

Article

Effect of Genotype and Microbial Inoculants on Quality, Fermentation, and In Vitro Digestibility of Barley at Early Dough Stage of Maturity

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Abstract

A $2 \times 2 \times 2$ factorial experiment was conducted to study the individual and combined effects of barley variety (Tarım 92 and Bravo), homofermentative inoculant (HMF; *Lactobacillus plantarum* and *Enterococcus faecium*), and heterofermentative inoculant (HTF; *Lactobacillus buchneri*) on the fermentation characteristics, chemical composition, nutritive value indices, and in vitro digestibility of whole-crop barley silages which were harvested at the early dough stage. Forty-eight laboratory silos (six replicates per treatment) were ensiled for 120 days. Tarım 92 silage exhibited superior fermentation stability across all treatments, with pH ranging from 4.11 to 4.30, Flieg points of 102.94–115.47, and lower $\text{NH}_3\text{-N}$ concentrations (142.01–173.72 mg/kg TN (total nitrogen)), reflecting better protein preservation than Bravo silage. In contrast, the poorest fermentation outcome (pH 5.24; Flieg 47.06; $\text{NH}_3\text{-N}$ 307.48 mg/kg TN) was exhibited by the Bravo silage inoculated with only homofermentative inoculants. Significant three-way interaction effects were detected among barley variety, homofermentative inoculant, and heterofermentative inoculant ($p < 0.05$). The dual-inoculated Tarım 92 silage showed the optimum in vitro digestibility and nutritional value (TDN 77.14%; NEL 1.81 Mcal kg^{-1} DM; RFV 176.25; ADF 18.75%; IVOMD 65.72%; predicted DMI 3.06% BW), while higher amounts of butyric acid and $\text{NH}_3\text{-N}$ suggested that better nutritional value was not always accompanied by better protein preservation. The combined inoculation also yielded the best outcomes within Bravo treatments (RFV 144.00), whereas HTF inoculation alone reduced IVDMD and IVOMD below uninoculated controls. Overall, these results show that the response to microbial inoculation is genotype-dependent and a combination of homofermentative and heterofermentative inoculation for high-dry-matter barley varieties can enhance nutritive value and digestibility. However,



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fermentation quality indicators, particularly butyric acid and $\text{NH}_3\text{-N}$, require consideration in the selection of technique.

Keywords: Barley silage; microbial inoculant; lactic acid bacteria; fermentation quality; in vitro digestibility; cultivar interaction; relative feed value

1. Introduction

Barley (*Hordeum vulgare*, L.) is an extensively cultivated whole-crop forage due to its high biomass yield, excellent energy density, resilience to a variety of climatic conditions including cold and drought, and adaptability to poor soil conditions compared to other forages [1,2]. Barley ensiled at the early dough stage has high intake characteristics, better nutrient digestibility and maximum economic yield [3,4]. At this stage of development, there is increased starch accumulation and desirable dry matter content, resulting in a higher energy density of the grain that supports microbial synthesis in the rumen [5]. Moreover, at this stage, sufficient lactic acid is produced due to the presence of adequate water-soluble carbohydrates (WSC), ensuring silage with good fermentation quality [6]. Successful preservation of whole-crop barley silage depends on rapid acidification. Insufficient amounts of naturally occurring lactic acid bacteria can lead to the growth of unwanted microbes, which lowers the quality of silage. Microbial inoculants are therefore frequently utilized to enhance fermentation and nutrient storage [7,8].

