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Effects of pasture grazing and fermented feed on growth performance, fecal microbiota, and carcass characteristics of Hanwoo steers and fermented characteristics of triticale silage

초지 방목 및 발효사료급여가 한우 거세우의 생산성,
분내 미생물 균총 및 도체특성에 미치는 영향과
트리티케일 사일리지 발효특성에 관한 연구

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농 학 박 사 학 위 논 문

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Overall summary

South Korea relies almost entirely on imported grain and animal feed to meet the requirements for livestock feed. As a result, fluctuations in international forage crop prices and feed price increases directly impact domestic livestock farms` income. One efficient method to reduce feed costs is to provide high-quality domestic forage. There are three approaches to incorporating domestic forage in beef cattle production. Firstly, expanding the use of grassland for beef cattle grazing is effective in reducing feed costs. However, it is necessary to solve the problem of reducing livestock productivity by beef cattle grazing. Secondly, expanding the use of domestic forage in total mixed rations (TMR) can also be useful. However, the variance in moisture content of domestic forage silage poses a risk of spoilage of TMR (Choi et al., 2019). Fermented feed (FF) is an alternative method that involves aerobic fermentation to improve storability. Finally, lactic acid bacteria (LAB) inoculants are widely used to improve the fermentation process and aerobic stability of silage.

Study 1 aimed to investigate changes in the composition of fecal microbiota and the differences between grazing and housing feeding systems of Hanwoo steers. Grazing steers on natural pastures increased the diversity of bacterial communities in the fecal microbiota, both at the phylum, family, and genus levels. Moreover, *Firmicutes* levels were higher in the feces of grazing steers grown on grasslands than in those of housed steers. *Firmicutes* consist of two dominant bacterial families: *Ruminococcaceae* and *Lachnospiraceae*. Meanwhile,

Sphingobacteriaceae, *Bacteroidaceae*, *Prevotellaceae*, and *Rikenellaceae* dominated *Bacteroidetes*, with higher numbers observed among housed steers. The changes in microbiota may have an impact on serum metabolic profiles (gammaglutamyl transpeptidase, glucose, total cholesterol, and triglyceride) and feeding behavior. The findings of this study enhance our current understanding of the gut microbiota of Hanwoo steers and provide evidence of the potential effects of different forages on the rumen microbiota of naturally fed animals.

Study 2 aimed to compare the growth efficiency, carcass quality, meat quantity, and quality characteristics of Hanwoo steers fed fermented feed with forage silage (FF) and concentrates and rice straw separately (CRS). This study is the first to document the effects of FF on the growth output, carcass characteristics, and meat quality of Hanwoo steers during early and late fattening periods. The results showed that feeding FF not only improved feed intake and growth performance but also enhanced carcass characteristics, meat quality, and fatty acid profiles of Hanwoo steers compared to those fed CRS. In addition, the study contributes to the meat industry by providing evidence that FF fed steers have superior meat quality than those fed CRS. Nevertheless, further research is required to determine the mechanisms by which FF affects meat quality.

In Study 3, the effect of LAB (*Lacticaseibacillus rhamnosus*) on the nutrient profiles, fermentation profiles, and microbial diversity of high-moisture triticale silage (before and after heading) was investigated. The results showed that the addition of *L. rhamnosus* INO-52 (novel inoculants of *Lacticaseibacillus rhamanosus*-52) and INO-54 (novel inoculants of *Lacticaseibacillus rhamanosus*-54)

had a positive impact on all the evaluated parameters. The inoculated silage had enhanced lactic acid production and lower pH, leading to a decrease in the richness and diversity of other bacterial species and a reduction in the concentration of acetic acid. These findings suggest that the tested strains have the potential to improve silage fermentation when used as inoculants. However, further research is needed to determine their efficiency for different types of grass and legume silages and their combinations with other additives, including homo-fermentative bacteria and artificial additives. The results of this study provide a foundation for future research in this area.

In conclusion, the most efficient way to reduce feed costs is to supply high-quality domestic forage to beef cattle. There are several ways to preserve the quality of forage and to use high-quality forage more effectively. Grazing steers on natural pastures increased the diversity of bacterial communities in the fecal microbiota at the phylum, family, and genus levels and influenced animal health and serum metabolic markers. The FF feed can be effective and profitable for the early and late fattening periods of Hanwoo steers without causing negative effects. The addition of *L. rhamnosus* can enhance the fermentation of silage inoculants. In summary, to expand the utilization of domestic forage, it is important to enhance its preservation and choose an appropriate method for feeding cattle.

Keyword : fermented feed, grazing, Hanwoo steers, inoculants, NGS, silage

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AA: acetic acid

ADF: acid detergent fiber

ADP: adenosine diphosphate

ALB: albumin

ALP: alkaline phosphatase

ATP: adenosine triphosphate

BA: butyric acid

BUN: blood urea nitrogen

Ca: calcium

CF: crude fiber

CIE: international commission of Illumination

CLA: conjugated linoleic acid

CP: crude protein

CRE: creatinine

CRS: concentrate and rice straw separately feed

CS: cornstalk

DM: dry matter

DMI: dry matter intake

DNA: deoxyribo nucleic acid

EE: ether extract

EMP: Ebden-Meyerhof-Parnas

FA: fatty acid

FF: fermented feed with Italian ryegrass silage and whole crop corn

FTMR: fermented total mixed ration

GIT: gastrointestinal tract

GLU: glucose

GS: grazing steers

HCN: hydrocyanic acid

HPLC: high performance liquid chromatography

HS: housing steers

INO-52: novel inoculants of *Lactobacillus rhamanosus*-52

INO-54: novel inoculants of *Lactobacillus rhamanosus*-54

IRG: Italian ryegrass

IVDMD: *in-vitro* dry matter digestibility

LA: lactic acid

LAB: lactic acid bacteria

LDH: lactate dehydrogenase

MAFRA: Ministry of Agriculture, Food and Rural Affairs

MFF: microbially fermented feed)

Mg: magnesium

MUFA: monounsaturated fatty acid

NAD+: nicotinamide adenine dinucleotide

NADH: nicotinamide adenine dinucleotide

ND: not detected

NDF: neutral detergent fiber

NEFA: non-esterified fatty acids

NFC: non-fiber carbohydrates

NGS: next-generation sequencing

No: non-inoculants silage

NSC: non-structural crbohydrates

OTUs: operational taxonomic unites

P: inorganic phosphorus

PBS: phosphate buffer saline

PCA: principal component analysis

PCR: polymerase chain reaction

PK: phosphoketolase pathway

PUFA: polyunsaturated fatty acids

SEM: standard error of the mean

SFA: saturated fatty acids

SGOT: serum glutamic oxaloactic transaminase

SGPT: serum glutamic pyruvic transaminase

SST: serum separating tube

STD: standard deviation

TB: total bacteria

Tbil: total bilirubin

TCA: tricarboxylic acid cycle

T-CHO: total cholesterol

TDN: total digestible nutrient

TG: triglyceride

TMF: total mixed fermented

TMR: total mixed ratio

Tpro: total protein

UFA: unsaturated fatty acid

WCC: whole crop corn

WSC: water-soluble carbohydrate

γGTP: gammaglutamyl transpeptidase

ω3: omega-3

ω6: omega-6

Units and marks

% : percent

CFU: colony forming unit

dL: deciliter

g: gram

IU: international unit

kg: kilogram

L: liter

mg: milligram

no.: number

ha: hectare

μEq: microequivalent

CHAPTER ONE

Ⅰ. General introduction

Feed is a major cost in beef cattle production, accounting for approximately 35% of the total cost to produce Hanwoo beef (Korean beef cattle) (KOSIS, 2023; Chang et al., 2018). South Korea heavily relies on imported grain and animal feed to meet livestock feed requirements, making the price of international crops and increasing feed prices have a direct impact on the income of domestic livestock farms. To reduce feed costs efficiently, supplying high-quality domestic forage is the most practical method. However, the domestic forage self-sufficiency rate is high (82.7%), and only 25% of it constitutes high-quality forage, including winter forage (Italian ryegrass, rye, barley, triticale), summer forage (forage sorghum, forage corn), and grass (MAFRA, 2022). The government (Korean Ministry of Agriculture, Food, and Rural Affairs; MAFRA) has implemented forage-based expansion programs to expand the production base for domestic forage and utilize resources. These programs include three main policies: forage production subsidies, assistance with forage distribution and processing, and the development of forage-producing lands. These policies directly or indirectly affect domestic forage production (Chang, 2018; Kim et al., 2020). The various subsidies and equipment (tractor, baler, mower, tedder, and others) for forage production provided by the Korean government have contributed to the rapid expansion of silage production among farmers (Kim et al, 2012). In recent years, there has been a significant increase in the use of round bale silage. However, challenges related to inconsistent moisture content and soil

contamination have led to a degradation in silage quality. To overcome these issues, new technologies must be developed to expand the use of domestic forage.

There are three main methods for utilizing domestic forage in beef cattle production. Firstly, increasing the grazing area for beef cattle can effectively reduce feed costs. Secondly, the use of domestic forage in total mixed rations (TMR) must be expanded, but the variability in the moisture content of domestic forage silage poses a risk for TMR spoilage (Choi et al., 2019). To improve the storability of TMR, TMF (total mixed fermented) can be produced under aerobic conditions (Nishino et al., 2003; Subepang et al., 2019). Finally, lactic acid bacteria (LAB) inoculants are widely used to enhance the fermentation process and aerobic stability of silage.

Study 1 investigated changes in the composition of the fecal microbiota community and the dynamics between grazing and housing feeding systems for Hanwoo steers. To achieve this, next-generation sequencing (NGS) was used to analyze the fecal microbiota of steers by targeting the V4 region of the 16S rRNA gene. Fecal samples were collected from both housed and grazing cattle after seven months, and these were correlated with animal welfare and serum biochemical parameters. In the second study, growth efficiency, carcass quality, meat quantity, and quality characteristics of Hanwoo steers were compared between those fed fermented feed supplementation with forage silage (FF) and those fed concentrates and rice straw separately (CRS) feeds. No studies have investigated the benefits of feeding Hanwoo steers FF/CRS feed from the early to late fattening period. Therefore, it is important to conduct feeding trials to test the efficacy of the FF silage/concentrate feeding system for Hanwoo steers, and

compare it to a conventional rice straw combined with a concentrate-based system. In the third experiment, the effect of LAB (*L. rhamnosus*) on the nutrient profiles, fermentation profiles, and microbial diversity of high-moisture triticale silage (before and after heading) was investigated. NGS was used to characterize microbial populations in the non-inoculated and inoculated whole-crop triticale silage.

CHAPTER TWO

Ⅱ. Literature review

1. The current situation of forage and grassland in South Korea

1.1. Forage production and supplementation in South Korea

In 2021, the total domestic forage consumption is 5,218 thousand tons, of which domestic forage is 4,315 thousand tons, and the forage self-sufficiency rate is 82.7%. Although the domestic forage self-sufficiency rate seems high, 58% of them are rice straw (low quality forage), and high-quality forage including winter forage (Italian ryegrass, rye, barley, triticale, and others), summer forage (forage sorghum, forage corn, and others), and grass account for only 25%. Imported forage account for 17.3% (903 thousand tons) of total forage demand in 2021. Over the past 10 years, the average forage self-sufficiency rate has been 78.7%, remaining in the 80% range except for poor harvest year due to drought in the spring in 2017 (MAFRA, 2022; Kim et al., 2020b). Although the forage cultivation area is increasing every year, the main reason for the stagnant self-sufficiency rate is the decrease in forage productivity due to climate change and the increase in the number of ruminants by increasing meat consumption. In particular, imported hay was mainly supplied to dairy farms or total mixed ratio (TMR) companies, but the recent increase in the use of imported forage for beef cattle had a significant impact on the self-sufficiency rate (MAFRA, 2022; Lee et al., 2022). Domestic forage is mainly distributed in the form of round bale silage, however, imported forage is distributed in the form of square bale hay suitable for long term storage and distribution.

Table 1. Proportion of domestic pasture, forage crop, and rice straw produced per year, with overlay of total domestic and imported annual dry forage tonnage (source: MAFRA, 2022)

Unit: thousand tons, thousand hectares

Item	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021
Forage required for rumiants feeding (Number)	**4,506 (3,274)**	**4,489 (3,330)**	**4,563 (3,431)**	**4,430 (3,274)**	**4,296 (3,239)**	**4,287 (3,303)**	**4,441 (3,373)**	**4,687 (3,481)**	**4,999 (3,783)**	**5,218 (3,974)**
Total domestic forage production (cultivation area)	**3,517 (108)**	**3,649 (103)**	**3,520 (120)**	**3,334 (91)**	**3,334 (105)**	**3,060 (98)**	**3,470 (120)**	**3,742 (123)**	**4,102 (134)**	**4,315 (140)**
Domestic forage crop production (cultivation area)	742 (78)	704 (75)	869 (92)	623 (66)	779 (80)	721 (74)	960 (97)	997 (101)	1,102 (112)	1,146 (118)
Domestic pasture production (cultivation area)	207 (30)	200 (29)	193 (28)	177 (25)	173 (25)	169 (24)	162 (23)	159 (23)	158 (23)	153 (22)
Rice straw	2,567	2,592	2,586	2,720	2,383	2,169	2,347	2,587	2,842	3,016
Total imported forage	**989**	**993**	**914**	**909**	**961**	**1,228**	**971**	**944**	**896**	**903**
Alfalfa	144	164	169	167	182	192	198	197	191	191
Grass hay	790	787	712	719	755	1,019	757	744	695	707
Others	55	42	33	22	24	17	16	3	11	6
Forage self-sufficiency rate	**78.1**	**77.9**	**80.0**	**79.5**	**77.6**	**71.4**	**78.1**	**79.9**	**82.1**	**82.7**

1.2. Grassland production and utilization in South Korea

Grazing studies have a long history of applying data and technologies to livestock producers, and have helped to strengthen our understanding of the biology and natural ecology of grasslands (Aiken et al., 2016). In addition, grazing animals are one of the most competitive and suitable feeding systems for cows worldwide. Pasture grazing *in situ* has a low environmental footprint, is beneficial for animal health, and is relatively inexpensive to produce and utilize (Wilkenson et al., 2020). Cattle can utilize a variety of structural polysaccharides, such as cellulose and hemicellulose, found in grass. Grasslands not only supply feed to livestock but also provide habitats for biodiversity and deliver many cultural services (Bai et al., 2022). Moreover, grasslands have a positive influence on the recharge of water tables, and water quality improvement, and have good potential for soil carbon sequestration (Peeters, 2009). Recent studies have shown that grassland soil organic carbon sequestration varies depending on factors such as improved grazing management, fertilization, sowing legumes, improved grass species, and irrigation. The amount can range from 0.105 to more than 1 Mg C ha^{-1} yr^{-1} (Conant et al., 2017). Carbon-rich grasslands have been frequently converted into croplands, which store less carbon per unit area. Grasslands, which account for about one-third (approximately 34%) of the global terrestrial carbon stocks, store most of their carbon (approximately 90%) underground as root biomass and soil organic carbon (Bai et al., 2022). Grass root carbon sequestration has a stabilization efficiency that is five times greater than that of aboveground carbon inputs (Jackson et al., 2017). Particularly, grazing animals have been found to enhance soil carbon sequestration through livestock

management practices like rotational grazing (Conant et al., 2017). Although many studies have examined the impact of grazing on grasslands and the environmental benefits of grasslands in the EU and the US, there are relatively few studies in South Korea. Therefore, it is essential to optimize the use of grasslands to maximize the productivity and economic value of their multiple functions (Kim and Kim, 2018) (Figure 1).

The domestic grassland area has decreased by 51% from 66,301 ha in 1995 to 32,388 ha in 2021. The primary reasons for this decline were the conversion of agricultural land, various industrial developments, and forest exploitation, which have continued to decrease since 1995. In 2021, only 100 ha of land was exclusively designated for grassland use, and most of this land was diverted for purposes such as agriculture (66.6 ha), urban development, and road construction. Recently, the MAFRA has initiated eco-pastoral system development projects to reduce production costs of raising cattle by improving the self-sufficiency rate of forage and expanding eco-friendly livestock farming. The eco-pastoral system serves several purposes, including raising the self-sufficiency rate of forage, improving animal welfare, enhancing farmers' income, and promoting grassland tourism (MAFRA, 2022).

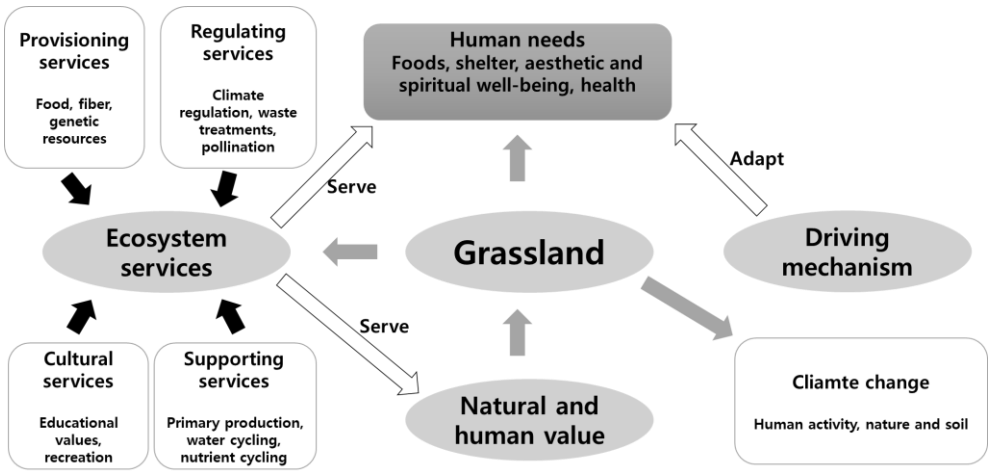

Figure 1. Goods and services provided by grasslands (modified from White et al., 2000; Zhao et al., 2020).

2. Composition of fecal microbiota in cattle

2.1. Fecal microbiota analysis in the feces of bovine

The optimal approach for the nutritional management of grazing animals is to maximize forage utilization and promote the growth and activity of fibrolytic microbes in their gastrointestinal tracts. The foundation of grazing animal nutrition should be based on a strategy that utilizes forage to provide all the necessary nutrients. The primary objective of protein nutrition for ruminant grazing animals is to maximize microbial protein yield. This is because cows rely on gastrointestinal microflora to ferment forage and derive nutrients from it (Lardy, 2020).

According recent studies, fecal microbiota is commonly used as a proxy for gastrointestinal microbiota because it is noninvasive, practical, and can be collected repeatedly from animals (Callway et al., 2010; Mao et al., 2012; Oikonomou et al., 2013; Kim et al., 2014). Ruminal gut microbes are associated

with their host, and a variety of host activities, such as nutrient absorption, metabolism, and environmental conditions, are closely related to the growth performance of ruminants. Additionally, the fecal bacterial communities of cows are markedly influenced by various factors, including the composition and nutrition of the feed (Zhang et al., 2021). Among these factors, diet is the most influential factor in altering ruminal gut microbes, as compared to other factors, such as breed, sex, and age (Kim and Wells, 2016; Callaway et al., 2010; Rice et al., 2012; Shanks et al., 2011; Shah et al., 2022; Mote et al., 2019). Therefore, it is essential to understand the fecal microbiota in different diets to enhance bovine productivity. Traditional culture-based studies are limited since they account for only a small portion of the total microbial population (Janssen, 2006). However, culture-independent NGS studies have recently increased our understanding of the correlation between animal performance and bovine microbial communities (Mote et al., 2019).

2.2. Changes in fecal microbiota according to diets

Firmicutes, *Bacteroidetes*, *Proteobacteria*, and *Ancionobacteria* are the dominant phyla in bovines, and the microbial composition of feces can be further affected by diet (Kim and wells, 2016; McCann et al., 2014a). Kim et al. (2013) reported that *Firmicutes* and *Bacteroidetes* were the most dominant phyla in fecal samples, and the microbial composition of the feces was greatly affected by dietary differences (moderate grain, high grain, and silage/forage). The difference in diets (grain-based diets vs. forage-based diets) had the greatest effect on the fecal microbiota of cattle. Rice et al. (2012) reported that the dominant phyla observed

were *Firmicutes* (61%, 19–83%), *Bacteroidetes* (28%, 11–63%), *Spirochaetes* (5%, 0–23%), and *Proteobacteria* (3.03%, 0.34–17.5%). The proportion of *Bacteroidetes* increased when fed more than 10% of the corn diet. Zhang et al. (2021) reported that the most common bacterial phyla of microbial composition in beef cattle are *Firmicutes* (60.61%), *Bacteroidetes* (28.32%), *Verrucomicrobia* (0.25%), *Tenericutes* (0.22%), *Actinobacteria* (0.11%), and *Elusimicrobia* (0.04%), respectively. This study found that concentrate-based diets (66.26%) had a larger abundance of *Firmicutes* than forage-based diets (54.96%). The results showed that animal feeding practices appeared to have a far greater impact on the phylum and family taxonomic levels of bovine fecal bacterial communities than any other factor. Fecal starch concentrations appeared to have a significant impact on the makeup of the entire bacterial population, and *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the phyla most sensitive to diet. *Firmicutes* decreased when fecal starch concentrations increased; however, *Bacteroidetes* increased in relative abundance as fecal starch levels increased.

According to these earlier investigations, changes in food, breed types, climate, and farming practices across a large geographical range are responsible for the dominance of *Firmicutes* and *Bacteroidetes*. In addition, it appears that grazing pastures, forage varieties, and forage quality are more favorable for the abundance of *Firmicutes* or *Bacteroidetes* pyrotypes (Henderson et al., 2015). A forage-based diet contains several secondary metabolites that function as prebiotics and enhance bacterial diversity. Because the bacterial communities of steers feeding on natural pastures are quite diverse, forage species and biomass may affect the variety of microbial communities in bovines (Chen et al., 2008;

Latham et al., 2018).

In dairy cows, the most crucial factor in explaining fecal microbiota richness is diet, and the fecal microbiota from mixed diets (forage and concentrate) is richer than that from only forage diets (Albonico et al., 2020). However, some studies failed to find any correlation between the *Firmicutes*:*Bacteroidetes* ratio and other factors such as diet. Therefore, although the *Firmicutes*:*Bacteroidetes* ratio is a simple and useful indicator of health, its correlation in domestic livestock has yet to be adequately investigated (Albonico et al., 2020).

2.3. *Bacteroidetes*

In recent years, many different types of bacteria in the phylum *Bacteroidetes* have undergone numerous name changes (Woese et al., 1990; Thomas et al., 2011). Bacteria have colonized virtually every habitat on Earth, including compost, soil, dead animals, decomposing plants, and the animal gastrointestinal tract (GIT), where they perform various functions (Bernardet et al., 2006; Reichenbach, 1992). While the GIT microbiota mainly consists of species from the Bateroidia class, environmental *Bacteroidetes* predominantly consist of *Flavobacteria*, *Cytophagia*, and *Sphingobacteria* classes (Thomas et al., 2011).

Bacteroidetes are highly represented at the phylum level in the fecal microbiota (Moore and Holdeman, 1974; Sghir et al., 2000; Hamilton et al., 2013; Wood et al., 1998). They are well known for breaking down polymeric organic materials. Numerous studies have been conducted on the biological role of symbionts in the breakdown of polysaccharides in the large intestine. Carbohydrates, which represent the majority of a typical human diet, serve as a major source of

nutrition for both the host and microbiota. Additionally, *Bacteroidetes* process enzymes that degrade starch, host-derived carbohydrates (such as N-glycans found in mucins or chondroitin sulfates), and other dietary polymers, such as the components of plant cell walls (cellulose, pectin, and xylan) (Salyers et al., 1977; Thomas et al., 2011).

Bacteroidetes also interact with the immune system to activate T cell-mediated responses and prevent the colonization of the GIT by potentially harmful bacteria, both of which contribute to the health of their hosts (Wen et al., 2008; Mazmanian et al., 2008). Gut *Bacteroidetes* typically produce butyrate, a byproduct of colonic fermentation, which has anti-cancer properties and contributes to the maintenance of a healthy gut (Mahowald et al., 2009). They are also involved in the metabolism of bile acids and the transformation of chemicals that are toxic or mutagenic (Ghosh et al., 2021).

In bovines, the diet consists largely of plant cell wall compounds that are resistant to host digestive enzymes. Therefore, the assimilation of short-chain fatty acids produced by microbial fermentation of polysaccharides can provide more than 50% of the total caloric supply (Ortega and Mendoza, 2003; Carroll and Hungate, 1954). Several studies have demonstrated that the GIT microbiota is strongly influenced by diet. For instance, *Prevotella*, the largest genus, is frequently found in the feces of cattle fed a diet high in corn. *Bacteroides* are the dominant genus in the feces of bovines fed a corn-based diet (Kim et al., 2014). In 1983, Yabuuchi proposed that a bacterium known as *Sphingobacteria* should be named after it was found in fresh bovine feces. The bacterium had a biochemical profile characteristic of an organism with a sphingolipid component

in its cell wall. Furthermore, it belonged to the dominant classes of bacteria found in fresh bovine feces, which have been shown to aid in infant gut development (Lambiase, 2014; Chen et al., 2021a; Nilsson, 2016).

2.4. *Firmicutes*

The name *Firmicutes* comes from the Latin word for "tough skin," and most of its members have a gram-positive cell wall structure. Recent studies have focused on humans, showing that *Firmicutes* can depolymerize different types of dietary fibers directly or indirectly, producing metabolites such as acetic acid, butyric acid, and lactic acid during the proliferation process (Cockburn and Koropatkin, 2016). *Firmicutes* are beneficial bacteria in the intestinal tract that help the host absorb energy from food. The intestinal *Firmicutes* richness of obese mice is significantly higher than that of slim mice (Ley et al., 2005). Thus, the *Firmicutes*:*Bacteroidetes* ratio is a potential biomarker of gut dysbiosis (Grigor'eva et al., 2020). The response of gut *Firmicutes* to dietary fiber has a significant impact on the host, including glucose metabolism, inflammation, gut permeability, and fatty acid oxidation synthesis (Sun et al., 2021; Gurug et al., 2020; Guo et al., 2020). Non-starch polysaccharides are diverse, and cellulose and hemicellulose are the most common plant constituents. Cellulose, formed from dehydrated glucose and linked by β-1,4-glycosidic bonds, is a linear polymer that can be broken down by *Ruminococcus* spp. and *Enterococcus* spp. (Chassard et al., 2010). *Firmicutes*, the most dominant phylum in cattle fecal samples (up to 50%), are more abundant in concentrate-based diets than in forage-based diets (Kim et al., 2014). Zhang et al. (2021) reported that the

Firmicutes of feedlot-fed cattle were higher than those of grazing-fed cattle, and the differences in microbiota composition between grazing and feedlot in Angus beef may have an impact on meat quality.

The genus *Oscillibacter* was found to be the most dominant in the *Firmicutes* phylum, which is consistent with the findings of Kim et al. (2014) in cattle fed a high-grain diet. The *Ruminococcaceae* family is known to be the predominant acetogen in the bovine rumen and is strongly associated with the degradation of fiber, such as cellulose and hemicellulose. Moreover, *Ruminococcaceae* is involved in energy metabolism and inflammation regulation (Jia et al., 2023; Ren et al., 2019). In both grazing and feedlot Angus cattle, *Ruminococcaceae* was the most abundant genus in the feces, and its relative abundance was significantly higher in grazing Angus beef (Zhang et al., 2021). *Lachnospiraceae*, on the other hand, are associated with pectin degradation (Cotta and Forster, 2006), and their relative abundance increases with high dietary grain content (Kotz et al., 2020). Finally, *Rikenellaceae* are known to produce acetate, propionate, and fatty acids with anti-inflammatory properties in the intestines of humans and chickens (Polansky et al., 2016; Parker et al., 2020; Andrade et al., 2022).

2.5. Minor phyla (*Proteobacteria*, *Spirochaetes*, *Verrucomicrobia*)

Typically, the gut microbial community is dominated by the phyla *Firmicutes* and *Bacteroidetes*, while less prevalent members include *Actinobacteria*, *Verrucomicrobia*, and *Proteobacteria* (Backhed et al., 2005). Despite the limited number of predominant phyla, they provide the host organism with increased genetic resources, including energy acquisition pathways, synthesis of vital

vitamins, maturation of the digestive system, and advancement of the immune system (Shin et al., 2015).

The presence of *Proteobacteria* in fecal matter contributes to the onset of intestinal inflammation, potentially as a result of dysregulation of the immune response. An increased abundance of *Proteobacteria* is considered a microbiological indicator of intestinal dysbiosis (Shin et al., 2015; Wang et al., 2022). Furthermore, high-production cows have shown a significant increase in the phylum *Proteobacteria* compared to low-production cows. *Proteobacteria* were abundant in the high-production group, according to the majority of prior studies (Jemi et al., 2014; McCann et al., 2014b). *Spirochaetes* are widely distributed in nature as free-living bacteria, commonly present in cattle feces, and are associated with cellulolytic activity (Nyonyo et al., 2014). *Verrucomicrobia* is closely related to the extremely strong disease resistance of cattle (Aricha et al., 2021) and is believed to contribute to intestinal health and glucose homeostasis (Johansson et al., 2011). Additionally, it has the potential to modulate adipose tissue metabolism, thereby regulating the storage of body fat (Solar et al., 2019).

3. Total mixed fermentation (TMF)

3.1. Characteristics of TMF

One of the significant challenges facing contemporary ruminant feeding systems is the need to reconcile the feeding of substantial quantities of cereal grains to facilitate high-performance production while ensuring the preservation of both rumen and body health. Moreover, high-grain diets are associated with a high incidence of metabolic disorders such as subacute ruminal acidosis (SARA), laminitis, and fatty liver (Abijaoudé et al., 2000; Plaizier et al., 2012;

Dong et al., 2013; Chen et al., 2021b). To promote cattle health, it is recommended to use a total mixed ration (TMR). TMR contains all the feed and nutrients required by cattle and ensures a fixed ratio of forage: concentrate (Opsi et al., 2012). Moreover, TMR can reduce the workload of feeding cattle, enable them to consume a fixed amount of forage and concentrate required for good production and health, and provide more control and accuracy over the amount of feed given than separate feeding systems. Due to these advantages, TMR is primarily used on dairy cattle farms; however, its use for feeding beef cattle has gradually increased in South Korea (Kim et al., 2007).

The total mixed fermented (TMF) is a comprehensive feed blend that includes forage and concentrate, designed to fulfill the precise nutrient requirements of cattle. This feed undergoes fermentation to enhance nutrient availability, feed intake, digestibility, and storability under aerobic conditions (Nishino et al., 2003; Subepang et al., 2019; Li et al., 2016; Kim et al., 2012). TMF is a concentrated mixture fermented under anaerobic conditions (ensiling), which may lead to a reduction in anti-nutritive compounds, specifically mimosine or hydrocyanic acid (HCN).

In South Korea, the limited availability of land and the dominance of rice farming have hindered the development of the cattle industry and the production of forage. Winter forage crops have been planted on marginal land, but this has not been sufficient to meet the demand for forage. To address this issue, the government is now encouraging farmers to grow forage crops on fallow rice paddies. This approach solves the problem of forage scarcity while also utilizing fallow land, helping to use land resources more efficiently and ensuring a stable

forage supply for long-term cattle production (MAFRA, 2022).

The majority of domestic forage in South Korea is in the form of silage, which has high moisture content and significant quality variation. However, the primary motivation for resuming TMF use is the abundance of wet residues produced by the agro-industry and silage. By utilizing endowed resources such as domestic forage with relatively high moisture content and agricultural by-products, TMF can enhance feed value and reduce the production costs of beef cattle (Kim et al., 2003; Cao et al., 2009).

3.2. Improvement of fermentation quality in TMF

The advantages of TMF include an increase in the population of LAB and the concentration of lactic acid in the silage, which inhibits the growth of mold and yeast, achieving a long storage time and the ability to deliver probiotics via silage (Wang et al., 2008; Han et al., 2014). Some studies have reported that adding LAB or enzymes enhances the fermentation quality of TMF. Additionally, mixed inoculations with enzymes have been found to enhance the quality of silage fermentation by converting fibers into water-soluble carbohydrates (WSC) for LAB, which can improve silage aerobic stability and fermentation quality. Moreover, studies have investigated the feeding of TMF to livestock, and the results demonstrate that TMF has higher nutritional digestibility and volatile fatty acid content than non-fermented total mixed ration (Nascimento et al., 2019; Soundharrajan et al., 2019; Kim et al., 2015).

3.3. Effects of TMF supplementation on beef cattle

Studies on feeding Hanwoo steers with TMF have been conducted since the 2000s, and they have shown that TMF is an effective feeding system for fattening Hanwoo steers. It enhances ruminal characteristics, total tract digestibility, productivity, and growth performance, and improves the biochemical metabolites and fatty and acetic acid profiles of steers, ultimately enhancing the meat quality of Hanwoo (Vasupen et al., 2005). Furthermore, the silage LAB inoculant persists in vitro in rumen fluid and alters ruminal bacterial populations, as observed in several studies. The relationship between production factors such as feed efficiency, beef production, and rumen microbiota has also been investigated (Song et al., 2023; Kim et al., 2012).

Kim et al. (2012) reported that TMF diets resulted in significant improvements in average daily and total live weight gain, as well as feed efficiency ($p < 0.05$), when compared to TMR diets. Additionally, certain metabolites such as blood urea nitrogen and serum glutamic-oxaloacetic transaminase were reduced in the TMF group, and blood profiles were also improved when compared to TMF diets. The TMF exhibited better performance in nutrient digestion, feed intake, blood profiles, and growth performance in beef cattle when compared to TMR. Kim et al. (2013) argued that feeding TMR (pH 6.0) could stabilize rumen pH and optimize rumen feed digestion compared to a separate feeding system (pH 5.7).

Han (2010) reported that feeding a TMF (total mixed fermentation) diet could reduce feeding costs by 6% and enhance ribeye area and carcass weight. Lee et al. (2009) found that TMF diets led to high feed intake, increased back fat

thickness, and higher marbling scores.

The present study investigated the effects of LAB addition and 21-day fermentation on TMF and observed its potential to enhance feed intake and growth efficiency in Hanwoo steers relative to the control group (Kim et al., 2018). However, there is currently no report on the benefits of feeding microbially fermented feed and control feed to Hanwoo steers throughout the early and late fattening periods. Therefore, conducting feeding trials to compare the formulated feed combined with silage and conventional rice straw combined with a concentrate-based system is important to determine the effects of the formulated feed/concentrate feeding system on Hanwoo steers.

4. Role of lactic acid bacteria in silage fermentation

4.1. Silage fermentation

Ensiling or ensilage refers to the process of preserving fresh forage by natural or artificial acidification and storing it under anaerobic conditions. This method is commonly used as animal feed due to insufficient feed supply (Stewart et al., 2018; Adesogan and Newman, 2010; Wilkinson et al., 2003). Silage preservation from various crops is based on microbial fermentation, typically with more than 50% moisture content. The preservation of forage through ensiling is of great interest because it provides a reliable, consistent, and predictable supply of feed for ruminant production (Soundharrajan et al., 2021). Forage crop or grass is retained as a substitute during times of scarcity when plant growth is limited or when forage conditions are poor, in order to supply animals. Ensiling is a vital biological process that occurs when fresh forage spontaneously ferments under

anaerobic conditions, allowing silage to be stored for an extended period. This process requires careful consideration of multiple factors, including plant growth, harvest, and storage. Fresh forage typically contains high moisture content (>80%), soluble proteins, and sugars in the liquid, making it more susceptible to mold, yeast, and bacterial growth. Enzymatic and microbial activities are therefore critical to ensuring proper silage fermentation. Adequate ensiling procedures create anaerobic conditions that encourage the growth of LAB while inhibiting the growth of unwanted microorganisms such as yeast and mold (Broberg et al., 2007; Liu et al., 2019; Wang et al., 2020). Natural bacteria can convert water-soluble carbohydrates into lactic acid, a significant acid found in fermented silages, which lowers the silage pH. The lactic acid content is a key indicator of silage quality. The primary objective of ensiling is to preserve forage for an entire year without spoilage, thereby enhancing the economic and environmental sustainability of silage production (Soundharrajan et al., 2021). The silage fermentation process is typically divided into four distinct phases: the early aerobic phase, log phase, fermentation phase, and stable phase (Muck et al., 2018; Pahlow et al., 2003) (Figure 2).

