THE EFFECTS of KEFIR ADDITION on FERMENTATION CHARACTERISTICS, AEROBIC STABILITY, and *IN VITRO* DIGESTION PROPERTIES of WHITE CLOVER SILAGES

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PhD Thesis
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TEKİRDAĞ NAMIK KEMAL UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

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ABSTRACT

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The aim of the present thesis was to examine the impact of kefir yeast on fermentation characteristics, aerobic stability, nutritive value, *in vitro* digestibility, some micro- and macronutrient concentration, and fatty acid composition of white clover silages. White clover silages were either uninoculated (Con) or inoculated with kefir yeast at the following application rates: 10 mg/kg (K10), 50 mg/kg (K50), 100 mg/kg (K100) on a fresh basis, and ensiled in laboratory-scale silos for 270 d, followed by 5 d of aerobic exposure. Changes in fermentation characteristics and nutritive value, *in vitro* digestibility and gas production, mineral concentrations, and fatty acid composition were assessed in terminal silages. The results showed that kefir yeast could effectively reduce neutral detergent fiber, hemicellulose, and ammonia-N contents. The increased acetic acid content of kefir yeast treated silages improved aerobic stability. Kefir yeast made no significant difference in cumulative gas production and estimated parameters except for 48 and 72 h. This study has also shown that kefir yeast reduced the potassium, sodium, selenium, and boron concentrations of white clover silages, whereas calcium, zinc, and iron concentration increased. In addition, kefir yeast significantly increased the proportion of saturated fatty acid in white clover silages, whereas the proportion of polyunsaturated fatty acid decreased. However, kefir yeast had no significant effect on the monounsaturated fatty acid proportion of white clover silages. Overall, it is concluded that adding 50-100 mg/kg of kefir yeast on a fresh basis is appropriate to improve the nutritional value of white clover silage.

Keywords: Kefir, White clover, Aerobic stability, Fatty acids, Gas production, Micronutrients

ÖZET

KEFİR İLAVESİNİN AK ÜÇGÜL SİLAJLARININ FERMANTASYON, AEROBİK STABİLİTE VE *İN VİTRO* SİNDİRİM ÖZELLİKLERİ ÜZERİNE ETKİLERİ

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Bu tezin amacı, kefir mayası ilave edilen ak üçgül silajlarının fermantasyon özellikleri, aerobik stabilitesi, besin değeri, *in vitro* sindirilebilirliği, bazı mikro ve makro besin elementleri konsantrasyonu ve yağ asidi kompozisyonu üzerindeki etkisini incelemektir. Kontrol grubu olarak kefir mayası eklenmemiş ak üçgül silajı (Kon) ve sırasıyla taze materyal üzerine 10 mg/kg (K10), 50 mg/kg (K50), 100 mg/kg (K100) kefir ilave edilerek laboratuvar tipi silolarda 270 gün boyunca silolanmış ve 5 gün aerobik ortamda bırakılmıştır. Terminal silajların fermentasyon özellikleri ve besin değeri, *in vitro* sindirilebilirlik ve gaz üretimi, mineral konsantrasyonları ve yağ asidi bileşimindeki değişiklikler değerlendirilmiştir. Sonuçlar, kefir mayasının nötral deterjanda çözünmeyen lif, hemiselüloz ve amonyak azotu içeriklerini etkili bir şekilde azaltabileceğini göstermiştir. Kefir mayası ile muamele edilmiş silajların artan asetik asit içeriği, aerobik stabiliteyi iyileştirmiştir. Kefir mayası kümülatif gaz üretimi ve tahmin edilen parametrelerde 48 ve 72 saat dışında önemli bir fark oluşturmamıştır. Bu çalışma ayrıca kefir mayasının ak üçgül silajlarının potasyum, sodyum, selenyum ve bor konsantrasyonlarını azalttığını; kalsiyum, çinko ve demir konsantrasyonlarını ise arttırdığını göstermiştir. Ayrıca kefir mayası, ak üçgül silajlarının doymuş yağ asidi oranını önemli ölçüde artırırken çoklu doymamış yağ asidi oranını ise azaltmıştır. Ancak kefir mayasının ak üçgül silajlarının tekli doymamış yağ asidi oranı üzerinde anlamlı bir etkisi olmamıştır. Genel olarak, ak üçgül silajının besin değerini iyileştirmek için taze materyele 50-100 mg/kg kefir mayası ilavesinin uygun olacağı sonucuna varılmıştır.

Anahtar Kelimeler: Kefir, Ak üçgül, Aerobik stabilite, Yağ asitleri, Gaz üretimi, Mikrobesinler

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LIST OF ABBREVIATIONS

AA	Acetic Acid
Abs	Absorbance
ADF	Acid Detergent Fiber
ADIN	Acid Detergent Insoluble Nitrogen
ADL	Acid Detergent Lignin
ADS	Acid Detergent Solution
a_w	Water Activity
B	Boron
BA	Butyric Acid
Bc	Buffering Capacity
BPW	Buffered Peptone Water
Ca	Calcium
CO_2	Carbon Dioxide
CP	Crude Protein Degradability
CPD	Crude Protein Degradability
CT	Condensed Tannins
CTAB	Cetyltrimethylammonium Bromide
Cu	Copper
$CuSO_4$	Copper Sulfate
DDM	Digestible Dry Matter
DM	Dry Matter
DMI	Dry Matter Intake
EE	Ether Extract
ELOS	Enzyme Soluble Organic Matter
EULOS	Enzyme Insoluble Organic Matter
FA	Fatty Acids
FAEs	Feruloyl Esterases
FAME	Fatty Acid Methyl Ester
FAO	Food and Agriculture Organization
Fe	Iron
FID	Flame Ionization Detector
FM	Fresh Material

GLM	General Linear Model
GP	Gas Production
H_2O	Water
H_2SO_4	Sulfuric Acid
HCl	Hydrochloric Acid
HNO_3	Nitric Acid
K	Potassium
K_2SO_4	Potassium Sulfate
KOH	Potassium Hydroxide
LA	Lactic Acid
LAB	Lactic Acid Bacteria
ME	Metabolizable Energy
Mg	Magnesium
min	Minute
MRS	De Man, Rogosa, and Sharpe Agar
MUFA	Monounsaturated Fatty Acid
N	Nitrogen
N_2O	Nitrous Oxide
N_2O_3	Dinitrogen Trioxide
N_2O_4	Dinitrogen Tetroxide
Na	Sodium
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NaOH	Sodium Hydroxide
NDF	Neutral Detergent Fiber
NDS	Neutral Detergent Solution
NEL	Net Energy Lactation
NH_3-N	Ammonia Nitrogen
NO	Nitric Oxide
NO_2	Nitrogen Dioxide
nonTP	Non-Tannin Phenols
NPN	Nonprotein Nitrogen
NSC	Nonstructural Carbohydrates
OMD	Organic Matter Digestibility

P	Phosphorus
PA	Propionic Acid
PDA	Potato Dextrose Agar
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated Fatty Acids
PVPP	Polyvinylpolypyrrolidone
RFV	Relative Feed Value
Se	Selenium
SE	Standart Error
SEM	Standart Error of Mean
SFA	Saturated Fatty Acids
T	Tannin
TDM	True Dry Matter
TP	Total Phenolic
TR	*Trifolium repens* L.
UNSFA	Unsaturated Fatty Acids
VRBG	Violet Red Bile Glucose Agar
WSC	Water Soluble Carbohydrates
Zn	Zinc

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1. INTRODUCTION

According to a recent report about world population prospects, it is expected that the human population will stand at 9.7 billion in 2050 and reach up to 10.9 billion in 2100 (United Nations, Department of Economic and Social Affairs, Population Division, 2019). This rapid population expansion also brings some interconnected chain problems such as nutrition, shelter, environmental pollution, and health. To cope with these issues brought increasing population, there is no doubt that humankind should make much more effort than humanity has made so far in the next few decades.

Livestock plays a pivotal role in global food security and nutrition, supplying approximately one-third of total protein intake, one-sixth of total calories, and more than half derived from ruminants (FAO, 2018). Ruminants can utilize forages and fibrous feedstuffs, which are indigestible by mammalian enzymes, converting them into high nutritional quality products such as meat and milk (van Gastelen, Hettinga, Klop, Alferink, and Hendriks, 2015). However, ruminants require large quantities of quality forages, not influenced by seasonal variation, throughout the year; and it seems not possible to supply this requirement without using one of two main storing approaches, ensiling or haymaking, considering the limited harvesting season and climatic conditions (McAllister et al., 2018; Muck, Kung Jr, and Collins, 2020).

Plant tissue contains various fodder nutrients that are a good environment for the growth of microbes and fungi. These organisms normally cause spoilage and mold quickly at high humidity. To prevent spoilage of plant tissues and long-term storage of moist forage, we profit ensiling. Various advantages of ensiling according to haymaking were also reported in the literature: low mechanical losses at harvest, low nutrient losses at wilting, less weather dependency, and ease of feeding management (Collins and Moore, 2018; van den Oever et al., 2021). However, undesirable microorganisms, such as clostridia and yeast, in poorly conserved silage increase dry matter (DM) losses and negatively affect the silage quality and palatability (Collins and Moore, 2018).

Awareness of ensiling as an agricultural practice is not recent and dates back approximately 3500 years ago. Possibly first been practiced in a coarse form of fresh material into a vertical stone structure covered by a lid by Egyptians and Greeks (McDonald, Henderson, and Heron, 1991; Nelson, Moore, and Collins, 2017). Indeed ensiling has

experienced unprecedented growth after the 1950s to meet the demands of intensified livestock sectors, thereby becoming more popular worldwide (Schmidt, 2008). The main reason behind this may be its simplistic technology: compressing forages, sealing airtight, and waiting for the conversion process of free sugar into lactic acid (LA), making it more accessible to a year-round livestock production system regardless of climatic conditions (Holzer, Mayrhuber, Danner, and Braun, 2003).

A closer inspection of producing high-quality silage shows that it is quite complex and influenced by several factors. In general, the microbiome of freshly harvested forages plays a crucial role in the ensiling process, and it is not always possible to alter ideal pH and inhibit clostridia and mold proliferation unless adequate epiphytic lactic acid bacteria (LAB) and water-soluble carbohydrates (WSC) are found. However, it is possible to improve silage quality by using a modern ensiling technique alone or in combination: co-ensiling with sugar-rich crops or by-products, e.g., oat, ryegrass, sugar beet pulp, citrus pulp (Łozicki et al., 2015; Yan et al., 2019; Chen et al., 2020a; Ülger et al., 2020); wilting to increase DM and sugar content (Rupp, Westreicher-Kristen, and Susenbeth, 2021); fermentation inhibitors for inhibition of microbial activity, e.g., sulphuric acid, formic acid, formaldehyde, sodium diacetate, potassium sorbate (Weinberg, 2008; Wen, Yuan, Wang, Desta, and Shao 2017; Yuan, Wen, Desta, Wang, and Shao, 2017b); fermentation enhancers to speed up silage fermentation and minimize losses, e.g., bacterial inoculants, enzymes, molasses, the fermented juice of LAB, pre-fermented juice of grasses, kefir (Bureenok, Suksombat, and Kawamoto, 2011; Okuyucu and Esen 2022; Koç, Özkan Ünal, Okuyucu, Esen, and Işık, 2021).

Extensive research has shown that legumes have unique advantages over grasses or cereals for maintaining the ecosystem and sustainable livestock production; these are: (i) reducing the dependency on industrial N-fertilizer, (ii) mitigation of greenhouse gases and nitrate, (iii) increasing nutritive value and protein content of herbage, (iv) increasing dry matter intake (DMI) and animal production, and (v) improving animal health status (Rochon et al., 2004; Dewhurst, 2013; Lüscher, Mueller-Harvey, Soussana, Rees, and Peyraud, 2014; Phelan et al., 2015; Castro-Montoya and Dickhoefer, 2018). However, despite these positive advantages, legumes are generally characterized by low resistance to grazing, high risk for bloating, and difficulty preserving as ensilage (Phelon et al., 2015).

White clover (*Trifolium repens* L.) (TR), belonging to the *Legumminaceae* family, has been widely grown in the temperate region and many parts of Europe with a growing trend toward sustainable, low-cost, low-input agricultural systems in the last three decades (Frame, Charlton, and Laidlaw, 1998). It is also well adapted to grazing systems and could be conserved as hay or silage. TR has similar protein quality to alfalfa (*Medicago sativa* L.) and contains more WSC than birdsfoot trefoil (*Lotus corniculatus* L.) and red clover (*Trifolium pratense* L.) (Kirchhof, Eisner, Gierus, and Südekum, 2010). Besides, the DM yield of TR is relatively lower than red clover but higher in digestibility (Kornfelt, Nørgaard, and Weisbjerg, 2013). Like other legume crops, TR is characterized by a lower structural fiber (NDF) than grasses (Dewhurst, Fisher, Tweed, and Wilkins, 2003). An increased ratio of polyunsaturated fatty acids (PUFA) in milk, particularly α-linolenic acid, was also indicated when dairy cows fed with TR (Steinshamn and Thuen, 2008; van Dorland, Kreuzer, Leuenberger, and Wettstein, 2008). However, the high N content, high buffering capacity (Bc), and low WSC content of TR make this crop difficult to ensile (Winters et al., 2004).

Promoting microbial fermentation in the desired way is an important aspect of controlling the ensiling process. LAB is among the most widely used for this purpose and plays a crucial role in producing organic acid, mainly LA and acetic acid (AA), which is fundamental to the conservation of silage with high quality (Wang et al., 2020; Jung, Ravindran, Soundharrajan, I., Awasthi, and Choi, 2022). Much of the current literature on silages focuses on bacterial inoculants and enzymes, which are safe, environmental-friendly, non-corrosive, non-toxic, and easy to use (Zhang et al., 2009; Wang et al., 2020; Rupp et al., 2021). A search of the literature also drew our attention to the possibility of an excellent starter culture for preserving high-quality silage using yeast and LAB in a combination (Schnürer and Jonsson, 2011). This assumption makes kefir an excellent candidate for an alternative silage additive due to its natural microbial flora.

On the one hand, kefir is one of the most well-known milk fermented beverages and has been used for a healthy and balanced life for centuries due to its natural probiotic properties (Miao et al., 2014; Satir and Guzel-Seydim, 2016). Kefir grain has a granular shape, semi-hard structure coated with polysaccharide matrix, and contains a diverse range of LAB, acetic acid bacteria, and yeast depending on the origin of fermented milk (Zhou, Liu, Jiang, and Dong, 2009; Gao, Gu, Abdella, Ruan, and He, 2012). On the other hand, kefir has been used as an alternative silage additive to improve alfalfa and wheat straw silage (Koç,

Karapınar, Okuyucu, Korucu Erdem, 2020; Koç et al., 2021; Okuyucu and Esen, 2022) as well as its avoid mold growth, *Aspergillus flavus*, in ensiled sorghum grains (Gonda et al., 2019).

Nonetheless, identifying kefir yeast as a silage additive would provide more useful information for its effects on *in vitro* digestion properties, mineral concentration, and fatty acid composition of leguminous crop silages. Therefore, this thesis seeks to understand and explain the role of the addition of kefir yeast not only on fermentation characteristics and aerobic stability but also on *in vitro* digestion properties, mineral concentration, and fatty acid composition of high moisture TR silage in long-term storage.

2. LITERATURE REVIEW

2.1 The Role of Forage Preservation and Ensiling in Ruminant Nutrition

The major contributor of ruminants to the food chain is converting non-edible structural fiber and proteins from plant biomass into high nutritional animal-sourced foods such as meat and milk, which are the primary sources of a well-balanced diet (Gislon et al., 2020; Zubairova, Tagirov, Mironova, Iskhakov, and Vagapov, 2022). Ruminants require a constant supply of high quantity and quality feed to optimize their productivity throughout the year, and forage crops play a crucial role in this optimization. In particular, the proportion of forages in a dairy cow's ration can be varied from 40 to 100%, and they are vitally important not only to derive their energy and nourishment but also to maintain their health and welfare (Adesogan et al., 2019). However, such climate-based factors, e.g., a shortage grazing period, maturity at harvesting, unavailability in the dry season, and preservation methods, are able to restrict the supply chain and affect forage quality significantly (Weinberg, 2008). It has also been conclusively shown that the maturity stage of the plant significantly affects the digestion, energy, and crude protein (CP) content (Figure 2.1) (Harris et al., 2017). Therefore we need to harvest forages when they reach the optimum yield and nutritional quality and preserve them with an appropriate method for further use.

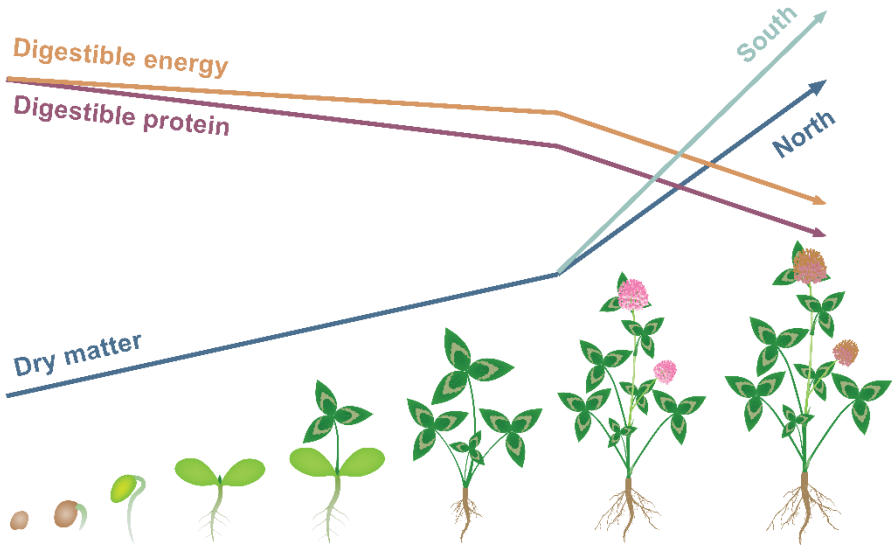


Figure 2. 1. Relationship between maturity stage of clover and its nutritional value in Northern Hemisphere (without scale).

The dry matter of clover is illustrated by two lines to show a dramatic increase of DM in the Southern part of the Northern Hemisphere (adapted from Harris et al. (2017) for clover).

There are two options for preserving forages: haymaking or ensiling, and in most cases, climatic conditions, traditions, and technology are the determining factors (Muck et al., 2020). Data from several studies suggest that it is not possible to reduce losses in both haymaking and ensiling completely; however, it is possible to minimize losses by applying a comprehensive approach from the initial crop selection stage to the final storage period (Hancock and Collins, 2006; Holmes and Muck, 2007; Zubairova et al., 2022).

The main goal of haymaking is the biological inactivation of plant enzymes and preventing microbial decomposition by drying the forages. In contrast, the main goal of ensiling is to protect moist forages from the spoilage of microorganisms by reducing the pH (Mehmood et al., 2020). It has been concluded that the greatest losses in haymaking occur during harvest (Muck et al., 2020). However, fewer losses during the storage period, ease of transportation, and more marketable than silage make haymaking more effective in dry climates (Mehmood et al., 2020; Muck et al., 2020). Unlike haymaking, ensiling is more popular and has become more common in industrialized animal husbandry, e.g., in Germany, Netherlands, Denmark, temperate climates, and wet climates (Filya, Ashbell, Hen, and Weinberg, 2000). Among the several advantages of ensiling compared to haymaking, perhaps the most attractive one for the producer is reducing the risk of weather damage. Another advantage of ensiling is reduced leaf shatter, which prevents nutritional losses and increases nutritional quality compared to haymaking (Polan, Stieve, and Garrett, 1998).

However, a successful ensiling requires some basic knowledge and proper application about the type of forage crops and their chemical characteristics, harvesting management (moisture at harvest, cut length, the temperature at harvest), silo types (bunker silos, pressed bag silos, tower silos, wrapped bales), sources of losses, and silage additives (inoculants, enzymes, chemicals).

2.2 Overview of the Ensiling Process

McDonald et al. (1991) point out that the following characteristics of a crop are needed to produce well-preserved and aerobically stable silage: (i) should contain an adequate soluble carbohydrate content, (ii) low Bc, and (iii) a DM content above 200 g/kg. Along the same lines, Weinberg (2008) suggests that the DM content of a crop should be ranged between 300-400 g/kg with low Bc for a successful ensiling. Minimum 30 to 50 g/kg DM soluble carbohydrate content was also required to support initial LAB growth (Weinberg, 2008). Moreover, Rooke and Hatfield (2003) underlined that both solubility and availability

of carbohydrates are equally important to provide sufficient fermentable material to the LAB in a silo; therefore, the greatest part of WSC should be glucose and fructose, which are readily fermentable.

Niyigena et al. (2021) concluded that cool-season crops have greater WSC content than warm-season forages; however, several factors are known to affect the total WSC content in both cool- and warm-season crops. Diurnal accumulation of WSC is one of them. Ruckle Bernasconi, Kölliker, Zeeman, and Studer (2018) noted that both grasses and legumes accumulate photosynthesis products during the daytime and are used for respiration and growth during the nighttime. McDonald et al. (1991) argued that the WSC content of grasses increases with the accumulation of photosynthesis products throughout the day and reaches a peak in the afternoon while legumes reach in the midday. Pelletier et al. (2010) concluded that a concomitant decrease in soluble carbohydrates after midday in legumes was possibly related to the conversion of hexose to nonstructural carbohydrates (NSC) and starch. Likewise, Andueza, Delgado, and Muñoz (2012) assert that the NSC content is higher in legumes harvested at sunset than at sunrise. Ruckle et al. (2018) noted that grasses could deposit approximately 200-250 g/kg DM of WSC in their stem and leaves, whereas legumes deposit approximately 250-350 g/kg DM of starches in their biomass. Starches in the seed of grasses and legumes could be neglectable due to the small seed size and growth stage and when harvested for ensilage (Smith, Bula, and Walgenbach, 1986). In addition, Kagan et al. (2020) demonstrated that harvesting season and geographic regions are other important factors that affect the WSC content of forages.

Almost every paper that has been written on silage includes a section relating to buffering capacity (Bc). According to Muck (1988), the term "Buffering capacity" refers to "the amount of acid required to drop crop pH from 6 to 4 per unit DM". In other words, Bc measures the degree of resistance of a crop to change in pH (Kung Jr, 2010). In a study investigating the minimum sugar content of forage to maintain the silage pH between 4.2 to 3.9, Stanaćev and Vik (2002) indicated that Bc is directly correlated with the sugar content of forage and legumes need more WSC than grasses to produce more LA for reducing pH. This view is supported by Mannetje (2016), who writes that high organic acid and crude protein (CP) concentrations and relatively low WSC content make legumes difficult to ensile. It has also been suggested that silage material, especially legumes, should be wilted to 300 and 500 g/kg DM before ensiling to decrease the Bc and reduce butyric acid bacteria, which cause spoilage. Muck (1988) reported that a high buffering capacity in alfalfa is associated with the

maturity stage, the number of cutting, and potassium fertilization. It is concluded that ensiling immature, first cut, and fertilized alfalfa requires more WSC than unfertilized, later cut, or more mature alfalfa.

Rotz's research is valuable for understanding how each major field process influences the final quality of forage and its DM, such as mowing, wilting, and chopping (Rotz, 2003). Slottner and Bertilsson (2006) argued that proteolysis during harvest and wilting reduced with an increased DM content of forages. Similarly, Borreani et al. (2018) demonstrated that DM and nutritive value losses could be reduced by a rapid wilting in the field. In addition, Figure 2.2 provides an overview of how DM content affects the nitrogen and organic acid concentrations of forage at harvest. As stated by Pottier and Martin Rosset (2015), soluble nitrogen, ammonia nitrogen, and organic acid concentration could be reduced with an increased DM content.

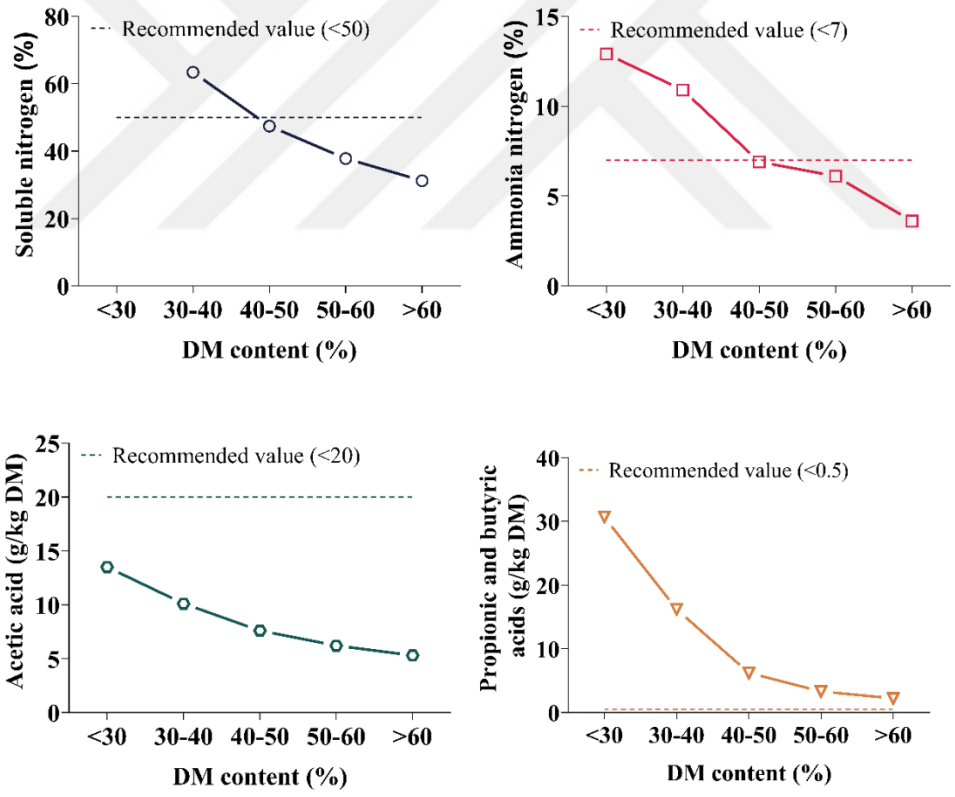


Figure 2. 2. Effect of dry matter content on nitrogen and organic acid concentration at harvest (adopted from Pottier and Martin Rosset, 2015).

Furthermore, Santos et al. (2014) showed that the DM, neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent insoluble nitrogen (ADIN) content of guinea

grass linearly increased with an increased regrowth interval while CP and ammonia nitrogen content linearly reduced; which is consistent with the data obtained by Snyman and Joubert (1996), who concluded that CP, nonprotein nitrogen (NPN), and *in vitro* digestibility of forages decreases as plants mature.

In the literature, the relative importance of DM on silage quality has been subject to considerable discussion. For example, Hu, Schmidt, Mcdonell, Klingerman, and Kung Jr (2010) highlighted that the overall fermentation process could be restricted due to the lack of moisture in dry forages. Muck (1987) showed that increasing DM at harvest decreased proteolysis rates, nonprotein nitrogen, free amino acids, and ammonia concentrations in alfalfa silages decreased. It was also reported that a lesser amount of AA production, higher ethanol fermentation, and more susceptibility to aerobic deterioration during the feed-out phase are common in high DM (>400 g/kg) silages (Kung Jr et al., 2018; Zhao et al., 2019a). In the same vein, Zhao, Tao, Wang, Li, and Shao (2021) noted that lower WSC, LA, and propionic acid (PA) concentrations were observed in low DM silages. Moreover, the DM content of forage also affects the effluent and fermentation losses of silage. In a study conducted by Randby and Bakken (2021), it was shown that effluent and fermentation losses in bunker silos were higher in low DM grass silage.

Proteolysis is a common term used to describe the hydrolyzation of proteins into peptides and free amino acids by indigenous plant proteases, followed by degradation into amides, amines, and ammonia by microbial activities during ensiling (He et al., 2019). It is commonly assumed that proteolysis occurs even though good management practices are applied during harvesting and storage periods (Grant and Ferraretto, 2018). Der Bedrosian, Nestor, and Kung Jr (2012) identified a positive correlation between *in vitro* starch digestibility and proteolysis in corn silages. In a follow-up study, Grant and Ferraretto (2018) attributed increased starch digestibility to the proteolysis of prolamin protein which is positively related to ammonia nitrogen concentration in corn silage. Thus, Ogunade et al. (2018) proposed that ammonia nitrogen could be used to monitor the degree of proteolysis during ensiling.