Bacterial inoculants are utilized to counter such challenges during the ensiling process because they provide efficient nutrient preservation and environmental sustainability. Moreover, they are easy to use, non-corrosive and are more beneficial compared to their chemical alternatives [8]. There is enough evidence suggesting that the use of bacterial inoculants during the ensiling process improves fermentation, preserves silage integrity, protects against pathogenic load, and improves aerobic stability of silage [9]. Most commonly utilized bacterial inoculants during ensiling are based on lactic acid bacteria (LAB) that can either be homofermentative (HMF) or heterofermentative (HTF) [10–12]. Homofermentative bacteria such as *Lactobacillus plantarum* and *Enterococcus faecium* are commonly utilized to accelerate lactic acid production and the reduction in silage pH [10]. This rapid drop in pH reduces the activity of undesirable microorganisms such as enterobacteria, clostridia and bacilli, thereby reducing DM losses and ammonia-N ($\text{NH}_3\text{-N}$) formation. However, as lactic acid is the primary product of homofermentative LAB, which can be utilized by spoilage yeast spp. as they can grow well below the pH of most silages, increasing the pH of silage makes it more susceptible to aerobic spoilage resulting from the growth of other spoilage (aerobic) microorganisms [13,14].

On the contrary, the main reason for the use of heterofermentative LAB such as *Lactobacillus buchneri* as silage inoculants is to enhance their aerobic stability against deterioration by yeast and molds when exposed to air. *L. buchneri* achieves this by converting lactic acid into acetic acid and 1, 2-propanediol during the fermentation process [15]. This aerobic stability is directly correlated to the amount of acetic acid present in silage, which acts as a growth inhibitor on spoilage microorganisms such as yeast and molds [15].

Recently, there has been an increased interest in the use of combination inoculants, which include both heterofermentative and homofermentative strains. These combinations aim to combine the benefits of both types of inoculants: an initial fermentation regulated by homofermentative bacteria, which results in good DM recovery and animal performance, and a subsequent conversion of the lactic acid to acetic acid by heterofermentative *L. buchneri*, which improves aerobic stability. One combination product includes an *L.*

buchneri strain that also generates ferulic acid esterase enzyme, which helps break down the linkages between lignin and carbohydrates in forage cell walls, potentially enhancing fiber digestibility [13].

Furthermore, the effectiveness of microbial silage inoculants is not solely affected by the microbial strains of the inoculant but is also influenced by the crop physiochemical characteristics [16]. Nair et al. [17] showed that variation in chemical composition and neutral detergent fiber (NDF) digestibility among barley varieties directly influences silage characteristics and animal performance. As a result, the fermentation dynamics and synergistic behaviors are most likely to differ depending on the variety of the barley being ensiled. However, there is not enough evidence describing how different barley varieties interact with different combinations of HMF and HTF inoculants.

In contrast to other research that assessed microbial inoculants or barley cultivars independently, this study used a $2 \times 2 \times 2$ factorial arrangement to examine their interaction. The findings offer a practical guide for choosing genotype-specific inoculation techniques to enhance the nutritional value, nutrient preservation, and fermentation quality of barley silage at the early dough stage of maturity. We hypothesized that dual inoculation would increase in vitro digestibility and improve nutritive value, but the extent of these improvements would be mainly influenced by inherent genotypic differences between the barley varieties. Therefore, the objective of this study was to analyze the effects of using HMF inoculants (*L. plantarum* and *E. faecium*), HTF inoculants (*L. buchneri*), or their combination during ensiling on the fermentation characteristics, volatile fatty acid (VFA) profile, chemical composition, and in vitro digestibility of two different barley varieties (Tarim 92 and Bravo) at the early dough stage of maturity.

2. Materials and Methods

2.1. Experimental Site and Barley Varieties

This study was conducted in November 2021 at the agricultural fields of the Center for Agriculture, Livestock and Food Research, Burdur Mehmet Akif Ersoy University, Turkey ($37^{\circ} 41' \text{ N}$, $30^{\circ} 20' \text{ E}$, 1200 m altitude). Barley was sown by broadcasting in mid of November 2021 (230 kg seed/hectare). The land was fertilized with 100 kg of diammonium phosphate per hectare. At the end of June 2022, barley was harvested at the early dough stage of maturity.

2.2. Study Design and Experimental Groups

The study followed a completely randomized design with $2 \times 2 \times 2$ factorial arrangement. Two barley varieties (Tarim 92 and Bravo) were inoculated with two levels (0 or 0.8 mg/kg) of HMF silage inoculant (*L. plantarum* and *E. faecium*; Pioneer[®] 1188; Corteva Agriscience, Inc., Indianapolis, IN, USA) and two levels (0 or 500 mg/kg) of HTF silage inoculant (*L. buchneri*; Pioneer[®] 11A44; Corteva Agriscience, IN, USA). The experiment consisted of eight treatment groups, each with six replicates (for a total of 48 silos).