The aerobic phase is characterized by the presence of oxygen trapped within the forage, which leads to an increase in ensiled temperature. During this phase, respiration by harvested plants and microbes results in nutrient oxidation and heat generation. The log phase follows the completion of the aerobic phase. As all available oxygen is consumed, bacteria begin to consume plant cells as an alternative source of nutrients. Plant enzymes aid in the hydrolysis of complex carbohydrates, starch, and fibers into simpler sugars that can be readily utilized

by bacteria. Enzymes also aid in breaking down plant-based proteins, increasing their solubility. During the fermentation phase, bacteria utilize the cellular nutrients and metabolites generated during the lag phase for growth. The pH of the silage decreases, leading to an increase in acidity, reaching levels of approximately 5.7–5.5.

During the fermentation phase, the primary microorganisms that facilitate the acceleration of the fermentation process are LAB, which grow, multiply, and produce lactic and acetic acids, leading to an increase in silage acidity (Soundharrajan et al., 2021). LAB populations and epiphytic diversity are highly variable, which can interrupt their growth process (Guo et al., 2023). Because lactic acid is stronger than acetic acid, it reduces the pH of silage to a greater extent than acetic acid (Danner et al., 2003). Generally, lactic acid fermentation begins when LAB dominates the process; however, homo-fermentative or hetero-fermentative fermentation may be more predominant due to variations. Homo-fermentative bacteria are preferred because they work faster, conserve more nutrients for cows, and enable better preservation of silage (Kung et al., 2018). The anaerobic fermentation phase typically lasts for approximately two weeks, during which time the silage cools to near ambient temperature (Okoye et al., 2022).

The stable phase begins after the anaerobic fermentation is completed. During this phase, most microbes become less active, and silage is preserved as bacteria slow down or stop growing. The quality of the forage can continue to improve for four to six months of storage due to ongoing bacterial action and kernel enzymes that aid in protein solubilization. The degree of degradation following

silage opening is determined by the chemical and microbiological characteristics of the silage during the stable phase. Fermented silages are exposed to air when they are opened for feed-out, leading to changes in environmental conditions, anaerobiosis, and conservation principles (Guo et al., 2023; Soundharrajan et al., 2021).

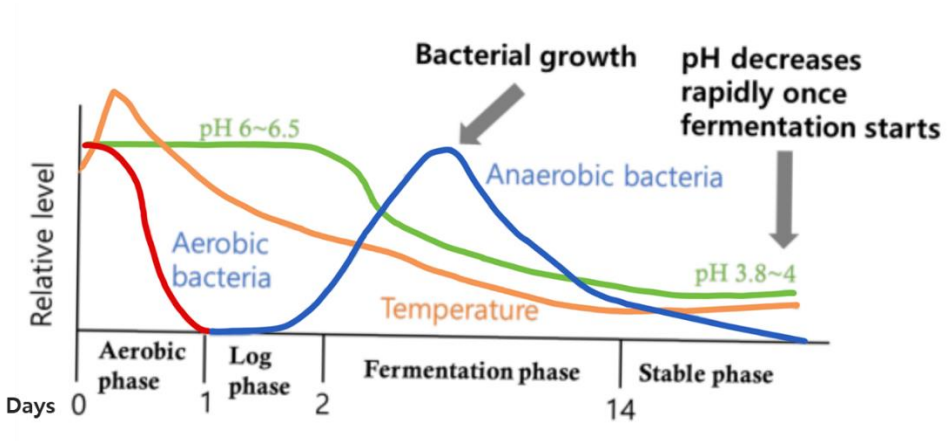

Figure 2. Four phases of the mechanism of silage fermentation (source: Soest Van, 1994; Soundharrajan et al., 2019).

4.2. Epiphytic microflora on forage

Epiphytic microflora, naturally present on the surface of grass or forage crops, are utilized in silage production and contain various types of aerobic and anaerobic microorganisms (Lin et al., 1992). This microflora also influences the efficacy of silage bacterial inoculation and plays a significant role in silage fermentation (McDonald, 1991). Microflora levels are influenced by factors such as the type of raw material (grass, forage crops, and legumes), stage of maturity, and environmental conditions such as soil, climate, and harvesting technique

(Muck, 2013; Burns et al., 2018). Native bacteria regulate the fermentation process and affect the stability of ensiled materials. The presence of LAB and other unwanted bacteria in plant components can influence the fermentation process and the quality of silage (Figure 3). LAB dominates the forage ensiling process, and changes in microbial populations are directly linked to silage fermentation (Driehuis et al., 2018). Epiphytic LAB is crucial for spontaneous fermentation of silage under anaerobic conditions. Thus, a higher level of epiphytic LAB in forage ensures good fermentation of silage (Zhang and Kumai, 2000), while undesirable microbes can produce low-quality silage (Muck, 2013). Epiphytic microflora makes up a relatively small percentage of the overall microflora, typically less than 1% of the forage. As a result, the impact of these inoculants on silage production may vary depending on the forage used.

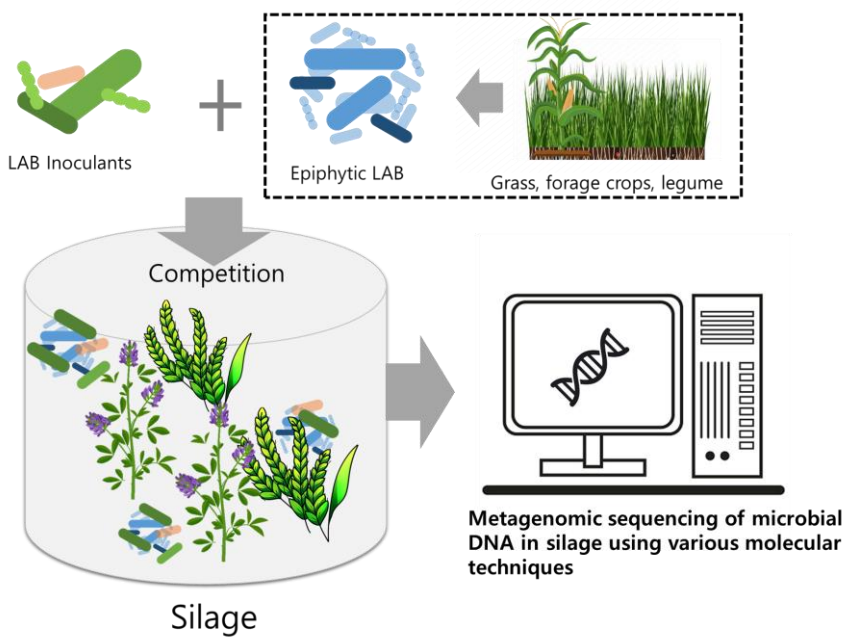

Figure 3. Associations among epiphytic lactic acid bacteria, lactic acid inoculants, silage fermentation (modified from Guo et al., 2023).

4.3. The roles of LAB in silage

Presently, research on silage LAB is motivated by innovative advancements to meet human needs, such as food safety, feed nutritional value, sustainable agriculture, and animal health (Amaral et al., 2020; Queiroz et al., 2018; Guo et al., 2023). The primary functions of LAB are to reduce pH levels and maintain nutrient quality in ensiled silages. LAB utilizes water-soluble carbohydrates (WSC) to generate beneficial organic acids, which can accelerate the acidification of the surrounding environment. This rapid acidification can improve the long-term quality of silage by inhibiting unfavorable microbial growth and preventing the production of harmful secondary metabolites (Soundharrajan et al., 2021). It is crucial to prevent unwanted bacterial, yeast, and mold growth by producing metabolites that enable the preservation of silage long-term without nutrient loss (Guo et al., 2023; Nazar et al., 2021; Soundharrajan et al., 2021; Kim et al., 2021).

LAB inoculants are widely used to improve the fermentation process and aerobic stability of silage (Okoye et al., 2022; Muck et al., 2018; Ávila and Carvalho, 2020). During ensiling, LAB inoculants can control the dynamics and functional alterations of microbial populations. Additionally, inoculants control intricate metabolic pathways during ensiling through metabolite transformation and the breakdown of forage substrates (Xu et al., 2017). LAB inoculants are considered more suitable as silage additives than other additives, such as chemical additives (sorbic, acetic, propionic, and benzoic formic acids) or cellulases because of their viability, environmental friendliness, increased dry matter recovery, fermentation properties, and impact on animal performance.

Adding LAB during the ensiling process increases lactic acid content while maintaining crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF) concentrations (Soundharrajan et al., 2019). According to another study, the addition of LAB significantly increases the nutritional characteristics of silage at various storage periods. WSC levels were lower in the silage that received LAB treatment because LAB can use WSC and convert it into organic acids (Borreani et al., 2018).

The current study focuses on both homo- and hetero-fermentative LAB. For silage production, two types of LAB additions are generally considered, namely homo-fermentative and hetero-fermentative LAB (Okoye et al., 2022). Homo-fermentative inoculants, including several species of *Lactobacillus*, *Pediococcus*, and *Lactococcus*, can increase lactic acid production, reduce pH levels, and prevent the degradation of proteins and sugar molecules in crops. Hetero-fermentative additives, such as *L. buchneri* and *L. brevis*, produce a combination of lactic acid and acetic acid that inhibits the growth of harmful substances like yeast and mold. Recent research on homo-fermentative LAB has demonstrated that they dominate during silage fermentation and can produce high-quality products (Li et al., 2022).

The most significant difference between homo-fermentative and hetero-fermentative bacteria is that homo-fermentative bacteria generate only lactic acid as the primary by-product of glucose fermentation, whereas hetero-fermentative bacteria produce ethanol, acetic acid, and CO_2 as by-products of glucose fermentation in addition to lactic acid (Giacon et al., 2021). Homo-fermentative LAB species have been extensively used to improve silage quality by hastening

the early stages of the ensiling process and quickly digesting water-soluble carbohydrates (WSC) to create lactic acid, which results in a rapid decrease in pH (Soundharrajan et al., 2021; Na et al., 2022). However, because of the possibility of pathogens rapidly metabolizing the lactic acid generated by homo-fermentative LAB during aerobic exposure, the limited conservation advantages were based only on the pH value that was reduced by homo-fermentative LAB (Weinberg and Muck, 1996) (Figure 4). In contrast, hetero-fermentative LAB inhibits aerobic degradation by producing large amounts of acetic acid. Combining LAB inoculants has proven advantageous for various crops and forage biomass because hetero-fermentative LAB is promising organisms for silage formation (Okoye et al., 2022). To fully understand their metabolic pathways, it is crucial to note that LAB inoculants have a finite biochemical profile, diverse metabolic profile, and relatively straightforward physiology. Furthermore, a complete understanding of the biochemistry, physiology, molecular biology, and genetics of these bacteria has helped increase the efficiency of their metabolic pathways (Nuryana et al., 2019). LAB produces many useful metabolites, such as organic acids, bacteriocins, and EPS, and has a variety of uses in creating, enhancing, and preserving fermented foods or silage. These pathways include glycolysis, lipolysis, and proteolysis (Bintsis, 2018).

4.4. The characteristics of triticale

Triticale is a hybrid of wheat and rye that was first developed in a German laboratory in the nineteenth century (Stace, 1987). Triticale (x *Triticosecale* Wittmack) is developed by crossing wheat (*Triticum* spp.) and rye

(*Secale cereal*) to combine the quality characteristics of wheat, and the adaptability, resistance to abiotic and biotic stresses of rye (Mergoum et al., 2019). The use of triticale depends on the specific characteristics of the plant species. Most triticale species have chemical compositions that are closer to wheat than to rye, making them suitable as a food source for both humans and animals. Triticale is commonly used in the production of silage for ruminants due to its high crude protein concentration (GlamoČLija et al., 2018; Soundharrajan et al., 2019; Lin et al., 2021). However, only a few studies have investigated silage production using triticale inoculated with LAB (Harper et al., 2017; Negi et al., 2022).

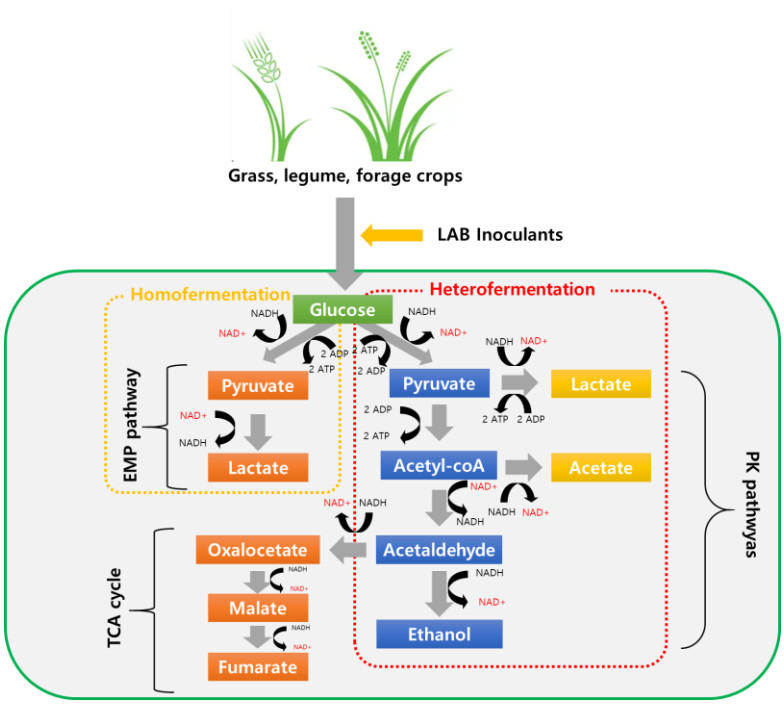

Figure 4. Schematic representation of LAB inoculants synthesizing organic acid metabolic pathway during silage fermentation (modified from Okoye et al., 2023; Kim et al., 2021). ATP: adenosine triphosphate; ADP: adenosine diphosphate; NAD+: nicotinamide adenine dinucleotide; NADH: nicotinamide adenine dinucleotide; EMP: Embden–Meyerhof pathway; PK: phosphoketolase pathway; TCA: tricarboxylic acid cycle

5. References

1. Abijaoudé, J, Morand-Fehr, P, Tessier, J, Schmidely, P,Sauvant, D. (2000). Influence of forage: concentrate ratio and type of starch in the diet on feeding behaviour, dietary preferences, digestion, metabolism and performance of dairy goats in mid lactation. *Animal Science*, 71(2), 359-368.
2. Adesogan, A, Newman, AT. (2010). Silage harvesting, soring, and feeding. University of Florida IFAS Extention SSAGR-177, https://edis.ifas.ufl.edu/publication/AG180(accessed July 22, 2023).
3. Aiken, G. (2016). Invited Review: Grazing management options in meeting objectives of grazing experiments. *The Professional Animal Scientist*, 32(1), 1-9.
4. Albonico, F, Barelli, C, Albanese, D, Manica, M, Partel, E, Rosso, F, Ripellino, S, Pindo, M, Donati, C,Zecconi, A. (2020). Raw milk and fecal microbiota of commercial Alpine dairy cows varies with herd, fat content and diet. *Plos one*, 15(8), e0237262.
5. Amaral, RC, Carvalho, BF, Costa, DM, Morenz, MJF, Schwan, RF,da Silva Ávila, CL. (2020). Novel lactic acid bacteria strains enhance the conservation of elephant grass silage cv. BRS Capiaçu. *Animal Feed Science and Technology*, 264, 114472.
6. Andrade, BGN, Bressani, FA, Cuadrat, RRC, Cardoso, TF, Malheiros, JM, de Oliveira, PSN, Petrini, J, Mourão, GB, Coutinho, LL, Reecy, JM, Koltes, JE, Neto, AZ, S, RdM, Berndt, A, Palhares, JCP, Afli, H,Regitano, LCA. (2022). Stool and ruminal microbiome components

associated with methane emission and feed efficiency in nelore beef cattle. *Front Genet*, 13, 812828.

7. Aricha, H, Simujide, H, Wang, C, Zhang, J, Lv, W, Jimisi, X, Liu, B, Chen, H, Zhang, C, He, L, Cui, Y, Gao, R,Aorigele, C. (2021). Comparative analysis of fecal microbiota of grazing Mongolian cattle from different regions in Inner Mongolia, China. *Animals (Basel)*, 11(7).
8. Ávila, C, Carvalho, B. (2020). Silage fermentation—updates focusing on the performance of micro-organisms. *Journal of Applied Microbiology,* 128(4), 966-984.
9. Backhed, F, Ley, RE, Sonnenburg, JL, Peterson, DA, Gordon, JI. (2005). Host-bacterial mutualism in the human intestine. *Science*, 307(5717), 1915-1920.
10. Bai, Y,Cotrufo, MF. (2022). Grassland soil carbon sequestration: current understanding, challenges, and solutions. *Science*, 377(6606), 603-608.
11. Bernardet, J-F, Nakagawa, Y. (2006). An introduction to the family Flavobacteriaceae. *The prokaryotes*, 7, 455-480.
12. Bintsis, T. (2018). Lactic acid bacteria as starter cultures: An update in their metabolism and genetics. *AIMS microbiology*, 4(4), 665.
13. Borreani, G, Tabacco, E, Schmidt, R, Holmes, B, Muck, R. (2018). Silage review: factors affecting dry matter and quality losses in silages. *Journal of Dairy Science*, 101(5), 3952-3979.
14. Broberg, A, Jacobsson, K, Strӧm, K, Schnürer, J. (2007). Metabolite profiles of lactic acid bacteria in grass silage. *Applied and environmental*

microbiology, 73(17), 5547-5552.

15. Burns, P, Borgo, MF, Binetti, A, Puntillo, M, Bergamini, C, Páez, R, Mazzoni, R, Reinheimer, J, Vinderola, G. (2018). Isolation, characterization and performance of autochthonous spray dried lactic acid bacteria in maize micro and bucket-silos. *Frontiers in Microbiology*, 9, 2861.
16. Callaway, T, Dowd, S, Edrington, T, Anderson, R, Krueger, N, Bauer, N, Kononoff, P, Nisbet, D. (2010). Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. *Journal of animal science*, 88(12), 3977-3983.
17. Cao, Y, Takahashi, T,Horiguchi, K-i. (2009). Effects of addition of food by-products on the fermentation quality of a total mixed ration with whole crop rice and its digestibility, preference, and rumen fermentation in sheep. *Animal Feed Science and Technology*, 151(1), 1-11.
18. Carroll, E, Hungate, R. (1954). The magnitude of the microbial fermentation in the bovine rumen. *Applied Microbiology*, 2(4), 205-214.
19. Chassard, C, Delmas, E, Robert, C, Bernalier-Donadille, A. (2010). The cellulose-degrading microbial community of the human gut varies according to the presence or absence of methanogens. *FEMS microbiology ecology*, 74(1), 205-213.
20. Chang, JB. (2018). The effect of forage policy on feed cost in Korea. *Agriculture*, 8(6), 72.
21. Chen, B, Tang, G, Guo, W, Lei, J, Yao, J, Xu, X. (2021a). Detection

of the core bacteria in colostrum and their association with the rectal microbiota and with milk composition in two dairy cow farms. *Animals (Basel)*, 11(12), 3363.

22. Chen, H, Wang, C, Huasai, S, Chen, A. (2021b). Effects of dietary forage to concentrate ratio on nutrient digestibility, ruminal fermentation and rumen bacterial composition in Angus cows. *Scientific Reports*, 11(1), 17023.
23. Chen, X, Wang, J, Wu, Y, Liu, J. (2008). Effects of chemical treatments of rice straw on rumen fermentation characteristics, fibrolytic enzyme activities and populations of liquid-and solid-associated ruminal microbes in vitro. *Animal Feed Science and Technology*, 141(1-2), 1-14.
24. Choi, YJ, Lee, SS. (2019). Effects of lactic acid bacteria inoculant on fermentation quality and *in vitro* rumen fermentation of total mixed ration. *Journal of the Korean socieity of grassland and forage science*, 39(3), 132-140.
25. Cockburn, DW, Koropatkin, NM. (2016). Polysaccharide degradation by the intestinal microbiota and its influence on human health and disease. *Journal of molecular biology*, 428(16), 3230-3252.
26. Conant, RT, Cerri, CE, Osborne, BB, Paustian, K. (2017). Grassland management impacts on soil carbon stocks: a new synthesis. *Ecological Applications*, 27(2), 662-668.
27. Cotta, M,Forster, R. (2006). The family *Lachnospiraceae*, including the genera *Butyrivibrio*, *Lachnospira* and *Roseburia*. *Prokaryotes*, 4, 1002-1021.
28. Danner, H, Holzer, M, Mayrhuber, E, Braun, R. (2003). Acetic acid

increases stability of silage under aerobic conditions. *Applied and environmental microbiology*, 69(1), 562-567.

29. Dong, H, Wang, S, Jia, Y, Ni, Y, Zhang, Y, Zhuang, S, Shen, X, Zhao, R. (2013). Long-term effects of subacute ruminal acidosis (SARA) on milk quality and hepatic gene expression in lactating goats fed a high-concentrate diet. *PLoS One*, 8(12), e82850.
30. Driehuis, F, Wilkinson, J, Jiang, Y, Ogunade, I, Adesogan, A. (2018). Silage review: Animal and human health risks from silage. *Journal of Dairy Science*, 101(5), 4093-4110.
31. Ghosh, S, Pramanik, S. (2021). Structural diversity, functional aspects and future therapeutic applications of human gut microbiome. *Archives of microbiology*, 203(9), 5281-5308.
32. GLAMOČLIJA, N, STARČEVIĆ, M, ĆIRIĆ, J, ŠEFER, D, GLIŠIĆ, M, BALTIĆ, M, MARKOVIĆ, R, SPASIĆ, M, GLAMOČLIJA, Đ. (2018). The importance of Triticale in animal nutrition. *Veterinary journal of Republic of Srpaka*, 1, 73-94.
33. Guo, P, Zhang, K, Ma, X, He, P. (2020). Clostridium species as probiotics: potentials and challenges. *Journal of animal science and biotechnology*, 11(1), 1-10.
34. Guo, X, Xu, D, Li, F, Bai, J, Su, R. (2023). Current approaches on the roles of lactic acid bacteria in crop silage. *Microbial Biotechnology*, 16(1), 67-87.
35. Gurung, M, Li, Z, You, H, Rodrigues, R, Jump, DB, Morgun, A, Shulzhenko, N. (2020). Role of gut microbiota in type 2 diabetes

pathophysiology. *EBioMedicine*, 51, 102590.

36. Hamilton, MJ, Weingarden, AR, Unno, T, Khoruts, A, Sadowsky, MJ. (2013). High-throughput DNA sequence analysis reveals stable engraftment of gut microbiota following transplantation of previously frozen fecal bacteria. *Gut microbes*, 4(2), 125-135.
37. Han, H, Ogata, Y, Yamamoto, Y, Nagao, S, Nishino, N. (2014). Identification of lactic acid bacteria in the rumen and feces of dairy cows fed total mixed ration silage to assess the survival of silage bacteria in the gut. *Journal of dairy science*, 97(9), 5754-5762.
38. Han, J, Hongdong Nonghyup, H, Hong, S. (2010). Economic Effects of Total Mixed Fermentation on Korean Beef Feeding Management. *Korean Journal of Agricultural Management and Policy*.
39. Harper, MT, Oh, J, Gialllngo, F, Roth, GW, Hristov, AN. (2017). Inclusion of wheat and triticale silage in the diet of lactating dairy cows. *Journal of Dairy Science*, 100(8), 6151-6163.
40. Henderson, G, Cox, F, Ganesh, S, Jonker, A, Young, W, Janssen, PH. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific reports*, 5(1), 14567.
41. Jackson, RB, Lajtha, K, Crow, SE, Hugelius, G, Kramer, MG, Piñeiro, G. (2017). The ecology of soil carbon: pools, vulnerabilities, and biotic and abiotic controls. *Annual review of ecology, evolution, and systematics*, 48, 419-445.
42. Jami, E, White, BA, Mizrahi, I. (2014). Potential role of the bovine

rumen microbiome in modulating milk composition and feed efficiency. *PloS one*, 9(1), e85423.

43. Janssen, PH. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and environmental microbiology*, 72(3), 1719-1728.
44. Jia, X, He, Y, Kang, Z, Chen, S, Sun, W, Wang, J, Lai, S. (2023). Comparison of fecal microbiota communities between primiparous and multiparous cows during non-pregnancy and pregnancy. *Animals*, 13(5), 869.
45. Johansson, ME, Larsson, JMH, Hansson, GC. (2011). The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host–microbial interactions. *Proceedings of the national academy of sciences*, 108(supplement_1), 4659-4665.
46. Kim, DH, Amanullah, SM, Lee, HJ, Joo, YH, Kim, SC. (2015). Effect of microbial and chemical combo additives on nutritive value and fermentation characteristic of whole crop barley silage. *Asian-Australasian Journal of Animal Sciences*, 28(9), 1274-1280.
47. Kim, DH, Lee, KD, Choi, KC. (2021). Role of LAB in silage fermentation: Effect on nutritional quality and organic acid production—An overview, *AIMS Agriculture and Food*, 6(1): 216–234, .
48. Kim, JD, Seo, M, Lee, SC,Han, K-J. (2020a). Review of the current forage production, supply, and quality measure standard in South Korea. *Journal of The Korean Society of Grassland and Forage Science*, 40(3), 149-155.

49. Kim, JH, Park, HS, Choi, KC. (2020b). Effect on different yields of drying rate of Italian ryegrass hay making during spring season. *Journal of The Korean Society of Grassland and Forage Science*, 40(4), 216-220.
50. Kim, K, Kim, K, Lee, S, Oh, Y, Chung, C, Kim, K. (2003). Effects of total mixed rations on ruminal characteristics, digestibility and beef production of Hanwoo steers. *Journal of Animal Science and Technology*, 45(3), 387-396.
51. Kim, M, Kim, J, Kuehn, L, Bono, J, Berry, E, Kalchayanand, N, Freetly, HC, Benson, AK, Wells, J. (2014). Investigation of bacterial diversity in the feces of cattle fed different diets. *Journal of animal science*, 92(2), 683-694.
52. Kim, M, Wells, JE. (2016). A meta-analysis of bacterial diversity in the feces of cattle. *Current microbiology*, 72, 145-151.
53. Kim, S, Alam, M, Gu, M, Park, K, Jeon, CO, Ha, JK, Cho, K, Lee, S. (2012). Effect of total mixed ration with fermented feed on ruminal in vitro fermentation, growth performance and blood characteristics of Hanwoo steers. *Asian-Australasian Journal of Animal Sciences*, 25(2), 213.
54. Kim, S, Jung, K, Kim, J, Lee, S, Baek, K, Choi, C. (2007). Effect of Feeding High Guality Hay on Performance and Physico-chemical Characteristics of Carcass of Hanwoo Steers. *Journal of Animal Science and Technology*, 49(6), 783-800.
55. Kim, SH, Kim, TK. (2018). Economic valuation of multi-functionality on an eco-pastoral system in Alpine grassland. *Journal of the Korean society of Grassland and Forage Science*, 38(4), 298-309.

56. Kim, TI, Mayakrishnan, V, Lim, DH, Yeon, JH, Baek, KS. (2018). Effect of fermented total mixed rations on the growth performance, carcass and meat quality characteristics of Hanwoo steers. *Animal Science Journal*, 89(3), 606-615.
57. Kotz, A, Azevedo, PA, Khafipour, E, Plaizier, JC. (2020). Effects of the dietary grain content on rumen and fecal microbiota of dairy cows. *Canadian Journal of Animal Science*, 101(2), 274-286.
58. Kosis (Korean Statostocal Information Sevice). (2023). Agriculture, forestary and fishery survey in 2023. https://kosis.kr/statHtml/statHtml.do?orgId=101&tblId=DT_1EO200&conn_path=I2 (accessed July 22, 2023)
59. Kung Jr, L, Shaver, R, Grant, R, Schmidt, R. (2018). Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *Journal of dairy Science*, 101(5), 4020-4033.
60. Lambiase, A. (2014). The family sphingobacteriaceae. In The prokaryotes: other major lineages of bacteria and the archaea, *Spinger Berlin hedelberg*, No 15412, 907-914.
61. Lardy, G, Waterman, R. (2020). Grazing animal nutrition: forages, *The Science of Grassland Agriculture*, 2, 815-826.
62. Latham, E, Weldon, K, Wickersham, T, Coverdale, J, Pinchak, W. (2018). Responses in the rumen microbiome of *Bos taurus* and *indicus* steers fed a low-quality rice straw diet and supplemented protein. *Journal of animal science*, 96(3), 1032-1044.
63. Lee, J-M, Kim, T-W, Kim, J-H, Cho, S-H, Seong, P-N, Jung, M-O,

Cho, Y-M, Park, B-Y, Kim, D-H. (2009). Comparison of chemical, physical and sensory traits of longissimus lumborum Hanwoo beef and Australian Wagyu beef. *Food Science of Animal Resources*, 29(1), 91-98.

64. Lee, SY, Cheon, DW, Park, HS, Choi, KC, Yang, SH, Lee, BH, Lee, BU, Jung, JS. (2022). Estimation on the consumption patterns and consciousness of domestic forage in Korean native cattle farmers. *Journal of The Korean Society of Grassland and Forage Science*, 42(1), 17-25.
65. Ley, RE, Bäckhed, F, Turnbaugh, P, Lozupone, CA, Knight, RD, Gordon, JI. (2005). Obesity alters gut microbial ecology. *Proceedings of the national academy of sciences*, 102(31), 11070-11075.
66. Li, Y, da Silva, EB, Li, J, Kung, L. (2022). Effect of homo-fermentative lactic acid bacteria inoculants on fermentation characteristics and bacterial and fungal communities in alfalfa silage. *Fermentation*, 8(11), 621.
67. Li, Y, Wang, F, Nishino, N. (2016). Lactic acid bacteria in total mixed ration silage containing soybean curd residue: their isolation, identification and ability to inhibit aerobic deterioration. *Asian-Australasian Journal of Animal Sciences*, 29(4), 516.
68. Lin, C, Bolsen, KK, Brent, BE, Hart, RA, Dickerson, JT, Feyerherm, AM, Aimutis, WR. (1992). Epiphytic microflora on alfalfa and whole-plant Corn. *Journal of Dairy Science*, 75(9), 2484-2493.
69. Lin, H, Lin, S, Awasthi, MK, Wang, T, Xu, P. (2021). Exploring the bacterial community and fermentation characteristics during silage fermentation of abandoned fresh tea leaves. *Chemosphere*, 283, 131234.

70. Liu, B, Huan, H, Gu, H, Xu, N, Shen, Q, Ding, C. (2019). Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages. *Bioresource Technology*, 273, 212-219.
71. MAFRA. (2022). The current situation of frage production and supplementation policy. *Ministry of Agriculture, food and Rural Affairs*.
72. Mahowald, MA, Rey, FE, Seedorf, H, Turnbaugh, PJ, Fulton, RS, Wollam, A, Shah, N, Wang, C, Magrini, V, Wilson, RK. (2009). Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. *Proceedings of the National Academy of Sciences*, 106(14), 5859-5864.
73. Mao, S, Zhang, R, Wang, D, Zhu, W. (2012). The diversity of the fecal bacterial community and its relationship with the concentration of volatile fatty acids in the feces during subacute rumen acidosis in dairy cows. *BMC veterinary research*, 8, 1-13.
74. Mazmanian, SK, Round, JL, Kasper, DL. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature*, 453(7195), 620-625.
75. McCann, JC, Wiley, LM, Forbes, TD, Rouquette Jr, FM, Tedeschi, LO. (2014a). Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures. *PloS one*, 9(3), e91864.
76. McCann, JC, Wickersham, TA, Loor, JJ. (2014b). High-throughput methods redefine the rumen microbiome and its relationship with

nutrition and metabolism. Bioinformatics and biology insights, 8, BBI. S15389.

77. McDonald, P, Henderson, A, Heron, SJE. (1991). The biochemistry of silage. *Chalcombe publications*, Marlow, UK.
78. Mergoum, M, Sapkota, S, Ahmed EAE, Sepehr, MN, Seyed, P, Mohammed, SA, Wesam, AH. (2019). Triticale (x *Triticosecale* Wittmack) Breeding. Advances in Plant Breeding Stractegies: Cereals: Volume 5, *Spinger*, 405-451.
79. Moore, W, Holdeman, LV. (1974). Special problems associated with the isolation and identification of intestinal bacteria in fecal flora studies. *The American journal of clinical nutrition*, 27(12), 1450-1455.
80. Mote, RS, Hill, NS, Skarlupka, JH, Turner, ZB, Sanders, ZP, Jones, DP, Suen, G, Filipov, NM. (2019). Response of beef cattle fecal microbiota to grazing on toxic tall fescue. *Applied and Environmental Microbiology*, 85(15), e00032-00019.
81. Muck, R, Nadeau, E, McAllister, T, Contreras-Govea, F, Santos, M, Kung Jr, L. (2018). Silage review: Recent advances and future uses of silage additives. *Journal of dairy science*, 101(5), 3980-4000.
82. Muck, RE. (2013). Recent advances in silage microbiology, *Agricultural and food science,* 22(1), 3-15.
83. Mullan, S, Bunglavan, SJ, Rowe, E, Barrett, DC, Lee, MR, Ananth, D, Tarlton, J. (2020). Welfare challenges of dairy cows in India identified through on-farm observations. *Animals*, 10(4), 586.
84. Na, N, Qili, M, Wu, N, Sun, L, Xu, H, Zhao, Y, Wei, X, Xue, Y, Tao,

Y. (2022). Bacterial community and fermentation quality of ensiling alfalfa with commercial lactic acid bacterial additives. *Front Microbiol*, 13, 836899.

85. Nascimento Agarussi, MC, Gomes Pereira, O, Paula, RAd, Silva, VPd, Santos Roseira, JP, Fonseca e Silva, F. (2019). Novel lactic acid bacteria strains as inoculants on alfalfa silage fermentation. *Scientific reports*, 9(1), 1-9.
86. Negi, T, Kumar, Y, Sirohi, R, Singh, S, Tarafdar, A, Pareek, S, Awasthi, MK, Sagar, NA. (2022), Advances in bioconversion of spent tea leaves to value-added products. *Bioresource Technology*, 346, 126409.
87. Nilsson, Å. (2016). Role of sphingolipids in infant gut health and immunity. *The Journal of Pediatrics*, 173, S53-S59.
88. Nishino, N, Harada, H, Sakaguchi, E. (2003). Evaluation of fermentation and aerobic stability of wet brewers' grains ensiled alone or in combination with various feeds as a total mixed ration. *Journal of the Science of Food and Agriculture*, 83(6), 557-563.
89. Nuryana, I, Andriani, A. Lisdiyanti, Yopi. (2019). Analysis of organic acids produced by lactic acid bacteria. IOP Conference Series: Earth and Environmental Science, *IOP Publishing*, 251, 012054.
90. Nyonyo, T, Shinkai, T, Mitsumori, M. (2014). Improved culturability of cellulolytic rumen bacteria and phylogenetic diversity of culturable cellulolytic and xylanolytic bacteria newly isolated from the bovine rumen. *FEMS Microbiology ecology*, 88(3), 528-537.
91. Oikonomou, G, Teixeira, AGV, Foditsch, C, Bicalho, ML, Machado,

VS, Bicalho, RC. (2013). Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth. *PloS one*, 8(4), e63157.