Several factors are known to be partially responsible for proteolysis during ensiling such as DM content, pH, and temperature. For example, in a study conducted by Valente, Borreani, Caredda, Cavallarin, and Sulas (2003), it was shown that proteolysis was reduced with an increased DM content. Zhao et al. (2019b) suggested that proteolysis caused by plant

proteinases and other proteolytic organisms could be inhibited by a rapid LA accumulation in the early fermentation phases. Likewise, it has been reported that rapid acidification by using formic acid in ryegrass silage results in reduced proteolysis and peptidases (Nsereko and Rooke, 1999). Wang et al. (2019b) noted a lower ammonia nitrogen concentration stored at 15 °C than stored at 30 °C silages due to reduced amino acid metabolism and enzyme activity of microorganisms like *Clostridia* and *Enterobacteriaceae*.

Similarly, Zhang, Yu, Wang, and Tian (2018) established that high temperature not only increases proteolysis but also increases butyric acid (BA) fermentation in silages stored at 40 °C. There has been substantial research undertaken on how DM content effect proteolysis in ensiling. Furthermore, many recent studies (e.g., Wang et al., 2019a; He et al., 2020) have shown that tannins could be used as an alternative treatment to restrict proteolysis, especially in legume silages.

2.3 Stages of Ensiling

In broad terms, ensiling can be defined as the inhibition of spoilage factors in any moisture plant material or industrial by-products via LA fermentation under anaerobic conditions in a silo. Such abiotic factors, including pH, temperature, moisture, or aerobic/anaerobic conditions, play a key role in successfully ensiling.

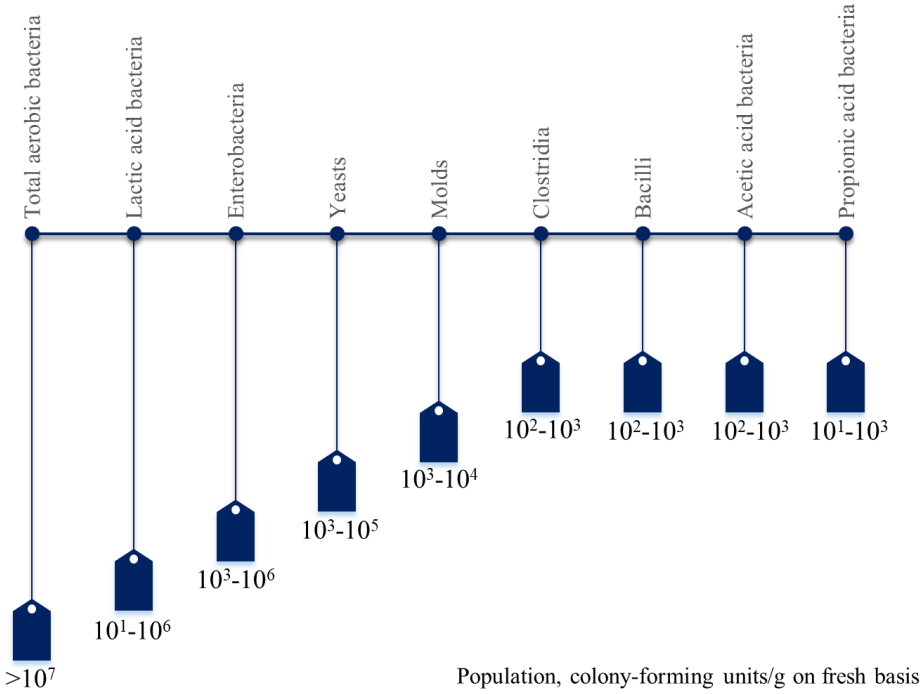


Figure 2. 3. Microbial population and abundance of a typical forage crop (adopted from Pahlow Muck, Driehuis, Oude-Elferink, and Spoelstra, 2003).

Epiphytic LAB and its abundance may be the most important ones in these abiotic factors due to acidifying the environment by converting WSC to organic acids (mainly LA) and reducing pH (Weinberg and Muck, 1996; Weinberg, 2008). Thus almost all studies underlined the importance of initial LAB count (Figure 2.3) and fermentable sugar contents.

The ensiling process can be divided into 4 distinct stages once the fresh material has been compacted and sealed to prevent air penetration (Weinberg and Muck, 1996):

2.3.1 Aerobic Phase

The initial phase of ensiling begins with compaction and consolidation of the material and sealing of the silo to cut atmospheric oxygen's re-entry (Yitbarek and Tamir, 2014). Chopping plant material into fine particles plays a significant role in this compaction (Meeske, 2005). It normally takes a few hours, and trapped oxygen in the silo is reduced by the respiration of plant material and aerobic and facultative aerobic microorganisms such as yeast and *Enterobacteria*. Furthermore, the enzymatic breakdown of protein and carbohydrates continues during this stage to provide a pH similar to fresh material to 6.0-6.5 (Oude Elferink, Driehuis, Gottschal, and Spoelstra, 2000; Weinberg, 2008).

2.3.2 Fermentation Phase

The fermentation phase of ensiling begins with the depletion of trapped oxygen in the silo and lasts for several days to weeks due to ensiling material's properties and environmental conditions. In a successfully ongoing fermentation, LABs become dominant in the silo, convert WSC into LA, and reduce the pH to 3.8-5.0 in this phase (Oude Elferink et al., 2000; Weinberg, 2008). Besides, the chemical breakdown of hemicellulose by releasing some sugars occurs at a slow rate in this phase, and they might be used if the LAB fermentation halt due to a lack of WSC (Bolsen, Ashbell, and Weinberg, 1996).

2.3.3 Stable Phase

Evidence suggests that the amount of aerobic loss is related to the permeability of silo and the density of silage material (Bolsen et al., 1996). If the silage material is well-squeezed and air penetration into ensiled material is prevented, relatively few changes occur in this phase. Furthermore, due to acidic environments, most of the fermentation phase microorganisms decrease in numbers. However, some inactive forms of acid-tolerant microorganisms and spore-formating clostridia and bacilli can survive during this stage. Also,

some acid-tolerant plant enzymes (e.g., proteases and carbohydrases) and LAB (e.g., *Lactobacillus buchneri*) continue to be active in these severe acidic conditions at a lower rate (Oude Elferink et al., 2000; Schmidt, Emara, and Kung Jr, 2008).

2.3.4 Feed-out Phase

Among the fermentation phases of silage, perhaps the most neglected phase is the feed-out phase, which begins when the silage is re-exposed to air. The reactivation of aerobic microorganisms (generally yeast and mold, but in some cases, *Enterobacteria* and bacilli) in the silage increases the consumption of WSC, fermentation end-products, and soluble nutrients. The largest DM (up to 50%) and nutrient losses can occur during the feed-out phase due to the respiration of these soluble products into carbon dioxide and water, raising pH and producing heat (Bolsen et al., 1996; Oude Elferink et al., 2000). Research arguments against heating in the silo also reveal that number of epiphytic microorganisms found in the silage, air exposure time, fermentation characteristics, filling and compaction rate of the silo and ambient temperature play a significant role in reducing DM losses.

2.4 Silage Bacteria

2.4.1 Lactic Acid Bacteria

LAB are facultative anaerobes that belong to the epiphytic microflora of ensiling materials. Thus far, several studies have well documented the essential role of LAB in the ensiling process of reducing pH and preserving forages (Blajman, Páez, Vinderola, Lingua, and Signorini, 2018; Ávila and Carvalho, 2020; Carvalho, Sales, Schwan, and Ávila, 2021). According to Nishino (2011), desired LA fermentation in the silo could be done with LAB, which is well adapted to low pH. This author also stated that surviving LAB might have a great chance to suppress the other bacteria after the silo opening.

Most of the LAB isolated in silage are mesophilic. They grow well at temperatures between 20 and 40 °C, with an optimum of around 30 °C (Driehuis and Oude Elferink, 2000). However, Amaral et al. (2020) stated that there had also been some LAB strains isolated in sugarcane and elephant grass silages that have the ability to grow at a low temperature of around 15 °C. It is believed that these strains could be used effectively to improve the fermentation process in cold climate zones where low temperatures may obstruct forage preservation (Bernardes et al., 2018).

Generally, fermentation pathways in the silo depend on available substrates, the presence/absence of oxygen, and the enzymatic structure of LAB (Weinberg, 2008). As shown in Figure 2.4, fermentation products of LAB could be LA (mainly), AA, 1,2-propanediol, ethanol, and other compounds depending on the used metabolic pathways and carbohydrates (Carvalho et al., 2021). Driehuis and Oude Elferink (2000) highlighted factors associated with the competitiveness of LAB flora during silage fermentation in two groups, which are (i) crop characteristics (e.g., DM and WSC content of ensiling material) and (ii) LAB characteristics (e.g., maximum growth rate, osmotolerance, acid tolerance, and substrate utilization).

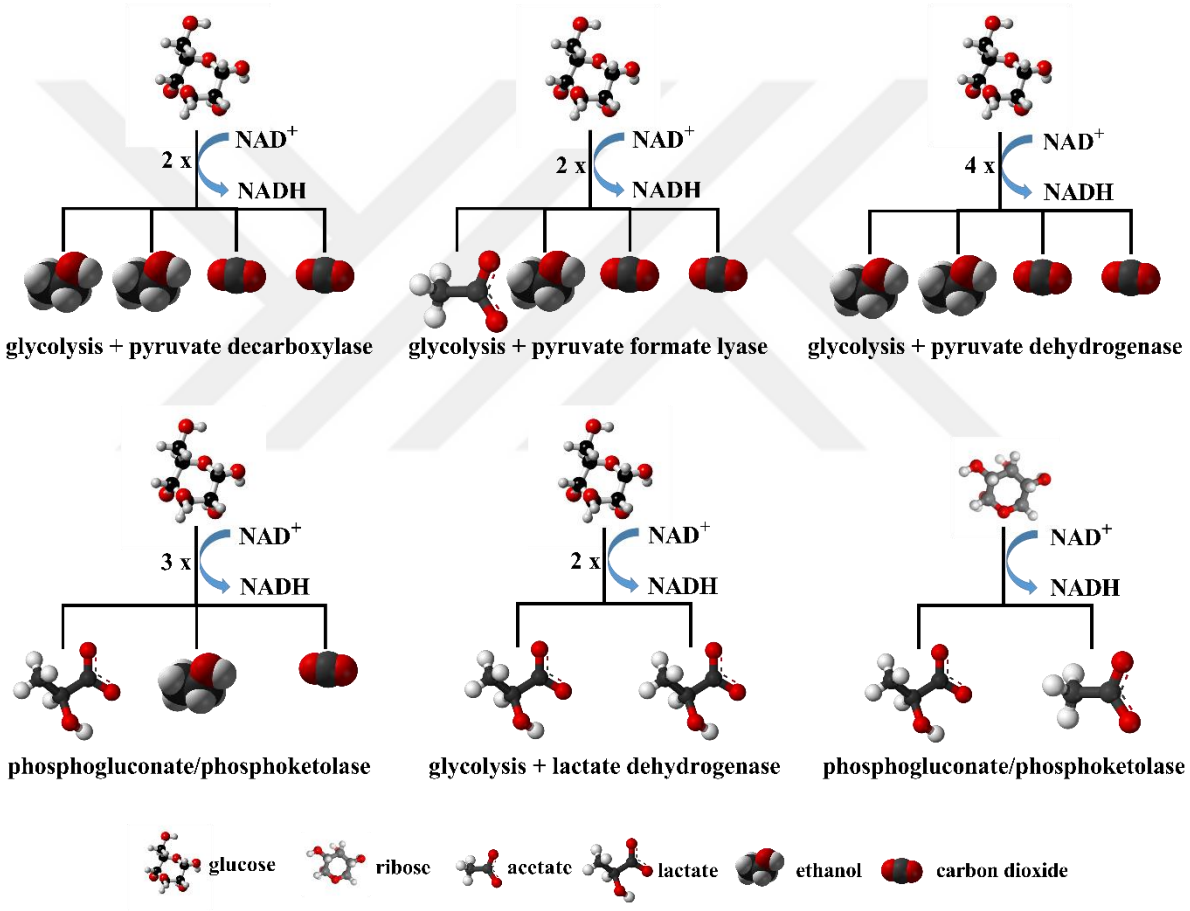


Figure 2. 4. Possible anaerobic fermentation pathways and end products when glucose is used as a substrate during ensiling (adopted from Rooke and Hatfield, 2003).

On the other hand, LAB could be classified into 3 groups according to their fermentation products and benefits, which are (i) obligate homofermentative (produces LA as the primary product of fermentation), (ii) facultative heterofermentative (ferment hexose into LA or pentose into AA and ethanol) (iii) obligate heterofermentative (ferment hexoses into

ethanol and LA and pentoses into LA and AA) (Von Wright and Axelsson 2012; Bernardi, Härter, Rabelo, Silva, and Reis, 2019). It has also been stated that homolactic fermentation in the silage leads to reduce pH, restricting the proliferation of undesirable bacteria, and decreases DM losses, whereas heterolactic fermentation inhibits yeast and mold growth and increases aerobic stability (Bernardi et al., 2019).

Carvalho et al. (2021) reviewed the literature to describe the selection criteria of LAB strain which might be used to enhance silage quality. The authors concluded that "*Lactobacillus plantarum, L. acidophilus*, *Enterococcus faecium, Pediococcus acidilactici,* and *P. pentosaceus*" are the main species of homofermentative or facultatively heterofermentative LAB to decrease pH and accelerate the LA production. They also underlined the importance of *L. buchneri*, obligately heterofermentative LAB, to improve aerobic stability. The phylogenetic relationship of the LAB species mostly used in silage inoculants is also given in Figure 2.5.

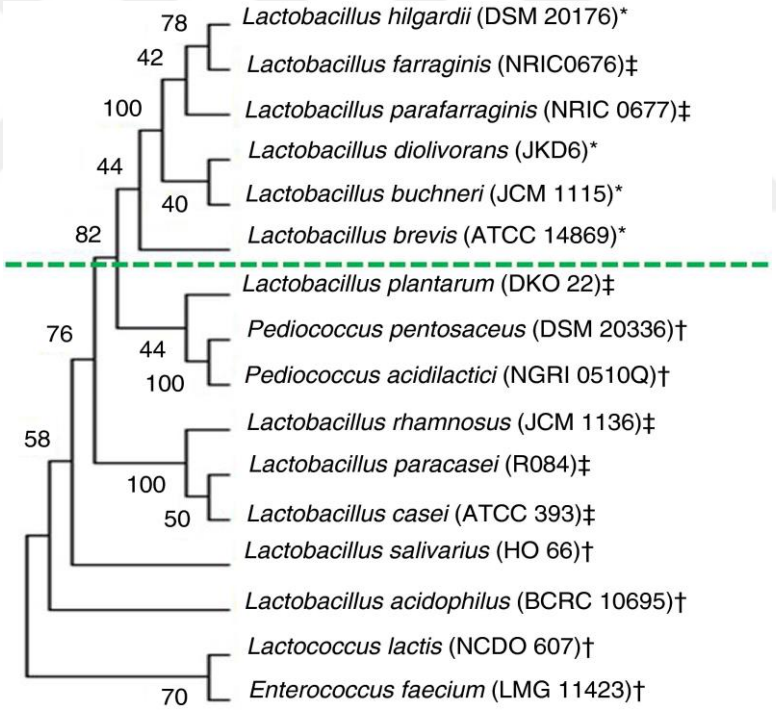


Figure 2. 5. Phylogenetic relationship of the lactic acid bacteria species mostly used in silage inoculants (Source: Carvalholho et al., 2021).

*†Homofermentative; *Obligately heterofermentative; ‡Facultatively heterofermentative. The upper part of the green dotted line indicates obligately heterofermentative LAB species, whereas the lower part indicates homofermentative LAB species. On the other hand, facultative heterofermentative LAB species could be in both parts of the phylogenetic group.*

A recent meta-analysis report, which was conducted to determine the role of both homo-and heterofermentative LAB on alfalfa silage, shows that homofermentative LAB decreases WSC and AA content and increases DM. On the contrary to homofermentative LAB, it has been concluded that heterofermentative LAB increases WSC and AA content and decreases DM of alfalfa silage (Blajman, Vinderola, Paez, and Signorini, 2020).

The literature on LAB has also highlighted several other beneficial effects during the ensiling and feed-out stage. For example, Gonda et al. (2019) demonstrated that LAB are able to adsorb mycotoxins and minimize gas losses and proteolysis in silage. Wilkinson and Rinne (2018) argued that LAB strains, which have the ability to produce cell wall-degrading enzymes, could have a beneficial effect on fermentation. In addition, Xie, Guo, Li, Wu, and Yu (2021) revealed that the synergetic effect of ferulate esterase producing LAB and main degrading enzymes in the rumen could facilitate the breakdown of polysaccharides. Gollop, Zakin, and Weinberg (2005) established that the bacteriocin-like antibacterial activity of LAB inoculated silages inhibits the detrimental microorganisms in silages and the rumen. Weinberg et al. (2007) highlighted the probiotic effect of LAB on animal performance.

2.4.2 Enterobacteria

Enterobacteria are gram-negative, non-spore-forming, and facultative anaerobic bacteria. As has already been noted, *Enterobacteria* is one of the two most active bacteria during the anaerobic fermentation stage of ensiling (Rooke and Hatfield, 2003). They are the main competitor of LAB for nutrients, fermenting them into a wide range of products such as lactate, acetate, formate, succinate, ethanol, acetoin, and 2,3 butanediol; however, they are inhibited by low pH (<4.5) and could be replaced with more acid-tolerant LAB under favorable conditions (Rooke and Hatfield, 2003; da Silva et al., 2020).

It has previously been observed that fresh crops were dominated by epiphytic *Enterobacteria* such as *Erwinia herbicola* and *Rahnella aquitilis*, then displaced with other *Enterobacteria* species such as *Serratia fonticola*, *Hafnia alvei*, and *Escherichia coli* (Driehuis and Oude Elferink, 2000). Some *Enterobacteria* species are often used as a hygienic marker to determine fecal contamination, e.g., *E. coli*. On the other hand, *E. coli* O157:H7 is one of the most notorious *Enterobacteria*, known as a foodborne pathogen, which may cause

several illnesses in children and the elderly (Queiroz, Ogunade, Weinberg, and Adesogan, 2018).

A strong relationship between Enterobacteria and AA at high temperatures has been reported in the literature (Wang et al., 2020). Previous studies by Kleinschmit and Kung Jr (2006) have indicated that *Enterobacteria* produce more AA at final silage. *Enterobacteria* species are also associated with proteolytic activity in silage, which may cause secondary fermentation, producing ammonia and biogenic amines (Ávila and Carvalho, 2020). Thus, due to their high proteolytic activity, Muck (2010) attributed the main gas losses in silos to nitrate-reducing *Enterobacteria*. If the silo is not completely anaerobic or decreasing rate of pH is low, *Enterobacteria* can still survive and deteriorate the silage once the pH increases at the feed-out stage (Driehuis and Oude Elferink, 2000).

2.4.3 Clostridia

Weinberg (2008) describes Clostridium as a "Gram-positive, endospore-forming strictly anaerobic rod" and underlined its importance in silage for two aspects: (i) clostridial fermentation results in higher DM and energy losses and (ii) clostridial silage carries a great potential risk to animal health and milk, and generally resulting in significant economic losses. Weinberg (2008) also noted that Clostridium species could be either saccharolytic (ferment carbohydrates), proteolytic (ferment proteins, protein hydrolysates, or amino acids), or both. Cato et al. (1986, cited in Zheng et al., 2018) point out that only 7 of the 84 bacteria species of the genus Clostridium have been most common in silage. Pahlow et al. (2003) demonstrated that *Clostridium tyrobutyricum*, lactate-assimilating bacteria, was the most abundant one. Zheng et al. (2018) indicated that *C. tyrobutyricum* and other butyric acid-producing Clostiridum, such as *C. butyricum* and *C. beijerinckii* might cause anaerobic deterioration with undesirable rancid odor. Additonaly, Driehuis and Oude Elferink (2000) established that most proteolytic Clostridium, such as *C. perfringens* and *C. sporogenes*, accumulate ammonia and amines, which are toxic compounds that have an adverse effect on silage palatability and intake during ensiling.

Jia, Liu, Hwang, and Huang (2020) assert that *C. perfringens* plays a pivotal role in the initial clostridial fermentation in alfalfa silage due to its saccharolytic characteristics and ability to grow at higher pH (5.5 to 8.0). To overcome this issue, Liu, Dong, and Shao (2018) suggest that *L. plantarum* and sucrose could be used to restrict clostridial fermentation by promoting LA fermentation in moist forages. Supporting this view, Li et al. (2020) write that

rate and extent acidification of silage could reduce clostridial fermentation. Data from several studies also suggest that the high DM content of forage suppresses the proliferation of Clostridium species in silage (Spoelstra, 1990; Pahlow et al., 2003; Zheng et al., 2018). Adesogan, Krueger, Salawu, Dean, and Staples (2004) established that a LAB and enzymes mixture or molasses could be used to inhibit clostridial fermentation, primarily associated with proteolysis and BA accumulation, in humid or moist conditions. It has been demonstrated that nitrate and its intermediates (nitrite and nitric oxide), degraded by *Enterobacteria* during the initial stage of ensiling, suppress the clostridial fermentation acting as an anticlostridial agent (Tabacco Piano, Cavallarin, Bernardes, and Borreani, 2009; König et al., 2017). Therefore it has been suggested that an additional nitrate is needed to inhibit the germination of clostridial spores in low DM silages (König et al., 2017).

2.4.4 Acetic Acid Bacteria

Driehuis and Oude Elferink (2000) describe acetic acid bacteria as obligate aerobic and acid-tolerant bacteria and highlight the genus Acetobacter's association with silage. Spoelstra, Courtin, and Van Beers (1988) argue that spoilage in aerobically unstable corn silages with a low yeast number could be initiated by Acetobacter. In contrast to Spoelstra et al. (1988), Nishino, Wang, Parvin, and Kan (2009) argue that Acetobacter could be used as an inoculant due to its ability to oxidize ethanol to AA aerobically, which could improve aerobic stability. Likewise, Kristensen et al. (2010) hold the view that AA, oxidized from ethanol, inhibits the proliferation of fungi and increases the aerobic stability of silage.

On the other hand, Pahlow et al. (2003) noted that Acetobacter could degrade lactate to acetate, which causes DM loss. Similarly, Muck (2013) highlighted that Acetobacter was the initiator of aerobic deterioration in a film with higher oxygen permeability. Drouin, Tremblay, Renaud, and Apper (2021) concluded that the growth of Acetobacter in silage might be related to outnumbered yeast or a high accumulation of PA. Recent research using next-generation sequence techniques has also revealed that initial aerobic deterioration in Napier grass, sugarcane, and corn silages is related to Acetobacter (Xu et al., 2019; Wang et al., 2020; Guan et al., 2020a).

2.4.5 Bacilli

Bacilli are endospore-forming, aerobic, or facultative anaerobic bacteria and are found in low numbers in fresh plant material. Bacilli are able to survive at low pH and ferment a

wide range of carbohydrates into lactate, acetate, butyrate, ethanol, 2,3-butanediol, and glycerol. *Bacillus licheniformis*, *B. pumilus*, *B. coagulans*, *B. sphaericus*, *B.cereus*, and *Paenibacillus polymyxa* are more isolated Bacilli species in silage (Driehuis, Wilkinson, Jiang, Ogunade, and Adesogan, 2018). Kung Jr, Shaver, Grant, and Schmidt (2018) thought that bacilli might cause severe heat, earthy odor, and high pH in silages after the initiation of aerobic spoilage by yeasts. However, it is possible to prevent bacilli growth in silage by avoiding primary contamination of fresh forages with soil or manure (Wang et al., 2020).

Queiroz et al. (2018) concluded that high temperatures, warm summer conditions, outnumbered on outer layers of bales, and after treatment with formaldehyde or antibiotics favor the increase of spore counts of *Bacillus* spp. in corn silage and the bulk tank of milk. Driehuis and Oude Elferink (2000) assumed that *B. cereus* is notorious and one of the major spoilage organisms in pasteurized milk and milk products and carries a high potential risk to animal health and milk safety. Kung Jr, Taylor, Lynch, and Neylon (2003) proposed potassium sorbate rather than sodium benzoate as an effective preservative to control *B. cereus* at pH 6.7 due to having higher pKa values (4.75 vs. 4.20). Furthermore, Amado, Fuciños, Fajardo, and Pastrana (2016) suggested that due to the inhibitory effect of nisin on pathogenic bacteria, e.g., *Listeria monocytogenes*, nisin-producing bacteria could be used in silage.

2.4.6 Yeasts

Yeasts are facultative anaerobic single-cell eukaryotes that play a pivotal role in the aerobic deterioration of silage. Several reports have shown that yeasts are the primary initiators of spoilage, resulting in high pH (Hafner et al., 2013; Kung Jr. et al., 2018; Ávila and Carvolho, 2020). Ogunade et al. (2018) attributed this spoilage and high pH to lactate assimilating yeasts which become active in the presence of air and metabolize lactate to CO_2. The most known lactate-assimilating yeasts belong to *Candida* genera and may cause aerobic deterioration of various silages such as maize, wheat, and alfalfa (Liu et al., 2019).

Weinberg et al. (2007) reported that a 40-60 g/kg of ethanol content at the initial stages of ensiling reflects an intensive yeast activity. This unusual ethanol production may be explained by the fact that the high WSC content of plant material could increase the alcoholic fermentation of yeast during the decreasing pH, resulting in more ethanol accumulation in the silo (Oude Elferink et al., 2001). Besides, this alcoholic fermentation leads to increase DM losses due to the formation of two molecules of CO_2 for each mole of oxidized glucose (Pinho

et al., 2015). A similar conclusion was also concluded by Ávila, Carvalho, Pinto, Duarte, and Schwan (2014).

Nishino, Hattori, and Kishida (2007) indicated that the structure of ensiling material, e.g., hollow stem, could increase the remaining air in the silo, facilitating yeast proliferation and ethanol production in the initial stages of ensiling. However, it is possible to eliminate air by wilting plant material to recommended DM, chopping to a correct length, compacting well, and packing quickly and tightly (Kung Jr., 2010).

Ávila et al. (2014) and Carvalho, Ávila, Pinto, Neri, and Schwan (2014) underlined the importance of LAB strain selection to reduce DM losses caused by the alcoholic fermentation of yeast, particularly in high WSC-rich plants such as sugarcane and recommended *L. buchneri* and *L. hilgardii* for this purpose. Borreani, Tabacco, Schmidt, Holmes, and Muck (2018) noted that temperature was another factor that affected fermentation speed and microbial community significantly, and they identified a decreasing trend of yeast count in silages stored at higher temperatures. This is consistent with the data obtained by Kim and Adesogan (2006) and Weiss, Kroschewski, and Auerbach (2016). However, Beltran, Rozes, Mas, and Guillamon (2007) reported lower DM losses and yeast counts in silage stored at low temperature; and concluded that it might be related to decreasing fermentation and growth rate of yeasts.

Hafner et al. (2015) revealed that potassium sorbate with an application rate of 0.5 g/kg or more reduces ethanol production by 58-85%. Nishino (2011) proposed AA and PA against yeast and molds due to their greater antifungal activity than LA. Likewise, Ávila and Carvalho (2020) recommended using short-chain fatty acids with greater pKa values for the inhibition of yeasts.

Several researchers considered yeast as "undesirable". A note of caution is due here since some yeasts are used as probiotics for ruminants (e.g., *Saccharomyces cerevisiae*) or used as antagonists for the biological control of mold growth in high moisture cereal silages (e.g., *Pichia anomala*, *P. guilliermondii*, and *S. cerevisiae*) (Vohra, Syal, and Madan, 2016; Wambacq, Audenaert, Höfte, De Saeger, and Haesaert, 2018). For example, Duniere et al. (2015) demonstrated that inoculation of *S. cerevisiae* or *S. paradoxus* with an application rate of 10^3 cfu/g did not change corn's nutritional, microbiological, or aerobic stability characteristics silages. Besides, it has been shown to improve ruminal fermentation and digestion in silages treated with yeast-containing inoculants (Owens and Smiley, 2018).

2.4.7 Molds

Molds are eukaryotic microorganisms known as strict aerobes, requiring oxygen to develop. They are characterized as "the slowest growers" microorganisms in the silage and are able to grow between temperatures 10-40 °C, between pH 4-8, and when water activity (a_w) is greater than 0.7 (Muck, 2010; Ogunade et al., 2018).

