²) on the edges at regular intervals; however, this system not guarantee a homogeneous weighting on the bunker surface (Borreani et al., 2015). Moreover, covering the plastic with a net and gravel bags is a suitable solution, even if using black and white plastic film.

Round bales

Preserving forage in round bales is mainly practiced in farms with small herds and because it allows to reduce environmental losses in comparison the haymaking technique (Coblentz and Akins, 2018). Applying a proper baling technique means to satisfy several recommendations such as harvest the forage crop at a good DM content (45 to 55 %DM), setting the chopping length (1 to

4 cm) related to the crop, apply the maximum possible compression, wrap as soon as the forage is baled in the field, applying a minimum of 6 layers to avoid yeast and mold growth, store the bales in the vertical position in a shady place until they are used (Benvenuti, 2021; Bisaglia et al., 2011).

The main advantages and disadvantages related to round bales silage have been reported by Coblenz and Akins (2018). The advantages of this preservation technique include i) lower risks of damage weather than the haymaking; ii) improved management flexibility due to it allows to use the same equipment applied for the haymaking; iii) lower fixed and operational costs due to no facilities are required; iv) reduced field losses (mainly legume leaves) in comparison to the dry hay; v) theoretically unlimited storage capacity without further investment, provided that the integrity of the plastic wrappings is maintained; vi) reduced effluent potential compared to chopped silage; and vii) greater flexibility in comparison to feeding options. The disadvantages of this technique regard i) limited performance of fermentation processes, resulting in a less stable final product often containing a higher concentration of unfermented sugars in case of a short wilting period; ii) as the result of unfinished fermentation, obtaining a high quality final product is closely linked to the integrity of the wrapping film which is essential for the establishment of an anaerobic condition within the bale; iii) polyethylene wrapping films represent an additional cost for farmers in terms of both expense and disposal; and iv) more specialized machinery in comparison to the previous equipment.

Round bale silages have a different moisture content which falls somewhat close to the 45 to 55% (Shinners, 2003). These suggested levels contrast the recommended targets for precision-chopped silages with high buffering capacity and low concentrations of WSC, such as alfalfa ($\leq 70\%$ DM at baling; Muck et al., 2003). Due to their high DM contents, fermentation in round bales is usually restricted (McDonald and Edwards, 1976). However, silage producers usually bale chopped forages with high DM content due to the additional bale weight causes justified safety concerns during handling and transport (Shinners, 2003). In this case, preservation is made possible by a reduced free water content, anaerobiosis, a reduction in pH and in many cases lower temperatures. In fact, the use of this technique is mainly for fodder in late summer and autumn cuts, when it has a lower sugar content than in spring, which is insufficient to guarantee the achievement of a pH that is useful for conservation in the presence of a high-water content. In addition, the approaching cold season makes it possible to assume a better conservation course. On the contrary, higher temperatures not only stimulate microbial activity, but also favoring gas exchange within the mass, promoting their expulsion from the bales during the day and then, drawing air and oxygen back inside as they cool down at night. The films, in fact, maintain an albeit limited gas permeability. The moisture content at harvest can be related to the sugar:protein

ratio; particularly, values close to 2 represent an ideal condition that allows ensiling at 70% water; for values below 2 (between 0.8 and 1) the water content must progressively decrease (Benvenuti, 2021).

Due to the limited rate and extent of fermentation for baled and wilted silages in the field, the improvement of fermentation through clever management strategies is encouraged (Coblentz and Akins, 2018). Management strategies may be adopted in the harvest period in order to enhance the rate of WSC and the fermentation process. Notably, Huntington and Burns (2007) compared the harvest of perennial warm-season (C4) grasses during the late afternoon to the early morning. They stated that harvesting in the late afternoon improves WSC concentrations; which could potentially improve the substrate pool for fermentation. Nonetheless, it has been thought that cutting alfalfa in the afternoon might lead to lose significant proportion of WSC during the night, thus losing its advantage over the morning cut. However, recent study has proved that nighttime WSC losses in the afternoon cut of alfalfa are low and that can be compensated by early morning photosynthesis of cut and unwilted alfalfa (Morin et al., 2012). Moreover, cutting later in the day may allow producers to obtain higher WSC yields in wilted silage if fresh forage harvested in the afternoon can consistently dry at an equal or faster rate than forage harvested in the morning (Owens et al., 1999).

Obtaining a level of density within the forage mass is a pre-condition for achieving an anaerobic environment, as high porosity levels within the ensiled mass are directly proportional to the rate of oxygen movement within it (Pitt and Muck, 1993). For chopped forage ensiled in horizontal silos, Holmes and Muck (2008) suggested an optimum density rate (bulk density) of 243 kg DM/m³ for chopped silages, but if different porosity is taken into account, this can be as high as 712 kg (fresh weight)/m³. On the other hand, in a study of Jennings (2011) the suggested that the optimum density rate was 162 kg DM/m³, but that can be exceeded if one adjusts the baling chamber pressure, swath density and baler forward speed. Tabacco et al. (2013) reported silage densities for bales comparable to those of some trench silages (e.g., 225 to 247 kg DM/m³).

Wrapping of forage material, as in the case of ensiled mass within the bunker silo, should be done after the chopping phase, as delays in the wrapping phase may compromise protein bioavailability (Goering et al., 1973; Rotz and Muck, 1994; Van Soest, 1982) and reduce energy density (Coblentz and Hoffman, 2010); partly due to the oxidation of non-structural carbohydrates (Rotz and Muck, 1994). Delaying the wrapping stages can lead to lower lactic acid production, which it means incomplete fermentations and high final pH levels (Coblentz et al., 2016a; Nia and Wittenberg, 2000). Furthermore, silages initially compromised by oxygen ingress and subsequently subjected to anaerobic conditions may be more susceptible to clostridia activity (Muck et al., 2003).

Wrapping operations are carried out using PE plastic films. Such polyethylene films have been the subject of several researches (Borreani and Tabacco, 2008; Coblenz et al., 2016b; Hancock and Collins, 2006) with the intention of improving the anaerobic conditions, and the nutritional and health profile of silages wrapped in high-density bales. Tabacco et al. (2013) demonstrated how a tying film (known as baletite, which replaces the common net) can reduce the number of holes on the bales and thus improve their internal anaerobic state, as the compression of the silage allows oxygen to be expelled faster, without negatively affecting farm costs. The preservation of the product inside the bales can be implemented by applying several layers of appropriate PE film (ideally 6 to 8, but depending on the product used); several types are commercially available with different thicknesses, colors and characteristics as they use components in different ratios; Undersander et al. (2003) demonstrated that the minimum cumulative thickness for preserving silage in bales is 0.152 mm (152 μ m). Oxygen ingress into the bales may also occur secondary to baling due to the action of insects, nematodes or birds; the only means of defense against these attacks is a good plastic film or the use of UV-stabilized protective netting that also preserves the integrity of the bales from attacks by wild animals (e.g. wild boar, crows, etc.), or intense weather phenomena (e.g. hailstorms).

Once the wrapping operations have been completed, it is necessary to move the bales by using special grabbers in order to prevent plastic damage and not to interfere with gas exchange. The bales should be stored and placed on the flat side in a well-drained site that is free of debris or sharp objects and that is not conducive to the possible presence of rodents or other pests that could perforate the wrapping film, if possible, it is also good to protect them from birds (Hersom and Kunkle, 2011; Jennings, 2011; Savoie and Jofriet, 2003). Some studies recommend that bales should be placed in a north-south orientation or on a north-facing slope (Garthe and Hall, 1992; Jennings, 2011) to avoid temperature fluctuations and possible movement of water inside; recommendations include suggestions for storage in shaded areas or stacking individually wrapped bales (Coblenz and Akins, 2018). The storing position of the bales has a crucial role in preventing molds development on the bale surface. Particularly, Bisaglia et al. (2011) observed that storing bales on their flat side allowed to reduce the amount of surface covered by mold in Italian ryegrass and lucerne on the curved surface of the bales.

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2. Plastic in silage production: staging and wrapping practices among the ensiling process

The main factors that affect the preservation of forage crops during the ensiling process are the degree of anaerobiosis achieved within the silo and its maintenance among the storage period (Woolford, 1990; Borreani et al., 2007). Air infiltration assists the growth of several spoilage microorganisms inducing aerobic deterioration and economical losses (Bernardes, 2016). Silage can be stored in different structures such as bunker silos, round bales, tower silos, and silo-bags. Storing silage in bunkers is widely practiced in livestock farms due to relatively low construction costs and safety, while allows for faster filling and unloading in comparison to tower silos (Savoie and Jofriet, 2003). In farms with small grassland swards, round bales are more present, due to their easier management and no need of specific storage structures. Overall, achieving a proper anaerobiosis can be attained using an appropriate plastic film placed properly. Therefore, this section gives an overview on the main plastic films used to seal and ensile chopped forage, with a focus on bunker silos and round bales.

Plastic films for bunker silos

Once completed the filling phase of the bunker silo, the forage mass should be sealed using a plastic film ideally in the same day of ensiling. The plastic film employed to seal the ensiled mass should present physical resistance to reduce the risk of damages by meteorological events and animals; should be ultra-violet (UV) stabilized due to the prolonged exposure to sunlight; and it should ensure a proper anaerobic condition along the storage period (Bernardes, 2016). Leaving the silo uncovered implies an organic matter (OM) losses close to 47% in the upper 0.5 m and 11% in the next 0.5 m below (Bolsen, 1997). Therefore, a proper sealing cover is crucial to reduce losses of highly digestible DM (Bolsen et al., 1993), the possible occurrence of mycotoxins (Cheli et al., 2013), and the arise of pathogenic microorganisms (Spadaro et al., 2015).

The mechanical proprieties of the films applied to seal the bunker silo can influence the degree of anaerobiosis reached within the ensiled mass. The color of the film may influence sealing efficiency due to the oxygen permeability is increased with the temperature of the plastic material

(Bernardes, 2016). Bernardes et al. (2009) compared two PE film with different colors (e.g., black and black-on-white, respectively) for sealing corn silage. High temperatures were detected in corn silage sealed with black film during storage. In addition, higher DM loss and yeast counts were observed in corn silage covered with black PE. Snell et al. (2003) stated that the effect of the color can affect the temperature of the film surfaces. They observed that at midday (e. g., 11:50 to 12:00 hours), temperatures were about 6-7°C for the dark-colored film in comparison to white films. Reducing oxygen permeability has always been thought to be related to the film thickness, due to a thinner film may constitute an inadequate barrier to oxygen flow being susceptible to laceration and perforation. However, enhancing film thickness is followed by increased production costs and difficulties in managing the film during sealing (Borreani and Tabacco, 2017), in addition to the amount of plastic for disposal. Furthermore, oxygen permeability greatly varies across different polymers (Borreani and Tabacco, 2017). Savoie (1988) proposed a model to predict the optimum thickness of PE film and its costs referring to the storage length, bulk density and DM content, film permeability, and the relative value of plastic and silage. In this simulation, PE cost increased with the thickness whereas silage losses decreased in thicker films; PE films with a 120 µm thickness were good for a storage length of 120 days, and for a storage period close to one year the optimum thickness was 200 µm. As expected, thicker films were more resistance to laceration and perforation than the thin films. Impact resistance is one important property of plastic films. The impact resistance is often measured through a dart impact test, involving different models to define the energy required for the films to fail below a range of defined impact-loading conditions (Mezghani and Furquan, 2012). Indeed, a thicker film should be more resistance to the perforation, environmental phenomena, and animal attacks than a film with a lower thickness, and also thicker film should ensure a proper anaerobic environment within the ensiled mass. However, new generation films produced in EVOH have ability to ensure a proper barrier effect against the oxygen permeability (Borreani and Tabacco, 2014), and in addition these films are made with new generation co-extruders that allow to produce multi-layer films with a reduced thickness (<90µm), an oxygen transmission rate (OTR) <2.5 cm³/m²/day, and a dart test score higher than a PE film (Mastroeni et al., unpublished data).

Preventing the aerobic deterioration in the upper layer of bunker silos requires proper management practices such as chopping the forage at an appropriate theoretical length, rapid filling, proper packing, efficient sealing, and good feed-out management (Mahanna et al., 2003; Wilkinson and Davies, 2013b). Among these management practices, the preparation and covering phases of a bunker silo are crucial to achieve a proper anaerobic environment. However, PE films have been frequently used to seal bunker silos, but PE films are not completely impermeable to

oxygen diffusion (Bernardes, 2016). In the first decade of 2000s the first generation of barrier films to cover bunker silos were made with a coextruded film using polyamide (PA) as a barrier polymer (Borreani et al., 2007; Wilkinson and Rimini, 2002). Although these barrier films had good mechanical characteristics in comparison to the conventional LDPE films, the PA barrier films appeared more rigid than LDPE films. To support farmers during the preparation and sealing practices in order to minimize the damage of the film, new oxygen barrier (OB) films were made by coextruding a layer of EVOH between two layers of PE bounded into a five-to-seven layer coextruded film (Borreani and Tabacco, 2014). Comparing these new OB films with the first-generation films, the EVOH layer improved the oxygen impermeability minimizing the spoilage phenomena, especially in the peripheral areas. In a meta-analysis led by Wilkinson and Fenlon (2014), it has been compared the using of PE films and OB barrier films in covering systems for bunker silos, piles, and bales. The aerobic stability for silage stored with PE films were lower than the OB films (75 h and 135 h, respectively), and the losses were reduced when OB films were applied to cover bunker silos, piles, and bales. Lima et al. (2017) evaluated two covering systems for corn silage stored in bunker silos. The two covering systems were applied in eight commercial bunker silos, which were divided in two parts lengthwise one-half was covered with OB film, whereas the other half with a PE film. Top silage covered with the OB covering system showed a well-fermented profile compared with the core section of the bunker, whereas the PE covering system led to lower lactic acid concentration and higher pH and mold counts in the top silage compared with the core silage. In addition to the fermentative profile, the authors estimated the milk yield per ton of DM using the Milk2006 spreadsheet (Shaver et al., 2006). The milk yield estimated for the PE system was 116 kg/ton less than the core, whereas the milk yield estimated for OB system was similar comparing to the core section (1258 and 1294 kg/ton, respectively). Some researchers have investigated the effect of different sealing methods on animal performances. Amaral et al. (2014) evaluated the effect of different covering methods on nutritive value of corn silage and performance of dairy cows. The authors observed that the utilization of OB films and the adoption of sustainable alternative sealing strategies such as sugar cane bagasse positioned on the plastic sheet can be considered as potential solutions to isolate and protect the silage surface, increase the recovery of digestible nutrients, and enhance production efficiency of lactating dairy cows. Parra et al. (2021) compared the performance of dairy heifers fed diets based on whole-plant corn silage stored in bunker silos sealed with either PE film (thickness $121 \pm 3.1 \mu\text{m}$) or OB film (thickness $46.7 \pm 2.5 \mu\text{m}$). In this study, heifers fed silage stored with the OB system had higher DM intake than animal fed silage with the PE system (i.e., 10.2 vs 9.39 kg/d).

Additionally, the OB system increased the digestible energy intake by 8% and average daily gain by 12%.

Preparation of the bunker

Isolating the bunker silos from the oxygen penetration is the main purpose to achieve a well-fermented product. The introduction of plastic sheet to cover bunker and pile silos has been defined as a great innovation, due to covering the silo allowed farmers to reduce the waste amounts (Wilkinson et al., 2003). However, the ensiled mass in the peripheral areas of the bunker is prone to aerobic deterioration because it is difficult to operate a proper packing condition. Borreani and Tabacco (2010) observed an increasing in yeasts counts from 3.64 to 4.97 log₁₀ colony form unit (cfu), and an increasing in mold counts from 1.76 to 3.71 log₁₀ cfu shifting from the central section (core) of a bunker to the peripheral areas. Honing (1991) reported that DM losses of silage were highest (76%) near the silo wall and lowest (16%) in the core. Considering this scenario, leaving the bunker uncovered after filling or not ensure a proper sealing trigs aerobic deterioration phenomenon. Notably, the microbial respiration will be responsible for the vast majority of DM loss during storage (Muck et al., 2003). Bolsen (1997) observed that the organic matter (OM) losses in uncovered bunkers presented an average of 47 and 11% in the first 0.5 m of the upper layer and in the following 0.5 m below, respectively, whereas when bunkers were covered with a LDPE film these losses were reduced by 20 and 5%, respectively.

The staging of a bunker silos is an important practice that needs to be led in order to prepare a proper condition to a well anaerobic environment. Lining the shoulders is not taken in consideration by farmers, due to it has always been considered as an additional cost and sometimes farmers prefer to spend more for a specific type of silage additive rather than trying to operate a proper staging and sealing of the bunker. Nonetheless, using silage additives and adopting proper management practices are complementary actions that define the quality of silage. In light of this contest, lining silo shoulders may be operated a day before of the ensiling process. The plastic film should be chosen considering the mechanical properties such as thickness, oxygen permeability, and UV-stabilized (Borreani et al., 2018). In addition, the plastic sheet should cover the shoulders of the bunker with the purpose to isolate the ensiled mass from the oxygen penetration in these critical areas of the bunker in which it is possible to observe aerobic deterioration phenomena (Borreani and Tabacco, 2007). After filling, the sidewall plastic should be put over the forage top (Bernardes et al., 2018), and suddenly it is possible to operate a first sealing of the stored mass through a transparent film. Concluding this operation, the bunker surface can be covered using another plastic film. Recently, it has been launched new generation films that contain within the same reel the transparent film and the covering film, in order to reduce the

staging time of the film and enhance the comfort of the operators during the staging phase. Mastroeni et al. (2023) compared different staging systems using a PE film and OB film: i) 1+1 solution, putting in two steps the transparent film and PE film; and ii) 2 in 1 solution, in which the transparent film and the OB film have been staged in a unique phase due to both films were presented in the same reel. In this trial, the authors stated that the solution 2 in 1 allowed to reduce the staging and covering time by the 60% in comparison to the other solution. Putting a safety net on the plastic film is a crucial practice that allows to maintain the mechanical properties of the film among the storage and feed-out phases. Notably, safety net especially UV-stabilized can prevent the status of the films from environmental events such as hail, snow or rainfall, and wild animals attack (Borreani and Tabacco, 2007). Mastroeni et al. (unpublished data) in a trial led in a commercial bunker divided in two sides lengthwise (a half covered with PE film, and the other half with OB film and safety net, respectively) observed that the silage covered with the OB film and the safety net showed a well-fermented profile then the other one.

Plastic films for round bales

Storing silage in round bales has been a good alternative to haymaking, due to it allows to reduce risk of weather damage, use the same equipment applied for the haymaking, support small-size farms in the formulation of the diet (Coblentz and Akins, 2018). Among the last 25 years, this technique has become an economical alternative in comparison to other harvesting systems in Europe (Tabacco et al., 2011), leading to a notable increase of herbage preserved as silage (Wilkinson and Toivonen, 2003).

Preserving silage in round bales consists in wilting forage in order to reach a proper moisture content, baling and wrapping it using a stretch films (Borreani and Tabacco, 2017). The plastic film is stretched, using a stretching unit attached to a bale wrapper, and wrapped around the bale. Generally, four to six layers of PE are applied in two or three successive complete bale rotations, with a 50% overlap between the layers. Reducing mold growth can be achieved by increasing the number of layers; however, this system leads to a waste of plastic film in conventional baling systems, due to the higher amount of plastic distributed on the flats ends (Borreani et al., 2019). Another system that can be applied to cover round bales is the adoption of an in-line wrapping machine that wraps the bales, loaded end to end in a long horizontal row, using a stretch film similar to the one used for single wrapped bales (Savoie and Jofriet, 2003).

Usually, stretch films applied to wrapped bales are made in PE due to its mechanical proprieties and low costs (Borreani and Tabacco, 2008). The standard plastic films employed in the production of bales are coextruded LDPE with a thickness of 25 µm before being stretched by 50%

(Lingvall, 1995). The main proprieties of the stretch applied to wrapped bale silage should satisfy several aspects, such as a maximum gas permeability ($<1800 \text{ cm}^3/\text{m}^2/24 \text{ h}$ with 0.02 MPa pressure; that corresponds to $<9000 \text{ cm}^3/\text{m}^2$ at 0.1 MPa pressure), elevated stretching capacity, resistance to perforation, UV-stabilized for at least 12 months, and proper adhesiveness to maintain the layers together (Borreani and Tabacco, 2017). Due to the higher volume: area ratio in round bales compared to bunker silos, environmental conditions can adversely affect the mechanical properties of a stretch film during the storage period; indeed, the permeability coefficient can exponentially increase with the temperature. Notably, the temperature of the stretch film affects the oxygen permeation rate and carbon dioxide within the film, and in addition the color of the film is a potential factor that influences the solar radiation reflectance and film temperature (Möller et al., 1999). Considering the value of solar reflectance, this parameter was investigated by Möller et al. (1999) evaluating the correlation between the fraction of the total solar energy reflected by 25 μm LDPE stretch films with different colors and the bale surface temperatures and within-bale temperatures. In this investigation, the solar reflectance detected in white, light-green, dark-green, and black was 31%, 28%, 13% and 4.4%, respectively. In addition, they measured the temperature for the same stretch films with an infrared camera reporting the following data: 32°C, 35°C, 46°C, and 53°C, respectively. They stated that the oxygen permeability for a 25 μm -thick black LDPE stretch film was twofold higher than a white stretch film with the same thickness. Frick and Tănîkon (2004) observed an increasing in oxygen permeability up to three-fold higher for black stretch films in comparison to white and light-green stretch films when exposed to sun. On the other hand, McEniry et al. (2011) observed that stretch film color had little effect on the gas composition within the baled-silage under temperate climatic conditions with moderate solar radiations in Ireland.

Operating a uniform film distribution on the lateral bale surface is crucial to achieve well-anaerobic conditions and an admissible risk of internal and external perforation of the bale (Borreani et al., 2007). Four, six or eight layers of film are usually applied in one, one-and-a-half or two successive rotations of the bale, with a 50 per cent overlap between successive film strips (Borreani et al., 2007; Wilkinson and Rinne, 2018). In order to reduce the growth of undesirable microorganisms and improve the conservation of silage increasing the number of layers may guarantee a successful preservation (Coblentz and Akins, 2018). Keller et al. (1998) evaluated the microbiological status of alfalfa baled-silage wrapped with 4, 6, 8 or 10 layers stating that it is important to apply at least 6 layers of stretch film to obtain a proper silage restricting undesirable microbial growth. However, increasing the number of layers can reduce the mold growth on the other hand it causes a waste of plastic film due to the higher proportion of plastic distributed on

the flat ends (Borreani et al., 2019). Recently to ensure a proper distribution of plastic, two different baling solutions have been launched on the market: a selective 3D wrapper and round-balers provided with a tying system to firm large round bales with PE tying-film in the baler chamber. The 3D wrapper concept consists on a biaxial rotation of film applicators which improves the homogeneity of the plastic film distribution on the surface increasing the number of layers on the corners of the bale (Borreani et al., 2007). On the other hand, the tying system allows to firm the bale in the press chamber through a twine, net or PE film. Bisaglia et al. (2011) investigated the possibility to replace net with PE film baling a total 48 round bales, half net-tied and the other half film-tied, consisting of half Italian ryegrass and half alfalfa. They observed that the film-tied bales allowed to reduce the mold presence on the bale surface. Stankiewicz (2019) proposed a mathematical model that describes the distribution and consumption of stretch film in baled-silage.

Enhancing the oxygen impermeability of the stretch film has been defined as one of the main goal to improve the conservation of baled-silage, in order to avoid the penetration of air within silage (Borreani and Tabacco, 2008). New plastics production technologies, combined with new low-oxygen permeability polymers that can be coextruded with PE, offer the possibility of producing multilayer stretch film for wrapping silage bales at costs that are as competitive as those of PE traditionally used on farms (Borreani and Tabacco, 2017). Most plastic films for the production of stretch-wrap silage are made coextruding linear LDPE with a thickness of 25 μm prior to being stretched by 50% or more when applied (Borreani et al., 2019). The first generation of OB stretch films to baled-silage was produced through the coextrusion of LDPE with PA (Borreani and Tabacco, 2017). However, due to the high ratio volume/ratio for baled-silage the oxygen permeability represents one of the main issues in this ensiling system, especially when the bales are stored for long period (Borreani and Tabacco, 2008). Recently, the new generation OB stretch films have been improved by coextruding a 2 μm thicker layer of a special EVOH grade between two or more PE layers with the purpose to improve the oxygen impermeability (Borreani and Tabacco, 2017). In several farm trials, using the new OB stretch films allowed to reduce DM losses by 2% during the storage period in alfalfa baled-silage with DM levels ranged between 55 to 65% (Borreani and Tabacco, 2010). On the other hand, several studies reported DM losses of 6% (Hancock and Collins, 2006), and 7% of the total harvested DM (Borreani and Tabacco, 2008; Shinnars et al., 2009) in alfalfa baled silage with similar DM contents and wrapped with conventional PE stretch films. In addition, the new OB stretch films have shown a remarkable influence in reducing the mold growth in the bale surface (Borreani and Tabacco, 2010).

Storage and management

After wrapping bales should be moved using clamps to maintain the stretch film integrity during the storage period. Choosing a well-drained storage site that is free of debris, sharp items, not favorable to the establishment of rodents or other pests that might perforate the PE film wrapping the bales is crucial to ensure the silage conservation (Coblentz and Akins, 2018). Generally, the stretch film are damaged by birds perforation causing quantitative and qualitative forage losses (McNamara et al., 2002). Whole cereal baled silages are attractive to rats, that can damage the stretch film (Kawamoto et al., 2012). It has been reported that fungal growth in baled silage due to film damage caused by birds or rats accounted for 40% of all deteriorated surface on several farms, with more than half of the damage caused by rats (Cai, 2004). Using physical barriers such as security net, possibly UV stabilized, positioned 1 m above and beside the bales may be a valuable solution to prevent birds-damage (McNamara et al., 2002); whereas it has been observed that creating open spaces between the bales and protecting the bottom with wire netting, without covering the bale with plastic films, reduce the hiding spaces that can be available for rats (Kawamoto et al., 2012).

The bales should be positioned with a north-south orientation or on a north-facing slope avoiding temperature fluctuations, and possible migration of water within the bales (Garthe and Hall, 1992; Jennings, 2011). Stocking in-line baled silage on the ground should be done in order to prevent mold growth on the bale surface. Bisaglia et al. (2011) observed that the storing position influenced the growth of mold in the Italian ryegrass and alfalfa forages. Notably, the visible area subjected by growth was more evident when bales were stored on the curved surface than on the flat end. Similar results have been reported by (McCormick et al., 2002) in ryegrass bales silage where the appearance of mold prevailed on bales stored on the curved surface, whereas it was absent on end-stored bales.

Conclusion

Producing high-quality silage is a challenge that involves a wide range of operations among the ensiling process (from field to bunk). Particularly, each phase of the ensiling process should be properly done to ensure the fermentation pathway preserving the nutritive value of the crop. Mitigating the aerobic deterioration is one of the main challenges in which silage producers are continuously involved. In this scenario, lining the bunker sidewalls, covering and weighting the plastic cover on the silo surface are management practices that can allow to minimize nutritive losses during the storage period and feed-out phase. On the other hand, preserving crops in round baled silage can be considered as a valuable solution for small-sized farms reducing the risk of weather damage compared with haymaking. However, the fermentation pathways are strictly

related to the number of layers and the integrity of the stretch film applied to wrap the bale. Notably, it is important to store the round bales considering strategies to prevent plastic damage. Future investigations need to be led at farm level in order to minimize losses and costs, and optimize the disposing practices of plastic films.

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3. Whole-plant corn silage: hybrid selection and management practices

Whole-plant corn silage (WPCS) is the main forage used to formulate dairy cattle diets due to it allows dairy nutritionists the opportunity to provide high energy (especially from starch in the kernel fraction) in addition to physically effective NDF (peNDF; provided by the stover fraction (Ferraretto et al., 2018)). Harvest practices, such as maturity stage at harvest, kernel processing, theoretical length of cut, and cutting height are important tools to improve physical, chemical characteristics and nutrient digestibility of WPCS (Ferraretto and Shaver, 2012; Johnson et al., 1999). The stage of maturity at harvest influences yield, nutrient composition, digestibility, and ensiling capacity of crops (Buxton and O'Kiely, 2003). Several crop management practices, such as planting density, nitrogen fertilization rates, harvest moment, cutting height, can influence WPCS yield and quality (Ferreira and Brown, 2016). However, uncontrollable factors related to climate conditions can affect silage production, dry matter (DM) composition and nutritional composition at harvest (Bernardes et al., 2018).

Whole-plant corn silage

Whole-plant corn is characterized by having a single erect stem which is divided in basic units known as phytomers. Each phytomer is characterized by a leaf blade, a leaf sheath, a node, an internode, and the axillary bud. In comparison to other grasses, whole-plant corn has two separate inflorescences per plant, the tassel and the ear, which correspond to the male and female inflorescences, respectively. The husks are leaves that cover the ear, where corn kernels develop after pollination. Corn kernels are organized and inserted in lines on a central cylinder called the cob, which is originated from the axillary bud from the phytomers (Ferreira and Brown, 2016).

The structure of the corn plant has a great impact on the chemical composition of corn silage. Carbohydrates synthesized in leaves are mobilized to the grain and stored as starch. Corn kernels comprise 30-52% of the total plant biomass (Boomsma et al., 2009), whereas starch constitutes 70-75% of the kernel dry weight (Liu et al., 2016). Considering the different composition of the grain and the vegetative portion of the plant (high and low concentrations of nonstructural carbohydrates, respectively), the proportion of grain in the corn plant has an important impact on the nutritional quality of WPCS (Ferreira and Brown, 2016).

The inflorescence development occurs during the vegetative growth stages, generally when corn plants have six fully exposed leaves (i.e., V6 phase) (Abendroth et al., 2017). At this stage, the axillary meristem of leaves differentiates into ears (Wu et al., 2009). These ears usually generate

rows of paired spikelets that produce one ovule-containing floret each. Once pollination is completed and the ovule is fertilized, each floret originates a single corn kernel.

In contrast to most other grasses, in corn the male inflorescence is spatially separated from the female inflorescence. Each ovule within the ear must be pollinated to become a mature grain. In the process, functional stigmas, known as setae connect the ovules to the outside of the ear to ensure pollination. The appearance and exposure of the silk to the environment is known as the silking stage and is known as the beginning of the reproductive stage of the corn crop. The first stage of the pollination process starts when the pollen grains emitted from the tassel during anthesis attach themselves to the silks of the ear (Ferreira and Brown, 2016). The synchrony between anthesis and the emergence of silks (generally known as anthesis-silking interval) is critical for proper kernel pollination and development (Oury et al., 2016).

Corn silage production

Producing high-quality WPCS is complex process that involves several practices from field to feed in order to minimize production costs in livestock systems. Management practices referred to planting date, plant density, and fertilization are essential for silage production.

Nitrogen is an essential nutrient which is generally applied to corn cultivations (Masoero et al., 2011). Nitrogen requirements for WPCS production ranged between 150 to 200 kg N/ha (Allen et al., 2003). The impact of N fertilization on corn yield has been investigated (Almodares et al., 2009; Cox et al., 1993). The main effect of N fertilization is linked to the capacity to increase the crude protein (CP) concentration (Sheaffer et al., 2006).

Plant density affects crop growth and development by changing light, water, and nutrient compositions among the growth stage altering the yield and quality of WPCS (Qian et al., 2023). The optimal plant density for corn silage differs depending on the environment and hybrid. Planting corn for silage at high densities may be attractive for farmers, because it allows to increase biomass yield and recover forage stocks. However, increasing plant density can lead to an increase in fiber concentrations and a reduction in CP content (Allen et al., 2003). High corn planting densities may decrease the energy concentration due to reduced kernel pollination or development (Ferreira et al., 2014). Exceeding by 8.0 plants/m² may decrease feed quality. Increasing in DM yields by 1.0 Mg/ha has been detected when plant density increased from 6.5 to 8.0 m² (Graybill et al., 1991).

Planting date for WPCS is generally scheduled later than corn planted for grain production, due to the less risk of frost before harvest for silage (Allen et al., 2003). Nevertheless, later planting date can enhance the risk of frost before maturity (Narasimhalu et al., 1986). Studies referred to

planting date state that delaying planting can adversely affect yields, DM, and forage quality. Overall, the optimum planting window should be chosen considering climatic and geographic conditions.

Hybrid selection

Selecting corn hybrids has been focused on grain yield to produce corn grain and WPCS (Bal et al., 2000). Initially, hybrid selections aimed to improve agronomic response without considering animal performance (Bal, 2006). Recently, specific corn hybrids have been selected to enhance the nutritional composition of WPCS and match the requirements of high-producing dairy cows. The selection of a corn hybrid for silage production should be done considering factors related to digestibility and intake. Altering the stalk or kernel characteristics can be considered as an opportunity to increase digestibility in WPCS (Ferraretto and Shaver, 2015). Increasing the content of cell wall pectin is crucial to enhance forage digestibility due to pectins are more digested than cellulose and hemicellulose (Hatfield and Weimer, 1995; Jung and Engels, 2002). In this section, an overview of the main corn hybrids selected for silage production will be reported.

Brown mid-rib

Corn brown midrib (BMR) manifests a reddish-brown pigmentation in the leaf midrib and stalk pith, related to the lignified tissues, which becomes evident when the plants have about five expanded leaves fading with the advancing of maturity (Barrière, 2017). The first investigations on BMR mutations reported that these mutations were originated from natural mutations and segregated as simple Mendelian recessive traits (Burnham and Brink, 1932; Jorgenson, 1931). The BMR genotypes are characterized by a low lignin content in stover tissues, which is usually related to an increasing in fiber digestibility, can be associated with an altered content of different hydroxycinnamates, even if the exact effects depend on the enzymes linked to specific BMR genes (Allen et al., 2003). Notably, researches on corn BMR mutants have been focused on *bm3* plants, because the *bm3* mutation enhances the cell wall digestibility in comparison to the other mutations improving cattle feeding value.

Hybrids for WPCS production with high NDF digestibility, such as BMR, have improved lactation performance in dairy cows (Oba and Allen, 2000a, 2000b). In a meta-analysis led by Ferraretto and Shaver (2015) the ruminal in vitro NDF digestibility measured at 30 and 48 hour was 9.4% greater for BMR than other hybrids; in addition, the authors observed significant reduction in lignin content for BMR related to a reduced cross-linking of lignin in comparison to other fibrous components as previously reported by Jung et al. (2012). Similar results were

observed by Ferraretto et al. (2015) evaluating the effect of feeding a TMR containing a floury-leafy corn silage hybrid in comparison with a BMR hybrid. In this study, in vitro NDF digestibility was higher for BMR compared with the other hybrid. In addition, cows fed with the BMR ration consumed 1.7 kg/d more DM than floury-leafy ration. Lim et al. (2015) evaluated the effects of corn silage hybrids (e.g., control vs BMR) and the proportion of corn silage used to formulate diets for high-producing cows. Lignin content was lower in BMR than control silage (2.19% vs 3.09% DM, respectively); in vitro NDF digestibility measured at 30 hour was higher for BMR hybrid (62.8% of NDF) than control (52.2%). Referring to the high-producing cows' performance, the group fed with the BMR hybrid produced more milk than control group.

On the other hand, the concern for BMR hybrid for silage production is referred to lower yield per hectare in comparison to the other hybrids (Eastridge, 1999). Gentinetta et al. (1990) evaluated 21 near-isogenic normal and *bm3* hybrids for forage quality and yields. The *bm3* had lower lignin content compared to the normal hybrids (38 and 60 g/kg DM, respectively); whereas DM yields for *bm3* hybrids were considerably lower than normal hybrids (14.4 and 17.9 Mg/ha, respectively). Peña et al. (2023) evaluated the effects of corn-planting population, using a conventional hybrid and BMR hybrids on DM yield, silage quality, and in vitro NDF digestibility. The authors observed that corn hybrids with the BMR trait did not improve or penalize DM yield but had a higher digestibility of NDF in comparison to conventional hybrid.

Waxy genotypes

Corn hybrids known as waxy are characterized by the *wx1* allele on the short arm of chromosome 9. The waxy genotype is characterized by the substitution of endosperm amylose by amylopectin (Alexander, 1988; Coe et al., 1988). In waxy corn, the main chemical components of starch in the kernels are amylose and amylopectin (Ketthaisong et al., 2013). Waxy hybrid used to produce WPCS have been resulted in lower milk yield in comparison to *bm3* when used to formulate diets with 500 g/kg forage to lactating cows (Moreira et al., 2000). Improving in fat-corrected milk yield for WPCS from waxy hybrid has been observed in comparison to conventional corn hybrid, even if no differences have been detected with other hybrid (Akay and Jackson Jr, 2001).

Leafy types of corn

Corn hybrids with the Leafy (*Lfy*) allele have the characteristic to delay flowering and increase the number of leaves above the ear respect to the normal hybrid (Shaver, 1983). Hybrids with the *Lfy* allele are named as superior hybrids because the additional leaves are more digestible, contain more protein than conventional hybrids, produce higher DM yields, grain yield equal to

normal hybrids (Allen et al., 2003). The gene action of this allele affects plant growth hormones producing more leaves above the ear (Glenn, 2013). Considering that leaves are more digestible than stalk, leafy hybrids may have a greater digestibility than conventional hybrids due to their greater leaf:stalk ratio. Lewis et al. (2004) examined the interactions of dual-purpose, leafy, and BMR corn hybrids, the stage of maturity at harvest, and cutting height management on yield and corn forage quality. In this study, leafy hybrids had better NDF digestibility than dual-purpose hybrids but not when compared to BMR hybrids. No differences in terms of lactation performance were observed in feeding trial where *Lfy* were compared to a normal hybrid (Bal et al., 2000; Kuehn et al., 1999).

Brachytic corn hybrid

Crop varieties affected by dwarfism or semi-dwarfism have different advantages, such as increased lodging resistance, denser growth, and higher harvest index (Hedden, 2003; Li et al., 2011). Many dwarf mutants have been classified by short internodes, which have been referred to impaired internode elongation (Zhang et al., 2019). The stem/internode elongation is mainly regulated by different plant hormones, such as gibberellins (GAs), brassinosteroids (BRs), strigolactones (SLs), and auxins (Wang et al., 2018). Referring to corn, only the category of brachytic mutants expresses a short stature caused by a shortening of the internode length without a reduction in the number of internodes or the size of other organs (Pilu et al., 2007). The classic corn dwarfing mutant brachytic 2 (*br2*) defects in transport of the auxin hormone (Multani et al., 2003; Salamini, 2003). The *br2* mutant phenotype results from loss of a P-glycoprotein (PGP) that generally regulates polar auxin transport in the stalk (Multani et al., 2003). This results in greater standability preventing losses in corn productivity (Barten et al., 2022). In particular, improving lodging resistance is crucial to convey resilience to severe weather conditions. Lodging resistance has been evaluated in a field trial during the 2020 Derecho event in the North Central U.S. Corn Belt, in which short-stature corn (SSC) plants carrying the *br2* mutant were compared to conventional corn hybrid (CCH) by Barten et al. (2022). The authors observed an average of 25% of plants damaged for SSC; on the other hand, an average of 50% was reported for CCH. The authors stated that cropping SSC may be a solution to prevent field losses in case of weather damage improving not only the economic value for farmers but also the sustainability of whole-plant corn system. Considering that whole-plant corn silage (WPCS) is one of the main forage used to formulate diet in dairy farms, the nutritive value of this new SSC hybrid has been recently investigated by Bourdoncle et al. (2023). In this study, the authors evaluated the effect of the *br2* mutation alone or in combination with the *bm3* mutation on fiber digestibility and lignification. The *br2* mutation enhanced fiber digestibility in near-isogenic inbreds by 4% units and hybrids by

2.7% units. These effects might be attributed to reduction in lignin concentration and alteration in the distribution of lignin within the stem.

Management factors

Adopting proper management practice among the whole ensiling process is crucial because significant amounts of DM are lost due to mechanical damage during harvest, plant and microbial respiration, deamination and proteolysis, seepage, aerobic spoilage, and feed-out phases (Grant and Adesogan, 2018). Crop genetics, harvest maturity, moisture content, chop length, and silage distribution and compaction are pre-ensiling components that can influence the fermentation pathway, storage losses, palatability, and nutritional quality of silage (Mahanna and Chase, 2003). In this section a description of the main pre-ensiling components affects the quality of silage are reported.

Maturity at harvest

The main factors that affect nutrients in whole-plant corn and its digestibility include, corn variety, maturity level, treatment, and weather conditions among cropping. The maturity of plant affects the number of fermentable sugars available for the pool of bacteria (e.g., lactic acid bacteria) involved in silage fermentation and nutritional value in formulating rations for the herd. On the other hand, maturity at harvest affects yield, nutrient composition, digestibility and ensiling potential of crops (Buxton and O'Kiely, 2003). As the maturity advances, several changes are associated with the extent of kernel development, which alters the proportion of DM contributed by its different fractions – generally, increased proportions of kernels and reduced proportions of stover (stalk, leaves, and husk; (Buxton and O'Kiely, 2003). During maturation of corn plant, sugars in kernels are converted to starch, which is followed by an increase in terms of DM within kernels (Allen et al., 2003). Generally, an increase in starch concentration corresponds to a decline in crude protein (CP), NDF, and ash content with delayed WPCS harvest (Allen et al., 2003; Buxton and O'Kiely, 2003; Johnson et al., 1999). Hence, delayed maturity at harvest has been suggested as a management strategy to increase DM and starch yields per hectare (Owens, 2014). Nevertheless, delayed WPCS maturity impairs nutrient digestibility (Ferraretto et al., 2018). In particular, cell wall digestibility of corn plant declines with the advance of maturity, which is related to greater stover lignification among the maturation of the corn plant. Therefore, targeting delayed maturity at harvest to increase starch concentration may affect stover digestibility (Allen et al., 2003). In a meta-analysis of feeding trials led by Ferraretto and Shaver (2012) greater NDF digestibility were reported when WPCS was harvested above 40% DM. This could be related to less negative effects of greater ruminal starch digestibility on NDF digestibility for drier, more mature WPCS (Russell

and Wilson, 1996). On the contrary, Owens (2014) suggested that this may be related to less adverse effects of delayed harvest on fiber digestibility with modern hybrids. Delayed maturity at harvest impairs the utilization of the kernel fraction. Notably, a reduction for total-tract starch digestibility (TTSD) was observed in diets containing WPCS with >40% DM in the meta-analysis of Ferraretto and Shaver (2012). This is likely related to an increase in the proportion of vitreous endosperm in kernel related to greater maturity. The increase in the proportion of vitreous endosperm in the kernels is related to the hardness of kernel (Correa et al., 2002) which may limit the percentage of fractured kernels during WPCS processing (Ferraretto et al., 2018). Besides, even the exposed endosperm is not fully digested due to the existence of a starch-protein matrix formed by the chemical bond between the zein protein and the starch granules (Kotarski et al., 1992). Lower ruminal in vitro starch digestibility was reported by Ngonyamo-Majee et al. (2009) in corn kernels harvested at intervals between one-half of milk line and black layer due to an increasing in proportion of vitreous endosperm in corn kernels with the advance of maturity.

Moisture content

Moisture allows silage compaction, supporting transition the silo to an anaerobic environment. Insufficient level of moisture is prone to yeast and mold issues, whereas excess moisture can lead to great effluent and/or prolonged fermentations, resulting in high acid levels and increased protein degradation (Mahanna and Chase, 2003). Silage producers should harvest WPCS at $\frac{1}{2}$ to $\frac{2}{3}$ milk line if it is included in rations with alfalfa or grass forages. On the other hand, if WPCS is the primary forage source, it is possible to schedule the harvest moment at a less mature stage, between $\frac{1}{3}$ to $\frac{1}{2}$ milk line maturity (considering the moisture limitation of the storage place), in order to reduce the rumen starch load and enhance total fiber levels in the ration. One factor that compensates for lower starch levels due to early harvesting is that kernels break more easily due to mechanical action when corn silage is harvested at higher moisture concentrations (Mahanna and Chase, 2003).

Theoretical length of cut

Matching a valuable compromise between silo fermentation and optimum rumen function needs to be done taking in consideration the theoretical length of cut (TLOC), because the forage component is mainly fed to provide adequate physically effective NDF (peNDF) (Ferraretto et al., 2018). This is desirable with increasing dietary concentrations of rapidly fermentable carbohydrates because peNDF is positively related to chewing activity, ruminal pH, and milk fat content (Mertens, 1997). Considering this scenario, longer particle length would stimulate chewing and maintain short dietary fractions, such as concentrate feeds, entrapped in the rumen mat (Allen

et al., 2003). Consequently, reduction in particle size through the use of processing rolls needs longer TLOC when cows are fed processed WPCS.