92. Okoye, CO, Wang, Y, Gao, L, Wu, Y, Li, X, Sun, J, Jiang, J. (2022). The performance of lactic acid bacteria in silage production: a review of modern biotechnology for silage improvement. *Microbiological Research*, 127212.
93. Opsi, F, Fortina, R, Tassone, S, Bodas, R, López, S. (2012). Effects of inactivated and live cells of Saccharomyces cerevisiae on in vitro ruminal fermentation of diets with different forage: concentrate ratio. *The Journal of Agricultural Science*, 150(2), 271-283.
94. Ortega Cerrilla, ME, Mendoza Martínez, G. (2003). Starch digestion and glucose metabolism in the ruminant: A review. *Interciencia*, 28(7), 380-386.
95. Pahlow, G, Muck, RE, Driehuis, F, Elferink, SJO, Spoelstra, SF. (2003). Microbiology of ensiling. *Silage science and technology*, 42, 31-93.
96. Parker, BJ, Wearsch, PA, Veloo, AC, Rodriguez-Palacios, A. (2020). The genus Alistipes: gut bacteria with emerging implications to inflammation, cancer, and mental health. *Frontiers in immunology*, 11, 906.
97. Peeters, A. (2009). Importance, evolution, environmental impact and future challenges of grasslands and grassland-based systems in Europe. *Grassland science*, 55(3), 113-125.

98. Plaizier, J, Khafipour, E, Li, S, Gozho, G, Krause, D. (2012). Subacute ruminal acidosis (SARA), endotoxins and health consequences. *Animal Feed Science and Technology*, 172(1-2), 9-21.

99. Polansky, O, Sekelova, Z, Faldynova, M, Sebkova, A, Sisak, F, Rychlik, I. (2016). Important metabolic pathways and biological processes expressed by chicken cecal microbiota. *Applied and environmental microbiology*, 82(5), 1569-1576.

100. Queiroz, O, Ogunade, I, Weinberg, Z, Adesogan, A. (2018). Silage review: Foodborne pathogens in silage and their mitigation by silage additives. *Journal of dairy science*, 101(5), 4132-4142.

101. Ren, H, Su, X, Bai, H, Yang, Y, Wang, H, Dan, Z, Lu, J, Wu, S, Cai, C, Cao, Y, Lei, X, Yao, J. (2019). Specific enrichment of microbes and increased ruminal propionate production: the potential mechanism underlying the high energy efficiency of Holstein heifers fed steam-flaked corn. *AMB Express*, 9(1), 209.

102. Rice, WC, Galyean, ML, Cox, SB, Dowd, SE, Cole, NA. (2012). Influence of wet distillers grains diets on beef cattle fecal bacterial community structure. *BMC microbiology*, 12(1), 1-13.

103. Salyers, A, Vercellotti, J, West, S, Wilkins, T. (1977). Fermentation of mucin and plant polysaccharides by strains of Bacteroides from the human colon. *Applied and environmental microbiology*, 33(2), 319-322.

104. Sghir, A, Gramet, G, Suau, A, Rochet, V, Pochart, P, Dore, J. (2000). Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Applied and Environmental*

Microbiology, 66(5), 2263-2266.

105. Shah, T, Ding, L, Ud Din, A, Hassan, F-u, Ahmad, AA, Wei, H, Wang, X, Yan, Q, Ishaq, M, Ali, N. (2022). Differential effects of natural grazing and feedlot feeding on yak fecal microbiota. *Frontiers in Veterinary Science*, 9, 252.

106. Shanks, OC, Kelty, CA, Archibeque, S, Jenkins, M, Newton, RJ, McLellan, SL, Huse, SM, Sogin, ML. (2011). Community structures of fecal bacteria in cattle from different animal feeding operations. *Applied and environmental microbiology*, 77(9), 2992-3001.

107. Shin, N-R, Whon, TW, Bae, J-W. (2015). Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends in biotechnology*, 33(9), 496-503.

108. Solar, C, Escalona, A, Garrido, D. (2019). The gut microbiome after bariatric surgery. *Microbiome and Metabolome in Diagnosis, Therapy, and other Strategic Applications*, 235-242.

109. Song, J, Ma, Y, Zhang, H, Wang, L, Zhang, Y, Zhang, G. (2023). Fermented total mixed ration alters rumen fermentation parameters and microbiota in dairy cows. *Animals*, 13(6), 1062.

110. Soundharrajan, I, Kuppusamy, P, Park, HS, Kim, JH, Kim, WH, Jung, JS, Choi, KC. (2019). Lactic acid bacteria mixture as inoculants on low moisture italian ryegrass silage fermentation. *Journal of The Korean Society of Grassland and Forage Science*, 39(3), 127-131.

111. Soundharrajan, I, Park, HS, Rengasamy, S, Sivanesan, R, Choi, KC. (2021). Application of future prospective of lactic acid bacteria as

natural additives for silage prodcution-a review. *Apllied Sciences*, 11(17), 8127.

112. Stace CA. (1987). Triticale: a case of nomenclatural mistreatment. *Taxcon*, 36(20), 445-452.

113. Stewart, RD, Auffret, MD, Warr, A, Wiser, AH, Press, MO, Langford, KW, Liachko, I, Snelling, TJ, Dewhurst, RJ, Walker, AW. (2018). Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nature communications*, 9(1), 870.

114. Subepang, S, Suzuki, T, Phonbumrung, T, Sommart, K. (2019). Enteric methane emissions, energy partitioning, and energetic efficiency of zebu beef cattle fed total mixed ration silage. *Asian-Australasian Journal of Animal Sciences*, 32(4), 548.

115. Sun, Y, Hu, J, Zhang, S, He, H, Nie, Q, Zhang, Y, Chen, C, Geng, F, Nie, S. (2021). Prebiotic characteristics of arabinogalactans during in vitro fermentation through multi-omics analysis. *Food and Chemical Toxicology*, 156, 112522.

116. Thomas, F, Hehemann, J-H, Rebuffet, E, Czjzek, M, Michel, G. (2011). Environmental and gut bacteroidetes: the food connection. *Frontiers in microbiology*, 2, 93.

117. Van Soest, PJ. (1994). Nutritional ecology of the ruminant, *Cornell University Press*, Ithaca and London, 140-155.

118. Vasupen, K, Yuangklang, C, Witayakun, S, Srinanuan, P. (2005). Effect of different moisture on quality of fermented total mixed ration. *Proceedings of 43rd Kasetsart University Annual Conference*, Thailand.

119. Wang, F, Nishino, N. (2008). Ensiling of soybean curd residue and wet brewers grains with or without other feeds as a total mixed ration. *Journal of Dairy Science*, 91(6), 2380-2387.

120. Wang, T, Teng, K, Cao, Y, Shi, W, Xuan, Z, Zhou, J, Zhang, J, Zhong, J. (2020). Effects of Lactobacillus hilgardii 60TS-2, with or without homofermentative Lactobacillus plantarum B90, on the aerobic stability, fermentation quality and microbial community dynamics in sugarcane top silage. *Bioresource Technology*, 312, 123600.

121. Wang, Y, Nan, X, Zhao, Y, Jiang, L, Wang, H, Zhang, F, Hua, D, Liu, J, Yang, L, Yao, J. (2022). Discrepancies among healthy, subclinical mastitic, and clinical mastitic cows in fecal microbiome and metabolome and serum metabolome. *Journal of Dairy Science*, 105(9), 7668-7688.

122. Weinberg, ZG, Muck, R. (1996). New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiology Reviews*, 19(1), 53-68.

123. Wen, L, Ley, RE, Volchkov, PY, Stranges, PB, Avanesyan, L, Stonebraker, AC, Hu, C, Wong, FS, Szot, GL, Bluestone, JA. (2008). Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature*, 455(7216), 1109-1113.

124. White, RP, Murray, S, Rohweder, M, Prince, S, Thompson, K. (2000). Grassland ecosystems. *World Resources Institute Washington*, DC, USA.

125. Wilinson, JM, Bolsen, KK, Lin CJ. (2003). History of silage. *Silage science and technology*, 42, 1-30.

126. Wilkinson, JM, Lee, MR, Rivero, MJ, Chamberlain, AT. (2020). Some challenges and opportunities for grazing dairy cows on temperate pastures. *Grass and Forage Science*, 75(1), 1-17.

127. Woese, C, Yang, D, Mandelco, L, Stetter, K. (1990). The flexibacter-flavobacter connection. *Systematic and applied microbiology*, 13(2), 161-165.

128. Wood, J, Scott, KP, Avgustin, G, Newbold, CJ, Flint, HJ. (1998). Estimation of the relative abundance of different *Bacteroides* and *Prevotella* ribotypes in gut samples by restriction enzyme profiling of PCR-amplified 16S rRNA gene sequences. *Applied and Environmental Microbiology*, 64(10), 3683-3689.

129. Xu, Z, He, H, Zhang, S, Kong, J. (2017). Effects of inoculants Lactobacillus brevis and Lactobacillus parafarraginis on the fermentation characteristics and microbial communities of corn stover silage. *Scientific Reports*, 7(1), 1-9.

130. Zhang, J, Kumai, S. (2000). Effluent and aerobic stability of cellulase and LAB-treated silage of napier grass (*Pennisetum purpureum Schum*). *Asian-Australasian Journal of Animal Sciences*, 13(8), 1063-1067.

131. Zhang, Z, Yang, L, He, Y, Luo, X, Zhao, S, Jia, X. (2021). Composition of fecal microbiota in grazing and feedlot angus beef cattle. *Animals*, 11(11), 3167.

132. Zhao, Y, Liu, Z, Wu, J. (2020). Grassland ecosystem services: a systematic review of research advances and future directions. *Landscape Ecology*, 35, 793-814.

CHAPTER THREE

Study 1

Microbiota and serum metabolic profile changes in Hanwoo steers in response to diets feeding system

1. Abstract

Diversity of bacteria and their function in cattle gastrointestinal tracts can influence animal welfare. Next-generation sequencing (NGS) was used to investigate microbial diversity in fecal of Hanwoo steers reared under natural grazing (GS) and housing (HS) systems. Additionally, serum metabolic parameters, such as liver and kidney markers, mineral and lipid content changes, and their correlation with pyrotags, were studied. A total 6,468 ± 87.86 operational taxonomic unites (OTUs) were identified in both steer groups, of which 3,538 ± 38.17 OTUs were from grazing steer and 2,930 ± 94.06 OTUs were from GS. Chao1 index analysis revealed a higher bacterial richness in GS. The dominant bacterial taxa were *Bacteroidetes* and *Firmicutes*. GS showed lower *Bacteroidetes* and higher *Firmicutes* abundance than HS. The serum of HS showed consistent increases in gammaglutamyl transpeptidase (γGTP), glucose (GLU), total cholesterol (T-CHO) and triglyceride (TG) levels. The impact of GS on animal health and serum metabolic markers was strongly correlated with microbiota. As shown in this study, grazing has a significant impact on the fecal microbiota at the phylum and family levels, as well as the serum biochemical metabolites of Hanwoo steers.

2. Introduction

Microbiota associated with the gut is important to maintaining the health of both animals and humans due to their symbiotic relationship. Several genes within the intestinal microbiota are involved in host health and survival, allowing optimal energy from diets to be harvested and stored (Turnbaugh et al., 2006). The genes also facilitate the production of vitamins, cofactors or biologically active molecules associated with microbial activity, including short-chain fatty acids, indole, tryptamine, peptidoglycans, and lipopolysaccharides, which are essential to human health (Gao et al., 2018; Lazar et al., 2018; Ríos-Covián et al., 2018).

A major component of rural landscapes is livestock forming, particularly ruminants (sheep and cattle), which provide essential services to society, including improved soil health, biodiversity management, recreational activities, and community support (Dumont et al., 2018; Rivero and Lee, 2022). As one of the most competitive and suitable feeding systems for cows globally, pasture grazing in situ has a low environmental footprint, is beneficial for animal health, and is relatively inexpensive to produce as well as to utilize (Wilkinson et al., 2020). In terms of animal welfare, it is believed that restricting animals' access to resources may have an adverse effect on their health. It has been shown that animals with an inability to access natural feeding behaviors are more likely to develop stereotypical and other abnormal behaviors (Smid et al., 2020). In a small study in Kerala, India, cattle restricted to accessing forages or grazing developed tongue rolling stereotypy, whereas cattle that were given free access to forages or grazing did not exhibit this condition (Mullan et al., 2020). Several

studies have shown that grazing in diverse vegetation improves mineral intake balance, reduces oxidative stress, and improves antioxidant levels in cattle (Haga et al., 2016; Nakajima et al., 2019).

The impact of grazing on animal welfare may vary depending on grazing conditions, including pasture status and environmental factors. Animal health, digestibility, growth rate, and meat quality differ significantly between grazing and feedlot systems. A grazing animal has a lower fatty acid content in its meat, and higher levels of vitamins than a feedlot animal (Prache et al., 2020). Previous report claimed that the housing animals produced more polyunsaturated fatty acids (Wilkinson et al., 2020), particularly omega-3 polyunsaturated fatty acids, and conjugated linoleic acid, which would enhance the nutritional value of the product while grazing animals had higher levels of total protein and casein as well as fat soluble vitamins (β-carotene and α-tocopherol) in pasture based organic milk (Manzi and Durazzo, 2017; O'Callaghan et al., 2016). In recent years, people have been expected to consume high-energy, balanced meals with a low fat content for their health preferences (Daley et al., 2010). Several studies have been conducted on the influence of the bovine microbiota on the host's energy and metabolism. Moreover, recent next-generation sequencing (NGS) studies have demonstrated that the enteric microbiota shifts in response to animal performance across multiple species. Particularly in cattle, diets/ feed additives/ feed methods are contributing to changes in rumen and fecal microbial communities (Shah et al., 2022; Mote et al., 2019; Niu et al., 2022; Shanks et al., 2011) as well as animal welfare. Nonetheless, potential changes in the microbial communities of grazing cattle, including orchard grass, perennial

ryegrass, Kentucky bluegrass, white clover, and tall fescue have not been thoroughly studied. Here, we investigated changes in the composition of the fecal microbiota community and the dynamics between grazing and housing feeding systems of Hanwoo steers. In order to accomplish this, NGS was used to analyze the fecal microbiota of steers by targeting the V4 region of the 16S rRNA gene. Fecal samples were collected from housing and grazing animals after seven months and correlated with animal welfare and serum biochemical parameters.

3. Materials and methods

3.1. Pastures and grassland management

At the Hanwoo farm in Jeongeup, Jeollabuk-do, South Korea, tall fescue (7.5 kg ha^{-1}), orchard grass (17 kg ha^{-1}), perennial ryegrass (3 kg ha^{-1}), Kentucky bluegrass (3 kg ha^{-1}) and white clover (2 kg ha^{-1}) were sown. Chemical fertilizers containing 21% nitrogen, 17% phosphoric acid, and 17% potassium were applied to the grassland. During the early spring, 20 bags of fertilizer were applied per hectare to grassland. Following the first grazing, 15 bags of fertilizer per hectare were applied, followed by 5 bags per hectare for each subsequent grazing period (2nd to 5th). Following the last grazing, 10 bags of fertilizer were applied per hectare. A total of seven paddocks were used for the experimental study.

3.2. Animals and feeding systems

A study was conducted at the Hanwoo farm in Jeongeup, Jeollabuk-do, South Korea. It was conducted in accordance with the animal care and standard guidelines of the National Institute of Animal Science, South Korea (Approval number NIAS-2020-443). We recruited a total of twenty-six Korean native breed cattle called Hanwoo steers for this study. The steers were representative of both the feedlot feeding system (n = 6, average initial bodyweight 260.8kg) and the grazing system (An average initial bodyweight 219.8kg; randomly six steers (n=6) were selected for data analysis out of 20 steers). A classification was made based on the practice of housing feedlot feeding- HS (concentrate and rice straw) and grazing feeding- GS (pastures containing tall fescue, orchard grass, perennial ryegrass, Kentucky bluegrass, and white clover). The composition of feed and

the concentrations of nutrients are presented in Table 2. The animals in housing feedlots were fed rice straw and concentrate between 9:00 a.m. and 04:00 p.m. Hanwoo steers were grazed using a rotational grazing system in which each pasture was divided into seven of 0.3~0.5 ha each. Depending on the season's forage growth, rotational pastures were grazed with differing grazing periods. There is an average grazing period of three to eight days followed by a rest period of 21 days in a rotational pasture. The growth of forage was slower during periods of hot summers. Thus, grazing was reduced and resting periods were lengthened (3-4 days grazing with 30-40 days resting periods). This resulted in a significant increase in forage regrowth. Grazing began in late April and ended in mid-November. Hanwoo steer grazed from 8 a.m. to 6 p.m. The steer grazed for 24 days in May, 25 days in June, 10 days in July, 25 days in August, 23 days in September, 27 days in October, and 7 days in November. When there was a drought or heavy rainfall, grazing cattle were fed hay harvested from pastures containing tall fescue, orchard grass, perennial ryegrass, Kentucky bluegrass, and white clover. We also determined the total bodyweight (every month) and average daily weight gain, the feed efficiency, and the feed intake.

Table 2. Ingredient composition and chemical analysis of concentrate

Items	Compositions (% DM)
Ingredients	
Cornflake	25.00
Wheat	18.00
Gluten feed	8.00
Tapioca residue	4.58
Wheat bran	11.79
Palm kernel meal	8.00
Coconut oil meal	3.00
Rapseed meal	5.00
Soybean meal	7.39
Disillers dried grains	2.22
Limestone	2.17
Molasses	3.00
Salt	0.81
Probiotics	0.05
Magnesium oxide	0.30
Sodium bicarbonate	0.30
Vitamin premix[a]	0.21
Mineral premix[b]	0.18
Total	100
Chemical compositions	
DM[1]	88.23
CP[2]	16.36
EE[3]	11.25
CF[4]	26.78
ADF[5]	22.50
NDF[6]	35.50
TDN[7]	74.68

[a]Vitamin premix contained the following ingredients diluted in cellulose (g/kg premix): L-ascorbic acid, 121.2; DL-α-tocopherol acetate, 18.8; thiamine hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myoinositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinal acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003. [b]Mineral premix contained the following ingredients (g/kg premix): Mg SO_4_$7H_2O$, 80.0; NaH_2PO_4 $2H_2O$, 370.0; KCl, 130.0; ferric citrate, 40.0; $ZnSO_4$_$7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$_$6H_2O$. 0.15; KI, 0.15; Na_2Se2O_3, 0.01; $MnSO_4$_H_2O, 2.0; $CoCl_2$_$6H_2O$, 1.0. Concentrate feed was provided by NongHyup Company. [c]Vitamin premix contained the following ingredients (Power Vitamine, Genobio, Republic of Korea) : Vitamin A, 6,000,000IU; Vitamin D3, 1,200,000 IU; Vitamin E, 1,000mg; Vitamin B1, 500mg; Vitamin B2, 500mg; Vitamin B6, 500mg; Vitamin B12, 10mg; Protected Vitamin C, 5,000mg; Pantothenic acid, 1,000mg; Niacin, 1,000mg; Biotin, 30mg; Folic acid, 600mg; Mn, 100mg. [1]DM: dry matter; [2]CP: crude protein; [3]EE: ether extract; [4]CF: crude fiber; [5]ADF: acid detergent fiber; [6]NDF: neutral detergent fiber; [7]TDN: total digestible nutrient.

3.3. Fecal sample collection and nutrient analysis of forages

Following a 12-hour fast, fecal samples were collected via the rectum using a disposable glove and transferred to sterile cryogenic tubes. For microbiome analysis, samples were frozen with liquid nitrogen and stored at -80°C. Pasture samples were collected at different times (May to November) from different locations within the same paddock before and after grazing, and were taken to the laboratory for forage intake and chemical analysis. The samples were dried at 60°C until a constant weight was achieved. We ground dry samples through a 1-mm screen to determine crude protein, acid detergent fiber (ADF), neutral detergent fiber (NDF), total digestible nutrient (TDN), and *in-vitro* dry matter digestibility (IVDMD). Nutrient compositions of pasture from grazed fields are presented in Table 3.

Table 3. Nutrient compositions, growth characteristics, productivity, and botanical composition of grassland during growing period and nutrition composition of rice straw

Item	Rice straw	Grass/Grazing						
		May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.
				Nutrient composition (% DM)				
DM[1]	88.82±0.36	22.50±2.04	30.00±4.02	34.66±2.94	31.32±0.17	27.32±0.82	29.73±0.29	30.02±0.44
CP[2]	4.45±0.02	12.37±0.47	12.05±1.08	14.51±0.04	13.57±0.47	18.60±0.27	15.37±0.42	15.63±0.49
ADF[3]	42.20±0.22	30.61±0.70	39.47±1.06	40.24±0.14	41.48±0.58	38.43±0.49	35.07±0.39	32.95±0.79
NDF[4]	68.99±0.08	54.22±0.04	64.32±0.72	61.29±1.24	60.57±0.85	60.04±0.87	57.26±0.36	55.68±0.72
TDN[5]	55.56±0.17	64.72±0.55	57.72±0.84	57.11±0.11	56.13±0.46	58.54±0.39	61.19±0.31	62.87±0.63
IVDMD[6]	40.07±0.87	75.58±1.28	70.62±2.38	71.19±0.61	71.39±0.56	73.18±0.65	74.56±0.57	74.70±0.74
				Growth characteristics and productivity of grassland				
Productivity (kg ha^{-1})	-	3,070±76	3,308±101	1,496±41	1,366±104	1,360±79	805±34	1,349 ± 31
Pasture utilization rate (%)	-	64	70	60	53	66	74	63
				Botanical composition of grassland(%)				
Grass	-	95	95	75	68	75	80	77
Weeds	-	5	-	20	32	25	10	-
Bare land	-	-	5	5	-	-	10	23

[1]DM: dry matter; [2]CP: crude protein; [3]ADF: acid detergent fiber; [4]NDF: neutral detergent fiber; [5]TDN: total digestible nutrient; [6]IVDMD: *in vitro* dry matter digestibility

3.4. Blood sampling and metabolic profile test

After fasting for 12 hours, blood was collected via the jugular vein using a classic needle and syringe after the sampling site had been cleaned with 70% alcohol. A serum-separating tube (SST) was used to collect the blood and it was then transferred to the National Institute of Animal Science, Cheonan, Korea. The blood sample was allowed to clot at room temperature without being disturbed. Afterward, the clot was centrifuged at 3,000rpm for 10 minutes to remove it. A biochemistry automatic analyzer (Hitachi 7180, Hitachi Ltd., Tokyo, Japan) was used to analyze serum biochemistry and minerals glucose (GLU), non-esterified fatty acids (NEFA), triglyceride (TG), total cholesterol (TCHO), total protein(Tpro), albumin (ALB), total bilirubin (Tbil), blood urea nitrogen (BUN), creatinine (CRE), serum glutamic oxaloactic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), gammaglutamyl transferase (γGTP), lactate dehydrogenase (LDH), calcium (Ca), magnesium (Mg), and inorganic phosphorus (P) were measured using a Hitachi 7180 after calibration and quality control assessments with commercial enzyme assay kits from Wako (Fujifilm Wako Pure Chemical Ltd., Osaka, Japan). Globulin was calculated by subtracting albumin from total protein. All biochemical analysis was completed in a single day.

3.5. Genomic DNA extraction

It was estimated that approximately 10 grams of each sample were added to 10 mL of 0.01% Tween 20 in PBS in a sterile Stomacher bag. A sonicator was used to sonicate the mixture for ten minutes. A pellet was collected by centrifugation

at 9000 g at 4°C for 10 minutes and DNA was extracted using the DNeasyPowerSoil Kit (Qiagen, Hilden, Ger-many) in accordance with the manufacturer's instructions. Quant-IT PicoGree kit (Invi-trogen) was used to measure genomic DNA.

3.6. Library construction and sequencing

To amplify the V3 and V4 regions, sequencing libraries were prepared according to the protocols of the Illumina 16S Metagenomic Sequencing Library. A total of two nano grams of gDNA was amplified using PCR with 5 x reaction buffer, 1 mM dNTP mix, 500 nM universal forward and reverse primers, and Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA). First, the PCR was run for 3 minutes at 95°C for heat activation, followed by 25 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, followed by a final 5minute extension at 72°C. For the first amplifications, the universal primer pair with Illumina adapter overhang sequences was as follows:

3V3-F: 5’ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTAC GGGNGGCWGCAG-3; V4-R:5’ TCTCGTGGGCTCGGAGATGTGTATA AGAGACAGGACTACHVGGGTATCTAATCC-3

AMPure beads were used to purify the first PCR products (Agencourt Bioscience, Beverly, MA). The first product was then amplified with PCR for further library construction containing the index using NexteraXT Indexed Primer. For the second PCR, the cycling conditions were the same as for the first

PCR, with the exception of 10 cycles. In or-der to quantify the final products, AMPure beads were used (KAPA Library Quantification kits for Illumina Sequencing platforms). The purified products were quantified using real time quantitative PCR following the qPCR Quantification Protocol Guide (Agilent Technologies, Waldbronn, Germany) and qualified using the TapeStation D1000 ScreenTape system (Agilent Technologies, Waldbronn, Germany). The pairedend (2 × 300 bp) sequence was determined by Macrogen using the MiSeqTM platform (Illumina, San Diego, USA). The poor quality sequences were removed using CD-HIT-OTU/rDnaTools. In or-der to calculate the bacterial diversity in different groups, alpha and beta diversity indices were calculated from the complete OTUs (operational taxonamic unites) table (alpha_diversity.py; UCLSUT/RDP (16S) or UNITE (ITS); alpha_rarefaction.py; make_2d_plots.py and make_otu_heatmap_html.py.

3.7. Statistical analysis

The student t-test was used to compare the microbiota and serum metabolic changes between fecal samples of grazing and housing steers using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Pearson correlation coefficients were generated using R software (Microgen) in order to understand the relationships between the bacterial taxonomic profiles and serum clinical parameters.

4. Results

4.1. Physiological and metabolic test of Hanwoo steer

Grazing steers (GS) had an average total weight gain of 157 kg, while housing steers (HS) had an average body weight of 154 kilograms. The average daily gain (ADG), feed intake, and feed efficiency remained unchanged. The GS consumed more crude protein and total digestibility nutrients (TDN) than the HS (p<0.01) (Table 4). We also examined the impact of GS and HS on serum profiles that are indicators of animal health. The following parameters such as albumin (ALB), creatinine kinase (CK), creatinine (CRE), gammaglutamyl transferase (γ-GTP), glucose (GLU), serum glutamate oxaloacetate transferase (SGOT), serum glutamate pyruvate transferase (SGPT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), total bilirubin (T-Bil), total protein (T-Pro), minerals such as calcium, phosphate, magnesium and lipid profiles includes total cholesterol (T-CHO), triglycerides (TG) and non-esterified fatty acids (NEFA) were analyzed. A month after the experimental trial, serum metabolic parameters had not changed significantly (Table 5). However, certain parameters such as ALB (p<0.03), Ca (p<0.01), γ-GTP (p<0.01), GLU (p<0.03), SGOT (p<0.09), LDH (p<0.09), Mg (p<0.03), Phosphate (p<0.05), T-CHO (p<0.04), and TG (p<0.03) were altered between the GS and HS after 3months experimental trail (Table 5). After 5months, GLU, γ-GTP, LDH, T-CHO, TG and NEFA levels were significantly increased in serum of GS compared to HS (Table 5). The serum levels of GLU, γ-GTP, Mg, T-CHO, TG, and NEFA were significantly higher in HS at the end of the experiment (Table 5) than in GS. As

compared to GS, γ-GTP, GLU, T-CHO, and TG increased consistently in serum of HS after three, five, and seven months of experimental trials.

Table 4. Effects of feeding systems on grazing on growth performance and feed intake in Hanwoo steers during growing period

Items	Total period			
	HS	**GS**	**SEM**	***P*-value**
Total body weight gain (kg)	154	157	4.98	0.59
Average daily gain (kg/day)	0.81	0.83	0.03	0.22
Feed intake (kg of DM/day)	7.80	8.22	0.20	0.09
Concentrate (kg of DM/day)	3.95	3.03	0.18	0.01
Rice straw (kg of DM/day)	3.85	-	0.07	-
Grass/grazing (kg of DM/day)	-	5.19	0.22	-
Feed efficiency[1]	0.11	0.09	0.01	0.03
Total energy intake (kg of DM)				
Crude protein	152.14	282.82	22.48	0.01
Total digestibility nutrients	932.87	1,173.05	46.94	0.01

SEM: standard error of mean, grazing steer (GS) vs housing steer (HS).
[1]Feed efficiency: total body weight gain / total feed intake

Table 5. Physiological parameters change in serum of grazing and housing at different periods

Parameters	Groups	1M	3M	7M
ALB (g/dl)	GS	5.10 ± 1.19	5.03 ± 0.82*	5.13 ±0.33
	HS	5.23 ± 1.16	6.97 ± 0.17	5.90 ± 0.54
CA (mg/dl)	GS	7.87 ± 2.12	7.57 ± 1.35*	7.20 ± 0.86
	HS	8.13 ± 2.37	11.7 ± 0.26	9.43 ± 0.97
CK (mg/dl)	GS	138.0 ± 19.1	129.3 ± 27.8	128.0 ± 14.9
	HS	189 3 ± 39.7	140.6 ± 20.9	108.6 ± 4.50
CRE (mg/dl)	GS	0.03 ± 0.05	0.03 ± 0.05	0.10 ± 0.08
	HS	0.03 ± 0.05	0.00 ± 0.00	0.00 ± 0.00
γGTP (IU/L)	GS	20.33 ± 2.05	13.67 ± 0.47*	7.67 ± 3.86 *
	HS	27.33 ± 9.46	24.67 ± 1.70	22.33 ± 2.62
GLU (mg/dl)	GS	100.6 ± 22.6	89.00 ±10.2*	85.67 ± 5.56*
	HS	95.67 ± 25.0	114.0 ± 3.74	108.0 ± 3.56
SGOT (IU/L)	GS	70.67 ± 17.7	83.00 ± 1.41*	101.6 ± 27.0
	HS	103.3 ± 17.7	124. 6 ± 12.3	83.33 ± 4.11
SGPT (IU/L)	GS	24.6 ± 5.46	23.6 ± 4.99	26.33 ± 2.49
	HS	24.6 ± 5.44	31.0 ± 3.27	30.67 ± 4.64
LDH (IU/L)	GS	1139 ± 232	1101 ± 243*	1385 ± 76.11
	HS	1193 ± 368	1566 ±179	1313 ± 103.1
Mg (mg/dl)	GS	2.53 ± 0.49	2.23 ± 0.41*	2.20 ± 0.16 *
	HS	2.47 ± 0.54	3.07 ± 0.12	2.87 ± 0.25
P (mg/dl)	GS	11.0 ± 3.00	11.2 ± 1.02*	8.47 ± 0.62
	HS	11.2 ± 2.43	14.8 ± 1.25	11.90 ± 1.77
T-Bil (mg/dl)	GS	0.07 ± 0.05	0.07 ± 0.02	0.22 ± 0.07
	HS	0.08 ± 0.04	0.08 ± 0.02	0.14 ± 0.13
T-Cho (mg/dl)	GS	147.0 ± 52.4	119.3 ± 6.24*	130.0 ± 13.7*
	HS	133.3 ± 29.3	164.3 ± 8.34	188.6 ± 22.8
T-Pro (g/dl)	GS	1.53 ± 0.45	1.53 ± 0.26	1.80 ± 0.08
	HS	1.43 ± 0.31	2.10 ± 0.00	1.87 ± 0.17
TG (mg/dl)	GS	13.6 ± 5.91	12.3 ± 0.47 *	16.33 ± 4.92*
	HS	10.3 ± 1.89	22.3 ± 8.99	37.33 ± 3.86
BUN (mg/dl)	GS	10.7 ± 2.13	12.3 ± 3.03	15.60 ± 1.95
	HS	13.3 ± 4.01	14.7 ± 1.24	12.03 ± 1.47
NEFA (μEq/L)	GS	132.6 ± 48.6	139.3 ± 47.2	36.0 ± 19.1*
	HS	229.0 ± 19.6	287.0 ± 28.0	112.0 ± 23.2

M (month), albumin (ALB), calcium (CA), creatinine kinase (CK), creatinine (CRE), gamma-glutamyl- transferase (γ-GTP), glucose (GLU), serum glutamate oxaloacetate transferase (SGOT), serum glutamate pyruvate transferase (SGPT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), total bilirubin (T-Bil), total protein (TP), minerals such as calcium, phosphate, magnesium and lipid profiles includes total cholesterol (T-CHO), triglycerides (TG) and non-esterified fatty acids (NEFA). *$p<0.05$, grazing steer (GS) vs housing steer (HS).

4.2. Fecal 16S rRNA gene sequencing report

Using Illumina sequencing, a total of 12 fecal samples from grazing and housing animals produced 3,214,142 raw reads. The CD-HIT-OTU/rDnaTools program was used to remove ambiguous, low quality, chimera, and other sequences. Finally, 717,056 high quality sequences were obtained from all fecal samples; the mean number of sequences per sample was 4,4816 ± 5,131 (mean ± standard deviation; the range was 36,381-56,347). OTUs determination resulted in 6,468 ± 87.86 OTUs across all samples (ranges between 384 - 637 OTUs). GS had an average of 589.66 ± 38.17 OTUs per sample whereas HS had an average of 488.33 ± 94.06 OTUs per sample (Figure 5a). A Phred score above 20 (Q20%) was 98.0% and a score above 30 (Q30%) was 93.0 % which indicated that the used samples had a significant quality level that is essential for NGS analysis.

4.3. Sequencing depth, coverage, and alpha diversity metrics

Figure 6 presents a rarefaction analysis of the fecal microbiota of GS and HS. Based on the graph, the analyzed samples displayed a flatter curve for OTUs than the right curve, indicating that a significant number of reads was used for this analysis. According to the calculated good average, the sampling depth captured most of the species diversity, with a mean coverage of 0.998 ± 0.0003 per sample. In terms of alpha diversity metrics, both GS and HS had almost the same number and evenness of species (Figure 5b). As a measure of richness, the GS fecal sample had a higher Cho1 value than the HS fecal sample (650.9 ± 32.1 vs 537 ± 105.4, respectively) (Figure 5a).

4.4. PCA analyses to grazing and housing related fecal microbiota shifts

A principal component analysis (PCA) was performed in order to investigate changes in the fecal samples from GS and HS. Two and three components contributed approximately 64.69% and 77.0 % to the overall variance, with the first component exhibiting the largest contribution (Figure 5c and d). This finding indicates that GS and HS had a significant influence on the feces of steers.