Molds could produce mycotoxins, which could be hazardous for animals and humans in spoiled silage. However, they produce mycotoxin under stress conditions. For example, the optimum growth temperature for *Fusarium* molds is 25 to 30 °C, and they grow aggressively at this temperature range without producing mycotoxins. However, under temperature stress, e.g., at freezing point, *Fusarium* molds produce large quantities of mycotoxins with a minimal growth rate (Whitlow and Hagler, 2005).

Ogunade et al. (2018) noted that most of the mycotoxin contamination in ensiled forage originated from *Fusarium* and *Aspergillus* at pre-harvesting or *Aspergillus* and *Penicillium* molds at post-harvesting periods. Moreover, Driehuis and Oude Elferink (2000) thought that *Aspergillus fumigatus*, *Penicillium roqueforti*, and *Byssochlamys nivea* are the most important mycotoxin-producing molds associated with aerobic spoilage of silage.

It has been suggested that total numbers of molds could be used as an indicator of mycotoxins; however, high numbers (>10^6 cfu/g wet silage) of molds should not be used as an indicator of mycotoxin production in silage due to two main reasons: (i) most of the common molds could not produce mycotoxins (Ogunade et al., 2018), and (ii) this could be a sign of aerobically spoiled silage (Kung Jr et al., 2018).

On the other hand, filamentous structures and colored spores of molds could be visible to the naked eye if the silage is heavily invested with molds. Borreani et al. (2018) established that approximately 16% of nutritive values of silages could be decreased before these filamentous structures or colored spores become visible. It is believed that the cell wall degrading ability of molds with other organisms could play a significant role in this nutritional loss (Zhang et al., 2018).

Queiroz et al. (2018) noted that AA inhibits the growth of yeast and molds; thereby, they proposed *L. buchneri* could be used not only to improve the aerobic stability of silage but also to prevent mold formation. Muck et al. (2018) argued that *B. subtilis*, which has the bacteriocin-producing ability, could also be used to inhibit yeast and molds in silage.

Similarly, Ädel Druvefors and Schnürer (2005) and Gonda et al. (2019) indicated that yeast could be used to reduce mycotoxin contamination risk via their inhibitory effect on mold growth. In addition, Boreani et al. (2018) proposed that yeast and molds growth could be inhibited effectively if organic acid (acetic, propionic, benzoic, and sorbic acids) or their salts (sodium propionate, ammonium propionate, calcium propionate, potassium sorbate, or sodium benzoate) could be used at a proper rate.

2.4.8 Listeria

Listeria is gram-positive, rod-shaped, aerobic, or facultatively anaerobic microorganisms. It is considered one of the main foodborne pathogens and causes listeriosis. Thus far, 20 species of *Listeria* have been identified in different sources, such as soil, sewage, water, plant, silage, milk, and meat. Although it is ubiquitous in the environment, it has been thought that silages are the primary sources of listeriosis (Driehuis and Oude Elferink, 2000; Duniere et al., 2017; Shourav, Hasan, and Ahmed, 2020).

Coblentz and Akins (2018) revealed a correlation between increased incidence of listeriosis and the development of baled-silage techniques, particularly in Europe. This correlation might be related to the slow fermentation rate and the microaerophilic environment in the sealed bale, creating favorable conditions for *Listeria* growth (Nucera et al., 2016). Moreover, it has been stated that a higher prevalence of *Listeria* was determined in visually contaminated areas (Nucera et al., 2016).

Among the identified *Listeria* species, *Listeria monocytogenes* is the most notorious one. There is some evidence that feeding poor-quality silage increases the contamination risk of raw milk by *L. Monocytogenes* (Driehuis and Oude Elferink, 2000; Nucera et al., 2016). It is also responsible for clinical mastitis, abortion, encephalitis, septicemia, conjunctivitis, and other sicknesses in animals and humans (Duniere et al., 2017; El Hag, El Zubeir, and Mustafa, 2021).

Dreyer, Thomann, Böttcher, Frey, and Oevermann (2015) recommend maximum care in farm environment hygiene to reduce the risk of *Listeria* outbreak. Amado, Fuciños, Fajardo, Guerra, and Pastrana (2012) proposed bacteriocin-producing LAB strains with antilisterial activity to inhibit *L. monocytogenes* during ensiling. These are *Lactococcus lactis* CECT 539 and *Pediococcus acidilactici* NRRL B-5627, which produce nisin and pediocin, respectively.

2.5 Losses

Nowadays, the main challenge is fabricating high-quality silage avoiding DM and quality losses as much as possible. Silage loss occurs during each ensiling stage from the field harvesting through the feed-out stage. The moisture content of a forage crop plays a fundamental role in assessing the size of harvest and storage losses.

Evidence from a number of experimental studies has established that in order to produce high-quality silages, all fermentation pathways facilitated by undesirable microorganisms in the silo should be eliminated until silo opening (Rooke and Hatfield, 2003; Borreani et al., 2018; Auerbach and Nadeau, 2020). It has also been stated that an additional DM loss of up to 20% might be incurred during the feed-out stage as a consequence of yeast activity (Auerbach and Nadeau, 2020).

A comparison of estimated harvesting and storage losses of legume-grass forage is presented in Figure 2.6. Harvesting losses and leaf shatter during mowing, chopping, bailing, and transporting are higher in dry crops (< 40% moisture), whereas microbial degradation and effluent losses are higher in wet crops (> 60% moisture) during the storage period (Emery and Mosier, 2012; Borreani et al., 2018). McDonald et al. (1991) argued that the risk of BA fermentation and effluent production is higher in wet crops, which results in higher fermentation losses, reduced feed value, and compromised hygienic quality of silage.

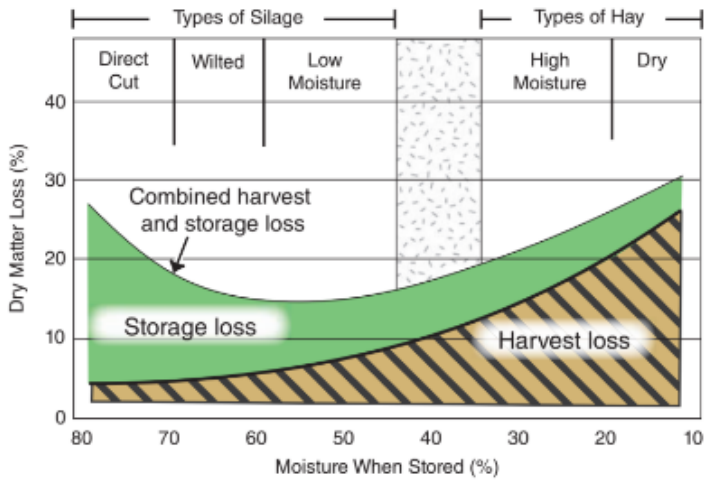


Figure 2. 6. Estimated harvesting and storage losses when legume-grass forage is stored as hay or silage (Source: Collins and Moore, 2018).

Clostridial fermentation of sugars, organic acids, or amino acids leads to increased BA and amine concentration in the silage, causing DM losses, reducing nutritive value, and reducing dry matter intake of ruminants (Muck et al., 2020). Therefore, it has been proposed that the moisture concentration of all forages should be arranged to recommended value with a field wilting to improve ensilability characteristics and avoid effluent losses (Borreani et al., 2018).

Carvalho et al. (2021) indicated that organic compounds are also eliminated together with effluents from the silo depending on the DM concentrations. Randby and Bakken's (2021) research is valuable for our understanding of which factors are associated with effluent losses. They sorted these factors as crop species, maturity at harvest, chop length, additives types, moisture content, and compaction rate.

Weinberg (2008) reported that around 10% of accumulated ensiling losses, which are considered inevitable, could be acceptable. The author also stated that DM losses in poorly managed silages could be higher than 40%, and spoiled feed could be hazardous for animal health. Figure 2.7 illustrates the factors affecting the energy losses (Weinberg, 2008).

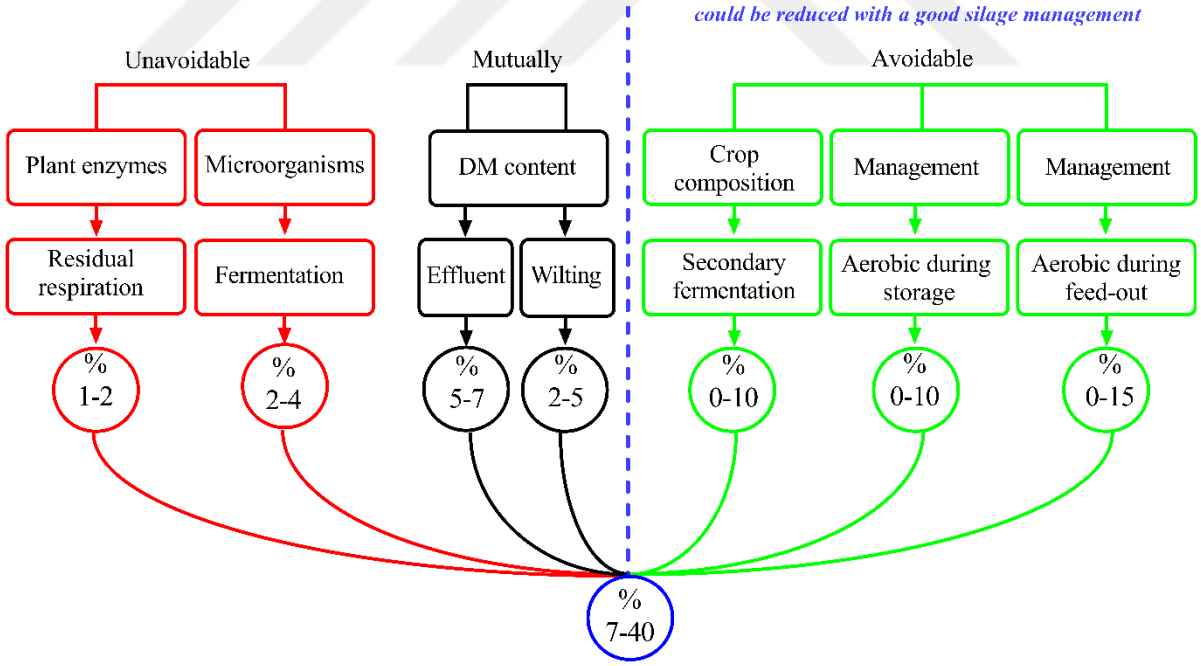


Figure 2. 7. Factors affecting the energy losses during ensiling and their estimated values (adopted from Weinberg, 2008).

It has been shown that DM losses in laboratory-scale silos could be increased by 1.7% in every 10 °C increase in temperature (Rees 1982, cited in Guan et al., 2020b). Adopting a

similar position, Guan et al. (2020b) indicated that the heterofermentative LAB community in the silo increases at higher ensiling temperatures, resulting in higher DM losses. In addition, Vervaeren, Hostyn, Ghekiere, and Willems (2010) highlighted that these increased heterofermentative LAB community in the silo also increases the AA content of silages, further enhancing methane production.

Avila and Carvalho (2020) argue that DM losses are significantly associated with the type of predominant microbes at the aerobic stage of ensiling. Lactate-assimilating yeasts, for example, are highly active at the aerobic stage of ensiling, and they can ferment WSC and fermentation end-products into carbon dioxide and water causing nutrient losses (Du, Risu, Gentu, Jia, and Cai, 2020).

According to Tian et al. (2020), silo density is another factor that affects DM losses. The amount of remaining oxygen in the silo could be decreased with increased silo density, which leads linearly to an increase in the silage's DM, WSC, and ammonia nitrogen levels. Furthermore, Savoie and Jofriet (2003) concluded that permeability and porosity of sealing materials are factors that affect DM losses in bunker or clamp silos during storage, causing 0.5-1.5% DM losses per month. Therefore, Randby, Halvorsen, and Bakken (2020) recommend bales sealed with tighter plastic due to lesser air exposure during the feed-out stage.

Filya (2003a) demonstrated that inoculation of *L. buchneri* + *L. plantarum* decreased pH, ammonia nitrogen, and fermentation losses in sorghum, whole-plant corn, and wheat silages compared to inoculated with *L. buchneri,* solely. In the same vein, Queiroz, Adesogan, Arriola, and Queiroz (2012) established that dual-purpose inoculants lowered spoilage by over 50% without affecting the nutritive value of farm-scale silos. Köhler et al. (2019) revealed that DM losses in the silo negatively correlate with the feed-out rate. Besides, Spörndly (2018) observed that yeast initiated spoilage during the feed-out stage, which is probably the main reason for high DM losses in big silos.

2.6 Silage Additives

Silage additives have been considered a flexible and strategically promising management tool due to alleviating spoilage bacteria, minimizing DM losses, and elevating the nutritional value of silage (Aurbach et al., 2020). Thus, producers generally use silage additives to overcome one of the following issues: prevent adverse effects on plant

respiration, restrict undesirable fermentation, prevent yeast and mold proliferation, handle air and temperature stress during storage, and ensure good fermentation quality (Arriola, 2010; da Silva, 2018).

According to their effect on fermentation, McDonald et al. (1991) categorized silage additives as being i) fermentation stimulants (LAB, enzymes, sugars), ii) fermentation inhibitors (strong acids or their corresponding salts, chemicals with strong antimicrobial properties), iii) aerobic deterioration inhibitors (LAB, or weak organic acids), iv) nutrients (minerals, urea, ammonia), and v) absorbents (straw, dried sugar beet pulp) (Figure 2.8).

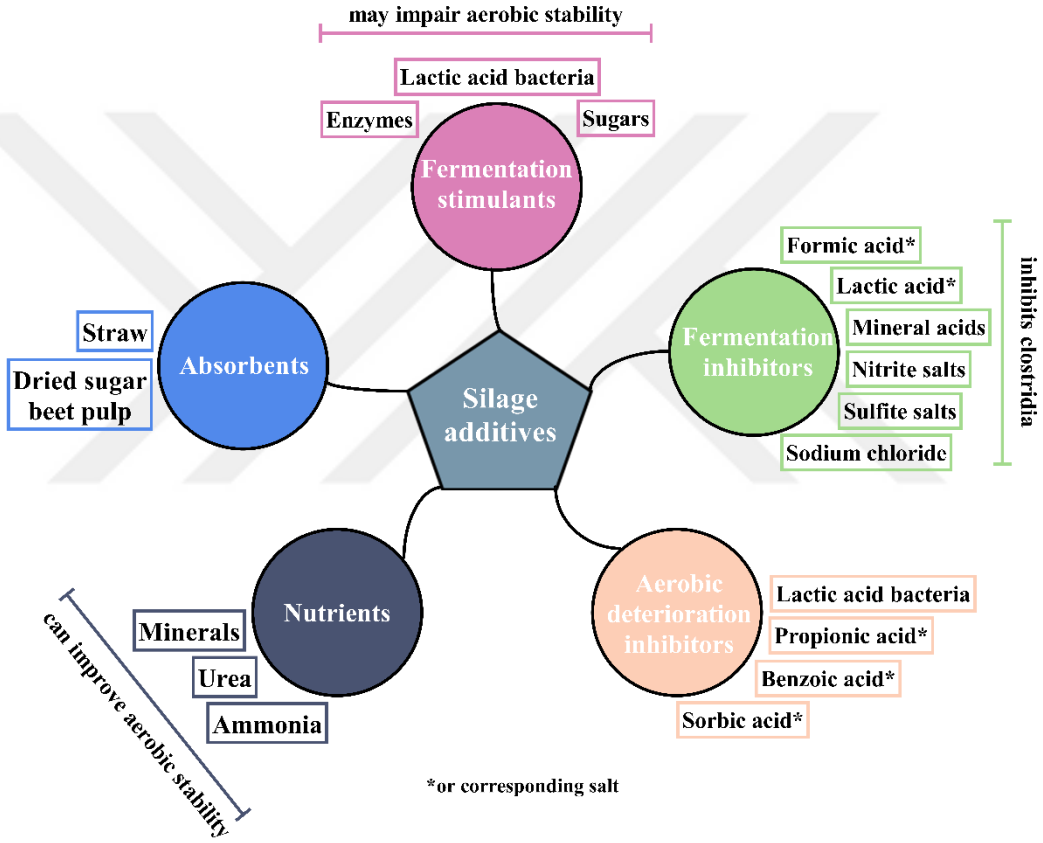


Figure 2. 8. Classification of silage additives (adopted from McDonald et al., 1991).

Silage additives should be effective, safe, suitable for certain types of crops, have ease of handling and application, non-pollutant, non-corrosive to equipment used, cost-effective, and easily accessible (Oude Eflerink, 2000; Weinberg, 2008).

2.6.1 Fermentation Stimulants

Fermentation stimulants can be categorized into microbial cultures (to increase LAB dominancy), enzymes (to increase available carbohydrate sources and to improve organic matter digestibility), and substrates (to stimulate fermentation) (Ndiweni, 1993). Many scholars believe that bacterial inoculants are the most suitable fermentation stimulants due to encouraging organic acid production and increase aerobic stability. They are often used to reduce the dependency of fermentation on epiphytic LAB flora of ensiling material. An external LAB fortification should be provided when the indigenous LAB population is less than 10^4 cfu/g on fresh material (Oladosu et al., 2016).

It has been shown that LAB strains in commercial products are generally isolated from silage or silage crops due to their effectiveness in converting sugars to predominantly LA (Curtis, 1996). Based on fermentation end-products, LAB could be either homofermentative or heterofermentative. The literature on LAB-based additives has highlighted that most of the bacteria in the homofermentative LAB group are acknowledged as "facultative heterofermentative LAB species" rather than "obligate homofermentative LAB species" (Pahlow et al., 2003). Nevertheless, facultative heterofermentative LAB species have phosphoketolase enzymes, which allow degrading pentoses into LA (primarily) and AA (Muck et al., 2018). Silages treated with one of the most used facultative heterofermentative LAB strains (*Lactobacillus plantarum*, *L. casei*, *Enterococcus faecium*, and *Pediococcus* spp.) had lower pH, AA, BA, ammonia-nitrogen (NH_3-N), and DM losses but higher LA compared to untreated silages (Muck et al., 2018). As well, Oliveiria et al. (2017)'s meta-analysis showed that the effect of LAB inoculation on these parameters varied by crop.

Enzymes can also be used as fermentation stimulants solely or combined with bacterial inoculants. There are two main goals for the addition of enzymes into silage: i) increase available carbohydrates and nutrients supply during ensiling to ameliorate fermentation quality, and ii) increase digestibility of forage by pioneering partial degradation (Ordaz, 2017). Breakdown of linkage between hemicelluloses and lignin provides more fermentable sugars (e.g., glucose) for LAB proliferation. Fijałkowska et al. (2020) established that the rapid proliferation of LAB in the silo increases LA and promotes a faster decrease in pH. Irawan et al. (2021) categorized enzymes incorporated in the inoculants into 2 groups: fibrolytic enzymes (e.g., cellulase, xylanase, and *β*-glucanase) and fungus or yeast-derived enzymes (e.g., *Aspergillus niger*, *Trichoderma viride*, and *Saccharomyces cerevisiae*). On the

other hand, a considerable amount of literature has been published on ferulate esterase-producing bacteria that are able to breakdown the linkage between lignin and the cell wall of forages and increase available carbohydrates for LAB proliferation (Nsereko et al., 2008; Addah, Baah, Okine, and McAllister, 2012; Comino et al., 2014; Lynch, Jin, Lara, Baah, and Beauchemin, 2014).

Molasses is one of the two most widely used silage additives with *L. plantarum.* Among various fermentation initiators, molasses have been effectively used to rapidly decrease silage pH and inhibit unwanted microorganisms via directly increasing fermentable substrate (Mu, Xie, Hu, Chen, and Zhang, 2021). Baytok and Muruz (2003) proposed that additional aqueous carbohydrate sources, such as molasses, fill the gaseous pores of fresh material and reduce oxygen flow in the silo. Nishino, Li, Wang, and Parvin (2012) demonstrated that the effect of molasses on LA fermentation in wilted silage was less than in direct-cut silages due to prolonged ensiling time. The authors also observed that AA concentration in wilted silage was higher than in direct-cut silage. Similar findings were also reported by Wu and Nishino (2015). Moreover, Hashemzadeh-Cigari et al. (2014) concluded that molasses could be added into silages to fortify resistance to spoilage and reduce yeast population after silo opening.

2.6.2 Fermentation Inhibitors

Acids and organic salts are mainly used to increase the acidity of silage and inhibit undesirable microorganisms. Muck et al. (2018) proved that formic acid and LA inhibit the undesirable microorganism and decrease proteolysis throughout ensiling. However, their inhibitory mechanisms differ from each other. Formic acid acidifies the environment, acts as an antimicrobial agent, and inhibits fermentation, whereas LA increases LAB count and ameliorates fermentation (He et al., 2019). In a study conducted to determine the effect of formic acid and *Lactobacillus farciminis* addition on *Neolamarckia cadamba* leaves silage, it has been reported that the relative abundance of "*Lactobacillus"* decreased with the addition of formic acid at the early stage of ensiling, whereas the relative abundance of "*Enterococcus, Acinetobacter, Pseudomonas, Exiguobacterium*, and *Bacillus*" increased (He et al., 2019). Furthermore, Yuan, Wen, Desta, Dong, and Shao (2017a) and He et al. (2019) exhibited that carboxypeptidase activity of formic acid-treated silage reduced, thus resulting in restricted peptide degradation. Similarly, Nsereko and Rooke (2000) demonstrated that applying formic

acid and formic acid-formaldehyde mixture on ryegrass silages reduced nonprotein nitrogen and ammonia concentrations.

It has been reported that sulphuric acid and hydrochloric acid are the most known mineral acids that could be used effectively to control undesirable microorganisms and improve fermentation quality. Mineral acids could rapidly reduce the pH; therefore, the microbial and enzymatic activity might be restricted at low pH, resulting in reduced plant respiration and nutrient loss (Direkvandi et al., 2020). It has also been demonstrated that this rapid pH reduction inhibits clostridial fermentation (Direkvandi et al., 2020). However, they were replaced with organic acid due to severe drawbacks such as high corrosivity, being harmful to human health, and requiring a large amount of mineral acid per ton, making them difficult to handle (König, 2020).

On the other hand, nitrite could be degraded to ammonia and nitrous oxide (N_2O) by *Enterobacteria* in the silage (Spoelstra, 1987). However, nitrite could also chemically be degraded to NO and nitrate and could have severely damaging effects on lung tissue in the presence of air by forming a mixture of nitrogen oxides gaseous (NO_2, N_2O_3, N_2O_4) (Oude Eflerink et al., 2000). Despite its safety concern, a minor reduction of nitrite in the silo positively affects the silage quality due to its inhibitory effect on clostridia (Oude Eflerink et al., 2000).

Degradation of sodium metabisulfite into sulfur dioxide could reduce the aerobic stress of fresh forage due to its strong reducing agent of oxygen. This sodium salt also acts as an antifungal agent and inhibits mold formation (Kleinschmit, Schmidt, and Kung Jr, 2005). Besides, increased WSC and pH and lowered fermentation acids could signify reduced heterofermentative LAB in sodium metabisulfite treated silages (Kleinschmit et al., 2005).

2.6.3 Aerobic Deterioration Inhibitors

Propionibacteria are able to degrade glucose and LA into AA and PA. Although this genus can produce two of three antimycotic acids, there is conflicting evidence about their effect on aerobic stability. For example, Dawson, Rust, and Yokoyama (1998) argued that inoculation of *Propionibacterium acidipropionici* improved the aerobic stability of corn silage. In contrast, Filya, Sucu, E., and Karabulut (2006) demonstrated *P. acidipropionici* alone or its combination with *L. plantarum* did not improve the aerobic stability of corn and

sorghum silages. Arriola, Kim, Staples, and Adesogan (2011) attributed this inconsistency to the acidic environment, which adversely affects the growth of *Propionibacteria*.

On the one hand, extensive research has shown that several organisms such as *L. buchneri*, *L. diolivorans*, and *L. reuteri*, have the ability to metabolize 1,2-propanediol to propanol and PA (Krooneman et al., 2002; Sriramulu et al., 2008; Zielińska, Fabiszewska, Świątek, and Szymanowska-Powałowska, 2017; Muck et al., 2018). It has been proved that inoculation of *L. buchneri* improved corn silage (Mari, Schmidt, Nussio, Hallada, and Kung Jr, 2009; Arriola et al., 2021), sorghum silage (Filya, 2003b; Ariola et al., 2021), alfalfa silage (Kung Jr et al., 2003; Besharati, Palangi, Nekoo, and Ayaşan, 2021). On the other hand, *L. buchneri* belongs to the heterofermentative LAB group; hence, little DM losses are inevitable throughout fermentation (Ranjit and Kung, 2000).

There are, however, other common chemical additives, such as weak organic acids, which are used to improve aerobic stability. Treating silage with a weak organic acid, e.g., AA and PA, increases available WSC in the silo. It has also been highlighted that weak organic acids are generally used to improve the aerobic stability of silage when ensiling material has high or low DM content or to prevent proteolysis when ensiling material has high protein content (Huhtanen, Jaakkola, and Nousiainen, 2013; Gheller et al., 2021). Piper Calderon, Hatzixanthis, and Mollapour (2001) revealed that weak acids, e.g., benzoic and sorbic acid, are able to disrupt the cell membrane of yeast by creating oxidative stress under aerobic exposure. Furthermore, da Silva (2018) reported that acid salts such as potassium sorbate and sodium benzoate are commonly used instead of their acid form due to being less corrosive and less dangerous.

2.6.4 Nutrients

Nonprotein nitrogen, such as urea and anhydrous ammonia, is used to increase CP and improve the aerobic stability of nitrogen-poor crops, e.g., corn, sorghum, and small grain crops (Muck and Kung Jr, 1997). Sun and Cheng (2002) established that alkaline treatments reduce cellulose's polymerization degree and crystallinity, which resulted in the breaking of linkages between lignin and carbohydrates. Breaking these linkages during ensiling might increase LAB's available nutrients and carbohydrates. This type of additive might also increase the pH and fermentation products in the silo and reduce DM recovery slightly (Muck and Kung Jr, 1997). Kebede, Mengistu, Assefa, and Animut (2018) emphasized the buffering capacity of alkalines, which increases the pH and restricts yeast proliferation at the initial

stages of ensiling. Morais et al. (2017) underlined that ammonium hydroxide, a water-bonded form of urea, reduces the fibrous fraction of ensiling material after degrading by urease. Similarly, Yitbarek and Tamir (2014) noted lesser mold counts, heat formation, and lower degraded protein in alkaline added silages. However, a note of caution is due here since the degradable and undegradable protein requirements of target animals should be considered whenever urea or anhydrous ammonia is added to the diet.

2.6.5 Absorbents

Generally, forage crops with DM less than 300 g/kg should be wilted or have absorbents added during ensiling to prevent nutritional and DM losses, inhibit clostridial fermentation, and reduce effluent production (Khorvash, Colombatto, Beauchemin, Ghorbani, and Samei, 2006; Nkosi et al., 2021). Although wilting is the cost-effective method to increase the DM content of forage, avoid effluent losses, and ameliorate the fermentation characteristics, adsorbents are the more effective choice in humid and rainy climates (Bernardes, Reis, and Moreira, 2005; Borreani et al., 2018). In the same vein, Oliveira et al. (2010, cited in Bezerra et al., 2019) highlighted that effluent production in the silo is primarily affected by DM content, and it is possible to minimize these losses by adding absorbents up to the DM content of silage reaches 300 g/kg. Several absorbents have been examined for their potential to reduce silage effluent losses, such as straw, dried sugar beet pulp, cereal grains, citrus pulp, and soybean curd residue (Khorvash et al., 2006; Bezerra et al., 2019; Yin, Wu, Tian, Wang, and Zhang, 2021). On the other hand, previous research has established that some of the absorbents rich in antinutritional factors, such as cotton hulls, rice hulls, and coffee hulls, reduced the digestibility of silage and decreased its quality (Bernardes et al., 2018).

3. MATERIAL and METHOD

3.1 Plant Material

TR was harvested at first cutting on May 23, 2019, and data collected were specific to that location and growing season. No irrigation was applied to supply precipitation during the growing season. The mean annual temperature and precipitation were 17.0 °C; 203.0 mm, respectively (Figure 3.1). On harvesting day, white clover was harvested by hand clipping at 10-25% of the blooming stage and immediately transferred into the laboratory. On arrival at the laboratory, fresh plant materials were chopped to a 1.5-2.0 cm length by hand, wilted for 20-24 hours at room temperature, and divided into five subfractions; each was about 2 kg. One subfraction was used to determine the chemical and microbiological values of the starting material and was immediately deep-frozen, subsequently dried at 60 ºC in a ventilated oven for at least 96 h, and milled through a 1.0 mm screen. The others were used for the treatments with kefir.