Referring to the advantages to achieve greater peNDF with forage particles of greater length, this condition may lead to a limitation in terms of intake reducing ruminal passage rate and increasing fill (Mertens, 1987). Minimal effects of TLOC settings on lactation performance were observed by Ferraretto and Shaver (2012). Digestibility of NDF was not affected by TLOC (Ferraretto and Shaver, 2012). Higher ruminal pH and retention time is often observed for long particles (Allen, 1997), which may enhance NDF digestibility. On the other hand, increased surface area for bacterial attachment when WPCS includes short particles may also increase NDF digestibility despite its faster passage rate (Johnson et al., 1999).

Depending on the crop, the best compromise suggests a chop length of 6- and 18-mm. Particle sizes in this TLOC range will allow for (i) proper silage compaction and exclusion of O₂ to prevent heating, molding, and poor bunklife; (ii) easier unloading from storage places; and (iii) adequate effective fiber to reduce the chances of digestive upsets, milk fat depression, and increased incidence of displaced abomasums. In particular, WPCS should be chopped at 9 to 12 mm TLOC. Notably, TLOC longer than 18 mm can create difficulties in (i) packing, (ii) proper functioning of silo unloaders, (iii) increased number of kernels passed in the manure, and (iv) preferential sorting of stover or larger cob pieces in total mixed rations (TMR). Chopping shorter than 9 mm TLOC allows to operate a proper compaction (especially in dry crops) and increase silo capacity. Nevertheless, it requires more power and may slow harvesting (Mahanna and Chase, 2003).

Kernel processing

The starch endosperm in corn kernels is protected by the pericarp, which, if intact, is greatly resistance to microbial attachment and enzymatic digestion (McAllister et al., 1994); breaking the seed coat is needed to enhance digestibility. The purpose of the processing rollers is to break the corn kernels into smaller particle sizes to ensure exposure of the starch endosperm, and thus starch digestion and utilization by ruminants animals is enhanced (Ferraretto et al., 2018). In a meta-analysis led by Ferraretto and Shaver (2012), using the processing rolls increased TTSD in diets containing WPCS with 32 to 40% at feed-out, but not when WPCS was above 40%. The proportion of vitreous kernel endosperm increases with increasing DM content of WPCS (Johnson et al., 1999); indeed, the greater hardness of kernels means that very dry WPCS kernels are less susceptible to breakage during kernel processing at harvest. Moreover, Allen et al. (2003) highlighted the potential benefits of mechanical processing of WPCS when a longer TLOC is desired to improve the peNDF content of silage. Harvesting silage with processing rolls increased

the TTSD of diet when TLOC was set at 0.93 to 2.86 cm, but not when the TLOC setting was shorter or longer (Ferraretto and Shaver, 2012a). Johnson et al. (2003) found that kernel processing reduced the amount of intact kernels when the TLOC was 11.1 mm, but not when it was 27.8 or 39.7 mm. Although recent technology (e.g., shredding rollers, increased roller speed differential) has attempted to reduce the problems associated with the proper processing of corn kernels when WPCS is harvested at longer TLOC settings (Ferraretto et al., 2018).

Cutting height

Managing cutting height at harvest allows to improve silage nutritive value through increased starch concentration and NDF digestibility, increasing milk production in dairy cows (Ferraretto et al., 2018). Lewis et al. (2004) observed a decrease in DM yields increasing the cutting height from 15 to 46 cm, even if authors stated that this decreasing trend was offset by an increase in milk yield at higher cutting height. In addition, the authors recommended to increase cutting height on leafy corn hybrids for silage purpose, but not for BMR hybrids. Neylon and Kung (2003) studied the effect of increasing cutting height from 12.7 to 45.7 cm on yield and nutritive value of leafy corn hybrids for silage. The authors observed that increasing the cutting height improved the nutritive value of WPCS by decreasing the concentration of ADF, NDF, and ADL followed by an increasing in starch content. On the other hand, DM yield decreased when cutting height increased, but milk yield per ton of DM was greater for the high-cut WPCS. Diepersloot et al (2022) evaluated the effects of cutting height, heterofermentative inoculation, and storage length on fermentative profile and nutrient composition of WPCS. Corn silage harvested with greater cutting height had higher starch concentration and NDF digestibility, lower NDF and lignin concentration. Considering the heterofermentative inoculants, authors observed that increasing cutting height may promote a fermentation that could improve aerobic stability and potentially create conditions that favor proteolysis, also increasing starch digestibility.

Snaplage

Snaplage includes the ear (cob and grain), husk, and shank and is harvested using a self-propelled forage harvesters (SPFH) equipped with a snapper head and an on-board kernel processor (Ferraretto et al., 2018). In the last decades the harvest techniques have been improved and the use of SPFH allows to harvest earlier and proceed more rapidly with kernel processing in snaplage in comparison to rehydrated or high moisture corn grain silages,, showing considerable logistic advantages to the production system (Daniel et al., 2019). In addition, corn harvested as snaplage could exhibit greater rumen degradability of starch (extent and rate) than dry ground corn as the starch-protein matrix in kernel endosperm is broken down during the ensiling process

(Hoffman et al., 2011). However, snaplage differs from rolled high-moisture corn (RHMC) in nutritional qualities and harvest management considerations, such as moisture content, forage harvester set-up, storage capacity, and impact of starch digestibility on milk production (Akins et al., 2014). Suggested moisture content for snaplage is between 32-40%. Kernel moisture of 28-35% is recommended with harvest beginning at black-layer (34-35% kernel moisture) in order to maximize starch accumulation (Mahanna, 2008). Gusmão et al. (2019) evaluated the nutritive value of snaplage affected by hybrid and maturity. The authors observed a decrease in NDF digestibility with the advance of maturity, and starch concentration increased in snaplage with the 35% of moisture. However, snaplage has a wide harvest window it is crucial to take in consideration that maturity can affect the nutritive value. Akins and Shaver (2014) evaluated lactation performance feeding corn snaplage alone or in combination with ground dry shelled corn (SPDC) versus RHMC. Cows fed diet with RHMC had a greater dry matter intake (DMI) than snaplage and SPDC which was likely due to greater starch digestibility for the snaplage. Milk yields were similar as well component-corrected milk yields, resulting in higher feed efficiency (kg of milk/kg of DMI) for both snaplage and SPDC in comparison to RHMC. Milk fat percentage was reduced by 0.27% in corn snaplage in comparison to RHMC which was likely due to greater starch digestibility of snaplage. These changes in milk composition along with reduced DMI were associated to greater ruminal starch digestibility in snaplage diets. Partial replacement of snaplage with dry ground shelled corn mitigated the depression in milk fat percentage.

Toplage

Nigon et al. (2016) changed a conventional ear-snapping corn header with cut-off knives in an effort to harvest more of the upper stalk and leaf portion of the corn plant. The aim was to produce silage that could be fallen between high-cut WPCS and snaplage and was referred to as toplage. Yields of DM were reduced by 23 to 28% compared to WPCS, but starch concentration was higher for toplage than WPCS (43 vs 33.9% of DM). Therefore, this novel approach allows a second operation to collect the remaining stalks. Cook et al. (2016) proposed this separation of corn plants at harvest as a tool in ration formulation; for instance, by splitting and feeding the stalks to animals of lower energetic demand (i.e., heifers, far-off dry cows).

Shredlage

Shredding WPCS at harvest is an advantageous method to change the physical characteristics of the silage. A new form of silage, called shredlage, is produced when whole-plant corn is harvested with a SPFH equipped with cross-grooved crop-processing rolls set with a roller speed differential that is around 20% higher and cut with a TLOC (22 to 26 mm) than the usual

(Ferraretto et al., 2018). The difference with conventional-processed WPCS regards the greater proportion of long stover particles in corn shredlage. Feeding longer particles forage in rations for lactating dairy cows, this can increase the peNDF content of the ration, which is crucial for the correct functioning of rumen, the health of cow and milk fat content (Mertens, 1997). Despite the TLOC, the shredlage process causes greater damage to stover particles and coarser grains than conventional harvesting. In relation to conventionally processed silage, 3.5 % fat corrected milk (FCM) yields and actual milk yields increased by 1 to 1.5 kg/cow per day when whole-plant was harvested as corn shredlage using either conventional (Ferraretto and Shaver, 2012a) or BMR hybrids (Vanderwerff et al., 2015). This response probably associated to greater kernel breakage during passage through rolls and the corresponding increased surface area allowing for enhanced bacterial attachment and digestion.

Conclusion

Whole-plant corn silage has become an important forage in dairy production. In particular, WPCS is a palatable, high-energy forage with high yield compared with other forages. The quality can be very uniform within a silo if the same hybrid is harvested and the silo is filled within a short time. However, the quality of corn silage varies depending on the environment, maturity at harvest, the genetics of the hybrid and fermentation in the silo. Over the past several years researches have been focused on manipulation of the physical and chemical characteristics of WPCS in an effort to improve its nutritive value and maximize milk production. Hybrid selection has a key role in enhancing the nutritional composition of WPCS and matching the requirements of high-producing dairy cows. The selection of a corn hybrid for silage production should be done considering factors related to digestibility and intake. The use of BMR hybrids has been one of the most consistent, cost-effective and adopted strategies to increase forage fiber digestion and milk production by dairy cows. Corn hybrids carrying the *br2* mutation can be considered as a potential opportunity to improve fiber and starch digestibility in lactating dairy cows. However, future researches have to be carried out to better understand the potential of these hybrids considering both agro-technical and nutritional factors. Whole-plant corn should be harvested at the appropriate maturity for the silo used, to optimize yield and quality and minimize loss of effluent. Adopting proper management practice among the whole ensiling process is crucial because significant amounts of DM are lost due to mechanical damage during harvest, plant and microbial respiration, deamination and proteolysis, seepage, aerobic spoilage, and feed-out phases.

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4. Review: Strategies and technologies in preventing regulated and emerging mycotoxin co-contamination in forage for safeguarding ruminant health

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Abstract

Ruminants are often considered less susceptible to mycotoxins than monogastrics, owing to rumen microflora converting mycotoxins to less toxic compounds or several compounds present in the rumen-reticulum compartment, being able to bind the mycotoxin “mother” molecule that make them unavailable for absorption process in the gastro-intestinal tract of host animals. However, if ruminants consume feed contaminated by the mycotoxins for long periods, their growth, development, and fertility can be compromised. Among regulated mycotoxins, the most studied and known for their effects are aflatoxins (AFs) AFB1, AFB2, AFG1 and AFG2, as well as the AFM1 for its high importance in dairy sector, deoxynivalenol (DON) and its metabolites 3/15 acetyl-DON and 3-glucoside DON, T-2 and HT-2 toxins, zearalenone (ZEN), fumonisins, in particular that belong to the B class and ochratoxin A. Furthermore, because of the emergence of multiple emerging mycotoxins that are detectable in feed utilized in ruminant diets, such as ensiled forage, there is now a growing focus on investigating these compounds by the scientific community to deepen their toxicity for animal health. Despite enhance of research, it is remarkable that there is a paucity of *in vivo* trials, as well as limited studies on nutrient digestibility and the impact of these molecules on rumen and intestinal functions or milk yield and quality. In this review, recent findings regarding occurrence of regulated and emerging mycotoxins in forage and their possible adverse effects on dairy cattle are described, with special emphasis on animal performance and on rumen functionality.

Keywords: animal health, feed safety, forage, fungi, volatile organic compounds

Implications

Ruminants are often considered less susceptible to mycotoxins than monogastrics, but this concept is outdated based on advancement of research on these aspects. In particular, ruminants are partially protected against some mycotoxins, due to the ability of rumen microflora to detoxify mycotoxins through enzymes production. However, mycotoxin contamination in livestock feed deserves attention because of the effect on animal health and its performance. Mycotoxin presence in feed and conversion to more toxic metabolites impact on rumen and intestinal functionality causing an imbalance in the bacterial population. This also triggers an immune response that impacts animal well-being, reproductive health, and the quality of milk. Aim of our review is to describe the main mycotoxins detected in silage and other ruminant feeds, the primary ensiling strategies to prevent or mitigate mycotoxins contamination during ensiling process and feed-out

phase, the effects on animals and finally the new technologies able to support the quantification of these metabolites.

Introduction

Mycotoxins comprise a different category of secondary metabolites naturally produced by many different phytopathogenic filamentous fungi, most of which are in the genera *Fusarium*, *Aspergillus*, and *Penicillium* spp. (Yiannikouris & Jouany, 2002). These compounds are chemically very stable and can be detected in animal feedstuffs, raw materials, and homegrown forage (Fink-Gremmels, 2008; Gallo et al., 2015; Ogunade et al., 2018). Ingestion of mycotoxins is responsible for several animal poisonings that can negatively impact feed intake, nutrient utilization, and absorption leading to disease and death, as well as subclinical conditions and immune suppression (Morgavi & Riley, 2007). The sensitivity of animals to different mycotoxins depends on the animal species, breed, age, sex, nutritional status, stress level, and other factors (Yiannikouris & Jouany, 2002). In general, ruminants are less sensitive to mycotoxins than monogastric due to the ability of rumen microorganisms ability to secrete enzymes able to detoxify mycotoxins to less toxic or non-toxic metabolites (Mobashar et al., 2010; Upadhaya et al., 2010), as deeply reviewed by Fink-Gremmels (2008b). However, this capacity of the ruminal microflora is limited, and is affected by diet and the presence of metabolic diseases (Jouany and Diaz 2005; Rodrigues, 2014; Spanghero et al., 2023).

Climate can affect growth, distribution, and production of mycotoxins by different fungal pathogens, and climate change may lead to alterations in the levels of mycotoxins in foods and animal feeds (Medina et al., 2017). The occurrence of new mycotoxin-matrix combinations is an additional concern. Eliminating or mitigating mycotoxin contamination in human foods and animal feeds is a crucial issue, and many countries have defined specific guidelines and regulations to safeguard consumers and livestock. Mycotoxins that are regulated in human foods and animal feeds are aflatoxins AFs and AFM1, that is the hydroxylated aflatoxin metabolite present in the milk arising from a diet contaminated with aflatoxin B1; trichothecenes of the type A such as T-2 and HT-2 toxin, and type B which include DON and diacetoxyscirpenol; fumonisins; zearalenone, ergot alkaloids; ochratoxin A; patulin; phomopsins; and sterigmatocystin that is the precursor of aflatoxin B1 in those situations in which cereals and/or food are contaminated by fungi able to produce aflatoxins (Van Egmond et al., 2007; Nieto et al., 2018). There are also many emerging mycotoxins that are less commonly found and are unregulated (Gruber-Dorninger et al., 2017).

Forage silage represents the main component of dairy cow diets and is an important source of mycotoxigenic fungi and mycotoxins. Based on this knowledge, silage safety needs attentions

because is a real risk both for animal and human health (Driehuis et al., 2018; Gallo et al., 2015). The contamination of silage can be affected by several factors that are referred to the whole farm environment from the field to the bunk. Some of these factors can be related to forage species, stage of maturity or moisture content at harvest time, ensiling processes, storage structure, use of silage additives or oxygen barrier films, feed-out methods and bunk management (Mahanna and Chase, 2003). Complying with the fixed concentration for mycotoxin limits in feeds, several measures can be adopted to reduce the production of mycotoxins during the field, pre-harvest, harvest, and ensiling phases (Ogunade et al., 2018). Among these strategies, the use of microorganisms or enzymes that degrade mycotoxins is an attractive solution. Many species of bacteria and fungi can detoxify feeds and foods. Researchers have isolated bacteria, yeast, and fungi that can detoxify mycotoxins from different sources; indeed, lactic acid bacteria LAB show an effective role in mycotoxins deactivation in silages due to their critical role during the ensiling process (Li et al., 2021).

This review describes the main mycotoxins and fungal pathogens involved in silage production, the main strategies used to prevent mycotoxin contamination to reduce farm economic loss and address animal and dairy products consumers' health, summarizing the known adverse effects of mycotoxins in ruminants, particularly lactating dairy cows.

Mycotoxins in silage

The greatest risk for exposure to regulated mycotoxins and other secondary metabolites in ruminants is forage contamination. Focusing on silages, mycotoxins that are detected in ensiled forages are produced by several species of fungi, such as *Alternaria* spp., *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillium roqueforti*, *P. paneum*, *Monascus ruber*, and others (Gallo et al., 2015). The three most important detected fungi genera present in pre-harvest time in cereals are *Aspergillus*, *Fusarium* and *Alternaria* spp. Indeed, *Alternaria* and *Fusarium* spp. are categorised as field fungi whereas *Aspergillus* spp. can be present both pre- and postharvest.

***Aspergillus* toxins**

Alternaria and *Fusarium* spp. are often categorized as field fungi genera due to their ability to proliferate among the growth and maturation of the plant (Ogunade et al., 2018). Some species of *Aspergillus* spp. can occur on preharvest and postharvest phases (Storm et al 2008), and other species such as *A. flavus* can contaminate crops in field and produce AFs (Storm et al., 2008) when the temperature is more than 32 °C, the relative humidity is higher than 80% and during periods of drought stress (Tsitsigiannis et al., 2012; Frisvad et al., 2005). El-Shanawany et al. (2005) stated that the growth of corn can be obstructed by some mycotoxins-producing fungi that produce

toxins before harvest, and apparently survive during the ensiling process, as reported for *A. flavus* in silages.

A. flavus and *A. parasiticus* are the two predominant toxigenic field species of *Aspergillus* in crops and silages. The main mycotoxins produced by *A. parasiticus* are AFB1, AFB2, AFG1, and AFG2, and the predominant mycotoxins produced by *A. flavus* are AFB1 and AFB2 Frisvad et al., (2005). AFs are a critical threat to human health due to their carry over in the milk. Indeed, lactating animals that consume contaminated silage can transform them into AFM1 and AFM2, toxic hydroxylated derivatives that can arise in dairy products (Masoero et al., 2007). Silages are often infected by toxins from *A. fumigatus* (Storm et al., 2008; Alonso et al., 2013; Frisvad et al., 2009). Gliotoxin is the most toxic of the potentially bioactive secondary metabolites produced by these species, occurred in corn silage and other silages (Pereyra et al., 2008; Richard et al., 2009). Kojic acid, cyclopiazonic acid, and β -nitropropionic acid are other specific mycotoxins produced by *A. flavus* and other *Aspergillus* species (Chang et al., 2009), however, are less widespread in silages (Storm et al., 2014). Boudra & Morgavi (2005) reported that gliotoxin, helvolic acid, and verruculogen are biologically active substances implicated in the pathogenesis of aspergillosis and were stable during storage phase, in contrast to fumagillin that was unstable under ensiling conditions. *A. fumigatus* can potentially produce two Clavine alkaloids, agroclavine and festuclavine (Frisvad et al., 2009), and maltoryzine had been recorded as a metabolite produced by *Aspergilli*. *A. clavatus* can produce maltoryzine, but there are no reports of these other mycotoxins in forages (Gallo et al., 2015).

Aflatoxins

The AFs are toxin with natural carcinogen potential produced by many strains of *A. flavus*, *A. parasiticus*, and *A. nomius*. AFB1 and AFG1, and their dihydro derivatives AFB2 and AFG2 are naturally found in food and feed ingredients. The reason for their widespread presence is their capacity to adjust to various environmental growth conditions, including a wide range of temperature and humidity (Tulayakul et al., 2005). These species are common in tropical and subtropical climate region (Cheli et al., 2013). Usually, the levels of AFs are lower than those of other mycotoxins in well-preserved silages (Kosicki et al., 2016). A survey by Rodrigues & Naehrer (2012) reported AFs were present in 33% of 7049 livestock feed samples, and the average concentration was 63 $\mu\text{g/kg}$ ppb. A study in France reported that the level of AFB1 was as much as 51 $\mu\text{g/kg}$ dry matter DM in corn silage that was stored in a bunker silo Richard et al., 2009. Moreover, several studies reported that the AF carry-over rates into cow milk ranged from 1% to 6% (Masoero et al., 2007). The Action Levels established by the US FDA for aflatoxins contaminations are 0.5 $\mu\text{g/kg}$ for liquid whole milk and 20 $\mu\text{g/kg}$ for feed ingredients offered to

dairy cattle (FDA, 2011); while the maximum levels recommended in Europe are 0.05 µg/kg for liquid whole milk and 5 µg/kg for feed ingredients (European Commission, 2006). Upadhaya et al. (2010) stated that a portion of ingested AFB1 can be transformed by ruminal microbes into aflatoxicol.

Ochratoxins.

The ochratoxins OTs are secondary metabolites produced by *Penicillium verrucosum*, *A. ochraceus*, *A. niger*, and *A. carbonarius* (Frisvad et al., 2005; 2009). Ochratoxin A OTA is considered the most toxic OT (Yiannikouris & Jouany, 2002).

Silages usually have lower levels of OTA than other mycotoxins produced by *Fusarium*, such as DON, ZEA, fumonisin, and T-2 toxin (Biomim, 2016). The lower presence of OTA is related to the conditions that characterize silage conservation phase such as high concentrations of acetic acid and carbon dioxide, are not endured by fungi that produce these toxins (Gallo et al., 2015). Biomim (2016) carried out a survey in several countries based on 124 silage samples in which only 1% was contaminated by OTA with a maximum concentration level about 3 µg/kg. As opposed to this, Kosicki et al. (2016) stated that the incidence of OTA in corn silage was higher than the levels previously reported and was approximately 47% of 61 samples collected in Poland, although the maximum concentration was only 7.5 µg/kg. Ruminants are relatively tolerant to OTA, and it is extensively and rapidly degraded in the rumen to a less toxic product, OTα (Fink-Gremmels, 2008). Protozoa present in rumen environment, is a central group able to degrade mycotoxins, moreover gut microbes also play a key role in OTA degradation and its detoxification in ruminants thanks to proteolytic enzymes activity. Moreover, OTA *in vitro* results also indicate that ochratoxin C (OTC) is the other form of ochratoxin present in feed and is the ethyl ester form of OTA. OTC would be rapidly metabolized to OTA in the rumen of cattle and finally degraded to OTα (Fink-Gremmels, 2008); that represent the biochemical pathway for detoxify OTA in ruminants. The bioavailability of dietary OTs may increase in animals given high-grain diets, probably because this leads to an increase in the ruminal pH from about pH 5.5 to 5.8 (Pantaya et al., 2016). This is related to changing in microbial population. The rumen microflora is highly influenced by the diet composition with an effect on protozoa population. Their presence is strictly related to diet composition, feeding level, and feeding frequency; these nutritional aspects consequently changing rumen habitat such as pH, the availability of nutrients and the turnover rate with an impact on protozoa and OT rumen detoxification.

Alternaria toxins.

Alternaria spp. is a huge and complex genus that can infect wheat worldwide, including a saprophytic and pathogenic species and is responsible for black point disease on grains.

Pathogenic species can cause damages to plants directly in field, while saprophytic species after harvesting phase to products. The taxonomy of this genus has not been settled, and some evidence suggest that this genus should be split into multiple genera (Lawrence et al., 2016; Somma et al., 2019). *Alternaria alternata* is the most common species, but its taxonomy is complicated due to the presence of different pathotypes or formae speciales that indicate pathogenicity (Woudenberg et al., 2015). Phylogenetic relationships may be related to toxin production.

Alternaria species produce more over 70 different mycotoxins, including alternariols, altertoxins, altenuene, tentoxin, and tenuazonic acid (Andersen et al., 2002) that have hematotoxic, genotoxic, and mutagenic effects. Specially, tenuazonic acid is the most acutely toxic molecule in the group of toxins produced by this fungi species. *Alternaria infectoria* produces several other mycotoxins or exometabolites, such as 4Z-infectopyrone, phomapyrones, novae-zelandins, dehydrocurvularin, pyrenochaetic acid, and alternarienic acid (Andersen et al., 2015; Ramires et al., 2018). *A. alternata*, *A. tenuissima*, and *A. arborescens* can all produce alternariol, alternariol-monomethyl ether, three altertoxins I, II, and III, and tenuazonic acid (Da Cruz Cabral et al., 2016; Logrieco et al., 2009; Patriarca et al., 2007).

Alternaria spp. detected in fruits, vegetables, cereals, and derived products, but a few studies have evaluated these species in silage (Escrivá et al., 2017). Notably, *A. alternata*, *A. arborescens*, and *A. tenuissima* were isolated from hay and silages (Mansfield & Kulda, 2007). Storm et al. (2014) reported low levels of alternariol and alternariol monomethyl ether in forages from Denmark and outlined the occurrence of other *Alternaria* secondary metabolites. Many studies conducted in Italy show that only 3% of silage samples investigated were contaminated by *Alternaria* mycotoxins and characterized by very low levels of these toxins (Gallo, unpublished data).

Yu et al. (1999) reported a high incidence of *A. alternata* type A mycotoxins in different feeds, such as hay, hay silages, and whole-crop forage corn silage, with concentrations exceeding 1000 µg/kg in some cases. However, this study also showed that only one strain of *A. arborescens* was related to the production of AAL mycotoxins, and that none of the other 98 analysed strains of *A. arborescens* or other *Alternaria* species produced these toxins.

***Fusarium* toxins.**

Fusarium is a wide and diverse fungal genus which contains many strains that produce mycotoxins in hay and silage (Gallo et al., 2015). The species most commonly associated with mycotoxins are *F. graminearum*, but other species, including *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. incarnatum* formerly *F. semitectum*, *F. langsethiae*, *F. oxysporum*, *F. poae*, *F. proliferatum*, *F. solani*, *F. sporotrichioides*, *F. subglutinans*, *F. tricinctum*, and *F. verticillioides* were also

isolated from infected wheat kernels (Leslie & Logrieco, 2006.; Logrieco et al., 2003). Most of these species are not common on small grains and are not regarded as pathogenic, although some might produce mycotoxins (Kim et al., 2006).

Fusarium mycotoxins are mostly produced by *F. proliferatum* and *F. verticillioides*, fumonisin B1 FB1 and FB2 are the predominant fumonisins, while FB1 is the most studied *Fusarium* mycotoxin. There are few studies reporting data about other fumonisin metabolites in corn silages such as FB4, FB6, FBA1, and FBA2, masked fumonisin and hydrolysed or partially hydrolysed forms. According to studies and surveys fumonisin contamination in corn silage is usually related to pre-harvest crop conditions (Driehuis et al., 2008). Despite this, the typical levels of fumonisin contamination in silage appear to be low and safe for lactating cows (Gallo et al., 2020).

Moreover, several *Fusarium* species produce trichothecenes in the type A and type B families (Cheli et al., 2013; McCormick et al., 2011). In the type B family, the most studied and known mycotoxin is deoxynivalenol (DON), while the knowledge about nivalenol (NIV) and fusarenon-X and their acetylated and deacetylated analogues 3-acetyl-DON, 15-acetyl-DON, and others are not well defined. These compounds are primarily produced by *F. culmorum* and *F. graminearum*. DON is considered the major mycotoxin in silages and other forages (Storm et al., 2008, EFSA 2013), and it can occur at different levels.

Boudra and Morgavi (2008) evaluated the stability of *Fusarium* toxins and reported that the concentrations of DON, FBs, and ZEN decreased during the time of ensiling for corn. Cheli et al. (2013) reported that the decrease in contamination was related to the DM content of silage, length of ensiling, and temperature, and that there was a 50% decrease in ZEN and a 100% decrease in DON during ensiling. Furthermore, plant enzymes can modify mycotoxins by conjugation to different polar substances after the infection has occurred (Berthiller et al., 2013; Nagl et al., 2014; Rychlik et al., 2014), and the modified forms of different *Fusarium* toxins ZEN, NIV, T-2 and HT-2 toxins, and FBs can also contaminate feeds (Dall'Asta et al., 2012).

Zearalenone and metabolites.

ZEN is a non-steroidal mycotoxin produced by *Fusarium graminearum*, *F. culmorum*, *F. crookwellense*, *F. equiseti* and *F. semitectum*, that occurs worldwide (Gupta et al., 2018). Although exposure to this mycotoxin does not cause death, it is considered toxic because it can lead to estrogenic syndromes in livestock. Another chemical derivative of ZEN, zearanol, has estrogenic effects and is signed as a growth promoter for cattle in feed lots (Hidy et al., 1977). In bovines, ZEN is mainly metabolized to β -zearalenol (β -ZEL), but we can find also β -zearalenol (α -ZEL) form present at very low levels. ZEN, α -ZEL, and β -ZEL differ in their estrogenic potency, and the capacity of rumen microbes to metabolize ZEN-contaminated feeds seems to be influenced by total

feed intake. The deactivation process could be related to biochemical pathway expression of lactonohydrolases in some microorganisms that convert ZEN into a non-estrogenic product (Takahashi-Ando et al., 2002). This enzyme acts in the rumen flora as well as by the bacterial flora in the large intestines of monogastric. For these reasons, studies are targeted on the potential application of these enzymes in the detoxification of food and feed commodities (Takahashi-Ando et al., 2004). ZEN and its metabolites, although not steroids, act similarly to 17 β -estradiol and can also bind to estrogen receptors (Lysøe et al., 2009). ZEN is most common in corn and wheat. Contaminated grains may contain DON in addition to ZEN, because both compounds can be synthesized by the same strains of *Fusarium*.

Silage is one of major sources of ZEN for cattle, but few information is available regarding the effect of ensiling on the levels of ZEN and its metabolites. González Pereyra et al. (2014) reported that ZEN and its metabolites were produced in field showing a steady performance during the whole ensiling process. If moderate levels of ZEN are present in vegetable material at harvest, these levels will not decrease during the ensiling phase because conversion of the ZEN (original molecule) to ZEL products apparently requires an acidic matrix (Gallo et al., 2015). Two studies reported that an average of 52% of collected corn silage samples had ZEN, and the average level was lower than 500 $\mu\text{g}/\text{kg}$ (Schollenberger et al., 2006 ; Storm et al., 2010). In contrast, Gallo et al. (2016) reported no detectable ZEN in corn silage.

Trichothecenes.

Trichothecenes are a huge and various class of mycotoxins produced by several genera of fungi (Grove et al., 2007). *F. graminearum* most commonly produces DON and NIV, although a newly identified trichothecene NX-2 was reported in some strains of *F. graminearum* and *F. culmorum* (Schiwek et al., 2022; Varga et al., 2015). DON, known as vomitoxin, is a type B trichothecene that can interfere with the eukaryotic protein biosynthesis (Foroud et al., 2019). Two similar compounds 3-ADON and 15-ADON are usually included with DON when determining toxicity, even though the *Fusarium* strains that produce these two other molecules may be in different natural populations or different species (Foroud et al., 2019; Liang et al., 2014 ; Miedaner et al., 2008 ; Ward et al., 2008 ; Zeller et al., 2003, 2004). Trichothecenes can cause a wide range of health problems in animals that lead to weight loss, including diarrhea, vomiting, reduced productivity, and reduced intake (Foroud et al., 2019; Wegulo, 2012). Studies in North America (Yu et al. 1999) and Northern Europe (Storm et al. 2014, Driehuis et al. 2008, Schollenberger et al. 2006, Storm et al. 2010, Oldenburg & Ellner, 2005) reported that DON concentrations were detected in 80% of tested forages, although the levels were highly variable. Comparable to several other mycotoxins, the level of DON differed within individual silos. For instance, Richard et al. (2007)

measured higher DON concentrations in the upper layer than in the lower layer of silos, even if they reported the opposite trend two years later (Richard et al., 2007). Driehuis, et al. (2008) and Gallo et al. (2015) reported that the levels of DON and ZEA were similar in the different zones of silos. The reported percentages of corn silage samples with NIV ranged from 100% (Oldenburg et al. 2005) to 4% (Schollenberger et al. 2006) of silages, with an average concentration of 131 µg/kg in hay silage.

Fumonisin.

Fumonisin are known as contaminants of corn and several other crops (Leslie et al., 2006). There are various fumonisin molecules, and FB1 is the most widely distributed and most studied one (Rheeder et al., 2002). Fumonisin inhibit sphingolipid biosynthesis, have diverse effects on the health of humans and animals (Kamle et al., 2019), and are most produced by *F. proliferatum* and *F. verticillioides* (Leslie et al., 2006). *F. proliferatum* is a cause of black point disease on wheat in several countries. Although wheat can be naturally contaminated with fumonisin in the field (Guo et al., 2016), there is poor evidence that fumonisin are present at relevant levels in silages.

Latorre et al. (2015) found that most of the fumonisin in corn silage were detected in modified form. These “hidden fumonisin” are missed in routine analysis but could be released upon digestion due to their transformation during the gastrointestinal digestion process in precursor mycotoxins (Dall’Asta et al., 2010). Among all mycotoxins, fumonisin are one of the most frequently reported threats to livestock. Rodrigues and Naehrer (2012) reported that fumonisin were detected in 64% of all silage samples, and the average concentration was 1965 µg/kg in 7049 livestock feed samples collected from the Americas, Europe, and Asia. Schmidt et al. (2015) stated that about the 48.6% of 327 corn silages sampled in Brazil had FB1, and the average concentration was 369 µg/kg. Pereyra et al. (2008) reported that the fumonisin level in corn silage ranged from 340 to 2490 µg/kg DM, with higher concentrations in samples from the top layer and close to the silo walls, regions that are susceptible to infiltration by air and have decreased fermentation.

Enniatins and beauvericin.

Enniatins and beauvericin are emerging mycotoxins produced mainly by fungi of the genus *Fusarium* that have attracted more attention due to their different toxic activities. The most frequently reported enniatins are A, A1, B, and B1 (Mahnine et al., 2011). These molecules were detected in cereals, such as wheat, barley, corn, and oats, as well as in cereal-based foods. Uhlig et al. (2006) conducted a survey collecting 228 grain samples oats, barley and wheat from Norway in which enniatins were detected in all samples and beauvericin was present in 32% of samples.

Sørensen et al. (2008) published a study of corn harvested in Denmark reporting that all samples were contaminated with enniatins and 98% were contaminated with beauvericin. Enniatin A1 levels were detected as 140-230 mg/kg in cereals from Spain, with a high concentration of 814 mg/kg in a rice sample (Meca et al., 2010). The same study reported beauvericin levels of up to 11.8 mg/kg (Meca et al., 2010). Like this, Mahnine et al. (2011) have noted high levels of both molecules in breakfast cereals from Morocco. New studies and additional data on the incidence and levels of these emerging mycotoxins in silage are needed.

Penicillium toxins

Species of *Penicillium* in the Roquefortorum section (ie., *P. roqueforti* and *P. paneum* ; Nielsen et al., 2006) are known as fungi that are commonly detected at postharvest in ensiled forages (Driehuis, 2013; Pereyra et al., 2008). Several factors, such as weather and storage conditions, influence the growth of these fungi and their mycotoxin productions (Boudra and Morgavi, 2005). Mycophenolic acid and roquefortines have been widely studied *Penicillium*-derived compounds in ensiled fodders. Several studies have reported mycophenolic acid, which is produced by *P. roqueforti* and *B. niviae* (Storm et al., 2008), in diverse percentages of corn silage samples from less than 3% (Storm et al., 2014) to 40% (Mansfield et al., 2008). Furthermore, mycophenolic acid levels higher than 20,000 µg/kg were mainly present in aerobic unstable silage samples (Medina et al., 2014; Schneweis et al., 2000, Gallo et al., 2015). Throughout roquefortines that are originated by different lines of the *Penicillium* section x, roquefortine C is the most studied toxin, and this molecule was detected in more than 40% of silage samples (O'Brien et al., 2006, Mansfield et al., 2008).

Other *Penicillium*-derived exometabolites were also discovered in silages, such as andrastin A, citreoisocumarin, agroclavine, festuclavine, and the *P. paneum* biomarker marcfortine A (O'Brien et al., 2006 ; Storm et al., 2014). Notably, O'Brien et al. (2006) reported that 2 of 5 collected corn silage samples were contaminated by agroclavine and festuclavine which are produced by different lines of *Penicillium*, and the concentration ranged from 100 to 1000 µg/kg.

Other fungal toxins

Several studies detected citrinin in silage that was contaminated with *Monascus ruber* (Garon et al., 2006; Richard et al., 2009; Schneweis et al., 2001). Among other exometabolites generated by this species, Schneweis et al. (2001) stated that monacolin was detected in about 20% of silage samples. Some zygomycete fungi that host endophytic bacteria can also develop secondary metabolites and toxic rhizonins (Jennessen et al., 2005) and rhizonines, but these have

not been detected in silages. Nonetheless, Jensen et al. (1994) reported that contamination by these fungi could cause zygomycosis in immunosuppressed animals.

Unspecified fungi toxins

Illicolins are fungal metabolites produced by several lines of *Fusarium* spp. and other fungal pathogens of cereal crops, such as *Ascochyta* spp. and *Cylindrocladium* spp. The illicolins have cytotoxic effects on HeLa cells, but only limited antibacterial activity. Posterior studies showed that these compounds have been considered toxic for many yeasts, indicating an antibiotic effect (Gutierrez-Cirlos et al., 2004). Illicolin A and B were discovered in some samples of corn silages. Ascochlorin is an isoprenoid antibiotic that is generated by the phytopathogenic fungus *Ascochyta viciae* (Berry et al., 2010). Macrosporin is a bioactive metabolite produced by *Stemphylium lycopersici*, a phytopathogen that induces leaf necrosis (Trigos et al., 2011). Although these compounds were detected in corn silages, their effects on animal health are unknown.

Field, pre-harvest, and harvest conditions to reduce mycotoxin contamination.

During the preharvest phase it is possible to adopt several interventions to prevent mycotoxin productions, such as using crop rotation with non-cereals, minimizing environmental stresses on crop plants, and adopting proper agronomic strategies. It is crucial to select hybrids that are adapted to local conditions, ensure that they have appropriate maturity stages, and confirm that they are less affected by abiotic stressors that increase the risk of mycotoxin contamination (Munkvold, 2014). Usually, earlier maturing hybrids have a lower risk for mycotoxin contaminations (Blandino et al., 2009). Thus, crop rotation is crucial because *Fusarium* spp. and *Aspergillus* spp. can survive in crop residue. Moreover, adopting crop rotation and tillage practices has a greater impact on *F. graminearum* and *F. culmorum* and their mycotoxins, especially DON and ZEA, than on fumonisin and AF-producing fungi. In corn, crop residues are also the major source of *F. graminearum* (Sutton, 1982). Scheduling an appropriate planting date is a necessary pre-harvest intervention that can reduce mycotoxin contamination because proliferation of fungal pathogens is linked to the developmental stage of the host plant, environmental conditions, and sometimes on vector activity. For growth of corn in temperate zones, an earlier planting date reduces the risk of fumonisin production (Blandino et al., 2009). Fertilization studies reported that adding a low level of nitrogen decreased the levels of fumonisins, AFs, and OTA relative to a non-fertilized control (Hasegawa et al., 2008), suggesting that nitrogen deficiency may promote mycotoxin production. Lastly, high density planting can enhance the risk of mycotoxin contamination for at least two reasons: i) it can stress plants and increase their susceptibility to fungal pathogens; and ii) it can create high-density crop canopies, which hold

more moisture and create suitable environment for fungal infection and production of DON, ZEA, and OTA (Blandino et al., 2009).

Preventing mycotoxin contamination of silage

Preventing mycotoxin contamination for forage crops it is a crucial issue. Several strategies can be applied during pre-harvest and post-harvest; indeed, it has been demonstrated by several research groups (Battilani et al., 2013; Nada et al., 2022) that it is possible to prevent fungal infections and mycotoxin productions at field level adopting different management practices, such as i) the use of crop rotation, tillage, and soil fertilizers; ii) scheduling of the crop variety and the seeding period; iii) decreasing fungal infestation using chemical and biological methods; iv) defining proper removal strategies; and v) monitoring insects and weed. Oude Elferink et al. (2000) reported that when storing fodder as silage, a low oxygen concentration and an increase of carbon dioxide concentration can prevent fungal growth. However, all ensiling phases should be strictly controlled and optimized to ensure proper conservation of the ensiled crops (Dell'Orto et al., 2015; Oude Elferink et al., 2000). However, silage making is not carried out following a standard procedure, indeed, farmers adopt different manners for ensiling and storing (Gallo et al., 2016 a, b). As widely reviewed by Muck et al. (2013) and Dunière et al. (2013), improper silo management can compromise silage making at any stage, leading to the proliferation of undesirable spoilage microorganisms.

Harvest

It is crucial to properly schedule the harvest date for preventing mycotoxin contaminations of fodders. The risk of mycotoxin contamination is lower when the harvest is earlier (Jones, 1981). Indeed, the cutting height of the harvesting machine should be set to minimize soil contamination, because *Fusarium* spores are common in agricultural soils (Jouany, 2007). Once harvested, farmers should rapidly store harvested forages, because delayed storage may expose the forage to high temperature and humidity, conditions that promote the growth of toxigenic fungi and the production of mycotoxins (Munkvold, 2014). Several studies reported that higher levels of AFs, DON, NIV, and fumonisins may be associated with delayed harvest in grains (Munkvold, 2003; Nordby et al., 2007) and silages (Mansfield et al., 2007).

Ensiling

Adopting proper ensiling practices allows to reduce or prevent the production of mycotoxins and the growth of undesirable microbes. Firstly, farmers should check the dry matter concentration of the crop, define a proper theoretical length of cut, carry out a rapidly filling of the

storage place, pack properly the silos to achieve the recommended bulk density, and avoid delaying the sealing operation preserving the anaerobic conditions (Borreani et al., 2018). Excluding *Penicillium*, which is considered a typical silage mold, the pathogenic strains of *Fusarium*, *Aspergillus*, *Monascus*, *Rhizopus*, and *Geotrichum* do not tolerate a low oxygen level or a low pH (Pahlow et al., 2003). Indeed, during ensiling moulds arise in unwell compacted areas, such as the top layer or sidewalls of a silo, because these areas are more prone to oxygen infiltration than the central section of the bunker (Borreani & Tabacco, 2010; Gallo et al., 2016a, b). Several studies found that sealing silos with oxygen-barrier plastic films reduced oxygen penetration, mould infestation, and spoilage of silage (Borreani & Tabacco, 2008), and other studies showed that these films decreased the production AFs (Cavallarin et al., 2011).

Several studies have examined the effects of silage LAB on improving silage fermentation and aerobic stability and in reducing fungal growth (Ranjit and Kung, 2000; Mari et al., 2009; Oliveira et al., 2017; Dalié et al., 2010) noted that LAB prevented mycotoxin contamination of silage *in vitro*. They classified several species in the *Lactococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc* genera as potential agents able to prevent or inhibit the growth of toxic fungi. For instance, they reported the highest production of antifungal metabolites by *Lc. lactis subsp. diacetylactis* after three to four days of incubation, and the peak yield of antimicrobial compounds was at 25 °C. Further, Piotrowska (2014) investigated the inoculation of silage with *Lb. brevis* and *Lb. plantarum* and reported that this reduced the OTA content by 16.9 to 35% when grown in de Man, Rogosa, and Sharpe broth and reduced the OTA content by 14.8 to 26.4% in PBS after 24 h. Inoculation of corn plants infested with southern rust *Puccinia polysora* using a mixture of *Pediococcus pentosaceus* and *Lb. buchneri* at ensiling enhanced the aerobic stability and prevented AF production (5200 vs. 0 µg/kg; Queiroz et al. 2012). Another study found that *Lb. buchneri* inoculation reduced the AF concentration without any effect on the concentrations of DON and ZEA in corn silage Iglesias et al., 2005. Ma et al. (2017) assessed the ability of LAB to bind AFB1 *in vitro* and in corn silage that has been contaminated with 30 µg/kg of AFB1. They found that certain non-LAB bacteria in silage bound to AFB1 *in vitro* decreasing the concentration of AFB1 in contaminated silage to a safe level within 72 h, with or without inoculation of LAB. Cavallarin et al. (2011) reported that inoculating corn silage with *Lb. buchneri* reduced the aerobic production of AF. Another study evaluated the capability of chemical and microbial additives in decreasing mycotoxin production into the silo. The purposes concerned in using microbial strains able to convert mycotoxins to fewer toxic compounds, addition of binding agents at the ensiling rather than the feeding phase, and inclusion of specific enzymes or chemicals for detoxification.

In well-preserved silage, the anaerobic conditions and production of organic acids inhibit the growth of fungi. Compaction is strictly linked to the DM content of an ensiled crop; so ensiling forage crops at an optimal DM content is crucial to achieve a proper feed with a high nutritive value 350–450 g/kg for grass and 300–350 g/kg for corn, with higher levels allowed for baled silage (Wilkinson & Davies, 2013). Packing silage in proper manner is strictly correlated to the sealing stage in the way to avoid dry matter losses during the storage period (Bernardes et al., 2012; S. C. Kim & Adesogan, 2006; Nelson, 1993; Weinberg & Ashbell, 2003). Petersson (1998) and Richard-Molard et al. (1980) confirmed these observations and reported that storage of *P. roqueforti* spores under airtight conditions for 3 months inhibited their germination.