Four strains of *L. plantarum*, i.e., 2.5×10^{10} cfu/g *L. plantarum* LP286 DSM 4784 ATCC 53187, 2.5×10^{10} cfu/g *L. plantarum* LP318 DSM 4785, 2.5×10^{10} cfu/g *L. plantarum* LP319 DSM 4786, and 2.5×10^{10} cfu/g *L. plantarum* LP346 DSM 4787 ATCC 55943 and two strains of *E. faecium* (1.25×10^{10} cfu/g *E. faecium* SF301 DSM 4789 ATCC 55593 and 1.25×10^{10} cfu/g *E. faecium* SF202 DSM 4788 ATCC 53519) (Pioneer[®] brand 1188; Corteva Agriscience, Inc., IN, USA) composed the HMF silage additive.

The HTF silage additive comprising 1.0×10^{11} cfu/g of *L. buchneri* LN4637 ATCC PTA-2494 (Pioneer[®] brand 11A44; Corteva Agriscience, Inc., Indianapolis, IN, USA).

2.3. Silage Preparation and Ensiling Process

Harvested barley fodder was cut into 1.5–2.5 cm particles and vacuum-packed in 250 g plastic bags for ensiling after silage supplement application. The control group received no additives, whereas the other groups received silage additives either HMF, HTF, or a combination of both, based on the manufacturer's recommendations before vacuum-packing. Barley forage was allowed to ensile for 120 days.

2.4. Physical Quality Assessment and Fermentation Characteristics of Ensiled Barley

The DLG scoring method developed by the German Agricultural Society (Deutsche Landwirtschafts Gesellschaft) was used to evaluate the physical characteristics of ensiled barley forages, including color (three points; 0 to 2), structure (four points; 0 to 4) and odor (15 points; 0 to 14). A panel of three specialists evaluated the physical quality of ensiled barley forage. The scores were categorized based on the panel's average score: bad (0 to 4 points), moderate (5 to 9 points), good (10 to 15 points), and excellent (16 to 20 points).

After assessing physical quality, we assessed the fermentation parameters of ensiled barley forages, including Flieg point, pH, and $\text{NH}_3\text{-N}$. The pH and dry matter (DM) of ensiled barley forages were evaluated to determine the Flieg point using the Dong et al. [18] method according to the following equation:

$$\text{Flieg point} = 220 + (2 \times \text{DM}\% - 15) - (40 \times \text{pH})$$

To determine pH, 100 g of silage was homogenized in 100 mL of distilled water for 5 min, then filtered through four layers of cheesecloth and measured with a glass-electrode pH meter (Apera Instruments, LLC, Columbus, OH, USA). The $\text{NH}_3\text{-N}$ concentrations were determined using the Meeske et al. [19] technique. Briefly, 50 g of silage were homogenized in 250 mL of 0.1 N sulphuric acid and filtered through four layers of cheesecloth; the filtrate was then analyzed by distillation and titration using the Kjeldahl method published by AOAC [20]. The volatile fatty acid (VFA) profile of ensiled barley forage, including acetic, propionic, isobutyric, butyric, and caproic acid, was determined by gas chromatography (GC; Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) fitted with a flame ionization detector (FID) and a 30 m $\times$ 0.53 mm i.d. capillary column (Teknokroma TRB-FFAP, Barcelona, Spain). VFAs were quantified using the same aqueous extract prepared for pH determination after performing centrifugation and filtration (0.45 μm) followed by the addition of boric acid. Total VFA was calculated as the sum of individual acid fractions, and all values were expressed as mmol/L [21].