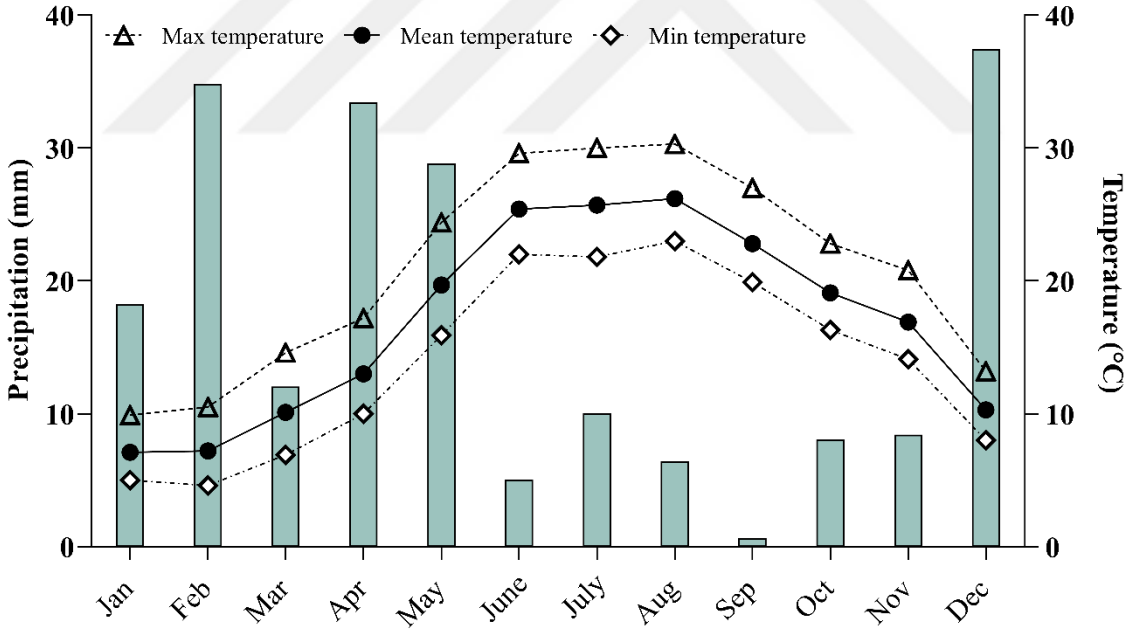


Figure 3. 1. Monthly total precipitation and mean temperature in 2019 at Erdek, Balikesir, Turkey.

In order to treat TR with a commercial dry natural kefir yeast (bran Sevdanem, Table 3.1) at the rate of 10, 50, and 100 mg/kg (fresh material, FM); Firstly, approximately 2 kg of wilted forage spread in a thin layer on a clean nylon cover with a 2 m^2 surface area, and then

inoculated with spraying the homogenized kefir solution prepared and in 20 ml of tap water by hand sprayer. Secondly, inoculated TR was homogenized well by inverting several times with clean nitrile gloves. The nylon covers and nitrile gloves were changed at each application rate to avoid contamination. For each of the four treatments (one control, four replication in each treatment), 250 g TR was packed into polythene bags (dimension 300 × 150 mm) and sealed by a vacuum sealer (CAS CVP-260PD). A total of 16 vacuum-packed silos were prepared with TR and kept at room temperature for 270 days.

Table 3. 1. Ingredients of kefir yeast*.

[1]*Lactobacillus kefiri*	[5]*Lactobacillus helveticus*
[1]*Lactobacillus parakefiri*	[6]*Lactobacillus plantarum*
[2]*Lactobacillus acidophilus*	*Leuconostoc mesentereoides*
[2]*Lactobacillus kefiranofaciens* subsp. *kefirgranum*	*Streptococcus thermophilus*
[2]*Lactobacillus bulgaricus*	*Lactococcus lactis* subsp. *lactis*
[3]*Lactobacillus casei*	*Bifidobacterium bifidum*
[4]*Lactobacillus fermentum*	*Kluyveromyces marxianus*
[4]*Lactobacillus reuteri*	*Saccharomyces cerevisiae*

**Includes at least 1× 10^8 cfu/g alive probiotic microorganisms according to manufacturer statement, [1] belong to the buchneri group, [2] belong to the delbrueckii group, [3] belong to the caseii group, [4] belong to the reuteri group, [5] belong to the helveticus group, [6] belong to the plantarum group.*

3.2 Determination of pH

Samples were prepared for pH analysis according to the procedure used by Anonymous (1986). A representative 20 g wet silage was taken and gently mixed in 100 ml of distilled water at room temperature for 1 h and then filtered through 4 layers of cheesecloths to measure the pH on opening each silo. The resulting pH was read on the WTW brand inoLab pH 730 model pH meter and recorded. The same procedure was applied to measure the pH of the pre-ensiled material.

3.3 Determination of Buffer Capacity

The first step in this process was to extract 20 g of pre-ensiled material with 250 ml of deionized water in a stomacher for 60 seconds and then filter through 4 layers of cheesecloths.

Once the sample was extracted, it was necessary to set the pH at 3.00 by using 0.1 N HCl and then standardized the pH at 4.00 using 0.1 N NaOH. After the standardization process, the volume of 0.1 N NaOH was recorded to increase the pH from 4.00 to 6.00, and the result was calculated as the following formula (Playne and Mc Donald, 1966):

$$Bc\ (meq\ NaOH/kg\ DM) = \frac{(T \times f) \times 100 \times 1{,}000}{DM\ (g/kg)} \quad (2.1)$$

Where;

Bc: Buffer capacity (meq NaOH/kg DM)

T: volume of titrant (ml)

F: factor of NaOH used for the titration

3.4 Ammonia Nitrogen

Samples were analyzed for NH_3-N content, as previously reported by Anonymous (1986). For the purpose of analysis, 10 g of copped silage was weighed into an opened neck glass bottle, added 100 ml of 0.3 M H_2SO_4, and then stored in the refrigerator for 24 h. In the follow-up phase of the NH_3-N analysis, 10 ml of filtered silage extract was taken into a Kjeldahl tube and added 10 ml of 10 M NaOH. The tube was then placed into the distilling unit, and the ammonia was collected in 50 ml of 4% boric acid solution. Then, collected ammonia was titrated with 0.1 M HCl using bromocresol green/methylene red mixed indicator solution, and the result was calculated as the following 3-step formula:

$$Af = \frac{(V_1 - V_0) \times M \times 1.401 \times V2}{W \times V_3} \quad (2.2)$$

Where;

Af: NH_3-N content of silage as fresh basis (g/kg)

V_0: blank, digested with samples (ml)

V_1: volume of titrant (ml)

V_2: volume of acid used to extract sample (ml)

V_3: volume of aliquot used in Kjehdal analysis (ml)

M: molarity of titrant

W: sample weight (g)

$$Ad = \frac{Af \times 100}{TDM} \quad (2.3)$$

Where;

Ad: NH_3-N content of silage as true DM basis

TDM: true DM content of silage (g/kg)

$$NH_3 - N\ (g/kg\ TN) = \frac{Ad \times 1{,}000}{N} \quad (2.4)$$

Where;

N: Total Nitrogen (g/kg) corrected for true DM content of the silage

3.5 Water-Soluble Carbohydrate

Samples were analyzed for WSC as previously reported by Anonymous (1986). In order to determine WSC concentration, 200 mg of dried samples were extracted in 200 ml of deionized water for 1 h, shaken several times manually, and filtered through the Whatman No:41 filter paper. Once the samples were filtered, 0.5 ml of clean extract was put into a clean test tube, and added 4 ml of 0.2% anthrone reagent (prepared in 98% H_2SO_4) and then allowed to boil for 10 minutes in a hot water bath. The solution was then transferred into a disposable polystyrene macro cuvette to read its absorbance at 620 nm in a Shimadzu brand 1208 model UV-visible spectrophotometer. A 50 ml stock solution was prepared by dissolving 50 mg glucose in distilled water to create a calibration curve. The solution was then diluted 10-fold, and a serial dilution was made to get 20, 40, 60, 80, and 100 µg/mL final

concentration. The same procedure was applied hereafter, described in detail above, and drawn a calibration curve using a Microsoft Excel sheet.

$$WSC\ (g/kg\ DM) = \frac{G \times D \times E \times 1{,}000}{W\ \times DM\ (g/kg)} \quad (2.5)$$

Where;

G: glucose concentration read from calibration graph (mg)

D: dilution factor

E: extract volume (200 ml)

W: sample weight used in extraction (mg)

3.6 Fleig score

Fleig scores of the silages were calculated according to Kilic (1984):

$$Fleig\ score = 220\ + (2\ \times\ DM\ - 15) - (40\ \times\ pH) \quad (2.6)$$

3.7 Lactic Acid

The spectrophotometric method previously described by Koç and Çoşkuntuna (2003) was used to determine the LA concentrations of samples. The first step in this process was diluting the samples 100 fold and pipette 1 ml into a clean test tube, and then adding 0.1 ml of $CuSO_4$ reagent (dissolved 5 g in 100 ml distilled water) and 6 ml of concentrated H_2SO_4. The mixture was then vortexed for 30 seconds and let to cool down in an ice-water bath for 5 minutes. The second step in this process was adding 0.1 ml of phenol solution (1.5 g of 4-phenylphenol and 5 g of NaOH dissolved in 100 ml) into the test tube, taking it out of the ice-water bath, and holding the resulting mixture at room temperature for 10 minutes and then in the hot water bath for 90 seconds, respectively. The final solution was then transferred into a disposable polystyrene macro cuvette to read its absorbance at 55 nm in a Shimadzu brand 1208 model UV-visible spectrophotometer. A 500 ml stock solution was prepared to create a calibration curve by dissolving 0.213 g lithium lactate in distilled water and adding 0.5 ml

concentrated H_2SO_4. The resulting solution was then diluted for 20-fold, and a serial dilution was made to get 2.5, 5.0, 10.0 and 15.0 µg/mL final concentration. The same procedure was applied hereafter, described in detail above, and drawn a calibration curve using a Microsoft Excel sheet.

$$LA\ (g/kg) = \frac{L \times D \times 100}{W \times DM\ (g/kg)} \quad (2.7)$$

Where;

L: lactic acid concentration read from calibration graph (mg)

D: dilution factor (generally samples diluted 100 fold with its weight)

W: sample weight used in extraction (g)

3.8 Quantification of Total Phenolics and Tannins

In order to measure the total phenolics (TP) and tannin (T) levels of samples, it was first necessary to extract samples either with 70% acetone or 50% methanol. Acetone was used as an extraction solvent in this dissertation. Approximately 200 mg of samples were weighed into a glass beaker, added 10 ml aqueous acetone (70%), and subjected to ultrasonic treatment for 20 minutes at room temperature. After ultrasonic treatment, the beaker contents were transferred into a centrifuge tube and subjected to the following 10 min centrifugation at 3,000 g at 4 ºC. Then the supernatant was collected in a clean test tube and stored at -20 ºC until further analysis.

Measurements of TP and T were performed according to Makkar, Blüemmel, Borowy, and Becker (1993). Polyvinylpolypyrrolidone (PVPP), an insoluble matrix, was used to precipitate tannins in the extraction, and the results were expressed as tannic acid equivalent.

A 50 µl tannin-containing extract and 450 µl distilled water were pipetted into a clean glass test tube, added 250 µl Folin-Ciocalteu reagent and 1,250 µl sodium carbonate solution, and then vortexed well. The final solution was then transferred into a disposable polystyrene micro cuvette to read its absorbance at 725 nm in a Shimadzu brand 1208 model UV-visible spectrophotometer after 40 min. A 50 ml stock solution was prepared by dissolving 50 mg tannic acid in distilled water to create a calibration curve. The solution was then diluted for

10-fold, and a serial dilution was made to get 20, 40, 60, 80, and 100 µg/mL final concentration. The same procedure was applied hereafter, described in detail above, and drawn a calibration curve using a Microsoft Excel sheet.

$$TP = \frac{C \times V_1 \times 1{,}000}{V_2 \times W \times DM(g/kg)} \quad (2.8)$$

Where;

TP: Total phenolics, g/kg DM

C: tannic acid concentration read from calibration graph (µg)

V_1: volume of 70% acetone used for extraction (ml)

V_2: volume of tannin-containing extract used for analysis (µl)

W: Weight of samples used for extraction (g)

To remove tannins from a tannin-containing extract, 100 mg PVPP was weighed into a clean test tube, pipetted 1 ml extract and 1 ml distilled water, and then vortexed. The test tubes were kept at 4 ºC for 15 minutes, vortexed once more, and then centrifuged at 3,000 g for 10 minutes to collect the supernatant. A 100 µl of supernatant and 400 µl distilled water were pipetted into a clean test tube, added 250 µl Folin-Ciocalteu reagent and 1,250 µl sodium carbonate solution, and then vortexed well. The final solution was then transferred into a disposable polystyrene microcuvette to read its absorbance after 40 min. The tannin content of samples was expressed as the differences between total phenolic and non-tannin phenols on a DM basis.

$$nonTP = \frac{C \times D \times 1{,}000}{V \times DM(g/kg)} \quad (2.9)$$

Where;

nonTP: non-tannin phenols (g/kg DM)

C: tannic acid concentration read from calibration graph (µg)

D: dilution factor

V: volume of non-tannin-containing extract used for analysis (µl)

$$T = TP - nonTP \tag{2.10}$$

Where;

T: tannin concentration (g/kg DM)

TP: Total phenolics (g/kg DM)

nonTP: non-tannin phenols (g/kg DM)

3.9 Quantification of Condensed Tannins

Measurement of condensed tannins (CT) was performed according to Porter, Hrsticch, and Chan (1986). Freshly prepared Butanol-HCl (95:5 v/v) and Ferric (2% ferric ammonium sulfate in 2N HCl) reagents were used. Briefly, 500 µl of the tannin-containing extract was pipetted into a clean test tube and added 3 ml Butanol-HCl and 100 µl Ferric reagents were heated in a hot water bath for 60 min. After cooling the test tube, the solution was transferred into a disposable polystyrene macro cuvette to read its absorbance at 550 nm. The unheated mixture was used as a blank in this assay, and the CT contents of samples were calculated as leucocyanidin equivalent by the given following equation[1]:

$$CTg/kg = \frac{(Abs_{550nm} \times DF)}{(DM\ g/kg)} \tag{2.11}$$

Where;

Abs_{550nm}: Absorbance read at 550 nm

DF: Dilution factor, equal to 1 if no 70% acetone was added into the tannin-containing extract.

[1] This formula assumes that the effective $\mathbf{E}^{1\%,\ 1\ cm,\ 550\ nm}$ of leucocyanidin is 460 (Porter et al., 1986)

3.10 Dry Matter and Ash

The analysis was based on the weight differences proposed by AOAC (1990). Clean crucibles, which hold at 102 ºC for at least 2 h and then transferred into the desiccator to come to constant weight, were used to determine the dry matter and ash content of samples. Approximately 2-3 g of sample was oven-dried at 102 ºC for 2 hours and then re-weight. Ash was determined by igniting a known dry sample in a muffle furnace at 550 ºC for 3 h and then re-weight.

$$DM\ (g/kg) = \frac{(W_f - W_i) \times 1{,}000}{W_s} \tag{2.12}$$

Where;

W_f: dry weight of sample and container (g)

W_i: tare weight of container (g)

W_s: sample weight (g)

$$Ash\ (g/kg) = \frac{(W_f - W_i) \times 1{,}000}{W_s} \tag{2.13}$$

Where;

W_f: ash weight of sample and crucible (g)

W_i: tare weight of crucible (g)

W_s: sample weight (g)

3.11 Crude Protein

The analysis was performed using a Gerhardt Kjeldatherm block digestion and Vapodest distillation unit. The nitrogen analysis of samples was determined according to the Kjeldahl method (AOAC, 1990). Approximately 1 g samples (to the nearest 0.0001 g) were accurately weighed onto N-free paper, transferred into a digestion tube, and then added 2

catalyst tablets (3.5 g K_2SO_4 + 3.5 mg Se) and 12 ml of concentrated H_2SO_4 and digested for about 1-3 h on digestion block. The addition of 10 M NaOH into the digestion tube was performed automatically in the distillation unit and separated ammonia trapped into 50 ml of 4% boric acid solution. Quantitative determination of Nitrogen was evaluated by titration with 0.01 M HCl using bromocresol green/methylene red mixed indicator solution. Crude protein (CP) was calculated by multiplying the nitrogen content of the samples by 62.5.

$$N(g/kg) = \frac{(V_s - V_B) \times N_{HCl} \times 14.007 \times 1{,}000}{W \times 1.000} \quad (2.14)$$

$$CP\ (g/kg) = \frac{N \times 62.5 \times 1{,}000}{DM\ (g/kg)} \quad (2.15)$$

Where;

V_s : volume of HCl required to titrate the sample (ml)

V_B: volume of HCl required to titrate blank (ml)

N_{HCl}: Normality of HCl (N)

14.007: equivalent weight of Nitrogen

W: sample weight (g)

3.12 Ether Extract

The EE analysis was performed using a soxhlet extraction unit according to the described procedure of AOAC (1990). Approximately 4-5 g of samples were weighed into an extraction thimble, hexane added instead of petroleum ether, and positioned on the heating block with the condenser water for at least 6 h. After extraction, the thimble was removed from the holder and allowed the spirit to air dry until any remaining spirit had evaporated. Then the beaker was placed in a 102 ºC oven for 2 hours, cooled in a desiccator, and re-weighed. The following formula was used to calculate the EE content of samples as g/kg on a DM basis.

$$EE\ (g/kg\ DM) = \frac{(W_f - W_i) \times 1{,}000 \times 1{,}000}{W_s \times DM\ (g/kg)} \quad (2.16)$$

Where;

W_f: weight of the beaker and fat residue (g)

W_i: tare weight of beaker (g)

W_s: sample weight (g)

3.13 Crude Fiber

The crude fiber (CF) content of samples was determined according to the described procedure by Naumann and Bassler (1993). Approximately 1 g of samples were placed into a 250 ml beaker and added 100 ml of 1.25% H_2SO_4. The beaker was then refluxed for 30 minutes after the onset of boiling and mixed several times manually. In the follow-up phase of the CF analysis, 10 ml 28% KOH was added to the beaker for the following 30 min boiling. The contents were then filtered into the crucibles, washed three times with hot 50 ml distilled water, and two times with 10 ml acetone. The crucibles were put in the oven at 102 ºC for overnight and weighed. The CF values were determined from the loss in weight after igniting a known dry residue in a muffle furnace at 550 ºC for 3 h. The following formula was used to calculate the CF content of samples as g/kg on a DM basis.

$$CF\ (g/kg) = \frac{(W_d - W_a) \times 1{,}000 \times 1{,}000}{W_s \times DM\ (g/kg)} \quad (2.17)$$

Where;

W_d: dry weight of crucible and dry fiber (g)

W_a: ash weight of crucible and dry fiber (g)

W_s: sample weight (g)

3.14 Neutral Detergent Fiber

A three-step method developed by Van Soest, Robertson, and Lewis (1991) was used to determine the Neutral Detergent Fiber (NDF) value of samples. The first step is the digestion of the feed sample in a neutral detergent solution (NDS) to separate NDS soluble and NDS insoluble fractions. The second step is washing the residue placed in crucibles to remove NDS residues. And the final step is defatting the samples by washing them with acetone.

The following NDS solution was prepared by dissolving 30 g sodium dodecyl sulfate, 18.61 g ethylenediaminetetraacetic disodium salt, 6.81 g sodium borate, 4.56 g anhydrous sodium phosphate dibasic, 10 ml triethylene glycol in 1 L distilled water and checked the pH from 6.9 to 7.1 before used.

Approximately 0.5 g of samples were placed into a 250 ml beaker, and added 0.25 g of sodium sulfite, 1 ml decalin, and 50 ml of NDS solution. The beaker was then refluxed for 60 minutes after the onset of boiling and mixed several times manually. The contents were then filtered into the crucibles, washed three times with hot 50 ml distilled water, and two times with 10 ml acetone. The crucibles were put in the oven at 102 ºC for overnight and then re-weighed. Ash-free NDF values were determined from the loss in weight after igniting a known dry residue in a muffle furnace at 550 ºC for 3 h. The following formula was used to calculate the ash-free NDF content of samples as g/kg on a DM basis.

$$NDF\ (g/kg) = \frac{(W_d - W_a) \times 1{,}000 \times 1{,}000}{W_s \times DM\ (g/kg)} \quad (2.18)$$

Where;

W_d: dry weight of crucible and dry fiber (g)

W_a: ash weight of crucible and dry fiber (g)

W_s: sample weight (g)

3.15 Acid Detergent Fiber

A similar three-step method developed by Van Soest et al. (1991), described in detail above, was used to evaluate the ADF value of samples. The NDS was replaced with an acid detergent solution (ADS), and sodium sulfite was omitted.

The following ADS solution was prepared by dissolving 20 g cetyltrimethylammonium bromide (CTAB) in 1.0 N H_2SO_4.

Approximately 1.0 g of samples were placed into a 250 ml beaker, added 2 ml dekalin and 100 ml of NDS solution. The beaker was then refluxed for 60 minutes after the onset of boiling and mixed several times manually. The contents were then filtered into the crucibles, washed three times with hot 50 ml distilled water, and two times with 10 ml acetone. The crucibles were put in the oven at 102 ºC for overnight and then re-weighed. The following formula was used to calculate the ADF content of samples as g/kg on a DM basis.

$$ADF\ (g/kg\ DM) = \frac{(W_f - W_i) \times 1{,}000 \times 1{,}000}{W_s \times DM\ (g/kg)} \quad (2.19)$$

Where;

W_f: dry weight of crucible and dry fiber (g)

W_i: tare weight of crucible (g)

W_s: sample weight (g)

3.16 Acid Detergent Lignin

The acid detergent lignin (ADL) content of samples was determined according to Van Soest et al. (1991). For the purpose of analysis, the crucibles containing ADF were placed into a large volume of a beaker, pipetted 30 ml of 72% of H_2SO_4, and manually stirred at hourly intervals. After 3 h, the acid was filtered with a vacuum and washed with hot distilled water until acid-free. The crucibles were put in the oven at 102 ºC for overnight and weighed, and then ignited in a muffle furnace at 550 ºC for 3 h and re-weighted. The following formula was used to calculate the ADL content of samples as g/kg on a DM basis.

$$ADL\ (g/kg) = \frac{(W_d - W_a) \times 1{,}000 \times 1{,}000}{W_s \times DM\ (g/kg)} \quad (2.20)$$

Where;

W_d: dry weight of crucible and dry fiber (g)

W_a: ash weight of crucible and dry fiber (g)

W_s: sample weight (g)

3.17 Hemicellulose and Cellulose

Hemicellulose and cellulose contents of samples were calculated using the NDF, ADF, and ADL values by the given following formulas (Okuyucu, Özdüven, and Koç, 2018):

$$Hemicellulose\ (g/kg\ DM) = NDF - ADF \quad (2.21)$$

$$Cellulose\ (g/kg\ DM) = ADF - ADL \quad (2.22)$$

3.18 Relative Feed Value

The digestible dry matter (DDM) and dry matter intake (DMI) of samples were calculated using NDF and ADF values by the given equations below (Rohweder, Barnes, and Jorgensen, 1978; Moore and Undersander, 2002). Then DDM and DMI values were used to calculate the relative feed value (RFV) of silages by the following formulas developed by Van Dyke and Anderson (2002).

$$DDM\ (\%) = 88.9 - (0.779 \times ADF) \quad (2.23)$$

$$DMI\ (\%) = 120/NDF \quad (2.24)$$

$$RFV = DDM \times DMI \times 0.775 \quad (2.25)$$

3.19 Enzyme Soluble Organic Matter

One of the most well-known tools for assessing enzyme soluble organic matter (ELOS) digestibility is the two-stage enzymatic digestion method previously described by Naumann and Bassler (1993). The cellulose obtained from *Trichoderma viride* (Onozuka R-10, Merck, Darmstadt, Germany) and pepsin (0.7 FIP-U/g, Merck, Darmstadt, Germany) enzymes were purchased and used in these two-stage enzymatic digestion studies. The first step in this process was to pre-treatment the sample with the pepsin enzyme. Approximately 300 mg of a sample was weighed into crucibles and then added to 30 ml pepsin solution (2 g of pepsin dissolved in 1 L of 0.1 N HCl). The resulting mixture was incubated at 40 °C for 24 h in an oven, hydrolyzed the starch content at 80 ºC in a water bath for 45 minutes, and then filtered under low vacuum pressure. In the second stage of the process, 30 ml cellulose-buffer solution (3.3 g cellulose dissolved in 1 L of acetate buffer solution; Solution A: 5.9 ml AA in 1 L distilled water; Solution B: 13.6 g sodium acetate + 1 L distilled water; w/w: 400/600) was added to crucibles and re-incubated at 40 °C for 24 h. At the end of the second incubation, crucibles were filtered, dried at 105 °C for at least 3 h, and burned at 550 °C (Esen et al., 2022). The ELOS was calculated between dried and burned residue weight differences after incubation. The following equations were used for estimating the ELOS and ME of samples (Cömert Acar, Özelçam, Şayan, and Soycan Önenç, 2018):

$$ELOS\ (g/kg) = \frac{[W_s - (W_1 - W_2) \times 1{,}000]}{W_s - W_a} \tag{2.26}$$

Where;

W_s: sample weight (g)

W_1: dry weight of crucible and dry sample (g)

W_2: ash weight of crucible and dry sample (g)

W_a: ash weight of sample (g)

$$EULOS\ (g/kg) = 1{,}000 - ELOS \tag{2.27}$$

Where;

EULOS: enzyme insoluble organic matter

$$ME = 14.27 - (0.0120 \times EULOS) + (0.0023 \times CP) - (0.0147 \times A) \quad (2.28)$$

Where;

ME: Metabolizable Energy (MJ/kg DM)

CP: Crude Protein (g/kg)

A: Ash (g/kg)

3.20 *In Vitro* Gas Production

The current dissertation employs the *in vitro* gas production technique according to the procedure used by Menke and Steingass (1998) to determine gas production. *In vitro* gas production technique was performed at the Animal Feed and Nutrition Laboratory of Atatürk University. For the purposes of analysis, rumen fluid was gathered from sheep freshly slaughtered at a local slaughterhouse and filtered through four layers of cheesecloth into a pre-warmed thermos, and immediately transferred to the laboratory. Anaerobic conditions were maintained throughout the preparation stages of rumen fluid and the conduct of the experiment. All the equipment was pre-warmed at 39 ºC before the injection. 200 mg dry weight of the samples was weighed into calibrated glass syringes of 100 ml, and then 30 ml rumen fluid-buffer mixture (1:2) was transferred into each syringe followed by incubation in a water bath at 39 ºC for 96 h. The total gas volume of TR silages was determined at 3, 6, 12, 24, 48, 72, and 96-h and corrected with blank syringes and standard alfalfa hay. The samples were incubated with a rumen fluid-buffer mixture in triplicate. An infrared methane analyzer was used to assess the methane content of gas produced previously described by Goel,

Makkar, and Becker (2008). Cumulative CP data of corn silages were fitted to the exponential model of Ørskov and McDonald (1979) by using Solver in MS Excel.

$$Y = a + b(1 - e^{-ct}) \quad (2.29)$$

Y: volume of gas produced at time t (ml)

a: volume of gas produced from the immediately soluble fraction of TR silages (ml)

b: gas produced volume from the insoluble fraction of TR silages(ml)

c: gas production rate constant (ml/h)

t: time of incubation

The ME and organic matter digestibility (OMD) of samples were calculated using equations recommended by Menke, Raab, Salewski, Steingass, and Fritz (1979) and Gheshlagh, Paya, Taghizadeh, Mohammadzadeh, Palangi, and Mehmannavaz (2021) as follows:

$$ME = 2.20 + 0.136 \times GP + 0.057 \times CP \quad (2.30)$$

$$NEL = -0.36 + 0.1149 \times GP + 0.0054 \times CP + 0.0139 \times CF - 0.0054 \times A \quad (2.31)$$

$$OMD = 14.88 + 0.889GP + 0.45\,CP + 0.0651 \times A \quad (2.32)$$

Where;

ME: Metabolizable Energy (MJ/kg DM)

NEL: Net Energy Lactation (MJ/kg DM)

OMD: Organic Matter Digestibility (g/kg DM)

GP: Gas Production (ml)

CP: Crude Protein (%)

EE: Ether Extract (%)

CF: Crude Fiber (%)

A: Ash (%)

3.21 Determination of *In Vitro* Protein Degradability

The *in vitro* protein degradability of samples was expressed as the subsequent loss in N content after hydrolyzing 1 h and 24 h. The hydrolysis of samples was done according to the procedure of Aufrere and Cartailler (1988) by using protease enzymes extracted from *Streptomyces griseus*, and borate-phosphate buffer. The buffer solution was prepared by dissolving 12.2 g sodium dihydrogen phosphate, and 8.91 g sodium tetra borax decahydrate in 1 L distilled water, and the pH was set to 8.0. Then, an enzyme-buffer solution was prepared by dissolving 20 mg protease, 10 mg nystatin, and 1 mg tetracycline in 1 L pre-warmed buffer solution at 40 ºC. Approximately 500 mg of a sample was weighed into falcon tubes and then added to 50 ml enzyme-buffer solution to incubate 1 h and 24 h in a water bath at 40 ºC. After stated incubation times, the mixtures were filtered through the Whatman filter paper No:54, and the residue was washed 3 times 50 ml of cold distilled water to inactivate the enzyme. The degraded N content of each sample was determined according to the Kjeldahl method by using sample residue.

$$CPD_{1h\ or\ 24h} = \frac{(CP_{0h} - CP_{1h\ or\ 24h}) \times 1{,}000}{CP_{0h}} \tag{2.33}$$

Where;

$CPD_{1h\ or\ 24h}$: Crude protein degradation of the sample after 1 h or 24 h (g/kg DM)

CP_{0h}: CP contents of the sample before enzymatic digestion (g/kg DM)

$CP_{1h\ or\ 24h}$: CP of residue after 1 h or 24 h of enzymatic treatment (g/kg DM)

3.22 Aerobic Stability Test

One of the most well-known tools for assessing aerobic exposure is the bottle system, based on trapping CO_2 gases into the KOH solution, described previously by Ashbell,

Weinberg, Azrieli, Hen, and Horev (1991). The 1.5 L gas-tight and corrosion-resistant polyethylene bottles were used in the aerobic stability test. At the end of the 5 days-aerobic stability test, the change in pH, carbon dioxide (CO_2) production, number of yeast, and molds were used as an indicator. The polyethylene bottles were cut into 2 parts to get a 0.5 and 1.0 L volume. A round hole, 0.5 cm diameter, was opened on the bottom side of the 1.0 L polyethylene bottles and then placed onto the 0.5 L part. Approximately 50 g of fresh silage was weighed into the upper part of the bottle unit, while 100 ml 20% KOH was filled in the lower part of the bottle unit and kept for 5 days at room temperature. After 5 days of aerobic exposure, 10 ml of aliquot was titrated with 1 N HCl and recorded to use volume to decrease the pH from 8.1 to 3.6. The following equation was used to estimate the CO_2 level of the silage sample:

$$CO_2(ml) = \frac{0.044 \times V_t \times V_{KOH}}{V_a \times W_f \times DM\ (g/kg)} \quad (2.34)$$

Where;

V_t: volume of 1 N HCl required to titrate the sample (ml)

V_{KOH}: total volume of 20% KOH used in the analysis (ml)

V_a: volume of KOH added in the lower part of the bottle (ml)

W_f: Fresh weight of silage weighted (kg)

The change in pH was determined as previously described above in section 2.2. The enumeration of yeasts and molds will be described in the following section.