Feed out phase

It is widely known that aerobic deterioration of silage during the feed-out phase is a notable problem that affects farm productivity and feed quality (Berger & Bolsen, 2006; Borreani and Tabacco, 2010). Indeed, the air effect during the feed-out phase is inevitable once the silo is opened because air can penetrate through the silage surface and travel up to 4 m within silage, especially along the peripheral areas of the silo (Pahlow et al., 2003; Borreani et al., 2007). To reduce mould infestation and mycotoxin contamination at feed-out, several measures can be applied to improve aerobic stability, such as maintaining a straight firm silo face, performing a feed out operation at a minimum rate of 10 to 16 cm per day during winter and 25 to 35 cm during summer, and using the silage immediately after removal from the silo (Borreani et al., 2018). A recent study Gallo et al. (2021) monitored the dynamics of bacterial, yeast, and fungal communities and mycotoxin production during ensiling of alfalfa and after exposure of this silage to air simulating the ensiling condition using mini silos. The authors scheduled the opening of the mini silos at different ensiling times 0, 1, 2, 3, 7, and 14 days and examined the fermentation parameters and microbiological populations colony forming units/g in the samples, with a specific focus on *Fusarium*, *Penicillium*, and *Aspergillus* spp.. The results showed that the homofermentative LAB and heterofermentative bacteria appeared dominant, but the levels of moulds, yeasts, and Enterobacteriaceae were much lower. These researchers didn't find *Acetobacter* spp., and noted that *Aspergillus*, *Fusarium* spp., and other fungi increased only during the first 2 days, and then decreased. In contrast, *Penicillium* spp. slightly declined over 14 days. Analysis of the different mycotoxins showed that DON increased to about 562 µg/kg DM, but the other *Fusarium* mycotoxins remained somewhat constant.

When silage was aired out, there were raises of moulds, yeasts, Enterobacteriaceae, *Acetobacter*, *Fusarium*, and *Penicillium* spp., and these trends showed different dynamics. The level of mycophenolic acid increased up to 2423 µg/kg DM, but the level of *Aspergillus* spp. decreased.

These results demonstrated that fungal species differ in their population dynamics during ensiling and feed out phases when there is exposure to air.

Although the aerobic deterioration of silage has been documented for about three decades, these results bear important implications (Wilkinson and Davies, 2013). When oxygen is introduced into the ensiled mass, aerobic microorganisms start to grow, first by respiration of soluble substrates and then it the respiration is led by other complex compounds. Usually, yeasts start this aerobic deterioration, and their consumption of sugars and production of acids enhance silage temperature and pH (Pahlow et al., 2003). The increased of pH supports the growth of bacilli and other aerobic bacteria, and silage temperature. Finally, the growth of moulds completes the deterioration of silage.

In a study on corn silage, it was found that acetic acid bacteria initiated aerobic deterioration in some cases, although the reasons for this are uncertain (Dolci et al., 2011). This deterioration takes place at different levels up to 20% of the total DM stored in silages in temperate (Borreani and Tabacco, 2010) and warm regions (Bernardes et al., 2012). These losses can reach up to 70% of the DM stored in peripheral areas and close to the side walls of bunker silos and are correlated with a decrease in digestible carbohydrates and organic acid fractions (Borreani et al., 2007; Bernardes et al., 2012), with the most digestible materials consumed first.

In addition to the direct economic loss of DM and nutrients, spoiled silage can lead to indirect losses, because the silage has a lower nutritional value and reduced palatability, thus affecting the performance and health of the animals (Kung et al., 1998). Many of these effects are correlated with the proliferation of potentially pathogenic or otherwise undesirable microorganisms (Driehuis, 2013) and with mycotoxin production (Richard et al., 2009; Cavallarin et al., 2011). Several research groups have highlighted the bearing of a uniform high-quality silage in the whole silo (Borreani and Tabacco, 2010; Muck, 2013; Wilkinson and Davies, 2013). Adding deteriorated silage from the top layers of the bunker in the total mixed ration could contaminate the ration with undesirable microorganisms, such as filamentous fungi, aerobic and anaerobic spores (Borreani et al., 2013; Dunière et al., 2013), and harmful mycotoxins (Cavallarin et al., 2011), reducing DM intake (Gerlach et al., 2013) as well as milk quantity and quality (Hoffman and Ocker, 1997).

Silage residues and microbiological risk

A recent study (Mosconi et al., 2023) investigated the effect of management practices linked to silage and total mixed ration TMR residues on a reservoir of *Clostridium tyrobutyricum* contamination during the following TMR preparation. The results showed that proper hygiene practices, such as the disposal of silage and TMR residues within the farm environment, was essential to reduce this contamination, prevent feed waste, and reduce economic losses. Although

there are no data documenting mycotoxin contamination of these residues, it should be strongly suggested that they are an important source of contamination at farm level, a topic that deserves further investigation in the future.

Production of volatile organic compounds by mycotoxigenic fungi

The VOCs are products of the main fermentative pathways and can be used to evaluate the quality of ensiled forages (Hafner et al., 2013; Sigolo et al., 2023). All fungi, including mycotoxigenic species, emit mixtures of VOCs during the different phases of ensiling (Daniel et al., 2013; Weiss, 2017). The specified molecules and concentrations produced during the conservation period, depend on the species of fungus and the environmental conditions during growth (Bennett & Inamdar, 2015). These fungal VOCs, which are blends of alcohols, aldehydes, acids, ethers, ketones, terpenes, thiols, and various derivatives, are responsible for the characteristic musty odours associated with damp indoor environments. Notably, 1700 VOCs have been identified as being emitted by plants (Dudavera et al. 2006), but only 300 VOCs have been associated with fungal metabolism, although most of the fungal VOCs appeared unique to fungi (Lemfack et al., 2014). Under laboratory conditions, individual fungal species produce a typical VOC pattern depending on the growth conditions (Bennett and Inamdar, 2015). The specific VOC profile that is produced by each species or microbial consortium depends on temperature, pH, moisture, nutrients, and age of the culture (Morath et al., 2012). Understanding the chemical composition of VOCs, their production during fermentation and their relationship to mycotoxin contamination of silage forage can help evaluate different storage procedures and the safety of the silage product (Kung et al., 2018).

Corn silage varies in nutritional and fermentative parameters. Notably, when several parameters are used to evaluate this silage, the interpretation of these data can be difficult for assessing the quality of the ensiled product (Tharangani et al., 2021). Therefore, the aggregation of parameters indicative of the overall quality of maize silage into a few fermentation-related dimensions may allow an easier interpretation of the microbiological dynamics during the ensiling and storage phases and a better evaluation of the overall silage quality. Ghilardelli et al. (2019) showed the principal component (PC) analysis can be used to grouped VOC profile in latent structures able to characterize corn silages fermentative quality and they can be used to assess silage quality and possible effects on farm performance. In particular, they used this multivariate technique to aggregate chemical and fermentative parameters with data on the main mycotoxins identifying the fermentative pathways that occurred in corn silages. To reduce the number of variables and identify the most important biological pathways, they extracted latent structures to provide a biological interpretation of the silage. The first PC was referred to the presence of

contamination from mycotoxins produced *Fusarium* spp. and *Alternaria* spp., two of the three most important toxigenic fungal genera the other being *Aspergillus* spp. that occur during the pre-harvest phase (Storm et al., 2008). The second PC was related to the presence of alcohols resulting from the degradation of phenylalanine by yeast or other aerobic spoilage microorganisms (Krizsan et al., 2007). This second PC was characterized by n-propanol and propyl acetate, which are derived from heterofermentative bacteria. Kristensen et al. (2010) described a rising of these fermentation end-products in forages treated with an obligate heterofermentative strain of *Lb. buchneri*. The fifth PC was related to mycotoxin contamination by NIV and enniatins, trichothecene mycotoxins produced by *Fusarium* spp. (Gallo et al., 2015). This preliminary study showed that the VOC profile in an ensiled mass was closely related to the presence of undesirable fungal contamination, desirable microorganisms such as LAB, and aerobic spoilage.

Near-infrared spectroscopy for rapid detection of mycotoxins in silage.

Due to the deleterious health effects of mycotoxins, rapid monitoring of animal feed contamination may help prevent economic losses and maintain animal health. Near Infra-Red Spectroscopy NIRS provides rapid feedback in farm, and measurements can be performed over time, so this technique may help to ensure the safety of cereals and silage that is consumed by animals and humans (Mallmann et al., 2020). Therefore, use of this “rapid method” has great potential for providing information about the presence of mycotoxins. However, due to the difficulty of using NIR spectroscopy to develop robust regression models for the quantification of individual mycotoxins, and because silages are usually contaminated by regulated and emerging mycotoxins, NIR spectroscopy results are currently used in qualitative models to analyze silage for determination of the concentration of all mycotoxins or different classes of mycotoxins. Notably, Ghilardelli et al. (2022) performed NIR spectroscopy calibration using a Random Forest RF model, a type of machine learning classification. The results indicated satisfactory classification of samples after applying different cut-offs to classify samples according to mycotoxin concentration ($\mu\text{g/kg DM}$) or count (n): i) total detectable mycotoxins; ii) regulated and emerging *Fusarium* toxins; iii) emerging *Fusarium* toxins; iv) fumonisins and their metabolites; and v) *Penicillium* toxins. This technique cannot directly determine the levels of different mycotoxins and fungi, but nonetheless provides important information regarding contamination level or risk.

Deactivation of mycotoxins by silage and ruminal microorganisms

The rumen effectiveness in degrading toxic compounds to less toxic metabolites has been widely investigated enhancing the aspect that intrinsic factors of rumen fluid e.g. bacteria and

yeast cell walls and feed constituents are capable in deactivating mycotoxins such as aflatoxins and zearalenone by binding (Gallo & Masoero, 2010). Fink-Gremmels (2008a) and Upadhaya et al. (2010) further characterized the effects of mycotoxins in ruminant animals. Specifically, i) dairy ruminants are fed complex diets, so mycotoxins may come from different food materials; ii) grazing animals may be exposed to different mycotoxin types than those feeding on pasture grasses; iii) the degradation and binding to mycotoxins is saturable and highly dependent on types of forage, so changes in feed composition to protein-rich concentrates may alter the ability of microorganisms in the rumen to metabolize mycotoxins; iv) the rumen converts certain mycotoxins into metabolites with similar or even greater toxicity, such as FAs and zearalenone; v) the antimicrobial activity of the various mycotoxins in the rumen and the consequent impairment of rumen function may decrease the ability of these microbes to degrade toxins vi) high-yielding cows, due to genetic breeding, are more susceptible to metabolic diseases; vii) cows in the transition period have a negative energy balance and are therefore more susceptible to mycotoxin contamination. Symptoms of mycotoxin poisoning are often unspecific and may include a reduced feed intake, feed refusal, "lack of appetite," a rough coat, poor body condition, reproductive issues, and immunosuppression (Gallo et al., 2015; Gallo et al., 2022). The lactation period, intestinal tract, and age can affect aflatoxin absorption efficiency. For instance, Coppock et al. (2018) stated that cows in early lactation may excrete 3.8-6.2% of the AFB1 dietary intake as AFM1 in the milk compared to 1.8-2.5% in late lactation; and the absorption rate of AF in duodenum, jejunum, and rumen is higher in rat and cattle than other tracts of the gut. Field investigations have outlined relationships of mycotoxin consumption with increased occurrence of problems in fresh cows, including displaced abomasum, ketosis, retained placenta, metritis, mastitis, and fatty liver (Whitlow et al., 1998). Gallo et al. (2022) summarized the results of 21 trials that examined the effects of the three major *Fusarium* mycotoxins on dairy cows. Durringer et al. (2020) and Roberts et al. (2021) have noted that beef cattle fed a diet contaminated with 1.7 mg/kg DON and 3.5 mg/kg FB tended to develop a decrease in rumen pH, body weight and crude protein digestibility, as well as alterations in immune cell chemotaxis, immunoglobulin production and cell membrane structure. In the same way, dairy cows fed diets contaminated with DON up to 0.9 mg/kg and FBs up to 1.3 mg/kg showed a decreasing trend in DM and neutral detergent fiber NDF digestibility, altered immune response, decreased milk production, and decreased quality of cheese production (Gallo et al., 2020). Moreover, in vitro studies reported a decrease in the ruminal fermentation during exposure to DON and FBs (Gallo et al., 2021). The combination of DON and ZEN in the diet of dry cows altered their leukocyte counts; in lactating cows, these contaminants altered the immune status (Kinoshita et al., 2015), decreased milk yield, and led to milk with a decreased fat

content. McKay et al. (2019) reported how a diet contaminated by 8.21 mg/kg of DON and 0.09 mg/kg of ZEA given to dairy cows for 4 weeks altered the rumen fermentation and reduced protein flow into the duodenum, and the toxic effects of ZEN and DON appeared to be synergistic.

Moreover, *in vitro* studies (Zhao et al., 2022) and *in vivo* studies (Borutova et al., 2008; Osselaere et al., 2013) outlined that DON increased oxidative stress, enhancing the cellular production of reactive oxygen species ROS and reactive nitrogen species RNS, and no counteracting of these effects by antioxidant systems or radical scavengers (Sordillo & Aitken, 2009). The effect of DON on milk nutrients were related to oxidative stress of the mammary glands and inflammation, and these cows developed decreased milk yield, casein synthesis, and milk fat content when fed a DON-contaminated diet (Lee et al., 2019).

Moreover, our research group recently evaluated the effects of contamination by several mycotoxins in the dairy cow diets at different stage of lactation. This research included *in vitro* studies and several *in vivo* trials that have been carried out at the dairy farm CERZOO s.r.l. (San Bonico, Piacenza, Italy), the experimental farm of the Catholic University of Piacenza. Gallo et al. (2020) examined 12 mid-lactation multiparous cows in a trial to evaluate the effects of a mycotoxin-deactivating feed product MDP on productive performance. Animals were divided in three experimental groups: the control group received a TMR contaminated with moderate contamination of *Fusarium* mycotoxins DON and FB; the MTX group received a TMR contaminated with slightly higher levels of *Fusarium* mycotoxins; and the MDP group received the MTX diet with a the MDP Mycofix, BIOMIN Holding GmbH, Tulln, Austria. The MTX group had 2.7% decreases in feed efficiency $P < 0.05$ and NDF digestibility $P < 0.05$ compared to the CTR and MDP groups. However, the dry matter intake DMI did not differ among the three groups. Milk yield decreased due to *Fusarium* mycotoxins, and the highest milk yield was detected in the control group $P < 0.05$. Rumination time was longer in the MTX group than in the other two groups $P < 0.05$, although we cannot explain this effect that deserve of future investigation. At the same time, mycotoxin level of diet impacted the rumen fermentation activity, being both NDF and DM digestibility decreased with higher mycotoxin contamination levels. These results led to the conclusion that *Fusarium* mycotoxins, in particular DON and FB, adversely affected diet digestibility, milk yield, and some parameters related to immune response and metabolism.

Another trial Catellani et al. (2023) was carried out at the same experimental farm investigated the effect of *Fusarium* mycotoxins on dairy cow performance over an extended period of 54 days. Cows were divided in three groups: i) the control group was fed with TMR with low contamination of DON 284.5 µg/kg DM, ZEA 43.2 µg/kg DM, and FB 129.6 µg/kg DM ; ii) the MTX group with TMR contaminated higher levels of DON 1021.0 µg/kg DM, ZEA 196.8 µg/kg

DM and FB 238.4 µg/kg DM, although these levels were below the U.S. and European Union guidelines; and iii) the MDP group was fed the MTX diet with DON 1009.6 µg/kg DM, ZEA 248.5 µg/kg DM, and FB 241.7 µg/kg DM supplemented with the MDP TOXO® XXL, Trouw Nutrition, Netherlands; 100 g/animal/day. The DMI was quite similar among the three experimental groups 26.0 kg/cow/day. The higher milk yield was detected in the MDP group 39.27 kg/cow/day, followed by control group 38.23 kg/cow/day and finally the MTX group 37.55 kg/cow/day. Analysis of milk quality showed that the milk fat concentration tended to be lower in the control group 3.52%, $P = 0.07$ than in the MTX group 3.74% and MDP group 3.80%. Thus, long-term exposure of dairy cows to moderate levels of Fusarium mycotoxins affected their performance. These negative effects may be mitigated by the dietary inclusion of an MDP.

Lastly, a recent trial of lactating dairy cows Gallo et al. (unpublished data) examined the impact of ZEN on productive and reproductive performances, especially evaluating the ovarian recovery activity after calving until 56 days in milk. The preliminary results showed that ZEN contamination impact cow performance, the number of corpora lutea in primiparous cows, the dimensions of follicular cysts, and the blood progesterone level, but these data are still being analysed.

Conclusions

This review provides a recent summary of the presence and concentrations of regulated and emerging mycotoxins in silages and other forage. We recommend that future studies provide more comprehensive and detailed data on this topic because of the limited amount of information currently available. Further, the correlation between the incidence of regulated and emerging mycotoxins with the main silage characteristics and microbial silage communities should be deeply investigated, due to relevant contribution of presence of these toxin compounds on silage safety evaluation and mycotoxin risk analysis for dairy cows. The role of different bacterial inoculants in preventing mycotoxin contamination during the different stages of ensiling appears controversial, and this intervention is still not fully supported by the scientific community. In any case, the results from several recent studies of this topic are intriguing. Testing the effects of different bacterial inoculants on mycotoxin contamination in animal feeds, especially silage, may lead to more effective methods for preventing mycotoxin ingestion by animals, and thereby safeguard their health, welfare, and performance. We also suggest that future trials examine different indicators of silage safety by measuring VOCs and mycotoxins and their relationships with animal performance and the quality of animal-derived products. Finally, to provide more information on the risk of mycotoxin contamination in silage and cattle diets and determine the effects of ingestion of regulated and emerging mycotoxins, we suggest creation of an international

network to monitor mycotoxins in silages and other forage crops worldwide. This network can document exposure and ingestion of mycotoxins by ruminants and verify the real-world effects of mycotoxin ingestion by livestock.

Ethics approval

Not applicable

Data and model availability statement

None of the data was deposited in an official repository. The data that support the mapped findings are available via the references cited.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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Marco Lapris: Conceptualisation, Writing - Review and Editing.

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Declaration of interest

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5. Improving the quality of whole-plant corn silage in the top layer of a silo by using an inoculant combination with sodium benzoate

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Abstract

The aim of this study was to determine the effect of a commercial bacterial inoculant in combination with sodium benzoate on aerobic spoilage of whole-plant corn silage (WPCS) in the upper layer of a bunker silo. More specifically, we compared control silage (CTR) that was treated with water alone and experimental silage (OS) that was inoculated with SiloSolve® OS (a combination of *Enterococcus faecium* [DSM 22502/1k20602], *Lactococcus lactis* [DSM 11037/1k2081], and *Lactiplantibacillus plantarum* [DSM 16568/1k20720]) in combination with sodium-benzoate. After harvest, 1000 kg of freshly chopped corn was divided into two equal groups (OS and CTR), followed by homogenization with a pitchfork. For each group, 12 silo-NET bags (SNBs; high density polyethylene, 27×120 cm, 200 µm mesh pores) were filled with approximately 5.5 kg of freshly chopped forage, and then buried at a depth of 0.30 m in different regions of a bunker silo (26 m long, 8.5 m wide, 3.5 m high) for 98, 142, or 217 days. The bunker silo was divided lengthwise into two equal parts, and a 2-m buffer zone separated the CTR group and the OS group. After application of each treatment, the SNBs were removed from the ensiled mass, weighed, and mixed thoroughly, followed by measurement of chemical, fermentative, and microbiological parameters. The OS group had a decreased loss of dry matter (5.3 ± 4.72 vs. 12.5 ± 4.72 g/kg DM, $P < 0.05$), greater aerobic stability (302.0 ± 64.5 vs. 225.7 ± 64.5 h, $P < 0.05$), a lower count of yeasts (2.33 ± 0.84 vs. 3.74 ± 0.84 log₁₀[CFU/g], $P < 0.05$), and a lower count of molds (2.24 ± 0.50 vs. 3.12 ± 0.50 log₁₀[CFU/g], $P < 0.05$). During the feed-out phase, the temperature of the core and top layer of the bunker and the amount of WPCS waste were determined for the two groups. The temperature was consistently lower in the OS group, and the total amount of waste (2.01% of total WPCS) was 43% lower in the OS group. We conclude that treatment of the top layer of WPCS in a bunker silo with a commercial inoculant and sodium benzoate can decrease spoilage and increase the economic value of silage.

Keywords: aerobic deterioration, *Lactiplantibacillus plantarum*, silage fermentation, silage additive, top layer spoilage

Abbreviations: ADFom, acid-detergent fiber exclusive of residual ash; ADICP, acid detergent insoluble CP; Ammonia N, Ammonia nitrogen expressed relative to total N (TN) basis; aNDFom, neutral-detergent fiber assayed with a heat stable amylase and exclusive of residual ash; AOAC, Association of Official Analytical Chemists; BEq, benzoate equivalent; CFU, colony forming units; CP, crude protein; CTR, control (WPCS treated without inoculant); DM, dry matter; EE, ether extract; GC, gas chromatography; GLM, general linear model; HDPE, high density polyethylene; HPLC, high performance liquid chromatography; LAB, lactic acid bacteria; LDPE, low-density

polyethylene; NDICP, neutral detergent insoluble CP; OS, WPCS treated with SiloSolve® OS (Chr. Hansen A/S, Hørsholm, Denmark) and sodium benzoate; PS, potassium sorbate; SB, sodium benzoate; SNB, silo-NET bag; SP, sodium propionate; TN, total N; VFA, volatile fatty acid; VOC, volatile organic compound; WPCS, whole plant corn silage

Introduction

Ensiling is a common practice used to preserve fodder for livestock, and is often preferred to haymaking because of decreased losses during the harvest and storage periods (Grant and Adesogan, 2018). The advantages of making silage from whole-plant corn rather than other crops (such as alfalfa) are that whole-plant corn silage (WPCS) has a lower buffering capacity and a higher content of water-soluble carbohydrates. In addition, the advantages of using corn for forage or grain are that it has low harvest costs, high yield per area, increased flexibility regarding harvest time, and high nutritional value (energy and fiber) for dairy cattle (Ferraretto et al., 2018).

Many studies of silage have examined the effects of different chemical and biological additives on fermentation and loss of dry matter (DM) (Muck et al., 2018; Kim et al., 2021; Soundharrajan et al., 2021). Biological additives, mainly lactic acid bacteria (LAB), are considered more suitable for silage than other additives because they lead to improved DM recovery, fermentation characteristics, and animal performance (Okoye et al., 2023). The use of an additive can be justified if it improves aerobic stability, and heterofermentative LAB can often improve the aerobic stability of silage more than homofermentative LAB (Muck et al., 2018). Previous researchers reported that treating silage with a bacterial mixture (i.e., *Lactiplantibacillus plantarum*, *Enterococcus faecium*, and *Lactococcus lactis*) improved its fermentative profile, although the aerobic stability occasionally decreased (Eisner et al., 2023; Jatkauskas et al., 2023).

An alternative approach for improving the aerobic stability of silage at feed-out is the addition of an antifungal chemical additive, such as a short-chain organic acid (Auerbach et al., 2020). Chemical additives typically have rapid effects after addition to a silo because they do not require the proliferation of a microorganism (which must also compete with epiphytic microflora) to produce antimycotic products (Da Silva and Kung, 2022). There are many commercially available chemical additives, and the common ingredients are potassium sorbate (PS), sodium propionate (SP), and sodium benzoate (SB) (Auerbach and Nadeau, 2013; Daniel et al., 2022). Adding these salts into an ensiled mass can inhibit the production of acids by yeasts and molds, thereby improving aerobic stability (Auerbach et al., 2012). A meta-analysis by Morais et al. (2017) showed that the effect of a chemical additive on increasing the aerobic stability of a grain silage was related to its antimycotic activity. Additional evidence demonstrated that the use of SB with other products (e.g., sodium nitrite and hexamine) improved the fermentation and aerobic stability of grass silages (Lingvall and Lättemäe, 1999; Conaghan et al., 2010).

The LAB activity during ensiling can be affected by several factors, such as DM at harvest and silage temperature. Therefore, the purpose of the present study was to assess the effects of a combination of SB with a select mixture of LAB on the fermentation profile, DM loss, counts of

yeasts and molds, and aerobic deterioration of the top layer of WPCS in a bunker silo. Our hypothesis was that the addition of SB with a mixture of LAB leads to improved aerobic stability. A secondary purpose was to evaluate the efficacy of a bacterial mixture with SB on the amount of waste and temperature of the ensiled WPCS mass during the feed-out phase.

Materials and methods

Silage preparation and treatments

Whole-plant corn hybrid Dekalb 7023 (Bayer Crop Science, Leverkusen, Germany) was harvested at the Centro Ricerche Zootecniche (CERZOO S.r.l, San Bonico, Italy) on August 10, 2022 when the kernel maturity stage was two-thirds of the milk line. The harvest was then chopped to an 8 mm theoretical length with a self-propelled forage harvester (John Deere 7780, John Deere, USA) that had a pick-up mechanical kernel processor roll (roll gap: 2.3 mm, model 375plus).

During harvest, approximately 1000 kg of freshly chopped forage was collected from the experimental field (45°00' 21.2"N 9°42' 20.8" E). The freshly chopped mass was divided into two equal amounts, which were then homogenized with a pitchfork, and individually sampled to determine the pre-ensiling characteristics. The two ensiling treatments were: CTR, control treatment with water alone; and OS, consisting of SiloSolve® OS (Chr. Hansen A/S, Hørsholm, Denmark; a combination of *Enterococcus faecium* [DSM 22502/1k20602], *Lactococcus lactis* [DSM 11037/1k2081], and *Lactiplantibacillus plantarum* [DSM 16568/1k20720]) with SB.

For each group (CTR and OS), 12 silo-NET bags (SNBs, consisting of high-density polyethylene [HDPE], size: 27×120 cm, pore mesh: 200 µm) were filled with approximately 5.50 kg of freshly chopped forage. Then, four SNBs of each group were buried in three different zones in the upper 0.30 m layer of the bunker silo (Figure 5.1). The bunker silo (26 m long, 8.5 m wide, 3.5 m high) was divided into 2 lengthwise regions (left: CTR, right: OS) with a buffer zone of 2 m between the groups. The ensiling durations were 98, 142, and 217 days. To characterize the pre-ensiled chopped whole plant corn, 3 samples from each group were collected for measurements of chemicals, pH, and microbiologic counts (Table 5.1).

Bunker staging

The sidewalls of the bunker were covered with a film protection consisting of low-density polyethylene (LDPE). After the filling and packing operations, 100 m² of the surface was sprayed with 25 L of the inoculum solution (according to the manufacturer's guidelines) in combination with SB or with 25 L of water. The ensiled mass was then covered using a double barrier (2 in 1) film. To keep the plastic film weighted down, 350 SNBs were filled with gravel, as an alternative method to sand or tiles, to facilitate the feed-out phase and prevent silage contamination.

Preparation of SNBs

The 25 L solution for SNBs in the OS group was prepared by diluting 1 g of the bacterial mix in 100 mL of water to reach a concentration of 1.5×10^5 CFU/g of forage, and this was added to 200 g of SB that was diluted in 1.9 L of water. The same amount of water was used for the SNBs in the CTR group.

Preparation of samples and recovery of SNBs

The bunker was opened after 39 days, removed after 98, 142, 217 days following the feed-out phase operation, weighed, mixed thoroughly, and representative samples (c.a. 2 kg) were collected. Each sample was then divided into 2 homogeneous subsamples (c.a. 1 kg each). One subsample was dried and used for chemical analysis, and the other subsample was stored at 4 °C prior to measurements of pH, lactic acid, ethanol, volatile fatty acids (VFA), ammonia N, microbiological counts (Gallo et al., 2021), and volatile organic compounds (VOCs) that were grouped as aldehydes, alcohols, ketones, and esters (Sigolo et al., 2023).

Chemical, biological, and fermentative parameters

For chemical analysis, each subsample was dried at 60 °C in a forced air oven for 48 h, milled, passed through a 1-mm screen (Thomas-Wiley, Arthur H. Thomas Co., Philadelphia, PA, USA), and then stored prior to analysis. The DM was determined as the gravimetric loss of residual free water after heating at 105 °C for 3 h (Association of Official Analytical Chemists [AOAC], 1995; method 945.15), with correction for volatile losses during oven drying using the equations of NorFor (2011). Ash was determined as the gravimetric residue after incineration at 550 °C for 2 h (AOAC, 1995; method 942.05); ether extract (EE) was determined using AOAC method 920.29; and crude protein (CP; $N \times 6.25$) was determined using the Kjeldahl method (AOAC, 1995; method 984.13). The soluble fraction of CP (expressed on a DM basis) was determined according to Licitra et al. (1996). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin (sulfuric acid [sa]) were sequentially determined using the Ankom II Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA), as described by Mertens et al. (2002) for aNDFom, and as described by Van Soest (1973) for ADFom and lignin (sa). The neutral detergent (ND) solution contained sodium sulfite and a heat-stable amylase (activity: 17.400 Liquefon-U/mL, Ankom Technology; Mertens, 2020). All fiber fractions were corrected for residual ash. Starch was determined by polarimetry (Polax 2 L, Atago®, Tokyo, Japan). Free sugars were determined following extraction in a phosphate buffer and acetonitrile, and quantification was performed using a high-performance liquid chromatography system (HPLC; Jasco, Tokyo, Japan) that had an LU-980 pump and an RI-2031 refractive index detector (Gallo et al., 2016).

To quantify volatile organic acids, lactic acid, ethanol and other alcohols, aldehydes, esters, and ketones, a wet sample (c.a. 50 g) was extracted using a Stomacher blender (Seward Ltd., West Sussex, Worthing, UK) for 3 min in distilled water (water:sample fresh weight ratio of 3:1) as

previously described (Sigolo et al., 2023). The mixture was filtered through gauze, and an aliquot was used to measure pH. To remove solid impurities, the mixtures were centrifuged at 4500 g for 15 min at room temperature, and the liquid phase was retained for determination of volatile fatty acids (VFAs) and volatile organic compounds (VOCs) using a gas chromatography (GC) system equipped with a flame ionization detector. The GC parameters and procedures used for identification and quantification of VFAs and VOCs were described by Sigolo et al. (2023). Lactic acid was determined using HPLC (Carvalho et al., 2012); when a compound was undetectable, its detection limit was reported as described in Sigolo et al. (2023). Ammonia nitrogen (ammonia N) was determined in a fresh sample (c.a. 20 g) in a slurry mix consisting of 150 mL of distilled water and magnesium oxide (10 g/sample; NorFor, 2011). The ammonia N concentration was expressed on a total N (TN) basis, and was determined after steam distillation using the Kjeldahl method (as above).

Determination of aerobic stability

The aerobic stability of silage was determined according to Ranjit and Kung (2000) using samples (c.a. 3 kg) that were placed in a 20 L bucket and allowed to undergo aerobic deterioration at room temperature (20.2 ± 0.82 °C). During this test, the temperature of the room and silage mass were recorded every 2 h using a data logger (Microlite, Dostmann Electronic GmbH, Wertheim, Germany) for 14 days. An ensiled mass was considered aerobically unstable when the difference between the room temperature and silage temperature was 2 °C or more.

Microbiological analysis

Microbial counts were determined as reported by Gallo et al. (2018). A sample (c.a. 30 g) of silage from a SNB was transferred into a sterile homogenization bag, suspended (1:10, w/v) in a peptone physiological salt solution (1 g/L of neutralized bacteriological peptone and 9 g/L of sodium chloride), and homogenized for 3 min in a Stomacher blender. Serial dilutions were then prepared. The numbers of LAB, yeasts, and molds were determined using a pour-plate technique with De Man, Rogosa and Sharpe (MRS) agar medium (LAB; Oxoid, Basingstoke, UK) or 40 g/L of rose-bengal chloramphenicol agar (yeasts and molds; Oxoid, Basingstoke, UK). The plates were incubated at 25 °C for 3 days (LAB and yeasts) or 5 days (molds). The colony forming units (CFUs) were enumerated separately, according to their macro-morphological features. Mean CFU values of duplicate subsamples were recorded for total yeasts and molds on plates that had 10 to 100 CFUs.

Statistical analysis

The levels of LAB, yeasts, and molds were log₁₀-transformed prior to statistical analysis, and the terms of the ANOVA (standard errors of the means and P values) refer to these log₁₀-transformed values.

Data were analyzed according to a completely randomized design, within a 2 × 3 factorial arrangement of treatments and using the general linear model (GLM) procedure of SAS Institute Inc. (2003):

$$Y_{ijk} = \mu + T_i + D_j + (T \times D)_{ij} + e_{ijk}$$

where Y_{ijk} is the dependent variable; μ is the overall mean; T_i is the fixed effect of treatments ($i = 2$); D_j is the fixed effect of ensiling duration ($j = 98, 142, \text{ or } 217$ days); the $(T \times D)_{ij}$ term is the first order interaction; and e_{ijk} is the residual error. A multiple comparison t-test was used to compare least square means to determine if a main effect or first order interaction was significant ($P < 0.05$). Figures were used to show effects of ensiling duration when interactions were statistically significant and biologically relevant.

Results

Effect of treatment and ensiling duration on chemical parameters

We first determined the characteristics of the freshly chopped corn before ensiling (Table 5.1), and then compared the effect of treatment (OS or CTR), ensiling duration (98, 142, or 217 days), and their interactions on the chemical characteristics of the WPCS (Table 5.2). The results show that treatment had significant effects on the levels of ash, CP, soluble CP, and starch. In particular, the CTR group had greater levels of ash (55.5 ± 2.03 vs. 52.9 ± 2.03 g/kg DM), CP (84.6 ± 2.43 vs. 81.3 ± 2.43 g/kg DM), and soluble CP (31.3 ± 2.16 vs. 28.4 ± 2.16 g/kg DM), and the OS group had a greater level of starch (269.0 ± 15.8 vs. 253.0 ± 15.8 g/kg DM). There were no significant effects of ensiling duration or the interaction of treatment with ensiling duration on any of these chemical parameters, including NDICP, ADICP, aNDFom, ADFom, lignin(sa), ether extract, and sugars.

Effect of treatment and ensiling duration on fermentative and microbiological parameters

We also determined the effect of treatment and ensiling duration on fermentative parameters (Table 5.3). Although the two groups had no significant differences in pH, treatment had a significant effect on the levels of many key fermentative parameters, including ammonia N, 1,2-propanediol, lactic acid, iso-valeric acid, iso-butyric acid, total esters, total glycols, lactic acid/acetic acid ratio, LAB, and dry matter content. Importantly, the OS group had greater levels of ammonia-N (18.6 ± 0.48 vs. 17.9 ± 0.48 g/kg TN) and 1,2-propanediol (12.9 ± 2.65 vs. 7.67 ± 2.65 g/kg DM), and the CTR group had a greater level of lactic acid (23.9 ± 5.97 vs. 18.0 ± 5.97 g/kg DM). The OS group had a greater level of acetic acid (29.8 ± 6.82 vs. 28.9 ± 6.82 g/kg DM) and the CTR group had a greater level of butyric acid (0.11 ± 0.06 vs. 0.09 ± 0.06 g/kg DM), but these differences were not statistically significant. We only detected iso-valerianic acid in the CTR group (36.0 ± 19.0 mg/kg DM) and the CTR group also had a significantly greater level of iso-butyric acid (30.0 ± 15.3

vs. 10.0 ± 15.3 mg/kg DM). The OS group had significantly more total esters (84.0 vs. 61.0 mg/kg DM), total glycols (13.3 vs. 8.23 mg/kg DM), ratio of lactic acid/ acetic acid (0.83 ± 0.1 vs. 0.61 ± 0.1), number of LAB (7.17 ± 1.06 vs. 5.94 ± 1.06 log₁₀ CFU/g), and DM losses (12.5 ± 4.72 vs. 5.3 ± 4.72 g/kg DM).

Ensiling duration had significant effects on multiple parameters, including total esters and dry matter loss. More specifically, the total ester content was greater at 217 days than at 98 and 142 days (99.0 ± 22.0 vs. 35.0 ± 22.0 and 83.0 ± 22.0 mg/kg DM) and the DM losses were greater at 217 days than at 98 and 142 days (11.2 ± 4.72 vs. 4.7 ± 4.72 and 10.8 ± 4.72 g/kg DM).

Analysis of the interaction of treatment with ensiling duration also showed significant effects on many parameters, including lactic acid, ethanol, iso-butyric acid, lactic acid/ acetic acid plus ethanol ratio, total alcohols, aerobic stability, yeasts, and molds (Fig. 5.2). The lactic acid content was slightly higher in the OS group at 98 days, but steadily decreased over time; in contrast, the lactic acid content in the CTR group increased over time and reached a peak at 217 days (Fig. 5.2). The OS group had an increasing level of ethanol over time, with a peak at 217 days; the CTR group also had an increasing level of ethanol, but lower levels at all three times. The CTR group had higher levels of iso-butyric acid at 98 and 142 days, but a lower level at 217 days. In both groups, the lactic acid/ acetic acid + ethanol ratio increased slightly over time, and the highest ratio was at 217 days in the CTR group. The total alcohols also increased over time in both groups, and these levels were greater at all times in the OS group. The aerobic stability increased over time in both groups, but was greater in the OS group at 98 days and very similar in the two groups at 142 and 217 days. Analysis of the counts of yeasts and molds showed that the CTR group had greater levels at 98 days, but the two groups had similar levels at 142 and 217 days.

Silage temperature and amount of waste during feedout

We measured the temperature of silage during feed-out at a depth of 0.30 m for the OS and CTR groups, in the middle of the bunker (core) at the same depth, and outside of the silo (environment) from October 24, 2022 to March 13, 2023 (Figure 5.3). The temperature of the CTR group was an average of 14.2 °C higher than that of the OS group, indicating the CTR group had greater spoilage.

Finally, we measured the weight of total silage waste in each group (Fig. 5.4). Considering the large volume of the bunker silo (773.5 m³), the staff of the experimental facility used a truck loader to remove spoiled silage from the top layer of each side of the bunker, and we then recorded the weights. The total amount of disposed silage was 9.4 metric tons and the OS group (3.4 metric tons) had 43% less waste than the CTR group (6.0 metric tons). Considering the total

amount of stored silage in the experimental bunker (460 metric tons), the total amount of disposed silage accounted for approximately 2.01% of the total.

Discussion

Preventing aerobic spoilage is one of the main challenges for silage producers. Two common management practices before ensiling can prevent aerobic spoilage: (i) harvesting crops at the correct DM level and particle length to optimize the bulk density and (ii) decreasing the porosity of the ensiled mass (Bernardes et al., 2018). Notably, air penetration within a silo mainly occurs in the periphery, a zone in which it is difficult to maintain proper compaction. Indeed, low compaction in these areas is linked to an increased silage temperature and spoilage during the storage and feed-out phases due to the production of mycotoxins (Gallo et al., 2016). Many silage producers now use additives to improve fermentative parameters and prevent the loss of nutrients (Muck et al., 2018).

Effect of the additive solution on chemical parameters

Silage inoculation with homofermentative strains is a widely applied procedure because it allows these bacteria to dominate during the first ensiling phase and minimizes DM losses (Wilkinson et al., 2003). In fact, our OS group had lower DM losses, and our CTR group had a higher CP concentration. Chilson et al. (2016) studied a combination of 4 homofermentative LAB and 4 fibrolytic enzymes in laboratory conditions and reported similar results. However, some bacteria (e.g., *Lactococcus*, which also occurs in SiloSolve® OS) have proteolytic activity and can hydrolyze peptides (Rooke and Hatfield, 2003). This might explain our results, although the contribution of LAB to proteolysis is minor compared to plant proteolysis during ensiling (Rooke and Hatfield, 2003). On the other hand, our addition of SB might have affected the level of CP (Yardimci et al., 2022). Our observation that soluble CP was higher in CTR group during ensiling requires further investigation to identify the responsible enzymes. Moreover, the quality of the WPCS we used in the current study was not compromised. A previous study showed that increased proteolytic activity in a silo led to increased levels of soluble CP and ammonia-N (Young et al., 2012), but this did not occur in our study, possibly because WPCS undergoes less proteolysis than legume silage (Rooke and Hatfield, 2003). The antimicrobial activity of SB might decrease proteolysis, which mainly occurs during fermentation by silage bacteria (Da Silva et al., 2015; Junges et al., 2017). Santos et al. (2019) observed a reduction of soluble CP and ammonia N in rehydrated sorghum grain silage that was treated with SB and developed for use as dairy cow feed. Although these authors did not report the CP content of the silage, it was probably lower. It has been speculated that the rapid acidification process of treated silage allows solubilization of nitrogen components, in particular the soluble fractions (Carpintero et al., 1979). We also found

that the ammonia-N level was higher in the OS group. Another study reported similar results in corn silage harvested at 2/3 milk-line with a combination of inoculants in comparison with an untreated group (Johnson et al., 2003). Besides, the levels of ammonia N detected in the present study were below the levels previously reported (Daniel et al., 2018; Saylor et al., 2020). Gallo et al. (2016) observed a similar decrease in soluble CP and ammonia N in the peripheral region of silage. Future studies are needed to investigate this phenomenon. Regardless, the quality of our final product was not compromised despite the greater level of soluble CP in our OS group.

Effect of the additive solution on fermentative and microbiological parameters

The pH values were lower in the OS group (3.87) than in the CTR group (4.25), although this difference was not statistically significant. Nonetheless, the pH level in the OS group was close to the optimum level reported by Kung et al. (2018a), suggesting that this group started fermentation earlier than the CTR group due to LAB activity (Li et al., 2022). We also observed that the OS group had a higher level of lactic acid at 98 days, although the CTR group had higher levels at 142 and 217 days. The decreasing level of lactic acid in the OS group might be because some strains of LAB can utilize lactic acid in anaerobic conditions when glucose is limiting (Lindgren et al., 1990). On the other hand, the addition of SB might inhibit the activity or growth of some microbes and lead to a shift of fermentation pathway (Guo et al., 2021).

The OS group had a higher concentration of acetic acid than CTR group, and this level increased over time. This can occur in silage inoculated with heterofermentative LAB that produce other fermented-end products, such as acetic acid, ethanol, and CO₂ (Muck et al., 2018). However, the additive solution in the present study only contained homofermentative LAB, and the production of organic acids by these LAB is related to the amount of sugars in the fresh crop and the LAB strain (Kim et al., 2021). Indeed, a shift from homolactic to heterolactic fermentation may occur under sugar-deficient conditions (Li et al., 2018). Some LAB species, such as *Enterococcus faecium* and *Lactococcus lactis*, are homofermentative and have a limited capacity to use different carbohydrates for growth (Kleerebezem et al., 2020; Li et al., 2018). For instance, the lack of appropriate nutrients may lead to the conversion of pyruvate into ethanol and acetate (Růžicková et al., 2020). Although SB inhibits undesirable microflora due to the release of benzoic acid during ensiling, benzoic acid can also cross the microbial cell membrane in its non-ionic form and acidify the cytosol, causing protein denaturation and interfering with cellular activity (Sabel et al., 2017; Santos et al., 2019). Conversely, some bacteria, such as *Lactiplantibacillus plantarum* (which was present in our additive), can proliferate in diverse environments and are considered to be “nomadic” (Martino et al., 2016). Notably, in low glucose environments or in presence of pentoses, *Lactiplantibacillus plantarum* undergoes a mixed acid fermentation to produce lactic acid, acetic

acid, and ethanol (Martino et al., 2016). Indeed, our OS group had higher levels of 1,2 propanediol and ethanol, so this could be explained by the activity of this species (Kung et al., 2018b).

A shift in fermentative pathways was reported by Jatkauskas and Vrotniakienė (2011), who tested the same additive solution used in the present study on a mixture of red clover and perennial ryegrass ensiled in micro-silos. These authors observed that treatment with homofermentative LAB and SB shifted the fermentation toward acetate and increased aerobic stability without compromising the quality of the silage. Our OS group had lower levels or undetectable levels of branched-chain fatty acids (iso-butyric, iso-valeric, and valeric acids), suggesting there was no significant clostridial fermentation or protein degradation during the ensiling. Prevention of clostridial fermentation is crucial for preserving silage because these bacteria can produce undesirable rancid odors, ammonia, and amines (Li et al., 2020).