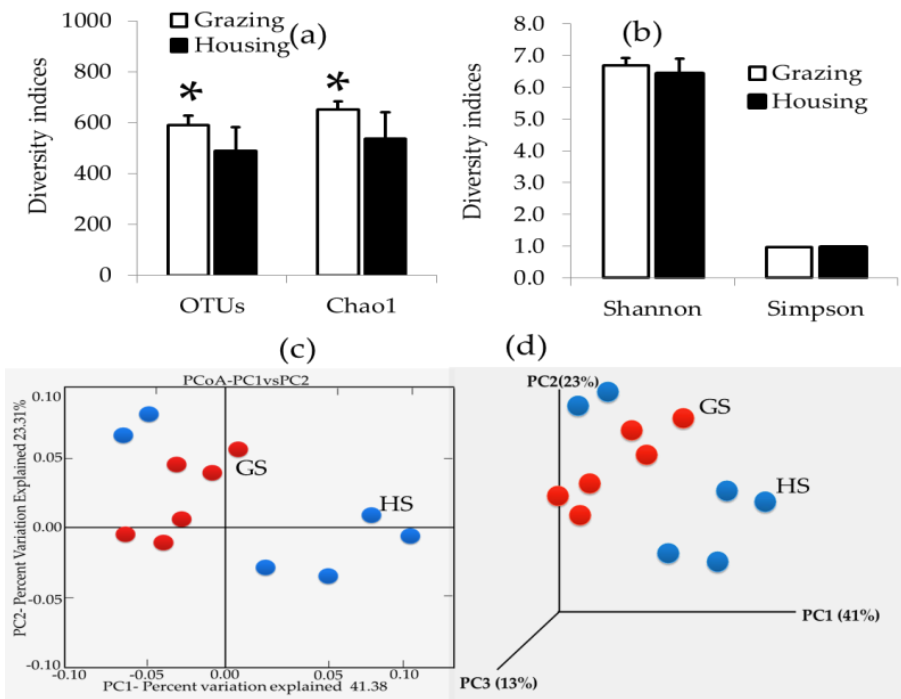

Figure 5. Diversity indices and principal coordinate plots operational taxonomic units (OTUs) level weighted Unifrac distance between steer groups. (a) OTUs and Cho1 indices of bacterial diversity; (b) shannon and simpson of bacterial diversity; (c) 2D PCA of steer groups; d) 3D PCA of steer groups. GS: grazing steer; HS: housing steer. *$p<0.05$, grazing steer (GS) vs housing steer (HS).

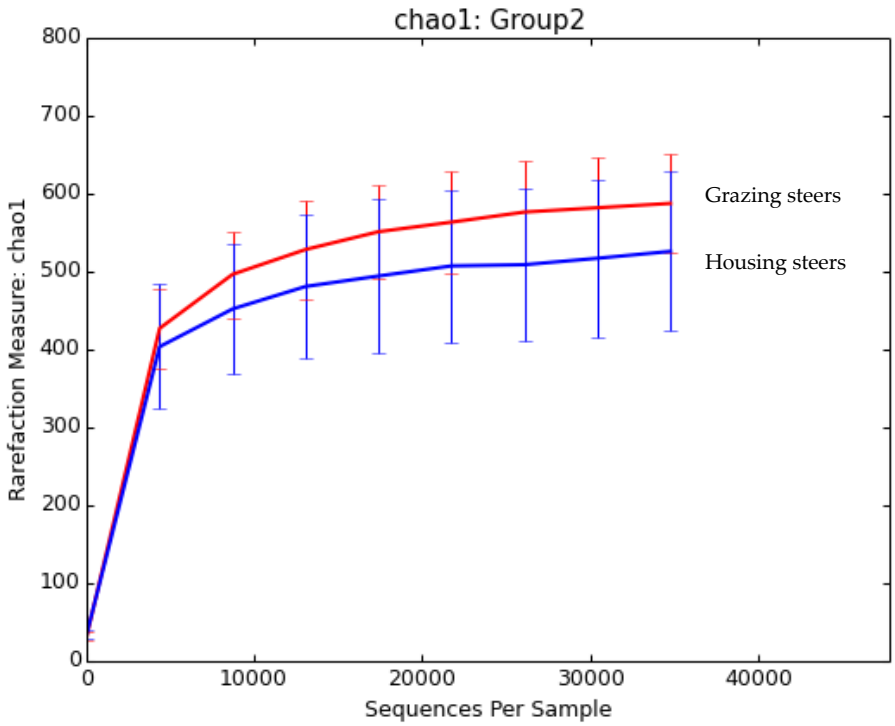

Figure 6. A rarefaction analysis of the fecal microbiota of grazing and housing steers.

4.5. Overall fecal microbiota compositions of the Hanwoo steer

The predominant phyla in both the GS and HS fecal samples were *Firmicutes* and *Bacteroidetes*, with combined sequences of these two phyla accounting for 91.8% of the entire microbial population in both GS and HS (ranges 34.5–40.8% and 49.9 - 57.2% respectively) (Figure 7). The remaining sequences were classified as *Spirochaetes*, *Verrucomicrobia*, and unclassified bacteria, which accounted for less than 2% of the total sequences. Phylum-level data showed a higher *Firmicutes* and a lower *Bacteroidetes* in the feces of the GS than in the feces of HS (*Firmicutes* 40.8 ± 3.0 vs 34.5 ± 2.4% respectively; $p<0.05$; *Bacteroidetes* 49.9 ± 4.3 vs 57.2 ± 4.4%, respectively; $p<0.047$).

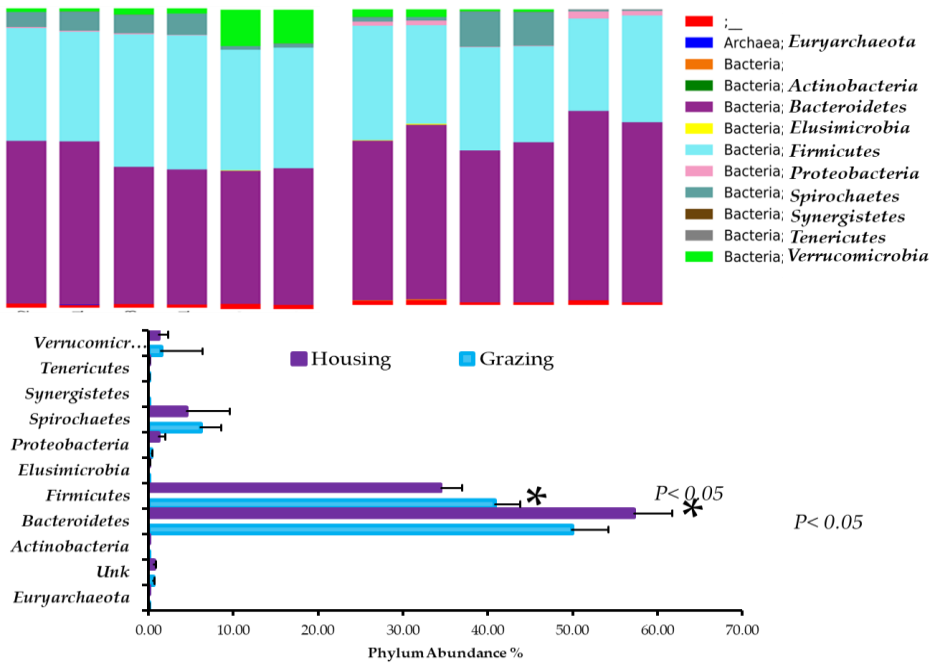

Figure 7. Relative abundance of microbiota changes in grazing and housing steers at phylum level.

There was a higher percentage of *Ruminococcaceae* and *Lachnospiraceae* bacteria in fecal samples from GS compared to HS (20.9 vs 16.9% and 5.1 vs 4.2%, respectively, of all *Firmicutes* sequences). Moreover, 6.8% of sequences were unclassified at the family level in the *Firmicutes* phylum, while all other families accounted for less than 8.0% in GS and 6.9% in HS. Within the *Bacteroidetes* phylum, *Bacteroidaceae* and *Sphingobacteriaceae* were the most prevalent families, followed by *Rikenellaceae* and *Prevotellaceae*. GS had lower levels of *Sphingobacteriaceae* (13.1 vs 18.4, $p < 0.009$) and *Bacteroidaceae* (12.9 vs 17.9%, $p < 0.002$) than HS (Table 6). Among the genus level, lower *Parapedobacter* ($p < 0.04$), and Bacteroides ($p < 0.01$) and higher *Porphyromonas* ($p < 0.013$), *Prevotella* ($p < 0.001$), *Ethanoligenens* ($p < 0.004$), and *Papillibacter* ($p < 0.002$) were observed in GS than in HS (Table 7). At species level, *Parapedobacter koreensis,*

Paludibacter propionicigenes, Paludibacter propionicigenes, Ethanoligenens harbinense, Alistipes finegoldii and *Papillibacter cinnamivorans* significantly varied among the experimental steers (Table 8).

Table 6. Microbiota changes at the family level between grazing and housing steers

S.No	Family	Grazing	Housing	STD	*P*-value
1	*Sphingobacteriaceae*	13.1	18.4	2.56	0.009
2	*Flavobacteriaceae*	0.78	1.31	0.76	0.360
3	*Non-classified*	6.86	6.65	1.27	0.422
4	*Bacteroidaceae*	12.9	17.9	1.85	0.002
5	*Porphyromonadaceae*	2.25	1.57	0.87	0.250
6	*Prevotellaceae*	3.93	6.19	2.70	0.280
7	*Rikenellaceae*	4.60	6.20	1.47	0.140
8	*Streptococcaceae*	0.02	0.03	0.02	0.530
9	*Christensenellaceae*	0.12	0.11	0.05	0.940
10	*Clostridiaceae*	1.02	0.68	0.26	0.090
11	*Clostridiales* Family	1.11	0.89	0.39	0.480
12	*Clostridiales* Family Ⅷ.	0.55	0.49	0.18	0.630
13	*Eubacteriaceae*	0.96	0.84	0.27	0.500
14	*Lachnospiraceae*	5.11	4.28	0.62	0.050
15	*Oscillospiraceae*	1.32	1.86	0.61	0.200
16	*Peptostreptococcaceae*	0.87	0.40	0.31	0.043
17	*Ruminococcaceae*	20.9	16.9	1.55	0.002
18	*Acidaminococcaceae*	0.33	0.20	0.17	0.274
19	*Selenomonadaceae*	0.14	0.09	0.06	0.330
20	*Kiloniellaceae*	0.00	0.04	0.02	0.050
21	*Rhodospirillaceae*	0.06	0.37	0.22	0.110
22	*Desulfovibrionaceae*	0.02	0.08	0.05	0.160
23	*Enterobacteriaceae*	0.04	0.13	0.10	0.310
24	*Succinivibrionaceae*	0.09	0.52	0.37	0.220

STD: standard deviation.

Table 7. Modulation of pyrotags at the genus level in grazing and housing steer feces

Genus	Grazing	Housing	STD	*P*-value
Parapedobacter	13.96	17.55	2.44	0.044
Bacteroides	12.76	16.84	2.03	0.014
Porphyromonas	3.173	1.572	0.81	0.013
Paraprevotella	3.068	5.296	2.54	0.250
Prevotella	0.694	0.083	0.17	0.001
Alistipes	4.237	6.059	1.44	0.090
Ethanoligenens	2.880	1.774	0.45	0.004
Papillibacter	9.173	6.020	1.23	0.002
Coprococcus	1.069	0.605	0.20	0.021
Dorea	0.281	0.145	0.07	0.02
Blautia	0.187	0.127	0.08	0.020
Treponema	4.467	4.471	3.75	0.990
Non-classified	4.113	4.286	0.51	0.610

STD: standard deviation.

Table 8. Species level changes in fecal microbiota of steers in response to diet systems

S.No	Species Name	Grazing	Housing	STD	*P*-value
1	Non-classified	1.264	1.153	0.003	0.510
2	*Parapedobacter koreensis*	4.351	6.196	0.006	0.002
3	*Parapedobacter soli*	12.14	11.35	0.030	0.700
4	*Muribaculum intestinale*	3.268	5.329	0.014	0.090
5	*Paludibacter propionicigenes*	1.793	0.263	0.002	0.000
6	*Bacteroides cellulosilyticus*	0.000	0.252	0.002	0.150
7	*Bacteroides clarus*	3.809	2.655	0.014	0.220
8	*Bacteroides plebeius*	4.665	5.339	0.018	0.600
9	*Porphyromonas pogonae*	2.062	1.406	0.009	0.270
10	*Paraprevotella clara*	3.068	5.296	0.025	0.250
11	*Prevotella shahii*	0.455	0.000	0.002	0.019
12	*Alistipes finegoldii*	1.110	2.329	0.009	0.069
13	*Alistipes onderdonkii*	1.161	1.138	0.005	0.940
14	*Alistipes putredinis*	0.993	1.283	0.005	0.440
15	*Flavonifractor plautii*	0.748	0.505	0.003	0.220
16	*Intestinimonas butyriciproducens*	2.519	2.409	0.006	0.810
17	*Acidaminobacter hydrogenoformans*	1.112	0.894	0.004	0.480
18	*Kineothrix alysoides*	2.128	1.746	0.006	0.420
19	*Eubacterium] tenue*	0.638	0.281	0.002	0.450
20	*Clostridium] cellobioparum*	1.298	0.766	0.005	0.130
21	*Clostridium] stercorarium*	0.141	0.340	0.001	0.070
22	*Ethanoligenens harbinense*	2.880	1.774	0.004	0.004
23	*Papillibacter cinnamivorans*	9.173	6.020	0.012	0.002
24	*Treponema porcinum*	3.584	3.973	0.038	0.880
25	*Akkermansia glycaniphila*	4.901	1.175	0.030	0.130

STD: standard deviation.

Next, specific microbiota were associated with the effects of GS and HS feeding systems on serum markers such as ALB, CK, CRE, G-GTP, GLU, SGOT, SGPT, LDH, BUN, T-Bil, TP, minerals including phosphate, and magnesium, as well as lipid profiles including T-CHO, TG and NEFA. Significant correlations were found between the microbiota at phylum/genus levels and serum clinical profiles. *Firmicutes* were positively associated with blood urea nitrogen and negatively associated with T-CHO and TG, whereas *Bacteroidetes* was positively associated with serum Ca, P, ALB, and NEFA (Figure 8).

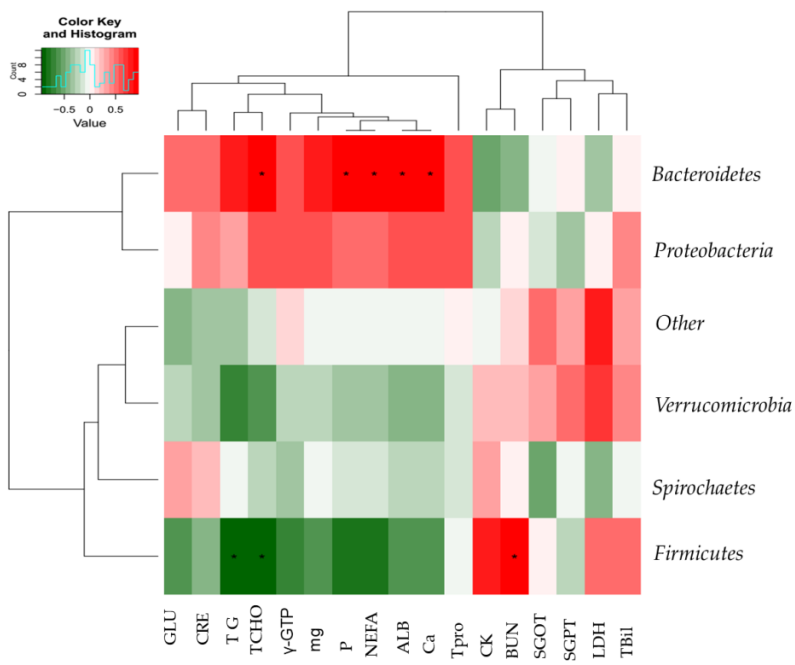

Figure 8. Heatmap correlation between the microbiota at the phylum level and serum metabolic profiles in experimental steers. Albumin (ALB), creatinine kinase (CK), creatinine (CRE), gamma-glutamyl- transferase (γ-GTP), glucose (GLU), serum glutamate oxaloacetate transferase (SGOT), serum glutamate pyruvate transferase (SGPT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), total bilirubin (T-Bil), total protein (TPro), minerals such as calcium (Ca), phosphate (P), magnesium (Mg) and lipid profiles includes total cholesterol (T-CHO), triglycerides (TG) and non-esterified fatty acids (NEFA). * Indicates statistically significant difference between grazing and housing steers at 0.05 level. **Indicates statistically significant difference between grazing and housing steers at 0.001 level.

Papillibacter and *Coprococcus* were negatively correlated with total protein, calcium and albumin at the genus level. CRE, GLU, and TG were negatively associated with *Barnesiella* and *Prevotella*. There were positive correlations between *Rikenellaceae* and CK, BUN, and γ-GTP at $p < 0.05$. The *Dorea* genus exhibited negative correlations with T-CHO, Ca, ALB, Mg, and NEFA and P at $p < 0.001$. SGPT was negatively correlated with *Blautia*, *Lachnospiraceae, Phascolarctobacterium*, and *Achnospiraceae,* but positively correlated with *Flavobacteriaceae. Ruminococcaceae* and *Clostridiales* were positively associated with T-Bil, whereas *Paraprevotella* and *Bacteroidales* were negatively associated with T-Bil (Figure 9).

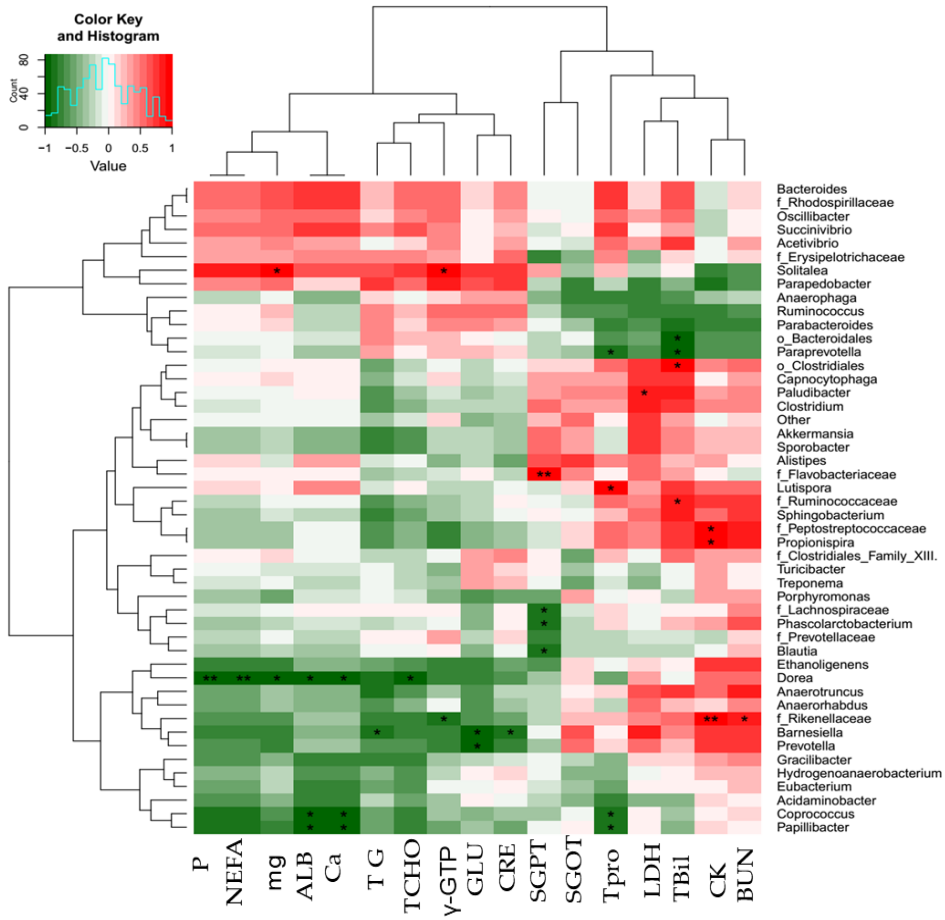

Figure 9. The correlation between genus level microbiota and serum metabolic profiles in experimental steers is represented by a heatmap. Albumin (ALB), creatinine kinase (CK), creatinine (CRE), gamma-glutamyl-transferase (γ-GTP), glucose (GLU), serum glutamate oxaloacetate transferase (SGOT), serum glutamate pyruvate transferase (SGPT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), total bilirubin (T-Bil), total protein (TPro), minerals such as calcium (Ca), phosphate (P), magnesium (Mg) and lipid profiles includes total cholesterol (T-CHO), triglycerides (TG) and non-esterified fatty acids (NEFA). * Indicates statistically significant difference between grazing and housing steers at 0.05 level. **Indicates statistically significant difference between grazing and housing steers at 0.001 level.

5. Discussion

In this study, we report changes in the fecal microbiota of Hanwoo steers as a result of grazing pastures, as well as its impact on serum metabolic profiles and animal performance. Final body weight (kg) and average daily growth rate (ADG) did not change significantly compared to housing steers. There were significant differences between grazing steers (GS) and housing steers (HS) in terms of feed intake, feed efficiency, and feed conversion ratio. It was found that the total intake of crude protein and total digestibility nutrients were higher for the GS than for the HS. The amount of hardly fermentable dietary fiber (ADF and NDF) found in grazing forages was higher than that found in diets fed to HS. As far as changes in physiological parameters in serum of both HS and GS are concerned, short-term feeding systems did not result in any changes in any physiological parameters. But, ALB ($p < 0.03$), Ca ($p < 0.01$), γ-GTP ($p < 0.01$), GLU ($p < 0.03$), SGOT ($p < 0.09$), LDH ($p < 0.09$), Mg ($p < 0.03$), P ($p < 0.05$), T-CHO ($p < 0.04$), and TG ($p <0.03$) were altered between the GS and HS after 3months experimental trail. The levels of GLU, G-GTP, LDH, T-CHO, TG and NEFA in serum of GS were significantly higher than those of HS at trail month five. By the end of the experimental months, serum GLU, G-GTP, Mg, T-CHO, TG and NEFA levels were higher in HS than in GS. In comparison to grazing steers, G-GTP, GLU, T-CHO, and TG were the most consistently increasing parameters in serum of HS after 3, 5, and 7 months of experimental trials. Ruminant concentrate diets are a major source of glucose, either through an increase in propionate production in the rumen (a gluconeogenic precursor) or an increase in intestinal glucose absorption (Park et al., 2018). Gluconeogenesis

produces glucose, which is the primary source of energy for ruminants (Yost et al., 1977). Excess glucose is converted into fatty acids that circulate throughout the body, particularly in the adipose tissue of the body (Rhoades et al., 2007). Increased energy intake leads to increased production of propionate in the rumen and gluconeogenesis in the liver. It may be the principal reason for the high level of serum glucose and lipid metabolites in the serum of HS. A lack of energy causes an increase in NEFAs in cattle (Beever, 2006) or pathological problems as ketosis and fatty liver (Douglas et al., 2006). In a state of negative energy balance, NEFAs are produced by lipolysis of triglyceride, which is stored in adipose tissue and transported to other organs and tissue (Teixeira et al., 2012). The hormone cortisol is an indicator of stress, which promotes lipolysis and stimulates the production of NEFAs in the blood (Samra et al., 1998). The present study found that the level of NEFA was consistently elevated throughout experimental periods in serum of HS, confirming that the housing of animals without natural feeding behaviors is closely associated with some abnormal behaviors (Smid et al., 2020). Limiting cattle's exploratory or forage activities could result in a significant reduction in animal welfare and could explain the increase in NEFA levels found in the serum of HS. The present study did not analyze the level of stress-related markers. Consequently, it is necessary to determine the level of stress-related markers and their impact on serum metabolites in GS and HS. Experimental steers were tested for serum levels of SGOT, SGPT and γ-GTP, which are produced in hepatocytes and released into the bloodstream if hepatocytes are damaged by high energy feeding and mold toxins. Steers fed with concentrate in housing conditions may have a significant

negative impact on liver markers when compared with steers that graze.

Alpha diversity indices (OTUs, Choa1) indicated that the fecal microbial diversity of GS was higher than that of HS. There has been research indicating that fiber-based diets improve microbial diversity because the fermentation of fiber stimulates microbial proliferation better than that of starch-based diets (Belanche et al., 2012; Fernandes et al., 2014). The fiber-based diet contains several secondary metabolites that can act as prebiotics and contribute to the improvement of bacterial diversity (Chen et al., 2008; Latham et a., 2018). During this study, animals grazed a variety of pastures containing large amounts of non-structural carbohydrates (NSC) and non-fiber carbohydrates (NFC) in tall fescue orchard grass, perennial ryegrass, Kentucky bluegrass, and white clover (Shah et al., 2022; Jensen et al., 2014). Microbial diversity depends on both NSCs and NFCs. Due to this; bacterial communities of steers grazing on natural pasture were highly diverse. Additionally, steer that grazed received higher levels of crude protein and TDN, which might contribute to microbial proliferation, and forage varieties and biomass may influence the diversity of microbial communities in cattle.

A significant difference was observed between the two steer groups regarding the relative abundance of microbes. The majority of pyrotags in fecal samples of GS and HS belong to the *Bacteroidetes* (49.90 ± 4.31 vs 57.23 ± 4.4%, respectively; $p < 0.047$) and *Firmicutes* (40.85 ± 2.99 vs 34.5 ± 2.4% respectively; $p < 0.09$). These phyla have previously been demonstrated to constitute the major gut-associated phylotypes in a variety of different mammalian species (Shah et al., 2022; Mote et al., 2019; Shanks et al., 2011), suggesting that *Firmicutes* and

Bacteroidetes (more than 90% of all high quality bacterial pyrotags) are critical to the microbial ecology of mammalian guts. Phyla such as *Spirochaetes* and *Verrucomicrobia* accounted for less than 2% of the total sequences, while unclassified as bacterial accounted for less than 1%.

It is essential to evaluate the *Firmicutes* to *Bacteroidetes* ratio in order to determine whether gut microbes have an effect on host energy needs (Jami et al., 2014). GS had a higher *Firmicutes* content ($p < 0.05$) and lower *Bacteroidetes* content ($p < 0.009$) in the fecal samples than HS. It is consistent with what has previously been reported for other animals, including Angus steer (Mote et al., 2019; Zhang et al., 2021) and Yaks (Shah et al., 2022). As compared to what we reported in the current study with other animals, there is a large variation in the ratio of *Firmicutes* to *Bacteroidetes* in cattle re-ported in the previous studies (Shah et al., 2022; Mote et al., 2019; Zhnag et al., 2021). According to previous studies, the dominance of *Firmicutes* or *Bacteroidetes* is due to variations in diets, breed types, climate, and forming practices across a wide geographical range (Henderson et al., 2015). Furthermore, forage varieties, forage quality, and forage locations in grazing pastures appear to be more favorable for abundance of *Firmicutes* or *Bacteroidetes* pyrotypes.

Ruminococcaceae, *Lachnospiraceae, Bacteroidaceae, Sphingobacteriaceae, Rikenellaceae*, and *Prevotellaceae* dominated both steer groups, with *Ruminococcaceae* and *Lachnospiraceae* being among the most abundant *Firmicutes* phylum in GS compared to housing steers in the fecal sample. The study has demonstrated that these bacteria play a critical role in the degradation of starch and fiber, as well as improving fiber digestibility (Lozupone et al., 2011). *Ruminococcaceae* have

also been reported to degrade protein (Wang et al., 2019). Adequate nutrients were available in grazing pastures, which were conducive to the relative abundance of fiber-degrading bacteria. *Ruminococcaceae* and *Lachnospiraceae* are important factors in stimulating growth of fibrolytic bacteria and are found in the rumens of Holstein cows (Godoy-Vitorino et al., 2012; Ziemer, 2014). It was found that *Sphingobacteriaceae, Bacteroidaceae, Prevotellaceae,* and *Rikenellaceae* represented the most dominant families in Bacteroidetes. The *Prevotellaceae* bacteria are a dominant bacterial species in the saccharolytic group in the rumen. They have a low protein binding ability and are capable of digesting a wide range of carbohydrate substrates (Stewart et al., 2018). HS feces contained slightly higher amounts of *Prevotellaceae,* indicating that the high carbohydrate ability could be attributed to a higher organic matter content. Recent studies have reported that the *Christensenellaceae, Ruminococcaceae*, and *Rikenellaceae* play a major role in forage degradation in the rumen due to their strong adhesion to forage grass after incubation (Liu et al., 2016; Shen et al., 2017). In the current study, higher proportions of *Ruminococcaceae* and *Lachnospiraceae* in GS are expected to improve fiber degradation. Among dominant genera, *Prevotella* ($p <$ 0.001), *Ethanoligenens* ($p < 0.004$), *Papillibacter* ($p < 0.002$), *Coprococcus* ($p < 0.002$), *Dorea* ($p < 0.002$) and *Blautia* ($p < 0.002$) were the most abundant genera in GS. *Parapedobacter* ($p < 0.04$), *Bacteroides* ($P < 0.01$), *Alistipes* ($p < 0.09$) and *Porphyromonas* ($p < 0.01$) were dominant genus in the HS group. The *Paraprevotella* was first identified in human faces and was found to produce succinic acid and acetic acid as end products of glucose metabolism in cattle (Morotomi et al., 2009); it has also been found in pigs' and humans' feces (Li et

al., 2012; Kim et al., 2011; Chen et al., 2012). It can utilize xylan as a growth substrate. It has been reported that *Paraprevotella* in the rumen of cattle fed cornstalk (CS) may have some beneficial effects on CS NDF degradation (Zhang et al., 2014). The genus *Bacteroides* is another well-known intestinal bacterium that can be both helpful and harmful (Wexler, 2007) and that participate in the natural transmission of antimicrobial resistance genes (Shoemaker et al., 1991). It has the capability of hydrolyzing complex organic compounds (Rismani-Yazdi et al., 2013), decomposing hemicellulose and xylan (Naas et al., 2014; Hopkins et al., 2003) and converting long chain fatty acids into short chain fatty acids (Shah et al., 1989), Among *Prevotella*, which belong to the *Alloprevotella* group, which has been characterized by significant genetic divergence, functional versatility, and is involved in the degradation of dietary proteins (Osborne et al., 1989), the metabolism of peptides (Wallace et al., 1997), as well as the utilization of hemicellulose (Gulino et al., 2013). In the rumen, Treponema is a common bacterial group that is associated with the degradation of soluble fibers (Bekele et al., 2011). The nutrient composition of pasture and concentrate may have favored the growth of fibrolytic bacteria.

As a final, we sought to determine if there is specific microbiota associated with the effects of GS and HS feeding systems on serum markers minerals, and if there is a significant correlation between the microbiota at the phylum-genus level and the serum clinical profiles. *Firmicutes* has been positively correlated with blood urea nitrogen and negatively correlated with T-CHO and TG at the phylum level, while *Bacteroidetes* has been positively associated with serum calcium, Phosphate, ALB, and NEFA at the phylum level, at a $p<0.05$ level. It

was observed that the abundance of *Firmicutes* in the feces of grazing steers was higher, which explained the role that *Firmicutes* might play in influencing the lipid profile of these animals and in particular the levels of T-CHO and TG

In terms of genus level correlations, *Papillibacter* and *Coprococcus* were negatively correlated with total protein, calcium, and albumin. CRE, GLU, and TG were negatively associated with *Barnesiella* and *Prevotella*. *Rikenellaceae* was positively correlated with CK at $p < 0.001$, BUN and negatively related to γ-GTP at $p < 0.05$. The genus *Dorea* was negatively correlated with T-CHO, Ca^{2+} ALB, Mg^{2+} at $p < 0.05$, NEFA and P at $p < 0.001$. SGPT was negatively correlated with *Blautia, Lachnospiraceae, Phascolarctobacterium* and *Achnospiraceae*, whereas *Flavobacteriaceae* was positively correlated with SGPT at $p < 0.001$. T-Bil was positively correlated with *Ruminococcaceae* and *Clostridiales*, whereas *Paraprevotella* and *Bacteroidales* were negatively correlated. Microbiota and serum clinical parameters demonstrated strong correlations, especially with regard to the impact of grazing on animal health and serum metabolic markers.

6. Conclusion

Grazing steers on natural pastures increased the diversity of bacterial communities in fecal microbiota at the phylum, family, and genus levels. Furthermore, *Frimicutes* levels were higher in the feces of grazing steers on natural pastures than in the feces of housing steers. There are two dominant bacterial families in Firmicutes: *Ruminococcaceae* and *Lachnospiraceae*. Meanwhile, *Sphingobacteriaceae, Bacteroidaceae, Prevotellaceae* and *Rikenellaceae* dominated the

Bacteroidetes, with higher numbers among the housing steers. The changes in microbiota may have an impact on serum metabolic profiles (G-GTP, GLU, T-CHO, and TG) and feeding behavior. The findings of this study contribute to the current understanding of the gut microbiota of Hanwoo steers and provide evidence for possible effects of various forages on the rumen microbiota of natural feeding animals.

7. References

1. Arthington JD, Spears JW, Miller DC. (2005). The effect of early weaning on feedlot performance and measures of stress in beef calves. *J Anim Sci.*, 83(4):933-9.
2. Beever, DE. (2006). The impact of controlled nutrition during the dry period on dairy cow health, fertility and performance. *Animal reproduction science*, 96(3-4), 212-226.
3. Bekele, AZ, Koike, S, Kobayashi, Y. (2011). Phylogenetic diversity and dietary association of rumen *Treponema* revealed using group-specific 16S rRNA gene-based analysis. *FEMS Microbiology Letters*, 316(1), 51-60.
4. Belanche, A, Doreau, M, Edwards, JE, Moorby, JM, Pinloche, E, Newbold, CJ. (2012). Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *The Journal of nutrition*, 142(9), 1684-1692.
5. Chen, W, Liu, F, Ling, Z, Tong, X, Xiang, C. (2012). Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLoS One*, 7(6), e39743.
6. Chen, X, Wang, J, Wu, Y, Liu, J. (2008). Effects of chemical treatments of rice straw on rumen fermentation characteristics, fibrolytic enzyme activities and populations of liquid and solid associated ruminal microbes in vitro. *Animal Feed Science and Technology*, 141(1-2), 1-14.
7. Daley, CA, Abbott, A, Doyle, PS, Nader, GA, Larson, S. (2010). A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. *Nutrition journal*, 9(1), 1-12.
8. Douglas, G, Overton, T, Bateman II, H, Dann, H, Drackley, J. (2006). Prepartal

plane of nutrition, regardless of dietary energy source, affects periparturient metabolism and dry matter intake in Holstein cows. *Journal of dairy science*, 89(6), 2141-2157.

9. Dumont, B, Ryschawy, J, Duru, M, Benoit, M, Chatellier, V, Delaby, L, Donnars, C, Dupraz, P, Lemauviel-Lavenant, S, Méda, B. (2019). Associations among goods, impacts and ecosystem services provided by livestock farming. *Animal*, 13(8), 1773-1784.
10. Fernandes, KA, Kittelmann, S, Rogers, CW, Gee, EK, Bolwell, CF, Bermingham, EN, Thomas, DG. (2014). Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. *PLoS One*, 9(11), e112846.
11. Gao, J, Xu, K, Liu, H, Liu, G, Bai, M, Peng, C, Li, T, Yin, Y. (2018). Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. *Frontiers in cellular and infection microbiology*, 8, 13.
12. Godoy-Vitorino, F, Goldfarb, KC, Karaoz, U, Leal, S, Garcia-Amado, MA, Hugenholtz, P, Tringe, SG, Brodie, EL, Dominguez-Bello, MG. (2012). Comparative analyses of foregut and hindgut bacterial communities in hoatzins and cows. *The ISME journal*, 6(3), 531-541.
13. Gulino, L-M, Ouwerkerk, D, Kang, AY, Maguire, AJ, Kienzle, M, Klieve, AV. (2013). Shedding light on the microbial community of the macropod foregut using 454-amplicon pyrosequencing. *PLoS One*, 8(4), e61463.
14. Haga, S, Nakano, M, Nakao, S, Hirano, K, Yamamoto, Y, Sasaki, H, Ishizaki, H. (2016). Seasonal foraging patterns of forest-grazing Japanese Black heifers with increased plasma total antioxidant capacity. *Animal Science Journal*, 87(2), 209-216.
15. Henderson, G, Cox, F, Ganesh, S, Jonker, A, Young, W, Janssen, PH. (2015).

Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific reports*, 5(1), 14567.

16. Hopkins, MJ, Englyst, HN, Macfarlane, S, Furrie, E, Macfarlane, GT, McBain, AJ. (2003). Degradation of cross-linked and non-cross-linked arabinoxylans by the intestinal microbiota in children. *Applied and environmental microbiology*, 69(11), 6354-6360.
17. Jami, E, White, BA, Mizrahi, I. (2014). Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. *PLoS One*, 9(1), e85423.
18. Jensen, KB, Harrison, P, Chatterton, NJ, Bushman, BS, Creech, JE. (2014). Seasonal trends in nonstructural carbohydrates in cool-and warm-season grasses. *Crop Science*, 54(5), 2328-2340.
19. Kim, HB, Borewicz, K, White, BA, Singer, RS, Sreevatsan, S, Tu, ZJ, Isaacson, RE. (2011). Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Veterinary microbiology*, 153(1-2), 124-133.
20. Latham, E, Weldon, K, Wickersham, T, Coverdale, J, Pinchak, W. (2018). Responses in the rumen microbiome of *Bos taurus* and *indicus* steers fed a low-quality rice straw diet and supplemented protein. *Journal of animal science*, 96(3), 1032-1044.
21. Lazar, V, Ditu, L-M, Pircalabioru, GG, Gheorghe, I, Curutiu, C, Holban, AM, Picu, A, Petcu, L, Chifiriuc, MC. (2018). Aspects of gut microbiota and immune system interactions in infectious diseases, immunopathology, and cancer. *Frontiers in immunology*, 9, 1830.
22. Li, RW, Connor, EE, Li, C, Baldwin, V, Ransom L, Sparks, ME. (2012). Characterization of the rumen microbiota of pre-ruminant calves using

metagenomic tools. *Environmental microbiology*, 14(1), 129-139.

23. Liu, J, Zhang, M, Xue, C, Zhu, W, Mao, S. (2016). Characterization and comparison of the temporal dynamics of ruminal bacterial microbiota colonizing rice straw and alfalfa hay within ruminants. *Journal of Dairy Science*, 99(12), 9668-9681.
24. Lozupone, C, Lladser, ME, Knights, D, Stombaugh, J, Knight, R. (2011). UniFrac: an effective distance metric for microbial community comparison. *The ISME journal*, 5(2), 169-172.
25. Manzi, P, Durazzo, A. 2017. Organic vs. conventional milk: Some considerations on fat-soluble vitamins and iodine content. *Beverages*, 3(3), 39.
26. Morotomi, M, Nagai, F, Sakon, H, Tanaka, R. (2009). *Paraprevotella clara gen. nov.*, *sp. nov*. and *Paraprevotella xylaniphila sp. nov*., members of the family '*Prevotellaceae*'isolated from human faeces. *International journal of systematic and evolutionary microbiology*, 59(8), 1895-1900.
27. Mote, RS, Hill, NS, Skarlupka, JH, Turner, ZB, Sanders, ZP, Jones, DP, Suen, G, Filipov, NM. (2019). Response of beef cattle fecal microbiota to grazing on toxic tall fescue. *Applied and Environmental Microbiology*, 85(15), e00032-00019.
28. Mullan, S, Bunglavan, SJ, Rowe, E, Barrett, DC, Lee, MR, Ananth, D, Tarlton, J. (2020). Welfare challenges of dairy cows in India identified through on-farm observations. *Animals*, 10(4), 586.
29. Naas, AE, Mackenzie, A, Mravec, J, Schückel, J, Willats, W, Eijsink, V, Pope, P. (2014). Do rumen *Bacteroidetes* utilize an alternative mechanism for cellulose degradation. *MBio*, 5(4), e01401-01414.
30. Nakajima, N, Yayota, M. (2019). Grazing and cattle health: a nutritional,

physiological, and immunological status perspective. *Animal Behaviour and Management*, 55(4), 143-153.

31. Niu, H, Feng, X-Z, Shi, C-W, Zhang, D, Chen, H-L, Huang, H-B, Jiang, Y-L, Wang, J-Z, Cao, X, Wang, N. (2022). Gut bacterial composition and functional potential of Tibetan pigs under semi-grazing. *Frontiers in Microbiology*, 13.
32. O'Callaghan, TF, Hennessy, D, McAuliffe, S, Kilcawley, KN, O'Donovan, M, Dillon, P, Ross, RP, Stanton, C. (2016). Effect of pasture versus indoor feeding systems on raw milk composition and quality over an entire lactation. *Journal of Dairy Science*, 99(12), 9424-9440.
33. Osborne, JM, Dehority, B. (1989). Synergism in degradation and utilization of intact forage cellulose, hemicellulose, and pectin by three pure cultures of ruminal bacteria. *Applied and Environmental Microbiology*, 55(9), 2247-2250.
34. Park, SJ, Beak, S-H, Kim, SY, Jeong, IH, Piao, MY, Kang, HJ, Fassah, DM, Na, SW, Yoo, SP, Baik, M. (2018). Genetic, management, and nutritional factors affecting intramuscular fat deposition in beef cattle—A review. *Asian-Australasian journal of animal sciences*, 31(7), 1043.
35. Prache, S, Martin, B, Coppa, M. (2020). Authentication of grass-fed meat and dairy products from cattle and sheep. *Animal*, 14(4), 854-863.
36. Rhoades, R, Sawyer, J, Chung, K, Schell, M, Lunt, D, Smith, S. (2007). Effect of dietary energy source on in vitro substrate utilization and insulin sensitivity of muscle and adipose tissues of Angus and Wagyu steers. *Journal of animal science*, 85(7), 1719-1726.
37. Ríos-Covián, D, Ruas-Madiedo, P, Margolles, A, Gueimonde, M, De Los Reyes-gavilán, CG, Salazar, N. (2016). Intestinal short chain fatty acids and their link

with diet and human health. *Frontiers in microbiology*, 7, 185.

38. Rismani-Yazdi, H, Carver, SM, Christy, AD, Yu, Z, Bibby, K, Peccia, J, Tuovinen, OH. (2013). Suppression of methanogenesis in cellulose-fed microbial fuel cells in relation to performance, metabolite formation, and microbial population. *Bioresource technology*, 129, 281-288.
39. Rivero, MJ, Lee, MR. (2022). A perspective on animal welfare of grazing ruminants and its relationship with sustainability. *Animal Production Science*, 62(18), 1739-1748.
40. Samra, JS, Clark, ML, Humphreys, SM, MacDonald, IA, Bannister, PA, Frayn, KN. (1998). Effects of physiological hypercortisolemia on the regulation of lipolysis in subcutaneous adipose tissue. *The Journal of Clinical Endocrinology & Metabolism*, 83(2), 626-631.
41. Shah, H, Collins, M. (1989). Proposal to restrict the genus Bacteroides (Castellani and Chalmers) to *Bacteroides* fragilis and closely related species. *International journal of systematic and evolutionary microbiology*, 39(1), 85-87.
42. Shah, T, Ding, L, Ud Din, A, Hassan, F-u, Ahmad, AA, Wei, H, Wang, X, Yan, Q, Ishaq, M, Ali, N. (2022). Differential effects of natural grazing and feedlot feeding on yak fecal microbiota. *Frontiers in Veterinary Science*, 9, 252.
43. Shanks, OC, Kelty, CA, Archibeque, S, Jenkins, M, Newton, RJ, McLellan, SL, Huse, SM, Sogin, ML. (2011). Community structures of fecal bacteria in cattle from different animal feeding operations. *Applied and environmental microbiology*, 77(9), 2992-3001.
44. Shen, J, Liu, Z, Yu, Z, Zhu, W. (2017). Monensin and nisin affect rumen fermentation and microbiota differently in vitro. *Frontiers in microbiology*, 8, 1111.
45. Shoemaker, NB, Anderson, K, Smithson, S, Wang, G, Salyers, A. (1991).

Conjugal transfer of a shuttle vector from the human colonic anaerobe *Bacteroides* uniformis to the ruminal anaerobe *Prevotella* (*Bacteroides*) ruminicola B (1) 4. *Applied and environmental microbiology*, 57(8), 2114-2120.

46. Smid, A-MC, Weary, DM, Von Keyserlingk, MA. (2020). The influence of different types of outdoor access on dairy cattle behavior. *Frontiers in veterinary science*, 7, 257.
47. Stewart, RD, Auffret, MD, Warr, A, Wiser, AH, Press, MO, Langford, KW, Liachko, I, Snelling, TJ, Dewhurst, RJ, Walker, AW. (2018). Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nature communications*, 9(1), 870.
48. Teixeira, D, Miranda-De La Lama, G, Villarroel, M, Garcia-Belenguer, S, Sañudo, C,María, G. (2012). Effect of straw on lamb welfare, production performance and meat quality during the finishing phase of fattening. *Meat Science*, 92(4), 829-836.
49. Turnbaugh, PJ, Ley, RE, Mahowald, MA, Magrini, V, Mardis, ER, Gordon, JI. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444(7122), 1027-1031.
50. Wallace, RJ, McKain, N, Broderick, GA, Rode, LM, Walker, ND, Newbold, CJ, Kopecny, J. (1997). Peptidases of the rumen bacterium, *Prevotella ruminicola*. *Anaerobe*, 3(1), 35-42.
51. Wang, L, Liu, K, Wang, Z, Bai, X, Peng, Q, Jin, L. (2019). Bacterial community diversity associated with different utilization efficiencies of nitrogen in the gastrointestinal tract of goats. *Frontiers in microbiology*, 10, 239.
52. Wexler, HM. (2007). *Bacteroides*: the good, the bad, and the nitty-gritty. *Clinical microbiology reviews*, 20(4), 593-621.

53. Wilkinson, JM, Lee, MR, Rivero, MJ, Chamberlain, AT. (2020). Some challenges and opportunities for grazing dairy cows on temperate pastures. *Grass and Forage Science*, 75(1), 1-17.

54. Yost, WM, Young, JW, Schmidt, SP, McGilliard, AD. (1977). Gluconeogenesis in ruminants: propionic acid production from a high-grain diet fed to cattle. *The Journal of Nutrition*, 107(11), 2036-2043.

55. Zhang, R, Zhu, W, Zhu, W, Liu, J, Mao, S. (2014). Effect of dietary forage sources on rumen microbiota, rumen fermentation and biogenic amines in dairy cows. *Journal of the Science of Food and Agriculture*, 94(9), 1886-1895.

56. Zhang, Z, Yang, L, He, Y, Luo, X, Zhao, S, Jia, X. (2021). Composition of fecal microbiota in grazing and feedlot angus beef cattle. *Animals*, 11(11), 3167.

57. Ziemer, CJ. (2014). Newly cultured bacteria with broad diversity isolated from eight-week continuous culture enrichments of cow feces on complex polysaccharides. *Applied and Environmental Microbiology*, 80(2), 574-585.

Study 2

Comparison between concentrate with rice straw and fermented feed supplementation with forage silage: growth performance and carcass characteristics in early and late fattening period of Hanwoo steers

1. Abstract

The present study was conducted to determine the effects of formulated feed (IRG: Italian ryegrass, WCC: whole crop corn, and fermented feed) on growth performance, carcass characteristics, and meat quality of Hanwoo steers during early and late fattening periods. Twelve Hanwoo steers were randomly assigned into two groups: concentrate with rice straw and fermented feed supplementation with forage silage (n = 6/group). The CRS group received concentrate with rice straw (CRS); the FF group received fermented feed supplementation with forage silage (FF) for 13 months. Results revealed that FF supplementation significantly ($p < 0.05$) increased effects on final body weight, average daily gain, and carcass yield than CRS feed. Hanwoo steers fed FF showed higher meat quality, carcass yield, and ribeye area than steers fed CRS feed. However, meat quality characteristics (such as cooking loss, fat thickness, marbling score, meat color, and crude fat), sensory characteristics, and pH values were similar between the two groups (all $p > 0.05$). There was no significant difference in fatty acids compositions of steers between the two groups ($p > 0.05$). Overall, these results indicate that feeding Hanwoo steers with FF can improve their growth efficacy and carcass yield during early and late fattening periods. Regarding an economic strategy, our research findings suggest that FF is effective and profitable for feeding Hanwoo steers during early and late fattening periods without causing adverse effects.

2. Introduction

In recent years, feed production costs have been increased by 70-80% in the livestock industry, largely due to dietary demands on raw materials. Particularly, Korea imports 60% of feed grains for livestock animals from other countries. This rising feed costs directly affect domestic livestock farms. It is well recognized that feed plays a key role in modulating fatty acid compositions of livestock, goats and sheep. It also considered as most important factor in livestock production, which is often higher that half of production cost. To overcome this issue, Korean government and livestock industry contributors have more attention on the production of new low-cost alternative feedstuffs for farmers engage in livestock (Jeong et al., 2016). Hanwoo is a major producer of cattle meat, producing extremely marbled beef, accounting for roughly 85% of all slaughtered cattle in 2017 (Chung et al., 2018). Hanwoo steers are typically fattened until nearly 30 months of age and since fattening period Hanwoo steers are feeding high-density concentrate diets. It is enhanced a high level of marbling, as showed by Lee et al, (2007) who evidenced that Hanwoo steers enhanced their marbling substantially between the ages of 12 and 27 months. Marbling is one of the key factors deciding Hanwoo quality grade. It can improve by feeding high-density concentrate diets (Lee et al., 2004). However, feeding high-density concentrate diets during the final fattening cycle might have a negative impact on digestive metabolism, feed quality, and feed intake (Lee et al., 2012). In addition, the demand for highly marbled beef has increased. Thus, beef producers have recently increased their use of grain-based feeds during the fattening season. Concentrate feed contains an excessive amount of fermentable carbohydrate, causing acidosis in cattle. During the fattening period, replacing animal-based materials with plant-based materials or microbially fermented feed can prevent acidosis and preserve the integrity and health of ruminal papillae and nutrient absorption (Neubauer et al., 2020). Nowadays, large amounts of concentrate are formulated with grass or whole-grain cereals, among other items, to provide energy, protein, and essential nutrients to animals. They have been used to fatten cattle in Korea despite the fact that the feed can cause excessive drops in ruminal pH during the late fattening period (Ogata et al.,

2019). Grain crops, such as maize, are increasingly being moved to the energy market in agricultural markets for food and feed. As a result, there is competition for biomass as bioenergy raises forecasts of international grain prices (Huang et al., 2011). Therefore, the use of other types of feeds, such as grass, to replace grain-based feeds must be investigated.

Grass and grass-based feeds are inexpensive and low-cost natural sources of high-quality roughage for ruminants. Furthermore, grass-fed cattle have a wide range of polyunsaturated fatty acids (PUFAs), saturated fatty acids (SFAs), and conjugated linoleic acid. Animal nutrition has a significant effect on FA composition and animal protein. Beef quality is primarily dependent on fat content and FA composition (Al-Thuwaini et al., 2019; Daley et al., 2010). Owing to strong market demand in South Korea, Hanwoo steers have a fattening period before slaughter. In general, beef skeletal muscles have large amounts of polyunsaturated fatty acids, such as alpha-linolenic acid, linoleic acid, and oleic acid, all of which have health benefits for consumers. Meat production processes should attempt to comprise grass-enriched beef production systems, while meeting consumer quality standards in terms of food quality at the same time (Chung et al., 2018) to satisfy retailer and customer demands. However, fat from grass-finished beef has been confirmed to have a yellowish appearance due to an increased level of carotenoid, suggesting that grass-rich feeds can adversely affect the quality of meat (Niderkorn et al., 2009). A higher concentrate in forage-based feeds for fattening ruminants can improve the quality of meat production. When concentrate is added to a forage feed, the efficiency of using absorbed nutrients to synthesis of animal tissues or products is typically enhanced. Furthermore, mixing concentrate and forage in the feed optimizes ruminant fattening and results in faster and more productive growth as well as heavier carcasses than either concentrate or forage alone. When fresh forage was added to the feed of lactating buffalo, the percentage of unsaturated fatty acids is increased while the percentage of short-chain fatty acids is decreased. Additionally, sensory characteristics are altered by forage without influencing consumes. It has been concluded that replacing green fodder in the feed is a low-cost feeding strategy for improving buffalo health (Uzun et al., 2018).

Forages, including Italian ryegrass (IRG), corn and wheat straw, are important for the healthy

production of the rumen in the ruminants. The quality of forage has a significant impact on productivity (Weiss et al., 2017). Total mixed ration (TMR) is commonly used for dairy cattle production in Korea. It was first introduced to Hanwoo in the 2000s. TMR is a complete feed consisting of forage, concentrate, minerals, vitamins, protein feeds and other additives in certain portions. It is of great interest to farmers because it has promising benefits for the management and development of ruminant (Bueno et al., 2020). Farmers raising domestic-bred fattening cattle have an interest in fibroid feed materials, such as TMR, over-concentrate because these cattle need more feed intake for rapid body weights (Kim et al., 2012). It has been evidenced that feedstuff has a key function in feed consumption, preservation of homeostasis of ruminant pH, minimize the occurrence of metabolic disease, maximizing milk production enhance their lifetime, and decrease labor input (MeGrath et al., 2018). Xie et al.(2020) stated that the performance of TMR feed and milk production was improved compared to individual feeds. Feeding TMR is appropriate with the addition of high humidity agriculture by-products. Silage, feed and straw are typical roughages used in the TMR.

Fermentation is a process that plays a significant role in the use of potential nutrients for improving their conservation and reducing anti-nutritional substances in TMR/silage process. Lactic acid bacteria (LAB) have been widely used as inoculants in most silage processes due to their higher efficiency for containing lower levels of acetic acid and butyric acid. Furthermore, LAB can control the unwanted microbial growth in ensiled silages. For example, *L. plantarum* is a lactic acid bacterium known to play an important role in the nutritional enhancement of silages through the production of lactic acid and other metabolites during the ensiling process as a preventive additive to silage and haylage production (Agarussi et al., 2019; Soundharrajan et al., 2019; Kim et al., 2015). Total mixed Fermented (TMF) is a concentrated mixture that is fermented under anaerobic conditions (i.e. ensiling). It may reduce anti-nutritive substances, such as mimosine or hydrocyanic acid (HCN). Kim et al. (2018) studied TMF, the addition of lactic characteristics acid bacteria and fermentation for 21 days and demonstrated that it could improve feed intake and growth efficiency in Hanwoo steers compared to fed with concentrate and rice

straw. However, the benefits of feeding fermented feed and rice straw and concentrates separately feed to Hanwoo steers from the early period to the late fattening period have not been reported yet. It is important to perform feeding trials to compare fermented feed combined with forage silage and compare it to a conventional rice straw combined with concentrate-based system in order to determine the efficacy of the fermented feed combined with forage silage/concentrate feeding system on Hanwoo steers. Thus, the main objective of this study was to compare the growth efficiency, carcass quality, and meat quantity and quality characteristics of Hanwoo steers fed between fermented feed combined with forage silage and rice straw and concentrates separately feed.

3. Materials and methods

3.1. Collection of crops and silage production with lactic acid bacteria

Italian ryegrass (IRG) and corn were cultivated at the Daum Hanwoo farm, Jeongeup, Jeollabuk-do, South Korea. IRG at early flowering and whole crop corn (WCC) at the yellow ripening stage were harvested and wilted in the field condition for two days. After reaching expected moisture (65–70%), the harvested samples were applied with lactic acid bacteria by automatic spraying method (mixture of three *Lacticaseibacillus plantarum* K46, KCC-10, and KCC-19, 100 g/50 tons, Top silage, Jeongeup, Korea) and packed in a round bale using the wrapping machine. LAB was prepared in sterile distilled water before silage preparation (Arasu et al., 2014).

3.2. Fermented feed preparation

Fermented feed was prepared following user guidelines of Innobio Corporation (Innobio Co, Shiheung, Korea) according to feed composition standard tables provided by the National Institute of Animal Science, which included cornflake, corn gluten feed, distillers dried grains, rice bran (fermented by microbes), limestone, and vitamin premix, with three microbial strains (*L. plantarum*, *B. subtilis*, and *S. cerevisiae*) maintained at an optimal temperature of 25 ◦C for 3 to 6 days under

aerobic conditions to accomplish proper fermentation. Subsequently, fermented feed was moved to a feed mixer (DW330-22, Dongwoo Solution Co., Changwon, Korea) through a conveyor system.

3.3. Animal and experimental design

This experimental study was designed and performed according to animal care and standard guidelines of the National Institute of Animal Science, South Korea (Approval Number NIAS-2020-443, Approval date: 17 February 2020). Twelve Hanwoo steers were obtained at 13 months old and were kept in individual cages located at the Daum Hanwoo farm, Jeongeup, Jeollabuk-do, South Korea. These animals were divided into two groups; the fed concentrate with rice straw separately (CRS) and fermented feed supplementation with forage silage (FF), each consist of 6 animals. The initial body weight for the CRS was 376.4 kg, and the FF was 415 kg. Feeding system: FF received concentrate with rice straw, FF received fermented feed supplementation with forage silage (IRG and WCC silages) for 13 months, according to growth steps following the Korean feeding standard (Figure 10). In detail, from the early fattening period (6 months), the CRS animals received rice straw (3.10 kg of DM/day), with concentrate (7.09 kg of DM/day), FF animals received fermented IRG (1.95 kg of DM/day), WCC (3.81 kg of DM/day), and feed (5.46 kg of DM/day) without concentrate. From the late fattening period (7 months), CRS group animals received rice straw (1.58 kg of DM/day), with concentrate (11.15 kg of DM/day), FF group animals received rice straw (1.58 kg of DM/day), Fermented IRG (1.29 kg of DM/day), WCC (0.43 kg of DM/day), feed (8.86 kg of DM/day) and concentrate (6.43 kg of DM/day). Water was provided to animals *ad libitum* every day. They were fed the experimental feed twice daily at 9.00 and 16:00. They had *ad libitum* access to water. During the study period, their average body weight, daily gain, and feed intake were measured.

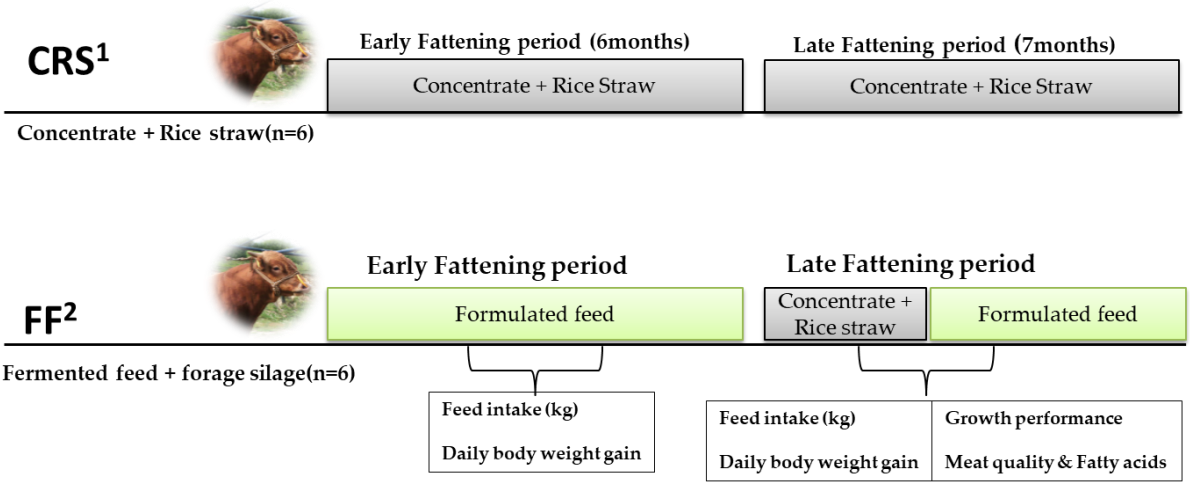

Figure 10. Experimental design and animal feeding system for concentrate and rice straw separately feed (CRS) and fermented feed with forage silage (FF).

[1]CRS: Hanwoo steers fed concentrate and rice straw separately; [2]FF: Hanwoo steers fed IRG silage + whole crop corn silage + fermented feed.

3.4. Nutrient profiles of rice straw and silage

Random grab samples of rice straw, IRG silage, and WCC silage were ground through a 1 mm screen for proximal determination. Dry matter content (DM) of rice straw was examined after drying in an air-dryer at 65 °C for 3 days using AOAC and DM of forage silage examined after drying in a freeze-dryer at −80 °C for 3–5 days (AOAC, 1995). Crude protein (CP), ether extract (crude fat), neutral detergent (NDF), and acid detergent (ADF) were determined (Serrapica et al., 2019; Al-Mentafji, 2005). Digestible energy values were calculated from total digestible nutrients (TDN) (Chang, 2018). Chemical compositions of rice straw, Italian ryegrass silage, and corn silage are shown in Table 9. Ingredients and chemical compositions of concentrate and fermented feed are presented in Table 10 and Table 11.

Table 9. Nutrient compositions of rice straw, Italian ryegrass (IRG) silage and whole crop corn (WCC) silage

Item (% DM)	Rice straw	IRG silage	WCC silage
Dry matter	88.50 ± 0.40[a]	34.96 ± 0.84[b]	31.97 ± 0.34[b]
Crude protein	4.40 ± 0.05[b]	8.40 ± 0.13[a]	8.69 ± 0.50[a]
Acid detergent fiber	42.10 ± 0.19[a]	39.41 ± 0.43[a]	31.20 ± 2.29[b]
Neutral detergent fiber	69.09 ± 0.12[a]	66.85 ± 0.43[a]	46.82 ± 2.12[b]
Total digestible nutrient	55.65 ± 0.15[b]	57.77 ± 0.34[b]	68.06 ± 0.97[a]

[a, b] different letters within a row indicates difference (p <0.05).

Table 10. Ingredient compositions and chemical analysis of concentrate and fermented feed

Concentrate Feed	(DM %)	Fermented feed (FF)	(DM %)
Ingredients		***Ingredients***	
Corn grain	27.47	Cornflake	73.50
Wheat grain	17.00	Corn gluten feed	10.00
Cane molasses	5.00	Distillers dried grains	5.00
Tapioca	6.00	Rice bran	10.00
Wheat flour	3.00	Probiotics	0.10
Corn gluten feed	20.00	–	–
Rapeseed meal	4.00	–	–
Palm kernel meal	8.82	–	–
Cottonseed hull	1.00	–	–
Tallow	0.62	–	–
Salt dehydrated	0.50	–	–
Limestone (1mm)	1.96	Limestone (1mm)	1.40
Vitamin premix[a]	0.10	Vitamin premix[c]	0.10
Mineral premix[b]	0.10	–	
Chemical compositions		***Chemical compositions***	
DM[1]	88.68	DM[1]	73.50
CP[2]	12.90	CP[2]	11.25
EE[3]	3.76	EE[3]	6.59
CF[4]	6.10	CF[4]	3.68
Calcium	0.91	Calcium	0.03
Phosphorous	0.41	Phosphorous	0.36
Crude ash	6.62	Crude ash	2.07
ADF[5]	22.50	ADF[5]	2.93
NDF[6]	35.50	NDF[6]	14.60
TDN[7]	73.00	TDN[7]	86.69

[a]Vitamin premix contained the following ingredients diluted in cellulose (g/kg premix): L-ascorbic acid, 121.2; DL-α-tocopherol acetate, 18.8; thiamine hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myoinositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinal acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003. [b]Mineral premix contained the following ingredients (g/kg premix): Mg SO_4_$7H_2O$, 80.0; NaH_2PO_4 $2H_2O$, 370.0; KCl, 130.0; ferric citrate, 40.0; $ZnSO_4$_$7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3$_$6H_2O$. 0.15; KI, 0.15; Na_2Se2O_3, 0.01; $MnSO_4$_H_2O, 2.0; $CoCl_2$_$6H_2O$, 1.0. Concentrate feed was provided by NongHyup Company. [c]Vitamin premix contained the following ingredients (Power Vitamine, Genobio, Republic of Korea): Vitamin A, 6,000,000 IU; Vitamin D3, 1,200,000 IU; Vitamin E, 1,000 mg; Vitamin B1, 500 mg; Vitamin B2, 500 mg; Vitamin B6, 500 mg; Vitamin B12, 10 mg; Protected Vitamin C, 5,000 mg; Pantothenic acid, 1,000 mg; Niacin, 1,000 mg; Biotin, 30 mg; Folic acid, 600 mg; Mn, 100 mg.; [1]DM: dry matter; [2]CP: crude protein; [3]EE: ether extract; [4]CF: crude fiber; [5]ADF: acid detergent fiber; [6]NDF: neutral detergent fiber; [7]TDN: total digestible nutrient.

Table 11. Feeding program and chemical analysis of concentrate and rice straw separately feed (CRS) and fermented feed with forage silage (FF)

Item	CRS[1]			FF[2]		
	Early fattening period	Late fattening period	Total fattening period	Early fattening period	Late fattening period	Total fattening period
			Feeds composition (% DM)			
Concentrate	69.57	87.56	79.56	-	36.14	18.87
Fermented feed	-	-	-	45.61	49.80	47.80
IRG[3] silage	-	-	-	18.42	7.23	12.58
WCC[4]	-	-	-	35.96	2.41	18.45
Rice straw	30.43	12.44	20.44	-	4.42	2.31
Total	100	100	100	100	100	100
			Chemical composition (% DM)			
DM[5]	88.63	88.66	88.64	69.98	78.91	74.44
CP[6]	10.31	11.84	11.08	9.80	11.28	10.54
ADF[7]	28.72	24.94	26.70	19.82	15.05	17.43
NDF[8]	45.72	39.68	42.70	35.81	29.11	32.46
TDN[9]	67.72	70.84	69.28	74.66	77.83	76.25

[1] CRS: Hanwoo steers fed concentrate and rice straw separately; [2] FF: Hanwoo steers fed IRG silage + WCC silage + FF; [3]IRG: Italian ryegrass; [4]WCC: whole crop corn; [5]DM: dry matter; [6]CP: crude protein; [7]ADF: acid detergent fiber; [8]NDF: neutral detergent fiber; [9]TDN: total digestible nutrient.

3.5. Carcass characteristics and meat quality

At the end of the experimental period, all steers were killed at a commercial abattoir regulated by National Agricultural Cooperative Federation, Korea (KOR) to determine carcass characteristics and meat quality. Immediately after the slaughter process, carcasses were weighed and then chilled for 48 hours at 4°C. Intramuscular samples were taken from the 13-14th ripples, ground, and mixed well to determine fatty acids of *M. longissimusdorsi* (LM). Grade of quantity (5 = grade A to 2 = grade D), marbling (1 = low-fat to 5 = high fat), color of meat (1 = very bright cherry red to 7 = very dark red), color of fat (1 = white to 7 = yellow), texture (1 = very smooth to 3 very coarse), maturity (1–15 mm to 15–26 mm old = 2), and grade of quality (7 = grade 1 + to grade 3 = 1) were assessed. Back fat thickness was measured perpendicular to the outside surface at a point two-thirds the length of the rib-eye between the last rib and the first lumbar vertebrae. Using a standard grid, the area of the rib-eye at the surface of the cut was calculated. Ten grams of chopped meat was combined with ten milliliters of deionized water and the pH of the slurry was determined using a pH meter (F-12, Horiba, Japan). Cooking loss was measured as de-scribed by Kang et al. (Kang et al., 2010). Cooking loss sample was then used to calculate shear force. The sample was cut into 1.5 cm length and 1 cm2 cross section with the fiber direction. A texture analyzer (TA-XT2i, Stable Microsystems Ltd., UK) equipped with a 25 kg load cell and a Warner-Bratzler shear blade at a test speed of 2.0 mm/s was used to determine the shear force. Only the maximum force (kg) was considered. A portion of the meat sample was cut into 2 cm wide, 4 cm long, and 0.5 cm thick pieces and roasted in a home-use electronic pan until an internal temperature reached 73°C.

3.6. Investigation of fatty acid

Lipids were extracted with chloroform/methanol and then evaporated using a solvent evaporator (PT-MR3100 Kinematica, Luzern, Switzerland). In a dry bath (Type 16500, USA), chloroform was dried using nitrogen gas at 50°C. Lipid methylation was performed before the sample was injected into a gas chromatograph (HP 5890 II fixed with a G1513A auto-sampler;

Hewlett Packard Co., Alto Palo, CA, USA). FA level was calculated with an ultra-pure helium as carrier gas (flux rate = 1.0 mL/min) using a silica capillary column (100 m 0.25 mm, i.e., 0.20-meter thickness, Supelco SP). Injector and detector temperatures were kept at 250°C. The original oven temperature was set at 140°C (30min). It was then increased to 220°C at 15°C/min(40min) (Arasu et al., 2014). The free fatty acids in samples were identified by comparing their retention times (RT) with standard RT of fatty acids (Sigma Aldrich, USA, # 47015U)

3.7. Statistical analysis

Significant differences between control and experimental samples were analyzed with linear model procedures of SAS (SAS version 9.4, SAS Institute, Cary, NC, USA). Samples were analyzed in triplicate. Data are presented as mean ± standard error of the means. Mean differences between two different feeding trials were analyzed using Dun-can's multiple range tests. Statistically significant difference was considered at $p < 0.05$.

4. Results and discussion

4.1. Nutrient compositions

In the current study, lactic acid bacteria (LAB) were added to the Italian ryegrass and whole crop corn silages and fermented feed as a biological additive. The nutrient profiles of silages and fermented feed such as dry matter content (DM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF) and total digestible nutrient (TDN) were analyzed. Impact of silages and fermented feed on growth performance, carcass characteristics and meat quality investigations during fattening periods in Hanwoo steers was studied and compared to commercial concentrate. Nutrient profiles such as CP content (8.40 %; 8.69 %) and TDN (57.77 %; 68.06 %) slightly varied between Italian ryegrass (IRG) silage and whole crop corn (WCC) silage. However, rice straw had very low CP content (4.40 %) and moderate level of TDN (55.65 %) than the silages. Dry matter content (DM) (65.04 %; 68.04 %), ADF (39.41 %; 31.20 %), and NDF level of (66.85 %; 46.82 %) of IRG and corn silage were lowered compared to rice straw (DM: 88.50 %; ADF: 42.10 %; NDF: 69.09 %) (Table 9). The nutrient profiles of IRG silage in this study was close to that of corn silage reported previously (Kim et al., 2003; Kim et al., 2015). LAB considered essential biological additives for silage production by rapid induction of fermentation process at ensiled condition. It can produce various organic acids, particularly lactic acid concentrations, which increase the acidification of silages. Rapid acidification prevented the yeast and mold growth in silages and preserved with high nutrients. Therefore, we used microbiological additives for animal feed production. It is a cost-effective strategy with high/similar effects compared to commercial feed. Hetero-fermentative lactic acid bacteria can promote acetic acid biosynthesis, thus improving aeration of silage and its quality. On the other hand, water-soluble carbohydrates are fermented by homo-fermentative bacterial inoculants into organic acids, especially lactic acid, which can quickly acidify silage and inhibit the growth of undesirable bacteria. To regulate the growth of yeast, homolactic inoculants such as lactobacilli known to produce acids such as acetic and propionic acids that inhibit the growth of microorganisms that trigger spoilage (Selwet, 2020) were added.