3.23 Microbiological Analysis

Microbiological analysis was performed immediately after opening the silos and after the aerobic stability test, as described previously by Seale et al. (1990). A 10 g sample was added to freshly prepared 90 ml of Buffered Peptone Water (BPW) and mixed accurately for 2 minutes. Also, serial dilutions were prepared by using 9 ml of BPW, stored in sterile dilution tubes. The pour-plate method and MRS Agar (Lactobacillus Agar according to De Man, Rogosa, and Sharpe) were used to determine LAB count. Petri dishes were incubated at

30 ºC in an anaerobic jar with CO_2 enriched atmosphere for 3 days and then counted. Similarly, bacteria of the *Enterobacteria*ceae family were determined pour-plate method and Violet Red Bile Glucose (VRBG) agar. *Enterobacteriaceae* was enumerated after incubation at 37 ºC for 24 h aerobically. On the other hand, diluted samples were inoculated by spreading on the Potato Dextrose Agar (PDA) surface and incubated aerobically at 30 ºC for 5 days to enumerate yeast and molds.

3.24 Determination of Mineral Concentrations

The present dissertation utilizes the microwave digestion method previously described by Eseceli, Ayaşan, Koç, Kader Esen, and Esen (2020) for the determination of 10 mineral concentrations (B, Ca, Cu, Fe, K, Mg, Na, P, Se, Zn) by inductively coupled plasma optical emission spectrometry (ICP-OES). For the purpose of analysis, approximately 250 mg of dried sample was extracted with 10 ml HNO_3 in a microwave digestion system (EthosOne, Milestone Srl, Sorisole-Italy) under constant pressure and temperatures (30 bar and 180 °C) for 15 minutes. After cooling at room temperature, the digested sample was transferred quantitatively into the clean falcon tubes and then completed to 25 ml final volume with deionized water. ICP-OES analysis was carried out on the equipment (SpectroBlue, Spectro, Kleve-Germany) at Kastamonu University Central Research Laboratory.

3.25 Determination of Volatile Fatty Acids Concentration

The volatile fatty acids (VFA) content of silages (AA, PA, and BA) was evaluated according to the procedure described by Ülger et al. (2020) at the Animal Feed and Nutrition Laboratory of Erciyes University. A 5 ml of clear silage extract was transferred into a 15 ml falcon tube and then added 1 ml metaphosphoric acid (25%)-formic acid solution (3:1, v:v) for deproteinization. The clear supernatant (1 µL) was injected onto a gas chromatograph (Shimadzu GC-2010+, Kyoto, Japan) using a capillary column (Restek, Bellefonte, PA, USA; 30 m, inner diameter: 0.25 mm, film thickness: 0.25 µm), and with flame ionization detector (FID) over a temperature range of 45-230 °C.

3.26 Determination of Fatty Acid Composition

Approximately 0.1 g extracted oil, via soxhlet extraction, was dissolved with 10 ml n-hexane and transferred into a 15 ml falcon tube shaken vigorously by hand and then added 0.5 ml 2 N KOH and shaken vigorously by hand once. The preparation was kept at 4 ºC for 2 h,

filtered through a 0.22 µm PTFE syringe filter, and stored at -20 ºC until chromatographic analysis (Esen et al., 2020). Fatty acid methyl ester analysis of samples was performed on a gas chromatograph (Agilent 6890N, Santa Clara, CA, USA) equipped with FID using a fused silica column (DB-23, Agilent, Santa Clara, CA, USA; 60 m, inner diameter: 0.25 mm, film thickness: 0.25 µm). The peaks were determined according to Supelco 37 Component FAME Mix certified reference material (Sigma-Aldrich Company LLC, Saint Louis, MO, USA) and area normalization.

3.27 Statistical Analysis:

The effect of treatments on fermentation quality and nutritive value of silages were analyzed using the GLM procedure of Minitab (2014) statistical package programs, and least-squares means were compared using Tukey's multiple comparison tests. The following statistical model was used:

$$y_{ij} = \mu + a_j + e_{ij} \quad (2.35)$$

Where y_{ij}= observed value; μ = overall mean; a_i= effect of treatment; e_{ij}= effect of the experimental error.

4. RESULTS and DISCUSSION

4.1 Chemical and Microbiological Composition of Pre-ensiled White Clovers

The chemical and microbiological composition of pre-ensiled TR is presented in Table 4.1 and Figure 4.1. The TR harvested at 10-25% of the blooming stage had a DM concentration of 212.1 g/kg. The NDF, ADF, and ADL values of pre-ensiled TR were 433.5, 251.8, and 61.0 g/kg on DM basis. The CP, EE, Ash, and WSC contents were 251.8, 25.6, 96.3, and 53.8 g/kg DM basis, whereas the initial pH and Bc were 5.03 and 361.9 mEq NaOH/kg DM. The yeast and LAB count of pre-ensiled TR were 6.35 and 6.50 log cfu/g FM, respectively.

Table 4. 1. Chemical and microbial composition of pre-ensiled white clover.

Chemical composition	**Mean±SE**
Dry matter, g/kg	212.1 ± 0.3
Buffering capacity, mEq NaOH/ kg DM	361.9 ± 1.2
Water-soluble carbohydrate, g/kg DM	53.8 ± 1.7
pH	5.03 ± 0.0
Crude protein, g/kg DM	251.8 ± 9.5
Ether extract, g/kg DM	25.6 ± 1.3
Ash, g/kg DM	96.3 ± 0.0
Neutral detergent fiber, g/kg DM	433.5 ± 0.7
Acid detergent fiber, g/kg DM	344.9 ± 13.8
Acid detergent lignin, g/kg DM	61.0 ± 2.9
Microbial composition	
Yeast, log cfu/g FM	6.35 ± 0.01
Lactic acid bacteria, log cfu/g FM	6.50 ± 0.02

SE: Standard error, DM: Dry matter, FM: Fresh material, cfu: colony-forming unit, mEq: milliequivalents, NaOH: Sodium hydroxide

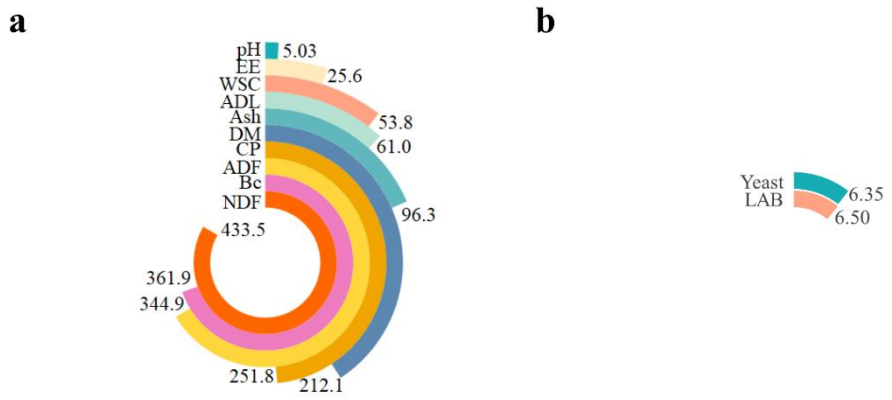


Figure 4. 1. Chemical (a) and microbial (b) composition of pre-ensiled white clover.

EE: Ether extract (g/kg DM), WSC: Water soluble carbohyrate (g/kg DM), ADL: Acid detergent lignin (g/kg DM), Ash (g/kg DM), DM: Dry matter: (g/kg), CP: Crude protein (g/kg DM), ADF: Acid detergent fiber (g/kg DM), Bc: Buffering capacity (mEq NaOH/kg DM), NDF: Neutral detergent fiber (g/kg DM), Yeast (log cfu/g), LAB: Lactic acid bacteria (log cfu/g)

In a study investigating the effects of feeding grass or TR silages on nitrogen metabolisms and amino acid concentration of lactating cows, Vanhatalo, Kuoppala, Ahvenjärvi, and Rinne (2009) reported that the DM, Ash, CP, NDF, WSC, and pH of early harvested TR as 214 g/kg, 102, 212, 375, 17 g/kg DM and 4.15, respectively. In another study conducted to determine the quality of TR and alfalfa silages, which were treated with the pre-fermented juice of TR and alfalfa, Sun et al. (2021) found that the DM, CP, WSC, NDF, ADF, ADL, and LAB of TR (harvested at early bud stage) as 401 g/kg, 195, 55.6, 350, 268, 106 g/kg DM and 4.15 log cfu/g, respectively. Furthermore, Ali et al. (2020) designed an experiment to evaluate the fermentation quality and microbial community of sterile TR silage treated with indigenous (fresh TR) and exogenous epiphytic (fresh maize, fresh sorghum) microbiota. They noted the DM, CP, WSC, NDF, ADF, ADL, Yeast, LAB, and pH of TR harvested at the early bloom stage as 170 g/kg, 241, 64.1, 424, 254, 87.8 g/kg DM, 6.30, 5.02 log cfu/g, and 5.85, respectively. Overall, the results obtained from this study were similar to Vanhatalo et al. (2009) for DM and ash; Ali et al. (2020) for Bc, CP, NDF, and yeast; Sun et al. (2021) for ADF and WSC. However, the ADL content was lower than that of the three studies. Furthermore, the WSC content of the current study was slightly lower than the results of Ali et al. (2020) but quite higher than the results of Vanhatalo et al. (2009). This discrepancy might be attributed to the harvest season, geographic region, temperature, maturity at harvest, and leaf-stem ratio. Kagan et al. (2020) make a similar point in their study, which compared the seasonal and diurnal variations of red and white clover's WSC in Kentucky.

It is common knowledge that legume forages are characterized by low WSC, high Bc, and low DM, whereas most gramineous grasses have optimal WSC content with low Bc and easily controllable moisture levels (Wang, Li, Zhao, Dong, and Shao, 2022). In the current study, the WSC (53.8 g/kg DM) content was sufficient to meet the theoretical requirement (>50 g/kg DM) for adequate fermentation. However, Bc (361.9 mEq NaOH/kgDM) was higher than the theoretical requirement (<300 mEq NaOH/kgDM), while DM content (212.1 g/kg) was lower than the theoretical requirement (300-400 g/kg) (Weinberg, 2008; Li et al., 2019; Wang et al., 2022). Prior studies have also noted the importance of epiphytic LAB in pre-ensiled material and recommended at least 5 log cfu/g FM required for sufficient fermentation (Zhao et al., 2021; Wang et al., 2022). In the present study, epiphytic LAB of fresh TR was higher than the minimum requirements of LAB for good quality silage. However, TR's higher Bc and low DM content make this crop challenging to ensile.

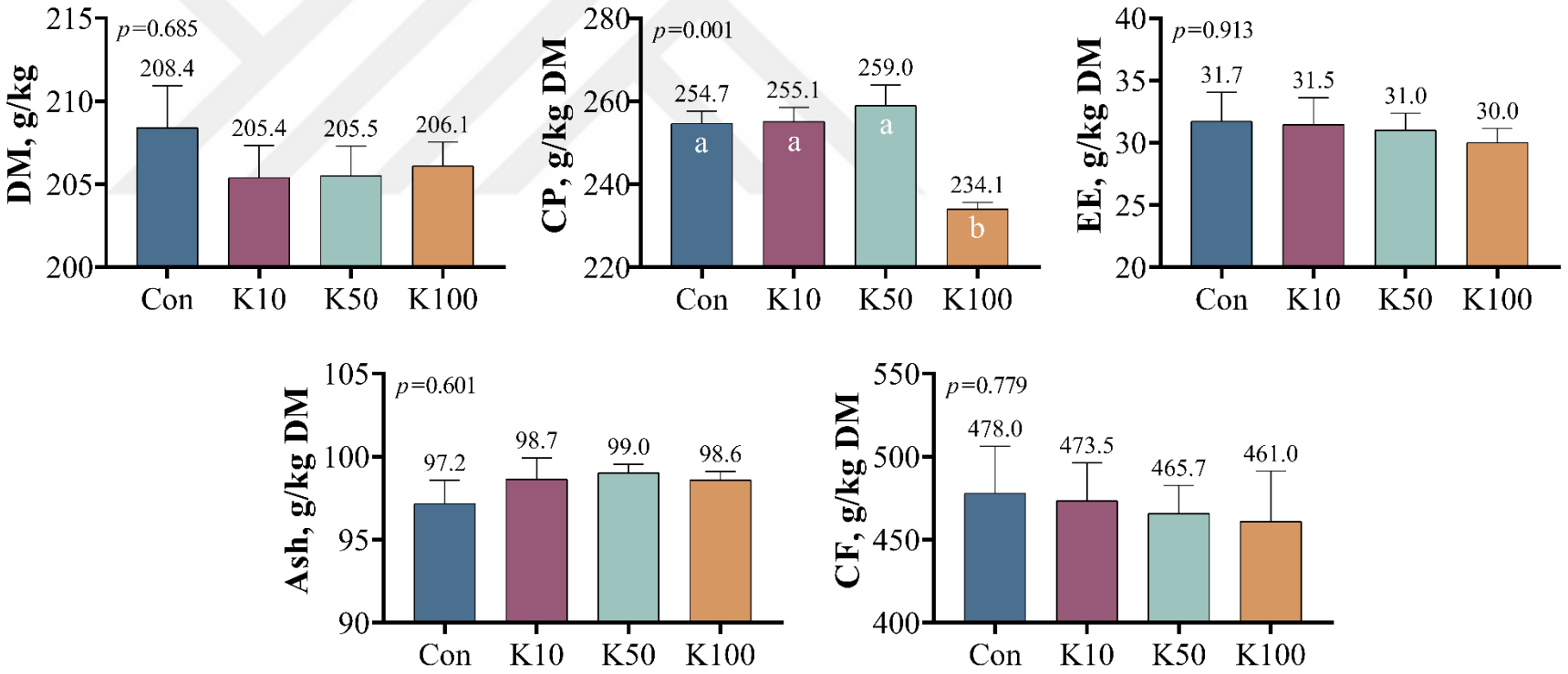
4.2 Chemical Characteristics of Kefir Treated White Clover Silages

After 270 d of ensiling, DM, EE, Ash, and CF content of TR silages have not been affected by kefir yeast inoculation (P>0.05; Table 4.2). A numerically decreasing trend was observed in EE and CF content of TR silages (Figure 4.2). Moreover, inoculation of kefir yeast with an application rate of 100 mg/kg significantly decreased the CP content of TR silages in the K100 group (P<0.001).

Table 4. 2. Chemical characteristics of white clover silages inoculated with kefir yeast.

Item	**Con**	**K10**	**K50**	**K100**	**SEM**	***P*-value**
DM, g/kg	208.4	205.4	205.5	206.1	1.96	0.685
CP, g/kg DM	254.7[a]	255.1[a]	259.0[a]	234.1[b]	3.43	0.001
EE, g/kg DM	31.7	31.5	31.0	30.0	1.82	0.913
Ash, g/kg DM	97.2	98.7	99.0	98.6	1.01	0.601
CF, g/kg DM	478.0	473.5	465.7	461.0	12.57	0.779

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, DM: Dry matter, CP: Crude protein, EE: ether extract, CF: Crude fiber. The values with different letters (a, b)in the same row are statistically different (P<0.05).



Con: Control, **K10**: 10 mg/kg kefir yeast, **K50**: 50 mg/kg kefir yeast, **K100**: 100 mg/kg kefir yeast, **DM**: Dry matter, **CP**: Crude protein, **EE**: Ether extract, **CF**: Crude fiber.

Figure 4. 2. Chemical characteristics of white clover silages inoculated with kefir yeast.

The values with different letters (a, b) in each graph is statistically different (P<0.05).

As is well known, kefir yeast contains yeast, such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, in their natural microbiome. Therefore the DM and CF content of TR silages are expected to decline with an increasing application rate of kefir yeast. Surprisingly, no differences were found in the DM and CF content of treated silages ($P>0.05$). A possible explanation for these results may be the low pH value (<4.1) of all kefir yeast inoculated TR silages after 270 d of ensiling. These results are consistent with data obtained by Dunière et al. (2015), who revealed the efficacy of yeast strains in a fourth-generation inoculant. Along the same lines, Xu et al. (2019) reported no changes in corn silage's nutritional composition and fermentation profile when the application of yeast rate increased from 10^3 to 10^4 and 10^5 cfu/g FM and combined with or without *L. buchneri*.

Accordingly, one major issue in producing high-quality silage is extensive protein degradation during wilting and ensiling (Krawutschke et al., 2013). It has been highlighted that extensive proteolysis could occur in legume forages, forming a large quantity of non-protein nitrogen (NPN) (Zhang et al., 2021). Moreover, a large volume of published studies describes the role of plant and microbial proteases on extensive proteolysis during wilting and ensiling (Sousa, Hansen, Nussio, and Nadeau, 2019; He et al., 2020). These proteases hydrolyze proteins into NPN intermediate products, such as peptides and free amino acids, which are further degraded into ammonia, amines, and amides (Kung Jr et al., 2018). As is well known, utilization of NPN is less efficient than true protein for ruminants and results in the reduced nutritional quality of silage (McDonald et al., 1991).

A significant change in CP content may be attributed to proteolysis (Chen, Li, Dong, and Shao, 2019). In the current study, the pH of TR silages in all groups was sufficient to restrict proteolytic activity; however, proteolysis occurred in the K100 group. The CP content was reduced by 7.97% in this group compared to the Con group. A possible explanation for this might be that the diversely epiphytic microorganisms in kefir yeast in the K100 group contributed much more to proteolysis than the other groups during ensiling.

Nevertheless, it is possible to reduce proteolysis in silage with some well-known applications. For example, Sousa et al. (2019) recommended rapid field wilting under ideal weather conditions and the use of silage additives to reduce the adverse effects of proteolysis during the ensiling process. Additionally, Fijalkowska, Pysera, Lipiński, and Strusińska (2015) underlined the importance of rapid reduction of silage pH and recommended the use of acidifying fermentation inhibitors.

4.3 Crude Protein and Enzyme Soluble Organic Matter Digestibility of Kefir Treated White Clover Silages

Deactivating protein-degrading enzymes increases the true protein concentration in silage resulting in increased ruminal-bypass protein, which could benefit ruminants (Broderick, Walgenbach, and Maignan, 2001). Existing research recognizes the critical role of plant enzymes on proteolysis during ensiling (Herrmann, Heiermann, and Idler, 2011; Ineichen, Marquardt, Wettstein, Kreuzer, and Reidy, 2019; Yin et al., 2021). Several experimental studies have established that aminopeptidase and protease are the two most crucial protein-degrading enzymes that degrade protein into nonprotein-N, peptides, free amino acids, and NH_3-N during ensiling (McKersie and Buchanan-Smith, 1982; McDonald et al., 1991). Nevertheless, Winters, Fychan, and Jones (2001) showed that protein hydrolysis during ensiling not only be mediated by plant proteases but also microbial protease might play a pivotal role. However, the activities of these enzymes differ from each other under different pH, temperature, and inhibitors, and it is possible to deactivate these enzymes under low pH (Yin et al., 2021). Therefore, thus far, numerous researchers have recommended silage additives to prevent proteolysis by enhancing a rapid decrease in pH and prohibiting protein-degrading bacterial growth (Ding, Long, and Guo, 2013; Li et al., 2018; He et al., 2020). Likewise, silage additives are also recommended for decreasing organic matter losses (Herrmann et al., 2011) in an extended ensiling period.

Table 4. 3. Crude protein and enzyme soluble organic matter digestibility of white clover silages inoculated with kefir yeast.

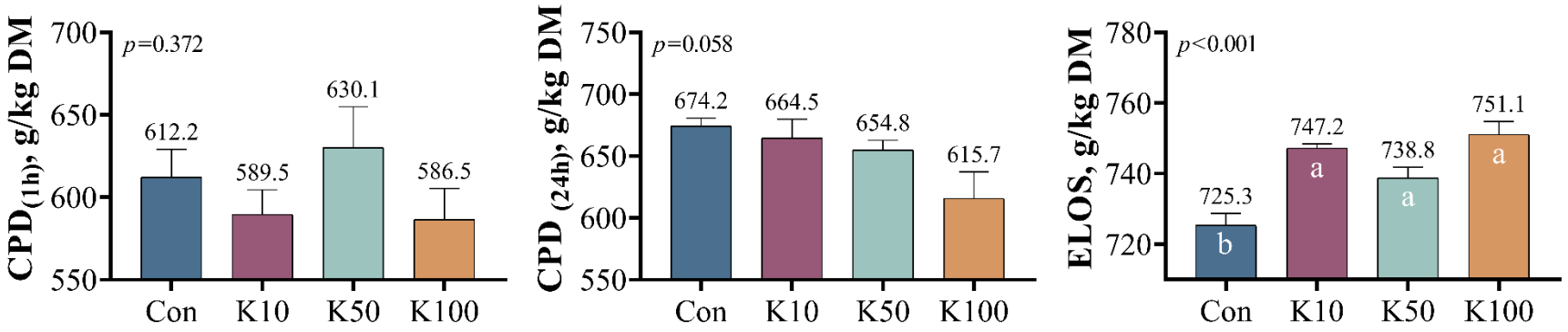
Item	Con	K10	K50	K100	SEM	*P*-value
$CPD_{(1h)}$, g/kg DM	612.2	589.5	630.1	586.5	19.24	0.372
$CPD_{(24h)}$, g/kg DM	674.2	664.5	654.8	615.7	14.13	0.058
ELOS, g/kg DM	725.3^{b}	747.2^{a}	738.8^{a}	751.1^{a}	2.98	<0.001

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, $CPD_{(1h)}$: Crude protein degradability after 1 hour, $CPD_{(24h)}$: Crude protein degradability after 24 hours, ELOS: Enzyme soluble organic matter, DM: Dry matter. The values with different letters (a, b)in the same row are statistically different (P<0.05).

An inspection of the data in Table 4.3 and Figure 4.3 reveals that an increase of CPD in the K50 group was observed in early fermentation; it disappeared as the fermentation time progressed. A comparison of the findings with those of other studies shows that there could be

a shift in the microbial community due to other species competing with the microbial species found in kefir yeast (Winters et al., 2001; Winters et al., 2004). The observed increment in CPD in the K50 group could also be attributed to the lower LA content and higher pH in this group. This is consistent with the data obtained by da Silva et al. (2020) and Cao et al. (2021). Besides, the reduction of CPD increased with an increased inoculation rate of kefir yeast after 24 h of the incubation period. The reduction rate of CPD between kefir yeast treated groups ranged between 1.44-8.68%. However, none of these differences were statistically significant ($P>0.05$). On the contrary, the ELOS content of TR silages was significantly affected by kefir yeast inoculation ($P<0.001$). These results are in agreement with those obtained by Winters et al. (2001), Hofmann et al. (2011), Li et al. (2018), He et al. (2020), and Yin et al. (2021).

It would be helpful at this stage to consider the effect of the ensiling period on proteolysis. For example, Hofmann et al. (2011) demonstrated that proteolytic activity in high moisture corn silage increased with an extended ensiling period due to degraded starch-protein matrix. Adopting a similar position, Der Bedrosian et al. (2012) and Ferraretto, Fredin, Muck, and Shaver (2016) reported increased ammonia-N and soluble protein concentration in whole-plant corn silages with a prolonged fermentation period. In addition, it was considered that extensive proteolysis in silage always results in inefficacious N utilization in ruminants, which increases the economic loss and environmental pollution risk (Wang et al., 2019). The research presented here confirms that kefir yeast could be used effectively in the long-term ensiling to prevent proteolysis in TR silages.



Con: Control, **K10**: 10 mg/kg kefir yeast, **K50**: 50 mg/kg kefir yeast, **K100**: 100 mg/kg kefir yeast, **CPD:** Crude protein degradation degretability, **ELOS**: Enzyme soluble organic matter .

Figure 4. 3. Crude protein and enzyme soluble organic matter digestibility of white clover silages inoculated with kefir yeast.

The values with different letters (a, b) in each graph is statistically different (P<0.05).

4.4 Fermentation Characteristics and Silage Quality of Kefir Treated White Clover Silages

Ensiling is a viable and effective alternative method to supply high-quality roughage for animals all year round (Zhang, Guo, Zheng, Chen, and Chen, 2021). Thus far, several studies have investigated the effects of different silage additives, such as LAB (homofermentative or heterofermentative; alone/combined), other inoculants, enzymes, and chemicals to improve the fermentation quality in different silages. All of these studies underlined the importance of a rapid pH reduction during the first few days of ensiling to inhibit the proliferation of undesirable microorganisms and reduce the proteolytic and lipolytic activity of plant tissue and microorganism under anaerobic conditions (Ding et al., 2013; Muck et al., 2018; Wang et al., 2019b).

In the current study, the pH of silages is expected to decline with an increased application rate of kefir yeast. However, as can be seen from Table 4.4 and Figure 4.4, inoculation of kefir yeast decreased the pH of TR in the K100 group, increased in the K50 group, and remained no change in the K10 group compared to the Con group ($P<0.001$). The highest and lowest pH was observed in the K50 (4.09) and K100 (3.92) groups, respectively. It seems possible that these results are due to higher yeast and lower LAB count of the K50 group. The trapped oxygen in the silo could facilitate lactate assimilating yeast activity and increase pH. In the same vein, the lower LA content of the K50 group confirms this assumption. In agreement, the pH of TR silages in all treated groups and untreated Con groups was in the recommended value of 4.2-3.9 (Stanaćev and Vik, 2002).