Our measurements of VOCs indicated higher levels of total alcohols and esters in the OS group. The production of total alcohols in silage may be related to amino acid catabolism by LAB (Hafner et al., 2013; Sigolo et al., 2023). On the other hand, the accumulation of esters can be attributable to biochemical production in silage, and this depends on DM, additives, and other variables (Sigolo et al., 2023). Notably, some LAB have enzymes, such as acyltransferase and esterase, that can produce esters from carboxylic acids and alcohols (Liu and Siezen, 2006). Moreover, some strains of *Lactiplantibacillus plantarum* can produce VOCs, such as alcohols and esters, that may play a subsidiary role in the inhibition of certain fungi, such as *Penicillium roqueforti*, *Aspergillus flavus*, and *Fusarium verticillioides* (Li et al., 2023).

We investigated the effect of inoculants in combination with SB, a chemical that is known to have an antimycotic effect and to improve aerobic stability of silage (Auerbach et al., 2012). The antimycotic effects of SB may therefore have delayed heat production within the silo (Da Silva et al., 2015). Indeed, the counts for yeasts and molds were lower in our OS group. Auerbach and Nadeau (2013) defined the benzoate equivalent (BEq) concept for comparing the composition of chemical products based on active ingredients and application rate. Daniel et al. (2022) also used the BEq concept in their studies of the effects of PS, SB, and SP in rehydrated corn grain silage, and concluded that the application of these additives improved the aerobic stability. Our results also showed that aerobic stability was greater in the OS group, and similar results were reported by Jatkauskas and Vrotniakienė (2011). This outcome may be because of the direct inhibition of lactate-assimilating yeasts, which are usually the first organisms to appear during aerobic deterioration after exposure to air, and these yeasts are responsible for an increase of silage temperature and production of CO₂ (Borreani et al., 2013; Da Silva et al., 2015; Li and Nishino, 2011).

Effect of the additive solution on silage temperature and amount of waste

Our data on silage mass temperature and waste over many months may have practical applications for measurement of aerobic deterioration of silage at farm level, because the temperature of a bunker silo can be easily measured using a thermic probe. Notably, our measurements of silo temperature provided insights about the environmental variables that can affect the temperature during ensiling. Indeed, as stated in the SNPA report 36/2023, 2022 was the warmest year in Italy since 1961 (SNPA, 2023). Importantly, our OS group had a much lower temperature than during the period of October 24, 2022 to March 13, 2023. High temperatures may compromise the digestibility of corn silage, reduce the starch concentration, increase lignin and fiber deposition, and cause a shift from homofermentative to a heterofermentative microflora (Bernardes et al., 2018).

Conclusions

The use of silage additives is a common practice because it promotes fermentation and improves the resulting nutritional quality of silage. Farmers are usually reluctant to treat crops that are to be ensiled, such as corn. However, a treatment consisting of chemical and biological additives that is added to the top layer of a bunker silo may allow farmers to take advantage of the combined effects of two different additives, minimize the time needed for application of the additive, decrease the amount of additive required, and consequently decrease the cost of ensiling. The present study showed that applying a combination of SB with a mixture of homofermentative LAB to the top layer of an experimental bunker with WPCS prevented aerobic deterioration. Even though our OS group had a lower level of lactic acid, it had a better microbiological profile and better aerobic stability than the CTR group. Indeed, the low yeast count in our OS group was due to the application of SB, which increased aerobic stability because of its antimycotic properties. In addition, our application of the combined solution decreased DM losses from the top layer. However, the data for silage temperature were only descriptive, and further studies are needed to determine how these data apply to economic losses in livestock. Nonetheless, the application of an inoculant in combination with SB to the top layer of silage may be considered a possible solution to reduce spoilage during the feed-out phase.

CRedit authorship contribution statement

Carmelo Mastroeni: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – Original Draft. **Erica Fiorbelli:** Methodology, Investigation, Writing – review & editing. **Samantha Sigolo:** Methodology, Investigation, Writing – review & editing. **Valentina Novara:** Methodology, Investigation, Writing – review & editing. **Eliana Carboni:** Methodology,

Investigation, Writing – review & editing. **Ivan Eisner:** Conceptualization, Investigation, Writing – review & editing. **Paolo Fantinati:** Conceptualization, Investigation, Writing – review & editing. **Antonio Gallo:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing- Original Draft, Supervision, Project administration, Funding acquisition.

Conflict of interest

The authors, Ivan Eisner and Paolo Fantinati, are employees at Chr Hansen A/S and they played no role in collection, analysis and interpretation of data. All authors declare no potential conflicts of interest.

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Table 5.1. Pre-ensiling conditions of corn (n = 6 samples)

Items ¹	Units of measurement	Mean	SD
Dry matter	g/kg as fed	355.6	19.7
Ash	g/kg DM	41.9	1.0
CP	g/kg DM	74.9	1.8
NDICP	g/kg DM	14.8	0.5
ADICP	g/kg DM	8.5	0.2
Soluble CP	g/kg DM	36.0	0.9
aNDFom	g/kg DM	427.5	6.7
ADFom	g/kg DM	277.0	7.1
Lignin(sa)	g/kg DM	31.2	0.8
Ether extract	g/kg DM	21.4	0.7
Starch	g/kg DM	252.2	10.4
Sugars	g/kg DM	18.2	0.6
pH	dmnl	5.58	0.14
LAB	log10 cfu/g	3.64	0.20
Mold	log10 cfu/g	4.22	0.89
Yeast	log10 cfu/g	3.76	0.87

From each freshly chopped masses 3 samples were collected to characterize the pre-ensiling conditions of corn. cfu, colony forming units; dmnl, dimensionless

¹CP, crude protein; DM, dry matter; NDICP, neutral detergent insoluble CP; ADICP, acid detergent insoluble CP; aNDFom, NDF assayed with sodium sulphite and a heat stable amylase and expressed exclusive of residual ash; ADFom, ADF expressed exclusive of residual ash; Lignin(sa), Lignin determined by solubilization of cellulose with sulphuric acid; LAB, Lactic Acid Bacteria.

Table 5.2. Effect of treatments (CTR and SiloSolve® OS) and ensiling duration (98, 142, and 217 days) on chemical parameters of whole-plant corn silage in the upper 0.30 m layer of the bunker silo

Items	Units of measurement	Treatment ¹		Duration (days) ²			SEM	Effect of the model ³		
		CTR	OS	98	142	217		Treatment	Duration	Treatment x Duration
Dry matter	g/kg as fed	303.3	314.6	301.4	316.9	308.5	19.4	0.17	0.30	0.21
Ash	g/kg DM	55.5	52.9	55.2	53.0	54.4	2.03	<0.05	0.11	0.48
CP	g/kg DM	84.6	81.3	84.1	82.2	82.6	2.43	<0.05	0.30	0.28
NDICP	g/kg DM	15.7	15.2	16.3	15.5	14.5	1.95	0.51	0.21	0.38
ADICP	g/kg DM	11.1	11.4	11.8	11.2	10.8	0.85	0.29	0.09	0.81
Soluble CP	g/kg DM	31.3	28.4	29.3	30.1	30.3	2.16	<0.05	0.62	0.10
aNDFom	g/kg DM	447	436	449.3	440.4	434.4	20.71	0.21	0.37	0.59
ADFom	g/kg DM	285	277	288	279.9	276.4	15.86	0.26	0.35	0.62
Lignin(sa)	g/kg DM	38.1	40.2	40.4	38.7	38.3	2.49	0.06	0.24	0.57
Ether extract	g/kg DM	25.4	24.6	24.7	25.4	25	0.98	0.06	0.45	0.48
Starch	g/kg DM	253.0	269.0	257.6	264.3	261.9	15.8	<0.05	0.70	0.77
Sugars	g/kg DM	10.4	10.5	10.1	10.0	11.2	1.28	0.93	0.14	0.60

CP, crude protein; NDICP, neutral detergent insoluble CP; ADICP, acid detergent insoluble CP; aNDFom, NDF assayed with sodium sulphite and a heat stable amylase and expressed exclusive of residual ash; ADFom, ADF expressed exclusive of residual ash; Lignin(sa), Lignin determined by solubilization of cellulose with sulphuric acid.

¹ CTR, control; OS, silage treated with SiloSolve® OS [*Enterococcus faecium* (DSM 22502/1k20602), *Lactococcus lactis* (DSM 11037/1k2081), *Lactiplantibacillus plantarum* (DSM 16568/1k20720)] plus Sodium benzoate (SB).

² Duration of ensiling period in days.

³ A p-value of < 0.05 was considered statistically significant.

Table 5.3. Effect of treatments (CTR and SiloSolve® OS) and duration (98, 142, and 217 days) on fermentative parameters of whole-plant corn silage in the upper 0.30 m layer of the bunker silo

Items	Units of measurement	Treatment ¹		Duration (days) ²			SEM	Effect of the model ³		
		CTR	OS	98	142	217		Treatment	Duration	Treatment x Duration
pH	dmnl	4.25	3.87	4.42	3.89	3.88	0.74	0.23	0.27	0.30
Ammonia N	g/kg TN	17.9	18.6	18.0	18.4	18.3	0.48	<0.05	0.31	0.26
Ethanol	g/kg DM	4.7	7.0	3.4	5.8	8.2	1.27	<0.05	<0.05	<0.05
1,2 propanediol	g/kg DM	7.67	12.9	9.4	10.4	11.0	2.65	<0.05	0.49	0.64
Acetic acid	g/kg DM	28.9	29.8	26.5	29.9	31.6	6.82	0.74	0.34	<0.05
Propionic acid	g/kg DM	1.0	0.8	0.7	0.9	1.1	0.3	0.13	0.17	<0.05
Iso-butyric acid	mg/kg DM	30.0	10.0	28.0	19.0	15.0	15.3	<0.05	0.24	<0.05
Butyric acid	g/kg DM	0.11	0.09	0.13	0.07	0.1	0.06	0.47	0.16	0.71
Iso-valerianic acid	mg/kg DM	36.0	n.d.	28.0	20.0	5.0	19.0	<0.05	0.07	0.07
Valerianic acid	mg/kg DM	1.2	n.d.	1.8	n.d.	n.d.	3.0	0.33	0.38	0.38
Lactic acid	g/kg DM	23.9	18.0	19.7	19.9	23.2	5.97	<0.05	0.43	<0.05
Total aldehydes	g/kg DM	0.20	0.10	0.16	0.16	0.13	0.25	0.34	0.95	0.78
Total alcohols	g/kg DM	5.93	8.94	4.47	7.38	10.4	1.63	<0.05	<0.05	<0.05
Total ketons	mg/kg DM	29.0	37.0	46.0	31.0	22.0	42.0	0.63	0.54	<0.05
Total esters	mg/kg DM	61.0	84.0	35.0 ^b	83.0 ^{ab}	99.0 ^a	22.0	<0.05	<0.05	0.28
Total glycols	g/kg DM	8.23	13.3	9.6	10.9	11.7	2.64	<0.05	0.28	0.53
Lactic/acetic	dmnl	0.83	0.61	0.77	0.66	0.72	0.1	<0.05	0.12	0.05
Lactic/acetic+ethanol	dmnl	1.3	1.31	1.12	1.24	1.55	0.12	0.91	<0.05	<0.05
LAB	log10 cfu/g	7.17	5.94	6.63	5.98	7.05	1.06	<0.05	0.15	0.05
Mold	log10 cfu/g	3.12	2.24	3.8	2.0	2.24	0.50	<0.05	<0.05	<0.05
Yeasts	log10 cfu/g	3.74	2.33	4.48	2.0	2.64	0.84	<0.05	<0.05	<0.05
DM losses	g/kg DM	12.5	5.3	4.7 ^b	10.8 ^{ab}	11.2 ^a	4.72	<0.05	<0.05	0.62
Aerobic stability	hour	225.7	302	119.5	336	336	64.5	<0.05	<0.05	<0.05

Ammonia N, Ammonia nitrogen expressed on a total N (TN) basis; cfu, colony forming units; DM losses, dry matter losses; LAB, Lactic Acid Bacteria.

n.d., no detected; dmnl, dimensionless

Superscripts indicate that the specific mean is lower (i.e., b) or higher (i.e., a) than the different ensiling length at a P <0.05, as evaluated with the multiple comparison t-test.

¹ CTR, control; OS, silage treated with SiloSolve® OS [*Enterococcus faecium* (DSM 22502/1k20602), *Lactococcus lactis* (DSM 11037/1k2081), *Lactiplantibacillus plantarum* (DSM 16568/1k20720)] plus Sodium benzoate (SB).

² Duration of ensiling period in days.

³ A p-value of < 0.05 was considered statistically significant.

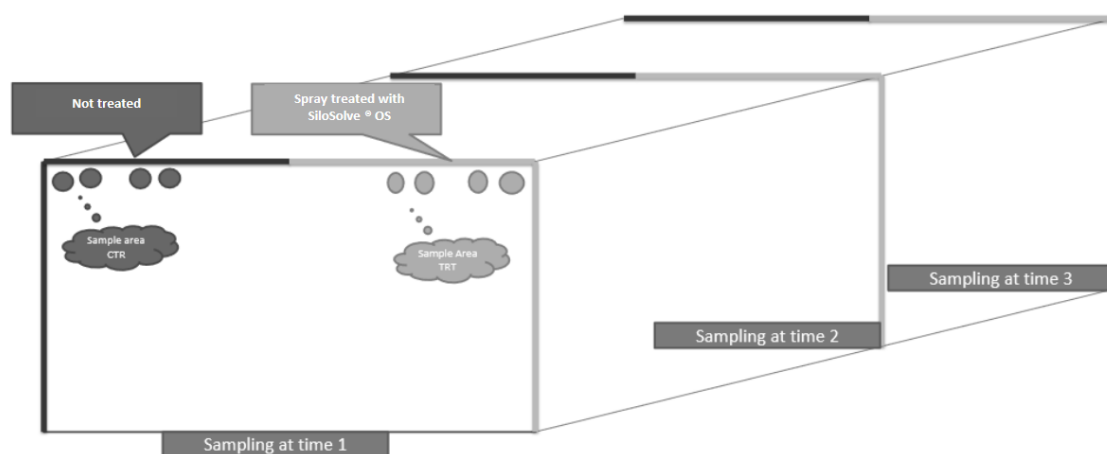


Figure 5.1 - Silo-NET bags disposition in the upper 0.30 m layer of the bunker silo

Graphs: Treatment * Duration

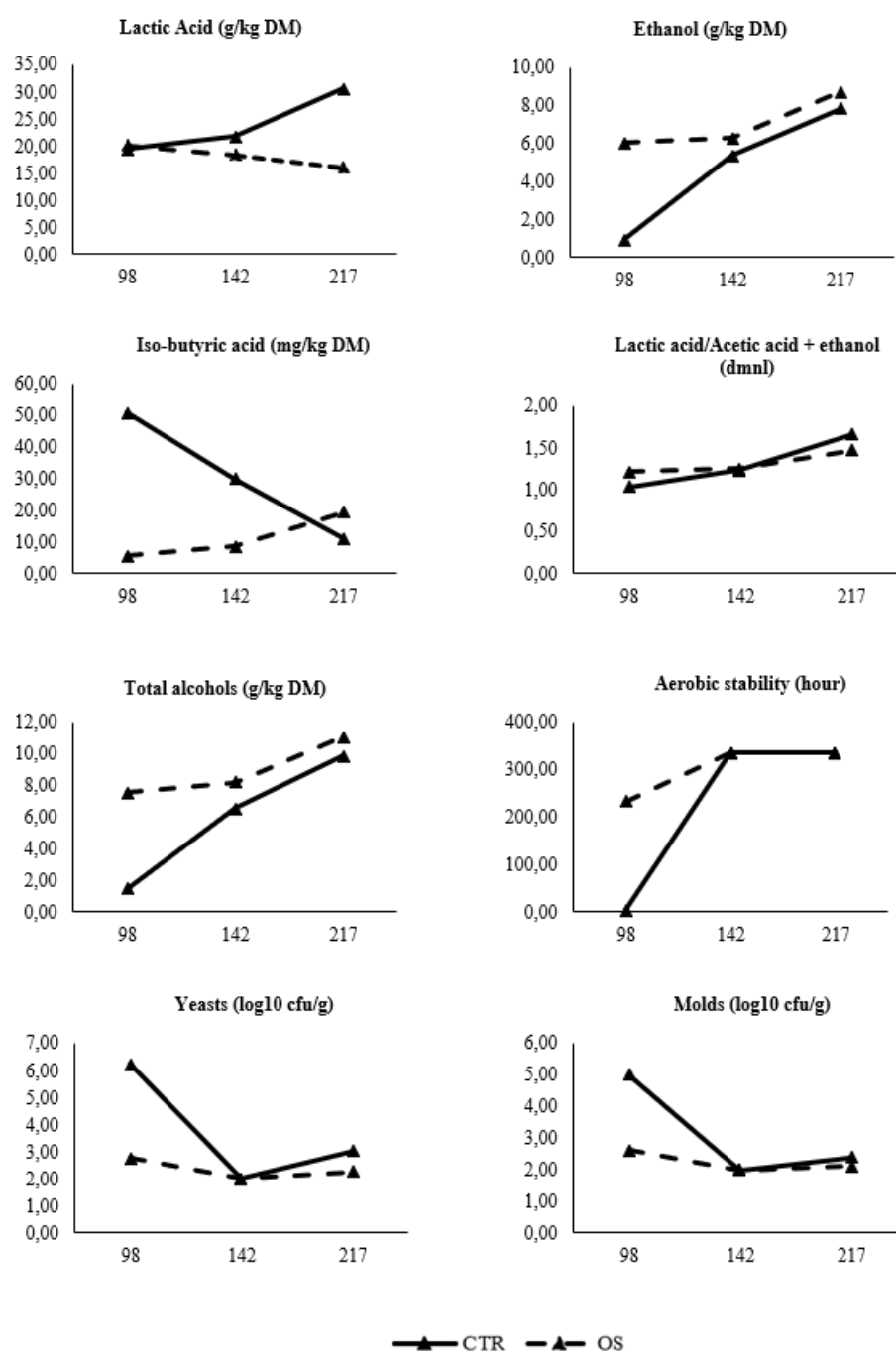


Figure 5.2 - Effect of treatments (CTR and SiloSolve® OS) and duration (98, 142, and 217 days) of whole-plant corn silage in the upper 0.30 m layer of the bunker silo on lactic acid, ethanol, iso-butyric acid, lactic acid/acetic acid + ethanol, total alcohols, aerobic stability, yeasts, and molds (Treatments x Duration interaction significant at $P < 0.05$; CTR and SiloSolve® OS)

Silage temperature and waste amounts

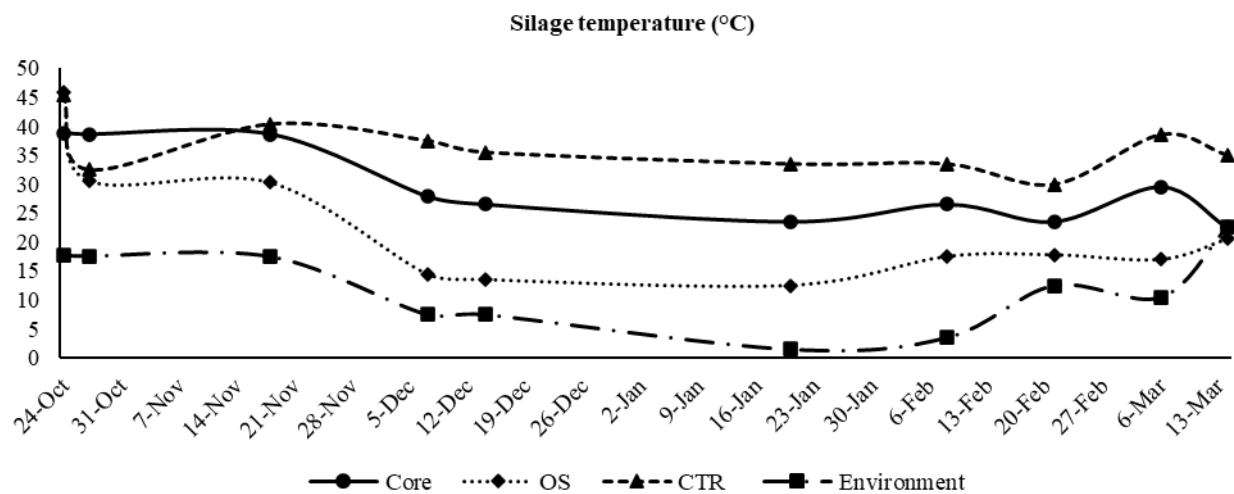


Figure 5.3 - Temperature trends measured among the trial in the core and top layer (CTR and SiloSolve® OS side, respectively) of the bunker silo, and environment

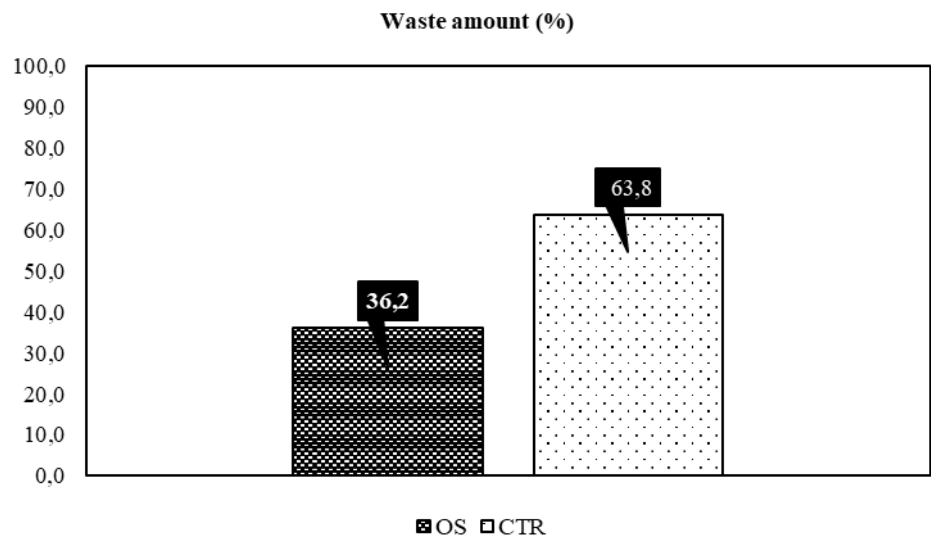


Figure 5.4 - The spoiled silage in the top layer of the bunker disposed for the two experimental theses (CTR and SiloSolve® OS side, respectively) during the trial by the staff of the experimental facility through a truck loader

6. Effects of *Limosilactobacillus mucosae* UNISS B81 compared to several commercial or pure lactic acid bacteria inoculants on different ensiling conditions of whole plant corn silage

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ABSTRACT

The inoculants for ensiling are generally isolated from plant-derived materials. Recently, some researchers proposed to use animal-derived lactic acid bacteria (LAB) to develop silage additives as a vehicle to convey inoculated LAB to animals, thus improving both silage quality and animal performances. In this work, we evaluated the effects of LAB inoculants on fermentative, chemical, and aflatoxin contamination in corn silage packed at different levels of density. These parameters were determined on late-season corn hybrid plants harvested at the $\frac{2}{3}$ milk line stage, with 6 ensiling treatments, 2 ensiling length (30 and 120 days), and under 3 ensiling conditions (i.e., high density, HD; low density, LD; or low density + *Clostridium* spp., LD + Cl). The ensiling treatments were a) an untreated control (CTR); b) a label dose of commercial inoculant containing *Lentilactobacillus buchneri* (DSM22501/1k20738) and *Lactococcus lactis* (DSM11037/1k2081) (BL); c) a label dose of commercial inoculant containing *Lactococcus lactis* (NCIMB30117/1k2083), *Lactiplantibacillus plantarum* (DSM16568/1k20720) and *Enterococcus faecium* (DSM22502/1k20602) (ELP); d) *Lactiplantibacillus plantarum* PB (LP PB); *Lacticaseibacillus rhamnosus* LR7 (LR LR7) *Lacticaseibacillus rhamnosus* RI (LR RI) in a ratio of 50%-25%-25% (R1); e) The same LABs as in previous point 4 in a ratio of 33%-33%-33% (R2); f) *Limosilactobacillus mucosae* UNISS B81 (Rumen LM). Each silage was ensiled in 20 L minisilos, compacted at three experimental conditions (high, low, and low density inoculated with *Clostridium tyrobutyricum* UC7086), stored at room temperature, and opened after 30 or 120 days of ensiling. The interaction of ensiling condition x ensiling length had a significant effect on silage pH (<0.05) with an increased pH in mini-silos with a low mass density inoculated with clostridia, respect to silage with high mass density. Even though the LAB counts were higher in low-density and low-mass-density inoculated with clostridia. The Rumen LM and BL treatments showed a similar profile for pH, ammonia N, propionic acid, lactic acid, and acetic acid. Acetic acid was higher (<0.05) in minisilos treated with facultative heterofermentative LAB (R1 and R2), and CTR silage. Different ensiling conditions affected the pH of silage. The ensiling length significantly affected the aerobic stability. Among inoculant tested, the mix R2 and Rumen LM showed the longer ($P < 0.05$) aerobic stability (216.7 and 200.7 h; respectively). In conclusion, Rumen LM was able to ferment corn silage under different ensiling conditions and to ensure good aerobic stability of the silage, although further ensiling field-scale experiments and on animal performances are necessary to support these preliminary results.

Keywords: aerobic stability; volatile organic compounds; heterolactic fermentation; homolactic fermentation.

Abbreviations: ADFom - ADF expressed exclusive of residual ash; ADICP - acid detergent insoluble CP; AF - aflatoxins; AFB1 - aflatoxin B1; AFB2 - aflatoxin B2; AFG1 - aflatoxin G1; aNDFom - NDF assayed with sodium sulphite and a heat stable amylase and expressed exclusive of residual ash; CFU - colony forming units; CP - crude protein; CTR - corn silage treated without inoculant; DM - dry matter; EE - ether extract; GC - gas chromatography; GLM - general linear model; HD - corn silage compacted at high density condition; HPLC - high performance liquid chromatograph; LAB - lactic acid bacteria; LA ratio - lactic acid/ acetic acid ratio; LA + Eth ratio, lactic acid/ acetic acid plus ethanol ratio; LD - corn silage compacted at low density condition; LD + Cl - corn silage compacted at low density condition and inoculated with *Clostridium tyrobutyricum* UC7086; BL - corn silage treated with *Lentilactobacillus buchneri* (DSM22501/1k20738) and *Lactococcus lactis* (DSM11037/1k2081); lignin(sa) - lignin determined by solubilization of cellulose with sulphuric acid; ELP - corn silage treated with *Lactococcus lactis* (NCIMB30117/1k2083), *Lactiplantibacillus plantarum* (DSM16568/1k20720) and *Enterococcus faecium* (DSM22502/1k20602); NDICP - Neutral detergent insoluble CP; TN - total N; R1 - corn silage treated with *Lactiplantibacillus plantarum* PB (LP PB), *Lacticaseibacillus rhamnosus* LR7 (LR LR7), and *Lacticaseibacillus rhamnosus* RI (LR RI) in a ratio of 50%-25%-25%; R2 - corn silage treated with *Lactiplantibacillus plantarum* PB (LP PB), *Lacticaseibacillus rhamnosus* LR7 (LR LR7), and *Lacticaseibacillus rhamnosus* RI (LR RI) in a ratio of 33%-33%-33%; Rumen LM - corn silage treated with *Limosilactobacillus mucosae* UNISS B81; VFA - volatile fatty acids; VOC - volatile organic compounds;

Introduction

The use of silage additives is a widespread practice that can enhance the preservation of forage into the silo (Muck et al., 2018). Indeed, the use of lactic acid bacteria (LAB), both homo- and hetero-fermentative LAB, alone or in combination at ensiling, allows to reduce spoilage onset phenomena and reduce the risk of a temperature rise during aerobic exposure of the silage. In particular, LAB can catabolize the water-soluble carbohydrates (WSC) present into the green fodder and drop the pH of silage exerting an antimicrobial activity, due to the production of organic acids such as lactic and acetic acid and other molecules including 1-2 propanediol, phenyllactic acid, bacteriocins, or 4-hydroxyphenyllactic acid (Chen et al., 2023; Nasrollahzadeh et al., 2022; Smaoui et al., 2022; Lu et al., 2022; Hirozawa et al., 2023). These molecules improve the aerobic stability of the silage inhibiting the onset of spoilage microorganisms such as enterobacteria, clostridia, yeasts, and molds (Muck et al., 2018) and preserving the nutritional value and palatability of the moist forage.

In several studies (Kristensen et al., 2010; Silva et al., 2017; Zhang et al., 2018), it has been demonstrated that LAB inoculants isolated from plant-derived materials can be used as silage additives to improve the preservation of the ensiled mass. An important aspect of LAB inoculums for widespread use is their ability to produce a probiotic impact on the host, boosting the animal's productive and reproductive functions (Weinberg et al., 2004). Recently, Guo et al. (2020) proposed to use animal-derived LAB to develop silage inoculants as a vehicle to convey inoculated LAB to animal (Han et al., 2014), thus improving both silage quality and animal performance, taking advantage from the probiotic characteristics of these LAB (Guo et al., 2020; Kuppusamy et al., 2020).

Several studies have been showed that *Limosilactobacillus mucosae* species is currently of interest as putative probiotics due to their metabolic capabilities and ability to colonize host mucosal niches (Valeriano et al., 2019); due to the apparent ubiquitous expression of mucus binding protein (mub) homologues within the species, as well as other adhesin-like proteins (Lee et al., 2012; Roos et al., 2000; Valeriano et al., 2014). *Limosilactobacillus mucosae*, a close relative of *Lim. reuteri* and *Lim. fermentum*, already used as silage inoculants (EFSA 2014; Ni et al., 2016; Andrada et al., 2023; Singh et al., 2023; Su et al., 2019), exerts different probiotic characteristics (Lee et al., 2024; Dang et al., 2024) such as reducing serum pro-inflammatory cytokines (Zhang et al., 2022), promoting intestinal barrier functions through the increased spermine production with a dependence on enhanced arginine metabolism (Zhou et al., 2024) and controlling inflammation by modulating macrophage polarization, thereby improving intestinal barrier function (Li et al., 2023).

In this trial, it has been studied the effect of *Limosilactobacillus mucosae* UNISS B81 by comparing it with other two commercial LAB inoculants and two previously tested and promising pure LAB mixtures (i.e., one strain of *Lactiplantibacillus plantarum* or two strains of *Lacticaseibacillus rhamnosus* as described in Gallo et al., 2021; 2022), on fermentation profile of silage ensiled at different ensiling conditions (i.e., high density, low density, and low density + *Clostridium* contamination, as detailed below). The idea behind this study is to understand how *Lim. mucosae* UNISS B81, an animal-derived LAB, can be used as possible potential probiotic inoculant to improve silages fermentative profile in relation to other commercial inoculants or pure LAB mixtures, with the aim of subsequently using it in *in vivo* trials to determine its beneficial effects on the host animal.

Materials and Methods

This paper presents results from a research project following the publication of previous related papers in the current journal (Gallo et al., 2021, 2022). All of the publications use similar analytical procedures and experimental designs. Therefore, the main traits of the current experiment are here reported, and the readers can refer to our previous papers for further details.

Silage preparation and experimental conditions

A late-season corn hybrid (Pioneer Hi-Bred International, Inc., Des Moines, IA, USA) was experimentally grown during the year 2020 at the Centro Ricerche Zootechniche (CERZOO S.r.L., San Bonico, Italy). The forage was harvested at the 2/3 milk line and then chopped to an 8 mm theoretical cut with a self-propelled forage harvester (John Deere model 7780, Moline, IL, USA) that had a pickup mechanical kernel processor roll (roll gap: 2.3 mm, model 375plus).

Approximately 25 tonnes of freshly chopped corn forage were collected in each of the triplicate plots from the same experimental field (45°00' 21.2"N 9°42' 20.8" E). A tractor with a bucket loader and a weighbridge at the farm was used to weigh, mix, and manage each 25 tonnes mass of freshly chopped forage. Each freshly chopped mass was divided into six approximately equal amounts, which were homogenized with a farm fork, and then individually sampled prior to application of the ensiling treatments (i.e., 1 untreated control and 5 other treated with bacterial inoculants, described below). A sample from each of the six amounts of every freshly chopped masses was collected before adding the treatments and characterized for pH before ensiling, chemical composition, mycotoxin contamination and microbiological analyses (Table 6.1). From each freshly chopped forage mass, 18 mini-silos (20 L plastic jars, in which about 12.0 kg of freshly chopped forage was weighed for high density condition and about 7.5 kg for low density condition, with or without clostridia contamination) were prepared and opened after 30 and 120

days. Thus, a total of 108 mini-silos, representing 6 ensiling treatments (including the control CTR), 2 ensiling length (30 and 120 days), 3 ensiling conditions (high density, or HD; low density, or LD; and low density + *Clostridium* spp., or LD + Cl) and 3 replicate for each specific minisilos, representing a specific treatment x ensiling length x ensiling condition. No minisilos with high density + *Clostridium* spp. were prepared, because preliminary experiments showed that *Clostridium* spp. is poorly able to grow in high density condition (data not showed).

The five lactic acid bacteria inoculant treatments (LAB treatments) were: a) a label dose of commercial inoculant containing *Lentilactobacillus buchneri* (DSM22501/1k20738) and *Lactococcus lactis* (DSM11037/1k2081) (BL treatment); b) a label dose of commercial inoculant containing *Lactococcus lactis* (NCIMB30117/1k2083), *Lactiplantibacillus plantarum* (DSM16568/1k20720) and *Enterococcus faecium* (DSM22502/1k20602) and advised for clostridial control (ELP treatment); c) *Lactiplantibacillus plantarum* PB (LP PB), *Lacticaseibacillus rhamnosus* LR7 (LR LR7) and *Lacticaseibacillus rhamnosus* RI (LR RI) in a ratio of 50%-25%-25% at a dose of 2.5×10^5 CFU/g of freshly chopped corn forage (R1); d) the same LABs as in previous point 4 in a ratio of 33%-33%-33% at a dose of 2.5×10^5 CFU/g of freshly chopped corn forage (R2); e) *Limosilactobacillus mucosae* UNISS B81 at a dose of 2.5×10^5 CFU/g (Rumen LM). An untreated silage was used as control (CTR), added with water.

The freshly chopped and mixed corn plants from each plot was weighed and transferred into 20 L plastic jars, compacted to a density of 198 ± 6 kg DM/m³ (HD), 123 ± 6 kg DM/m³ (the same for LD and LD +Cl), and then carefully sealed with plastic lids and stored at $20.3 \pm 0.63^\circ\text{C}$. A mixed culture made by cells and spores of *C. tyrobutyricum* UC7086, $1.00\text{-}1.50 \log_{10}$ cfu/g concentrated (Università Cattolica del Sacro Cuore Culture Collection, Piacenza-Cremona, Italy) isolated in our lab from a cheese sample with late blowing defect, was used to simulate the third ensiling condition (low density plus *Clostridium* spp.).

Application of bacterial inoculants

The two commercial inoculant products were used according to manufacturer recommendations, as freeze-dried powders diluted in 30°-heated water at 2 mg/kg of fresh forage using a sprayer to a final expected dose of approximately 1.5×10^5 CFU/g freshly-chopped forage. An equal amount of water without inoculant was sprayed into the CTR mass. The other LAB strains were grown overnight at 37°C in 350 mL flasks containing 250 mL of MRS broth (Oxoid, Thermo Scientific, Basingstoke, England) until the end of the log-growth phase ($\sim 1 \times 10^9$ CFU/mL). Cultures were then centrifuged for 5 min at $4600 \times g$ at room temperature and the supernatant was discarded. The pellet was washed three times with a saline solution (0.89 g/100 mL NaCl), diluted in the same solution, and applied in the same manner and dose as the commercial products. Cell

counts in MRS medium (Oxoid, Thermo Scientific, Basingstoke, UK) were used to confirm the inoculum dose.

Preparation of samples and mini-silos

Each silage was thoroughly mixed with a wheel loader and shovels. Three representative samples (~1 kg each) were collected from different parts of each pile, mixed and stored in airtight bags, immediately frozen, and used for subsequent chemical, fermentative, and microbiological analyses. The mini-silos were opened and weighed after 30 and 120 days. After opening, the mass of each mini-silo was mixed thoroughly, and representative samples (~2 kg) were collected. Samples were then divided into 2 homogeneous sub-samples (~1 kg each). One of these sub-samples was used for chemical and mycotoxin analysis as described by Gallo et al. (2016), and the other subsample was stored at 4°C prior to measurements of pH, lactic acid, ethanol, volatile fatty acids (VFA), volatile organic compounds (VOC, grouped as aldehydes, alcohols, ketones, esters), ammonia N, and microbiological counts (Gallo et al., 2021).

Chemical-biological-fermentative parameters and mycotoxin contamination

Chemical analysis on samples collected from fresh chopped material and from minisilos were DM, ash, ether extract, crude protein, soluble fraction of CP, Neutral detergent (ND), acid detergent (AD), and lignin (sulfuric acid [sa]) and starch by polarimetry (Polax 2 L, Atago®, Tokyo, Japan). Analyses were determined according to AOAC (Association of Official Analytical Chemists), 1995) methods and Licitra et al., (1996) and Mertens et al., (2002) and details on the analysis are given in Gallo et al., 2016 and 2021.

To quantify volatile organic acids, lactic acid, ethanol and other alcohols, aldehydes, esters, and ketones, a wet sample was extracted in distilled water, filtered, centrifuged and the liquid phase was retained for determination of VFA and VOC using a gas chromatography (GC) system that was equipped with a flame ionization detector (Sigolo et al., 2023). Lactic acid was determined using HPLC (Carvalho et al., 2012). When a compound was undetectable, its detection limit was reported (i.e., 0.1 g/kg DM). Ammonia N concentration was determined in about 20 g of fresh sample in a slurry mix composed of 150 mL of distilled water and magnesium oxide (10 g/sample; (NorFor, 2011)). The ammonia N concentration, expressed on a total N (TN) basis, was determined after steam distillation using the Kjeldahl method described earlier. The pH of the previously filtered water mixture was also measured.

Measurements of aflatoxins (AF) were according to (Gallo et al., 2010). Briefly, the AF were extracted from 10 g of dried samples with 100 mL of an acetone:water solution (70:30 vol/vol). The

mixture was shaken at 150 r.p.m. for 45 min (Universal Table Shaker 709, Enrico Bruno srl, Torino, Italy), passed through Schleicher & Schuell 595 ½ filter paper (Dassel, Germany), and an aliquot of 5 mL was then purified on an immunoaffinity column (Aflatoxin Easi-extract, R-Biopharm Diagnostics Technologies, Glasgow, UK). The column was then washed with 5 mL of water and slowly eluted with 2.5 mL of methanol. The eluate was concentrated under a flow of N² and brought to 2 mL by addition of an acetonitrile:water (41:59) solution, filtered into vials (Millipore Corporation, Bedford, MA, USA; HV 0.45 mm), and then analyzed by HPLC as described earlier. All the analyses were performed in duplicate.

Determination of aerobic stability of silages and microbiological analysis

Aerobic stability of silages was determined by placing approximately 3 kg of sample into a 20 L pail and allowing aerobic deterioration at 20.2 ± 0.82°C (Ranjit and Kung, 2000). Environmental and silage mass temperatures were monitored every 2 hours using a data recorder (Microlite, Dostmann Electronic GmbH, Wertheim, Germany). To prevent drying and contamination, the mini-silos were wrapped in a double layer of air-permeable cheesecloth. The aerobic stability test lasted 14 days, and the ensiled mass was declared aerobically unstable when the temperature differential between the ambient and the silage exceeded 2°C.

Microbial counts in mini-silos were determined according to Gallo et al. (2018) in samples of about 30 g of silage, which were transferred into a sterile homogenization bag, suspended (1:10, w/v) in a peptone physiological salt solution (1 g/L of neutralized bacteriological peptone and 9 g/L of sodium chloride), and homogenized for 3 minutes in a Stomacher blender. Serial dilutions were made. The counts of LAB and of yeasts and molds were determined using a pour-plate technique respectively with De Man, Rogosa, and Sharpe (MRS) agar medium and 40 g/L rose-bengal chloramphenicol agar (yeast and molds; Oxoid, Basingstoke, UK) respectively. For LAB (in anaerobic conditions) and yeasts, plates were incubated for 3 days at 25°C, whereas molds were incubated for 5 days. The colony-forming units (CFU) were counted separately, according to their macro-morphological features. Mean CFU values of duplicate subsamples were recorded for total yeasts and molds on plates that yielded 10–100 CFU per Petri dish.

Statistical analysis

The LAB, yeasts and molds were log₁₀-transformed prior to statistical analysis, and the terms of the ANOVA (standard errors of the means and P values) refer to these log₁₀-transformed values.

Data were analyzed according to a completely randomized design, within a $6 \times 3 \times 2$ factorial arrangement of treatments, using the GLM procedure (SAS Institute Inc., 2003), with the model:

$$Y_{ijkl} = \mu + T_i + C_j + D_k + (T \times C)_{ij} + (T \times D)_{ik} + (C \times D)_{jk} + (T \times C \times D)_{ijk} + e_{ijkl}$$

where: Y_{ijkl} is the dependent variable; μ is the overall mean; T_i is the fixed effect of bacterial inoculants ($i = 6$); C_j is the fixed effect of ensiling conditions ($j = 3$, being HD, LD, LD + Cl); D_k is the fixed effect of the ensiling length ($k = 30$ or 120 days); the $(T \times C)_{ij}$, $(T \times D)_{ik}$ and $(C \times D)_{jk}$ term are the first order interactions; $(T \times C \times D)_{ijk}$ term is the second order interaction and e_{ijkl} is the residual error. When there was no interaction and the treatment main effect was significant, the CTR mean was compared with other treatments using Dunnett's multiple comparison test, with a difference that was considered significant if the P value was below 0.05. When the interaction was significant (i.e., $P < 0.05$) and biologically relevant, data were presented to show effects of ensiling length for each response. When the second order interaction was significant, differences among specific means of interest were verified and reported in the text.

Results

The DM content, the pH and some microbiological counts (LAB, yeasts and molds) of freshly chopped corn prior to ensiling in mini-silos were assessed and are shown in Table 6.1. The DM (314 g/kg as fed) was within the recommended range for obtaining a proper silage. The total LAB count presented a value typically found in corn before harvest, whereas the number of yeast and molds were 6.5 and 3.5 log cfu/g, respectively. The aflatoxin B1 was detected at a contamination level of 0.51 ± 0.32 $\mu\text{g/kg DM}$.

The effects of ensiling conditions, treatments, and ensiling lengths on chemical parameters of corn silage are shown in Table 6.2. The ensiling conditions affected ($P < 0.05$) aNDFom, ADFom, and lignin(sa) contents. The corn silage ensiled in HD conditions had the lowest values aNDFom, ADFom, and lignin(sa) (373.0 ± 11.12 g/kg DM; 232.0 ± 10.04 g/kg DM; 27.1 ± 1.11 g/kg DM, respectively). The effect of treatment was significant ($P < 0.05$) for ash, ADICP, aNDFom, and ether extract. The lowest ash content was observed for the CTR group (47.9 ± 2.34 g/kg DM). The ADICP contents were lower in ELP and CTR in comparison to the other groups. The aNDFom levels were higher in CTR and Rumen LM compared to the other treatments. The lowest ether extract value was detected in silage treated with R2 (i.e., 29.0 ± 0.94 g/kg DM). The effect of ensiling length affected ($P < 0.05$) aNDFom, ADFom, lignin(sa), and starch. The mentioned parameters reported the highest values at 120 days (381.0 ± 11.12 g/kg DM; 239.0 ± 10.04 g/kg DM; 28.0 ± 1.11 g/kg DM; 324.0 ± 15.68 g/kg DM, respectively). Considering the effect of treatment and ensiling conditions on chemical parameters, an interaction ($P < 0.05$) for DM content was found and reported in Fig. 6.1.

The DM levels decreased in WPCS ensiled in LD and LD + Cl conditions. Rumen LM, BL, and R2 showed a higher DM decrease respect to CTR group. The second order interaction (Treatment x ensiling condition x ensiling length) was detected ($P<0.05$) for CP and NDICP.