2.5. Nutritive Value and in Vitro Digestibility of Fresh and Ensiled Barley Forage

Freshly harvested and ensiled barley forages were dried in a hot air oven at 105 °C for 8 h to extract DM. The crude protein (CP; method 954.01), ether extract (EE; method 920.39), and crude ash (method 942.05) were analyzed using AOAC techniques [20]. The ANKOM A2000 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA) was used to analyze crude fiber (CF), ash-free neutral detergent fiber after amylase treatment (aNDFom), ash-free acid detergent fiber (ADFom), and acid detergent lignin (ADL). Horrocks and Vallentine's [22] formulae were used to derive the values of non-fiber carbohydrates (NFC), hemicellulose (HEC), digestible dry matter, dry matter intake (DMI), relative feed value (RFV), net energy for lactation (NEL), total digestible nutrients (TDN), total carbohydrates (total CHO), and cellulose. The nutritional composition of freshly harvested barley varieties is presented in Table 1.

Table 1. Nutritional composition (%) of freshly harvested barley varieties ^{1,2}.

Variety	DM	CP	CF	EE	Ash	aNDFom	ADFom	NFC
Tarim 92	37.70	9.56	24.04	2.16	6.61	41.32	22.94	40.35
Bravo	37.12	10.41	23.09	1.90	7.56	45.17	23.34	34.96

¹ All values are presented on DM basis except the DM that is presented on as-fed basis. ² DM = dry matter; CP = crude protein; CF = crude fiber; EE = ether extract; aNDFom = ash-free neutral detergent fiber after amylase treatment; ADFom = ash-free acid detergent fiber; NFC = non-fiber carbohydrates.

Each sample's *in vitro* true digestibility was assessed using the ANKOM DaisyII incubator (Ankom Technology Corporation Fairport, New York, USA). Dried silage samples (0.25 g) were prepared in triplicate, placed in ANKOM F57 filter bags (4.5 × 4.0 cm; pore size 25 µm) and placed into the incubation vessels of the DaisyII system. Each jar was filled with 1330 mL of buffer solution A (KH₂PO₄, 10 g/L; MgSO₄·7H₂O, 0.5 g/L; NaCl, 0.5 g/L; CaCl₂·2H₂O, 0.1 g/L; urea, 0.5 g/L) and 266 mL of buffer solution B (Na₂CO₃, 15.0 g/L; Na₂S·9H₂O, 1.0 g/L). Fresh rumen fluid was collected in an insulated container under CO₂ environment and filtered through four layers of cheesecloth. Then, 400 mL of filtered rumen fluid was added to each incubation vessel. After 48 h of incubation at 39 °C, the bags were carefully rinsed with cold tap water to cease microbial activity, and then oven-dried at 105 °C to a constant weight. The contents of dried bags were then analyzed for organic matter (OM) and neutral detergent fiber (aNDFom).

2.6. Statistical Analysis

Data were subjected to statistical analysis using GLM procedures of SPSS (version 22.0; IBM Corp. Inc., Armonk, NY, USA). To this end, data were tested for normality using Levene's test followed by the normalization of non-normalized traits using logarithmic or square-root transformation. The GLM procedures were applied, and three-way ANOVA was conducted to assess the effect of variety, and HMF and HTF inoculants, followed by the separation of significant different means using Tukey's test as post hoc. Differences among the means were assumed significant at $p \leq 0.05$. Results were presented as mean ± SEM (pooled).