Inoculants include one or two groups of acidophilic bacteria. During co-fermentation, the synthesis of acetic acid, 1,2-propanediol and propionic acid is stimulated by bacterial strains of these species. They make sustainable feed silage more aerobically stable. The findings of the study could be used to establish bacterial preparations that can facilitate the ensilage of renewable raw materials.

4.2. Growth performance

A significant amount of dietary roughage is essential in TMR for healthy rumination (Jiang et al., 2017). To meet maintenance and production requirements, dietary intake is essential. It has been reported that the nature of feed and environmental conditions also plays a key role in the feed intake of animals. This study replaced the concentrate and rice straw with FF and fed it to animals for 13 months. Animals fed with FF showed higher feed intake during early fattening (11.22 kg of DM/day) as well as late fattening (14.02 kg of DM/day) period's than the animals fed with CRS feed (10.19 kg of DM/day vs 12.73 kg of DM/day) (Table 12). These data suggest that the animals had more interested in eating FF than the CRS. It might be due to microbially fermented silages. It contains various organic acids, which makes good smells and taste. A similar experiment with other silages was conducted in Hanwoo steers; this study author reported that the total feed intake was higher in animals fed with mixed silage and concentrate than the concentrate with rice straw (Lee et al., 2010). Previous experimental data also showed Hanwoo steers fed with IRG silage had a higher feed intake average compared to concentrate with rice straw (Kim et al., 2015). However, Teixeira Junior et al. (2015) reported that animals fed silage have lower feed consumption than control diet which is different from our findings. The increase of nutrient intake by the treatment group might have resulted in an enhanced metabolic balance by gain or changes in fermentation expansion. The initial body weight average of Hanwoo steers was 376 kg for CRS and 415 kg for FF during the early fattening period. In late fattening period an average body weight for CRS was 576 kg and for FF was 624.4 kg. The final body weight average of FF fed Hanwoo steers was 595 kg during the early fattening period and 752 kg during the late fattening period. As

shown in Table 12, the average daily gains of Hanwoo steers fed with FF was higher than that of Hanwoo steers fed with CRS feed during both early fattening periods ($p < 0.05$). These results showed that the average daily gain during the early fattening period was higher than that during the late fattening period. It indicates that the animals treated with our FF increased daily feed intake than the CRS fed animals; it might influence the increase of average daily gain during the experimental period. This data confirmed that the replacement of concentrate with fermented silages and feed had a significant impact on animal performance. This data consistent with Lin et al. (Lin et al., 2004) have also found that fermented feed supplementation could improve feed intake, average daily gain, and feed conservation ratio of Hanwoo steers. Cho et al. (2009) and Kim et al. (2018) also reported an increase in total feed intake and a decrease in the intake of concentrate, when cattle fed with fermented silage than those fed with a roughage feeding system. In fact, a lower nutritional value of the feed could not help but decrease nutrient concentration and rumen wealth. This research found that formulated feed could alter moisture content and nutrition consumption that potentially enhance body weight gain. Overall study reports confirmed that the replacement of silages and fermented feed significantly improve the animal's performances than the concentrated and rice straw fed animals.

4.3. Carcass and meat quality characteristics

The fermented feed shows significant positive impacts on body weight and feed intakes. Then we analyzed the effects of fermented feed supplementation on carcass yield and quality characteristics of Hanwoo steer during early and late fattening periods. Results are shown in Table 13. Carcass weight (CRS 401.8 kg vs FF 444 kg) and rib-eye area (CRS 87.6 cm^2 vs FF 91cm^2) of fermented fed Hanwoo steers were substantially increased compared to those of CRS (concentrate + rice straw+ whole crop silage) feed fed Hanwoo steers (both $p < 0.05$). However, backfat thickness (CRS 10 mm vs FF 13.20 mm), dressing percent, and quantity characteristics such as marbling score, meat color, fat color, texture, and quality grade were similar between FF and CRS of Hanwoo steers throughout the experimental period. The findings of the present study were

consistent with those of Kim et al. (2015). They reported that carcass weight and ribeye area of Hanwoo steers were enhanced by feeding IRG silage. Kim et al. (2018) have also reported similar results, showing that carcass weight of steer was significantly decreased when concentrate supplementation provided, consistent with our data. Table 14 summarizes chemical compositions (Percentage of moisture, crude fat, crude protein, and ash,) and meat quality (such as cooking loss, shear force, water holding capacity, surface color, and sensory evaluation) of FF/CRS group animals Hanwoo steers. A slight increase in moisture percentage was noted in animal fed with fermented feed compared to animals fed with concentrate. Crude fat and protein level remained unchanged between CRS and FF animals. For meat qualities study, less cooking loss was noted for animals treated with FF than the CRS animals ($p < 0.05$). Shear force, water holding capacity and pH were not changed significantly between CRS and FF animals. In addition, surface color and sensory evaluation were similar in CRS and FF animals. Overall acceptability is slightly higher for animals treated with FF than CRS animals fed with concentrate and rice straw. Duckett et al. (2013) have also reported that intramuscular fat content and palatability can be improved by moving from a forage feed to a high concentrated feed at 40 d prior to slaughter. Kerth et al. (2007) indicated that cattle finished on forage have subcutaneous fat that is more yellow in color compared to grain-finished cattle, which might be a drawback for the customer to consider forage-finished beef. Scheffler and Gerrard (2007) suggested that post-mortem glycogen is converted to lactate and H^+, resulting in a decrease in the pH of meat and that the amount of glycogen at slaughter is inversely linked to the ultimate pH. Wicks et al. (2019) proposed that a high energy feed could increase post-mortem glycolysis ability, leading to an extended pH decline and a lower final pH. Overall data confirmed that the low-cost silage and fermented feed could improve the animal performance and meat quality by either increasing/ similar to high-cost concentrate fed animals.

Table 12. Effects of feeding fermented feed supplementation with forage silage (FF) or concentrate and rice straw (CRS) separately feed on growth performance, total feed intake, and feed conversion ratio in Hanwoo steers during early and late feeding period

Items	Early fattening period				Late fattening period				Total period			
	CRS[1]	FF[2]	SEM[3]	*P*-value[4]	CRS[1]	FF[2]	SEM[3]	*p*-Value[4]	CRS[1]	FF[2]	SEM[3]	*P*-value[4]
Initial body weight (kg)	376	415	11.50	0.26	576	624	1.47	0.03	376	415	11.5	0.26
Final body weight (kg)	539	595	14.80	0.04	696	752	17.4	0.03	696	752	17.40	0.03
Average daily gain (kg)	0.83	0.93	0.04	0.05	0.76	0.83	0.05	0.20	0.79	0.88	0.02	0.22
Total feed intake (kg of DM)	10.19	11.22	0.20	0.51	12.73	14.02	0.37	0.01	11.46	12.62	0.28	0.02
Concentrate (kg of DM)	7.09				11.15	5.70			9.12	2.85		
Rice straw (kg of DM)	3.10				1.58	0.70			2.34	0.35		
IRG silage (kg of DM)		1.95				0.84				1.39		
WCC silage (kg of DM)		3.81				0.28				2.04		
Fermented feed (kg of DM)		5.46				6.51				5.99		
FCR[5]	14.04	13.19	0.43	0.26	16.88	20.84	1.15	0.31	15.10	16.18	0.43	0.73

[1]CRS: Hanwoo steers fed concentrate and rice straw separately; [2]FF: Hanwoo steers fed IRG silage + WCC silage + fermented feed; [3]standard error of the mean (*p*-value); [4]probability levels; [5]FCR: feed conversion ratio (total feed intake / total body weight grain); IRG: Italian ryegrass; WCC: whole crop corn.

Table 13. Carcass characteristics and quality traits of Hanwoo steers fed concentrate and rice straw separately feed (CRS) and fermented feed with forage silage (FF)

Items	CRS[1]	FF[2]	SEM[3]	*P*-value
		Carcass characteristics		
Carcass weight (kg)	402	444	12.9	0.02
Back fat thickness (mm)	10.00	13.20	0.9	0.18
Rib-eye area (cm^2)	87.60	91.00	2.1	0.05
Dressing percent (%)	59.80	61.80	0.8	0.24
Quantity grade [5]	67.00	64.60	0.8	0.2
		Quality traits		
Marbling score[6]	4.60	5.00	0.7	0.52
Lean color[7]	5.00	4.80	0.1	0.51
Fat color[8]	3.00	2.80	0.1	0.51
Texture[9]	1.40	1.20	0.2	0.13
Mature[10]	2.00	2.40	0.1	0.4
Quality grade[11]	3.00	3.40	0.3	0.57
		Economic analysis		
Total production cost[12] (KRW/steer)	8,863,074	8,459,352	31,782	0.05
Animal cost[13]	5,272,911	5,272,911	-	-
Feed cost[14]	2,118,853	1,715,130	93,450	0.05
Operating costs[15]	1,471,310	1,471,310	-	-

[1]CRS: concentrate + rice straw; [2]FF: IRG silage + WCC silage + fermented feed; [3]Standard error of the mean; [4] significance levels; [5]grade A (5 point) ~ grade D (2 point); [6]low fat (1 point) ~ high fat (5 point); [7]very light cherry red (1 point) ~ very dark red (7 point); [8]white (1 point) ~ yellow (7 point); [9]very fine (1 point) ~ very coarse (3 point); [10]below 15-month-old (1 point) ~from 15 to 26 months old (2 point); [11]grade 1^{++}(5 point), grade 1^{+}(4 point), grade 1(3 point), grade 2(2 point), grade 3(1 point). [12]Total production cost: animal cost + feed cost + operating costs; [13]animal cost: 13 month old Hanwoo steers (source: raising cost of Korean beef cattle per head, KOSIS, 2021); [14]Feed cost: CRS(concentrate + rice straw), FF(IRG silage + WCC silage + fermented feed); [15]operating cost: labor, livestock manure management, machine cost, and others(source: raising cost of Korean beef cattle per head, KOSIS, 2021); IRG: Italian ryegrass; WCC: whole crop corn.

Table 14. Chemical compositions and meat quality characteristics between concentrate with rice straw separately or fermented feed with forage silage

Items	CRS[1]	FF[2]	SEM[3]	*P*-value
Chemical compositions				
Moisture (%)	62.23	64.00	0.89	0.30
Crude fat (%)	14.38	14.34	1.18	0.47
Crude protein (%)	20.81	20.98	0.39	0.80
Ash (%)	0.85	0.85	0.02	0.32
Quality traits				
Cooking loss (%)	19.89	19.63	0.67	0.05
Shear force (kg)	4.43	3.12	0.43	0.59
Water holding capacity (%)	56.80	57.17	0.52	0.39
pH	5.51	5.51	0.03	0.19
Surface color				
CIE L*	39.07	38.49	0.65	0.99
a*	24.08	21.94	0.72	0.67
b*	11.50	9.83	0.52	0.66
Hunter L*	32.71	32.21	0.57	0.99
a*	19.24	17.35	0.68	0.76
b*	7.40	6.36	0.35	0.72
Sensory evaluation				
Color	3.33	3.81	0.10	0.05
Aroma	3.38	3.16	0.13	0.41
Juiciness	3.56	3.80	0.11	0.26
Texture	3.48	3.88	0.10	0.06
Overall acceptability	3.47	3.81	0.10	0.09

[1]CRS: Concentrate + rice straw; [2]FF: IRG silage + WCC silage + fermented feed; [3]standard error of the mean; [4]significance levels; *CIE: international commission on Illumination. IRG: Italian ryegrass; WCC: whole crop corn.

4.4. Fatty acid analysis

Many reports claim that forage feeding system as opposed to concentrate feeding resulted in beneficial changes in the fatty acids of meat, particularly a shift in n-6 to n-3 PUFA ratio and elevated levels of CLA (conjugated linoleic acid) (Dannenberger et al., 2007; Nuernberg et al., 2005). Lee et al. (2009), reported that red clover silage fed animals had higher proportions of C18: 3-n, total n-3 fatty acids and PUFA compared to animals fed with grass. Ye et al. (2020) reported that silage-based beef had higher polyunsaturated fatty acids (n3) than concentrate-based beef. In the current study, C14:0, C16:0, C16:1-n-7, C18:0, C18:1n-9, C18:3n-6, C18:2-n-6, C18:3n-3, C20:1n-9, and C20:4n-6, were analyzed in the meat of animal fed concentrated and animal fed formulated feed. These fatty acids profiles were not altered significantly between CRS and FF animals. However, we noted slight changes in C14:0 (myristic acid), C16:1n-7 (palmitic acid), and C20:1n-9 (eicosanoic acid) between experimental groups. The calculated saturated, unsaturated, monounsaturated and polyunsaturated fatty acid profiles remained unchanged in rice straw and concentrate separately and fermented feed animals. At the same time, a slight increase in unsaturated fatty acid was noted in the animals fed formulated feed compared to concentrate fed animals. The ω6/ω3 ratio was lower for animals fed formulated feed than the rice straw and concentrate separately feed (Table 15). Overall data suggested that the fermented feed supplementation did not alter fatty acid profiles in the meat compared to concentrate fed animals. This study was slightly inconsistent with previous studies, which may be due to differences in the animal breed, plant-based silages and production.

There were few limitations to the present study, I) sample size was relatively small, II) Blood parameters are essential to confirm the new feed impact on the clinical markers, particularly cytokines, but we did not analyses the clinical parameters change in the CRS and FF animals, III Gut microbiota plays a significant role in animal growth performances, so microbiome study is essential to confirm the microbial changes in the experimental animals. Advantages of the current study: This study replaced commercial concentrate with fermented feed. It prepared from grass, legume and other natural substrates by microbial fermentation. The fermented feed shows significant positive impacts on the animal's growth performance. It is a very cost-effective strategy that reduces the economic burden for livestock cultivars. Future aspect study: In-depth study is required to determine the changes in mRNA expressions in response to fermented feed supplementation, intramuscular fat deposition status, cytokines, adipokines and myokines changes and microbiome associated mechanisms.

Table 15. Fatty acid compositions in Hanwoo steers fed concentrate with rice straw separately (CRS) or fermented feed with forage silage (FF) (% of fat)

Items	CRS[1]	FF[2]	SEM[3]	*P* - value
C14:0 (myristic)	3.12	2.80	0.12	0.87
C16:0 (palmitic)	28.02	28.29	0.43	0.42
C16:1n-7 (palmitoleic)	4.91	4.31	0.18	0.31
C18:0 (stearic)	11.91	11.79	0.42	0.98
C18:1n-9 (oleic)	49.19	49.92	0.57	0.39
C18:2n-6 (linoleic)	0.11	0.13	0.01	0.08
C18:3n-6 (γ-linolenic)	2.04	1.98	0.11	0.51
C18:3n-3 (α-linolenic)	0.04	0.04	0.00	0.98
C20:1n-9 (eicosanoic)	0.51	0.60	0.04	0.76
C20:4n-6 (arachidonic)	0.15	0.15	0.02	0.97
SFA[3]	43.05	42.88	0.49	0.55
UFA[4]	56.95	59.12	0.49	0.55
MUFA[5]	54.61	54.83	0.55	0.48
PUFA[6]	2.34	2.30	0.14	0.63
ω3	0.11	0.13	0.01	0.08
ω6	2.23	2.17	0.13	0.65
ω6 / ω3	20.91	16.56	0.98	0.46

[1]CRS: concentrate + rice straw; [2]FF: IRG silage + WCC silage + fermented feed; [3]SFA: saturated fatty acids (C14:0, C16:0,18:0, C20:1n-9, C20:4n-6); [4]UFA: unsaturated fatty acid (C16:1n-7, C18:1 n-9, C18:2n-6, C18:3n-6, and C18:3n-3); [5]MUFA: monounsaturated fatty acid (C16-1n-7 and C18:1n-9) [6]PUFA: polyunsaturated fatty acid (C18:2n-6, C18:3n-6, C18:3n-3, C20:1n9, and C20:4n-6); ω3: omeaga-3 fatty acid (C18:3n-3); ω6: omeaga-6 fatty acid (C18:2n-6, C18:3n-6, and C20:4n-6).

5. Conclusion

The feed cost indicates the most extensive single variable in beef production. Therefore, in the current study, we partially replaced concentrate feed with naturally fermented IRG, corn and feed for animal performances in Hanwoo steers. The data suggest that fermented feed supplementation with forage silage fed animals had significant daily body weight gain, carcass weight, and rib-eye area than the concentrate with rice straw separately animals ($p < 0.05$). In addition, carcass characteristics, meat quality, and fatty acid profiles remained unchanged in the concentrate with rice straw separately and fermented feed with forage silage animals ($p > 0.05$). It confirmed that replacement of concentrate with low-cost fermented feed plays a significant impact on the animal performance higher or similar to the concentrate with rice straw separately animals diet. It suggested that our fermented feed with forage silage usage could be considered a cost effective strategy to improve animal performance. Further, in-depth study in fermented feed and its impact on animal performance will require in future.

6. References

1. Al-Mentafji, HN. (2005). Ofiicial methods of analysis of AOAC international. *AOAC*, Washionton, DC, USA.
2. Al-Thuwaini, T, Al-Shuhaib, MB. (2019). The effects of grass-based versus grain-based feeding of ruminants on the human hygienic status, a review. *World`s Veterinary journal*, 9(3), 174-180.
3. Bueno, AV, Lazzari, G, Jobim, CC, Daniel JL. (2020). Ensiling total mixed ration for ruminants: a review. *Agronomy*. 10(6), 879.
4. Cho, W-M, Chang, S-S, Cho, YM, Kim, HC, Kwon, EG, Yang, SH, Paek, BH. (2009). Effects of forage source and shipping time on growth performance and carcass characteristics of Hanwoo steers. *Journal of The Korean Society of Grassland and Forage Science*, 29(4), 375-382.
5. Choi, CW. (2018). Nutrient requirement for maintenance and nutritional changes of the Hanwoo steers in early-fattening stage under heat stress. *Korean Journal of Agricultural Science*, 45(1),74-83.
6. Chung, KY, Lee, SH, Cho, SH, Kwon, EG, Lee, JH. (2018). Current situation and future prospects for beef production in South Korea - A review. *Asian-Australasian journal of animal sciences*, 31(7), 951-60.
7. Daley, CA, Abbott, A, Doyle, PS, Nader, GA, Larson, S. (2010). A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. *Nutrition Journal*, 9, 10.
8. Duckett, S, Neel, J, Lewis, R, Fontenot, J, Clapham, W. (2013). Effects of forage species or concentrate finishing on animal performance, carcass and meat quality. *Journal of animal science*,91(3), 1454-1467.

9. Fisher, AV, Enser, M, Richardson, I, Wood, JD, Nute, GR, Kurt, E, Sinclair, LA, Wilkinson, RG. (2000). Fatty acid composition and eating quality of lamb types derived from four diverse breed × production system. *Meat science*, 55(2),141-147.

10. Folch, J, Lees, M, Sloane Stanley, GH. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *The Journal of biological chemistry*, 226(1), 497-509.

11. Huang, H, Von Lampe, M, Van Tongeren, F. (2011). Climate change and trade in agriculture. *Food Policy*. 36, S9-S13.

12. Jeong, C-D, Mamuad, LL, Ko, JY, Sung, HG, Park, KK, Lee, YK, Lee, SS. (2016). Rumen fermentation and performance of Hanwoo steers fed total mixed ration with Korean rice wine residue. *J Anim Sci Technol.*, 58(4).

13. Jiang, FG, Lin, XY, Yan, ZG, Hu, ZY, Liu, GM, Sun, YD, Liu, XW, Wang, ZH. (2017). Effect of dietary roughage level on chewing activity, ruminal pH, and saliva secretion in lactating Holstein cows. *Journal of dairy science*, 100(4), 2660-71.

14. Jo, C, Cho, SH, Chang, J, Nam, KC. (2012). Keys to production and processing of Hanwoo beef: a perspective of tradition and science. *Animal Frontiers*. 2(2), 32-8.

15. Kang, SN, Song, Y-M, Kim, C-W, Kim, TW, Chu, GM, Yang, BS, Jin, SK, Kim, IS. (2010). Effect of feeding high carbohydrate-low fat fermented feed on the meat quality characteristics in finishing pigs. *Korean J Food Sci Anim Resour*. 30(5), 826-832.

16. Kerth, C, Braden, K, Cox, R, Kerth, LK, Rankins, DL. (2007). Carcass,

sensory, fat color, and consumer acceptance characteristics of Angus-cross steers finished on ryegrass (*Lolium multiflorum*) forage or a high-concentrate diet. *Meat science*, 75(2), 324-31.

17. Kim, DH, Amanullah, SM, Lee, HJ, Joo, YH, Kim ,SC. (2015). Effect of microbial and chemical combo additives on Nutritive Value and fermentation characteristic of whole crop barley silage. *Asian-Australas J Anim Sci*. 28, 1274-1280.
18. Kim, SH, Alam, MJ, Gu, MJ, Park, KW, Jeon, CO, Ha, JK, Lee, SS. (2012). Effect of total mixed ration with fermented feed on ruminal *in vitro* fermentation, growth performance and blood characteristics of Hanwoo steers. *Asian-Australas J Anim Sci*., 25, 213-223.
19. Kim, TI, Mayakrishnan, V, Lim, DH, Yeon, JH, Baek, KS. (2018). Effect of fermented total mixed rations on the growth performance, carcass and meat quality characteristics of Hanwoo steers. *Animal science journal*., 89, 606-615.
20. Kim, W, Seo, S, Yoon, SH, Kim, KY, Cho, YM, Park, TI, Go, JM, Park, GJ. (2003). Selection of promising barley cultivar for silage 2. nutrient value and total digestible nutrient yield. *Journal of The Korean Society of Grassland and Forage Science,* 23(4), 283-288.
21. Kim, WH, Kang, SN, Arasu, MV, Chu, GM, Kim, DH, Park, JH, Oh, YK, Cho, KC. (2015). Profile of Hanwoo steer carcass characteristics, meat quality and fatty acid composition after feeding Italian ryegrass silage. *Korean J Food Sci Anim Resour*., 35(3), 299-306.
22. Lee, DH. (2004). Methods for genetic parameter estimations of carcass

weight, longissimus muscle area and marbling score in Korean cattle. J *Anim Sci Technol*. 46(4), 509-516.

23. Lee, SH, Park, EW, Cho, YM, Kim, SK, Lee, JH, Jeon, JT, Lee, CS, Im, SK. (2007). Identification of differentially expressed genes related to intramuscular fat development in the early and late fattening stages of Hanwoo steers. *Journal of biochemistry and molecular biology*. 40(5), 757-764.
24. Lee, SM, Kim, JY, Kim, EJ. (2012). Effects of stocking density or group size on intake, growth, and meat quality of Hanwoo steers (*Bos taurus coreanae*). *Asian-Australas J Anim Sci*. 25(11), 1553-1558.
25. Lin, H, Decuypere, E, Buyse, J. (2004). Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*) 2. Short-term effect. Comparative biochemistry and physiology. *Biochemistry & molecular biology*. 139, 745-751.
26. McGrath, J, Duval, SM, Tamassia, LFM, Kindermann, MK, Stemmler, RT, Gouvea, VN, Acedo, TS, Immig, I, Williams, SN, Celi, P. (2018). Nutritional strategies in ruminants: a lifetime approach. *Research in Veterinary Science*., 116, 28-39.
27. Nascimento Agarussi, MC, Gomes Pereira, O, Paula, RAd, Silva, VP, Roseira, JPS, Silva, FF. (2019). Novel lactic acid bacteria strains as inoculants on alfalfa silage fermentation., *Scientific Reports*, 9, 8007.
28. Neubauer, V, Petri RM, Humer E, Kröger, I, Reisinger, N, Baumgartner, W, Wagner, M, Zebeli, Q. (2020). Starch-rich diet induced rumen acidosis and hindgut dysbiosis in dairy cows of different lactations. *Animals*. 10(10), 1727.

29. Niderkorn, V, Baumont, R. (2009). Associative effects between forages on feed intake and digestion in ruminants. *Animal : an international journal of animal bioscience*. 3, 951-60.
30. Ogata, T, Makino, H, Ishizuka, N, Iwamoto, E, Masaki, T, Ikuta, K, Kim, YH, Sato, S. (2019). Long-term high-grain diet altered the ruminal pH, fermentation, and composition and functions of the rumen bacterial community, leading to enhanced lactic acid production in Japanese Black beef cattle during fattening. *PLOS ONE*. 14, e0225448.
31. Santos-Silva, J, Mendes, IA, Portugal, PV, Bessa, RJB. (2004). Effect of particle size and soybean oil supplementation on growth performance, carcass and meat quality and fatty acid composition of intramuscular lipids of lambs. Livestock Production Science., 90:79-88.
32. Scheffler, TL, Gerrard, DE. (2007). Mechanisms controlling pork quality development: the biochemistry controlling postmortem energy metabolism. *Meat science*. 77, 7-16.
33. Selwet, M. (2020). Influence of inoculation with *Lactobacillus* on fermentation, production of 1,2-propanediol and 1-propanol as well as maize silage aerobic stability. *Open Life Sciences*, 15, 373-378.
34. Serrapica, F, Masucci, F, Raffrenato, E, Sannino, M, Vastolo, A, Barone, CMA, Di Francia, A. (2019). High fiber cakes from mediterranean multipurpose oilseeds as protein sources for ruminants. *Animals : an open access journal from MDPI*, 9(11), 918.
35. Shahidi, F, Rubin, LJ, D'Souza, LA, Teranishi, R, Buttery, RG. (1986). Meat flavor volatiles: a review of the composition, techniques of analysis,

and sensory evaluation. *C R C Critical Reviews in Food Science & Nutrition*., 24(2), 141-243.

36. Soundharrajan, I, Kuppusamy, P, Park, H, Kim, JH, Kim, WH, Jung, JS, Choi, KC. (2019). Lactic acid bacteria mixture as inoculants on low moisture Italian ryegrass silage fermentation. *Journal of The Korean Society of Grassland and Forage Science*. 39(3), 127-31.
37. Teixeira, Junior, D, Missio, RL, Rossi, Sforcini, MP, Oliveira, MDS. (2015). Productive performance of dairy cows fed with hydrolyzed sugarcane. *Ciência Rural*. 45(10), 1848-1853.
38. Uzun, P, Masucci, F, Serrapica, F, Napolitano, F, Braghieri, A, Romano, R, Manzo, N, Esposito, G, Francia, D. (2018). The inclusion of fresh forage in the lactating buffalo diet affects fatty acid and sensory profile of mozzarella cheese. *Journal of dairy science*. 101(8), 6752-6761.
39. Weiss, CP, Gentry, WW, Meredith, CM, Meyer, BE, Cole, NA, Tedeschi, LO, McCollum, FT, Jennings, JS. (2017). Effects of roughage inclusion and particle size on digestion and ruminal fermentation characteristics of beef steers. *J Anim Sci.*, 95(4), 1707-1714.
40. Wicks J, Beline M, Gomez JF, Luzardo, S, Silva, SL, Gerrard, D. (2019). Muscle energy metabolism, growth, and meat quality in beef cattle. *Agriculture*. 9(9), 195.
41. Wood, J, Enser, M, Richardson, I, Whittington, F. (2007). Fatty acids in meat and meat products. *Fatty acids in foods and their health implications*, 87-107.
42. Xie Y, Xu S, Li W, Wang, M, Wu, Z, Bao, J, Jia, T, Yu, Z. (2020). Effects

of the application of *Lactobacillus plantarum* inoculant and potassium sorbate on the fermentation quality, *in vitro* digestibility and aerobic stability of total mixed ration silage based on alfalfa silage. *Animals : an open access journal from MDPI*.10(12), 2229.

41. Ye, Y, Eyres, GT, Reis, MG, Schreurs, NM, Silcock, P, Agnew, MP, Johson, PL, Maclean, P, Realini CE. (2020). Fatty acid composition and volatile profile of *M. longissimus thoracis* from commercial lambs reared in different forage systems. *Foods (Basel, Switzerland)*, 9(12), 1885.

Study 3

Effects of lactic acid bacteria inoculation on performance and microbial community dynamics in anaerobic fermentation of triticale silages at different stages

1. Abstract

Production of high-quality grass-based silages by microbial-mediated anaerobic fermentation is an effective strategy in livestock farms. In the present study, an ensiling process was used to preserve and enhance fermentative metabolites in triticale silages with novel inoculants of *Lacticaseibacillus rhamanosus*-52 and, *Lacticaseibacillus rhamanosus*-54. Triticale silages treated with LAB lowered pH values than non-inoculated silages due to rapid changes of microbial counts. LAB addition improved anaerobic fermentation profiles, showing higher lactic acid, but lower acetic acid and butyric acid concentrations. A background microbial dynamic study indicated that the addition of *L. rhamanosus-52* and *L. rhamanosus-54* improved silage fermentation, enriched *Lacticaseibacillus* spp., and decreased microbial richness with diversity, leading to increased efficiency of lactic acid fermentation. In conclusion, LAB treatment can increase silage quality by enhancing the dominance of desirable *Lacticaseibacillus* while inhibiting the growth of undesirable microbes.

2. Introduction

Silages, which are produced from a range of differenced grasses, legumes and other plants, are considered the major foods for most ruminant animals (Wood, 2003). The most common crops such as corn and alfalfa are used for silage production worldwide (Chen et al., 2020). Also, small grains based cereals including barley oats, triticale and other legumes plants have been used for forage and silage production (Lithourgidis et al., 2007). Conservation of silage from various crops relies on microbial fermentation. Lactic acid bacteria (LAB) have a critical role in important organic acid productions and are thus considered essential for the preservation of silage with high quality (Liu et al., 2019; Wang et al., 2020). The selection of inoculants is based on their capability to induce acidification rapidly by the higher amount of organic acids production, particularly lactic acid, thereby increasing the aerobic stability and silages digestibility (Muck et al., 2018)

Microbial diversity has been observed in forage samples, certain undesirable microorganisms can grow in forage which produces high amounts of butyric acid, decreasing the quality silage by reducing its aerobic stability, dry matter content, water-soluble carbohydrate concentration and ease of digestibility (Pedroso et al., 2005; Vissers et al., 2007). Among such undesirable microbes, *Listeria* sp. and *Clostridia*, (Duni`ere et al., 2013) and mycotoxin fungi can produce pathogenic substances (Driehuis et al., 2018). Furthermore, yeast is the major microorganism which strongly involved in the reduction of silage quality via degradation of lactic acid and increased silage pH, which favors undesirable microbial growth (Borreani et al., 2018; Negi et al., 2022). As a biological

additives, LAB have a crucial production of legume, grass and other silages forage preservation via ensiling is becoming more widely used due to its reliable and foreseeable feed production for livestock. Digestible nutrients lost due to oxidation process in plants, activities of undesirable microbes, proteolytic activity, amino acid deamination and decarboxylation by microbes may undesirably affect preservation efficiency by increasing energy and nutrient loss and accumulate anti-nutritional compounds in silages (Oliveira et al., 2017). LAB inoculation to a forage during ensiling promotes silage acidification by production of various organic acids, includes lactic, acetic, butyric, propionic acid and other essential acids. The lower pH environments could prevent spoilage microbial growth inhibition of yeast, mold and listeria species could improve the aerobic stability.

Understanding the microbial community's contributions to silage production is a critical factor in identifying microorganisms that are suitable for silage production and controlling the growth of pathogens; it could also provide novel insights that will aid in preserving silage quality (Frank, 2013). Therefore, high-throughput next-generation sequencing (NGS) tools provide a practical means of conducting amplicon-specific investigations of microbial ecology in different environments (Adams et al., 2009) including silage (McAllister et al., 2018). The ever expanding applications of NGS techniques provide an excellent opportunity to identify and estimate the effects of different physical, chemical and biological factors (type of crop, moisture, addition of inoculants and organic acids, enzymes, and DM concentration) on the microbial population in silage samples.

Triticale (X *Triticosecale* Wittmack) is a hybrid plant from wheat and rye. First bred was developed in German Lab in the ninetieth century (Stace, 1987). Uses of triticale is depends on the features of the particular plant species. Most of the triticale species chemical compositions are closer to wheat compared to rye; these are used as a food source for both humans and animals. Triticale is used in the preparation of silage for ruminant animals due to its high crude protein concentration (GlamoC˘Lija et al., 2018; Soundharrajan et al., 2019; Lin et al., 2021). Few studies have focused on silage production using triticale inoculated with LAB (Harper et al., 2017; Negi et al., 2022). The current study is to investigate the impact of LAB (*L. rhamnosus*) on the nutrient profiles, fermentation profile and microbial diversity of high-moisture triticale silage (before heading and after heading). NGS was used to characterize the microbial populations in non-inoculated vs. inoculated whole crop triticale silage.

3. Materials and methods

3.1. Lactic acid bacteria (LAB) isolation and identification

Triticale was obtained from an agriculture farm, Jangsoo, South Korea and taken to the laboratory for isolation of LAB using MRS and BCP agar medium (Soundharrajan et al., 2020). The carbohydrates fermentation and extracellular enzyme production were determined using API 50 CH and API-ZYM kits, respectively. The 16s rRNA sequencing was performed at Solgent Co. (Seoul, South Korea). The 16srRNAsequences of strains were submitted to the NCBI gene data bank (INO-52: MW015791 and INO-54: MW015792).

3.2. Inoculants preparation

The inoculants were cultured in MRS broth (CONDA, Madrid, Spain) at 30±2 ◦C for 24 h. Then pellets were collected by centrifugation for 30 min at 4000g, in 4 ◦C and then bacterial population were counted by QUANTOMTM live cell staining kit (Logos biosystem, Gyeonggi-do, South Korea) (Soundharrajan et al., 2020). For silage production, LAB colonies were suspended in sterile distilled water at the density of 1×10^4/mL.

3.3. Triticale forage crop collection and silage production

The Joseong triticale cultivar cultivation site is located in Jangsoo, Chunbuk, South Korea (Latitude: 35.6185318 and longitude: 127.5107881). Triticale seed was sowed in soil on 23 October 2018 (seeding rate : 220 kg ha^{-1}). The cultivation conditions were: soil pH, 5.8 (1:5 H_2O); total nitrogen (0.18%); organic matter

(45 g/kg), P_2O_5 (255 mg/kg), and cation exchange capacity (cmol(+)/kg) for K (0.4), Ca (3.7) and Mg (1.0). When the triticale reached 19 and 24% DM content in the early heading (May 3) and heading (May 10) stages, respectively, the triticale was manually harvested and wilted under field condition for 8–10 h, so it reached 35–40% DM content. Then triticale was chopped (250 g from each stage) to a theoretical cut length (1.5–2.5 cm) using a forage cutter. The samples were divided into 3 groups, with 6 bags per group per stage. The treatments were No (ensiling forage without bacterial inoculants), 2 inoculants (INO-52: novel inoculants of *Lacticaseibacillus rhamanosus*-52 and INO-54: novel inoculants of *Lacticaseibacillus rhamanosus*-54) and 2 stages (early heading and heading stage); the experiment had a 2×3 factorial arrangement. Group I denotes the non-inoculated samples (non-inoculated, added 3 mL sterile water only); Group II, the samples treated with INO- 52; and Group III, the samples treated with INO-54 (1 mL of inoculant (10^4 CFU/mL) with 2 mL of sterile water). Then the air was extracted and sealed using a vacuum (Food saver V48802, MK Corporation, Korea). Total 36 bags were prepared and stored at room temperature for 180d. After fermentation periods; the samples were opened and used for microbiological and nutritional changes.