The effect of ensiling conditions, treatments, and ensiling length on fermentative parameters of corn silage are reported in Table 6.3. The effect of ensiling conditions was significant ($P<0.05$) for propionic acid, total alcohols, lactic/acetic (LA) ratio, and lactic/acetic + ethanol (LA + Eth) ratio. Propionic acid and total alcohols reported the lowest values in corn silage ensiled in HD conditions (1.97 ± 1.53 g/kg DM; 17.6 ± 4.61 g/kg DM, respectively). High levels of LA and LA + Eth ratios were detected in HD conditions (4.65 ± 1.25 ; 2.34 ± 0.70 , respectively). The effect of treatment was significant ($P<0.05$) for pH, acetic acid, propionic acid, total alcohols, and LA ratio. Rumen LM had the highest pH level (4.06 ± 0.88). Corn silage treated with inoculants containing obligate heterofermentative LAB, (i.e., BL and Rumen LM) had a higher acetic acid content respect to the other groups. The highest content of propionic acid was found in masses inoculated with BL and Rumen LM (i.e., 25.0 ± 4.68 g/kg DM; 23.0 ± 4.68 g/kg DM, respectively). Total alcohols were lower in corn silage treated with R1 and R2 in comparison with the other groups. The BL corn silage had the lowest level of LA ratio (3.0 ± 1.25). The effect of ensiling length was significant ($P<0.05$) for 1,2 propanediol, propionic acid, iso-valerianic acid, ethyl propionate, total aldehydes, total alcohols, LA ratio, LA + Eth ratio, yeasts, and aerobic stability. The 1,2 propanediol and iso-valerianic acid were only detected at 30 days (0.9 ± 0.27 g/kg DM; 0.7 ± 1.79 mg/kg DM, respectively). Propionic acid was characterized by an increase among the ensiling length reaching the value of 5.08 ± 1.53 g/kg DM at 120 days. Ethyl propionate was only detected at 120 days (0.9 ± 0.24 mg/kg DM). The content of total aldehydes decreased by 40% from 30 to 120 days. The total alcohol content increased with time (i.e., 20.2 vs 24.6 ± 4.61 g/kg DM, respectively). The LA ratio, LA + Eth ratio, and yeast counts decreased from 30 to 120 days by 49.5%, 32.4%, and 15.8% respectively. The aerobic stability increased among the ensiling length reaching the maximum level at 120 days (267.7 ± 47.75 hour).

Regarding the effect of treatment and ensiling conditions on fermentative parameters, interactions ($P<0.05$) for ethyl acetate, ethanol, butyric acid, total esters, and LAB were observed and reported in Fig. 6.1. Corn silage treated with R1 had lower ethyl acetate when ensiled in HD and LD conditions in comparison to the other treatments. On the other hand, corn silage treated with BL reached a peak in ethyl acetate when ensiled in LD + Cl conditions. The ethanol content showed an increasing trend within the different ensiling conditions between treatments; on the other hand, only corn silage treated with R2 had a lower ethanol content in LD + Cl conditions compared to the other treatments. Butyric acid decreased in corn silage treated with R2, and BL

among the different ensiling conditions; on the other hand, R1 group reached a peak when ensiled in LD conditions. The total esters values were higher in poor ensiling conditions (i.e., LD and LD + Cl, respectively) than HD for all the treatments. Corn silage treated with R1 had lower total esters content in HD and LD conditions compared to the other treatments. The LAB were higher in poor ensiling conditions (i.e., LD and LD + Cl) than HD. The highest LAB count was observed in BL group across all ensiling conditions. Interactions ($P < 0.05$) for the effect of treatment and ensiling length for ammonia N, ethyl acetate, ethanol, butyric acid, total esters were found and reported in Fig. 6.2. The CTR group had higher ammonia N levels than the other groups at 120 days. Ethyl acetate increased with the advance of ensiling length in all treatments, even if corn silage treated with R1 showed a steady trend among the trial and it had a lower ethyl acetate content at 120 days in comparison to the other treatments. A similar trend was observed for total esters concentration. Ethanol concentrations decreased among the ensiling length for all the treatments, especially for R1 and R2. Butyric acid increased from 30 to 120 days, especially R1 was the group with the highest value at 120 days. Regarding the effect of ensiling conditions and ensiling length, interactions ($P < 0.05$) for lactic acid, acetic acid, LAB, and molds were observed and reported in Fig. 6.3. The LD and LD + Cl caused a more pronounced decrease in lactic acid over time, while this reduction was not observed for HD conditions. The acetic acid increased in three experimental conditions from 30 to 120 days; indeed, this value was higher in corn silage ensiled in LD and LD + Cl conditions. The LAB count detected between the ensiling lengths decreased in corn silage packed in HD conditions; on the other hands, LAB counts increased in LD and LD + Cl conditions among the all trial. Mold counts detected in corn silage ensiled in HD decreased from 30 to 120 days; on the other hand, mold counts in LD + Cl increased with the advance of ensiling length. The second order interaction (Treatment x ensiling conditions x ensiling length) was significant ($P < 0.05$) for ethanol, iso-butyric acid, butyric acid, lactic acid, and LAB.

Regarding aflatoxin contents (Table 6.4), no significant interactions for treatment and ensiling conditions were found in this trial, even if there is a slightly increasing trend for aflatoxin B1 and aflatoxin G1 at 120 days of mini-silos opening (i.e. 0.97 and 0.17, respectively) in comparison to 30 days.

Discussion

The aim of ensiling is to create the best anaerobic conditions safeguarding ensiled mass quality. It is important to know that the main microorganisms present at the harvest period are aerobic forms (Pahlow et al., 2003). Indeed, using LAB inoculants at ensiling is crucial to reduce spoilage onset phenomena by achieving a final product with the highest nutritional values.

In this trial, the use of a strain of obligate heterofermentative *Lim. mucosae* species, isolated from liquid ruminal ewe, to ferment whole-plant corn has been tested. In a preliminary assay, this strain demonstrated a good resistance to low pH and high bile salt concentrations (essential for passing through the gastric barrier unharmed), as well as strong antibacterial activity against *Listeria monocytogenes* and *Staphylococcus aureus* (Fancello et al., results not published). Several strains belonging to these LAB species, as above mentioned, exert different probiotic characteristics on the host (Curiel et al., 2024) as well they can be successfully used in the production of functional products (de Oliveira et al., 2021; Viana et al., 2024).

The silage fermented with *Lim. mucosae* UNISS B81 had final fermentative properties almost equal to the BL treatment in terms of pH, ammonia N, acetic acid, propionic and lactic acid content, and aerobic stability. The treatments with commercial inoculants (BL and ELP, respectively) were not different in 1,2-propanediol content. However, this is in contrast with Muck et al. (2018), because both inoculants contain *Lentilactobacillus buchneri*, which is well known to degrade lactic acid into acetate and 1,2-propanediol increasing pH level in silage after long ensiling times. In contrast to Muck et al. (2018), our study found similarities with several researches (Kleinschmit and Kung, 2006; Schmidt et al., 2009), which also reported no production of 1,2 propanediol in silage stored for 5 or 42 days. Although, further investigation into this topic is needed. The ethanol content was lower in CTR than in BL and ELP. However, numerical differences in yeast counts were observed for the mentioned treatments, high concentration of ethanol may find an explanation in the metabolic pathways. Notably, bacteria belonging to *Lactiplantibacillus plantarum* are able to proliferate in different environments due to the fact that they are ubiquitous bacteria (Martino et al., 2016). This statement can explain our results regarding the concentration of ethanol in ELP. On the other hand, obligate heterofermentative LAB such as *Lentilactobacillus buchneri* are known to be involved in ethanol production (Kung et al., 2018), which confirms our findings in mini-silos treated with BL.

Comparing the two microbial mixes used in our work (i.e., R1 and R2) we did not observe substantial differences in the fermentative parameters. However, it has been detected a high content of butyric acid in R1 mix, but this datum requires a specific interpretation and deserves to be investigated in further studies. Moreover, the concentration of butyric acid in corn silage treated with R1 did not compromise the profile of the final product.

Considering VOCs emission, both at farm and laboratory scale, it is known that the emission of these compounds is an issue that needs to be investigated with the aim of mitigating their impact at farm level (Hafner et al., Sigolo et al., 2023). Indeed, VOCs formation in silages has a negative impact on feed intake in dairy cows and goats (Weiss, 2017). Focusing on BL and

Rumen LM, in this trial it was observed a similarity in the VOC production pathway between these treatments. Despite this statement, after 120 days of fermentation, the concentrations of total esters and ethanol were similar between the control and the group inoculated with *Lim. mucosae* UNISS B81. Given this scenario, it is possible to take into account the use of these animal-derived LAB as a possible and potential vehicle to mitigate VOCs.

LAB and ensiling conditions

Achieving a well preserved and safe silage is crucial to maximize animal performance; notably, it is known that this is only the last step of the whole ensiling process. Indeed, a factor that farmers must consider during the ensiling process regards the packing operation which is crucial to improve silage density. Hence to highlight the action of LAB, in this trial we decided to simulate different ensiling conditions such as low density, low density with clostridia cells and spores' presence, and high density and evaluate how different ensiling conditions can affect silage fermentation at farm level (Kung et al., 2021). Low-density conditions are usually detected in correspondence of the side walls of the horizontal bunker silos, in which the compacting action is less efficient (Gallo et al., 2016). The density and oxygen concentration of forage mass are proportionally linked to silo porosity, which is one of the most critical factors determining forage preservation and nutritive value and poor packing densities have been clearly demonstrated to be unfavorable in farm-scale silos (McDonald et al., 1991; Ruppel et al., 1995). These factors may negatively influence the fermentation processes and silage quality, affecting animal performances (Sucu et al., 2016).

Normally, inoculation had no effects on the nutrient compositions of silages, as reported before (Tabacco et al., 2011; Queiroz et al., 2013). Although poor ensiling conditions usually decreased the dry matter content, a less marked reduction in R2 group was observed, even if inoculations were able to carry out their protective action also in low density silages as supported by a longer aerobic stability of inoculated silage compared to the CTR (with the exception of the R1 group). Similarly, Kung et al. (2021) observed that silage density interacted with air stress on its effect on aerobic stability and this markedly decreased when low-density silos were air stressed in comparison with high-density silos in the same situation. The pH was lowest in high density silage, which was accompanied by a high lactate content, LA and LA + Eth ratios. The high content of lactate in high density silage was in accord with the results achieved by other authors (Kung et al., 2021; Sucu et al., 2016). Similarly, the lactic acid content and LA and LA +Eth ratio was higher in the silages fermented with R1 and R2 respect to the others microbial treatments. The reduction of ammonia-N in high-density silages is indicative of lower proteolytic activity, which may have resulted in improved protein-N utilization in the rumen (Sharp et al., 1994). Likewise, in

inoculated silage the ammonia N content was low respect to CTR silage group. Similar results were obtained by other authors (Gallo et al., 2018).

Referring to ethanol contents, both in low density and low density plus clostridia silage, these data were higher than high density silage as reported by Sucu et al. (2016); whereas in trial conducted by Kung et al. (2021) on air stressed silage, the ethanol contents were low. It is possible that in our experiments the air level detected into the ensiled mass was relatively low inhibiting *Acetobacter* growth, indeed in this condition ethanol cannot be metabolized to acetic acid (Spolestra et al., 1988). Moreover, *Acetobacter* spp. are common detected at farm level in corn silage (Gallo et al., 2020) but not at laboratory-scale silos that are usually well sealed (Kung et al., 2021; Guan et al., 2018).

The content of acetic acid was high in poorly packed silage comparable with the results of other authors (Kung et al., 2021). Therefore, considering the low number of yeasts and the highest level of ethanol recorded in low packed silage and excluding any activity of *Acetobacter* and *Enterobacteria*, it is possible to state that at laboratory scale the amount of air trapped into the ensiled mass at sealing was higher in low density corn silage than in high packed silage, and in presence of high water soluble carbohydrates the epiphytic and inoculated homo and facultative heterofermentative LAB might metabolize pyruvate aerobically producing more acetic acid than in high packed silage. Indeed, air present may alter the use of sugars by some LAB (Condon, 1987). The acetic acid content was highest in silage inoculated with obligate heterofermentative bacteria, namely BL and *Lim. mucosae* UNISS B81, which means a better aerobic stability in comparison with the other treatments. The low-density silage in the absence or in the presence of inoculated clostridia had the highest content of butyric acid. Zhang et al. (2015) evaluated the effects of two levels of ensiling wet densities (500 and 600 kg/m³ respectively) on *Leymus chinensis* silage quality observing that pH value, butyric acid, ammonia-N concentration, and the coliform bacteria were lower in high-density silage than low-density silage. Therefore, it is crucial to operate a well compaction of the forage reducing the porosity rate and avoiding the consumption of available carbohydrates and acids operated by aerobic microorganisms, causing DM losses (Borreani et al., 2018).

Conclusions

In this work, although the different combinations of LAB did not change the final fermentative parameters profile of silage, it is important to underline that, silages obtained with *Lim. mucosae* UNISS B81 used alone presented a higher content of acetic acid, propionic acid, ethanol, and total alcohols, and a good aerobic stability. These results showed similar fermentative performances that can be compared to those obtained using the BL treatment. High density

conditions allowed to achieve a well-fermented silage with an optimal aerobic stability. The inoculated corn silage ensiled in high density conditions had a lower proteolytic activity when compared with low-density silage ensiled in absence or presence of clostridia. Thus, reducing porosity level is crucial to minimize the activity of aerobic microorganisms that can predispose silage to spoilage. We can conclude that *Lim. mucosae* UNISS B81, used for the first time in corn silage production, was able to ferment the corn with good results in terms of nutritive value and aerobic stability. Further farm studies will be therefore needed to assess the ability of this strains to improve also the animal performances by exerting probiotic activity.

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Table 6.1. Characteristics (mean $\pm$ standard deviation) of freshly chopped whole corn plant (n=6).

Item ¹	Units of measurement ²	Mean	Standard deviation
Dry matter	g/kg as fed	314	8.7
pH	dmnl	4.1	0.12
LAB	log ₁₀ cfu/g	8.9	0.1
Molds	log ₁₀ cfu/g	3.5	1.0
Yeasts	log ₁₀ cfu/g	6.5	0.9
AFB1	µg/kg DM	0.51	0.32
AFB2	µg/kg DM	0.05	0.00
AFG1	µg/kg DM	0.05	0.00
AFG2	µg/kg DM	0.05	0.00

¹ AFB1, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2; LAB, lactic acid bacteria.

² cfu, colony forming unit; DM, dry matter; dmnl, dimensionless.

Table 6.2. Effects of ensiling conditions (density and *Clostridium* addition), addition of lactic acid bacteria inoculants and length of ensiling on chemical parameters of corn silage.

Item	Units of measurement	Ensiling conditions			Treatments ¹						Ensiling length ²			Effects of model (P < 0.05) ³						
		High density	Low density	Low density + <i>Clostridium</i>	CTR	ELP	BL	Rumen LM	R1	R2	30	120	SEM	Treatment	Ensiling conditions	Treatment x ensiling conditions	Ensiling length	Treatment x Ensiling length	Ensiling conditions x Ensiling length	Treatment x ensiling conditions x Ensiling length
Dry matter	g/kg as fed	317.0	293.0	300.0	308.0	304.0	300.0	301.0	306.0	300.0	307.0	300.0	5.72	<0.05	<0.05	<0.05	<0.05	0.95	<0.05	0.34
Ash	g/kg DM	50.3	50.4	50.1	47.9 ^b	50.9 ^a	50.3 ^a	50.0 ^{ab}	51.0 ^a	51.7 ^a	50.3	50.3	2.34	<0.05	0.83	0.58	0.94	0.75	0.20	0.94
CP	g/kg DM	86.6	88.4	87.9	86.6	87.9	89.6	86.5	86.8	88.4	86.2	89.1	1.88	<0.05	<0.05	0.43	<0.05	0.19	0.22	<0.05
NDICP	g/kg DM	9.30	9.90	9.91	9.50	9.47	10.0	9.74	9.70	9.80	9.82	9.56	0.37	<0.05	<0.05	0.69	<0.05	<0.05	<0.05	<0.05
ADICP	g/kg DM	7.90	8.00	7.99	7.84 ^b	7.80 ^b	8.11 ^a	7.94 ^{ab}	8.00 ^{ab}	8.10 ^a	7.83	8.08	0.25	<0.05	0.13	0.30	<0.05	0.55	<0.05	0.73
Soluble CP	g/kg DM	44.9	45.0	44.4	44.5	45.5	45.1	44.4	44.3	44.8	42.9	46.6	1.37	0.09	0.15	<0.05	<0.05	0.89	0.06	0.10
aNDFom	g/kg DM	373.0 ^b	381.0 ^a	380.0 ^a	384.0 ^a	380.0 ^{ab}	374.0 ^b	382.0 ^a	375.0 ^b	375.0 ^b	375.0	381.0	11.12	<0.05	<0.05	0.12	<0.05	0.80	0.94	0.63
ADFom	g/kg DM	232.0 ^c	238.0 ^a	237.0 ^b	241.0	236.0	233.0	237.0	234.0	234.0	232.0	239.0	10.04	0.17	<0.05	0.39	<0.05	0.30	0.59	0.67
Lignin(sa)	g/kg DM	27.1 ^c	28.0 ^a	27.9 ^a	27.4	27.1	28.1	27.7	27.8	28.0	27.3	28.0	1.11	0.09	<0.05	0.65	<0.05	0.75	0.06	0.99
Ether extract	g/kg DM	29.6	29.8	29.8	30.3 ^a	29.3 ^{bc}	29.6 ^{bc}	30.3 ^a	29.9 ^{ab}	29.0 ^c	29.6	30.0	0.94	<0.05	0.40	0.51	0.06	0.52	0.10	0.93
Starch	g/kg DM	321.0	319.0	322.0	323.0	320.0	325.0	317.0	319.0	319.0	317.0	324.0	15.68	0.69	0.75	0.33	<0.05	0.21	0.55	0.39

CP, crude protein; NDICP, neutral detergent insoluble CP; ADICP, acid detergent insoluble CP.

Superscripts indicated that the specific mean was higher (i.e., a) or lower (i.e., b) than CTR at a P < 0.05, as evaluated with Dunnett’s multiple comparison test.

¹ CTR, control; ELP, *Lactococcus lactis* (NCIMB30117/1k2083), *Lactiplantibacillus plantarum* (DSM16568/1k20720) and *Enterococcus faecium* (DSM22502/1k20602); BL, *Lentilactobacillus buchneri* (DSM22501/1k20738) and *Lactococcus lactis* (DSM11037/1k2081); Rumen LM, *Limosilactobacillus mucosae* UNISS B81; R1, *Lactiplantibacillus plantarum* PB (LP PB), *Lacticaseibacillus rhamnosus* LR7 (LR LR7), and *Lacticaseibacillus rhamnosus* RI (LR RI) in a ratio of 50%-25%-25%; R2, as in R1 in a ratio of 33%-33%-33%.

² Ensiling lengths, 30 and 120 days respectively.

³ The Effects of the model were significant at a P < 0.05.

Table 6.3. Effects of ensiling conditions (density and Clostridium addition), addition of lactic acid bacteria inoculants and length of ensiling on fermentative parameters of corn silage.

Item	Units of measurement	Ensiling conditions			Treatments ¹						Ensiling length ²			Effects of model (P < 0.05) ³						
		High density	Low density	Low density + Clostridium	CTR	ELP	BL	Rumen LAB	R1	R2	30	120	SEM	Treatment	Ensiling conditions	Treatment x Ensiling conditions	Ensiling length	Treatment x Ensiling length	Ensiling conditions x Ensiling length	Treatment x Ensiling conditions x Ensiling length
pH	dmnl	3.88	4.04	4.03	3.99	3.93	3.98	4.06 ^a	3.98	3.96	3.91	4.06	0.88	<0.05	<0.05	0.49	<0.05	0.66	<0.05	0.77
Ammonia N	g/kg TN	61.6	66.9	68.1	75.6	64.3	66.0	63.2	59.1	65.0	46.6	84.4	16.48	0.09	0.21	0.32	<0.05	<0.05	0.77	0.73
Ethyl acetate	mg/kg DM	76.7	143	135	125	102	141	133	98.3	110	85.5	151	35.65	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.06
Ethanol	g/kg DM	14.2	18.4	18.0	16.8	17.0	18.2	16.9	16.3	15.8	17.9	15.9	2.21	<0.05	<0.05	<0.05	<0.05	<0.05	0.61	<0.05
1,2 propanediol	g/kg DM	0.5	0.4	0.4	0.5	0.4	0.5	0.5	0.5	0.4	0.9	n.d.	0.27	0.92	0.41	0.13	<0.05	0.92	0.41	0.13
Acetic acid	g/kg DM	16.0	24.8	23.0	22.2 ^{ab}	21.3 ^{bc}	25.0 ^a	23.0 ^{ab}	18.6 ^{cd}	17.6 ^d	15.3	27.2	4.68	<0.05	<0.05	0.10	<0.05	0.20	<0.05	0.31
Propionic acid	g/kg DM	1.97 ^b	3.20 ^a	3.19 ^a	3.03 ^{ab}	3.06 ^{ab}	3.29 ^a	3.36 ^a	2.08 ^{bc}	1.92 ^c	0.57	5.08	1.53	<0.05	<0.05	0.16	<0.05	0.32	0.10	0.29
Iso-butyric acid	mg/kg DM	n.d.	1.7	0.7	n.d.	1.45	1.24	n.d.	2.1	n.d.	n.d.	1.6	2.65	0.06	<0.05	<0.05	<0.05	0.06	<0.05	<0.05
Butyric acid	g/kg DM	0.005	0.014	0.019	0.004	0.023	0.002	0.004	0.036	0.009	n.d.	0.026	0.021	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Iso-valerianic acid	mg/kg DM	0.2	0.5	0.4	n.d.	0.4	n.d.	1.3	0.6	n.d.	0.7	n.d.	1.79	0.23	0.79	0.90	<0.05	0.23	0.79	0.90
Ethyl lactate	mg/kg DM	145	140	146	136	156	146	139	145	140	172	114	27.4	0.35	0.62	0.41	<0.05	0.37	<0.05	0.27
Ethyl propionate	mg/kg DM	n.d.	0.6	0.7	n.d.	n.d.	n.d.	1.2	0.7	0.8	n.d.	0.9	2.24	0.43	0.33	0.14	<0.05	0.43	0.33	0.14
Lactic acid	g/kg DM	69.1	52.1	56.0	56.3	64.4	58.1	55.2	58.7	61.6	68.1	50.0	9.90	0.08	<0.05	0.71	<0.05	0.18	<0.05	<0.05
Total aldehydes	g/kg DM	0.08	0.08	0.01	0.08	0.09	0.09	0.09	0.07	0.09	0.1	0.06	0.03	0.62	0.18	0.34	<0.05	0.12	0.24	0.97
Total alcohols	g/kg DM	17.6 ^b	25.4 ^a	24.2 ^a	23.4 ^a	23.2 ^a	25.0 ^a	23.6 ^a	19.8 ^b	19.5 ^b	20.2	24.6	4.61	<0.05	<0.05	0.17	<0.05	0.08	0.38	0.11
Total ketones	mg/kg DM	10.7	19.9	11.3	0.6	12.7	17.7	15.3	22.2	15.5	0.7	27.2	19.81	<0.05	0.10	0.28	<0.05	<0.05	0.09	0.26
Total Esters	mg/kg DM	255	378	361	335	322	376	356	293	307	275	388	77.54	<0.05	<0.05	<0.05	<0.05	<0.05	0.22	0.50
Lactic/acetic	dmnl	4.65 ^a	2.73 ^b	3.16 ^b	3.12 ^{bc}	3.49 ^{bc}	3.00 ^c	3.10 ^{bc}	3.95 ^{ab}	4.41 ^a	4.67	2.35	1.25	<0.05	<0.05	0.39	<0.05	0.67	0.13	0.45
Lactic/acetic+ethanol	dmnl	2.34 ^a	1.32 ^b	1.61 ^b	1.62	1.79	1.53	1.57	1.84	2.22	2.10	1.42	0.70	0.06	<0.05	0.27	<0.05	0.34	0.11	0.46
LAB	log ₁₀ cfu/g	7.85	8.43	8.45	8.17	8.15	8.41	8.27	8.23	8.22	8.22	8.26	0.18	<0.05	<0.05	<0.05	0.34	0.36	<0.05	<0.05
Molds	log ₁₀ cfu/g	2.31	2.17	2.17	2.05	2.21	2.22	2.40	2.25	2.16	2.25	2.19	0.36	0.14	0.16	0.28	0.36	0.68	<0.05	0.11
Yeasts	log ₁₀ cfu/g	2.31	2.23	2.09	2.47	2.22	2.04	2.21	2.20	2.12	2.40	2.02	0.43	0.11	0.10	0.28	<0.05	0.07	0.24	0.15
Aerobic stability	hour	207.5	191.9	184.9	185.6	191.0	195.4	200.7	179.3	216.7	121.8	267.7	47.75	0.25	0.13	0.72	<0.05	0.59	0.91	0.36

n.d., not detected; dmnl, dimensionless.

Superscripts indicated that the specific mean was higher (i.e., a) or lower (i.e., b) than CTR at a P < 0.05, as evaluated with Dunnett's multiple comparison test.

¹ CTR, control; ELP, *Lactococcus lactis* (NCIMB30117/1k2083), *Lactiplantibacillus plantarum* (DSM16568/1k20720) and *Enterococcus faecium* (DSM22502/1k20602); BL, *Lentilactobacillus buchmeri* (DSM22501/1k20738) and *Lactococcus lactis* (DSM11037/1k2081); Rumen LM, *Limosilactobacillus mucosae* UNISS B81; R1, *Lactiplantibacillus plantarum* PB (LP PB), *Lacticaseibacillus rhamnosus* LR7 (LR LR7), and *Lacticaseibacillus rhamnosus* RI (LR RI) in a ratio of 50%-25%-25%; R2, as in R1 in a ratio of 33%-33%-33%.

² Ensiling length, 30 and 120 days respectively.

³ The Effects of the model were significant at a P < 0.05.

Table 6.4. Effects of ensiling conditions (density and Clostridium addition), addition of lactic acid bacteria inoculants and length of ensiling on aflatoxin contamination during fermentation of corn silage.

Item	Units of measurement	Ensiling conditions			Treatment ¹						Ensiling length ²			Effects of model (P < 0.05) ³						
		High density	Low density	Low density + Clostridium	CTR	ELP	BL	Rumen LAB	R1	R2	30	120	SEM	Treatment	Ensiling conditions	Treatment x Ensiling conditions	Ensiling length	Treatment x Ensiling conditions	Ensiling conditions x Ensiling length	Treatment x Ensiling conditions x Ensiling length
AFB1	µg/kg DM	0.76	0.66	0.79	0.40	0.55	1.00	1.09	0.69	0.69	0.52	0.97	0.981	0.29	0.84	0.23	<0.05	0.38	0.89	0.10
AFB2	µg/kg DM	0.06	0.05	0.06	0.05	0.05	0.06	0.06	0.05	0.05	0.05	0.06	0.022	0.49	0.52	0.15	0.05	0.49	0.52	0.15
AFG1	µg/kg DM	0.14	0.09	0.10	0.05	0.10	0.15	0.13	0.09	0.15	0.05	0.17	0.262	0.85	0.74	0.17	<0.05	0.85	0.74	0.17

¹ CTR, control; ELP, *Lactococcus lactis* (NCIMB30117/1k2083), *Lactiplantibacillus plantarum* (DSM16568/1k20720) and *Enterococcus faecium* (DSM22502/1k20602); BL, *Lentilactobacillus buchneri* (DSM22501/1k20738) and *Lactococcus lactis* (DSM11037/1k2081); Rumen LM, *Limosilactobacillus mucosae* UNISS B81; R1, *Lactiplantibacillus plantarum* PB (LP PB), *Lacticaseibacillus rhamnosus* LR7 (LR LR7), and *Lacticaseibacillus rhamnosus* RI (LR RI) in a ratio of 50%-25%-25%; R2, as in R1 in a ratio of 33%-33%-33%.

² Ensiling length, 30 and 120 days respectively.

³ The Effects of the model were significant at a P < 0.05.

Graphs: Treatment x Ensiling conditions

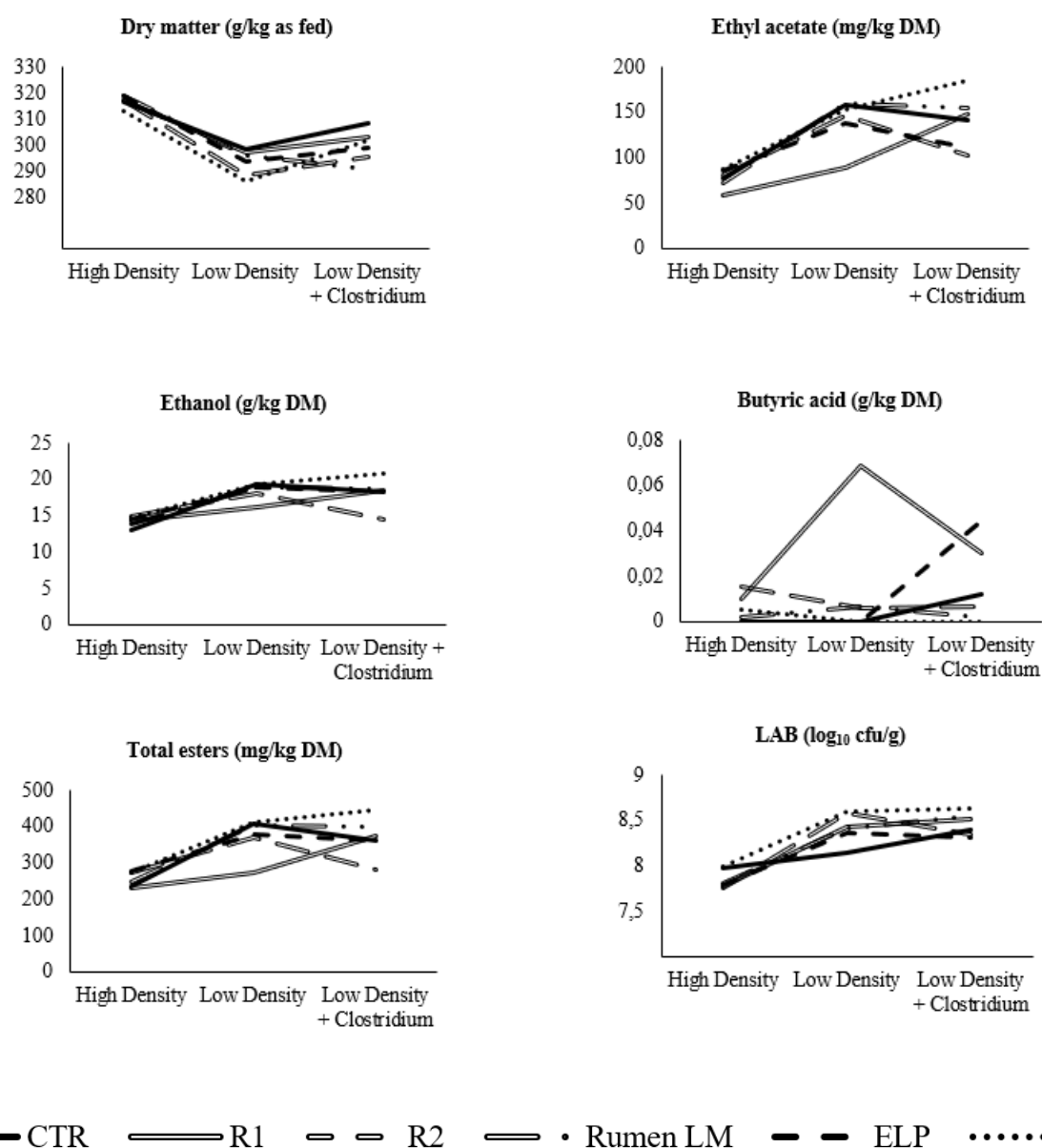


Fig. 6.1. Effect of treatment and ensiling conditions on dry matter, ethyl acetate, ethanol, butyric acid, total esters, and LAB concentrations of corn silages (Treatments x Ensiling conditions interaction significant at $P < 0.05$; CTR, R1, R2, Rumen LM, ELP, BL treatments below of the figure).

Graphs: Treatment x ensiling length

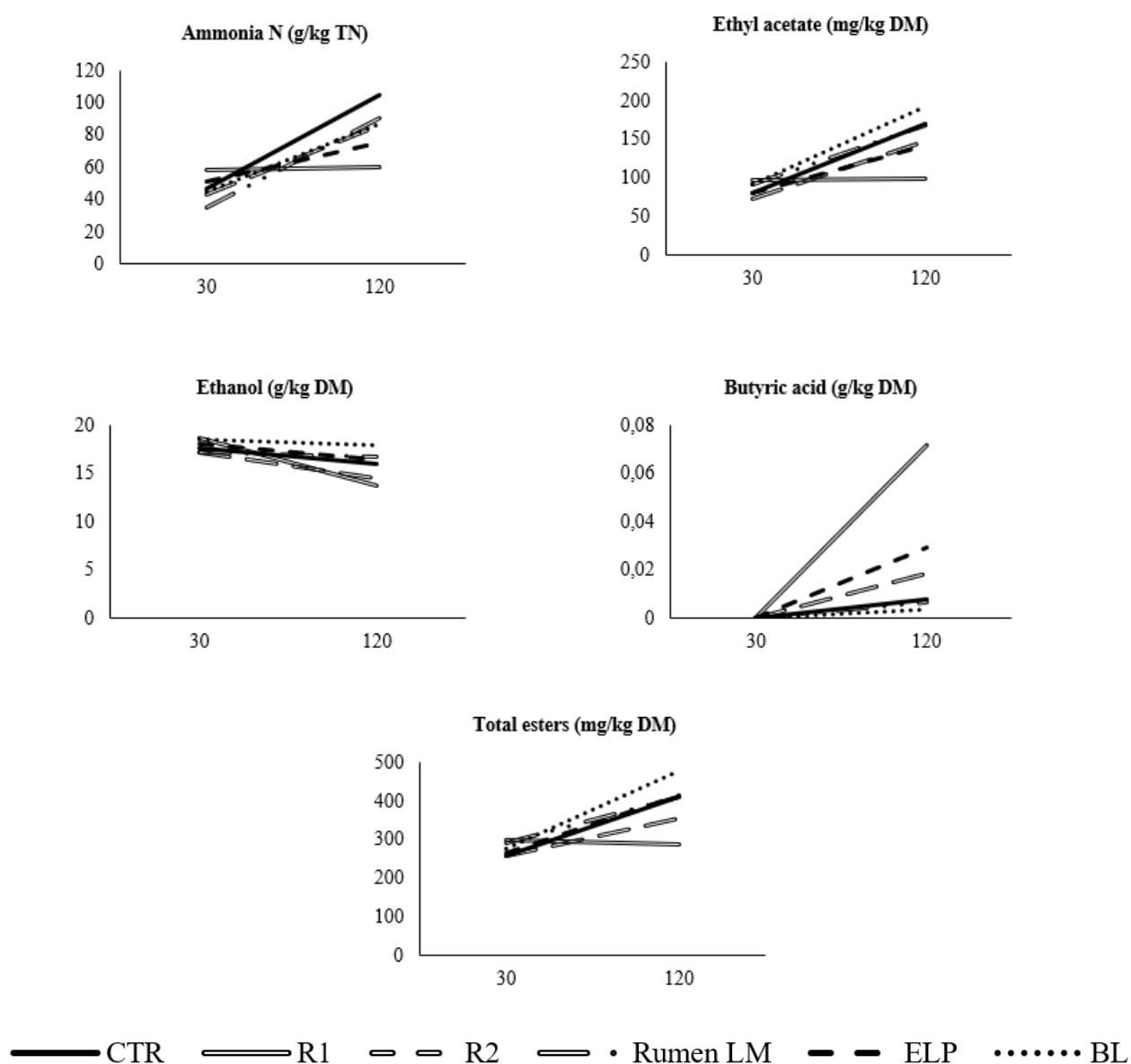


Fig. 6.2. Effect of treatment and ensiling length on ethyl acetate, ethanol butyric acid, total esters, and LAB concentration of corn silages (Treatment x Days interaction significant at $P < 0.05$; CTR, R1, R2, Rumen LM, ELP, BL treatments below of the figure).

Graphs: Ensiling conditions x ensiling length

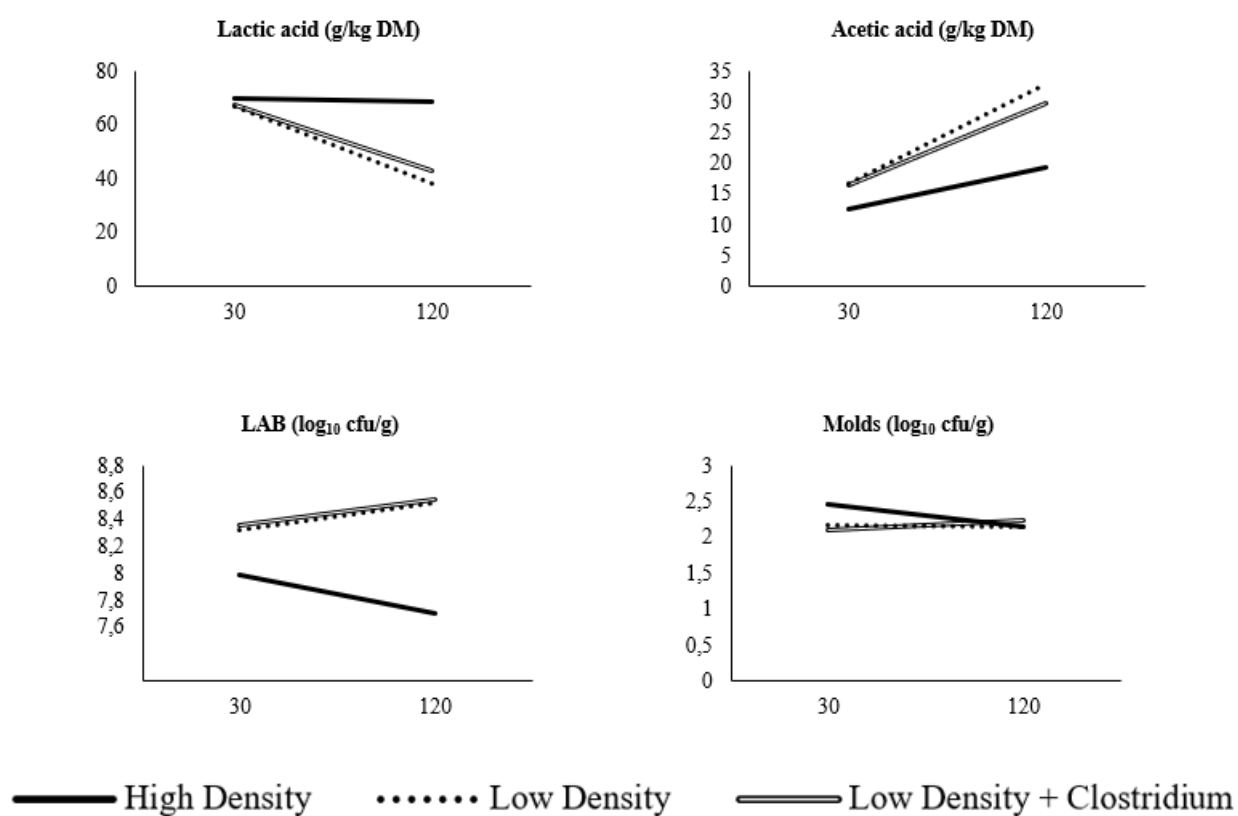


Fig. 6.3. Effect of ensiling conditions and ensiling length on lactic acid, acetic acid, LAB and Molds of corn silages (Ensiling conditions x Ensiling length interaction significant at $P < 0.05$; High Density, Low Density, and Low Density + Clostridium conditions below of the figure).

7. Assess the agro-technical factors and forage feeding value of corn hybrids for silage production carrying the *brachytic2* mutation harvested at different stage of maturity

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Abstract

The aim of this study was to evaluate the forage feeding value of three smart corn hybrids (SCH) with a brachytic2 mutation (i.e., *br2*) mutation in comparison to two conventional corn hybrids (CCH) harvested at 5 different harvest moments [HM (i.e., 114, 119, 125, 135, and 149 days)]. Crop production performances and nutritional values, fiber digestibility, starch digestibility and estimate the energy content (milk/ton) of corn hybrids through MILK2006 and MILK2024 were evaluated. In particular, the five corn hybrids selected for whole-plant silage were grown in an experimental field and harvested at 5 different HM. The field was divided in 18 experimental plots (30 m long x 4 rows x 0.75 m spacing between rows, with 1 row empty buffer zone). The data were analyzed accordingly to a split-plot design with HM (main plot), hybrids (sub-plot) and their first order interaction as main effects. Specific contrasts were used to verify linear or quadratic effects of HM. Notably, CCH (i.e., DKC6731 and DKC7023, respectively) were grown at a density rate of 8.5 plants/m², whereas SCH (i.e., DKC7236SC, EW6558XKL1, and EX7210XKL1, respectively) were grown at a density rate of 13 plants/m². The harvest period started when the earlier hybrid EW6558XKL1 showed the milk line; whereas, the last HM was carried out when EX7210XKL1 showed the black layer. There were 3 intermediate HM where the experimental hybrids were harvest at 1/3, 1/2, and 2/3 milk line, respectively. Fresh yield linearly decreased ($P<0.05$) among the HM, whereas DM yield quadratically increased ($P<0.05$) with HM. The energy content (milk/ton) calculated with the MILK2006 and MILK2024 was different among hybrid ($P<0.05$), with SCH hybrids being characterized by higher values than CCH. Fibrous fractions decreased ($P<0.05$) with the advance of the maturity. The effect of hybrid was significant ($P<0.05$) for NDF, ADF, and ADL, with higher values in CCH than SCH (i.e., +3.85%, 11.0% and 16.2%, respectively). Starch concentration quadratically increased ($P<0.05$) between the HM. The SCH had higher starch content than CCH by 12.3%. NDF digestibility at 12 and 120 hours decreased ($P<0.05$) with the maturity stage and differed among hybrids, with SCH characterized by higher NDF digestibility than CCH. A quadric reduction ($P<0.05$) was detected for the in-situ starch digestibility 7 hour (isSD7 h) when referred to the HM. The isSD7 h showed a significant effect ($P<0.05$) for hybrids; indeed, SCH had a higher isSD7 h than CCH by 12.1%. Considering these results, cropping SCH may be considered as a potential strategy due to the high forage feeding value that can meet the energy requirements of dairy cows. Basing on the results of this trial, harvesting at 135 days after planting (HM 4) may be a potential suggestion to achieve a compromise in terms of DM yield, starch content, and NDF and starch digestibility. Further studies are warranted to elucidate the agronomical advantages of these new corn hybrids and their effects on lactation performance.

Keywords: Corn hybrid, *brachytic2* mutation, fiber digestibility, starch digestibility

Introduction

Whole-plant corn silage (**WPCS**) is the predominant forage used to formulate diets for dairy cattle worldwide (Gallo et al., 2016; Ferraretto et al., 2018). The WPCS production has always been focused on planting hybrids with high grain yield characteristics or dual-purpose hybrids to allow farmers the possibility to harvest corn grain or WPCS based on feed stock availability (Ferraretto and Shaver, 2015). Traditionally, selection aimed to characterize traits such as yield, pest and disease resistance, leafiness, standability, relative maturity, drought tolerance, stay-green ranking and dry-down (Adesogan, 2006). But over the years, specific corn hybrids for silage production have been selected to enhance the nutritional value of WPCS and match the requirements of high-producing dairy cows. The selection of a corn hybrid for silage production should be done considering factors related to digestibility and potential intake by animals. Altering the stalk or kernel characteristics and/or their ratio can be considered as an opportunity to increase digestibility in WPCS (Ferraretto and Shaver, 2015). Increasing the content of cell wall pectin is crucial to enhance forage digestibility due to pectins are more digested than cellulose and hemicellulose in rumen-reticulum compartment (Hatfield and Weimer, 1995; Jung and Engels, 2002). Cell wall composition has an important role in forage digestibility that can impact stalk quality by affecting cell wall structure and strength. Maximizing digestible carbohydrate intake and/or enhance neutral detergent fiber digestibility (**NDFD**) from ensiled crops is crucial due to the energy needed for maintenance and milk production exceeds the amount of energy that high-producing cows can consume, especially in early lactation (Refat and Yu, 2016). Fiber digestibility is strictly dependent on plant maturity, in WPCS the fibrous fraction decline by the advance of maturity stage with no significant change in lignin concentration from early dent to black layer (Owens, 2005). Coors et al. (1997) assumed that the observed decrease in fiber concentration with maturity is due to the dilution effect with increasing grain content as corn plant matures.

Crop varieties affected by dwarfism or semi-dwarfism have different advantages, such as increased lodging resistance, denser growth, and higher harvest index (Hedden, 2003; Li et al., 2011). Many dwarf mutants have been classified by short internodes, which have been referred to impaired internode elongation (Zhang et al., 2019). The stem/internode elongation is mainly regulated by different plant hormones, such as gibberellins, brassinosteroids, strigolactones, and auxins (Wang et al., 2018). Referring to corn, only the category of brachytic mutants expresses a short stature caused by a shortening of the internode length without a reduction in the number of

internodes or the size of other organs (Pilu et al., 2007). Notably, corn hybrids carrying the *brachytic2* (**br2**) mutation are characterized by a reduced plant height through the shortening of internodes and more erected leaves, and a greater standability which allows to prevent yield losses (Pilu et al., 2007; Barten et al., 2022). The *br2* mutation affects the lignin distribution within the stem of whole-plant corn, increasing fiber digestibility and standability of smart corn hybrids (**SCH**) in comparison to conventional corn hybrids (**CCH**) (Bourdoncle et al., 2023).