3. Results

3.1. Physical Quality and Fermentation Characteristics of Barley Silage

The effects of varieties, HMF inoculant, HTF inoculant, and their interactions on the silage fermentation characteristics and physical quality of barley are presented in Table 2. Variety had a significant effect on pH ($p < 0.001$), with Tarim 92 silage having a pH of 4.238, compared to 4.808 for Bravo silage. Higher odor scores, greater Flieg points, lower NH₃-N concentrations, and higher DLG scores were observed for Tarim 92 silage compared to Bravo silage. Significant V × He ($p = 0.006$), V × Ho ($p = 0.005$), and V × He × Ho ($p = 0.011$) indicated that silage fermentation quality was influenced not only by inoculant treatments but also by barley genotype as addition of inoculants resulted in different effects in different varieties. Within Tarim 92 silages, all treatments achieved pH 4.11–4.30 and Flieg scores of 102.94–115.47. Among all the treatments of Bravo silage, HMF inoculant resulted in the poorest fermentation quality. Inoculation of barley with HMF bacteria increased silage pH to 5.24 and reduced Flieg point to 47.06, while dual inoculation partially improved the quality (pH 4.97; Flieg 51.20). Lower NH₃-N values were observed in Tarim 92 silages, whereas markedly higher concentrations were observed in Bravo silages. The highest NH₃-N concentration was observed in Bravo treated with HMF alone, while the lowest value was recorded in untreated Tarim 92 silage. Tarim 92 silages were consistently rated excellent, whereas lower and more variable scores were observed with Bravo silages. Across all

treatments, Tarım 92 silages showed better fermentation quality and more consistent silage characteristics than Bravo silage.

Table 2. Effect of genotype and microbial inoculants on physical quality of barley ensiled at early dough stage of maturity.

Item	He ¹	Ho ²	pH	Odor	Structure	Color	Flieg Point	NH ₃ -N ³ (mg/kg TN)	DLG ⁴
Variety (V)									
Tarım 92			4.238	11.75	4.00	1.25	107.97	152.33	17.00
Bravo			4.808	6.83	4.00	1.33	62.70	256.12	12.17
Heterofermentative (He)									
Not added			4.528	9.17	4.00	1.25	86.37	205.53	14.42
Added			4.518	9.42	4.00	1.33	84.30	202.92	14.75
Homofermentative (Ho)									
Not added			4.400	7.92	4.00	1.25	91.50	195.97	13.17
Added			4.646	10.67	4.00	1.33	79.17	212.48	16.00
SEM			0.095	0.787	0.000	0.102	6.992	17.982	0.787
Three-way interactions (V × He × Ho)									
Tarım 92	—	—	4.300 c	11.00	4.00	1.17	108.00	142.01 c	16.17
Tarım 92	—	+	4.113 c	12.67	4.00	1.17	115.47	146.79 c	17.83
Tarım 92	+	—	4.280 c	11.33	4.00	1.50	105.46	149.05 c	16.83
Tarım 92	+	+	4.260 c	12.00	4.00	1.17	102.94	173.72 b	17.17
Bravo	—	—	4.460 bc	4.33	4.00	1.33	74.94	225.83 bc	9.67
Bravo	—	+	5.240 a	8.67	4.00	1.33	47.06	307.48 a	14.00
Bravo	+	—	4.560 b	5.00	4.00	1.00	77.60	269.26 a	10.00
Bravo	+	+	4.970 a	9.33	4.00	1.67	51.20	221.92 b	15.00
SEM			0.063	0.539	0.000	0.167	2.526	7.166	0.591
<i>p</i> -values									
V			<0.001	0.006	—	0.102	<0.001	0.009	0.028
He			0.010	0.444	—	0.265	0.281	<0.001	0.282
Ho			<0.001	<0.001	—	0.035	<0.001	0.010	<0.001
V × He			0.006	0.284	—	0.426	0.006	<0.001	0.208
V × Ho			0.005	0.008	—	0.026	<0.001	0.005	0.002
He × Ho			0.012	1.000	—	0.122	0.781	<0.001	0.607
V × He × Ho			0.011	0.565	—	0.102	0.142	<0.001	0.282

¹ He = *Lactobacillus buchneri* (heterofermentative inoculant); ² Ho = *Lactobacillus plantarum* + *Enterococcus faecium* (homofermentative inoculant). + = added; — = not added; — = not applicable. SEM = standard error of the mean. ³ NH₃-N = ammonia nitrogen (mg/kg TN (total nitrogen)); ⁴ DLG = Deutsche Landwirtschafts-Gesellschaft physical quality score; — in the *p*-value block = not tested (structure score showed zero variance across all treatments). a–c: Within each column, treatment means not sharing a common superscript differ significantly (*p* < 0.05).