3.4. Sampling procedure and laboratory analysis of silage

On days 0 and 180, samples were taken from each treatment group in order to determine their nutritional profiles and microbial diversity (total bacteria, lactic acid bacteria, yeast and molds).

On days 0 and 180, samples were processed to determine DM concentration by drying at 60 ◦C until reaching a constant weight. Then, samples were ground and passed through a 1 mm size of the sieve. Nitrogen (N) was determined via the Dumas combustion method, using Elementar Analyzer (Vario MAX cube, Elementar, Germany) and crude protein (CP) was calculated as N x 6.25. The contents of Acid detergent fiber (ADF) (AOAC, 1990) and Neutral detergent fiber (NDF) (Van Soest et al., 1991) were determined. The total digestible nutrient (TDN) content was calculated from the following formula: TDN = 89.9 - (ADF×0.79) (Holland et al., 1990).

3.5. Quantification of fermentative metabolites

Ten grams of ensiled triticale silages was added to 90 mL of water and kept in an orbital shaker for an hour. Then, the supernatant was filtered via multiple layers of cheesecloth and a 0.2 μm filter. A portion of the samples was used to determine the pH by a pH meter (Inolab pH Meter, Thomas Scientific, NJ, USA). Another part of the sample was acidified to pH 2 with 50% H_2SO_4 and frozen at 20 ◦C for HPLC analysis. Then, the frozen samples were thawed and centrifuged at 8000g for 20 min at 4 ◦C and the supernatant was used to analyze the lactic, acetic and butyric acid concentrations of the experimental silages with a high-performance liquid chromatography system (HP1100, Agilent Co., USA; Column: Agilent Hi-Plex HL 1170-6830, 7.7 x 300 mm, 8 μm HPLC column, Mobile phase: 0.005 M H_2SO_4 0.7 mL/min, detector: UV, 55℃, Injection volume 20 μL). For HPLC analysis; the flow rate was 0.5 mL/min at a column

wavelength of 220 nm. The loading volume was 0.01 mL. The lactic, acetic and butyric acid contents were quantified using peak area and calculated using equivalents of the respective standards (Arasu et al., 2014).

3.6. Enumeration of total bacteria, LAB, molds and yeast

An aliquot of each sample was taken after filtering through sterilized cheesecloth and used to enumerate LAB, total bacteria, mold and yeast. Ten-fold serial dilutions of samples were performed in distilled water, 100 µl diluted samples were spread on Man Rogosa Sharpe Agar for LAB counts, 3 M Petri film for yeast and mold counts, A QUANTOM TXTM staining kit used to count the total bacteria (Soundharrajan et al., 2020).

3.7. Genomic DNA extraction and library construction and sequencing of triticale silage

Total genomic DNA was extracted from homogenized experimental silages using a Qiagen (Qiagen, Hilden, Germany) according to the manufactures protocol. The Genomic DNA was quantified using a Quant-IT PicoGree Kit (Invitrogen, USA). Sequencing libraries were prepared to amplify the V3 and V4 regions according to the protocols of the Illumina 16S Metagenomic Sequencing Library by Macrogen Pvt Ltd using the MiSeq™ platform (Illumina, San Diego, USA) (Park et al., 2020).

3.8. Statistical analysis

SPSS16 software was used to determine the significant differences between experimental silages. The following parameters were included during statistical analysis; one-way ANOVA, analysis of multivariate with post-doc, Duncan and descriptive options. 5% levels were considered as least significant between non-inoculated and inoculated samples. Pearson correlation coefficients were generated using R software (Microgen) in order to understand the relationships between the bacterial taxonomic profiles and silage quality variables. The correlation coefficients of the relationship between the microbial population and silage quality parameters were determined. The top 20 independent variables were determined based on the relative abundance of microbes at the species level, and the dependent variables were determined from the silage quality parameters, lactic acid, acetic acid, butyric acid, lactic acid to acetic acid ratio. The correlation coefficients between species and groups were also measured ($p < 0.05$).

4. Results and discussion

4.1. Isolation and characterization of lactic acid bacteria

Potent isolates (INO-52 and INO-54) were selected based on their capacity for rapid pH reduction, enhancement of lactic acid production, and effects on other essential parameters, such as growth rate, antimicrobial activity, and probiotic potential. The selected isolates were gram-positive stain, non-spore-forming, and rod-shaped with negative-catalase activity (Table 16). Antibacterial activity of silages extract fermented with INO-52 and INO-54 at different moistures levels is shown in Table 17. They often appear in a chain form and it grew well at 32± 2 ◦C with shaking at 150 rpm. These isolates could ferment different carbohydrate-containing substrates (Table 18) and secrete industrially important enzymes (Table 19). Physiochemical and molecular characterization results revealed that these strains belonged to *Lacticaseibacillus rhamnosus* (NenBank Accession No: MW015791 and MW015792).

Table 16. Biochemical and physiological characteristics of selected strains

Strain code	Gram stain	Shape	Motility	Growth	Genus	Species
INO-52	Postive	Rod	Non	Facultative aerobic	*Lacticaseibacillus*	*rhamnosus*
INO-54	Postive	Rod	Non	Facultative aerobic	*Lacticaseibacillus*	*rhamnosus*

Table 17. Antibacterial activity of silages extract fermented with INO-52 and INO-54 at different moisture levels

Pathogenic bacteria		*E.faecalis*	*E.coli*	*P.aeruginosa*	*S.aureus*
Control silage extract	40%	3.7±1.1	4.4±1.0	2.4±0.4	4.2±0.9
	50%	7.5±0.8	8.2±0.7	6.2±1.1	8.0±0.5
	60%	10.5±1.5	9.0±1.0	5.5±0.2	7.5±0.8
	70%	7.8±1.2	8.2±1.0	8.7±1.6	8.5±0.5
INO-52 inoculated extract	40%	13.5±3.3	13.7±2.4	12.8±1.4	12.9±0.4
	50%	18.1±3.3	18.3±0.8	17.4±1.4	17.5±0.4
	60%	22.0±3.4	27.0±1.7	26.0±1.3	27.6±2.1
	70%	18.9±1.3	24.0±1.5	19.9±0.8	23.0±2.9
INO-54 inoculated extract	40%	10.3±0.3	13.3±0.1	9.8±0.8	8.5±0.2
	50%	15.6±0.9	19.6±0.3	13.4±0.4	13.8±0.2
	60%	27.6±0.5	26.6±0.5	20.0±0.2	26.6±1.1
	70%	25.4±0.3	23.6±0.6	19.3±1.2	18.8±3.0

* Data were expressed as mean of standard deviation (mean± STD, n=3).

Inhibitory zone was mentioned as millimeter (mm).

Table 18. Carbohydrates fermentation ability of INO-52 and INO-54

S.No	Substrates	INO-52[1]	INO-54[2]
1	Glycerol	+	+
2	Erythritol	0	+
3	D-Arabinose	+	+
4	L-Arabinose	+	+++
5	D-Ribose	++	+++
6	D-Xylose	+	+
7	L-Xylose	0	0
8	D-Adonitol	0	0
9	Methyl- ß-D-xiloside	0	0
10	D-Galactose	+++	+++
11	D-Glucose	+++	+++
12	D-Fructose	+++	+++
13	D-Mannose	+++	0
14	L-Sorbose	+++	++
15	L-Rhamnose	++	0
16	Dulcitol	0	0
17	Inositol	+	0
18	D-Mannitol	+++	+++
19	D-Sorbitol	+++	+++
20	Methyl- αD-mannoside	0	0
21	Methyl- α-D-glucoside	++	0
22	N-acetyl glucosamine	+	++
23	Amygdalin	++	++
24	Arbutin	++	+++
25	Esculin ferric citrate	+++	+++
26	Salicin	++	+++
27	D-Celiobiose	+++	+++
28	D-Maltose	+	+++
29	D-Lactose	+++	+++
30	D-Melibiose	+	0
31	D-Saccharose	+	+
32	D-Trehalose	+++	+++
33	Inulin	0	+
34	D-Melezitose	+++	++
35	D-Raffinose	0	+
36	Amidon	0	+
37	Glycogen	0	+
38	Xylitol	0	+
39	Gentiobiose	0	+
40	D-Turanose	+++	+++
41	D-Lyxose	0	0
42	D-Tagatose	+++	+++
43	D-Fucose	+	0
44	L-Fucose	+	0
45	D-Arabitol	+	0
46	L-Arabitol	+	0
47	Potassium gluconate	+	++
48	Potassium 2-Ketogluconate	+++	+++
49	Potassium 5-Ketogluconate	+	+

[1]INO-52: novel inoculants of *Lacticaseibacillus rhamanosus*-52; [2]INO-54: novel inoculants of *Lacticaseibacillus rhamanosus*-54

Table 19. Extracellular enzymes production by INO-52 and INO-54

Enzymes	**INO-52**[1]	**INO-54**[2]
No	0	0
Alkaline phosphatase	0	+++
Esterase (C_4)	+++	+++
Esterase lipase (C_8)	+++	+++
Lipase (C_{14})	++	++
Leucine arylamidase	++	+++
Valine arylamidase	+++	+++
Cystine arylamidase	++	++
Trypsin-like serine protease	+	++
αChymotrypsin	+	+++
Acid phosphatase	+++	+++
Naphthol-AS-biphosphohydrolase	+++	++
$\alpha\alpha$--alactosidase	+	+
$\beta\beta$-Galactosidase	+++	+++
β-Glucuronidase	+	+
$\alpha\alpha$--Glucosidase	+++	+++
$\beta\beta$-lucosidase	++	0
N-Acetyl-β-glucosaminidase	0	0
$\alpha\alpha$-Mannosidase	0	0
$\alpha\alpha$-Fucosidase	+++	+++

[1]INO-52: novel inoculants of *Lacticaseibacillus rhamanosus*-52; [2]INO-54: novel inoculants of *Lacticaseibacillus rhamanosus*-54; No: non-inoculants.

4.2. Microbiological and chemical compositions of forage samples before ensiling (d0)

Chemical compositions such as crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF), and microbiological compositions such as yeast, mold, and lactic acid bacteria (LAB) of early and heading-stages of triticale are presented (Table 20). Chemical compositions of early-stage triticale had lower ADF (26.4%), and NDF (53.9%) than heading-stage triticale (ADF 35.7%, NDF 61.5%), but higher CP (22.2%) and TDN (67.4%) than heading stage (CP 16.7%, TDN 61.6%) triticale. There was no significant difference in pH between early heading (pH 6.2) and heading (pH 6.1) stage ($p < 0.01$). Microbial analysis revealed that the population of total bacteria (7.75 log CFU g^{-1} vs 6.58 log CFU g^{-1}; $p < 0.039$) was higher in the early heading stage than in the heading stage, whereas mold (3.29 log CFU g^{-1} vs 4.81 log CFU g^{-1}) was less abundant in the early heading than in the heading stage (Table 20). Epiphytic populations of mold and yeast were within their ranges typically reported before ensiling (Romero et al., 2017).

Table 20. Physiochemical characteristics and microbial profiles whole crop forage triticale at different stages on day 0

Items	Early heading	Heading stage	*P*-value between stages(n=3)
ADF[1] (%)	26.4 ±0.19	35.7±0.25	<0.01
NDF[2] (%)	53.9±0.17	61.5±0.27	<0.01
TDN[3] (%)	69.0±0.15	61.6±0.43	<0.01
CP[4] (%)	22.2±0.36	16.7±0.11	<0.01
pH	6.23±0.01	6.14±0.07	<0.01
TB[5] (log CFU g^{-1})	7.75±0.07	6.58±0.05	<0.039
LAB (log CFU g^{-1})	5.04±0.06	3.81±0.14	<0.061
Mold (log CFU g^{-1})	3.29±0.04	4.81±0.07	<0.01
Yeast (log CFU g^{-1})	4.33±0.15	3.74±0.24	<0.023

[1]ADF: acid detergent fiber; [2]NDF: neutral detergent fiber; [3]TDN: total digestible nutrients; [4]CP: crude protein; [5]TB: total bacteria ND: not detected; CFU: colony forming unit

4.3. Chemical compositions, pH, microbial population's and organic acids profiles of silages at 180d

Dry matter content (DM) of fermented silages showed no significant difference between the non-inoculated and NO-52 or INO-54 inoculated silages. Percentages of assessed nutrients (ADF, NDF, TDN, and CP) in non-inoculated and inoculated silage samples remained unchanged within a stage. To monitor the quality of silages, pH is one of major parameters. pH in the range of 3.8 – 4.2 is desirable (Ahmadi et al., 2019). In addition, pH of 4.20 is considered a key marker for well- conserved silage (He et al., 2020). Both early heading and heading silages after LAB treatment had lower pH values (pH 4.10–4.20) than

non-inoculated silages due to rapid changes of microbial counts (Table 21). This result suggested that total bacteria and lactic acid bacteria (LAB) were lower in non-inoculated silages at both stages. These microbial populations particularly LAB plays a major role in the reduction of pH of silages. Higher pH in non-inoculated silages was positively correlated with lower numbers of total bacteria and LAB (Table 21). Both inoculants grew rapidly in the early and heading stages of ensiled silages.

Table 21. pH, nutrient profiles, and microbial populations (CFU)[1] of chopped whole crop triticale silages on day 180 at different stages

Parameters analyzed	Early heading stage			Heading stage		
	No	INO-52[1]	INO-54[2]	No	INO-52[1]	INO-54[2]
			Moisture and nutritive profiles, % DM[1]			
Moisture[3]	64.40±0.27	62.60±0.22	63.0±0.36	59.9±0.60	59.90±0.55	58.7±0 .13
ADF[4]	26.67±0.12 [b]	26.62±0.07 [b]	26.01±0.16 [b]	36.18±0.12 [a]	35.58±0.13 [a]	35.3±0.13 [a]
NDF[5]	47.48±0.15 [b]	47.69±0.21 [b]	47.55± .07 [b]	61.65±0.29 [a]	61.61±0.13 [a]	62.1±0.24 [a]
TDN[6]	68.83±0.09 [a]	68.87±0.06 [a]	69.35±0.13 [a]	61.32±0.09 [b]	61.79±0.10 [b]	62.0±0.10 [b]
CP[7]	22.80±0.21 [a]	23.20±0.05 [a]	23.30±0.07 [a]	18.30±0.13 [b]	17.80±0.17 [b]	18.0±0.28 [b]
			Microbial populations (CFU)			
pH	6.20 ± 0.08[a]	4.20 ± 0.03 [b]	4.20±0.028[b]	6.10 ± 0.01[a]	4.10 ± 0.01 [b]	4.20 ± 0.03 [b]
TB (10^7)[8]	5.85 ± 0.17 [c]	70.0 ± 4.17 [a]	46.0 ± 2.00[b]	12.7 ± 0.28 [c]	150 ± 5.98 [a]	32.4 ± 1.05[b]
LAB(10^7)[9]	3.23 ± 0.26 [c]	60.1 ±1.92 [a]	42.0 ± 2.23 [b]	3.63 ± 0.28	137 ± 7.91	30.0 ± 1.92
Yeast(10^3)	37.1 ± 2.37 [a]	12.2 ±1.09 [b]	12.7 ± 0.88[b]	31.8 ± 2.30 [a]	9.20 ± 0.33[b]	5.90 ± 0.24 [c]

[1]INO-52: novel inoculants of *Lacticaseibacillus rhamanosus*-52; [2]INO-54: novel inoculants of *Lacticaseibacillus rhamanosus*-54; [3]moisture; [4]acid detergent fiber; [5]neutral detergent fiber; [6]total digestible nutrients; [7]crude protein; [8]total bacteria; [9]lactic acid bacteria; CFU: colony forming unit. The data were expressed as the mean ± SEM (n=6), [a, b, c] different letters within a row indicates difference (p <0.05). No: non-inoculants silage.

However, INO-52 inoculated silages exhibited higher numbers of LAB ($p < 0.05$) in both stages than INO-54. Besides, INO-52 and INO-54 inoculated silages showed significant reduction in yeast counts than non-inoculated silages. There was no mold growth in any experimental silage. Similar to previous studies (Peng et al., 2021; Soundharrajan et al., 2020) have also reported that the addition of *L. rhamnosus* as inoculants can increase silage quality by decreasing pH values of grass and legume silages. Several other researchers have demonstrated the benefits of using LAB for silage production as an inoculant. LAB can convert water soluble carbohydrates (WSC) into organic acids in ensiled silages, resulting in pH reduction and preservation of forage (Ahmadi et al., 2019; Soundharrajan et al., 2019; Lin et al., 2021). Among LAB, *Lacticaseibacillus* sp. is present at very low concentrations in most forage samples. When LAB is unable to produce a sufficient level of lactic acid during fermentation, the pH of silage increases, leading to undesirable microbial growth. Higher LAB and lower year counts were observed in INO-52- and INO- 54-treated silage than in the non-inoculated silages, indicating that LABs dominated other epiphytic bacterial communities. During the fermentation of silage, enterobacteria disappear and dominant LABs are produced. These changes are closely associated with the rate of pH reduction and production of higher lactic acid in LAB-inoculated silage (Nascimento Agarussi et al., 2019; Negi et al., 2022).

Fermentative metabolites such as lactic acid, acetic acid, and butyric acid concentrations of ensiled triticale indicate the quality of ensiled silages. Non-inoculated silages had higher pH but lower organic acids, total bacteria, and

LAB with higher yeast counts, indicating that LABs found naturally in silages were not sufficient to induce silage fermentation rapidly (Table 22). By contrast, INO-52 and INO-54 addition enhanced lactic acid levels in silages at both stages than non-inoculated silages ($p < 0.05$). In the early heading silage, INO-52 and INO-54 inoculation increased lactic acid concentrations (% of DM basis) in silages than non-inoculated silages ($p < 0.05$). Particularly INO-54 treatment resulted in greater amount of lactic acid but reduced the level of acetic acid compared to INO-52 treatment (INO-54: 4.31 ± 0.19 vs INO-52: 4.83 ± 0.11% DM; $p < 0.05$). Interestingly, butyric acid production was not detected in INO-52, and INO-54 treated silages whereas it was detected in non-inoculated silage. Ratio of lactic acid to acetic acid (LA/AA) were higher in INO-52 and INO-54 inoculated silage compared to non-inoculated silage ($P < 0.05$). INO-54 inoculation produced a higher LA/AA ratio than INO-52 (38.1 ± 1.14 vs 23.9 ± 3.38% DM; $p < 0.05$). Besides, those inoculants strongly enhanced lactic acid production in heading stage silages compared to non-inoculated silages ($p < 0.05$). Lactic acid was not found in the non-inoculated silages at the heading stage. However, INO-52 and INO-54 inoculation potentially increased lactic acid level (4.30 ± 0.32 vs 4.48 ± 0.08%DM), and LA/AA ratio (24.0 ± 2.54 vs 31.1 ± 7.83%DM) and slightly reduced acetic acid content compared to the non-inoculated silage (Table 22). Lactic, acetic and butyric acids are main acids present in silage. They indicate the highest concentration of organic acid production from WSC by *Lacticaseibacillus* (Li et al., 2019; Yang et al., 2019). Lactic acid is dominant acid in fermented silages

than the other acids during the ensiling process and it lower pH of silages. Lactic acid is approximately 10-20 times stronger than other acids (Kung et al., 2018). The lower lactic acid concentration and the lactic to acetic acid ratio in the non-inoculated silage reflected its lower LAB count and the inability of LAB to dominate the fermentation process, as discussed above. High acetic and butyric acid contents are considered negative indicators of silage quality (Kung et al., 2018). They are responsible for dry matter content reduction and energy production during fermentation (Nascimento Agarussi et al., 2019). At the same time, INO-52- and INO-54 addition significantly reduced acetic acid concentrations in silages at both stages than the non-inoculated. In addition, butyric acid was found in the non-inoculated silage in the early heading stage but not found in INO-52 or INO54 treated silage. This suggests that the addition of LAB could reduce acetic acid and butyric acid concentrations in ensiled silages. Enterobacteria are the second most prevalent members of epiphytic microbes in most silage. A reduction in levels of these bacteria is considered an indicator of good quality silage because bacteria are major competitors of LAB for utilization of water-soluble sugars and their activity results in loss of gas and nutritional content of silage (Muck, 2010). Fermentative metabolites in different stages of silages in response to LAB treatment.

Table 22. Lactic acid, acetic acid, butyric acid, and ratio between lactic acid and acetic acid in early heading stage and heading stage of chopped triticale silages in response to LAB treatments (No, INO-52, INO-54)

Parameters	Early heading stage			Heading stage		
	No	INO-52	INO-54	No	INO-52	INO-54
			Organic acids production (% DM basis)			
Lactic acid	0.15 ± 0.04[c]	4.31 ± 0.19[b]	4.83 ± 0.11[a]	ND	4.30 ± 0.32[a]	4.48 ± 0.08[a]
Acetic acid	0.32 ± 0.05[a]	0.18 ± 0.03[b]	0.12 ± 0.02[b]	0.19 ± 0.03	0.17 ± 0.01	0.14 ± 0.03
Butyric acid	0.07 ± 0.01[a]	ND	ND	ND	ND	ND
Lactic acid/Acetic acid	0.45 ± 0.09[c]	23.9 ± 3.38[b]	38.1 ± 1.14[a]	ND	24.0 ± 2.54[b]	31.1 ± 7.83[a]

ND: not detected. The data were expressed as the mean ± SEM (n=6), [a, b, c] different letters within a row indicates statistically significant between groups (p<0.05). INO-52: novel inoculants of *Lacticaseibacillus rhamanosus*-52; INO-54: novel inoculants of *Lacticaseibacillus rhamanosus*-54; No: non-inoculants silage.

4.4. **Metagenomics analysis of bacterial community dynamics in ensiled silages**

Relative abundance and diversity of bacteria in triticale silage treated with INO-52 and INO-54 were investigated using next- generation sequencing (NGS). Illumina sequencing analysis is a powerful method to study the diversity and compositions of complex microbial community in environmental samples (Degnan and Ochman, 2012). It has been documented that most of dominant bacterial populations involved in silages belong to *Firmicutes* and *Bacteroides* (Liu et al., 2019; Zhao et al., 2017) including *Lacticaseibacillus*, *Pediococcus*, *Lactococcus*, *Weissella* and *Leuconostoc* (A´vila and Carvalho, 2020; Zeng et al., 2020).

The microbial community richness and diversity of experimental silages were determined based on alpha diversity indices. Reads ranged from 51,236 to 64,507. All samples had good coverages of nearly 1 (>0.99994), indicating that the sampling depth had adequately captured most microbial communities. OUT, Chao1 and Simpson indices of non-inoculated silages were higher than those of LAB inoculated silages (Table 23). The microbial community richness and diversity were sharply reduced after INO-52 and INO-54 treatments. This suggests that the addition of INO-52 and INO-54 during the ensiling process can prevent other microbial diversity and richness compared to the non-inoculated. Furthermore, almost all 16S sequences belonged to the phylum of *firmicutes* in both non-inoculants (S1-96.82% vs S2-95.12% and inoculated (S1: INO-52-99.77% vs INO-54-99.54% and S2: INO-52-99.86% vs INO-54-98.12%) silages of early heading and heading stages. *Bacteroidetes* and *Proteobacteria* were the next most dominant phylum found in the experimental silage samples (Fig. 11a).

Table 23. Community richness and diversity of species in experimental silages

Groups	OTUs	Chao1	Shannon	Good's coverage	Read counts
			Early heading stage		
No	75	82.5	1.628058	0.999935921	56966.5
INO-52	26	26.875	0.095425	0.999946363	56,172
INO-54	25	26	0.579067	0.999953799	51236
			Heading stage		
No	47.5	48	2.002347	0.999984102	55887.5
INO-52	18.5	19.26667	0.109076	0.999945383	54881.5
INO-54	41	41	0.301752	1	64507

OTU: Operational Taxonomic Unit (species or group of species often used when only DNA sequences data is available; Chao1: returns the Chao1 richness estimate for an OUT; Shannon: indicates numbers and evenness of species; No: non-inoculants silage.

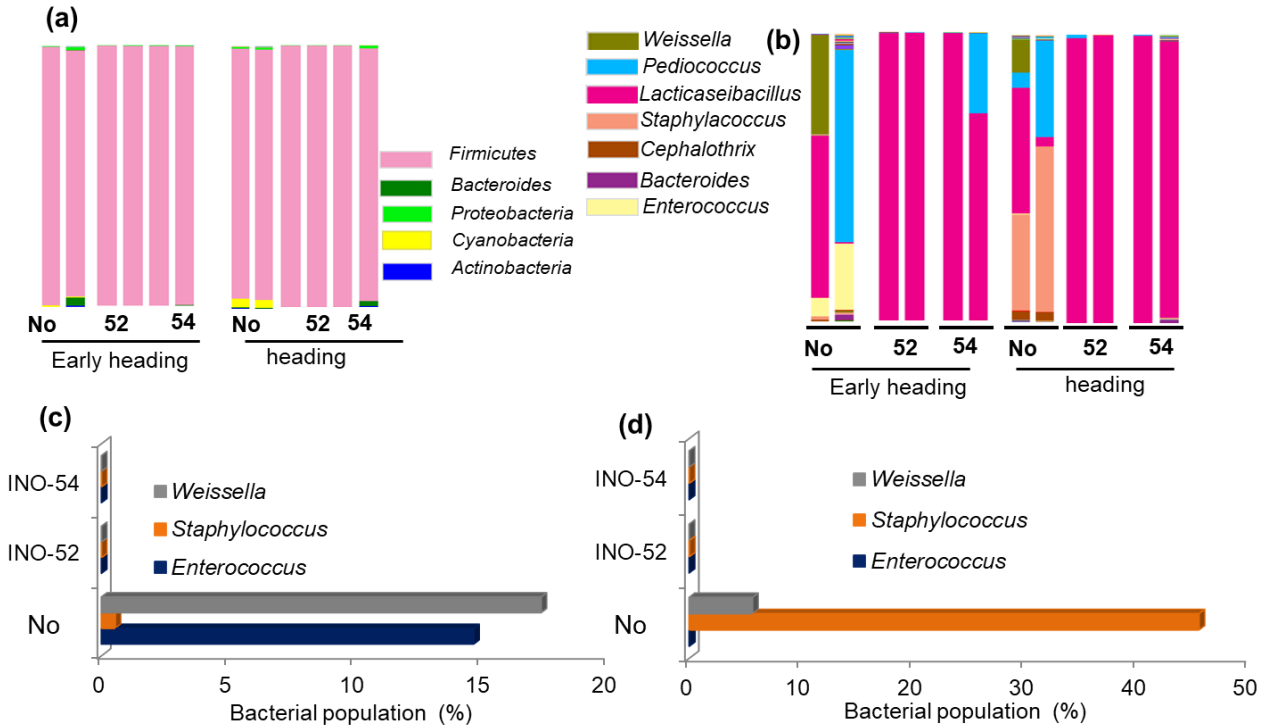

Figure 11. Relative abundances of bacterial community dynamics at day 180 in the early heading and heading stages of triticale silages with INO-52 (novel inoculants of *L. rhamanosus*-52) and INO-54 (novel inoculants of *L. rhamanosus*-54) (a) relative abundances of microbial dynamics at phylum level; (b) relative abundances of microbial dynamics at genus level; (c) Percentage of variations in INO-52 (novel inoculants of *L. rhamanosus*-52) and INO-54 (novel inoculants of *L. rhamanosus*-54) of early heading stage of experimental silages; (d) Percentage of variations in INO-52 (novel inoculants of *L. rhamanosus*-52) and INO-54 (novel inoculants of *L. rhamanosus*-54) of heading stage of experimental silages; Percentage of variations in *Weissella*, *Staphylococcus* and *Enterococcus sp*. at early heading (c) and heading (d) stages of triticale silages. No: non-inoculants silage.

Reduction of *Bacteroidetes* and *Proteobacteria* microbial communities were observed in early heading silage inoculated with INO-52 and INO- 54 compared to non-inoculated silage. In heading stage silage, *Bacteroidetes* were not found in INO-52 treated silages whereas *Bacteroidetes* in INO-54 treated silage showed slight increases (0.95%) compared to the non-inoculated (0.42%). The *proteobacteria* population was reduced in INO-52 or INO-54 (0.11% vs 0.61%) treated group compared to the non-inoculated (0.71%). At the genus level, *Lacticaseibacillus* spp. was the dominant one in all experimental silages followed by *Enterococcus*, *Staphylococcus*, *Weissella*. *Enterococcus*, *Staphylococcus* but *Weissella* were less abundant or not detected in INO-52 or INO-54 treated silages (Fig. 11b–d). *Lacticaseibacillus* was less abundant in non-inoculated silages while silages treated with INO-52 and INO-54 showed higher percentage of *Lacticaseibacillus* in both early and heading stages. Almost all 16S sequences of *Lacticaseibacillus* were major part of the *L. rhamnosus* (>95% in early heading stage and >80% in heading stage) in INO-52 and INO-54 treated silages at both stages (Fig. 12a–c). The majority of genus detected in the present study was *Lacticaseibacillus* in all experimental silages; this result was consistent with previously published data (He et al., 2020; Wang et al., 2019). The diversity of bacterial and composition of taxonomy in LAB-inoculated samples differed from those of the non-inoculated after 180 days of ensiling; indicating that triticale at early heading and heading stages silage fermentation underwent a shift from *Enterococcus*, *Staphylococcus* and *Weissella* genera to *Lacticaseibacillus*. The added LAB can compete with other microbes. LAB inoculants can survive and multiply in inoculated silage. Most of *Lacticaseibacillus* 16S sequences

belonged to *L. rhamnosus*. It is indicating that inoculation of LAB to the silage inhibited indigenous microbial growth in samples. It has been reported that grass and maize silage inoculated with LAB show a reduction in *E. coli*, *Enterobacteria*, *Staphylococcus*, and *Bacillus* with increased LAB levels (Li and Nishino, 2013; Queiroz et al., 2018). Microbial inoculants can dominate the silage ecosystem and regulate the microbial process during fermentation (Duniere et al., 2017).

Unweighted UniFrac emperor PCA analysis showed a perfect separation and differences in the distribution and structures of bacterial population between non-inoculated and inoculated silages at both stages (Fig. 12d-e). Non-inoculated silages showed less variation in microbial diversity at both early heading and heading stages than INO-52 and INO-54 treated silages. INO-52 and INO-54 treatments showed a high level of variation in microbial diversity at both early and heading stages. The higher abundance of dominant bacteria can decrease microbial richness and diversities (Ali et al., 2020; Liu et al., 2019; Yan et al., 2019). The reduction in microbial diversity might have occurred because *L. rhamnosus* -52 and *L. rhamnosus*-54 outcompeted other bacteria or reduced silage pH by increasing lactic acid and acetic concentrations (Ogunade et al., 2018), which could inhibit undesirable bacterial growth in silages (Nyambe et al., 2017). Bacterial community interactions at species levels between non-inoculated and inoculated (INO-52 and INO- 54) silages were revealed by establishing correlations between different samples, shown in heat maps. *L. rhamanosus* showed strong positive correlations with INO-52 and INO-54 inoculated silages. However, it was negatively correlated with non-inoculated silages at both early (Fig. 13a) and heading stages (Fig. 14a). *L. plantarum*

exhibited a slight positive correlation with the non-inoculated group but negative correlations with both INO-52 and INO-54 treatment groups. These findings indicate that *L. rhamanosus-52* and *L. rhamanosus-54* inoculants play a significant role in modulating the relative abundances of various microbial populations in early and heading stages ($p < 0.05$). Next, interactions of microbial populations at species levels with fermentative metabolites between non-inoculated and inoculated silages were determined. Treatment with inoculants INO-52 and INO-54 lowered silages pH as compared to non- inoculated silage. This finding was confirmed by heat map correlation analysis.

The pH of the early heading stage silage was negatively correlated with *L. rhamanosus* at $p < 0.01$ but positively correlated with *Weissella* minor at $p < 0.01$. *Enterococcus hirae* ($p < 0.05$) *Aerococcus viridans* ($p < 0.01$), and *Cephalathrix komarekiana*. Lactic acid ($p < 0.01$) and the ratio of lactic acid to acetic acid ($p < 0.05$) were positively correlated with L. *rhamanosus* ($p < 0.01$), *Aquabacterium parvum* ($p < 0.05$) but negatively correlated with *Weissella* minor ($p < 0.01$), *Enterococcus hirae* ($p < 0.05$) *Aerococcus viridans* ($p < 0.01$), and *Cephalathrix komarekiana* ($p < 0.05$). Acetic acid and butyric acid production were positively associated with *Weissella* minor ($p < 0.01$), *Enterococcus hirae* ($p < 0.05$) *Aerococcus viridans* ($p < 0.01$), *Cephalathrix komarekiana*, and *L. rhamanosus* was negatively associated with acetic acid ($p < 0.01$) and butyric acid ($p < 0.05$) (Fig. 13b). In the heading stage, *L. rhamanosus* was negatively associated with pH but positively correlated with lactic acid and lactic acid/ acetic acid ratio. *Cephalothrix komarekiana* ($p < 0.0001$), *Aero sakkonema funiforme* ($p < 0.0001$), *Staphylococcus warneri* ($p < 0.01$) and *Lawsonella clevelandensis* ($p < 0.01$) were correlated

positively with pH, and acetic acid, but negatively with lactic acid and ratio of LA/AA (Fig. 14b). These results suggest that *L. rhamnosus* could play a major role in silage fermentation under the right conditions. Many studies have shown that *L. rhamnosus* has the potential to improve grass and legume silage fermentation by increasing lactic acid content, thereby inhibiting the growth of mold, yeast and undesirable bacteria (Guan et al., 2020; Li and Nishino, 2011). In the present experiments, LAB dominated the bacterial community in inoculated triticale silage and induced rapid pH reduction by producing higher concentrations of lactic acid and organic acids with synergistic effects in inhibiting *enterobacterial* and yeast growths.

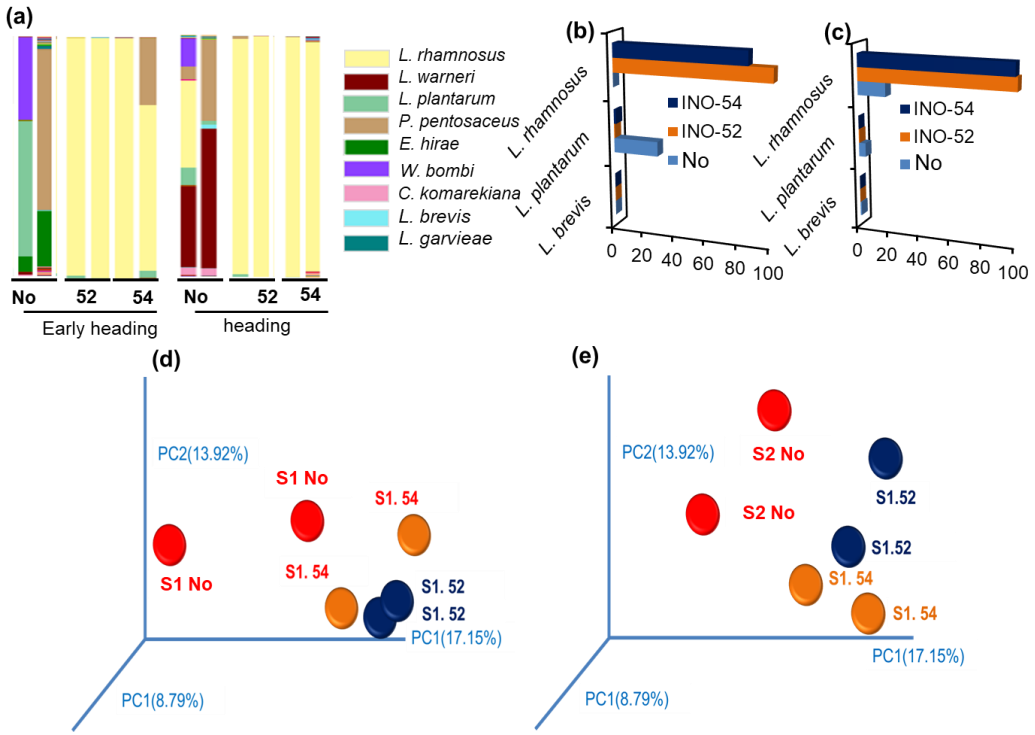

Figure 12. Relative abundances of bacterial community dynamics at the species level and Principal coordinate analysis. (a) relative abundance of bacterial ecology at species level in non-inoculants (No) and inoculants (INO-52 and INO-54) applied silages; Differences in *L. rhamnosus*, *L. plantarum* and *L. brevis* in non-inoculants silage and inoculants treated silages of triticale at early heading (b) and heading (c) stages; Principal coordinate analysis plots for bacteria from early heading (d) and heading (e) stages of triticale ensiled with inoculants or without an inoculant (No) and opened at 180d of fermentation. Red circles indicate non-inoculants silage replicates; Navy circles indicates INO-52 replicates; Orange circles indicate INO-54 replicates; S1- early heading stage of triticale; S2- heading stage of triticale.