The objectives of this study aimed to i) assess the nutritive value of three SCH carrying the *br2* mutation in comparison to two CCH harvested at 5 different harvest moments (**HM**, being 114, 119, 125, 135, and 149 days from sowing); ii) evaluate the effect of the *br2* mutation on fiber digestibility, starch digestibility, and lignification of SCH and CCH; and iii) estimate the energy content (milk/ton) of corn hybrids through MILK2006 and MILK2024 feed evaluation systems.

Materials and methods

Five corn hybrids (Bayer AG, Leverkusen, Germany) selected for whole-plant silage were grown in an experimental field located in Cascina Alta-Cumignano sul Naviglio near Cremona, Italy (45°21'31.6"N 9°49'37.2"E). Notably, there were two CCH (DKC6731 and DKC7023) and three SCH (DKC7236SC, EW6558XKL1, and EX7210XKL1).

Field trial site and harvest management

Planting date was April 6th, 2023. The experimental field was fertilized with 100 t/ha of swine slurry; no pre-sowing manuring was applied. The field was divided into 15 experimental plots (30 m long x 4 rows x 0.75 m spacing between rows, with 1 row empty buffer zone). The experimental site was treated with pre- and post-emergence herbicide to prevent weed development. Hybrids, nested within each plant type (e.g., conventional and smart), were then randomized within the conventional and smart blocks. Conventional corn hybrids were grown at a density rate of 8.5 plants/m² and the smart hybrids at a density rate of 13 plants/m², according to manufacturer recommendations. The hybrids were irrigated three times by flood on May 3, June 1, and July 10, 2023; respectively.

The harvest period was scheduled considering five different harvest moments (**HM**) from planting (i.e., 114, 119, 125, 135, and 149 days). For each HM sample moment, 3 samples of fresh chopped plant material were collected. Particularly, the first HM started when the earlier hybrid EW6558XKL1 showed the milk line; whereas the last HM was carried out when EX7210XKL1 showed the black layer. There were 3 intermediate HM where the experimental hybrids were harvest at 1/3, 1/2, and 2/3 milk line, respectively. Each hybrid from plots was harvested with a Kemper Champion C1200 (Maschinenfabrik, Stadtlohn, Germany) chopping the 2 central rows between the five HM. The plot chopping was carried out in 2 steps for each time as follows: only

cobs + only plants (5 plants on lateral rows) and whole plants (5 m length – 2 central rows). In addition, the biomass yield and dry matter (**DM**) yield were detected for each HM and hybrid.

Sample preparation, nutrient and yield analysis

After harvesting, samples were dried in a forced-air oven set at 60°C for 48 h to determine DM concentration and ground to pass a 1-mm Wiley mill (Arthur H. Thomas, Philadelphia, PA). About 250 g of each dried and ground sample was sent to a commercial laboratory (Rock River Laboratory Inc.; Watertown, WI) for analysis of crude protein (**CP**), soluble CP, acid detergent insoluble CP (**ADICP**), neutral detergent insoluble CP (**NDICP**), acid detergent fiber (**ADF**), acid detergent lignin (**ADL**), undigested neutral detergent fiber after 240 h of incubation (**uNDF240**), starch, water-soluble carbohydrates (**WSC**), ether extract (**EE**), and ash using near-infrared reflectance spectroscopy (**NIRS**) with a Foss 5000 (Foss North America Inc., Eden Prairie, MN). Calibration equations for NIRS analysis referred to CP, WSC, starch, ADF, ADL, soluble CP, ADICP, NDICP, EE, and ash were based on procedures described in AOAC method 990.03 (AOAC International, 2012), Dubois et al. (1956), AOAC method 920.39 (AOAC International, 2012), Hall (2015), Möller (2009), AOAC 942.05 (AOAC International, 2012), AOAC method 920.29, respectively. Calibration equations for NIRS analysis of uNDF were based on in vitro incubation procedures reported in Goeser et al. (2009) for 240 h. Yield analysis data for the corn hybrids were run through Milk2006 (Shaver and Lauer, 2006) and Milk2024 (Diepersloot et al., 2024) to estimate milk/ton and were converted to the metric system prior to statistical analysis.

In vitro NDF digestibility estimate

The in vitro NDF digestibility (**ivNDFD**) was evaluated using rumen fluid inoculum collected and pooled from 2 ruminally cannulated lactating dairy cows. Following the method described by Goeser and Combs (2009). The in vitro procedure was conducted under an approved protocol by the Animal Care and Use Committee of the College of Agriculture and Life Sciences, University of Wisconsin-Madison.

Approximately 0.5 g of dried, 1-mm ground whole-plant corn sample was weighed into tared, labeled filter bags with a mean pore size of 35 µm (F57, Ankom Technology, Macedon, NY), which then were heat-sealed. Three repetitions were completed and each repetition included 0-h, 5 digestion time points (12, 24, 30, 48, and 120 h), and filter bag blanks analyzed in duplicate.

At approximately 0630 h on the day of incubation, 0.3125 g of primer was combined with 250 mL of buffer solution and 40 mL of reducing solution in each of five 1,000-mL side-arm Erlenmeyer flasks. The buffer and reducing solutions were prepared as described by Goering and Van Soest (1970). All 1,000-mL flasks were gassed with CO₂ for 15 min while rumen fluid inoculum was collected. At 0630 h, approximately 1 L of rumen fluid was collected from each of 2 ruminally-cannulated, lactating cows into prewarmed, glass-lined thermoses. The donor cows

were fed a 60% forage (alfalfa and corn silage) and 40% concentrate diet at libitum once daily at 0700 h. The rumen fluid inoculum was transported to the laboratory and, in a warm (39°C) room, strained through 4 layers of cheesecloth while being gassed continuously with CO₂. At 0645 h, 250 mL of strained, pooled rumen fluid was added to each of 1,000-mL flasks. All flasks were then sealed with rubber stoppers, set in an incubating (39°C) shaker, and allowed to reach 37.4 mmHg of pressure, which correspond to 30 mL of gas production or 0.12 mL of gas production/mL of rumen fluid inoculum. The amount of gas production to reach 37.4 mmHg was determined through manual calibration by forcing known amounts of gas into a sealed 1,000-mL side-arm flask and measuring corresponding pressures through an electronic pressure sensor. Once flasks averaged 37.4 mmHg per flask, the contents of the 1,000-mL Erlenmeyer flasks were recombined under CO₂ and used to inoculate samples with 50 mL of inoculum solution per flask.

Each day around 1500 h, all remaining corn bags were deflated using a plastic rod while purging the in vitro flask with CO₂. Samples were removed at 12, 24, 30, 48, and 120 h after inoculation. Fermentations finished by rinsing the bags with ambient-temperature distilled water until the effluent was clear, as described by Eun et al. (2007). The neutral detergent fiber (NDF) concentration (%DM) was determined by using a neutral detergent solution containing α-amylase and sodium sulfite, following the procedure reported by Goering and Van Soest (1970) adapted for an Ankom 200 Fiber Analyzer (Ankom Technology). The NDF percentages were determined using the following equation:

$$\text{NDF (\% of DM)} = \frac{[(\text{bag wt} + \text{residue}) - (\text{bag wt} \times \text{bag correction factor})]}{[(\text{bag wt} + \text{sample}) - (\text{bag wt})]} \times 100.$$

The bag correction factor is the weight of an empty, sealed bag divided by the weight of the same bag after undergoing the ruminal in vitro and NDF procedure. Neutral detergent fiber digestibility was determined using the following equation:

$$\text{NDFD (\% of NDF)} = 100 \times \frac{(\text{NDF}_{0\text{h}} - \text{NDF}_{\text{residue}})}{(\text{NDF}_{0\text{h}})}.$$

In situ starch digestibility 7 h

Measurements of in situ starch digestibility (**isSD**) were carried out at the University of Wisconsin campus tie-stall barn (Dairy Cattle Center, Madison, WI) under a protocol approved by the Animal Care and Use Committee of the College of Agricultural and Life Sciences. To evaluate isSD, the dried whole-plant corn samples were ground to pass through a 4-mm screen in a Willey mill (Arthur H. Thomas, Philadelphia, PA). Dacron polyester cloth bags (R1020, 10 x 20 cm and 50 ± 10 microporosity; Ankom Technology, Macedon, NY, USA) containing 5.00 ± 0.25 g of DM were used. Two ruminally-cannulated lactating cows fed a 60% forage (alfalfa and corn silage) and 40%

concentrate diet were used. The in situ bags were placed in a nylon laundry bag (30 x 40 cm) and then positioned in the ventral rumen for 7 h. Each laundry bag was attached to the inside of the rumen cannula with a 0.75 m long nylon rope and contained a rubber weight to ensure they remained submerged in the ruminal contents. Bags were moistened in warm water prior to incubation. Blank bags were also incubated to allow correction for any infiltration of DM into samples bags. After removal, samples were soaked in ice water for 15 min to inhibit ruminal microbial activity and rinsed with room temperature tap water to wash off any large particles adhered to the bags. The bags were then placed in clean laundry bags for further rinsing in a washing machine (Roper RTW4516F*, Whirlpool Corp., Benton Harbor, MI, USA) set on the rinse and spin cycle with room temperature water for 30 min. The bags were dried in a forced-air oven at 60°C for 48 h. Residues were ground through a 1 mm Udy mill screen (Udy Corp., Boulder, CO, USA) for starch analysis. Duplicates within cows were composited into 1 sample before nutrient analysis. The mean starch disappearance value of the 2 bags on a treatment per incubation time was calculated for each cow. Samples were submitted to the same commercial laboratory and analyzed for starch as described previously.

Statistical analysis

Data were analyzed using a split-plot design with HM (five levels) as the main plot factor and Hybrid (five levels) as the subplot factor. A mixed model approach was employed to account for the random effects of replications (**rep**). Analyses were conducted using the PROC GLIMMIX procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). The fixed effects included HM, hybrid, and their interaction (HM x Hybrid). The random effect of rep was included to appropriately model the experimental design. The statistical model was formulated as follows:

$$Y_{ijk} = \mu + HM_i + Hybrid_j + (HM \times Hybrid)_{ij} + rep_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} = the response variable, μ = the overall mean, HM_i = represents the fixed effect of harvest moment, $Hybrid_j$ = denotes the fixed effect of corn hybrid, $(HM \times Hybrid)_{ij}$ = indicates the interaction between HM and Hybrid, rep_{ij} = the random effect of replication, ε_{ijk} = the residual error. Specific contrasts were tested to examine differences among hybrids and HM. Orthogonal contrasts were used to test specific hypotheses. Additionally, linear and quadratic trends for harvest time were evaluated using pre-specified orthogonal coefficients, obtained by using IML procedure of SAS (version 9.4). Least squares mean (**LSMEANS**) were estimated for the levels of HM, hybrid, and their interaction. Pairwise comparisons were conducted using the diff option to identify significant differences. Additionally, the slice option was used to investigate simple effects of HM within each level of hybrid and vice versa. Statistical significance was declared at $P < 0.05$.

Results

Fresh yield and dry matter yield

Data for fresh and DM yields are reported in Table 1. The fresh yield decreased ($P < 0.05$) with the advance of maturity. Notably, the highest value of fresh yield at 114 days from planting (i.e., 97.4 t/ha) and the lowest at 149 days from planting (i.e., 73.9 t/ha). The DM yield increased ($P < 0.05$) among the HM. Interaction of HM with hybrids showed significant effects ($P < 0.05$) on fresh and DM yields (Fig. 1). Notably, the conventional hybrids decreased fresh yield from HM 1 to 3 and had higher fresh yield than smart hybrids at HM 5. Smart hybrids fresh yield production peaked at HM 3. Regarding DM yield, both conventional and smart hybrids quadratically increased ($P < 0.05$) with HM, except DKC6731 and EX7210XKL1 that showed the lowest ($P < 0.05$) DM yield at HM 2. In addition, greater ($P < 0.05$) DM yields for EX6658XKL1 and DKC7236SC were reported in comparison to conventional hybrids at HM 3. No effects of hybrids on fresh and DM yield were reported.

Nutrient composition and MILK2006-2024 calculation

Table 2 reports the effect of HM, hybrids and their interaction on nutrient composition and MILK2006 or MILK2024 index calculations.

The HM had an effect ($P < 0.05$) on several nutritional parameters as well as MILK2024. The highest DM content was detected after 149 days from planting (i.e., 47.0%), whereas the lowest after 114 days from planting. Notably, DM quadratically increased ($P < 0.05$) among the HM. The CP concentration quadratically decreased ($P < 0.05$) between the different HM; in particular, CP concentration decreased by the 8.7% from 114 to 149 days. Indeed, the interaction of HM and hybrid showed significant effects ($P < 0.05$) on CP (Fig. 2). All the hybrids performed a decreasing trend among the different HM. Specially, EX6658XKL1 and EX7210XKL1 had higher CP content than the other hybrids at HM 2 and 3 (9.2% and 9.1%, respectively; 9.3% and 8.8%, respectively). On the other hand, conventional hybrids had the higher CP concentration than smart hybrids at HM 5. A quadratic decrease ($P < 0.05$) soluble CP was detected; particularly, soluble CP decreased by the 17.8% from 114 to 135 days; however, an increase by the 12.4% between 135 and 149 days was observed. The NDICP linearly decreased ($P < 0.05$) among the HM. A quadratic decrease ($P < 0.05$) in NDF fraction was observed by the advance of maturity; especially, NDF decreased by the 16.9% from 114 to 135 days. The ADF quadratically decreased ($P < 0.05$) among the different HM; in particular the lowest ADF content was reported at 135 days after planting (i.e., 20.9 %DM). The ADL quadratically decreased ($P < 0.05$) by the 14.6% between 114 and 135 days. Starch quadratically increased ($P < 0.05$) by advancing the stage of maturity, reaching the highest value at 135 days after planting (i.e., 39.1 %DM). The WSC quadratically decreased ($P < 0.05$) by the 41.8%

from 114 to 135 days. The EE quadratically increased ($P < 0.05$) with the advance of maturity. The concentration of ash showed a quadratic decrease ($P < 0.05$) among the HM. Calculation of the new MILK2024 reported a quadratic increase ($P < 0.05$) by advancing the maturity stage, reaching a peak at 135 days (i.e., 3023.4 kg/ton DM).

The hybrid had a significant effect ($P < 0.05$) on several nutritional parameters as well as MILK indexes. The EX6658XKL1 was the hybrid with the highest DM content (i.e., 37.7%). Regarding the CP, the DKC7236SC and DKC6731 were the hybrids with the lowest CP content (i.e., 8.5 and 8.6 %DM, respectively). Soluble CP was on average higher for CCH than SCH (e.g., 41.1 ± 1.06 and 36.1 ± 1.06 %CP, respectively). The NDF fraction was higher in CCH in comparison to SCH; particularly, DKC6731 was the hybrid with the highest NDF concentration (i.e., 44.6 ± 0.65 %DM), whereas EX6658XKL1 had the lowest value (i.e., 40.5 ± 0.65 %DM). The ADF was higher in CCH than SCH; in particular, the ADF concentration for SCH was on average 11.0% lower than CCH. The ADL content was lower by the 16.2% in comparison to CCH. The SCH were characterized by high starch content; notably, the starch concentration was higher in SCH by the 12.3% in comparison to CCH. The CCH had higher WSC in comparison to SCH; especially, the DKC6731 had the highest values (6.0 ± 0.23 % DM, respectively). The SCH had higher EE content than CCH. Minimal differences for ash content were observed between hybrids; moreover, smart hybrids had lower ash content than conventional. Especially, the DKC7236SC had the lowest ash content (4.0% DM). For the MILK2006 calculation, the DKC7236SC reported the highest milk yield per ton of DM (2622.9 kg/ton DM); whereas, the DKC7023 the lowest (2403.3 kg/ton DM). Referring to MILK2024, SCH showed a higher milk yield per ton in comparison to CCH; specially, the DKC7236SC was the hybrid with the highest value (2744.8kg/ton DM).

Analysis of the interaction of HM with hybrid showed significant effects ($P < 0.05$) on ADICP (Fig. 2). Particularly, a decreasing trend from 125 to 149 days was observed in all hybrids. Moreover, CCH had the higher ADICP content in the last three HM (e.g., 125, 135, and 149 days, respectively).

Nutrients digestion

Values related to the effects of HM, hybrids and their interaction on nutrients digestion are reported in Table 3.

Except for NDF digestibility after 120 hour of incubation, the HM had a significant effect ($P < 0.05$) on the other parameters. The NDF digestibility after 12 hour of incubation quadratically decreased ($P < 0.05$) by the 21.7% from 114 to 125 days. A quadratic decrease ($P < 0.05$) in NDF digestibility after 24 hour of incubation was observed between 114 and 135 days; particularly, the NDF digestibility during this period decreased y the 19.1%. Contrarily, a quadratic and linear increase ($P < 0.05$) was observed in NDF digestibility after 30 and 48 hour of incubation from 114

to 149 days (e.g., 17.8% and 19.8%, respectively). Starch digestibility quadratically decreased ($P < 0.05$) among the HM. Notably, the highest isSD7h was detected at HM 1 (i.e., 73.6% starch); whereas the lowest at HM 5 (i.e., 31.9% starch). The uNDF quadratically decreased ($P < 0.05$) between 114 and 135 days by the 7.0%.

Referring to the hybrid, significant effect ($P < 0.05$) was observed on NDF digestibility after 30, 48, and 120 hour after incubation, isSD7h, and uNDF. Considering the NDF digestibility for the mentioned time point, SCH had higher digestible fiber than CCH. Particularly, the EX6658XKL1 hybrid had the highest values of digestible fiber at 30 and 48 hour (i.e., 43.4% and 58.8% NDF, respectively); whereas, the DKC6731 hybrid reported the lowest values of digestible fiber at 30, 48 and 120 hour (i.e., 38.5%, 52.6% and 63.9 %NDF, respectively). On the other hand, the EX7210XKL1 had the highest value of digestible fiber at 120 hour (i.e., 69.7 %NDF). The SCH had higher isSD7h compared to CCH. Especially, the EX7210XKL1 had the highest value for isSD7h (i.e., 65.9% starch). On the other hand, DKC6731 was the hybrid with the lowest isSD7h (i.e., 52.7% starch). Regarding the uNDF, SCH had lower uNDF than conventional; especially, the EX7210XKL1 had the lowest uNDF at 240 hours (8.9% DM).

Discussion

Choosing hybrid to make silage that can fulfill yield and nutritive value to formulate diets for dairy cows is crucial. Hybrid selection, specially corn hybrids for silage production, has been done with the purpose to improve agronomic response without considering animal performance (Bal, 2006). Understanding the effects of WPCS hybrids in dairy cow diets may provide dairy producers to make better decisions on hybrid selection indicating potential strategies for the seed corn industry in relation to WPCS hybrids (Ferraretto and Shaver, 2015). In the last decades, many seed corn industries have been selected corn hybrids to enhance the nutritional composition of WPCS and match the requirements of high-producing dairy cows. Corn brown mid-rib (BMR) hybrids carrying the *bm3* mutation are known to have a low lignin content in stover tissues, which is usually related to an increasing the cell wall digestibility in comparison to other mutations improving cattle feeding value (Allen et al., 2003). Corn hybrids carry the *br2* mutation are characterized by a reduced plant height due to the shortening of the internodes and the presence of more erected leaves (Pilu et al., 2007). In the current study, three SCH carrying the *br2* mutation have been compared with two CCH selected for silage production to evaluate productivity (e. g., fresh and dry yield, and milk production), nutrient composition, ivNDFD and isSD7h in five different harvest moments.

Fresh yield and dry matter yield

Maturity at harvest influences several crop factors such as yield, nutrient composition, digestibility and ensilability of crop (Ferraretto et al., 2018). Fresh yield decreased among the HM for all the hybrids, but no difference for the hybrid effects were observed in the study. The DM yield increased during the HM reaching a peak at HM 5, as expected. Indeed, delaying harvest moment has been reported as a possible strategy to increase DM yields per hectare (Neumann et al., 2020). No differences between hybrids have been detected during the trial. Similar results were reported in different trials in which different corn hybrids were compared. For instance, Peña et al. (2023) evaluated the effects of corn-planting population, comparing different corn hybrids. The authors observed that corn hybrids with the BMR trait did not improve or penalize DM yield.

Nutrient composition and MILK2006-2024 calculation

Enhancing DM content at harvest is crucial due to during the maturation of the corn plant, sugars in kernels are converted to starch increasing the DM concentration of kernels (Allen et al., 2003). In this trial a linear increase in DM content was observed among the HM. Moreover, harvesting whole-plant corn hybrid for silage with a proper DM content is recommended to optimize nutritive value of the ensiled crop and dairy cows performance (Ferraretto et al., 2018). The CP concentration decreased with the advance of maturity, with high concentration in the first HM and a low content for HM 5. Indeed, crops harvested too early present low concentrations of DM, high CP and fiber content, and low level of starch in kernel (Ferreira and Brown, 2016). Nevertheless, differences between hybrids have been reported in this study, values for CP ranged among the data present in interested literature (Gallo et al., 2016; Tharangani et al., 2021). The HM and hybrid affected soluble CP content. The soluble CP was higher in the early stage of maturity which finds an explanation in high CP content in the first two HM of the trial (Tamminga et al., 1991; Van Vuuren et al., 1991). The SCH had lower soluble CP than conventional. This variation may be explained considering some different mutations in endosperm due to a different zein levels that may affect starch digestibility of WPCS. The WPCS quality depends on stover portion, due to 40 to 50% of the whole-plant consists in stover, that is high in NDF, ADF, and lignin (Hunt et al., 1992). The fibrous fractions decreased with the advance of maturity. Delaying the harvest period for WPCS has been related to an increase in DM and starch content followed by a decline in CP, NDF, and ash concentration (Allen et al., 2003; Buxton and O'Kiely, 2003). In this study SCH exhibited lower NDF and ADF concentration than conventional hybrids. Similar results have been observed by Bourdoncle et al. (2023), in which smart corn had a lower NDF and ADF content than tall and BMR hybrids. Coons et al. (2019) evaluated the inclusion of a *bm3* corn hybrid with floury kernel genetics in comparison to conventional and *bm3* corn hybrids in dairy cow diets. In this study, the hybrids carrying the *bm3* mutation had lower NDF and ADF concentration than the conventional hybrid. Ferraretto and Shaver (2015) observed that concentration of NDF and ADF

were similar between conventional, BMR, high fiber digestibility, and leafy corn hybrids. Peña et al. (2023) reported that the concentration of NDF was slightly lower in conventional corn hybrid than BMR. The lignin concentration decreased with the advance of maturity. Gentinetta et al. (1990) evaluated 21 near-isogenic normal and *bm3* hybrids for forage quality and yields. The *bm3* had lower lignin content compared to the normal hybrids (38 and 60 g/kg DM, respectively); whereas DM yields for *bm3* hybrids were considerably lower than normal hybrids (14.4 and 17.9 Mg/ha, respectively). This decreasing trend in WPCS is generally followed by an increase in DM and starch concentration. Xu et al. (1995) documented the change in yield, plant physical and chemical composition, and whole-plant and stover digestibility for WPCS hybrids harvested at three stages of maturity. The authors concluded that as maturity advanced from milk to black layer the NDF, ADF, and lignin decreased. Referring to hybrids effect, lower lignin concentrations were found in SCH compared to CCH. Lignin has always been considered a limiting factor in ruminants nutrition due to its relationship with cellulose and hemicellulose, which creates a physical barrier, obstructing the degradation process of the rumen microorganisms (Hatfield, 1993). The low concentration in SCH may find an explanation in the suppression of the internode elongation which is operated by the *br2* mutation. Notably, the *br2* has the ability to encode a P-glycoprotein1 protein, regulating the auxin flux from node to internode and avoiding the auxin accumulation in the intercalary meristem region to suppress the elongation of internodes (Zhang et al., 2019). Bourdoncle et al. (2023) observed that the lignin composition in corn hybrids carrying the *br2* mutation was 4.3% points lower than conventional corn. Starch concentration increased with the advance of maturity due to the conversion of sugars in kernels, which finds an explanation with the increase of DM content (Johnson et al., 2002). As maturity progresses, the kernel proportion increases and sugar in the amyloplasts celles of kernels is converted to starch during ear development until physiological maturity, at which time the kernel reaches its maximum in terms of DM weight (Gusmão et al., 2021). Differences in starch content have been observed between hybrids; specially, starch concentration was higher in SCH than CCH. The change in starch composition is related to the selection of hybrids with starch with a higher amylopectin content than amylose or hybrids with a more floury endosperm (Giuberti et al., 2014). For the best our knowledge, only Bourdoncle et al. (2023) evaluated the nutrient composition of these smart hybrids which present higher starch concentration in comparison to BMR and conventional corn hybrids. The concentration of sugars declined with the advance of maturity due to their conversion in starch within kernel followed by an increase in DM of the crop (Allen et al., 2003). Considering sugars composition between the tested hybrids, CCH had a higher sugars concentration in comparison to SCH; on the other hand, this difference can explain the lower

starch concentration in the conventional corn respect to the smart hybrids. However, data for ether extract presented differences between HM and hybrid effects, these values were not considered as biologically relevant. Ash slightly decreased among the HM. Nevertheless, ash content differed between hybrids, these values were not considered as biologically relevant, even if ash content ranged among the data reported by Hoffman (2005). Energy content of corn hybrids (milk/ton) were predicted using the MILK2006 and MILK2024 indexes. For both Milk indexes, SCH reported a higher energy content (milk/ton) in comparison to CCH. Bourdoncle et al. (2023) reported no difference in terms of energy content between SCH and BMR; however, energy content was higher in SCH than BMR (3627 and 3569 kg/ton, respectively).

Nutrients digestion

Differences in ruminal ivNDFD at 12, 24, 30 and 48 hour were detected for the HM effect. Fiber digestibility is affected by the stage of maturity because the tissues of crop have a different digestibility rates in the early and advanced phase (Adesogan et al., 2019). Regarding the hybrid effect, differences in NDFD at 30, 48, and 120 hours were observed. High NDFD in SCH is related to a higher leaves/stalk ratio and a different deposition of lignin (Bourdoncle et al., 2023). Catellani et al. (unpublished data) evaluated the NDF digestibility after 48 h of rumen incubation testing a smart corn hybrid carrying the *br2* mutation in comparison to a conventional corn hybrid. The authors observed an increasing in NDFD for the smart hybrid in comparison to the conventional hybrid by 6-7% NDFD 48h. Bourdoncle et al. (2023) observed that SCH had a high ivNDFD at 30 h when compared to conventional hybrids; however, no differences were observed in comparison to BMR corn hybrids. Ferraretto and Shaver (2015) reported a better ivNDFD at 30 h and 48 for BMR corn hybrids, but no differences were observed in conventional, dual-purpose or normal fiber digestibility hybrids compared with high fiber digestibility hybrids. Nevertheless, it has been examined that there are some BMR varieties that are less or equal digestible than conventional hybrids (Bean and McCollum III, 2006). Bourdoncle et al. (2023) investigated the forage feeding value of corn hybrids with the *br2* mutation evaluating fiber digestibility and stem lignification. The authors concluded that hybrids with the *br2* mutation increased fiber digestibility due to the ability to affect the distribution of lignin within the stem of corn. The isSD7 declined with the advance of the maturity which it is related to an accumulation of starch in the kernel fractions with a consequently increase of DM content and a reduction in NDF (Allen et al., 2003). Late stage of maturity in WPCS impairs starch digestibility in lactating dairy-cows (Ferraretto and Shaver, 2012), which is explained by the increase in kernel vitreousness (Correa et al., 2002) and the vitreous to floury endosperm ratio, that might be related to increased zein proteins cross-linked to starch granules (Giuberti et al., 2014). Jensen et al. (2005) evaluated the effects of maturity stage of WPCS harvested with three different levels of DM on digestion of starch and NDF. The authors

stated that increase maturity at harvest corresponded to a high starch content, but starch digestibility decreased with maturity. Similar results have been reported by Saylor et al. (2021) stating a greater isSD for the early maturity stage (1/4 milk line) in comparison to the other harvest maturities (1/2 and 3/4 milk line, respectively). The type of hybrid also affected the isSD. Particularly, SCH had the greater starch degradability. Variations in isSD 7h between corn hybrids for snaplage production with different proportions of vitreous and floury endosperm were reported by Gusmão et al. (2021). In this study, hybrids with lower vitreous endosperm had greater starch degradability. Actually, there are no studies about starch digestibility for corn hybrids carrying *br2* mutation, therefore more studies are needed to clarify this scenario. Hybrids and HM uNDF affected at 240 h. The uNDF declined as maturity advanced. Considering the hybrids effect, SCH had lower values than conventional hybrids for uNDF 240h. The low uNDF content can be explained considering the lower lignin levels in smart hybrids that might explain the greater NDF digestibility (Oba and Allen, 1999). Low uNDF contents were reported by Ferraretto et al. (2015) in BMR corn hybrids used to formulate dairy cows diets in comparison to floury-leafy corn hybrids.

Conclusion

Selecting corn hybrids for silage production taking in care both yield and forage feeding value is crucial for dairy farmers and silage producers. Strategies to enhance fiber and starch digestibility of corn hybrids for silage production have been carried out in the last decades. Corn hybrids carrying the *br2* mutation characterized by a reduced plant height through the shortening of internodes and more erected leaves, and conventional corn hybrids harvested at 5 different harvest moments were compared to assess the forage feeding value, evaluate fiber and starch digestibility, and estimate the energy content. Fresh yield showed a decreasing trend with the advance of maturity; on the other hand, DM yield increased the maturity. However, no differences between hybrids were detected for the yield parameters. The SCH reported higher energy content (milk/ton) than CCH. Fibrous fractions of SCH were lower than CCH due to the high leaves/stalk ratio. The SCH were characterized by a higher starch content compared to CCH. The ivNDFD at 30, 48, and 120 hour was higher in SCH than CCH. The SCH had a greater isSD7 h than CCH by 13.7%, that may be related to a better conversion within the kernel fractions that makes starch of smart hybrids more digested than conventional hybrids. Considering these results, cropping SCH may be considered as a interesting strategy due to the high forage feeding value that can meet the energy requirements of dairy cows. In this study, SCH had low fibrous fractions, high starch content, a better isSD when compared with CCH. In addition, low uNDF at 240 h were observed in SCH, highlighting the fact that enhancing fiber digestibility is a key factor to improve WPCS.

Basing on the results of this trial, harvesting at 135 days after planting (HM 4) may be a potential suggestion to achieve a compromise in terms of DM yield, starch content, and NDF and starch digestibility. Further studies are warranted to elucidate the agronomical advantages/disadvantages of these new corn hybrids and their effects on lactation performance.

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Table 7.1. Effect of harvest moment and hybrid on fresh yield, dry matter (DM) yield, MILK2006-2024 calculation of whole-plant corn silage (WPCS)

Item	Units of measurement	Harvest Moment (Days)					Hybrid					SEM	Effect of the model (P<0.05)			Contrasts	
		114	119	125	135	149	DKC6731	DKC7023	DKC7236SC	EX6658XKL1	EX7210XKL1		Harvest	Hybrid	HM x H	Linear	Quadratic
Fresh Yield*	t/ha	97.4 ^a	87.7 ^b	87.8 ^b	79.5 ^c	73.9 ^d	82.4	87.2	85.5	83.2	88.1	1.76	<0.05	0.11	<0.05	<0.05	n.s.
DM Yield*	t/ha	25.7 ^c	25.5 ^c	30.2 ^b	34.1 ^a	34.7 ^a	29.6	30.4	29.5	30.5	30.1	0.55	<0.05	0.62	<0.05	<0.05	<0.05

DM, dry matter

* Data from Bayer Italy

Superscripts indicated that the specific mean was higher (i.e., a) or lower (i.e., b, c, and d) than a different harvest moment or hybrid at a P<0.05. When the interaction was significant (i.e., P < 0.05) and biologically relevant, data were presented to show effects of harvest moment for each response.

Table 7.2. Effect of harvest moment and hybrid on nutrient composition of whole-plant corn silage.

Items	Units of measurement	Harvest Moment (Days)					Hybrid					SEM	Effect of the model (P<0.05)			Contrasts	
		114	119	125	135	149	DKC6731	DKC7023	DKC7236SC	EX6658XKL1	EX7210XKL1		Harvest	Hybrid	HM x H	Harvest Moment	
																Linear	Quadratic
DM*	%	26.4 ^a	29.0 ^d	34.4 ^c	42.9 ^b	47.0 ^a	36.4 ^b	35.2 ^c	35.3 ^c	37.7 ^a	35.3 ^c	0.37	<0.05	<0.05	0.16	<0.05	<0.05
CP [§]	%DM	9.2 ^a	8.9 ^b	8.7 ^c	8.4 ^d	8.3 ^d	8.6 ^b	8.7 ^a	8.5 ^b	8.9 ^a	8.8 ^a	0.06	<0.05	<0.05	<0.05	<0.05	<0.05
Soluble CP [§]	%CP	43.1 ^a	36.6 ^c	35.9 ^c	35.4 ^c	39.8 ^b	42.0 ^a	40.3 ^a	36.5 ^b	35.5 ^b	36.4 ^b	1.06	<0.05	<0.05	0.95	n.s.	<0.05
ADICP [§]	%DM	0.53	0.51	0.52	0.57	0.51	0.51	0.54	0.53	0.55	0.52	0.017	0.10	0.54	<0.05	n.s.	n.s.
NDICP [§]	%DM	1.4 ^a	1.5 ^a	1.5 ^a	1.3 ^b	1.2 ^b	1.5	1.4	1.3	1.4	1.3	0.04	<0.05	0.08	0.83	<0.05	n.s.
ADF [§]	%DM	23.8 ^a	22.9 ^a	23.1 ^a	20.9 ^b	23.2 ^a	24.2 ^a	24.6 ^a	21.8 ^b	21.6 ^b	21.7 ^b	0.50	<0.05	<0.05	0.88	n.s.	<0.05
NDF [†]	%DM	46.6 ^a	44.1 ^b	41.5 ^c	38.7 ^d	40.0 ^{cd}	44.6 ^a	44.3 ^a	40.7 ^b	40.5 ^b	40.8 ^b	0.65	<0.05	<0.05	0.73	<0.05	<0.05
ADL [§]	%DM	4.1 ^a	3.9 ^{ab}	3.8 ^b	3.5 ^c	3.9 ^{ab}	4.2 ^a	4.3 ^a	3.6 ^b	3.6 ^b	3.5 ^b	0.08	<0.05	<0.05	0.85	n.s.	<0.05
Starch [§]	%DM	29.5 ^c	33.2 ^b	33.7 ^b	39.1 ^a	34.3 ^b	31.4 ^b	31.8 ^b	35.4 ^a	35.6 ^a	35.6 ^a	0.80	<0.05	<0.05	0.89	<0.05	<0.05
WSC [§]	%DM	5.5 ^a	3.5 ^c	3.7 ^{bc}	3.2 ^c	4.2 ^b	6.0 ^a	4.2 ^b	3.5 ^c	3.3 ^c	3.2 ^c	0.23	<0.05	<0.05	0.41	<0.05	<0.05
Ether extract [§]	%DM	2.5 ^b	2.6 ^b	2.8 ^a	2.7 ^a	2.8 ^a	2.5 ^b	2.5 ^b	2.8 ^a	2.7 ^a	2.8 ^a	0.04	<0.05	<0.05	0.80	<0.05	<0.05
Ash [§]	%DM	4.3 ^a	4.3 ^a	4.3 ^a	3.8 ^c	4.0 ^b	4.1 ^b	4.3 ^a	4.0 ^b	4.1 ^b	4.2 ^{ab}	0.06	<0.05	<0.05	0.25	<0.05	<0.05
Milk2006 [†]	kg/ton DM	2502.6	2496.4	2553.2	2559.0	2570.5	2475.4 ^b	2403.3 ^c	2622.9 ^a	2580.8 ^a	2599.2 ^a	30.71	0.05	<0.05	0.53	<0.05	n.s.
Milk2024 [†]	kg/ton DM	2893.4 ^b	2924.8 ^b	2956.0 ^b	3023.4 ^a	2935.5 ^b	2599.3 ^b	2569.2 ^b	2744.8 ^a	2719.9 ^a	2732.4 ^a	46.33	<0.05	<0.05	0.81	n.s.	<0.05

ADF, acid detergent fiber; ADL, acid detergent lignin; NDF, assayed with a heat stable amylase and expressed inclusive of residual ash; CP, crude protein; ADICP, acid detergent insoluble CP; NDICP, neutral detergent insoluble CP; DM, dry matter; WSC, water soluble carbohydrates.

* Data from Bayer Italy

[†] Data from UW Madison

[§] Data from Rock River Laboratory

Superscripts indicated that the specific mean was higher (i.e., a) or lower (i.e., b, c, and d) than a different harvest moment or hybrid at a P<0.05. When the interaction was significant (i.e., P < 0.05) and biologically relevant, data were presented to show effects of harvest moment for each response.

Table 7.3. Effect of harvest moment and hybrid on nutrients digestion of whole-plant corn silage.

Item	Units of measurement	Harvest Moment (Days)					Hybrid					SEM	Effect of the model (P<0.05)			Contrast	
		114	119	125	135	149	DKC6731	DKC7023	DKC7236SC	EX6658XKL1	EX7210XKL1		Harvest Moment	Hybrid	HM x H	Harvest Moment	Quadratic
NDFD _{12h} [†]	%NDF	19.3 ^a	18.9 ^a	15.1 ^b	16.4 ^b	15.8 ^b	16.4	16.7	18.4	17.0	17.0	0.89	<0.05	0.57	0.90	<0.05	<0.05
NDFD _{24h} [†]	%NDF	30.8 ^a	29.1 ^{ab}	25.9 ^c	24.9 ^c	27.7 ^{bc}	26.5	25.5	28.4	29.4	28.7	1.06	<0.05	0.06	0.07	<0.05	<0.05
NDFD _{30h} [†]	%NDF	39.7 ^c	41.8 ^b	38.9 ^c	40.4 ^b	46.8 ^a	38.5 ^b	40.8 ^{ab}	42.8 ^a	43.4 ^a	42.0 ^a	0.93	<0.05	<0.05	0.62	<0.05	<0.05
NDFD _{48h} [†]	%NDF	52.0 ^c	54.5 ^{bc}	55.8 ^b	57.0 ^b	62.3 ^a	52.6 ^b	55.5 ^{ab}	56.4 ^a	58.8 ^a	58.3 ^a	1.23	<0.05	<0.05	0.55	<0.05	n.s.
NDFD _{120h} [†]	%NDF	66.2	66.8	67.3	67.9	68.4	63.9 ^b	64.3 ^b	69.6 ^a	69.2 ^a	69.7 ^a	0.57	0.06	<0.05	0.47	<0.05	n.s.
isSD7h [†]	%starch	73.6 ^a	68.0 ^b	60.8 ^c	64.4 ^{bc}	31.9 ^d	52.7 ^d	57.7 ^c	63.1 ^{ab}	59.4 ^{bc}	65.9 ^a	1.29	<0.05	<0.05	0.64	<0.05	<0.05
uNDF _{240h} [§]	%DM	10.0 ^{ab}	9.9 ^{bc}	9.6 ^{bc}	9.3 ^c	10.7 ^a	11.1 ^a	11.5 ^a	9.1 ^b	9.0 ^b	8.9 ^b	0.37	<0.05	<0.05	0.82	n.s.	<0.05

isSD7h, *in situ* Starch disappearance 7 hour; NDF, neutral detergent fiber; NDFD_{12,24,30,48,120h} (NDF digestibility after 12, 24, 30, 48, and 120 hour of incubation); uNDF_{240h}, indicates the part of the fiber that has not been digested after 240 hours.

[†] Data from UW Madison

[§] Data from Rock River Laboratory

Superscripts indicated that the specific mean was higher (i.e., a) or lower (i.e., b, c, and d) than a different harvest moment or hybrid at a P<0.05. When the interaction was significant (i.e., P < 0.05) and biologically relevant, data were presented to show effects of harvest moment for each response.

Graphs: Harvest moment x Hybrid

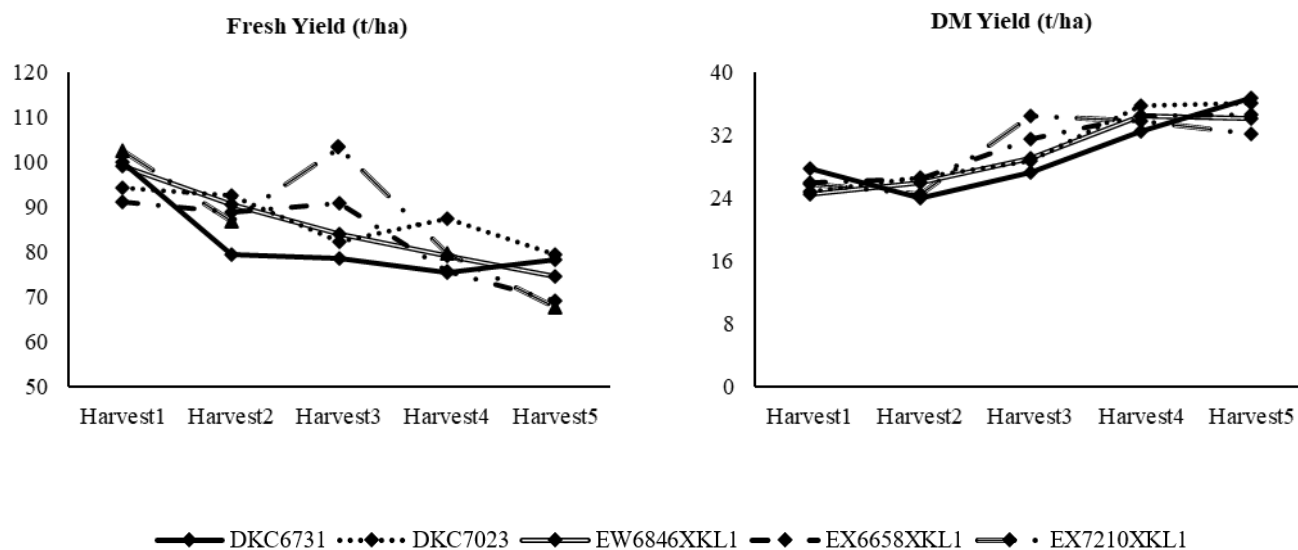


Fig. 7.1. Effect of harvest moment and hybrid on fresh and DM yield of whole-plant corn silage.

Graphs: Harvest moment x Hybrid

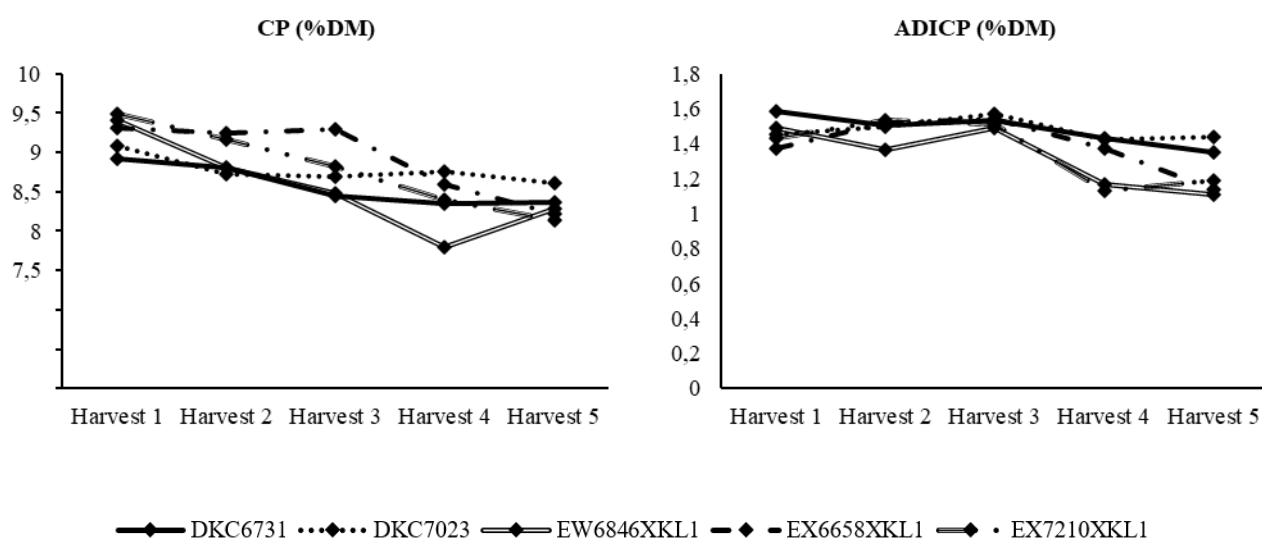


Fig. 7.2. Effect of harvest moment and hybrid on CP and ADICP of whole-plant corn silage.