3.2. Volatile Fatty Acids

The VFA profile of ensiled barley is affected by both the genotype of barley as well as by microbial inoculants. This is clearly presented in Table 3. Variety had a significant effect on isobutyric acid (*p* < 0.001) and butyric acid (*p* = 0.023). Bravo silage exhibited lower isobutyric acid concentration compared to Tarım 92 silage. However, Bravo silage had higher butyric acid and higher total VFA concentrations. Acetic acid concentration (*p* < 0.001) and total VFA concentration were significantly increased in silages treated with HMF inoculation. In contrast, silages with HTF inoculation exhibited significantly reduced propionic acid (*p* = 0.048) and significantly increased butyric acid concentration (*p* < 0.001). Acetic acid (*p* = 0.042) and butyric acid (*p* < 0.001) exhibited a significant V × He × Ho interaction. Within the three-way interaction, the highest acetic acid concentration and the highest total VFA concentration were recorded in dual-inoculated Bravo silage.

Table 3. Effect of genotype and microbial inoculants on volatile fatty acid (mmol/L) composition of barley ensiled at early dough stage of maturity.

Item	He ¹	Ho ²	ACE	PRO	ISO-BUT	BUT	CAP	Total
Variety (V)								
Tarım 92			16.35	0.27	0.31 a	1.19	0.05	18.17
Bravo			18.4	0.32	0.13 b	1.97	0.04	20.85
Heterofermentative (He)								
Not added			17.67	0.33	0.22	0.93	0.05	19.19
Added			17.08	0.27	0.21	2.22	0.05	19.83
Homofermentative (Ho)								
Not added			16.31	0.35	0.12	1.87	0.06	18.71
Added			18.44	0.24	0.31	1.28	0.03	20.31
SEM			0.77	0.02	0.01	0.22	0.01	0.82
Three-way interactions (V × He × Ho)								
Tarım 92	—	—	17.14 ab	0.29	0.29	0.22 c	0.07	18
Tarım 92	—	+	15.34 ab	0.36	0.09	1.90 ab	0.06	17.75
Tarım 92	+	—	18.29 ab	0.21	0.35	0.23 c	0.05	19.13
Tarım 92	+	+	14.64 b	0.23	0.5	2.40 a	0.04	17.81
Bravo	—	—	16.80 ab	0.45	0.06	2.95 a	0.06	20.31
Bravo	—	+	15.96 ab	0.31	0.05	2.42 a	0.03	18.77
Bravo	+	—	18.46 ab	0.36	0.19	0.32 c	0	19.32
Bravo	+	+	22.39 a	0.16	0.2	2.18 ab	0.05	24.98
SEM			1.53	0.04	0.02	0.44	0.01	1.64
<i>p</i> -values								
V			0.082	0.094	<0.001	0.023	0.412	0.034
He			0.581	0.048	0.812	<0.001	0.654	0.591
Ho			0.064	<0.001	<0.001	0.067	0.11	0.183
V × He			0.21	0.115	0.054	0.041	0.33	0.215
V × Ho			0.154	0.812	0.032	0.012	0.45	0.192
He × Ho			0.312	0.541	0.11	0.003	0.612	0.411
V × He × Ho			0.042	0.213	0.154	<0.001	0.591	0.081

¹ He = *Lactobacillus buchneri* (heterofermentative inoculant); ² Ho = *Lactobacillus plantarum* + *Enterococcus faecium* (homofermentative inoculant). + = added; — = not added. SEM = standard error of the mean. ACE = acetic acid; PRO = propionic acid; ISO-BUT = isobutyric acid; BUT = butyric acid; CAP = caproic acid. a–c: Within each column, treatment means not sharing a common superscript differ significantly ($p < 0.05$).

The lowest acetic acid concentration was observed in dual-inoculated Tarım 92 silage. Butyric acid concentration was lowest in uninoculated and HMF-only treatments of both varieties but its concentration increased significantly with HTF inoculation. It reached its highest values in HTF-only Bravo silage and dual-inoculated Tarım 92 silage. Propionic and caproic acid concentrations remained low and comparatively stable across treatments. They exhibited no clear genotype or inoculant driven pattern. Overall, Bravo silage consistently exhibited a higher total VFA load than Tarım 92 silage, and this difference was most significant in dual-inoculated silages. Bravo's total VFA concentration exceeded that of Tarım 92 by approximately 40%.