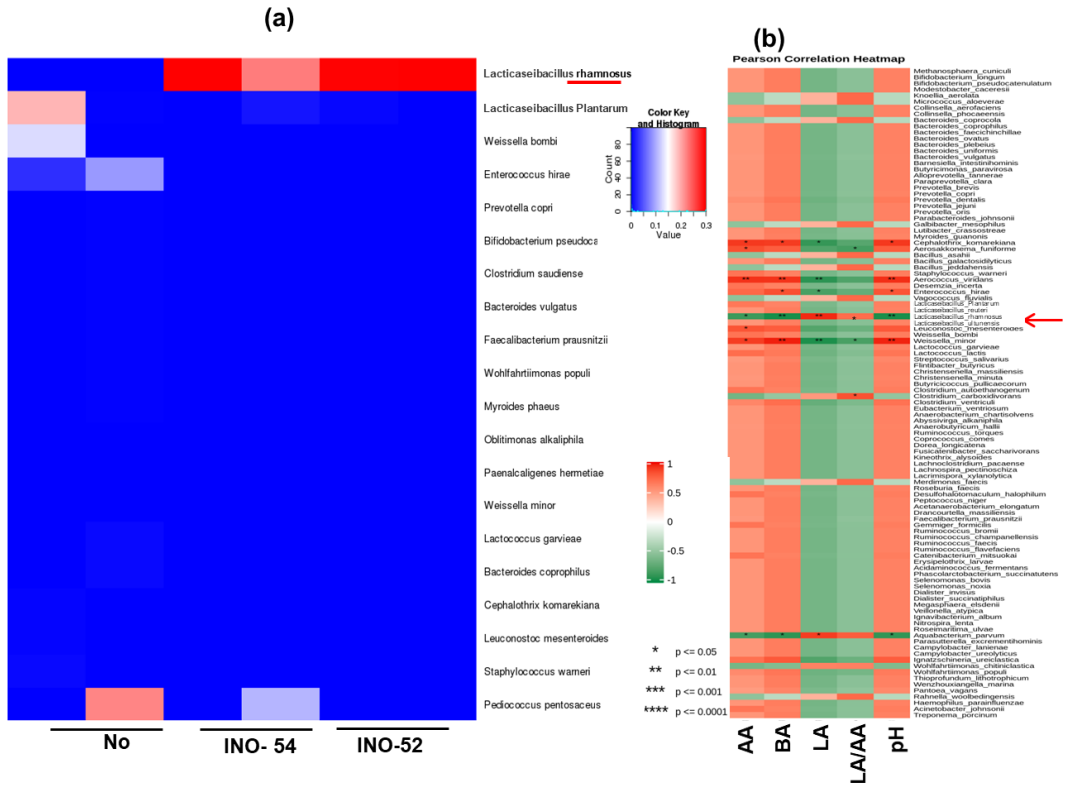

Figure 13. Spearman correlation heatmap of bacterial dynamics at species and fermentative metabolites of early heading stages triticale silage at 180d. (a) correlation analysis bacteria at species level between Inoculated and non-inoculated silages; (b) correlation analysis between bacteria community at the species level and fermentative metabolites. AA: acetic acid; BA: butyric acid; LA: lactic acid; LA/AA: lactic acid and acetic acid ratio, %DM; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. No: non-inoculants silage.

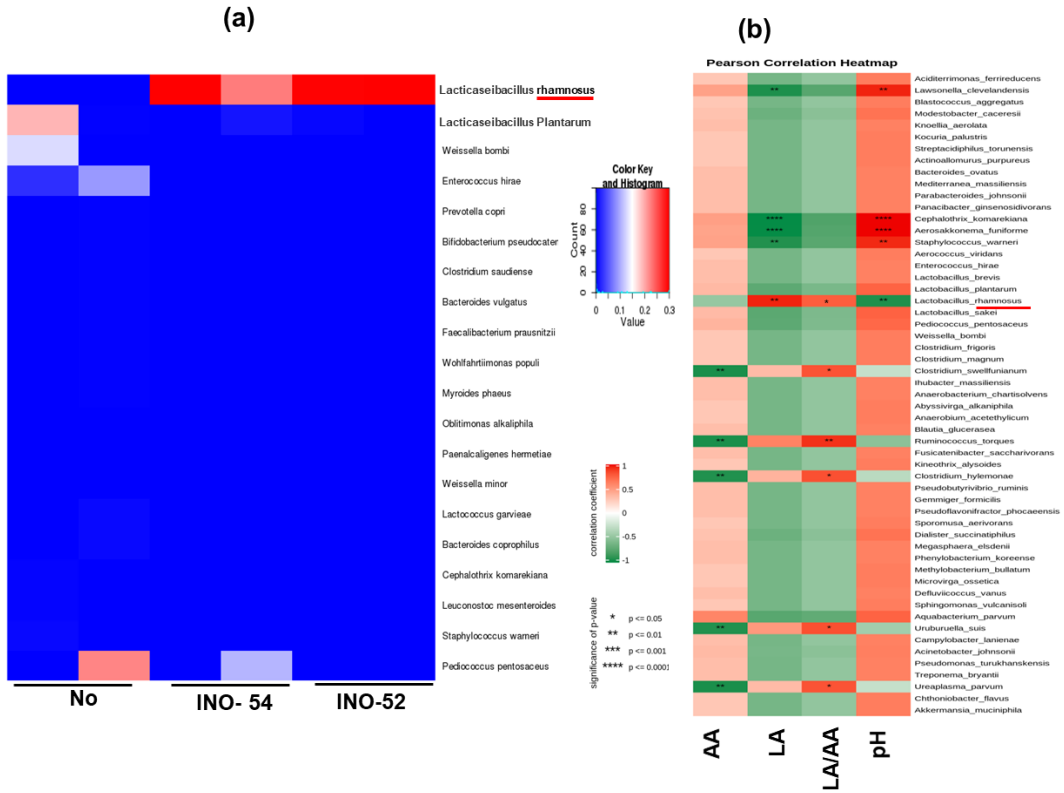

Figure 14. Spearman correlation heatmap of bacterial dynamics at species and fermentative metabolites of heading stages triticale silage at 180d. (a) correlation analysis bacteria at species level between Inoculated and non-inoculated silages; (b) correlation analysis between bacteria community at the species level and fermentative metabolites. AA - acetic acid; BA- Butyric acid; LA: lactic acid; LA/AA: lactic acid and acetic acid ratio, %DM; **p*<0.05; ***p*<0.01; ****p*<0.001.

5. Conclusion

The addition of *L. rhamnosus* INO- 52 and INO-54 had a positive effect on all evaluated parameters. Lactic acid production was enhanced and pH was reduced in inoculated silage, thereby decreasing the richness and diversity of other bacterial species and reducing the concentration of acetic acid. Overall, current data suggest that these strains have the potential to enhance silage fermentation as silage inoculants. However, their efficiencies for different types of grass and legume silages and their combination with other additives including homo-fermentative bacteria and artificial additives need to be examined in further studies.

6. References

1. Adams, I.P., Glover, R.H., Monger, W.A., Mumford, R., Jackeviciene, E., Navalinskiene, M., Samuitiene, M., Boonham, N., (2009). Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. *Mol. Plant Pathol*. 10 (4), 537–545.
2. Ahmadi, F., Lee, Y.H., Lee, W.H., Oh, Y.-K., Park, K., Kwak, W.S., (2019). Long-term anaerobic conservation of fruit and vegetable discards without or with moisture adjustment after aerobic preservation with sodium metabisulfite. *Waste Manage*. 87, 258–267.
3. Ali, N., Wang, S., Zhao, J., Dong, Z., Li, J., Nazar, M., Shao, T., (2020). Microbial diversity and fermentation profile of red clover silage inoculated with reconstituted indigenous and exogenous epiphytic microbiota. *Bioresour. Technol*. 314, 123606.
4. AOAC. (1990). Official method of analysis. 15thed. *AOAC*, Washington, DC.
5. Arasu, M.V., Jung, M.-W., Kim, D.H., Ilavenil, S., Jane, M., Park, H.S., Al-Dhabi, N.A., Jeon, B.T., Choi, K.C., (2014). Enhancing Nutritional Quality of Silage by Fermentation with *Lactobacillus plantarum*. *Indian J. Microbiol*. 54 (4), 396–402.
6. A´vila, C.L.S., Carvalho, B.F., (2020). Silage fermentation—updates focusing on the performance of micro-organisms. *J. Appl. Microbiol*. 128 (4), 966–984.

7. Borreani, G., Tabacco, E., Schmidt, R.J., Holmes, B.J., Muck, R.E., (2018). Silage review: Factors affecting dry matter and quality losses in silages. *J. Dairy Sci*. 101 (5), 3952–3979.
8. Chen, L., Qu, H., Bai, S., Yan, L., You, M., Gou, W., Li, P., Gao, F., (2020). Effect of wet sea buckthorn pomace utilized as an additive on silage fermentation profile and bacterial community composition of alfalfa. *Bioresour. Technol.* 314, 123773.
9. Degnan, P.H., Ochman, H., (2012). Illumina-based analysis of microbial community diversity. *Isme J.*, 6 (1), 183–194.
10. Driehuis, F., Wilkinson, J.M., Jiang, Y., Ogunade, I., Adesogan, A.T., (2018). Silage review: Animal and human health risks from silage. *J. Dairy Sci.*, 101 (5), 4093–4110.
11. Duni`ere, L., Sindou, J., Chaucheyras-Durand, F., Chevallier, I., Th´evenot-Sergentet, D., (2013). Silage processing and strategies to prevent persistence of undesirable microorganisms. *Anim. Feed Sci. Technol.*, 182 (1), 1–15.
12. Duniere, L., Xu, S., Long, J., Elekwachi, C., Wang, Y., Turkington, K., Forster, R., McAllister, T.A., (2017). Bacterial and fungal core microbiomes associated with small grain silages during ensiling and aerobic spoilage. *BMC Microbiol.*, 17 (1), 50.
13. Frank, D., (2013). Silage and the safety and quality of dairy foods: a review. Agric. *Food Sci*. 22 (1), 16-34.
14. GlamoC˘Lija, N., StarC˘EviC´, M., C´iri´c, J., Sefer, D., Glisic, M., Baltic, M., Markovic, R., SpasiC´, M., GlamoC˘Lija, Đ., (2018). The

importance of triticale in animal nutrition. *Veterinary Journal of Republic of Srpska*, 18(1), 73-94.

15. Guan, H., Shuai, Y., Yan, Y., Ran, Q., Wang, X., Li, D., Cai, Y., Zhang, X., (2020). Microbial community and fermentation dynamics of corn silage prepared with heat-resistant lactic acid bacteria in a hot environment. *Microorganisms*, 8 (5), 719.
16. Harper, M.T., Oh, J., Giallongo, F., Roth, G.W., Hristov, A.N., (2017). Inclusion of wheat and triticale silage in the diet of lactating dairy cows. *J. Dairy Sci.*, 100 (8), 6151–6163.
17. He, L., Wang, C., Xing, Y., Zhou, W., Pian, R., Chen, X., Zhang, Q., (2020). Ensiling characteristics, proteolysis and bacterial community of high-moisture corn stalk and stylo silage prepared with Bauhinia variegate flower. *Bioresour. Technol.*, 296, 122336.
18. Holland, C., Kezar, W.P., Lazowski, E.J., Mahanna, W.C. and Reinhart, R. (1990). The pioneer forage manual: A nutritional guide. Pioneer Hi-Bred International, INC., Des Moines, IA., *Pioneer Hi-Bred International*, pp. 1-55.
19. Kung, L., Shaver, R.D., Grant, R.J., Schmidt, R.J., (2018). Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *J. Dairy Sci.*, 101 (5), 4020–4033.
20. Li, P., Zhang, Y., Gou, W., Cheng, Q., Bai, S., Cai, Y., (2019). Silage fermentation and bacterial community of bur clover, annual ryegrass and their mixtures prepared with microbial inoculant and chemical additive. *Anim. Feed Sci. Technol.*, 247, 285–293.

21. Li, Y., Nishino, N., (2013). Effects of ensiling fermentation and aerobic deterioration on the bacterial community in Italian ryegrass, guinea grass, and whole-crop maize silages stored at high moisture content. *Asian-Australas. J. Anim. Sci.*, 26, 1304–1312.
22. Li, Y., Nishino, N., (2011). Effects of inoculation of *Lactobacillus rhamnosus* and *Lactobacillus buchneri* on fermentation, aerobic stability and microbial communities in whole corn silage. *Grassl. Sci.* 57, 184–191.
23. Lin, H., Lin, S., Awasthi, M.K., Wang, Y., Xu, P., (2021). EXploring the bacterial community and fermentation characteristics during silage fermentation of abandoned fresh tea leaves. *Chemosphere*, 283, 131234.
24. Lithourgidis, A.S., Dhima, K.V., Vasilakoglou, I.B., Dordas, C.A., Yiakoulaki, M.D., (2007). Sustainable production of barley and wheat by intercropping common vetch. *Agron. Sustain. Dev.*, 27 (2), 95–99.
25. Liu, B., Huan, H., Gu, H., Xu, N., Shen, Q., Ding, C., (2019). Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages. *Bioresour. Technol.*, 273, 212–219.
26. McAllister, T.A., Duni`ere, L., Drouin, P., Xu, S., Wang, Y., Munns, K., Zaheer, R., (2018). Silage review: Using molecular approaches to define the microbial ecology of silage. *J. Dairy Sci.* 101 (5), 4060–4074.
27. Muck, R.E., (2010). Silage microbiology and its control through additives. *Rev. Bras. de Zootec.*, 39, 183–191.
28. Muck, R.E., Nadeau, E.M.G., McAllister, T.A., Contreras-Govea, F.E., Santos, M.C., Kung, L., (2018). Silage review: recent advances and future

uses of silage additives. *J. Dairy Sci.*, 101 (5), 3980–4000.

29. Nascimento Agarussi, M.C., Gomes Pereira, O., Paula, R.A.d., Silva, V.P.d., Santos Roseira, J.P., Fonseca e Silva, F., (2019). Novel lactic acid bacteria strains as inoculants on alfalfa silage fermentation. *Sci. Rep.*, 9(1), 8007.
30. Negi, T., Kumar, Y., Sirohi, R., Singh, S., Tarafdar, A., Pareek, S., Awasthi, M.K., Sagar, N. A., (2022). Advances in bioconversion of spent tea leaves to value-added products. *Bioresour. Technol.*, 346, 126409.
31. Nyambe, S., Burgess, C., Whyte, P., O'Kiely, P., Bolton, D., (2017). The fate of verocytotoXigenic *Escherichia coli* C600φ3538(ΔvtX2:cat) and its vtX2 prophage during grass silage preparation. *J. Appl. Microbiol.* 122 (5), 1197–1206.
32. Ogunade, I.M., Jiang, Y., Pech Cervantes, A.A., Kim, D.H., Oliveira, A.S., Vyas, D., Weinberg, Z.G., Jeong, K.C., Adesogan, A.T., (2018). Bacterial diversity and composition of alfalfa silage as analyzed by Illumina MiSeq sequencing: Effects of Escherichia coli O157:H7 and silage additives. *J. Dairy Sci.*, 101 (3), 2048–2059.
33. Oliveira, A.S., Weinberg, Z.G., Ogunade, I.M., Cervantes, A.A.P., Arriola, K.G., Jiang, Y., Kim, D., Li, X., Goncalves, M.C.M., Vyas, D., Adesogan, A.T., (2017). Meta-analysis of effects of inoculation with homo-fermentative and facultative hetero-fermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. *J. Dairy Sci.*, 100 (6), 4587–4603.
34. Park, J., Kim, S.J., Kim, E.B., (2020). Changes in the microbial

community of the mottled skate (*beringraja pulchra*) during alkaline fermentation. *J. Microbiol. Biotechnol.*, 30 (8), 1195–1206.

35. Pedroso, A.D.F., Nussio, L.G., Paziani, S.D.F., Loures, D.R.S., Igarasi, M.S., Coelho, R.M., Packer, I.H., Horii, J., Gomes, L.H., (2005). Fermentation and epiphytic microflora dynamics in sugar cane silage. *Sci. Agric.*, 62, 427–432.
36. Peng, C., Sun, W., Dong, X., Zhao, L., Hao, J., (2021). Isolation, identification and utilization of lactic acid bacteria from silage in a warm and humid climate area. *Sci. Rep.*, 11 (1), 12586.
37. Queiroz, O.C.M., Ogunade, I.M., Weinberg, Z., Adesogan, A.T., (2018). Silage review: Foodborne pathogens in silage and their mitigation by silage additives. *J. Dairy Sci.*, 101 (5), 4132–4142.
38. Romero, J.J., Zhao, Y., Balseca-Paredes, M.A., Tiezzi, F., Gutierrez-Rodriguez, E., Castillo, M.S., (2017). Laboratory silo type and inoculation effects on nutritional composition, fermentation, and bacterial and fungal communities of oat silage. *J. Dairy Sci.*, 100 (3), 1812–1828.
39. Soundharrajan, I., Kim, D., Kuppusamy, P., Muthusamy, K., Lee, H.J., Choi, K.C., (2019). Probiotic and triticale silage fermentation potential of *Pediococcus pentosaceus* and *Lactobacillus brevis* and their impacts on pathogenic bacteria. *Microorganisms*, 7 (9), 318.
40. Soundharrajan, I., Muthusamy, K., Han, O.-K., Lee, H.J., Purushothaman, S., Kim, D., Choi, K.C., (2020). Effects of microbial inoculants on the fermentation and preservation of triticale silages at high

and low moisture levels. *Appl. Sci.*, 10 (21), 7855.

41. Stace, C.A., (1987). Triticale: A case of nomenclatural mistreatment. *Taxon* 36 (2), 445–452.
42. Van Soest, P.J., Robertson, J.B., Lewis, B.A., (1991). Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *J. Dairy Sci.*, 74(10), 3583–3597.
43. Vissers, M.M.M., Driehuis, F., Te Giffel, M.C., De Jong, P., Lankveld, J.M.G., (2007). Concentrations of butyric acid bacteria spores in silage and relationships with aerobic deterioration. *J. Dairy Sci.* 90 (2), 928–936.
44. Wang, T., Teng, K., Cao, Y., Shi, W., Xuan, Z., Zhou, J., Zhang, J., Zhong, J., (2020). Effects of *Lactobacillus hilgardii* 60TS-2, with or without homo-fermentative *Lactobacillus plantarum* B90, on the aerobic stability, fermentation quality and microbial community dynamics in sugarcane top silage. *Bioresour. Technol.*, 312, 123600.
45. Wang, Y., He, L., Xing, Y., Zhou, W., Pian, R., Yang, F., Chen, X., Zhang, Q., (2019). Bacterial diversity and fermentation quality of Moringa oleifera leaves silage prepared with lactic acid bacteria inoculants and stored at different temperatures. *Bioresour. Technol.*, 284, 349–358.
46. Wood, B.J.B., (2003). Fermented foods: origins and applications. In: Caballero, B. (Ed.), Encyclopedia of Food Sciences and Nutrition (Second Edition). *Academic Press, OXford*, pp. 2330–2338.
47. Yan, Y., Li, X., Guan, H., Huang, L., Ma, X., Peng, Y., Li, Z., Nie, G., Zhou, J., Yang, W., Cai, Y., Zhang, X., (2019). Microbial community and fermentation characteristic of Italian ryegrass silage prepared with corn

stover and lactic acid bacteria. *Bioresour. Technol.*, 279, 166–173.

48. Yang, L., Yuan, X., Li, J., Dong, Z., Shao, T., (2019). Dynamics of microbial community and fermentation quality during ensiling of sterile and nonsterile alfalfa with or without *Lactobacillus plantarum* inoculant. *Bioresour. Technol.*, 275, 280–287.
49. Zeng, T., Li, X., Guan, H., Yang, W., Liu, W., Liu, J., Du, Z., Li, X., Xiao, Q., Wang, X., Zhang, X., Huang, L., Xiang, Q., Peng, Q., Yan, Y., (2020). Dynamic microbial diversity and fermentation quality of the miXed silage of corn and soybean grown in strip intercropping system. *Bioresour. Technol.*, 313, 123655.
50. Zhao, X., Liu, J., Liu, J., Yang, F., Zhu, W., Yuan, X., Hu, Y., Cui, Z., Wang, X., (2017). Effect of ensiling and silage additives on biogas production and microbial community dynamics during anaerobic digestion of switchgrass. *Bioresour. Technol.*, 241, 349–359.

CHAPTER FOUR

General conclusion

South Korea almost relies on imported grain feed and forage to meet increasing livestock feed requirements, making the price of international crops and increasing feed prices have a direct impact on the income of domestic livestock farms. To reduce feed cost efficiently, supplying high quality domestic forage is the most practical method. However, domestic high-quality forages account for only 25% of total supplying forage and it is necessary to solve the inconsistent domestic forage quality. To overcome these issues, following technologies must be developed to expand the use of domestic forage.

Study 1 aimed to investigate changes in the composition of the fecal microbiota community and the dynamics between grazing and housing feeding systems in Hanwoo steers. In Study 2, the growth efficiency, carcass quality, meat quantity, and quality characteristics of Hanwoo steers fed FF (fermented feed combined with forage silage) and CRS (concentrates combined with rice straw separately) feeds were compared. Study 3 focused on investigating the effect of LAB (*L. rhamnosus*) on the nutrient profiles, fermentation profiles, and microbial diversity of high-moisture triticale silage, both before and after heading.

Study 1 investigated the impact of grazing and housing feeding systems on the composition of fecal microbiota communities and the dynamics between them in Hanwoo steers. The results indicated that grazing on natural pastures led to an increase in bacterial community diversity at the phylum, family, and genus levels, with higher levels of *Firmicutes* observed in the feces of grazing

steers than housed steers. These microbiota changes were found to have potential effects on serum metabolic profiles such as G-GTP, GLU, T-CHO, and TG, as well as feeding behavior. Overall, the findings from this study contribute to our understanding of the gut microbiota of Hanwoo steers and the impact of different forages on rumen microbiota in naturally fed animals.

In Study 2, feeding Hanwoo steers with fermented feed (FF) showed improvement not only in feed intake and final body weight but also in carcass weight, ribeye area, compared to those fed with concentrate combined with rice straw (CRS) ($p < 0.05$). However, the meat quality characteristics results suggested that the cooking loss, fat thickness, marbling score, meat color, fatty acids profiles, crude fat and sensory characteristics and pH values were not altered significantly between rice straw and concentrate separately feed and fermented feed ($p>0.05$). However, additional research is necessary to determine the mechanisms through which FF affects meat quality.

In Study 3, the addition of *L. rhamnosus* INO-52 and INO-54 had a positive effect on all evaluated parameters. Lactic acid production was enhanced, and pH was reduced in the inoculated silage, resulting in a decrease in the richness and diversity of other bacterial species and a reduction in the concentration of acetic acid. Overall, the data suggest that these strains have the potential to improve silage fermentation when used as inoculants. However, future studies should examine their efficiency in different types of grass and legume silages and their combination with other additives, such as homo-fermentative bacteria and artificial additives.

In conclusion, the most effective method of reducing feed costs for beef

cattle is by providing them with high-quality domestic forage. There are several ways to preserve the quality of forage and to use it more efficiently. Grazing steers on natural pastures has been shown to increase the diversity of bacterial communities in the fecal microbiota at the phylum, family, and genus levels, with potential effects on animal health and serum metabolic markers. FF feed has demonstrated its effectiveness and profitability for the early and late fattening periods of Hanwoo steers without any negative effects. Additionally, the addition of *L. rhamnosus* can enhance the fermentation of silage inoculants. In summary, to increase the utilization of domestic forage, it is essential to enhance its preservation and choose an appropriate method for feeding cattle.

Ⅷ. Summary in Korean

국내 배합사료에 사용되는 사료 원료와 조사료 중 건초는 대부분 수입에 의존하고 있기 때문에 기후변화에 따른 이상기상 빈발, 물류비상승, 국제 지정학적 위험 등으로 인한 국제 곡물가격 상승이 국내 축산업에 미치는 영향이 크게 나타난다. 따라서 한우, 젖소, 염소 등 축산 농가의 경쟁력 향상을 위해서는 국내산 양질조사료의 생산과 가축 이용확대를 통하여 생산비를 절감하는 것이 무엇보다 중요하다.

국내 조사료 자급률은 2021년 기준 82.7%로 높지만 이탈리안 라이그라스(IRG), 호밀, 청보리, 트리티케일, 사일리지용 옥수수 등 양질의 조사료는 전체 조사료 공급량의 25%로 낮은 수준에 머물고 있다. 이에 따라 정부에서는 '조사료생산기반 확충사업'을 통하여 국내 조사료 생산기반 구축과 함께 양질의 조사료 유통확대를 목표로 정책을 추진하고 있다. 본 정책의 3가지 주요 핵심내용은 (1) 국내산 조사료 생산 및 이용을 위한 조사료 제조비와 조사료 수확·제조장비 지원, (2) 국내산 조사료 유통확대를 위한 유통·가공 지원, (3) 간척지, 유휴지, 농경지, 산지 등 조사료 생산 기반을 확대하기 위한 것이다. 정부의 이와 같은 노력에도 국내산 양질 조사료 재배면적은 답보상태에 있다. 따라서 국내조사료 생산과 이용 활성화를 위해서는 국내산 조사료를 활용한 TMR산업 활성화 및 초지 가축 방목이용 기술 개발과 국내산 저장 조사료(사일리지)의 품질문제 해결이 필요하다.

따라서 본 연구는 초지방목과 발효사료 급여가 한우 거세 비육우에 미치는 영향과 사일리지의 저장성 향상을 위한 첨가제 처리에 따른 영향을 조사하기 위하여 수행하였다.

연구 1은 한우 거세 비육우의 방목과 우사 사육에 따른 장내 미생물 균총변화와 대사물질 변화를 분석하기 위하여 시험축의 분변에 있는 미생물 균총 변화를 NGS 를 이용하여 분석하였고 대사체 분석 결과와 연계하였다.

연구 2는 한우 거세우 발효사료 비육기 급여에 따른 가축의 생산성, 도체특성, 육질특성에 미치는 영향을 알아보기 위하여 수행되었다.

연구 3은 트리티케일 사일리지의 저장성 향상을 위해서 젖산균 첨가제 2종(*Lacticaseibacillus rhamanosus*-52, *Lacticaseibacillus rhamnosus*-54)을 트리티케일 출수초기와 출수기로 나누어 첨가하여 그 효과를 분석하였다.

연구 1. 방목과 우사 사육에 따른 한우 거세 비육우 분뇨 미생물 균총 및 대사체 변화

본 연구는 한우 거세 비육우의 방목과 우사 사육에 따른 장내 미생물 균총과 대사물질 변화를 분석하기 위하여 수행되었다. 방목과 사사의 장내 미생물 균총 다양성을 분석한 결과 방목이(3,538±38.17 OTUs)로 우사 사육(2,930±94.06 OTUs)보다 높게 나타났다. Chao 1 분석에서도

역시 방목이 우사 사육보다 높은 결과를 보였다. 방목과 우사 사육 모두 *Bacteroidetes*와 *Firmicutes*가 높게 나타났으며 방목에서 우사 사육보다 *Bactreroidetes*는 낮게 나타났고 상대적으로 *Firmicutes*는 높게 나타났다. 혈액분석에서 우사 사육은 gammaglutamyl transpeptidase(γGTP), glucose(GLU), total cholesterol (T-CHO) 그리고 triglyceride (TG) 수치가 증가하는 결과를 보였다. 결과적으로 방목에 따른 한우 거세우의 혈중대사물질과 장내 미생물과의 상관성이 나타났고 분변을 통하여 방목에 따른 미생물 균총 변화를 확인할 수 있었다.

연구 2. 한우 거세우에서 조농분리 급여와 발효사료 혼합 급여가 가축의 성장 및 도체 특성에 미치는 영향

한우 거세우 발효사료 비육기 급여에 따른 가축의 생산성, 도체특성, 육질특성에 미치는 영향을 알아보기 위하여 수행되었다. 13개월령의 한우 거세우 12두를 2개의 그룹으로 나누어 한쪽에는 FF(발효사료 + IRG 사일리지 + 옥수수 사일리지)를 다른 쪽에는 CRS(볏짚 + 농후사료)사료를 급여하였다. FF급여에서 비육전기 일당 증체량은 0.93kg으로 관행(0.83kg) 대비 유의적으로 높았으나 비육후기에는 유의적인 차이가 나타나지 않았다. 도체중은 FF급여에서 444kg로 관행 401.8kg대비 높게 나타났고 등심단면적은 FF급여가 91cm^2으로 관행 87.60cm^2대비 높았다(p<0.05). 하지만, 가열감량, 등지방두께, 도체등급,

육색, 조지방 등에서는 처리에 대한 차이가 나타나지 않았다(p>0.05). 결과적으로 한우 거세우 비육초기 및 후기 FF 급여에따른 고기품질, 도체 특성, 지방산 조성 등에 대한 차이가 크지않았다. 따라서 발효사료를 이용하여 기존 농후사료를 대체할 수 있으며 생산성 개선과 사료비 절감을 통하여 농가소득 향상에 기여 할 수 있을 것으로 판단된다.

연구 3. 젖산균 접종이 혐기조건에서 숙기별 트리티케일 사일리지의 발효특성 및 미생물상 변화에 미치는 영향

사일리지의 품질을 높이기 위해서는 혐기조건에서 미생물 발효가 잘 이루어져야 한다. 본 연구에서 트리티케일 사일리지의 저장성 향상을 위해서 젖산균(LAB: Lactic acid bacteria) 첨가제 2종 (*Lacticaseibacillus rhamanosus*-52, *Lacticaseibacillus rhamnosus*-*54*)을 트리티케일 출수초기와 출수기로 나누어 첨가하여 그 효과를 분석하였다. 모든 첨가제 처리에서 사일리지 내 미생물 변화를 촉진하여 미첨가 보다 사일리지 내 pH를 낮추는 능력이 높아졌다(p<0.05). LAB 사일리지 첨가는 혐기발효를 촉진시켜 lactic acid를 증가시키고 acetic acid와 butyric acid 를 감소시켰다(p<0.05). 또한, *L. rhamanosus*-52와 *L. rhamanosus*-54는 사일리지 미생물 발효를 통한 저장성 개선 효과가 나타났다. *Lacticaseibacillus* spp. 의 비율이 높아지면 미생물 다양성은 감소되었지만 젖산발효 효율은 증가하였다. 결과적으로 트리트케일

사일리지에 대한 LAB 처리는 젖산균의 증식을 촉진시키고 사일리지 품질에 악영향을 주는 미생물균의 증식 억제 효과가 있었다.

종합결론

한우 거세 비육우의 생산비를 낮추고 가축의 생산성을 향상시키기 위한 연구를 추진하였다. 육성기 한우 거세우 초지 방목에 따른 장내 미생물 다양성이 증가하였으며 *Firmicutes* 가 장내 우점하였다. 한우 육성기 방목은 우사 사육 대비 일당증체량이 유사하게 나타났고 혈중 대사물질 분석결과 방목에서 대사성 질병 관련 물질들이 우사 사육보다 적게 나타나 더 건강한 밑소 생산이 가능할 것으로 보인다. 한우 거세 비육우에서 국내산 양질 조사료를 활용한 사료비 절감, 고기 품질과 가축의 생산성 향상이 무엇보다 중요하다. 본 연구에서 젖산균 첨가제 처리를 통하여 국내산 트리티케일 사일리지의 발효 품질 문제를 해결할 수 있었다. 또한, 발효 사료 및 발효 조사료 혼합 급여는 가축의 생산성을 향상시킬 수 있고 사료비 측면에서 절감효과가 있을 것으로 사료된다.

주요어: 발효사료, 방목, 한우거세우, 첨가제, NGS, 사일리지

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처음 학문적 열의로 교수님 방문을 두드렸던 저를 맞아주시고 바쁘신 일정에도 저의 논문 완성을 위해 많은 시간과 조언을 아끼지 않으셨던 백명기 교수님께 진심으로 깊은 감사를 드립니다. 교수님의 학문적 열정과 가르침을 평생 본 받으며 자랑스러운 제자가 될 수 있도록 끊임없이 노력하겠습니다.

연구를 하면서 현장의 중요성을 알려주시고 심사위원장으로써 학위논문을 지도해주신 김유용 교수님 감사드립니다. 앞으로 근무를 하면서 교수님께서 하신 말씀처럼 현장을 중심에 놓고 연구를 하겠습니다. 논문의 부족한 부분을 세심하게 지도하여 주신 김영훈 교수님 감사드립니다. 이번 학위논문뿐만 아니라 제가 어려울 때마다 고민도 들어주시고 따뜻한 격려를 해주셨던 김종근 교수님 감사드립니다. 논문 지도와 직장 선배로서 아낌없는 조언을 해주신 최연호 박사님 감사드립니다. 또한 시험 시작부터 논문 작성까지 같이 함께해주신

최기춘 박사님 감사드립니다. 논문 작성이 서툴었던 저에게 사소한 것부터 하나하나 가르쳐 주시고 연구가 잘 안 풀릴 때 마다 방향을 제시해 주셨습니다. 항상 박사님의 가르침 잊지 않고 살아가겠습니다.

제가 연구직생활을 처음 시작하면서 가장 본받고 싶었던 분이 지금 이상훈 과장님과 박형수 연구관님 입니다. 부족한 저를 항상 이끌어주시고 격려해주셔서 정말 감사드립니다. 그리고 우리 초지사료과 이기원 박사님, 이배훈 박사님, Ilavenil 박사님 그리고 박권순 선생님께 깊은 감사의 마음을 표합니다. 본 논문 실험에 도움을 주신 백열창 선배님과 동기인 이유경 박사님 그리고 많은 조언을 해주셨던 하승민 박사님 정말 감사드립니다. 또한 박사과정동안 많은 도움을 준 반추동물영양생리학 실험실 정다진솔 박사와 재성이에게도 감사를 표합니다. 또한 본 논문 실험을 할 수 있도록 흔쾌히 허락해주신 다움목장 손영수 대표님께 감사의 마음을 전합니다.

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2023 년 8 월 5 일

정종성 올림