Conclusion and future perspectives

Producing high-quality WPCS is the main challenges in which silage producers and farmers are involved to fulfill the requirements of dairy and beef cattle. To obtain a high-quality silage it is crucial to properly manage the whole ensiling process from field to silo. Notably, both farmers and silage producers have to consider four key factors:

Harvest and store in a prompt manner to achieve high quality;

Avoid dry matter losses;

Take care during the staging and sealing practices of the bunker or wrapping practice for round bale;

Manage the silo surface during the feed-out operations.

Among the whole process, silage production is dependent on both controllable and uncontrollable factors. The management factors that considered as controllable are related to forage species, stage of maturity at harvest, harvesting and ensiling methods, storage structures, use of silage additives, feed-out and bunk management operations, and diet formulation. On the other hand, uncontrollable factors are linked to climate conditions in different part of the world, such as warm or cold regions that can negatively affect silage production and utilization.

This Thesis aimed to highlight the main challenges in which farmers and silage producers are involved to produce high-quality silage. In particular in this Thesis all the abovementioned aspects have been discussed through i) a review referred to mycotoxins occurrence in ruminant systems; ii) a farm trial in which an inoculant combination with sodium benzoate was tested to reduce the aerobic deterioration of the top layer of bunker; iii) evaluating the effects of lactic-acid bacteria inoculants on fermentative, chemical, and aflatoxin contamination in whole-plant corn silage packed at different levels of density; and iv) determining the forage feeding value of three smart corn hybrids carrying the *br2* mutation in comparison to two conventional corn hybrids harvested at 5 different harvest moment.

The review provided a summary referred to the presence and concentrations of regulated and emerging mycotoxins in silages and other forages. The correlation between the incidence of regulated and emerging mycotoxins with the main silage characteristics and microbial silage communities should be deeply investigated. In particular, evaluating the role of different bacterial inoculants in preventing mycotoxin contamination during the different stages of ensiling should be studied to prevent mycotoxin ingestion by animals, safeguarding their health, welfare and performance. In addition, future trials may examine different indicators of silage safety by measuring VOC and mycotoxins and their relationships with animal performance and the quality of animal-derived products.

The farm trial was carried out to testing a combination of LAB mixture and sodium benzoate to reduce the aerobic spoilage in the top layer of an experimental bunker silo. Considering the experimental groups of the trial, the upper layer of the bunker treated with the additive combination had lower yeasts count and higher aerobic stability than the control group. In addition, the application of the combined solution allowed to reduce the DM losses from the top layer. The trial was extended to evaluate the efficacy of bacterial mixtures and chemical additive combination monitoring the disposal of waste amounts and the temperature of the ensiled mass during the feed-out phase. However, data reported for the silage mass temperature and waste amounts have a numeric weightiness, they may be considered as a starting point to measure the aerobic deterioration at farm level and monitor the condition of the farm bunker using thermic probe. Notably, measuring the temperature of the silo during this trial has been helpful to understand how the environmental conditions can affect the temperature during the storage period. Therefore, it may be possible to figure out how data referred to temperature and waste amounts can be considered as a way to evaluate the dynamics of the economic losses in livestock.

The third study aimed to evaluate the effectiveness of an animal-derived LAB (i.e., *Lim. mucosae* UNISS B81) in comparison to commercial inoculants on fermentative, chemical, and aflatoxin contamination in WPCS ensiled in minisilos packed at different levels of density. In this work, although the different combinations of LAB did not change the final fermentative parameters profile of silage, it is important to underline that, silages obtained with *Lim. mucosae* UNISS B81 used alone presented a higher content of acetic acid, propionic acid, ethanol, and total alcohols, and a good aerobic stability. High density conditions allowed to achieve a well-fermented silage with an optimal aerobic stability. Thus, reducing porosity level is crucial to minimize the activity of aerobic microorganisms that can predispose silage to spoilage. Moreover, *Lim. mucosae* UNISS B81 was able to ferment the corn with good results in terms of nutritive value and aerobic stability. Further farm studies are needed to assess the ability of this strains to improve also the animal performances by exerting probiotic activity.

The fourth study aimed to evaluate the forage feeding value of three smart corn hybrids carrying the *br2* mutation in comparison to two conventional corn hybrids harvested at 5 different harvest moments. Fiber digestibility, starch digestibility and estimate the energy content (milk/ton) of corn hybrids through Milk2006 and Milk2024 were evaluated. Fresh yield showed a decreasing trend with the advance of maturity; on the other hand, DM yield increased the maturity. Smart corn hybrids reported higher energy content (milk/ton) than conventional corn hybrids. Fibrous fractions of smart corn hybrids were lower than conventional corn hybrids due to the high leaves/stalk ratio. In particular, smart corn hybrid showed a higher ivNDFD than

conventional corn at 120 h. In addition, SCH had a greater isSD7 h than CCH, that may be related to a better conversion within the kernel fractions that makes starch of smart hybrids more digested than conventional hybrids. Further studies are warranted to elucidate the agronomical advantages/disadvantages of these new corn hybrids and their effects on lactation performance.

Moreover, considering the importance of silage, and particular WPCS as predominant forage used in ruminant diets worldwide, this Thesis aimed to evaluate all the aspects involved among the ensiling process highlighting the strength and weakness points. Future studies are needed to shed light on the aspects related to the sustainability of ensiling considering controllable and uncontrollable factors related to regions of production.

Appendix: Assessing agronomic and nutritional performance of alfalfa (*Medicago sativa* L.) ley: impact of year, cultivar and mowing time in successive cuts

Abstract

Alfalfa (*Medicago sativa* L.), a perennial legume crop, is renowned in livestock system for its high biomass yield and nutritional value for ruminants. This study evaluated yield, nutritional value, and morpho-physiological traits of four alfalfa cultivars (i.e., La Rocca, Prosementi, PR57Q53, Miranda), cut three times, with five different mowing timings, in an experimental field following the traditional management of the alfalfa ley. Results demonstrated that dry matter (DM) yield increased with later mowing times. Significant differences in crude protein (CP) yield were observed across cultivars, mowing timings, and years. Miranda exhibited the highest CP concentration but lower overall DM yield, while La Rocca excelled in later cuts, indicating differential adaptability. Later mowing times resulted in higher DM yield but lower CP concentration and increased lignification, reducing fiber digestibility. These findings underscore the importance of strategic cultivar selection and adaptive mowing practices to balance forage yield and quality. Selecting multifoliolate cultivars may improve forage quality, and identifying new cultivars with reduced lignin content or increased cellulose digestibility could further enhance ruminant utilization. This study highlights the need for adaptive management strategies that consider yearly climatic variations and specific interactions between cultivar, mowing time, and environmental conditions to optimize alfalfa forage yield and quality.

Keywords: alfalfa, haymaking, maturity, fiber fractions, multi-leaf trait

Introduction

Alfalfa (*Medicago sativa* L.) stands as a cornerstone in livestock systems, widely recognized for its major role in forage production, due to its high nutritional value and efficient extraction of water and nutrients from the subsoil via its deep root system (Raes et al., 2023). Such a leguminous forage crop is extensively integrated into dairy cow rations, contributing to the optimization of livestock systems. However, as alfalfa plants reach maturity, there is a concurrent increase in cell-wall material deposition, resulting in diminished digestibility (Albrecht et al., 1987). This phenomenon is primarily attributed to a rising lignin concentration, more pronounced in stems than leaves.

To optimize alfalfa forage quality, the implementation of a judicious harvest management is essential. Various factors exert significant influence on forage quality, including growth stage (Nordkvist & Åman, 1986; P. Yu et al., 2003), cultivar (Griffin et al., 1994), and growing conditions (i.e., rainfall, temperature, and soil composition) (Cox et al., 1994; Mathison et al., 1996). A nuanced understanding and effective manipulation of these factors are imperative for maximizing alfalfa forage quality.

Among these, the harvest period merges as a pivotal factor shaping forage quality, as an increase in plant maturity correlates with higher dry matter yield but lower forage quality. The morphological stage of maturity at harvest significantly affects digestibility, with advancing maturity leading to an increase in fiber content and a decrease in digestibility and intake potential (Albrecht & Beauchemin, 2015; Mertens, 1987).

Consideration of the correct harvest period, often achieved at 10% flowering, is crucial for balancing forage quantity, protein content, and acceptable levels of digestible fiber during haymaking (Palmonari et al., 2014). Factors such as temperature, light and soil conditions, alongside fertilization, water supply, cutting frequency, and the growth year, further influence the yield and quality of alfalfa (Fan et al., 2016; Hakl et al., 2017; Macolino et al., 2013; Palmonari et al., 2014; Santos et al., 2020). Selecting the appropriate cultivar is crucial for maximizing crop yields per unit area (Monirifar et al., 2020), influencing yield, quality, pest tolerance, and stand life (Poole et al., 2003).

Cultivar selection involves considerations of adaptability to the environment, farm management practices, and the intended forage use (Rauf et al., 2016; Annicchiarico et al., 2020; Pecetti et al., 2006).

Recent development in genetic selection have led to multileaf with a substantial percentage of leaves composed of more than three leaflets (up to 7-9), aiming to increase protein percentage

while minimizing fiber concentration (Cherniavskih et al., 2021). However, realizing these benefits hinges on effective haymaking and preservation techniques, emphasizing the need to preserve leaf presence in the hay. Suboptimal haymaking operations, such as baling excessively dry hay, pose the risk of increased leaf loss and diminished forage quality (Lamb et al., 2007).

The nutritional value of alfalfa is strictly tied to harvest management, necessitating a careful selection of the harvest period based on desired forage quality (Undersander, 2011). Harvest schedule decisions consider parameters such as the number of cuts per season, date of cut, stage of maturity, interval between cuts, and cutting height. Harvesting in earlier stages of maturity (full budding) contributes to higher forage quality and digestibility at the expense of minor yield performance. Alternatively, harvesting at full bloom aims for higher dry matter yields but may compromise forage quality (Weir et al., 1960; P. Yu et al., 2003).

Developing an appropriate cutting schedule is crucial for alfalfa growers to achieve a balance between yield and quality. Cultivar selection should be pursued considering the harvest period to enhance both yield and cultivar persistence (Milić et al., 2019). Care in the haymaking yard is essential to minimize dry matter losses caused by mechanical handling and weather damage in the field (Undersander et al., 2011).

This study focuses on the evaluation of four different alfalfa cultivars, assessment of cutting frequency based on maturity stages, and analysis of the nutritional composition of cultivars concerning the stage of maturity. The objective is to understand how various haymaking operations can affect the yield and quality of the four alfalfa cultivars, providing valuable insights into the optimization of alfalfa cultivation for enhanced forage productivity and quality in livestock systems.

Materials and methods

Site description

The field experiment was set up at the Beccastecca experimental farm, located in San Cesario sul Panaro (44°34'52.1"N 11°02'40.8"E; 42 m above sea level), Modena, Emilia-Romagna region, Northern-Italy. The site is characterized by a temperate climate, with an average annual temperature of 14.2 °C and annual rainfall of 750 mm (30-year average). Rainfall and air temperature during the trial period were obtained from an automated meteorological station positioned near the experimental field.

The soil is a fine-silty, mixed, mesic Fluventic Ustochrepts (Soil Survey Staff 2014), with a silty clay loam texture (sand 122 g kg⁻¹, silt 489 g kg⁻¹, clay 389 g kg⁻¹). The initial physical-chemical properties of the topsoil layer (0-30 cm) were as follows: pH H₂O 8.4; organic C 10.4 g kg⁻¹; total N (Kjeldahl) 1.2 g kg⁻¹; available P (Olsen) 12.6 mg kg⁻¹; exchangeable K (NH₄⁺ Ac) 188 mg kg⁻¹; and cation exchange capacity 25 cmol⁺ kg⁻¹.

Experimental treatments and field management

The field experiment was established in 2010, although measurements and data collection occurred in 2011 and 2012. The experimental design was arranged as a split-plot with four replicates (blocks), one main factor (alfalfa [*Medicago sativa* L.] cultivar) and one secondary factor (mowing timing). In detail, the main factor was represented by the alfalfa cultivar, with four levels: (i) La Rocca, a traditional cultivar with early and fast growth, half-dormant with resistance to low temperatures (fall dormancy class = 7), wide adaptability to a range of soil types, and good leafiness; (ii) Prosementi, a reference cultivar in the Po Valley environment, well adapted to the non-irrigated conditions, half-dormant with resistance to low temperatures (fall dormancy class = 6.5), adaptability to a range of soil types, and excellent leafiness; (iii) PR57Q53, of American origin (Pioneer), with high productive performance in the Po Valley environment, half-dormant with resistance to low temperatures (fall dormancy class = 6.5), and good leafiness; (iv) Miranda, a multifoliate cultivar with a high leaf-to-stem ratio, good longevity and adaptability, half-dormant with resistance to low temperatures (fall dormancy class = 7), and excellent forage quality. The secondary factor was represented by the mowing timing of the first, second, and third cut, with five levels according to Kalu & Fick, (1981): (i) green buds (Stage 1), (ii) purple buds (Stage 2), (iii) 10% flowering (Stage 3), (iv) 50% flowering (Stage 4), and (v) 100% flowering (Stage 5). As a results, 20 treatments were included in the field experiment (four alfalfa cultivars five mowing timings). Each plot was 1.4 m wide and 6 m long (8.4 m²). The field layout was replicated three times across three sectors, each designated for the three annual cuts, to prevent overlapping effects with previous cuts. Within each sector, the four blocks were organized, followed in hierarchical order by the four cultivars and the five mowing timings, all randomized. A noteworthy exception occurred during the first cut of 2012, where the number of timings was reduced from 5 to 3. The fourth and fifth timings could not be executed due to meteorological and organizational challenges.

Seeding took place on March 31st, 2010, while the application of experimental treatments began in the spring of 2011, on a consolidated one-year-old alfalfa ley and continued for two years (2011 and 2012). In the days before seeding, a basal fertilization was performed with 100 kg ha⁻¹ of P₂O₅ and 150 kg ha⁻¹ of K₂O, distributed and incorporated by plowing on February 25th. The seeding was done with a mechanical row seeder, spaced 15 cm apart, using a seed rate of 40 kg ha⁻¹. On May 30th, 2010, the trial was weeded with Imazamox at a rate of 40.0 g ha⁻¹ and with 48.0 g ha⁻¹ of 2,4-DB. During 2010, four undifferentiated mowings were carried out on the entire experimental field, for which no measurements or weights were collected. The timing and method of these mowings follow the traditional management of the alfalfa ley.

Biomass measure and chemical analysis

Forage yields were assessed in 2011 and 2012, for each cut, each cultivar and mowing time. In detail, alfalfa above-biomass coming from each plot was completely collected and weighted with a mechanical forage harvester. Samples of alfalfa from each cut, each cultivar and mowing time (around 200 per year) were collected and transported to the laboratory for successive evaluations as detailed below. In particular, the samples were dried in a forced-air oven (60 °C, until constant weight) for DM determination. These samples were ground to pass at 0.5mm for near-infra-red spectroscopy (NIR) analysis and a 1mm screen for gas production test. In particular, the samples were characterized by a Foss (Hilleroed, Denmark) NIR systems 5000 spectrophotometer equipped with a monochromator and transport module, scanning over the wavelength range 400–2500 nm, every 2 nm. The calibrations used to obtain sample characterizations were produced by a commercial forage testing laboratory (CRPA Lab, Reggio Emilia, Italy) and samples were analyzed for ash, sugar, crude protein (CP), soluble CP, aNDFom, ADF, ADL, NDICP, ADICP, NDF degradability evaluated after 24 h of rumen incubation (dNDF), and undigested NDF (uNDF) as reported by (Brognia et al., 2009). Morpho-phenological traits were measured at harvest in each cut as follows: (i) the phenological stage was assessed following Kalu & Fick, 1981; (ii) the plant height was determined by average 10 plants measurements per plot from soil surface to the tip of the tallest tiller; (iii) the internode amount was determined by counting the number of internodes of 10 plants per plot; (iv) the internode average was determined as the mean internode length of 10 plants per plot.

Calculations and statistical analyses

Average annual values for alfalfa forage yield, nutritional value, and morpho-phenological traits were calculated for each factor (i.e., cultivar, mowing time, and year) and for each factor interaction. Calculations were as follows: (i) the mean of cumulative annual yields (sum of yield from the three cuts), for DM yield and CP yield; (ii) the mean of the weighed means (on a DM yield basis) for CP, aNDFom, ADF, ADL, dNDF, uNDF, and Sugars; (iii) the mean of the arithmetic means for phenological stage, plant height, internode number, and internode average length.

Relationship between variables (i.e., phenology, response, and weather) and objects (cultivar and mowing time) was assessed by performing three principal component analyses (PCA) with type I scaling for each cut, classifying objects by either cultivar or mowing time factors. This procedure generated loading (correlations between common latent factors and original variables) vectors.

We conducted a repeated measures analysis of variance (ANOVA) on alfalfa forage yield, nutritional value, and morpho-phenological traits with cultivar, mowing time, year and block as fixed factors and replicate as random effect. Shapiro-Wilk and Levene's tests were performed to check normality and homogeneity of variances of measured variables. When the ANOVA assumptions were violated, data were log-transformed prior to analysis and back-transformed after the post-hoc test. Tukey's honestly significant difference (HSD) was used as post-hoc to test significant differences among treatments with a p-value of 0.05 as threshold.

We used R 4.3.2. (R Core Team, 2020) with nlme (Pinheiro et al., 2013), multcomp (Hothorn et al., 2007), and FactoMineR (Lê et al., 2008) packages for the mixed model, HSD test and PCA, respectively.

Results

Preliminary analyses and significance according to ANOVA

The results of the ANOVA investigating the impact of experimental factors, namely alfalfa cultivar (C), mowing time (MT), and experimental year (Y), along with their interactions, on alfalfa forage yield and quality across the first to third cuts, are comprehensively outlined in Table A.1.

At the first cut, significant effects of C, MT, and Y factors were observed on variables related to alfalfa forage yield (i.e., DM yield and CP yield), nutritional value (i.e., CP, aNDFom, ADF, ADL, dNDF, uNDF, and Sugars), and morpho-phenological traits (i.e., phenological stage, plant height, internode number, and internode average length). However, two-factor and three-factor interactions did not show significant effects, except for MT $\times$ Y, which exhibited P-values lower than 0.05 for most variables, including CP yield and all variables related to forage nutritional value. Additionally, C $\times$ MT was significant for aNDFom and ADF, and C $\times$ Y was significant for CP yield, while other interactions did not yield significant differences.

The ANOVA at the second and third cut showed a similar pattern to that of the first cut concerning P-values related to individual factors, with C, MT, and Y factors significantly affecting most of variables related to alfalfa forage yield, nutritional value and morpho-phenological traits. On the other hand, 2-factor and 3-factor interactions showed a distinctive pattern between the second and the third cut: (i) both C $\times$ Y and MT $\times$ Y significantly impacted variables related to alfalfa forage yield and nutritional value (and morpho-phenological traits to a lesser extent) at the second cut; (ii) at the third cut, MT $\times$ Y was the sole interaction exerting significant effects, albeit with minor significance compared to other interactions.

Finally, the ANOVA conducted on variables concerning average annual alfalfa forage yield, nutritional value and morpho-phenological traits, demonstrated consistent significant effects

of individual factors (C, MT, and Y). Additionally, there was an intermediate pattern observed due to 2-factor and 3-factor interactions compared to single cuts.

The results of the principal component analysis (PCA) as a preliminary analysis are presented in Figure A.1 for cultivars and Figure A.2 for MT. The first two principal components of PCA (Dim1 and Dim2) explained 77.0%, 58.3%, and 74.7% of the total variance for the first, second, and third cuts, respectively. The relationships between variables varied slightly to highly across the three cuts.

Specifically, at the first cut, DM yield was closely associated with CP yield, both of which were positively correlated with rainfall. Conversely, fibers (i.e., aNDFom, ADF, ADL) exhibited close associations among themselves and with temperature, while being negatively correlated with sugars and dNDF. No relationship was observed between fibers and DM yield/CP yield.

At the second cut, a similar relationship between DM yield and CP yield as observed in the first cut was noted, with temperature replacing precipitation as the associated weather variable. Fibers continued to show positive associations among themselves and negative correlations with sugars, and dNDF. Once again, no relationship was observed between DM yield/CP yield.

In contrast to the first and second cuts, DM yield and CP yield were positively correlated with fibers at the third cut. Additionally, a positive relationship was observed between CP and dNDF, as well as between phenological stage and internode number. No relationship between weather variables and response variables was evident at the third cut. Generally, minor relationships were observed between morpho-phenological variables and other variables across all cuts.

The PCA analysis further indicates that PR57Q53 and Prosementi exhibited the closest relationship among cultivars, whereas La Rocca and Miranda were consistently more distant from the former pair. Specifically, at the first cut, La Rocca appeared to (i) achieve maximum phenological stage and (ii) minimize DM yield and CP yield compared to the other cultivars. Conversely, Miranda maximized CP at the second cut. Furthermore, the PCA indicates that transitioning from mowing time number 1 to mowing time number 5 resulted in a progressive (i) increase in variables associated with alfalfa forage yield and morpho-physiological traits, and (ii) decrease in variables associated with nutritional value.

Effect of cultivar on alfalfa forage yield, nutritional value, and morpho-physiological traits

The effect of cultivar on alfalfa forage yield, nutritional value, and morpho-physiological traits is reported in Table A.2. At the first cut, Prosementi and Miranda had the highest DM yield, while La Rocca had the lowest. Conversely, Miranda reduced DM yield at the second and third cuts. The average annual DM yield was highest for three of the four cultivars: Prosementi,

PR57Q53, and La Rocca, while Miranda had a significantly lower yield (11-13% less) compared to the others. Miranda consistently exhibited the highest CP concentration, followed by PR57Q53 and Prosementi, with La Rocca having the lowest CP concentration. The annual average CP yield was higher for PR57Q53 and Prosementi, while La Rocca and Miranda had the lowest CP yields in the first and subsequent cuts, respectively.

In terms of fiber fractions (aNDFom, ADF, and ADL), La Rocca showed the highest values in the second and third cuts, except for ADF in the first cut where PR57Q53 and Prosementi had higher values than Miranda and La Rocca. Miranda had the lowest annual average fiber fraction values compared to the other cultivars.

La Rocca had the lowest values for dNDF all cuts and on an annual average. Miranda had the highest values for these parameters in the first and third cuts, as well as the annual average, whereas PR57Q53 and Prosementi exhibited slightly higher values across the cuts. The uNDF was annually low for Prosementi, whereas Miranda was the cultivar with the highest value in second and third cut. La Rocca had the highest sugar content in the first cut, while Miranda had the highest in the second and third cuts. PR57Q53 and Prosementi had lower sugar content compared to the other cultivars, with Miranda having the highest annual average sugar content and PR57Q53 the lowest.

Regarding morpho-physiological traits, La Rocca had the highest values except for the phenological stage and internode number in the first cut, where Miranda had the lowest values, particularly in the second and third cuts for the phenological stage. PR57Q53 and Prosementi showed intermediate values across the cuts. La Rocca exhibited the greatest plant height across all cuts, especially in the second cut, whereas Miranda had the lowest height, particularly in the third cut. In the third cut, La Rocca had higher internode numbers compared to PR57Q53 and Miranda. For internode average, Prosementi had generally higher values in the first and second cuts compared to the other cultivars, while only Miranda reduced internode average in the third cut. La Rocca had the highest annual average for most morpho-physiological traits, except for the internode average, where Prosementi had the highest value, despite not being statistically different. Miranda consistently had the lowest values across all four morpho-physiological traits.

Effect of mowing time on alfalfa forage yield, nutritional value, and morpho-physiological traits

The effect of mowing time on alfalfa forage yield, nutritional value, and morpho-physiological traits is reported in Table A.3.

The highest values of DM yield were detected for the 3 MT of the first cut, the 5 MT of the second cut, and the 4 MT of the third cut. Conversely, the lowest values were observed in the 1 MT for all three cuts. The average annual DM yield was highest in the third MT.

Considering the MT within the three cuts, the highest concentrations of CP were detected in the 1 MT. The CP concentration exhibited a decreasing trend among the MTs, with the lowest concentration in the last MT of all cuts. The annual average CP concentration was higher in the 1 MT compared to the other MTs. CP yields increased with the advance of the MTs in the second and third cuts.

The values for fiber fractions increased among the MTs within the three cuts. Notably, the highest values for aNDFom, ADF, and ADL were in the 3 MT of the first cut, the 4 MT of the second cut, and the 5 MT of the third cut. Conversely, lower fiber concentrations were detected in the 1 MT compared to the others. The annual average for the fiber fractions was higher in the 2 and 3 MTs than in the 1 MT.

Values for dNDF, and sugars followed the same trend among the MTs. Specifically, the highest values for these parameters were in the 1 MT of each cut, and decreased with the advance of the MTs. The lowest dNDF values were reported in the 4 MT of the second cut and the 5 MT of the third cut. The lowest sugar content was detected in the 2 MT of all the cuts. The annual average for dNDF, and sugars was higher in the 1 MT than in the other MTs. Among the MTs, values detected for uNDF were always low at the 1 MT. Similar results were observed in the annual average for uNDF.

The phenological stage, plant height, and internode number exhibited an increasing trend among the MTs, reaching the highest values in the last MT of all cuts. The same trend was observed for the annual average, where the highest value was reported in the 3 MT. The internode average was higher in the 3 MT of the first cut compared to the other MTs. In the second and third cuts, the internode average was highest in the 1 MT and lowest in the 5 MT.

Effect of year on alfalfa forage yield, nutritional value, and morpho-physiological traits

The effect of year on alfalfa forage yield, nutritional value, and morpho-physiological traits is reported in Table A.4.

The DM yield increased from 2011 to 2012 in the first and second cuts, but in the third cut, the DM yield was higher in 2011 than in 2012. The average annual DM yield was higher in 2012 compared to 2011.

The CP concentration was higher in the first and second cuts of 2011 compared to 2012, although the third cut of 2012 had a higher CP concentration than 2011. The highest CP yield was recorded for the first cut of 2012, whereas the lowest was in the third cut of 2012. The average annual CP yield was higher in 2012.

In the first cut of 2011, fiber fraction values were lower than in 2012. However, except for ADF, values for aNDFom and ADL were higher in the second and third cuts of 2011 compared to

2012. The average annual values for aNDFom and ADL were higher in 2011 than in 2012, in contrast to ADF.

In the first cut of 2011, dNDF, and sugars were higher than in 2012. Conversely, these values were higher in the second and third cuts of 2012 than in 2011. The average annual dNDF was higher in 2012 than in 2011, whereas sugars were higher in 2011 than in 2012. Regarding the uNDF, values for this parameter were lower in the first and third cut of 2011 than 2012; on the other hand, only in the second cut uNDF was higher in 2011 than 2012.

Data for the phenological stage and internode number were higher in the first and third cuts of 2012 compared to 2011, while in the second cut, these values were higher in 2011 than in 2012. Plant height values were higher in the second and third cuts of 2011 than in 2012, whereas higher values were detected in the first cut of 2012 compared to 2011. The internode average was higher in the first and second cuts of 2012 than in 2011. Conversely, in the third cut of 2011, the internode average was higher than in 2012. The average annual plant height and internode average were higher in 2011 than in 2012. On the other hand, the average annual internode number was higher in 2012 than in 2011.

Effect of interaction between cultivar and year on alfalfa forage yield, nutritional value, and morpho-physiological traits

The effect of the interaction between cultivar and year on alfalfa forage yield, nutritional value, and morpho-physiological traits is reported in Table A.5.

A significant interaction between cultivar and year for DM yield was found in the second cut. In 2011, Prosementi had the highest value, while in 2012, DM yield was slightly higher for La Rocca than for Prosementi, although not statistically relevant. In both years, PR57Q53 showed an intermediate value between the cultivars, whereas Miranda consistently had the lowest DM yields.

CP yields for Miranda were higher in the first cuts of 2011 and 2012 compared to the other cuts; notably, the lowest CP yields for Miranda were recorded in the third cut of 2012. Miranda had the highest average annual CP concentration for 2011. An increase in CP yields was observed for La Rocca, PR57Q53, and Prosementi in the first and second cuts of both years, although the yields were lower in the third cut compared to the second cut for both years.

La Rocca had the highest fiber fractions in the second cut of 2011, while Miranda had the lowest values. The aNDFom was slightly higher for Prosementi compared to PR57Q53 in the second cuts of 2011 and 2012. The highest average annual fiber fraction was observed for La Rocca in 2011. The dNDF values were higher for all cultivars in 2012 than in 2011 for the second cut. Low uNDF concentrations were detected for Prosementi in the second and third cut of 2011. Prosementi

was the cultivar with the lowest uNDF concentration for 2011 considering the average annual values.

Regarding morpho-physiological traits, an interaction effect was observed for the phenological stage, with data for 2011 being higher than for 2012 across all cultivars. The internode number showed an effect in the third cut, with values increasing from 2011 to 2012; La Rocca had the highest values in both years.

Effect of interaction between mowing time and year on alfalfa forage yield, nutritional value, and morpho-physiological traits

The effect of the interaction between mowing time and year on alfalfa forage yield, nutritional value, and morpho-physiological traits is reported in Table A.6.

An increase in DM yield was observed with the advance of the MT across the years. Notably, in the second cut, the highest DM yields were reported in the 5 MT and the lowest in the 1 MT for both 2011 and 2012.

CP yields showed an increasing trend between the MT and years for all cuts. However, the lowest CP yields were detected in 2012 for the first two MTs of the third cut. The average annual CP yields increased between the MTs in 2011, whereas a decreasing trend was reported for 2012. Regarding CP concentrations, the earlier MTs (e.g., 1 and 2) had higher concentrations compared to the other MTs. High CP concentrations were detected for the average annual in the 1 MT across the years.

Fiber fractions increased with the advance of the MTs over the years of the trial. Specifically, aNDFom levels were higher in the 4 and 5 MT of the second cut in 2012, and the third cut in both 2011 and 2012. The ADF contents were higher in the second cut of 2011 across all MTs. The highest ADL concentrations were reported in the 5 MT of 2011 and the 4 MT of 2012, respectively. The 1 MTs had the highest dNDF values throughout the trial, while the lowest were reported in the last MTs, except for the 4 MT of 2012 in the second cut. Low uNDF contents were reported in each 1 MT of all the trial, except for the 1 MT of 2012 in the first cut.

The effect of the interaction between MT and year was observed in the third cut for the phenological stage, which increased with the advance of maturity. Plant height values were higher in the second and third cuts of 2011 than in 2012. The highest values were observed in the MTs of 2011 for the second cut, while the lowest values were reported in the MTs of 2012 for the third cut. Internode numbers were higher in the 5 MT of the second and third cuts for both 2011 and 2012. The interaction for the internode average was observed in the third cut, where the 1 MT had the highest average.

Discussion

The results presented in this study provide a comprehensive analysis of the effects of alfalfa cultivar, mowing time, and experimental year on forage yield, nutritional value, and morpho-phenological traits. The findings are derived from ANOVA and PCA analyses across three cuts within the growing seasons, shedding light on the interplay between genetic and environmental factors in determining alfalfa performance.

Impact of cultivar

Our results underscore the significant influence of cultivar choice on alfalfa forage yield and quality. Specifically, Prosementi and Miranda demonstrated the highest DM yield at the first cut, while La Rocca showed superior performance in subsequent cuts, indicating a potential adaptation to later growth stages or environmental conditions encountered later in the season. This variation suggests that different cultivars may possess distinct growth patterns and stress responses, which can be strategically utilized to optimize forage production throughout the growing season (Prasad et al., 2008).

Selecting alfalfa cultivars to increase yields in different cropping areas should consider many agrotechnical factors within the farming system. The choice of comparing the four mentioned cultivars has been done considering the longevity, productivity, and weather resistance, as it is widely known that alfalfa cultivars may have an effect on quality as well as yield (L.-X. Yu & Kole, 2021). Selecting multifoliate cultivars is linked to the potential for improving alfalfa quality (Cherniavskih et al., 2021). Multifoliate cultivars, which generate more than three leaflets per leaf, generally result in higher quality forage, though this is not always consistent (Alam et al., 2009). These cultivars have shown higher nutritional value and perform better *in vitro* digestibility in comparison to trifoliate cultivars (Juan et al., 1993; Petkova & Panayotova, 2007).

Indeed, our study confirms that the multifoliate cultivar Miranda consistently exhibited the highest level of CP concentration, highlighting its potential as a high-protein forage option as reported by Yancheva et al. (2012). However, Miranda's lower overall DM yield compared to other cultivars may limit its utility in scenarios where biomass production is paramount. This lower yield performance in terms of DM and CP, especially in comparison to Prosementi and PR57Q53, was related to the morpho-physiological traits such as the plant height and the length of internodes. This was also previously suggested by earlier studies (Jing et al., 2023).

Influence of mowing time

Mowing time had a profound effect on forage yield and quality, with later mowing times generally associated with higher DM yield but lower CP concentrations. This inverse relationship

aligns with the understanding that as alfalfa matures, there is a trade-off between biomass accumulation and nutrient concentration. Notably, in order to optimize yield and quality forage crops have to be mown at the right maturity (Rotz, 2005). On the other hand, several constraints should be considered when crop is mown, such as weather, dryer capacity, and the number of hours that the mower could operate in the field (Savoie et al., 1995) in order to maximize yields and avoid nutritive losses. The highest DM yields observed in the third MT of the first cut, fifth MT of the second cut, and fourth MT of the third cut reflect the cumulative effect of prolonged growth periods. An increase in DM yield as result of mowing was observed in a field study conducted to investigate the impact of different patterns of raking and baling operations in the Eastern region of Saudi Arabia (Al-Gaadi, 2018). However, as plant maturity increases, the digestibility and nutritional quality of forage decreases (Ball et al., 2001). The CP concentration was high in the first two MTs and tended to decrease delaying the MT, as previously reported (Atis et al. 2019; Nelson & Satter, 1990; Palmonari et al., 2014). The decline in CP concentration with advancing mowing times suggests a dilution effect due to increased fiber content, which also aligns with the observed increase in fiber fractions (aNDFom, ADF, ADL) over time. This phenomenon may be related to a gradual thickening of the crop cell wall with the advance of maturity, in which lignin and other structural components of the cell wall increase, and consequently the cell content decreased following by a reduction of the nutritional quality (Liu et al., 2023). Both aNDFom and ADF levels were affected by the stage of maturity with an accumulation of lignin in the stems. Albrecht et al. (1987) observed that digestibility of alfalfa decreased with maturity due to a deposition of the cell-wall material that increases stem proportions. Considering this scenario, we highlight that it is important to operate the MT in the pre-bloom stage to harvest forage with a proper protein content and a good value for NDF digestibility (Palmonari et al., 2014). Feeding total mixed rations (TMR) with alfalfa hay mown in the afternoon in the early stage of maturity has been observed to improve nutrient digestibility (e.g., DM, organic matter, and CP) and nitrogen efficiency in dairy cows when included at level of 200 g/kg DM in TMR with 650 g/kg DM concentrates (Yari et al., 2014).

Yearly variations

The significant yearly variations observed in forage yield and quality metrics indicate the critical role of environmental conditions, particularly weather patterns, on alfalfa performance. Higher DM yields and lower CP concentrations in 2012 compared to 2011 for most cuts (and on average) may suggest more favorable growing conditions in 2012, possibly due to variations in temperature, precipitation, and other climatic factors (Thivierge et al., 2016). Environmental

variations from year to year and season to season alter forage quality, even when forages are harvested at the same stage of maturity (Buxton, 1996).

However, the lower cumulative rainfall and higher air temperature recorded over the alfalfa growing season (until the third cut) in 2012 than in 2011 suggest that factors other than environmental variations were the main drivers of the observed differences in DM yields and CP concentrations in our study. Specifically, our alfalfa crop in 2012 likely developed a deeper rooting system than in 2011, thereby providing more water and nutrients to the plants and consequently increasing DM yield. Previous studies indicate that alfalfa roots can reach depths of up to 2 meters after three years (Edwards et al., 2019). Additionally, the increase in biomass production is often inversely correlated with CP concentration due to the competing use of glucose during photosynthesis, which supports our findings of lower CP concentrations in 2012 compared to 2011 (Chand et al., 2022).

Similarly, the fibrous fractions, especially aNDFom and ADL, followed that same pattern observed for CP concentration; on the other hand, dNDF had an opposite trend and was on average higher in 2012 than in 2011 by 5.4%. The increase in fibrous fractions and the decrease in digestibility as a consequence of reduced water absorption by alfalfa have been previously reported (Undersander et al., 1987). Overall, these variations highlight the need for adaptive management strategies that account for yearly climatic differences to optimize alfalfa forage yield and quality.

Moreover, the environmental conditions that influence alfalfa are not constant among the growing season, it might be suggestable to schedule the timing of cutting considering the environmental factors that can influence the yield and nutritive value of forage. In particular, Brink et al. (Brink et al., 2010) evaluated the variation of yield and fiber components among spring, early summer, late summer, and fall of 2004 and 2005 in Pennsylvania, Wisconsin, and Idaho. The authors observed that DM, nutrient composition and fiber digestibility were affected by the environmental conditions, especially for harvest timing made in spring and early summer in humid environments, and during early and late summer in arid regions.

Interaction between factors

The interactions between cultivar and year, as well as mowing time and year, further elucidate the complexity of alfalfa growth dynamics. The significant interaction between cultivar and year for DM yield in the second cut, where Prosementi outperformed other cultivars in 2011 while La Rocca excelled in 2012, highlights the differential adaptability of cultivars to yearly climatic variations. This suggests that cultivar selection should consider not only inherent genetic traits but also historical climate data to predict performance under varying conditions.

Similarly, the interaction between mowing time and year for CP yield and fiber fractions underscores the necessity of adaptive management strategies. The varying trends in CP yield and fiber fractions across years and mowing times suggest that optimal mowing schedules may need to be adjusted annually based on prevailing climatic conditions to achieve desired forage quality outcomes. For instance, an increase in DM and CP yields was observed during the second cut for both 2011 and 2012, with the greatest productions detected at the fifth mowing time in the second cut of 2012 compared to 2011. This variation necessitates flexible management practices that respond to annual climatic conditions.

Regarding nutritional parameters, Miranda consistently exhibited a desirable nutritional profile across years, with high CP concentration and low fibrous fractions, as confirmed by Yancheva et al. (2012). Although dNDF values varied numerically, Miranda had higher dNDF in both 2011 and 2012 compared to other cultivars, suggesting its potential for maximizing digestible portions of the forage and dry matter intake.

The decline in nutritive values, such as dNDF, with advancing mowing times is attributable to the accumulation of cell wall material within stems, which limits feed intake (Jung & Allen, 1995). These findings highlight the importance of adaptive management strategies that consider yearly climatic differences and the specific interactions between cultivar, year, and mowing time to optimize alfalfa forage yield and quality.

Practical implications and future recommendations

The PCA results provide additional insights into the relationships between different forage traits and environmental variables. The strong correlation between DM yield and CP yield with rainfall at the first cut, and temperature at the second cut, emphasizes the critical influence of weather conditions on these key forage attributes. The negative correlation between fiber fractions and sugars/dNDF across cuts further highlights the trade-offs between structural and non-structural carbohydrates, which are modulated by environmental factors.

Distinct patterns observed in the third cut, where DM yield and CP yield were positively correlated with fiber fractions, suggest a shift in plant physiology towards the end of the growing season. This could be attributed to changes in environmental conditions, such as reduced water availability or lower temperatures, influencing plant metabolic pathways and resource allocation.

Maximizing yields and quality of forages is crucial to improve farm performances, as yield and quality are interdependent factors (Putman, 2021). Management practices should encompass multiple aspects, including selecting well-drained fields; opting for cultivar with proper fall dormancy, winter hardiness, and yield potential, and mowing in large swaths to enhance drying

rates (Undersander, 2021). Traditional cultivars with early and fast growth, and wide adaptability, like La Rocca, which showed high DM yield in the last cut, can enhance yields in late cuts.

A compromise between quality and yield is essential to harvest a forage that meets herd requirements. In high-producing ruminants, alfalfa quality represents the ability of forage to provide digestible energy and support voluntary feed intake (Lin et al., 2021). Desirable traits in alfalfa include increased cellulose and decreased lignin in cell walls, maintaining a CP content of 20%, and 30% non-fiber carbohydrates, combined with essential amino acids and a slower rate of protein degradation in the rumen (Bhandari et al., 2023). Delaying the harvest reduces the leaf mass, which contains most of the protein, and increases stem development, leading to higher cellulose and lignin content (Barsukov et al., 2024). Therefore, maintaining proper CP levels can be achieved by harvesting multifoliolate cultivars and scheduling mowing times strategically, as leaves contribute significantly to feed value compared to other alfalfa tissues.

The digestibility of fiber is affected by forage characteristics such as lignification, leaf-to-stem ratio, and plant maturity, along with dietary characteristics and feed intake (Lin et al., 2021). In this study, lignification increased with advancing mowing times, leading to decreased dNDF. In addition, morphophysiological traits such as plant height and internode numbers were positively correlated with fibrous fractions and negatively related to dNDF, especially in the last mowing time of the third cut, as reported in the PCA.

Future research should investigate the interactions between rumen microflora and plant cell wall components to improve forage digestibility and feed efficiency, particularly for forages harvested during the last cuts. This research is essential to develop adaptive management strategies that account for yearly climatic differences to optimize alfalfa forage yield and quality.

Conclusions

The present study highlights the complex interplay between alfalfa cultivar, mowing time, and environmental conditions in determining forage yield and quality. Strategic selection of cultivars and adaptive mowing practices, informed by a deep understanding of environmental influences, can optimize alfalfa production. Among the cultivars tested, the multifoliolate Miranda showed higher CP content and lower fibrous fractions. In contrast, the more common Prosementi and PR57Q53 excelled in yield, and the well-adapted La Rocca performed well in later cuts. The study confirms that dry matter yields increase at later maturity stages but at the cost of reduced digestibility due to lignification.

Refining agrotechnical practices, including optimal mowing timings and haymaking processes, is essential for achieving a balance between yield and quality. These findings provide a robust foundation for developing targeted management strategies that enhance the productivity

and sustainability of alfalfa-based forage systems in intensive and temperate agricultural context (as that of the Po Valley), supporting at the same time resilient agricultural practices in the face of environmental variability.