It is common knowledge that the primary aim of adding exogenous LAB into silage is to enhance the reduction of silage pH by degrading WSC to organic acid, particularly LA (Chen et al., 2020b). Likewise, the present study aimed to reduce silage pH by using kefir yeast, which contains several LAB species naturally. The WSC content of TR silages was reduced by 50.07-55.84%, and residual WSC contents ranged between 23.77 to 26.86 g/kg DM after 270 d of ensiling. Furthermore, no significant difference between the groups was evident ($P>0.05$). In accordance with the present results, previous studies have also demonstrated that increased residual WSC content in silage indicates the partial preclusion of fermentation due to insufficient amounts of metabolic water for microbial growth, resulting in

no more formations of organic acids (da Silva, Smith, Barnard, and Kung Jr, 2015; Wei, 2020). These results also accord with earlier observations, which showed that higher residual WSC content indicates lesser DM losses and higher nutritive value of silages (Weinberg, Ashbell, Hen, and Azrieli, 1993; Wang, Yuan, Dong, Li, and Shao, 2018). Additionally, these findings might further indicate that the high residual WSC content of silages is a splendid substrate for yeast, molds, and aerobic bacteria after silo opening (Silva et al., 2018; Auerbach et al., 2020).

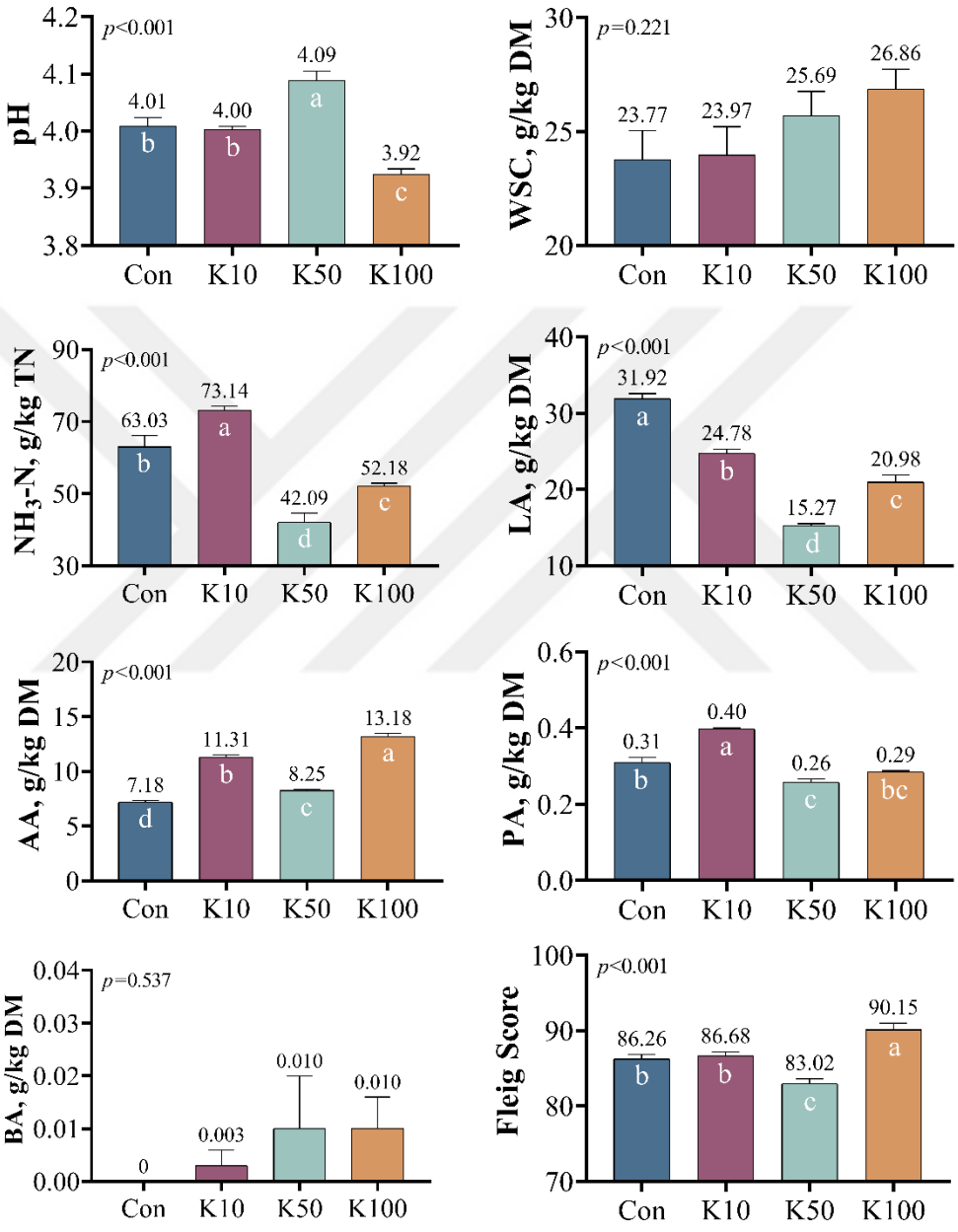
Table 4. 4. Fermentation characteristics and silage quality of white clover silages inoculated with kefir yeast.

Item	**Con**	**K10**	**K50**	**K100**	**SEM**	***P*-value**
pH	4.01^{b}	4.00^{b}	4.09^{a}	3.92^{c}	0.01	<0.001
WSC, g/kg DM	23.77	23.97	25.69	26.86	1.13	0.221
NH_3-N, g/kg TN	63.03^{b}	73.14^{a}	42.09^{d}	52.18^{c}	2.10	<0.001
LA, g/kg DM	31.92^{a}	24.78^{b}	15.27^{d}	20.98^{c}	0.31	<0.001
AA, g/kg DM	7.18^{d}	11.31^{b}	8.25^{c}	13.18^{a}	0.20	<0.001
PA, g/kg DM	0.31^{b}	0.40^{a}	0.26^{c}	0.29^{bc}	0.01	<0.001
BA, g/kg DM	-	0.003	0.010	0.010	0.006	0.537
Fleig score	86.26^{b}	86.68^{b}	83.02^{c}	90.15^{a}	0.66	<0.001

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, WSC: Water-soluble carbohydrate, NH_3-N: Ammonia nitrogen, LA: Lactic acid, AA: Acetic acid, PA: Propionic acid, BA: Butyric acid. The values with different letters (a, b, c, d)in the same row are statistically different (P<0.05).

Previous studies have considered NH_3-N as the main product of undesirable fermentation and indicate proteolysis in silage (Pahlow et al., 2003; He, Zhou, Chen, and Zhang, 2021). A high accumulation of NH_3-N during ensiling is attributed to the deamination of amino acids by microbial activity (Lv et al., 2020; Zhou, Pian, Yang, Chen, and Zhang, 2021). It has also been noted that LA fermentation in the silo is negatively affected by the NH_3-N produced from proteolysis due to the increased buffering effect (Dong, Chen, Li, Yuan, and Shao, 2019). Furthermore, several researchers evaluated the legume silage as well preserved when the NH_3-N content is lesser than 150 g/kg TN (de Rezende, Rabelo, da Silva, Härter, and Veiga, 2015; Nkosi et al., 2016). In the present study, NH_3-N contents of TR

silages were significantly affected by kefir yeast inoculation (P<0.001). In all groups, including the Con group, the NH_3-N content of TR silages was lower than the recommended value for legume silages, whereas the highest and lowest were seen in the K10 (73.14 g/kg TN) and K50 (42.09 g/kg TN) groups (P<0.001).



Con: Control, **K10**: 10 mg/kg kefir yeast, **K50**: 50 mg/kg kefir yeast, **K100**: 100 mg/kg kefir yeast, **DM**: Dry matter, **CO_2**: Carbon dioxide.

Figure 4. 4. Fermentation characteristics and silage quality of white clover silages inoculated with kefir yeast.

The values with different letters (a, b,c,d) in each graph is statistically different (P<0.05).

In a well-designed and robust study, Dong et al. (2019) examined the transformation dynamics of nitrogen in alfalfa and red clover. Contrary to expectations, the authors demonstrated that lower production of NH_3-N in red clover was chiefly related to lower pH rather than polyphenol oxidase enzyme activity. Likewise, Gandra et al. (2019) ensiled rehydrated maize with α-amylase or glucoamylase and evaluated the chemical composition, fermentation profile, total losses, and amylolytic activity of rehydrated maize. They found higher NH_3-N content in glucoamylase-treated silage and assumed it might be related to the solubility of hydrophobic zein proteins found in the starch-protein matrix of maize grains in LA and AA by enzymes. However, the findings of the current study do not support this research. The evidence from this study suggests that the total amount of LA and AA is inversely related to NH_3-N content in leguminous silage. A possible explanation for this might be the high Bc of TR. These results are in agreement with those obtained by Dong et al. (2019).

One major theoretical issue in using kefir yeast as silage inoculants is how acetic acid bacteria, naturally found in kefir grain, would affect the silage quality. The greater part of the literature described acetic acid bacteria as an initiator of aerobic spoilage. Acetic acid bacteria are able to grow at low pH on ethanol, and they can continue to grow when ethanol is fully consumed, converting AA to CO_2 and H_2O (Muck, 2010). However, thus far, they have only been reported in corn silages (Muck, 2010). Furthermore, a considerable amount of literature has been published on acetic acid bacteria that could be used as an inoculant (Nishino et al., 2009; Kristensen et al., 2010). These studies suggest that acetic acid bacteria are able to oxidize ethanol to AA, thus inhibiting the proliferation of fungi and increasing the aerobic stability of silage.

On the other hand, considerable literature has grown up around the theme of dual-purpose inoculants containing homo- and hetero-fermentative bacteria to cope with the limitations of inoculants containing either type of bacteria solely (Adesogan, Krueger, Salawu, Dean, and Staples, 2004; Li et al., 2017; Zhao et al., 2021). These studies suggest that the combination of homo- and hetero-fermentative microorganisms have the potential to enhance the speed of fermentation and increase aerobic stability. In addition, previous research has been shown that kefir grain contains a diverse range of LAB, acetic acid bacteria, and yeast (Zhou et al., 2009; Gao et al., 2012) and could be used effectively to improve wheat straw and alfalfa silage (Koç et al., 2021; Okuyucu and Esen, 2022).

In the current study, as expected, inoculation of kefir yeast into TR silages decreased the LA and increased AA content ($P<0.001$). The highest and lowest LA content was found in the Con (31.92 g/kg DM) and K50 (15.27 g/kg DM) groups, whereas the highest (13.18 g/kgDM) and lowest AA (7.18 g/kg DM) content were observed in the K100 and Con group, respectively. The BA content of TR silages appeared to be unaffected by kefir yeast inoculation ($P>0.05$). Only trace amounts of BA were detected in kefir yeast treated groups. Contrary to the BA content of TR silages, the PA content was significantly affected by the inoculation of kefir yeast ($P<0.001$). The highest and the lowest PA was observed in K10 (0.40 g/kg DM) and K50 (0.26 g/kg DM) groups ($P<0.001$).

Several studies have revealed that AA plays a crucial role in the prevention of aerobic deterioration of silage by inhibiting fungi (McDonald et al., 1991; Li et al., 2016), and at least 8 g/kg of DM AA is required to inhibit these undesirable organisms (Wilkinson and Davies, 2013). The evidence presented in this section suggests that in TR silages inoculated with kefir yeast, AA accumulates at the expense of LA, and the figure above illustrates that kefir yeast inoculated TR silages had a greater AA content than the recommended value at silo opening after 270 d of ensiling. The LA contents of the K50 group were lower than that of recommended value (20 g/kg DM) for good-quality silages, whereas the LA content of the K100 group was close to the recommended value (Pottier and Martin Rosset, 2015). According to Kleinschmit and Kung Jr (2006), the ratio of LA/AA below 3 was an indication of heterolactic dominant fermentation. In the current study, the ratio of LA/AA was lower than 2.19 except Con group (4.45). Thus, fermentation in all kefir yeast inoculated groups was dominated by heterofermentative bacteria. A broadly similar point has also recently been made by Koç et al. (2021), who studied the effect of different kefir sources (commercial and homemade) on fermentation characteristics, aerobic stability, and microbial profile of alfalfa silage.

As stated in the previous section, no significant differences were observed in the DM content of TR silages; thus, an increase or decrease in pH directly affected the Fleig score. The increased pH in the K50 group decreased the Fleig score of this group, while decreased pH in the K100 group increased the Fleig score of this group ($P<0.001$). These results corroborate the findings of a great deal of the previous work, which evaluated the Fleig score as quality parameters and underlined the importance of DM and pH (Karakozak and Ayaşan, 2010; Öten, Kiremitci, and Çınar, 2016).

4.5 Cell Wall Components of Kefir Treated White Clover Silages

During ensiling, acidic conditions promoted by silage microorganisms and enzymatic hydrolysis of structural carbohydrates play a pivotal role in the overall nutritive value of silage. To date, factors thought to be influencing the degradation of fiber and improvements in digestibility have been explored in several studies (Wang and McAllister, 2002; Mendoza, Loera-Corral, Plata-Pérez, Hernández-García, and Ramírez-Mella, 2014; Ordaz, 2017). So, to conclude, all of these studies underlined that soluble carbohydrate concentration, degree of lignification, enzyme interaction, and microbial community of ensiled forage significantly affect silage fermentation.

Digestible cell wall fraction of plant biomass could be hydrolyzed with the extension of ensiling period by hydrolytic activities such as acidolysis, microbial activities, and enzymatic actions (Zhao et al., 2018; Ren et al., 2021). Thus, the DM losses during ensiling may be attributed to the degradation of soluble mass and digestible cell wall fractions to gas and volatile substances due to the synergetic effects of enzymes and microorganisms (Sarteshnizi et al., 2018). Adopting a similar position, Williams and Shinners (2012) argued that respiration of plant cells and the acid hydrolysis of cell walls during ensiling could be other possible sources of DM losses.

Previous research comparing the cell wall of grasses and legumes has revealed that pectins and cellulose are more abundant than xylans in the legume cell wall, whereas phenolic acids are more plentiful in the grass cell wall (Jung and Allen, 1995). Contreras-Govea et al. (2011) noted that the hemicellulosic fraction of alfalfa was well-degraded at lower DM (arabinose, galactose, and in particular, rhamnose), while cellulose, part of ADF fraction, was not so. Additionally, Herrmann et al. (2011) indicated that the low pH and prolonged storage period of silage led to increasing hydrolysis of hemicellulose by organic acid, produced during fermentation at a slow rate but continuously. It has also been demonstrated that carbohydrate pools in silage are mostly related to the pectic and hemicellulosic fraction of the cell wall, which generally increases with the degradation of arabinosyl fraction linked to xylose and galactose (Rooke and Hatfield, 2003; Contreras-Govea et al., 2011).

On the one hand, lignin, one of the three main polymers found in plant biomass and significantly affects forages' digestibility, is not a polysaccharide. It is a polymer derived from the phenylpropanoid pathway forming monolignols in vascular plants (Moore and Jung,

2001). On the other hand, lignin reduces the digestion of cell walls acting as a physical barrier to the microbial enzymes hindering them from reaching their target polysaccharides (Moore and Jung, 2001; Ordaz, 2017). Saliba et al. (1999, cited in Faria Júnior et al. 2011) offered an explanatory view for reducing the digestibility of forages. They attributed this issue to vanillin, which is derived from ferulic acid and linked lignin to hemicellulose. Similarly, it has been established that lignin and hemicellulose are interconnected in the monocot's cell wall mainly by ferulic acid. Specifically, the ferulic acid is linked to arabinose residues of xylan with carboxylic ester bonds on one side and lignin with ether bonds on the other side (Wong, 2006; Mogodiniyai Kasmaei, Schlosser, Sträuber, and Kleinsteuber, 2020). Therefore, it has been hypothesized that it is possible to increase the bioavailability of fiber constituents for the fermentation process by degrading carboxylic ester bonds between lignin and hemicellulose with feruloyl esterases (FAEs) (Mogodiniyai Kasmaei et al., 2020; Carvalho et al., 2021).

In recent years there has been an increasing amount of literature on ferulic acid esterase-producing LAB to increase NDF degradation and reduce fiber concentration during ensiling (Comino et al., 2014; Lynch et al., 2014; Ding et al., 2019; Xie et al., 2021). The LAB species associated with the production of FAEs are *L.buchneri*, *L. plantarum*, *L. brevis*, *L. reuteri*, and *L. crispatus* (Ding et al., 2019; Carvalho et al., 2021). However, studies undertaken so far provide conflicting evidence concerning the impact of FAE-producing LAB strains on reducing fiber contents. For example, Ding et al. (2019) isolated 2 FAE-producing LAB strains (*L. plantarum* and *L. brevis*) from ensiled *Elymus nutans* and inoculated them into alfalfa silage. They found that both FAE-producing LAB strains increased fermentation quality, whereas only *L. plantarum* could reduce alfalfa fiber content. In addition, they reported that none of them could effectively reduce the degradation of silage protein. In another example, Xu et al. (2020) concluded that *L. plantarum* strain A1 did not get advantageous for hemicellulose degradation in mixed corn stalk and potato pulp silage. Mogodiniyai Kasmaei et al. (2020) remarked that these inconsistent results could be attributed to readily available substrates in silage that obstruct the inoculant's FAE activity, such as glucose.

In the current study, following the inoculation of kefir yeast, a significant reduction in the NDF ($P<0.001$) and hemicellulose ($P<0.05$) contents were recorded (Table 4.5 and Figure 4.5). The reduction rate of NDF and hemicellulose between groups ranged between 1.33-

5.54% and 10.32-30.55%, respectively. However, no evidence was found for linear reduction between application rate and these parameters. It is difficult to explain, but it might be related to the relatively higher ADL and hemicellulose content of the K50 group (151.1 g/kg DM vs. 127.2 and 133.9 g/kg DM in the K10 and K100 groups, respectively), which acted as a protective layer and reduced the degradation rate of cellulose content. This is consistent with the data obtained by Mussatto, Fernandes, Milagres, and Roberto (2008) and Zhao et al. (2018). Another possible explanation of our findings is that cell wall degrading enzyme activity may be restricted at an early stage of fermentation, resulting in insufficient substrates required for efficient fermentation. The lowest LA content (15.27 g/kg DM) in the K50 group confirms this assumption. Similar results were reported by Fijałkowska et al. (2020) in a study of grass silage inoculated with three different *Lactobacillus* spp. (*L. paracasei*, *L. brevis*, and *L. plantarum*) isolated from a fresh mass of sugar beet and fodder beet silage.

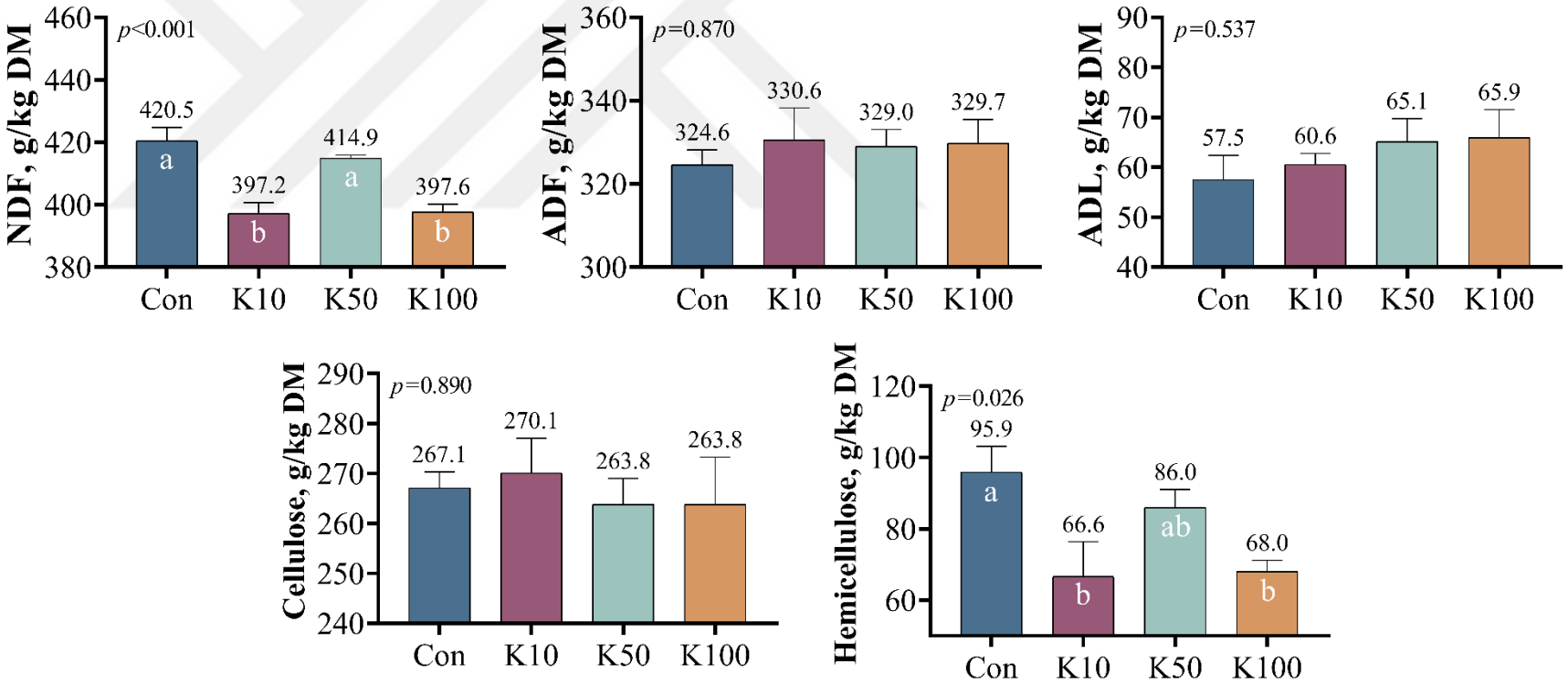
Table 4. 5. Cell wall components of white clover silages inoculated with kefir yeast.

Item	**Con**	**K10**	**K50**	**K100**	**SEM**	***P*-value**
NDF, g/kg DM	420.5^{a}	397.2^{b}	414.9^{a}	397.6^{b}	3.10	<0.001
ADF, g/kg DM	324.6	330.6	329.0	329.7	5.52	0.870
ADL, g/kg DM	57.5	60.6	65.1	65.9	4.53	0.537
Cellulose, g/kg DM	267.1	270.1	263.8	263.8	6.62	0.890
Hemicellulose, g/kg DM	95.9^{a}	66.6^{b}	86.0^{ab}	68.0^{b}	6.77	0.026

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, ADL: Acid detergent lignin. The values with different letters (a, b)in the same row are statistically different (P<0.05).

Furthermore, the inoculation of kefir yeast affected none of the ADF, ADL, and cellulose contents (P>0.05). This finding is consistent with Sun, Trevorah, and Othman (2018), who indicated that ADL degradation is difficult due to the close association between hemicellulose and/or Cell by covalent, hydrogen, and ester bonds. A possible explanation for these results may be the inhibition of cellulolytic enzymes with an increased organic acid concentration and decreased pH. This view is supported by Siegert and Banks (2005), who writes that cellulolytic enzymes could become inactivated, and the rate of cellulose hydrolysis dramatically decreases when the total organic acid concentration is higher than 2 g/L. On the contrary, Zhao et al. (2018) argued that removing hemicellulose using xylanase or acid

treatment could facilitate the enzymatic hydrolysis of cellulose via increased porosity and surface area and decreased cellulose crystallinity. Therefore, the results obtained in the K100 group might be related to these acidic conditions.



Con: Control, **K10**: 10 mg/kg kefir yeast, **K50**: 50 mg/kg kefir yeast, **K100**: 100 mg/kg kefir yeast, **NDF**: Neutral detergent fiber, **ADF**: Acid detergent fiber, **ADL**: Acid detergent lignin.

Figure 4. 5. Cell wall components of white clover silages inoculated with kefir yeast.

The values with different letters (a, b) in each graph is statistically different (P<0.05).

4.6 Relative Feed Value of Kefir Treated White Clover Silages

It is common knowledge that digestibility and energy obtained from forage crops decrease with maturity. In other words, indigestible parts of plant structural carbohydrates, such as NDF and ADF fractions, increase with the increasing maturity of forages (Jeranyama and Garcia, 2004). Although many systems have been developed to predict forage quality to date, RFV, developed by Rohweder et al. (1978), is the most widely used due to reflecting both the digestibility and intake potential of forages (Jeranyama and Garcia, 2004; Hackmann, Sampson, and Spain, 2008). It is evident that acknowledging forage's nutritional value is crucial in allocating forage to animals (according to their physiological state) and marketing.

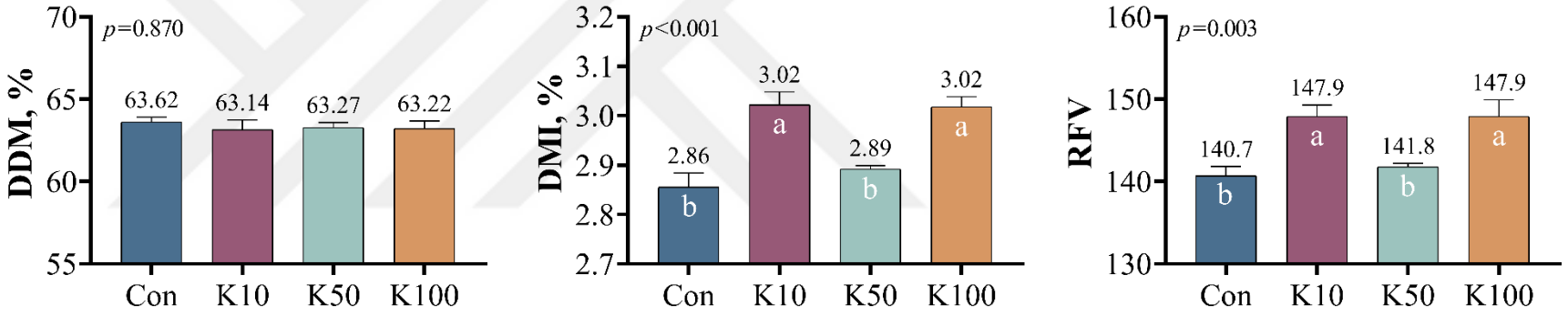
Table 4. 6. Relative feed value of white clover silages inoculated with kefir yeast.

Item	Con	K10	K50	K100	SEM	*P*-value
DDM, %	63.62	63.14	63.27	63.22	0.43	0.870
DMI, %	2.86^{b}	3.02^{a}	2.89^{b}	3.02^{a}	0.02	<0.001
RFV	140.7^{b}	147.9^{a}	141.8^{b}	147.9^{a}	1.35	0.003

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, DDM: Digestible dry matter, DMI: Dry matter intake, RFV: Relative feed value. The values with different letters (a, b)in the same row are statistically different (P<0.05).

In the present study, inoculation of kefir yeast improved the DMI (P<0.001) and RFV (P<0.01) of TR silages after 270 d of ensiling. The DMI and RFV of the K10 and K100 groups were 5.59% and 4.34% higher than the Con group, whereas only numerically higher values were obtained in the K50 group according to the Con group (Table 4.6 and Figure 4.6). This result may be explained by the fact that higher NDF values which observed in the K50 group compared to K10 and K100 groups. This is consistent with the data obtained by Li, Zhou, Zi, and Cai (2017), who reported that LAB and cellulase-treated silages had higher RFV than the control silages in a study investigating the effect of LAB and cellulase enzymes on stylo (*Stylosanthes guianensis*) silages. In that study, the authors also concluded that LAB and cellulose, either used solely or combined, improved the RFV and nutritional quality of stylo silages. In accordance with the present results, similar findings have also been reported by Djordjevic, Mandic, and Stanojevic (2016), who have argued that treatment of first-cut alfalfa silages with Silko (a mixture of homofermentative *L. plantarum* strains which commercially available in Croatia) had higher RFV than untreated alfalfa silages.

However, this system also has a number of serious shortcomings. Firstly, it assumes all forages' DDM and DMI have constants. Secondly, it uses only NDF and ADF values obtained in the laboratory. Thirdly, the CP content of forages is ignored. Fourthly, RFV is not able to use in ration formulation or assessment (Jeranyama and Garcia, 2004). These shortcomings mean that study findings need to be interpreted cautiously. In the same vein, Hackmann et al. (2008) concluded that RFV does not explicitly reflect plant-related factors on DMI and DDM due to using only NDF and ADF values. Previous research has indicated that numerous factors could be changed DMI and DDM independently of NDF and ADF content of forages, such as forage species, application of fertilization, harvesting date, harvesting methods, maturity, disease, conservation, and processing methods (Van Soest, 1994; Hackmann et al., 2008; Filik and Filik, 2021).



Con: Control, **K10**: 10 mg/kg kefir yeast, **K50**: 50 mg/kg kefir yeast, **K100**: 100 mg/kg kefir yeast, **DDM**: Digestible dry matter, **DMI**: Dry matter intake, **RFV**: Relative feed value.

Figure 4. 6. Relative feed value of white clover silages inoculated with kefir yeast.

The values with different letters (a, b) in each graph is statistically different (P<0.05).

4.7 Microbiological Compositions of Kefir Treated White Clover Silages

Most researchers investigating the microbial biome of various silages concluded that fermentation significantly changed the microbial community and abundances (Duniere et al., 2017; Zhou, Chen, Guo, Shen, and Yang, 2019; Guan et al., 2020). This view is supported by Ni et al. (2018), who writes that silage quality is significantly affected by the variation of the microbial community. For example, it has been established that silage fermentation is generally initiated by *Weissella*, *Pediococcus*, and *Lactococcus*, whereas more acid-tolerant *Lactobacilli* become dominant with reduced pH (Cai et al. 1998). Similarly, in a recent study, Zhou et al. (2019) demonstrated that *Enterobacteriaceae* and *Trichocomaceae* were the abundant genera in the early stage of fermentation in a mixture of citrus waste, wheat bran, and wheat leaves silage. In contrast, they were replaced with *Lactobacillus*, *Pediococcus*, *Sphingobiu*m, *and Saccharomycetaceae* for further ensiling stages.

As previously discussed by Auerbach and Nadeau (2020), all metabolic pathways, which increase the nutrient and energy losses during storage, should be suppressed to maintain the silage quality at a high level up to silo opening. The authors also highlighted that up to 20% of additional DM and energy losses would occur if the yeast and mold activity were not suppressed up to silo opening. Additionally, Tian et al. (2020) have established that yeast and mold counts are significantly affected by the compaction of silage material, additive types, and interaction between them.

As expected, in the present study, both LAB and yeast count increased compared to the Con group ($P<0.001$; Table 4.7 and Figure 4.7). These results mirror those of the previous studies that have examined the effects of different kefir sources on fermentation characteristics, aerobic stability, and microbial communities of alfalfa silages (Koç et al., 2021). The obtained results also agree with Okuyucu and Esen's (2022) findings which showed that kefir could be used effectively to improve wheat straw silages. Furthermore, no molds were detected in all silages. A possible explanation for this might be higher LAB counts, which inhibit molds. This finding was also reported by Filya, Muck, and Contreras-Govea (2007). Another possible explanation for this is that a higher AA concentration could avoid the mold count of kefir-treated silages. These findings are in agreement with those obtained by Muck (2010).

Table 4. 7. Microbiological compositions of white clover silages inoculated with kefir yeast.

Item	Con	K10	K50	K100	SEM	*P*-value
LAB, log cfu/g FM	5.84^{d}	6.50^{b}	6.42^{c}	6.59^{a}	0.014	<0.001
Yeast, log cfu/g FM	6.13^{c}	6.47^{a}	6.45^{a}	6.30^{b}	0.012	<0.001
Mold, log cfu/g FM	ND	ND	ND	ND	-	-
***Enterobacteriaceae*, log cfu/g FM**	5.95^{a}	5.36^{b}	4.19^{c}	3.17^{d}	0.008	<0.001

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, LAB: Lactic acid bacteria, cfu: colony-forming unit: FM: Fresh material, ND: Not detected. The values with different letters (a, b, c, d)in the same row are statistically different (P<0.05).

Previous research has established that *Enterobacteriaceae* was one of the two most abundant families with *Lactobacillaceae* in all silages (Mu, Xie, Hu, Chen, and Zhang, 2020). Henderson (1993) noted that *Enterobacte*r, *Escherichia*, *Salmonella*, *Erwinia*, *Klebsiella*, and *Proteus* are the most isolated genera associated with silage. Most of the isolated *Enterobacteriaceae* in silage is evaluated as nonpathogenic; however, their development in silage is unwanted due to competing with LAB for carbohydrate sources (McDonald et al., 1991). Queiroz et al. (2018) reported that one possible cause of the slow reduction of pH was the proliferation of *Enterobacteriaceae*. Additionally, Ali et al. (2020) concluded that AA-producing *Enterobacteriaceae* or heterofermentative LAB plays a significant role in reducing LA/AA ratio in silage.

According to some researchers, the NH_3-N content of silage could be used as an indicator of *Enterobacteriaceae* inhibition (McDonald et al., 1991; Oladosu et al., 2016; da Silva et al., 2021). In the current study, the findings clearly indicate that an increased application rate of kefir yeast decreased *Enterobacteriaceae* count (P<0.001), and a 50 mg/kg FM or higher application rate of kefir yeast reduced the deamination of TR silages (P<0.001; Table 4.3 and Figure 4.3). In accordance with the present results, previous studies have demonstrated that an increased abundance of LAB could reduce *Enterobacteriaceae* count in silage (Mu et al., 2021). On the other hand, this outcome contradicts Curtis (1996), who highlighted that *Enterobacteriaceae* activity is inhibited when the silage pH falls lower than 5.0. This contradiction could be attributed to the presence of acid-tolerant epiphytic *Enterobacteriaceae* found in ensiling material. A similar point has already been made by Bearson, Bearson, and Foster (1997).

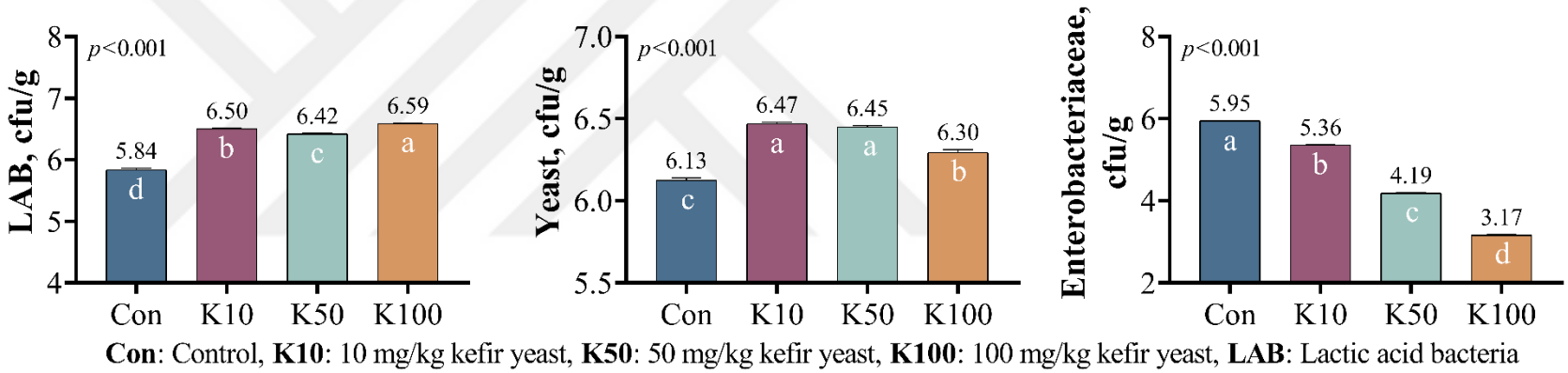


Figure 4. 7. Microbiological compositions of white clover silages inoculated with kefir yeast*.

**No molds detected. The values with different letters (a, b, c, d) in each graph is statistically different (P<0.05).*

4.8 Total Phenolics, Tannin, and Condensed Tannin Contents of Kefir Treated White Clover Silages

Legume forages, such as alfalfa and clover, are commonly placed into ruminant diets to meet their high protein demands. Preserving these forage crops to optimize their use in ruminant nutrition is crucial because two-thirds of the annual production of these forage crops are obtained at the first spring cut, and only about one-fifth of their CP escapes the rumen intact in some of them (Coblentz and Grabber, 2013; Rufino-Moya, Blanco, Bertolín, and Joy, 2019). Several researchers have noted that these legume proteins might be degraded via proteolysis during ensiling or ruminal fermentation after they are ingested (Rooke and Hatfield, 2003; Tabacco et al., 2006; Coblentz and Grabber, 2013).

Tannins, on the other hand, are one of the most frequently used responses to overcome these problems in recent years (He, Lv, Xing, Chen, and Zhang, 2020; Chen et al., 2021). They are a diverse range of secondary plant metabolites which generally classified into two types: hydrolyzable tannins, which are hydrosoluble in the digestive tract, and condensed tannins, which are able to form a complex with dietary protein and digestive enzymes (Wang, 2012; Zhang, Cheng, Elsabagh, Lin, and Wang, 2021).

Table 4. 8. Total phenolics, tannin, and condensed tannin contents of white clover silages inoculated with kefir yeast.

Item	Con	K10	K50	K100	SEM	*P*-value
TP, g/kg DM	11.80	11.65	13.03	11.48	0.63	0.330
T, g/kg DM	2.00	2.83	4.28	3.55	0.68	0.160
CT, g/kg DM	6.40	5.89	6.07	6.83	0.40	0.390

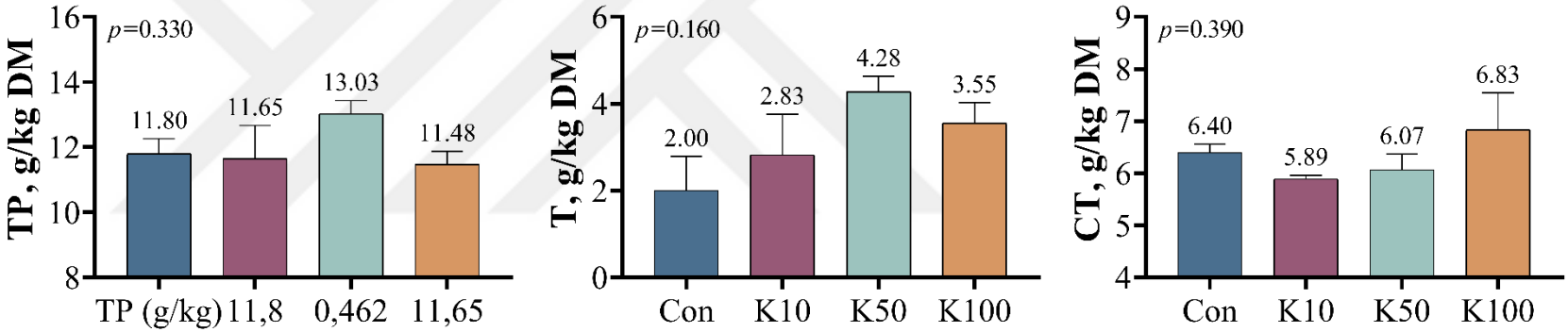
Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, TP: Total phenolics, T, Tannin, CT: Condensed tannin.

In the literature, it is widely acknowledged that tannins protect proteins from enzyme hydrolysis by binding to the proteins or free amino acids reversibly, deactivating enzymes, or inhibiting microorganisms (Salawu, Acamovic, Stewart, Hvelplund, and Weisbjerg, 1999; Chai et al., 2018; He et al., 2020). It has been demonstrated that plant proteases catalyze the hydrolysis of peptide bonds constituting free amino acids and peptides, which further deaminated to form NH_3-N and amines as a consequence of microbial activities (Kung Jr et al., 2018; He et al., 2020). As is well known from the previous studies, high content of

nonprotein-N in silage is undesirable, causes nutritional loss of animal feed, and increases nitrogen emission in animal production (McDonald et al., 1991; Li et al., 2018; He et al., 2020). Furthermore, Bunglavan and Dutta (2013) established that its higher binding capacity makes tannins a potential protein protector from degradation during ensiling. A strong negative correlation has also been reported by several researchers between tannin and soluble nitrogen concentration in legume silages (Albrecht and Muck, 1991; Salawu et al., 1999).

Similarly, well-designed and robust studies have well documented that tannins inhibit methane production via modifying rumen fermentation, deactivating digestive enzymes, or having a direct inhibitory effect on methanogens and/or protozoa (Patra and Saxena, 2010; Wang, 2012). For example, Min et al. (2020) argued that the effect of tannins on rumen microbes, ruminal protein degradability, and methanogenesis could be attributed partially to the antimicrobial properties of the tannins, which reduce fiber digestion and result in incomplete digestion of the feed by the ruminal microbial population. In the same vein, McAllister, Bae, Jones, and Cheng (1994) noted that tannins act as a protective barrier to prevent the attachment of microorganisms to the cell wall of plants, which is essential for degradation. However, all these effects depend on the source of tannins and concentration (Hagerman and Butler, 1991). Contrary to its beneficial effect of reducing proteolysis during ensiling and inhibitory effect on methane formation, higher levels than 50 g/kg of DM tannins are generally considered antinutritional factors for ruminants (Makkar, 2003; Theodoridou et al., 2012).

As Table 4.8 and Figure 4.8 show, there is no significant difference between groups in terms of total phenolic, tannin, and condensed tannin concentration of TR silages ($P>0.05$). A possible explanation for this might be that the reduced pH value (<4.2) could be deactivated the plant peptidase activities in all TR silages. These results further support the idea that plant peptidase activities could be restricted with a rapid reducing pH because the optimum pH for their activities is between 5 to 7 (Salawu et al., 1999). Another possible explanation for this might be that tannins could deactivate plant proteases by binding them, contributing to reducing protein degradation in the silo. These results are consistent with data obtained by Chai et al. (2018) and Chen et al. (2019).



Con: Control, **K10**: 10 mg/kg kefir yeast, **K50**: 50 mg/kg kefir yeast, **K100**: 100 mg/kg kefir yeast, **TP**: Total phenolics, **T**: Tannin, **CT**: Condensed tannin.

Figure 4. 8. Total phenolics, tannin, and condensed tannin contents of white clover silages inoculated with kefir yeast.

4.9 Aerobic Stability of Kefir Treated White Clover Silages

Aerobic stability is generally assessed by analyzing the chemical (pH, CO_2) and microbiological (LAB, yeast, and mold count) changes after 5 to 7 days of aerobic exposure (Ashbell et al., 1991) or recording temperature by data loggers set 5 min to 1 h interval (da Silva et al., 2019; Carvalho et al., 2021). In those studies in which a data logger was used, aerobic stability was broadly defined as the duration of time in h before the silage temperature raised 2 °C above the baseline temperature (Kung Jr et al., 2003; Barmaki, Alamouti, Khadem, and Afzalzadeh, 2018). Although recording temperature with the data logger is simple, this method can not give any information about the silage's chemical and microbial composition. Therefore, the current study used the bottle system to obtain robust information about the silage's chemical and microbiological composition after 5 d of aerobic exposure. As has already been noted, changes in pH, production of CO_2, and yeast and mold counts were used as spoilage indicators in the current study (Ashbell et al., 1991; Weinberg, Khanal, Yildiz, Chen, and Arieli, 2010).

There is a large number of published studies that describe the primary initiator of aerobic spoilage during the feed-out phase as yeasts (e.g., Santos et al., 2015; Duniere et al., 2017; Kung Jr et al., 2018). Some of this research has also highlighted that aerobic deterioration of feed not only reduces the nutritional quality but also presents hazards to the environment when disposing of spoiled feed. Therefore, any improvements in the aerobic stability of silage could offer a great advantage for producers (Ranjit and Kung Jr, 2000). Thus far, several studies have attempted to improve silage's aerobic stability by using different additives, including acid-based additives, nutrients, and inoculants (Kung Jr and Ranjit, 2001; Bernardes et al., 2018; Kung Jr et al., 2018).

After 5 d of aerobic stability test, increasing pH, yeast counts and CO_2 production in all TR silages indicate that aerobic spoilage has already begun. However, in the current study, there was a tendency for an increased reduction of pH ($P<0.05$) and yeast count ($P<0.001$) with an increased inoculation rate of kefir yeast after 5 d of aerobic exposure (Table 4.9 and Figure 4.9). Similarly, a negative correlation was observed between total organic acid concentration and CO_2 production. In addition, no mold was detected in all TR silages. Considering all of this evidence, it seems that these results are likely related to total organic acid concentration with an increased AA proportion of TR silages treated with kefir yeast ($P<0.001$; Table 4.4 and Figure 4.4). In accordance with the present results, previous studies

have demonstrated that the increase in AA leads to improved aerobic stability of silage (Muck et al., 2018; Carvalho et al., 2021). A comparison of the findings with those of other studies also confirms that kefir yeast improves the aerobic stability of silage (Koç et al., 2021; Okuyucu and Esen, 2022). Furthermore, no significant differences were observed in kefir yeast treated silage compared to the Con group in terms of DM content (P>0.05). This is consistent with the data obtained by Koç et al. (2021), who make a similar point in their study of alfalfa silage treated with different kefir sources after 7 d of aerobic exposure.

Table 4. 9. Microbiological compositions of white clover silages after 5 d of aerobic exposure.

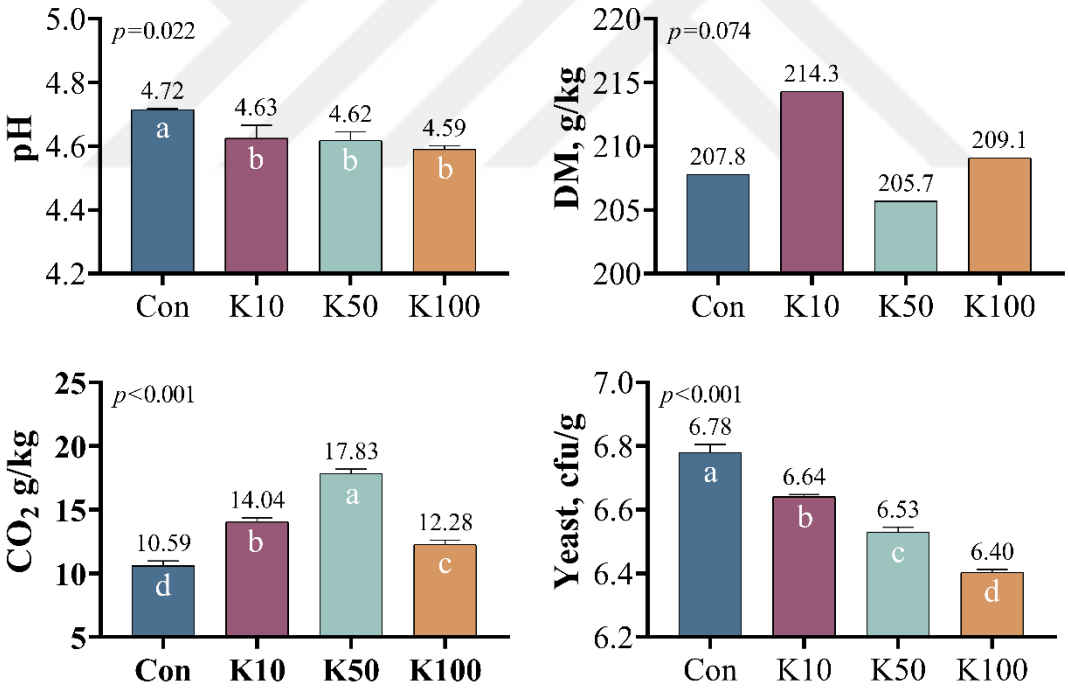
Item	**Con**	**K10**	**K50**	**K100**	**SEM**	***P*-value**
DM, g/kg	207.8	214.3	205.7	209.1	2.13	0.074
pH	4.72^{a}	4.63^{ab}	4.62^{ab}	4.59^{b}	0.03	0.022
Yeast, log cfu/g FM	6.78^{a}	6.64^{b}	6.53^{c}	6.40^{d}	0.02	<0.001
Mold, log cfu/g FM	ND	ND	ND	ND	-	-
CO_2, g/kg DM	10.59^{d}	14.04^{b}	17.83^{a}	12.28^{c}	0.35	<0.001

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, CO_2: Carbon dioxide, cfu: colony-forming unit: FM: Fresh material, ND: Not detected. The values with different letters (a, b, c, d)in the same row are statistically different (P<0.05).

The existing body of research on the aerobic stability of the silage suggests that the selection process of strains plays an important role in the feed-out phase when inoculants are used as silage additives. For example, obligately heterofermentative LAB are chiefly used to enhance the aerobic stability of silage due to the production ability of AA and PA, whereas homofermentative LAB are used to improve fermentation quality (Muck et al., 2018; Carvalho et al., 2021). On the one hand, different heterofermentative LAB strains exist, producing AA and 1,2-propanediol and improving aerobic stability, such as *L. buchneri, L. diolivorans*, and *L. reuteri* (Krooneman et al., 2002; Sriramulu et al., 2008). On the other hand, *L. buchneri* is among the most widely used heterofermentative LAB strains to improve aerobic stability; several researchers have suggested that a combination of different species or strains promotes simultaneous improving fermentation and aerobic stability of silage (Krooneman et al., 2002; da Silva et al., 2019; Carvalho et al., 2021). In addition, Duniere et al. (2017) concluded that different factors play a crucial role in selecting inoculants, such as

improving the fermentation quality during ensiling, increasing aerobic stability, and enhancing silage digestibility.

It is well known from previous studies that DM losses during ensiling are higher when heterolactic fermentation becomes dominant (McDonald et al., 1991; Bernardes et al., 2018). However, most of these studies agree that small losses of DM conceived during the initial fermentation stages are less important (Ranjit and Kung Jr, 2000; Kung Jr and Ranjit, 2001; Carvalho et al., 2021). Furthermore, the relationships between aerobic stability and temperature have been explored by several authors (Koc, Coskuntuna, Ozduven, Coskuntuna, and Samli, 2009; Bernardes et al., 2018). These authors concluded that CO_2 production and yeast and mold counts increased at higher temperatures. Additionally, it has been highlighted by several researchers that lower yeast and mold counts and higher aerobic stability were observed with an increased ensiling period regardless of the using inoculants or additives (Comino et al., 2014; Ferrero, Piano, Tabacco, and Borreani, 2019).



Con: Control, **K10**: 10 mg/kg kefir yeast, **K50**: 50 mg/kg kefir yeast, **K100**: 100 mg/kg kefir yeast, **DM**: Dry matter, **CO_2**: Carbon dioxide.

Figure 4. 9. Aerobic stability of white clover silages after 5 d of aerobic exposure*.

**No molds detected. The values with different letters (a, b, c, d) in each graph is statistically different (P<0.05).*

4.10 *In Vitro* Gas Production Kinetics, Metabolizable Energy, Net Energy Lactation, and Organic Matter Digestibility of Kefir Treated White Clover Silages

It has been proved that fermentation of fibrous and non-fibrous carbohydrates plays a crucial role in the feed utilization process of ruminants (Pitt, Cross, Pell, Schofield, and Doane, 1999). During this process, the amount of received metabolic protein by an animal is equal to the sum of the undegraded rumen escaped protein and the microbial protein produced from ruminal fermentation (Zhao, Li, Ren, Li, and Guo, 2005; Westreicher-Kristen, Blank, Schulz, and Susenbeth, 2017). Some characteristics can indicate the nutritional value of the feed, such as ruminal and intestinal degradability, degradation and passage rate, which are the primary factors that affect the extent of carbohydrate digestion in the rumen (Tafaj, Zebeli, Junck, Steingass, and Drochner, 2005).

Yet, numerous studies have attempted to determine the degradation characteristics of feedstuffs in rumen fluid using *in situ*, *in vitro*, and *gas production* techniques (such as Liu, Lai, Lu, Guo, and Luo, 2016; Costa et al., 2020; Eseceli et al., 2020; Ayasan et al., 2021; Esen et al., 2022; Olomonchi, Garipoğlu, Ocak, and Kamalak, 2022). However, many scholars believe that *in vitro* techniques are the most cost-effective and relevant tools to evaluate the fermentation characteristics of feedstuffs. A high correlation between *in vitro* and *in situ* techniques indicates that this technique is a suitable replacement for *in situ* method (Taghizadeh, Safamehr, Palangi, and Mehmannavaz, 2008). These techniques also give valuable information to explain and support the results of *in vivo* techniques, which are difficult to implement and require a high number of animals, laboriousness, and financial costs (Rodrigues, Cone, Ferreira, Blok, and Guedes, 2009; Elghandour et al., 2017; Westreicher-Kristen et al., 2017).

Previous research has established that the correlation between the cumulative GP and nutritional content of feeds could effectively be used to predict the OMD and ME content of feeds (Menke and Steingass, 1988; Li, Zi, Zhou, Hou, and Cai, 2014). It has also been indicated that GP is positively correlated with VFA produced during microbial fermentation in the rumen (Blümmel et al., 1997). Likewise, Palangi (2021) highlighted that *in vitro* GP technique provides valuable information to estimate the required substrate for producing VFA and energetic values of feedstuffs. The Hohenheim gas technique, on the other hand, is one of the most common *in vitro* techniques for simulating rumen fermentation. In this technique,

the volume of gas produced reflects the fermentation profile of feed in the rumen and allows for estimating rumen fermentation kinetics (Menke and Steingass, 1988).

Table 4. 10. *In vitro* gas production kinetics, metabolizable energy, net energy lactation, and organic matter digestibility of white clover silages inoculated with kefir yeast.

Item	Con	K10	K50	K100	SEM	*P*-value
3 h, ml	18.13	17.86	18.04	18.71	0.73	0.856
6 h, ml	30.76	29.70	31.04	32.42	0.78	0.158
12 h, ml	39.78	38.71	39.62	41.21	0.73	0.166
24 h, ml	42.96	43.43	42.06	45.70	1.16	0.202
48 h, ml	45.28^{b}	46.53^{ab}	45.18^{b}	50.09^{a}	1.18	0.040
72 h, ml	46.57^{b}	47.81^{ab}	46.74^{b}	52.68^{a}	1.41	0.031
96 h, ml	46.83	47.04	46.74	52.42	1.66	0.084
a, ml	0.61	2.63	1.07	5.66	1.92	0.289
b, ml	45.05	44.04	44.40	45.49	0.92	0.693
a+b, ml	45.66	46.67	45.47	51.15	1.44	0.054
c	0.173	0.152	0.172	0.129	0.013	0.101
CH_4, %	16.73	16.57	16.48	16.74	0.16	0.609
ME, MJ/kg DM	9.50	9.56	9.40	9.75	0.16	0.500
NEL, MJ/kg DM	5.33	5.37	5.31	5.60	0.14	0.283
OMD, % DM	65.17	65.62	64.57	66.68	1.08	0.579

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, a: gas production volüme of readily soluble fraction, b: gas production volume of insoluble fraction, c: gas production rate constant for the insoluble fraction, a + b: potential gas production volüme, CH_4: Methane, ME: Metabolizable energy, NEL: Net energy lactation OMD: Organic matter digestibility. The values with different letters (a, b, c, d)in the same row are statistically different (P<0.05).

Evidence suggests that microbial inoculants, particularly LAB, which survived during fermentation and entered the rumen alive, could improve animal performance (Weinberg, Muck, and Weimer, 2003; Weinberg, Chen, and Gamburg, 2004). Along the same lines, it has been subsequently argued that silage inoculants often imparted their antibacterial activity to

inoculated silages (Gollop et al., 2005; Contreras-Govea, Muck, Mertens, and Weimer, 2011). However, in the current study, inoculation of kefir yeast did not significantly affect the cumulative GP and estimated parameters except for 48 and 72 h (P>0.05; Table 4.10). These results agree with some researchers' reports that LAB inoculation did not always significantly affect the *in vitro* gas production profile and degradability of silage (Contreras- Govea et al., 2011; Guo et al., 2019).