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Table A.1. The results of the ANOVA investigating the impact of experimental factors, namely alfalfa cultivar (C), mowing time (MT), and experimental year (Y), along with their interactions, on alfalfa forage yield and quality across the first to third cuts

Source of variation		DM yield Mg ha ⁻¹	CP yields kg ha ⁻¹	CP % DM	aNDFom %DM	ADF %DM	ADL %DM	dNDF %DM	uNDF %DM	Sugars %DM	Phenological stage Kalu & Fick, 1981	Plant height cm	Internode number No.	Internode average length
P-value														
1st cut	Cultivar (C)	0.004	< 0.001	< 0.001	0.108	0.036	0.146	< 0.001	< 0.001	0.009	0.309	0.002	0.075	< 0.001
	Mowing timing (MT)	< 0.001	0.122	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.012
	Year (Y)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.031	< 0.001	< 0.001	0.001
	C x MT	0.118	0.156	0.257	0.036	0.031	0.261	0.077	0.468	0.350	0.720	0.791	0.844	0.135
	C x Y	0.206	0.036	0.226	0.825	0.786	0.537	0.549	0.110	0.250	0.694	0.264	0.810	0.428
	MT x Y	0.525	0.027	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.012	< 0.001	0.835	0.224	0.131	0.585
	C x MT x Y	0.684	0.893	0.594	0.687	0.642	0.802	0.145	0.433	0.391	0.496	0.696	0.830	0.619
2nd cut	Cultivar (C)	< 0.001	< 0.001	0.001	0.004	0.012	0.008	< 0.001	< 0.001	0.001	0.002	< 0.001	0.143	< 0.001
	Mowing timing (MT)	< 0.001	0.002	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Year (Y)	< 0.001	0.650	< 0.001	< 0.001	0.076	< 0.001	< 0.001	< 0.001	0.004	< 0.001	< 0.001	< 0.001	< 0.001
	C x MT	0.398	0.379	0.047	0.235	0.326	0.580	0.206	0.852	0.430	0.383	0.407	0.938	0.252
	C x Y	0.005	< 0.001	0.001	0.002	0.015	0.006	0.007	< 0.001	0.216	0.005	0.462	0.449	0.204
	MT x Y	0.002	< 0.001	< 0.001	< 0.001	0.014	< 0.001	< 0.001	< 0.001	< 0.001	0.340	< 0.001	< 0.001	0.420
	C x MT x Y	0.178	0.470	0.347	0.161	0.169	0.187	0.235	0.323	0.464	0.320	0.325	0.650	0.197
3rd cut	Cultivar (C)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002	< 0.001
	Mowing timing (MT)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Year (Y)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	C x MT	0.340	0.251	0.191	0.528	0.506	0.141	0.448	0.223	0.417	0.036	0.022	0.244	0.044
	C x Y	0.326	0.011	0.053	0.082	0.010	0.152	0.088	0.012	0.752	0.073	0.303	0.045	0.326
	MT x Y	0.093	0.002	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.001	0.039	0.007
	C x MT x Y	0.127	0.067	0.434	0.371	0.652	0.534	0.148	0.042	0.414	0.043	< 0.001	0.328	0.015
Total	Cultivar (C)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.026	< 0.001
	Mowing timing (MT)	< 0.001	0.074	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.003
	Year (Y)	0.002	0.005	< 0.001	0.016	0.002	< 0.001	< 0.001	0.049	0.041	0.758	0.025	0.026	0.002
	C x MT	0.285	0.262	0.165	0.266	0.287	0.327	0.244	0.241	0.399	0.380	0.407	0.675	0.144
	C x Y	0.325	0.081	0.015	0.026	0.006	0.020	0.162	0.025	0.797	0.022	0.291	0.060	0.436
	MT x Y	0.103	0.003	< 0.001	0.009	0.003	< 0.001	0.001	0.002	0.044	0.023	< 0.001	0.021	0.989
	C x MT x Y	0.330	0.477	0.458	0.406	0.488	0.508	0.176	0.445	0.423	0.286	0.511	0.603	0.277

ADF, acid detergent fiber; ADL, acid detergent lignin; aNDFom, NDF expressed exclusive of residual ash; CP, crude protein; DM, dry matter; dNDF, NDF degradability evaluated after 24 h of rumen incubation; uNDF, undigested NDF.

Effect of the model significant P<0.05.

Table A.2. Effect of cultivar on alfalfa forage yield, nutritional value, and morpho-physiological traits

Cultivar		DM yield Mg ha ⁻¹	CP yields kg ha ⁻¹	CP % DM	aNDFom %DM	ADF %DM	ADL %DM	dNDF %DM	uNDF %DM	Sugars %DM	Phenological stage Kalu & Fick, 1981	Plant height cm	Internode number No.	Internode average length
1st cut	La Rocca	4.67 ^b	825 ^b	18.0 ^c	36.7	32.2 ^b	6.68	38.1 ^b	43.5 ^a	9.40 ^a	2.44	80.9 ^a	11.8	6.88 ^{ab}
	Miranda	5.13 ^a	954 ^a	18.9 ^a	37.0	32.3 ^b	6.58	39.7 ^a	42.7 ^b	9.19 ^{ab}	2.35	74.1 ^b	10.9	6.75 ^b
	PR57Q53	5.04 ^{ab}	954 ^a	19.2 ^b	37.3	32.8 ^a	6.73	39.5 ^a	43.2 ^{ab}	8.79 ^b	2.28	79.7 ^{ab}	11.1	7.19 ^a
	Prosementi	5.17 ^a	952 ^a	18.7 ^b	37.5	32.8 ^a	6.66	39.6 ^a	42.5 ^b	9.06 ^{ab}	2.26	81.7 ^a	11.3	7.24 ^a
2nd cut	La Rocca	5.21 ^a	950 ^a	18.3 ^b	42.8 ^a	37.9 ^a	8.17 ^a	33.4 ^b	45.8 ^{ab}	6.46 ^b	5.22 ^a	95.4 ^a	11.8	8.33 ^{ab}
	Miranda	4.42 ^b	837 ^b	19.1 ^a	41.2 ^b	36.7 ^b	7.92 ^b	34.3 ^a	46.1 ^a	6.87 ^a	4.59 ^b	80.4 ^c	11.3	7.25 ^c
	PR57Q53	5.23 ^a	975 ^a	18.7 ^{ab}	42.1 ^{ab}	37.7 ^{ab}	8.03 ^{ab}	34.7 ^a	45.7 ^{ab}	6.71 ^{ab}	4.86 ^{ab}	87.9 ^b	11.4	7.88 ^b
	Prosementi	5.36 ^a	1001 ^a	18.8 ^{ab}	42.5 ^a	37.5 ^{ab}	8.00 ^{ab}	34.4 ^a	45.2 ^b	6.57 ^b	4.69 ^b	90.6 ^b	11.2	8.42 ^a
3rd cut	La Rocca	4.59 ^a	831 ^a	18.2 ^c	38.7 ^a	35.0 ^a	7.66 ^a	33.8 ^b	47.6 ^c	7.16 ^b	6.60 ^a	79.0 ^a	12.4 ^a	6.60 ^a
	Miranda	3.46 ^c	680 ^b	19.8 ^a	35.3 ^c	32.1 ^c	7.16 ^b	35.1 ^a	48.7 ^a	7.61 ^a	5.60 ^c	65.3 ^c	11.4 ^b	5.90 ^b
	PR57Q53	4.49 ^{ab}	841 ^a	18.9 ^b	37.8 ^b	34.5 ^{ab}	7.56 ^a	34.6 ^a	48.2 ^b	7.16 ^b	6.12 ^b	70.7 ^b	11.6 ^b	6.35 ^a
	Prosementi	4.33 ^b	809 ^a	18.8 ^b	37.8 ^b	34.2 ^b	7.51 ^a	34.7 ^a	47.9 ^{bc}	7.10 ^b	6.01 ^b	74.2 ^b	12.1 ^{ab}	6.32 ^a
Average annual	La Rocca	14.5 ^a	2606 ^b	18.1 ^c	39.9 ^a	35.5 ^a	7.59 ^a	35.0 ^b	45.6 ^b	7.49 ^b	4.76 ^a	85.1 ^a	12 ^a	7.27 ^a
	Miranda	13.0 ^b	2471 ^b	19.1 ^a	38.4 ^b	34.1 ^b	7.27 ^b	36.5 ^a	51.9 ^a	7.84 ^a	4.18 ^b	73.3 ^c	11.2 ^b	6.63 ^b
	PR57Q53	14.8 ^a	2770 ^a	18.8 ^{ab}	39.5 ^a	35.4 ^a	7.51 ^a	36.2 ^a	43.4 ^{bc}	7.40 ^b	4.42 ^b	79.5 ^b	11.4 ^{ab}	7.14 ^a
	Prosementi	14.9 ^a	2763 ^a	18.7 ^b	39.9 ^a	35.4 ^a	7.47 ^a	36.2 ^a	39.7 ^c	7.44 ^b	4.32 ^b	82.2 ^{ab}	11.5 ^{ab}	7.33 ^a

ADF, acid detergent fiber; ADL, acid detergent lignin; aNDFom, NDF expressed exclusive of residual ash; CP, crude protein; DM, dry matter; dNDF, NDF degradability evaluated after 24 h of rumen incubation; uNDF, undigested NDF.

For each row mean values with different letters are significant at $P < 0.05$.

Table A.3. Effect of mowing time on alfalfa forage yield, nutritional value, and morpho-physiological traits

Mowing time		DM yield Mg ha ⁻¹	CP yields kg ha ⁻¹	CP % DM	aNDFom %DM	ADF %DM	ADL %DM	dNDF %DM	uNDF %DM	Sugars %DM	Phenological stage Kalu & Fick, 1981	Plant height cm	Internode number No.	Internode average length
1st cut	1	4.6 ^b	892	19.5 ^a	34.1 ^c	29.6 ^c	5.96 ^c	41.5 ^a	41.7 ^c	10.22 ^a	2.08 ^b	69.2 ^c	10.1 ^c	6.87 ^b
	2	5.14 ^a	934	18.5 ^b	38.0 ^b	33.4 ^b	6.82 ^b	39.0 ^b	43.1 ^b	8.55 ^b	2.29 ^b	80.2 ^b	11.5 ^b	6.98 ^{ab}
	3	5.26 ^a	939	18.1 ^c	39.3 ^a	34.6 ^a	7.22 ^a	37.1 ^c	44.1 ^a	8.56 ^b	2.63 ^a	87.9 ^a	12.3 ^a	7.19 ^a
2nd cut	1	4.43 ^c	905 ^b	20.5 ^a	39.7 ^c	35.6 ^b	7.35 ^c	37.0 ^a	44.4 ^b	7.22 ^a	3.81 ^c	79.6 ^d	9.31 ^d	8.67 ^a
	2	4.68 ^c	909 ^b	19.5 ^b	42.5 ^{ab}	37.9 ^a	8.11 ^b	34.6 ^b	45.7 ^a	6.15 ^c	4.18 ^c	86.4 ^c	11.0 ^c	7.91 ^{bc}
	3	5.10 ^b	933 ^{ab}	18.3 ^c	41.9 ^b	37.4 ^a	8.05 ^b	34.1 ^{bc}	46.1 ^a	6.59 ^b	4.85 ^b	89.7 ^{bc}	11.5 ^{bc}	7.95 ^b
	4	5.34 ^b	959 ^{ab}	18.0 ^c	43.6 ^a	38.5 ^a	8.43 ^a	32.1 ^d	46.4 ^a	6.58 ^b	5.06 ^b	92.4 ^{ab}	12.0 ^b	7.99 ^b
	5	5.74 ^a	998 ^a	17.4 ^d	43.0 ^{ab}	37.9 ^a	8.21 ^{ab}	33.3 ^c	45.8 ^a	6.72 ^b	6.31 ^a	94.9 ^a	13.2 ^a	7.34 ^c
3rd cut	1	3.64 ^c	737 ^b	20.3 ^a	35.8 ^d	31.9 ^c	6.86 ^d	37.5 ^a	46.1 ^c	7.68 ^a	4.93 ^e	64.9 ^b	9.34 ^e	7.13 ^a
	2	3.93 ^b	777 ^b	19.8 ^b	36.8 ^{cd}	33.8 ^b	7.53 ^{bc}	34.1 ^c	49.4 ^a	6.69 ^c	5.52 ^d	68.0 ^b	10.7 ^d	6.53 ^b
	3	4.13 ^b	762 ^b	18.6 ^c	37.5 ^{bc}	34.2 ^b	7.46 ^d	35.0 ^b	48.0 ^b	7.28 ^b	6.12 ^c	74.0 ^a	12.05 ^c	6.28 ^{bc}
	4	4.75 ^a	850 ^a	18.0 ^d	38.0 ^{ab}	34.6 ^{ab}	7.67 ^{ab}	33.6 ^c	48.6 ^b	7.29 ^b	6.68 ^b	76.7 ^a	13.17 ^b	5.92 ^{cd}
	5	4.64 ^a	827 ^a	18.0 ^d	39.0 ^a	35.3 ^a	7.85 ^a	32.5 ^d	48.5 ^b	7.37 ^b	7.17 ^a	77.9 ^a	14.07 ^a	5.60 ^d
Average annual	1	12.7 ^c	2533	20.0 ^a	37.0 ^b	32.8 ^b	6.80 ^b	38.6 ^a	44.0 ^b	8.23 ^a	3.61 ^c	71.3 ^c	9.58 ^c	7.56 ^a
	2	13.7 ^b	2619	19.1 ^b	39.6 ^a	35.5 ^a	7.53 ^a	36.1 ^b	45.7 ^a	7.03 ^c	4.00 ^b	78.2 ^b	11.1 ^b	7.14 ^b
	3	14.5 ^a	2633	18.2 ^c	40.1 ^a	35.8 ^a	7.64 ^a	35.3 ^c	45.9 ^a	7.38 ^b	4.53 ^a	83.9 ^a	11.9 ^a	7.14 ^b

ADF, acid detergent fiber; ADL, acid detergent lignin; aNDFom, NDF expressed exclusive of residual ash; CP, crude protein; DM, dry matter; dNDF, NDF degradability evaluated after 24 h of rumen incubation; uNDF, undigested NDF.

For each row mean values with different letters are significant at $P < 0.05$.

Table A.4. Effect of year on alfalfa forage yield, nutritional value, and morpho-physiological traits

Year		DM yield Mg ha ⁻¹	CP yields kg ha ⁻¹	CP % DM	aNDFom %DM	ADF %DM	ADL %DM	dNDF %DM	uNDF %DM	Sugars %DM	Phenological stage Kalu & Fick, 1981	Plant height cm	Internode number No.	Internode average length
1st cut	2011	3.78 ^b	747 ^b	19.8 ^a	34.1 ^b	29.1 ^b	6.04 ^b	40.5 ^a	42.3 ^b	10.6 ^a	2.27 ^b	68.5 ^b	10.1 ^b	6.79 ^b
	2012	6.22 ^a	1096 ^a	17.7 ^b	40.1 ^a	36.0 ^a	7.29 ^a	37.9 ^b	43.6 ^a	7.65 ^b	2.40 ^a	89.7 ^a	12.5 ^a	7.23 ^a
2nd cut	2011	4.93 ^b	937	19.1 ^a	42.8 ^a	37.7	8.40 ^a	31.7 ^b	47.1 ^a	6.54 ^b	5.2 ^a	92.4 ^a	12.4 ^a	7.58 ^b
	2012	5.18 ^a	943	18.3 ^b	41.5 ^b	37.2	7.66 ^b	36.7 ^a	44.3 ^b	6.76 ^a	4.48 ^b	84.7 ^b	10.4 ^b	8.37 ^a
3rd cut	2011	4.88 ^a	904 ^a	18.7 ^b	41.0 ^a	36.2 ^a	7.97 ^a	33.9 ^b	46.6 ^b	6.77 ^b	5.84 ^b	82.0 ^a	11.4 ^b	7.38 ^a
	2012	3.56 ^b	677 ^b	19.2 ^a	33.8 ^b	31.7 ^b	6.98 ^b	35.2 ^a	49.6 ^a	7.75 ^a	6.33 ^a	62.6 ^b	12.3 ^a	5.21 ^b
Average annual	2011	13.6 ^b	2589 ^b	19.2 ^a	39.8 ^a	34.7 ^b	7.59 ^a	35.0 ^b	45.6 ^a	7.75 ^a	4.44	81.0 ^a	11.3 ^b	7.25 ^a
	2012	15.0 ^a	2716 ^a	18.2 ^b	39.1 ^b	35.4 ^a	7.34 ^b	36.9 ^a	45.3 ^b	7.36 ^b	4.40	79.0 ^b	11.7 ^a	6.94 ^b

ADF, acid detergent fiber; ADL, acid detergent lignin; aNDFom, NDF expressed exclusive of residual ash; CP, crude protein; DM, dry matter; dNDF, NDF degradability evaluated after 24 h of rumen incubation; uNDF, undigested NDF.

For each row mean values with different letters are significant at $P < 0.05$.

Table A.5. Effect of interaction between cultivar and year on alfalfa forage yield, nutritional value, and morpho-physiological traits

Source of variation		DM yield Mg ha ⁻¹	CP yields kg ha ⁻¹	CP % DM	aNDFom %DM	ADF %DM	ADL %DM	dNDF %DM	uNDF %DM	Sugars %DM	Phenological stage Kalu & Fick, 1981	Plant height cm	Internode number No.	Internode average lenght	
Cultivar x Year															
1st cut	2011	La Rocca	3.59	691 ^b	19.3	33.7	28.6	6.00	39.6	42.6	11.1	2.38	71.4	10.75	6.66
		Miranda	4.00	796 ^a	19.9	34.0	28.9	6.01	40.9	42.3	10.5	2.29	60.8	9.61	6.36
		PR57Q53	3.74	752 ^{ab}	20.1	34.5	29.5	6.12	40.8	42.5	10.3	2.17	70.0	9.94	7.04
		Prosementi	3.71	750 ^{ab}	19.7	34.4	29.3	6.04	40.6	42.0	10.5	2.25	71.8	10.11	7.12
	2012	La Rocca	5.74	959 ^b	16.7	39.7	35.7	7.36	36.5	44.4	7.71	2.51	90.4	12.86	7.1
		Miranda	6.25	1111 ^a	17.9	39.9	35.6	7.15	38.5	43.0	7.90	2.42	87.4	12.28	7.14
		PR57Q53	6.35	1156 ^a	18.3	40.1	36.2	7.34	38.1	44.0	7.30	2.39	89.5	12.31	7.34
		Prosementi	6.52	1155 ^a	17.7	40.7	36.4	7.29	38.6	43.0	7.67	2.28	91.6	12.50	7.37
2nd cut	2011	La Rocca	4.93 ^b	901 ^{bc}	18.4 ^c	44.0 ^a	38.6 ^a	8.67 ^a	30.4 ^b	47.3 ^a	6.22	5.90 ^a	98.5	12.9	7.74
		Miranda	4.43 ^c	876 ^c	19.9 ^a	41.0 ^b	36.2 ^b	8.18 ^b	31.9 ^a	47.9 ^a	6.83	4.70 ^b	83.7	12	7.04
		PR57Q53	5.09 ^{ab}	971 ^{ab}	19.1 ^b	42.8 ^a	38 ^a	8.40 ^b	32.3 ^a	47.0 ^{ab}	6.64	5.05 ^b	91.8	12.5	7.52
		Prosementi	5.28 ^a	1002 ^a	19.1 ^b	43.3 ^a	37.9 ^a	8.34 ^b	32.4 ^a	46.2 ^b	6.47	5.15 ^b	95.8	12.2	8
	2012	La Rocca	5.49 ^a	998 ^a	18.2 ^a	41.5 ^a	37.3 ^a	7.68 ^a	36.5 ^a	44.4 ^a	6.7	4.55 ^a	92.3	10.6	8.93
		Miranda	4.41 ^b	798 ^b	18.2 ^a	41.5 ^a	37.1 ^a	7.65 ^a	36.7 ^a	44.3 ^a	6.9	4.47 ^a	77.1	10.5	7.47
		PR57Q53	5.38 ^a	978 ^a	18.3 ^a	41.3 ^a	37.4 ^a	7.65 ^a	37.1 ^a	44.4 ^a	6.78	4.67 ^a	84.1	10.4	8.24
		Prosementi	5.44 ^a	999 ^a	18.4 ^a	41.6 ^a	37.1 ^a	7.65 ^a	36.5 ^a	44.1 ^a	6.66	4.23 ^a	85.5	10.1	8.84
3rd cut	2011	La Rocca	5.18	922 ^a	17.9	42.6	37.6 ^a	8.24	32.8	46.4 ^b	6.64	6.55	89.9	12.2 ^a	7.63
		Miranda	4.17	818 ^b	19.8	38.5	33.7 ^c	7.60	34.4	47.4 ^a	7.14	5.20	74.5	10.9 ^{bc}	6.98
		PR57Q53	5.11	949 ^a	18.7	41.4	36.8 ^{ab}	8.03	34.3	46.5 ^b	6.64	5.80	79.5	10.7 ^c	7.57
		Prosementi	5.04	926 ^a	18.5	41.5	36.5 ^b	8.00	34.1	46.2 ^b	6.66	5.80	84.3	11.9 ^{ab}	7.33
	2012	La Rocca	3.99	740 ^a	18.6	34.8	32.3 ^a	7.08	34.8	48.9 ^b	7.68	6.64	68.2	12.5 ^a	5.57
		Miranda	2.75	542 ^b	19.8	32.2	30.5 ^b	6.72	35.8	50.1 ^a	8.08	6.01	56.1	11.9 ^a	4.82
		PR57Q53	3.87	733 ^a	19.1	34.1	32.2 ^a	7.10	34.9	50.0 ^a	7.68	6.43	62.0	12.4 ^a	5.13
		Prosementi	3.63	693 ^a	19.2	34.0	31.9 ^a	7.03	35.2	49.6 ^a	7.54	6.22	64.0	12.3 ^a	5.3
Average annual	2011	La Rocca	13.7	2514	18.4 ^c	40.8 ^a	35.6 ^a	7.80 ^a	33.7	45.7 ^{ab}	7.66	4.94 ^a	86.6	12.0	7.34
		Miranda	12.6	2490	19.9 ^a	38.0 ^b	33.1 ^b	7.30 ^c	35.6	45.9 ^a	8.09	4.06 ^b	73.0	10.9	6.79
		PR57Q53	13.9	2673	19.2 ^b	40.1 ^a	35.3 ^a	7.65 ^{ab}	35.3	45.6 ^{ab}	7.61	4.34 ^b	80.4	11.0	7.38
		Prosementi	14.1	2678	19.0 ^b	40.3 ^a	35.1 ^a	7.60 ^b	35.2	45.1 ^b	7.61	4.40 ^b	84.0	11.4	7.48
	2012	La Rocca	15.2	2697	17.8 ^b	39.1 ^a	35.4 ^a	7.40 ^a	36.1	45.6 ^{ab}	7.33	4.57 ^a	83.6	12.0	7.20
		Miranda	13.4	2452	18.4 ^a	38.9 ^a	35.1 ^a	7.23 ^a	37.4	44.9 ^b	7.61	4.30 ^a	73.5	11.6	6.48
		PR57Q53	15.6	2867	18.5 ^a	39.0 ^a	35.6 ^a	7.39 ^a	37.0	45.6 ^a	7.21	4.50 ^a	78.5	11.7	6.90
		Prosementi	15.6	2847	18.3 ^a	39.5 ^a	35.6 ^a	7.36 ^a	37.1	44.9 ^{ab}	7.29	4.25 ^a	80.3	11.6	7.17

ADF, acid detergent fiber; ADL, acid detergent lignin; aNDFom, NDF expressed exclusive of residual ash; CP, crude protein; DM, dry matter; dNDF, NDF degradability evaluated after 24 h of rumen incubation; uNDF, undigested NDF.

For each row mean values with different letters are significant at $P < 0.05$.

Table A.6. Effect of interaction between mowing time and year on alfalfa forage yield, nutritional value, and morpho-physiological traits

Source of variation		DM yield Mg ha ⁻¹	CP yields kg ha ⁻¹	CP % DM	aNDFom %DM	ADF %DM	ADL %DM	dNDF %DM	uNDF %DM	Sugars %DM	Phenological stage Kalu & Fick, 1981	Plant height cm	Internode number No.	Internode average lenght	
Mowing time x Year															
1st cut	2011	1	3.43	683 ^b	19.9 ^a	29.9 ^c	24.7 ^c	4.96 ^c	43.8 ^a	39.8 ^c	12.3 ^a	2.00	58.2	8.96	6.56
		2	3.84	762 ^{ab}	19.9 ^a	35.4 ^b	30.3 ^b	6.33 ^b	39.7 ^b	42.9 ^b	10.1 ^b	2.25	68.2	10.1	6.79
		3	4.09	796 ^a	19.4 ^a	37.1 ^a	32.1 ^a	6.83 ^a	38.0 ^c	44.2 ^a	9.4 ^c	2.56	79.1	11.3	7.03
	2012	1	5.78	1100 ^a	19.1 ^a	38.2 ^b	34.4 ^b	6.95 ^c	39.2 ^a	43.6 ^a	8.11 ^a	2.17	80.2	11.2	7.18
		2	6.45	1105 ^a	17.1 ^b	40.6 ^a	36.5 ^a	7.31 ^b	38.4 ^a	43.2 ^a	7.06 ^b	2.33	92.3	13.0	7.17
		3	6.43	1081 ^a	16.8 ^b	41.5 ^a	37.0 ^a	7.60 ^a	36.2 ^b	44.0 ^a	7.77 ^a	2.70	96.7	13.2	7.35
2nd cut	2011	1	4.39 ^b	896 ^a	20.4 ^a	41.2 ^b	36.6 ^b	7.91 ^b	33.7 ^a	46.1 ^c	6.39 ^b	4.25	81.2 ^c	9.81 ^d	8.36
		2	4.45 ^b	908 ^a	20.5 ^a	43.9 ^a	38.3 ^a	8.55 ^a	31.4 ^b	46.8 ^{bc}	5.91 ^c	4.31	86.2 ^c	11.4 ^c	7.6
		3	5.18 ^a	978 ^a	18.9 ^b	42.2 ^{ab}	37 ^{ab}	8.21 ^b	32.3 ^b	46.8 ^{bc}	6.82 ^{ab}	5.19	95.6 ^b	12.9 ^b	7.54
		4	5.14 ^a	964 ^a	18.8 ^b	43.6 ^a	38.4 ^a	8.75 ^a	29.6 ^c	48.1 ^a	6.66 ^{ab}	5.44	95.9 ^b	13.2 ^b	7.36
		5	5.5 ^a	942 ^a	17.1 ^c	43.1 ^a	38.1 ^{ab}	8.57 ^a	31.7 ^b	47.8 ^{ab}	6.94 ^a	6.81	103.2 ^a	14.8 ^a	7.03
	2012	1	4.47 ^d	914 ^b	20.5 ^a	38.1 ^c	34.6 ^b	6.79 ^c	40.2 ^a	42.7 ^c	8.05 ^a	3.36	78.1 ^b	8.81 ^c	8.98
		2	4.91 ^c	909 ^b	18.5 ^b	41.2 ^b	37.5 ^a	7.66 ^b	37.8 ^b	44.7 ^{ab}	6.39 ^b	4.05	86.6 ^a	10.7 ^{ab}	8.22
		3	5.02 ^c	887 ^b	17.7 ^{bc}	41.7 ^b	37.7 ^a	7.89 ^{ab}	36.0 ^c	45.5 ^a	6.35 ^b	4.51	83.7 ^{ab}	10.2 ^b	8.36
		4	5.53 ^b	953 ^b	17.2 ^c	43.6 ^a	38.6 ^a	8.11 ^a	34.7 ^d	44.7 ^{ab}	6.5 ^b	4.69	88.8 ^a	10.8 ^{ab}	8.62
		5	5.98 ^a	1054 ^a	17.6 ^c	42.9 ^{ab}	37.7 ^a	7.84 ^{ab}	34.9 ^{cd}	43.9 ^b	6.51 ^b	5.8	86.5 ^a	11.6 ^a	7.66
3rd cut	2011	1	4.17	838 ^b	20.2 ^a	38.1 ^c	33.3 ^d	7.08 ^c	38.3 ^a	44.6 ^b	7.14 ^b	4.62 ^c	72.0 ^b	8.67 ^d	8.4 ^a
		2	4.64	932 ^a	20.2 ^a	40.9 ^b	36.5 ^{bc}	8.08 ^b	34.1 ^b	47.4 ^a	5.91 ^d	4.81 ^c	76.6 ^b	10.1 ^c	7.69 ^b
		3	4.79	865 ^b	18.1 ^b	41.9 ^{ab}	37.2 ^{ab}	8.14 ^b	34.1 ^b	46.6 ^a	6.03 ^d	5.94 ^b	85.9 ^a	11.7 ^b	7.48 ^{bc}
		4	5.38	950 ^a	17.7 ^{bc}	40.9 ^b	35.9 ^c	8.00 ^b	32.5 ^c	47.0 ^a	8.06 ^a	6.44 ^b	85.8 ^a	12.5 ^b	6.91 ^{cd}
		5	5.4	935 ^a	17.4 ^c	43.3 ^a	37.9 ^a	8.54 ^a	30.7 ^d	47.4 ^a	6.74 ^c	7.38 ^a	89.8 ^a	14.3 ^a	6.39 ^d
	2012	1	3.11	635 ^c	20.4 ^a	33.4 ^{bc}	30.5 ^b	6.64 ^d	36.8 ^a	47.7 ^c	8.22 ^{ab}	5.23 ^c	57.9 ^b	10.0 ^c	5.87 ^a
		2	3.21	621 ^c	19.4 ^b	32.6 ^c	31.0 ^b	6.99 ^{bc}	34.2 ^b	51.4 ^a	7.47 ^c	6.23 ^b	59.3 ^b	11.3 ^b	5.36 ^{ab}
		3	3.48	659 ^{bc}	19.0 ^{bc}	33.0 ^c	31.1 ^b	6.78 ^{cd}	35.9 ^a	49.4 ^b	8.52 ^a	6.3 ^b	62.1 ^{ab}	12.5 ^b	5.08 ^b
		4	4.11	750 ^a	18.3 ^d	35.1 ^a	33.3 ^a	7.34 ^a	34.7 ^b	50.2 ^b	6.52 ^d	6.92 ^a	67.6 ^a	13.8 ^a	4.93 ^b
		5	3.88	720 ^{ab}	18.6 ^{cd}	34.7 ^{ab}	32.7 ^a	7.16 ^{ab}	34.3 ^b	49.5 ^b	8 ^b	6.96 ^a	66.0 ^a	13.9 ^a	4.81 ^b
Average annual	2011	1	12	2417 ^b	20.2 ^a	36.9 ^b	32 ^b	6.78 ^b	38.2 ^a	43.8 ^b	8.35 ^a	3.62 ^b	70.5 ^c	9.2 ^c	7.77
		2	12.9	2603 ^a	20.2 ^a	40.3 ^a	35.3 ^a	7.72 ^a	34.8 ^b	45.9 ^a	7.14 ^b	3.79 ^b	77.0 ^b	10.5 ^b	7.36
		3	14.1	2638 ^a	18.8 ^b	40.6 ^a	35.7 ^a	7.79 ^a	34.5 ^b	46.0 ^a	7.28 ^b	4.56 ^a	86.9 ^a	11.9 ^a	7.35
	2012	1	13.4	2649 ^a	19.9 ^a	37.1 ^b	33.6 ^b	6.82 ^c	39.0 ^a	44.3 ^b	8.12 ^a	3.59 ^b	72.1 ^b	10.0 ^b	7.34
		2	14.6	2635 ^a	18.1 ^b	39.0 ^a	35.6 ^a	7.36 ^b	37.3 ^b	45.5 ^a	6.93 ^c	4.20 ^a	79.4 ^a	11.7 ^a	6.92
		3	14.9	2628 ^a	17.6 ^c	39.6 ^a	35.9 ^a	7.51 ^a	36.0 ^c	45.7 ^a	7.47 ^b	4.50 ^a	80.8 ^a	12.0 ^a	6.93

ADF, acid detergent fiber; ADL, acid detergent lignin; aNDFom, NDF expressed exclusive of residual ash; CP, crude protein; DM, dry matter; dNDF, NDF degradability evaluated after 24 h of rumen incubation; uNDF, undigested NDF.

For each row mean values with different letters are significant at P < 0.05.

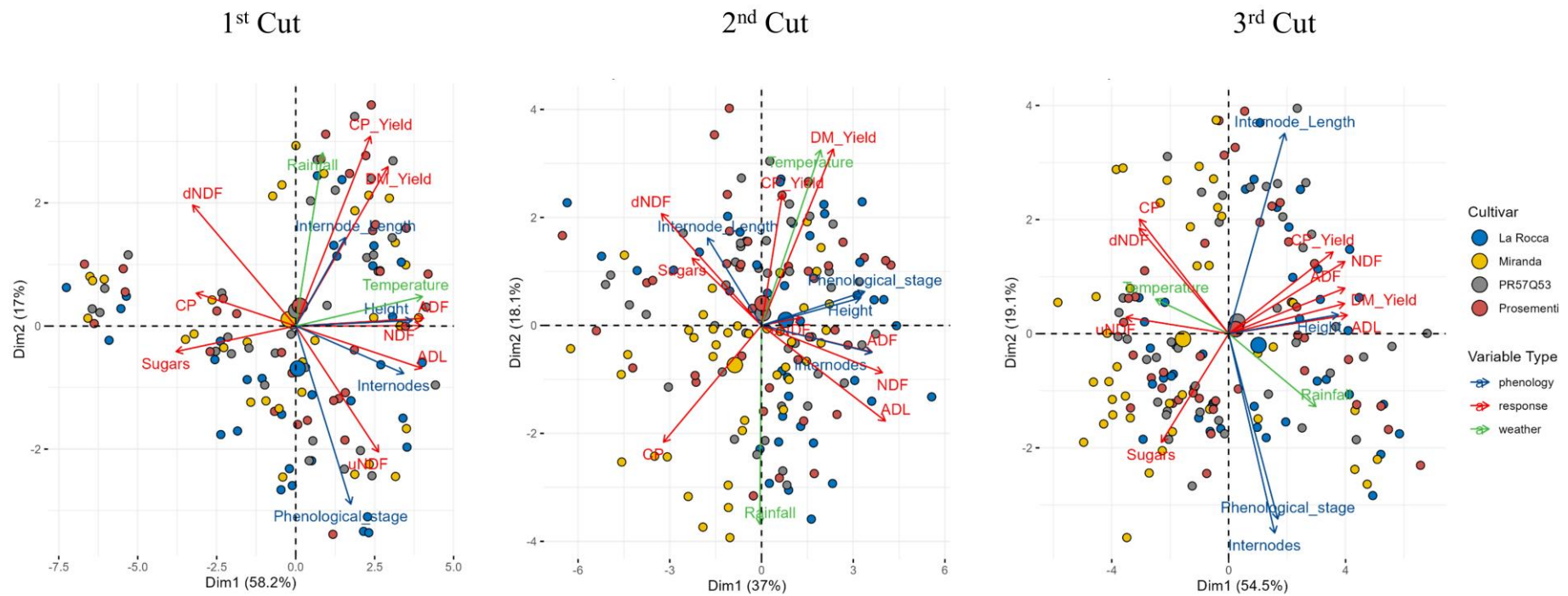


Figure A.1: Principal component analysis (PCA) performed on cultivars (La Rocca, Miranda, PR57Q53, Prosementi) among the three cuts referred to the phenology (e.g., phenological stage, plant height, internode number, and internode average length), response (e.g., DM yield, CP yield, CP, aNDFom, ADF, ADL, dNDF, uNDF, and sugars), and weather (e.g., temperature and rainfall).

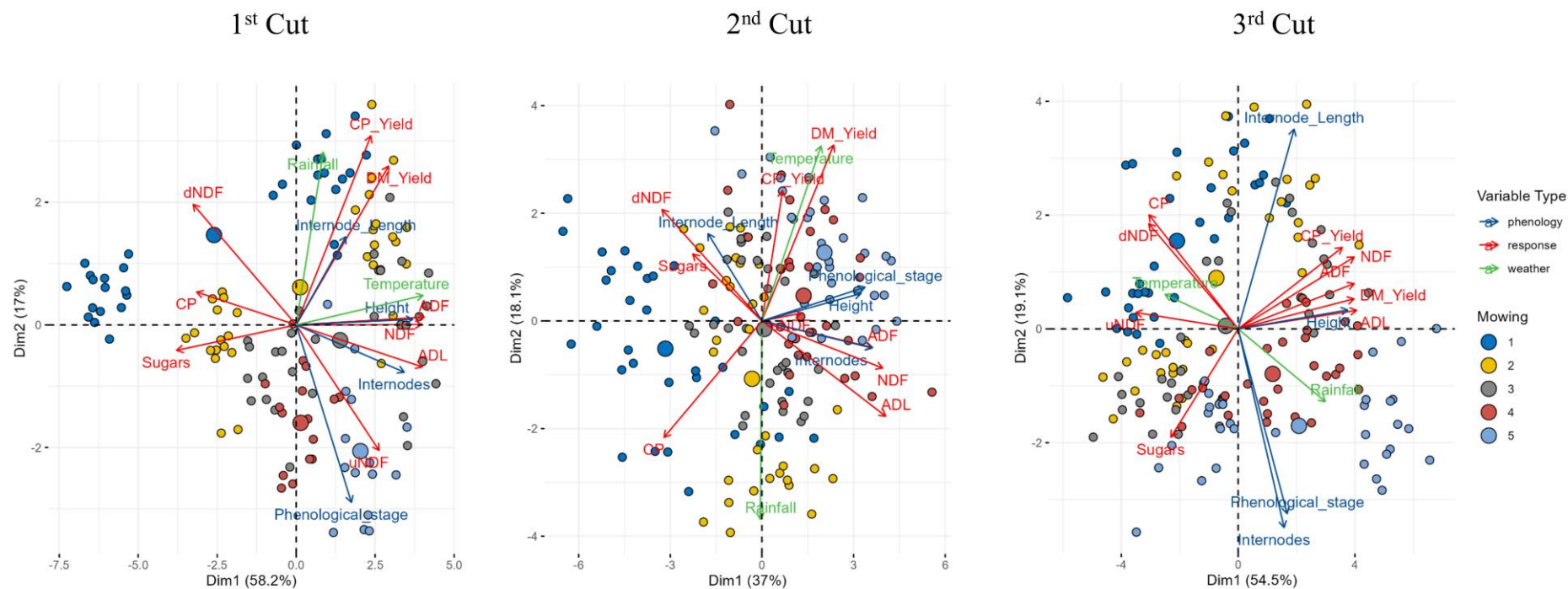


Figure A.2: Principal component analysis (PCA) performed on mowing time (1, 2, 3, 4, and 5) among the three cuts referred to the phenology (e.g., phenological stage, plant height, internode number, and internode average length), response (e.g., DM yield, CP yield, CP, aNDFom, ADF, ADL, dNDF, uNDF, and sugars), and weather (e.g., temperature and rainfall).

List of publications, scientific communication, and submitted paper

Ridurre COSTI E PLASTICA con nuovi materiali di copertura delle trincee - C.Mastroeni, F.Masoero, A.Gallo, M.Colombo, A.Garavaldi, MTPacchioli (2022). StalledaLatte 5/2022.
<http://www.ediagroup.it/BDO/RST223662.asp>

Plastiche «intelligenti» PER INSILARE FORAGGI: vantaggi e costi - C.Mastroeni, M.Colombo, F.Masoero, A.Garavaldi, A.Gallo (2023). StalledaLatte 3/2023.
<http://www.ediagroup.it/BDO/RST233737.asp>

Oxygen barrier plastic films: an opportunity to improve silage quality and reduce the use of plastic - Carmelo Mastroeni, Francesca Ghilardelli, Samantha Sigolo, Martina Mosconi and Antonio Gallo (Oral Presentation - ASPA 2023, Monopoli);
<https://doi.org/10.1080/1828051X.2023.2210877>

Guida alla conservazione dei foraggi in rotoballe fasciate ad alta densità (HD) in collaborazione con Manifatture Norberto Pardini & Figli Spa

Regulated and emerging mycotoxins in silage: Occurrence, effects on animal and prevention Strategies - A. Gallo, A. Catellani, M. Lapris, F. Ghilardelli, C. Mastroeni (Contents - XIX International Silage Conference (ISC) 2023, Beijing)
<https://www.isc2021.cn/uploads/19th%20ISC%20proceedings%20Beijing.pdf>

Evaluating fermentative profile in corn silage using chemical and biological additives - C. Mastroeni, S. van Kuijk, F. Ghilardelli, S. Sigolo, E. Fiorbelli and A. Gallo (Poster Presentation; EAAP, Lyon 2023); <https://doi.org/10.3920/978-90-8686-936-7>

Oxygen barrier plastic films: an opportunity to improve silage quality and reduce plastic used at farm level - Carmelo Mastroeni, Francesca Ghilardelli, Samantha Sigolo, Martina Mosconi and Antonio Gallo (Poster Presentation - AGRIFOODPLAST 2023, Piacenza). In Proceedings of the International Conference on Micro and Nano-Plastics in the Agri-Food Chain, Piacenza, Italy, 10-12 September 2023.

Rotoballe fasciate ad alta densità: indicazioni pratiche e operative per migliorare la conservazione dei nostri foraggi. Carmelo Mastroeni (2023).

<https://www.ruminantia.it/rotoballe-fasciate-ad-alta-densita-indicazioni-pratiche-e-operative-per-migliorare-la-conservazione-dei-nostri-foraggi/>

Review: Strategies and technologies in preventing regulated and emerging mycotoxin co-contamination in forage for safeguarding ruminant health - A. Gallo, A. Catellani, F. Ghilardelli, M. Lapris, C. Mastroeni (published on Animal: The international journal of animal biosciences; <https://doi.org/10.1016/j.animal.2024.101280>)

Il foraggio insilato: Riduzione dell'uso di materiali plastici impiegati nella conservazione dei foraggi per una produzione sostenibile del latte. 2024. Antonio Gallo, Carmelo Mastroeni, Marco Colombo. https://plasticless.crpa.it/nqcontent.cfm?a_id=32207

Improving the quality of whole-plant corn silage in the top layer of a silo by using an inoculant in combination with sodium benzoate - Carmelo Mastroeni, Erica Fiorbelli, Samantha Sigolo, Valentina Novara, Eliana Carboni, Ivan Eisner, Paolo Fantinati, and Antonio Gallo (submitted on Animal Feed Science and Technology; (Manuscript number ANIFEE-D-24-00711R1) pre-print <http://dx.doi.org/10.2139/ssrn.4901944>)

Effects of Limosilactobacillus mucosae UNISS B81 compared to several commercial or pure lactic acid bacteria inoculants on different ensiling conditions of whole plant corn silage - Francesco Fancello, Mauro Spanghero, Carmelo Mastroeni, Valentina Novara, Alessandro Catellani, Severino Zara, Angela Bianco, Daniela Bassi, and Antonio Gallo (submitted on Animal Feed Science and Technology; (Manuscript number ANIFEE-D-23-01009) pre-print <http://dx.doi.org/10.2139/ssrn.4592582>)

List of conferences, seminars, and other activities

- Internship at “Manifatture Norberto Pardini & Figli SpA” from April 1st to September 30th 2023.
- 25th ASPA Congress - Bari June 13rd to 16th 2023. <https://www.assaspa.org/25-congresso-aspa>
- Quality, Safety and Sustainability of the dairy sector PhD Research school. Piacenza June 19th to 23rd 2024. <https://www.phooder.unipr.it/schools/universita-cattolica-del-sacro-cuore/quality-safety-and-sustainability-of-the-dairy-sector-phd-research-school/33/>
- 1^o Agri-Food Plast Conference, Piacenza, Italy, 10–12 September 2023.
- 1^o Ruminant Feed Efficiency Academy in Milan (Italy).
- Visiting student at Animal and Dairy Sciences Department – UW Madison from January 18th to July 14th 2024.
- AN SCI 865 Design and Analysis of Biological studies (UW Madison; January – May 2024).
- AN SCI 420 Microbiomes of Animal Systems (UW Madison; January – May 2024).
- Seminar activity for the Bachelor degree course “Alimentazione, morfologia e benessere animale” in Agricultural Sciences and Technology (L25) – Università degli Studi di Catania. April 29th 2024 (online session). <https://www.di3a.unict.it/corsi/l-25#insegnamenti>
- Dairy Symposium, Madison (WI, USA) May 15th 2024. <https://dairyinnovationhub.wisc.edu/dairy-symposium/>
- Practical nutrition and ration information for an aspiring nutritionist with GPS Dairy Consulting (WI, USA) May 31st 2024.
- Four-State Dairy Nutrition and Management Conference. Dubuque (IA, USA), June 5th and 6th 2024. <http://fourstatedairy.org/>
- Theoretical-practical course on ruminant nutrition with the support of the dynamic software, AMTS – Verona, November 7th and 8th 2024. <https://dairyinnovations.it/amts-course-2024-en>
- Referee activity as junior revisor for the paper “Mitigating aflatoxin B1 in high-moisture sorghum silage: *Aspergillus flavus* growth and aflatoxin B1 prediction”, Front. Microbiol., 23 May 2024, Sec. Food Microbiology, Volume 15 - 2024 | <https://doi.org/10.3389/fmicb.2024.1360343>
- Relator for the conference “Fasciati di erba medica: valorizzare la proteina aziendale” September 17th 2024 – Progetto “Energy&Protein Independence Farm”, cofinanziato dal FEASR. Operazione 16.1.01 “Gruppi Operativi PEI” del Programma di Sviluppo Rurale 2014 – 2020 della Regione Lombardia. <https://dipartimenti.unicatt.it/diana-la-ricerca->

[tecniche-agro-zootecniche-gestionali-per-incrementare-autosufficienza-e-competitivita#content](https://dipartimenti.unicatt.it/diana-la-ricerca-tecniche-agro-zootecniche-gestionali-per-incrementare-autosufficienza-e-competitivita#content)

- Dissemination and field activities for Progetto 'Energy&Protein Indipendence Farm', cofinanziato dal FEASR. Operazione 16.1.01 "Gruppi Operativi PEI" del Programma di Sviluppo Rurale 2014 - 2020 della Regione Lombardia.
<https://dipartimenti.unicatt.it/diana-la-ricerca-tecniche-agro-zootecniche-gestionali-per-incrementare-autosufficienza-e-competitivita#content>
- Co-relator for a Master degree student in Science of Animal Production (LM86) - Università degli Studi di Sassari.