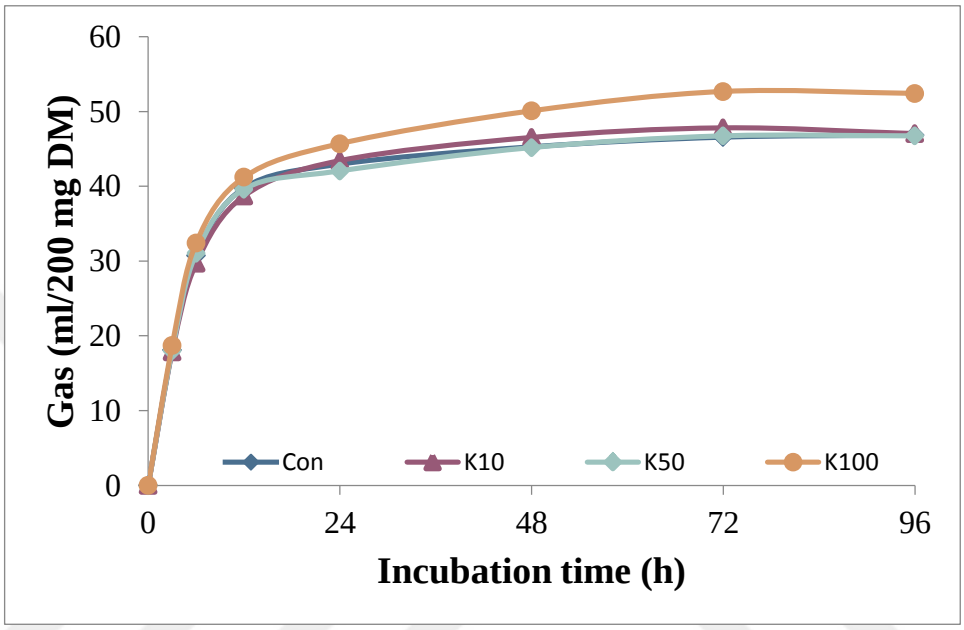


Figure 4. 10**.** Fitted curves of cumulative gas production of white clover silages inoculated with kefir yeast.

The fitted GP curves of kefir yeast treated TR silages are also presented in Figure 4.10. The highest GP values were observed in the K100 group at all incubation times. These results might be attributed to this group's numerically higher WSC content than others. The cumulative GP volumes of kefir yeast treated TR silages varied between 46.74-52.42 ml/200mg DM after the 96 h incubation period. Moreover, none of the CH_4, ME, NEL, and OMD were significantly affected by the inoculation of kefir yeast (P>0.05). Contrary to expectation, the reduced NDF content of TR silages did not alter the cumulative GP values and CH_4 ratios in the K10 and K100 groups (P>0.05). A possible explanation for this might be the higher AA:PA ratio of these groups. Similar findings were also reported by Navarro-Villa, O'Brien, López, Boland, and O'Kiely (2013) and Guo et al. (2019), who revealed the importance of hydrogen in CH_4 formation.

4.11 Macro- and Micronutrients of Kefir Treated White Clover Silages

A greater part of the literature on forages focuses on changes in major nutrient concentrations, e.g., protein and fiber, from the initial field harvesting to the final feed-out stages, including managemental practices, mechanical harvesting treatments, and preservation methods (Schlegel, Wyss, Arrigo, and Hess, 2018). Although the vast majority of studies have not practiced mineral analysis, this is recommended to evaluate how much of a mineral requirement of livestock is met. Some studies have postulated a decrease in Ca, P, and K concentration of forages when preserved as hay compared to fresh material (Meschy, Baumont, Dulphy, and Nozieres, 2005; Arrigo, 2007). A similar reduction in P has also been reported when forages are preserved as silage (Meschy et al., 2005; Arrigo, 2007). In contrast, an increase in Ca, Cl, and Mg concentration has been reported when forages are preserved as silage (Meschy et al., 2005; Arrigo, 2007). Likewise, Schlegel et al. (2018) reported an increase in P, Mg, Na, Fe, Mn, Zn, Co, and Se concentrations in grass-clover silage (*Lolium perenne*, *Poa pratensis*, *Dactylis glomerata*, *Phleum pratense*, *Festuca rubra*, *Trifolium repens*, and *Trifolium pratense*) compared to fresh form. The authors also noted that Ca, Mg, and Zn concentrations in this silage form of grass-clover were significantly higher than in its hay form.

Previous studies established that supplying the optimal macro- and micronutrients in the silo is crucial for the desired fermentation process (Vintiloiu, Lemmer, Oechsner, and Jungbluth, 2012; Romero-Güiza, Vila, Mata-Alvarez, Chimenos, and Astals, 2016). It has been argued that macronutrients act as a buffering agent in the silo, whereas micronutrients play a crucial role as a cofactor in enzymatic reactions (Romero-Güiza et al., 2016). For example, iron, cobalt, and nickel are essential in enzyme systems to enhance the degradation of the substrate in the silo (Wahid et al., 2018). It has also been demonstrated that iron, cobalt, nickel, copper, and zinc play a pivotal role in methanogens in the anaerobic fermentation process (Demirel and Scherer, 2011; Wahid et al., 2018). Cobalt is necessary to synthesize corrinoid proteins and plays a significant role in the vitamin B_{12} enzyme (Vintiloiu et al., 2012).

On the other hand, molybdenum is required to oxidize and reduce CO_2 and has a key function in forming enzymes and synthesizing various anaerobic microorganisms (Jarrell and Kalmokoff, 1988). Iron is used as a bonding element in sulfide precipitation and prevents sulfide toxicity in the silo, whereas selenium stimulates numerous methanogens and plays a

significant role in the formation of selenocysteine or selenomethionine proteins (Vintiloiu et al., 2012). Nickel is involved in several enzymes and enhances the growth of methanogens during the anaerobic fermentation process (Wahid et al., 2018). Copper is a metallic enzyme activator that plays a key role in superoxide dismutase and hydrogenase; and is able to inhibit metabolism (Schattauer, Abdoun, Weiland, Plöchl, and Heiermann, 2011).

Table 4. 11. Macro- and micronutrients of kefir treated white clover silages.

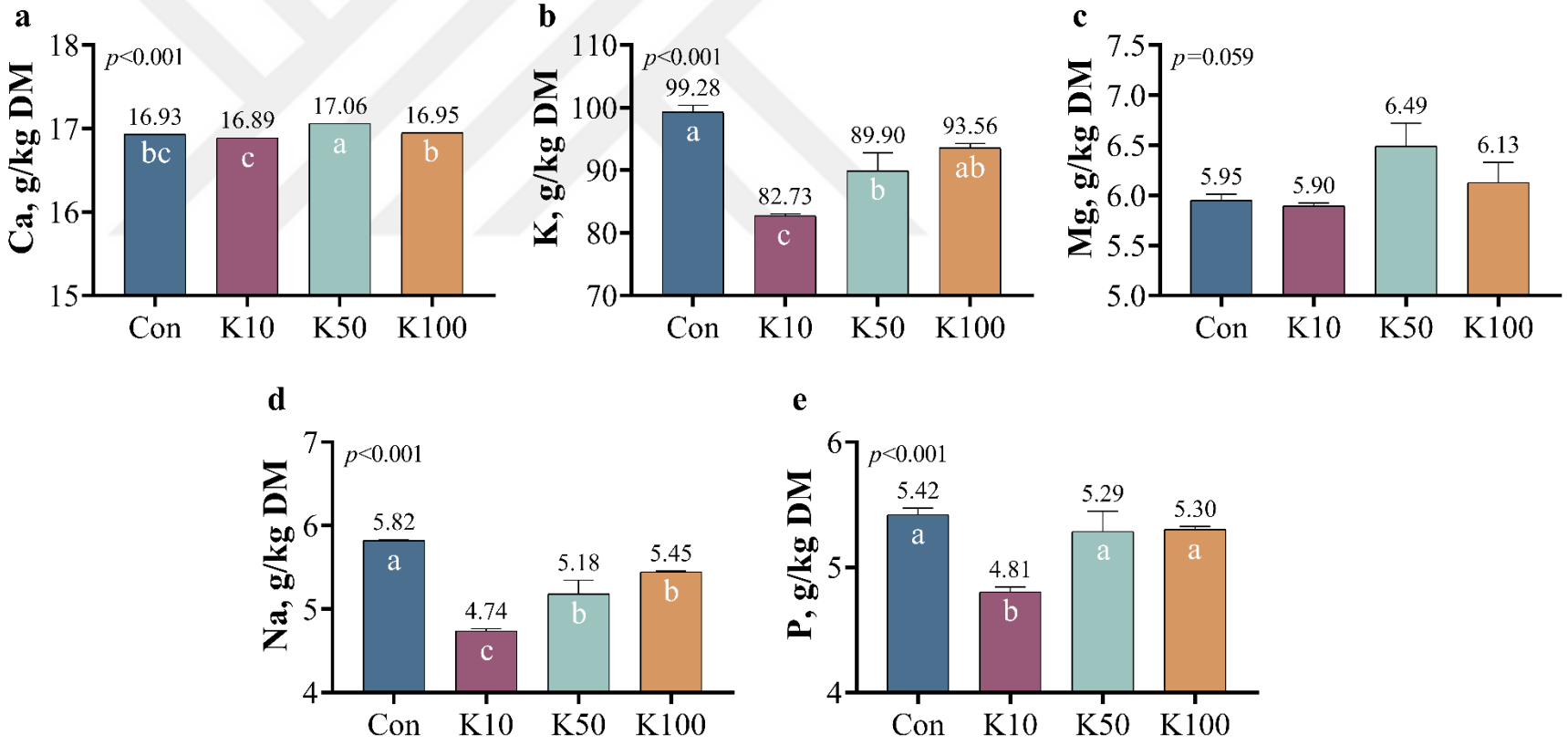
Item	Con	K10	K50	K100	SEM	*P*-value
Macronutrients						
Ca, g/kg DM	16.93^{bc}	16.89^{c}	17.06^{a}	16.95^{b}	0.01	<0.001
K, g/kg DM	99.28^{a}	82.73^{c}	89.90^{b}	93.56^{ab}	1.60	<0.001
Mg, g/kg DM	5.95	5.90	6.49	6.13	0.16	0.059
Na, g/kg DM	5.82^{a}	4.74^{c}	5.18^{b}	5.45^{b}	0.08	<0.001
P, g/kg DM	5.42^{a}	4.81^{b}	5.29^{a}	5.30^{a}	0.09	<0.001
Micronutrients						
B, mg/kg DM	50.18^{a}	43.61^{b}	51.35^{a}	48.29^{ab}	1.60	0.014
Cu, mg/kg DM	20.05	19.20	19.04	19.97	0.32	0.079
Fe, g/kg DM	0.49^{ab}	0.44^{b}	0.51^{a}	0.51^{a}	0.02	0.019
Se, mg/kg DM	14.39^{a}	6.96^{c}	7.93^{c}	11.51^{b}	0.67	<0.001
Zn, mg/kg DM	55.46^{b}	43.42^{c}	44.93^{c}	73.29^{a}	2.02	<0.001

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, SEM: standard error of mean, Ca: Calcium, K: Potassium, Mg: Magnesium, Na: Sodium, P: Phosphorus, B: Boron, Cu: Copper, Fe: Iron, Se: Selenium, Zn: Zinc. The values with different letters (a, b, c, d) in the same row are statistically different (P<0.05).

Furthermore, previous research highlighted the importance of manganese in several enzymatic functions of *L. plantarum*, such as lactate dehydrogenase, manganisuperoxide dismutase, xylose isomerase, manganicatalase, RNA, polymerase, malolactic enzyme (Haag, Steinbrenner, Demmig, Nägele, and Oechsner, 2016). Pobeheim, Munk, Lindorfer, and Guebitz (2011) argued that inadequate nickel and cobalt concentration in the silo limits AA and PA metabolism. Furthermore, Zhang, Cheng, Huang, and Jin (2022) noted that zinc is essential for enzyme and protein structure.

In the current study, inoculation of kefir yeast reduced the potassium, sodium, selenium ($P<0.001$), and boron ($P<0.05$) concentrations, whereas calcium, zinc ($P<0.001$), and iron ($P<0.05$) concentrations of TR silages increased (Table 4.11, Figures 4.11 and 4.12). There was no evidence that inoculation of kefir yeast has an influence on magnesium and copper ($P>0.05$) concentration of TR silages. These results are in line with those of previous studies (Schattauer et al., 2011; Schlegel et al., 2018). Although inoculation of kefir yeast into TR silages reduced selenium concentration, with an increasing application rate of kefir yeast, selenium concentrations tended to increase. In accordance with the present results, previous studies have demonstrated that the addition of sodium selenite into the growth medium of LAB reduced growth rates (Lee, Fleming, Cogan, Hodgson, and Davies, 2019).

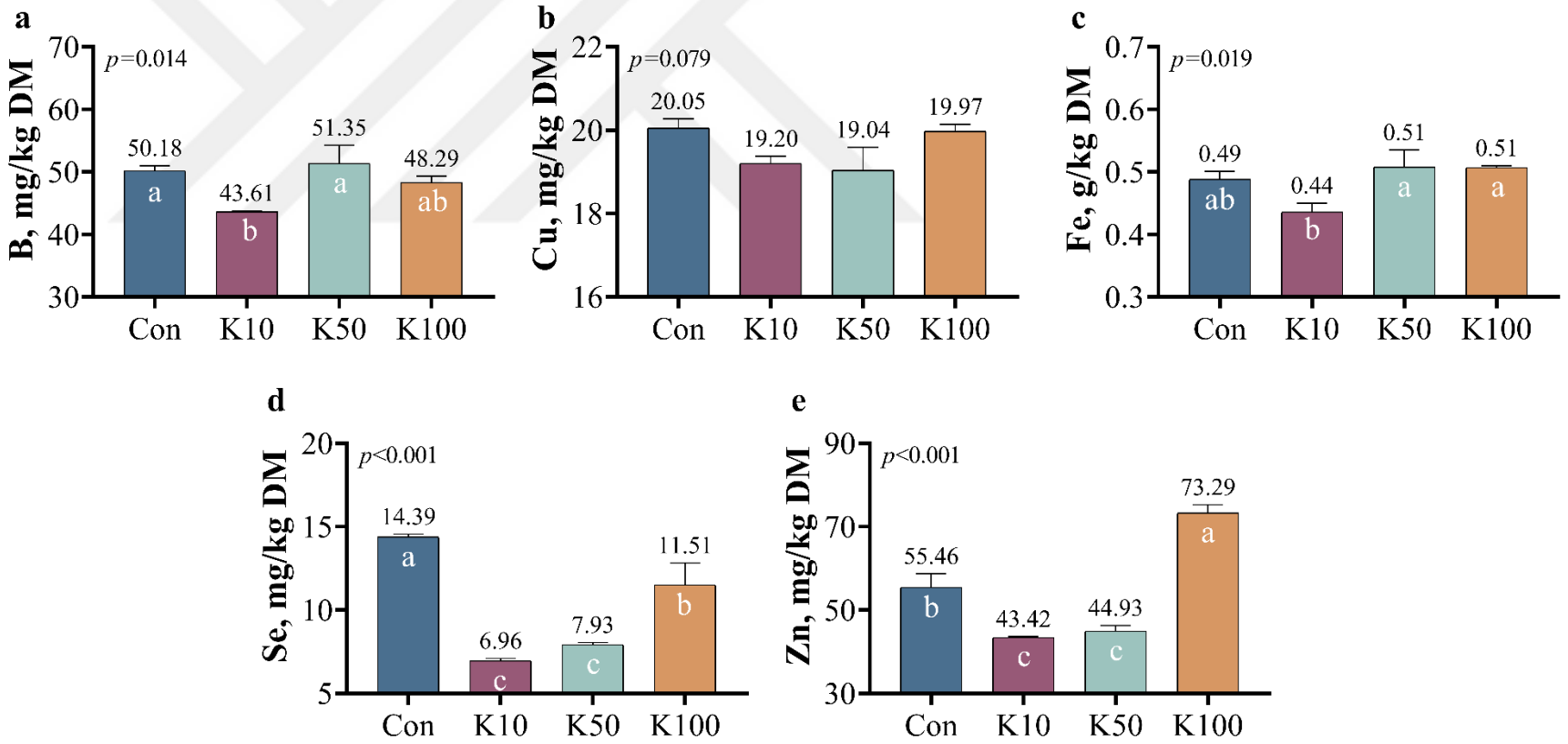
On the other hand, the concentration of potassium (10 g/kg DM), magnesium (1.9 g/kg DM), sodium (0.6-0.8 g/kg DM), copper (9-11 mg/kg DM), and zinc (43-60 mg/kg DM) concentration of TR silages meets the daily requirements of dairy cattle in all treated groups (NRC, 2001). In contrast, the calcium (53-67 g/kg DM) and phosphorous (32-44 g/kg DM) concentration of TR silages did not meet the daily requirements of dairy cattle in all treated groups (NRC, 2001).



Con: Control, **K10**: 10 mg/kg kefir yeast, **K50**: 50 mg/kg kefir yeast, **K100**: 100 mg/kg kefir yeast, **DM:** Dry matter, **Ca**: Calcium, **K**: Potassium, **Mg**: Magnesium, **Na**: Sodium, **P**: Phosphorus.

Figure 4. 11. Macronutrients of kefir treated white clover silages.

The values with different letters (a, b, c, d) in each graph is statistically different (P<0.05).



Con: Control, **K10**: 10 mg/kg kefir yeast, **K50**: 50 mg/kg kefir yeast, **K100**: 100 mg/kg kefir yeast, **DM:** Dry matter, **B**: Boron, **Cu**: Copper, **Fe**: Iron, **Se**: Selenium, **Zn**: Zinc.

Figure 4. 12. Micronutrients of kefir treated white clover silages.

The values with different letters (a, b, c, d) in each graph is statistically different (P<0.05).

4.12 Fatty Acid Composition of Kefir Treated White Clover Silages

The existing literature on the fatty acid (FA) composition of silages focuses on increasing linoleic and linolenic acid in animal diets and aims to transfer them into milk and meat due to their beneficial effect on human nutrition. It is believed that a diet containing any long-chain FA supplement increases the linoleic and linolenic acid and decreases the short- and medium-chain FA concentration in milk. In contrast, a diet rich in palmitic and palmitoleic acids increases the total FA in milk greater than linoleic and linolenic acids (Palmquist and Jenkins, 2017; Agarussi et al., 2020).

In addition, it is well known from previous studies that the proportion of n-3 PUFA affecting milk fat in clovers is significantly higher than in grasses. Research showed that TR's DMI and ruminal passage rates are higher than those of grass when fed to cattle, thus resulting in a significant change in FA composition (Dewhurst et al., 2003; van Dorland et al., 2008). Besides, several researchers noted that reduced lipolysis in clovers due to increased polyphenol oxidase activities results in an increased escaping rate of biohydrogenation and enhances the n-3 PUFA ratio in milk fat (Lee, Harris, Dewhurst, Merry, and Scollan, 2003; Lee et al., 2004). During the ensiling process, oxidation and biohydrogenation of unsaturated fatty acids (UNSFA) cause significant changes in the FA composition (Łozicki et al., 2015; Koczoń et al., 2019). For example, Alves, Cabrita, Jerónimo, Bessa, and Fonseca (2011) reported a decrease in α-linolenic and linoleic acid ratios in maize silage compared to fresh form. Similarly, another article established by Agarussi et al. (2020) showed that palmitoleic and arachidic acid ratios increased, whereas margaric and gondoic acid ratios decreased in whole-plant corn silages.

Vlaeminck, Fievez, Cabrita, Fonseca, and Dewhurst (2006) argued that microorganisms that produced odd- and branched-chain FA during ensiling might increase the total FA concentration. By contrast, Alves et al. (2011) highlighted that field manipulation, such as wilting and shading, accelerates oxidative degradation and results in the loss of FA. Another source of FA losses is DM losses due to the respiration of organic compounds (Alves et al., 2011). Similarly, Dewhurst and King (1998) reported that maturity and light exposure significantly affect the proportion of α-linolenic acid in grasses. The authors also showed that the extended wilting period dramatically reduces the total FA and a-linolenic acid proportion, whereas shading has a similar but lesser effect.

Table 4. 12. Faty acid composition of kefir treated white clover silages.

Item	Con	K10	K50	K100	*P*-value
Myristoleic	ND	1.01±0.13^{a}	0.46±0.09^{b}	0.33±0.09^{b}	0.010
Pentadecylic	3.38±0.32^{a}	1.95±0.32^{b}	2.89±0.32^{ab}	2.53±0.32^{ab}	0.047
Pentadecenoic	16.73±1.26	15.99±1.78	15.04±1.26	15.02±1.26	0.744
Palmitic	ND	0.48±0.16	0.41±0.12	ND	0.770
Palmitoleic	ND	1.00±0.28	0.40±0.20	0.66±0.20	0.288
Heptadecenoic	ND	0.92±0.13	0.63±0.13	0.57±0.18	0.256
Stearic	2.97±0.33^{a}	0.86±0.33^{b}	0.49±0.33^{b}	1.14±0.38^{b}	0.001
Oleic	4.81±1.83^{c}	20.92±1.83^{a}	16.57±1.83^{ab}	10.56±1.83^{bc}	<0.001
Linoelaidic	19.70±1.67	ND	ND	17.68±2.36	0.523
Arachidic	43.72±2.92^{b}	66.12±1.92^{a}	56.51±2.92^{a}	54.50±2.92^{ab}	0.001
Eicosenoic	1.53±0.10	ND	1.43±0.13	1.40±0.13	0.694
Linolenic	ND	ND	ND	0.19±0.003	-
Eicosadienoic	ND	ND	0.57±0.02	0.59±0.02	0.583
Behenic	0.97±0.07	ND	ND	ND	-
Dihomo-γ-linolenic	ND	ND	0.46±0.14	0.28±0.14	0.484
Eicosatrienoic	7.05±1.01	ND	6.34±1.43	6.15±1.43	0.853
Eicosapentaenoic	ND	ND	4.41±0.04	4.24±0.04	0.078
ΣSFA	50.56±3.00^{b}	69.17±3.00^{a}	60.30±3.00^{ab}	57.89±3.00^{ab}	0.007
ΣMUFA	22.69±2.58	30.83±2.58	33.81±2.58	27.55±2.58	0.053
ΣPUFA	26.75±2.45^{a}	0.00±2.45^{c}	5.89±2.45^{bc}	14.56±2.45^{b}	<0.001
ΣUnsaturated	49.44±3.00^{a}	30.83±3.00^{b}	39.70±3.00^{ab}	42.11±3.00^{ab}	0.007
ΣUNSFA/ΣSFA	1.04±0.11^{a}	0.45±0.11^{b}	0.66±0.11^{ab}	0.74±0.11^{ab}	0.023

Con: Control, K10: 10 mg/kg kefir yeast, K50: 50 mg/kg kefir yeast, K100: 100 mg/kg kefir yeast, Σ: Total, SFA: Saturated fatty acid, MUFA: Monounsaturated fatty acid, PUFA: Polyunsaturated fatty acid, ND: Not detected. The values with different letters (a, b, c, d) in the same row are statistically different (P<0.05).

Prior studies have also explored the relationships between FA and LAB and found that linoleic and linolenic acid biohydrogenation in LAB-treated silages leads to a decrease in long-chain fatty acids (>18C) concentration (Alves et al., 2011; Yu, Wang, Tian, Li, and Zhang, 2013). Moreover, Jalč, Lauková, Simonová, Váradyová, and Homolka (2009) demonstrated that corn silages inoculated with *Enterococcus faecium*, had a higher SFA (3-5%) and PUFA (3-7%) proportion.

In the current study, the FA composition was significantly affected by the inoculation of kefir yeast, most likely because the exogenous microorganisms transformed the fatty acids present in TR silages. In accordance with the present results, previous studies indicated that decomposition, degradation, or conversion of FA during ensiling occurs only through the saturation, desaturation, homologation, and degradation of oleic acid (Koczoń et al., 2019). The increased saturated arachidic acid content of kefir yeast treated silages possibly indicates the conversion of oleic acid to form arachidic acid by homologation reaction (Table 4.12 and Figure 4.13). Moreover, degradation is the one pathway for the conversions of unsaturated oleic acid (C18:1 cis 9) to the other FA present in the silage during fermentation. Thus, it seems likely that some of the unsaturated oleic acids degraded to form shorter FA, such as myristoleic, palmitic, palmitoleic, and heptadecenoic acids (Table 4.12). These results are consistent with data reported by Ogawa et al. (2005), Kishino, Ogawa, Yokozeki, and Shimizu (2009), and Koczoń et al. (2019). In addition, inoculation of kefir yeast significantly increased the SFA proportion in TR silages ($P<0.01$), whereas the PUFA proportion decreased ($P<0.001$), and monounsaturated fatty acid (MUFA) remained unchanged ($P>0.05$). Similar findings were also reported by Jalč et al. (2009), Jalč, Lauková, and Kišidayová (2010) and Łozicki et al. (2015).

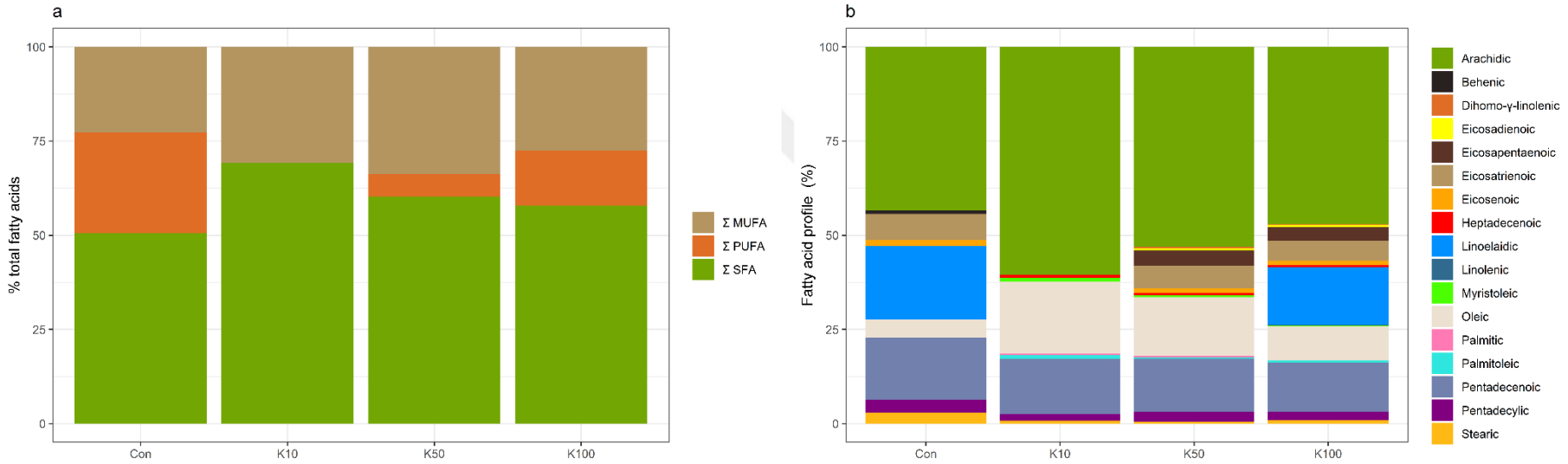


Figure 4. 13. Fatty acid composition of kefir treated white clover silages.

5. CONCLUSION and RECOMMENDATIONS

This thesis aimed to understand and explain the role of the kefir yeast addition on fermentation characteristics, aerobic stability, *in vitro* digestibility, mineral concentration, and fatty acid composition of high moisture TR silage in long-term storage. The findings clearly indicate that inoculation of kefir yeast could help reduce NDF and hemicellulose contents, thereby improving the digestibility of TR silages. The research has also shown that inoculation of kefir yeast could also be used to reduce NH_3-N and protein degradability. In addition, heterofermentative characteristics of kefir yeast led to increased AA content and decreased LA content of TR silages. Contrary to the observed high yeast count at silo opening, yeast count was significantly reduced after 5 d of aerobic exposure.

Prior to this study, there were no data on how the addition of kefir yeast effect *in vitro* digestibility, mineral concentrations, and fatty acid composition of a forage crop. Inoculation of kefir yeast did not significantly affect the cumulative GP and estimated parameters except for 48 and 72 h. The micro- and macronutrient analysis results show that inoculation of kefir yeast reduced the K, Na, Se, and B concentrations of TR silages, whereas Ca, Zn and Fe increased. Moreover, inoculation of kefir yeast significantly increased the SFA proportion in TR silages, whereas the PUFA proportion decreased. However, inoculation of kefir yeast did not significantly affect MUFA proportion of TR silages.

Overall, these findings suggest that adding 50-100 mg/kg FM kefir yeast is appropriate to improve the nutritional value of TR silage. In terms of future work, it would be interesting to repeat the experiments described here using 16S rRNA sequencing techniques in farm-scale silos to characterize the microbial community of kefir-treated silages. Future studies should explore the effects of kefir-treated silages on *in vivo* digestibility and animal performance.

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