3.3. Chemical Composition

Chemical composition of barley silage was primarily influenced by variety ($p < 0.001$) and its interaction with HMF and HTF inoculants ($p < 0.001$ for CP, Ash, CF, ADF, ADL, and EE) (Table 4). Tarım 92 silage's improved fermentation quality was exhibited by its superior DM (43.73 vs. 32.49%), reduced aNDFom, ADFom, and CF. Within the three-way interaction, the highest CP was recorded in the Bravo silage treated with HMF inoculant alone. Lowest CF, aNDFom, and ADFom were observed in dual-inoculated Tarım

92 silage. A significant He $\times$ Ho interaction for aNDFom ($p < 0.001$) confirmed that regardless of genotype, dual inoculation decreased aNDFom in both varieties below any single-inoculant outcome.

Table 4. Effect of genotype and microbial inoculants on chemical composition of barley ensiled at early dough stage of maturity.

Item	He ¹	Ho ²	CP	EE	Ash	CF	aNDFom	ADFom	ADL	DM
Variety (V)										
Tarim 92			9.82	2.21	7.35	19.97	42.20	20.76	4.18	43.73
Bravo			10.29	2.47	9.05	23.74	47.98	26.20	4.21	32.49
Heterofermentative (He)										
Not added			10.40	2.17	8.36	22.57	46.33	24.01	3.90	38.74
Added			9.71	2.52	8.04	21.14	43.85	22.95	4.49	37.48
Homofermentative (Ho)										
Not added			9.84	2.63	8.05	22.00	45.66	24.09	4.78	38.73
Added			10.26	2.06	8.35	21.71	44.52	22.87	3.61	37.49
SEM			0.12	0.08	0.19	0.58	0.71	0.62	0.18	1.79
Three-way interactions (V $\times$ He $\times$ Ho)										
Tarim 92	—	—	9.77 c	2.00 d	7.32 d	18.96 c	42.59	20.98 d	3.81 c	45.00
Tarim 92	—	+	10.17 b	1.83 d	8.05 c	23.90 a	44.34	22.36 c	4.14 b	45.00
Tarim 92	+	—	9.26 d	2.57 b	6.78 e	18.77 c	42.68	20.96 d	5.65 a	43.30
Tarim 92	+	+	10.07 b	2.46 c	7.23 d	18.24 c	39.20	18.75 e	3.14 d	41.61
Bravo	—	—	10.24 b	2.81 b	9.17 a	25.21 a	48.69	27.30 a	5.23 a	31.63
Bravo	—	+	11.41 a	2.03 d	8.91 b	22.19 b	49.70	25.42 b	2.42 e	33.35
Bravo	+	—	10.10 b	3.15 a	8.91 b	25.06 a	48.66	27.12 a	4.44 b	35.00
Bravo	+	+	9.41 d	1.91 d	9.21 a	22.50 b	44.86	24.96 b	4.74 b	30.00
SEM			0.06	0.06	0.06	0.21	0.25	0.24	0.13	0.81
<i>p</i> -values										
V			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
He			<0.001	0.267	0.002	0.558	<0.001	0.248	<0.001	0.015
Ho			<0.001	<0.001	0.002	<0.001	<0.001	<0.001	0.140	<0.001
V $\times$ He			<0.001	<0.001	<0.001	<0.001	0.585	<0.001	<0.001	0.984
V $\times$ Ho			<0.001	<0.001	0.238	0.008	0.544	0.935	<0.001	0.077
He $\times$ Ho			<0.001	0.003	<0.001	0.535	<0.001	0.610	<0.001	0.001
V $\times$ He $\times$ Ho			<0.001	0.017	<0.001	<0.001	0.585	<0.001	<0.001	0.059

¹ He = *Lactobacillus buchneri* (heterofermentative inoculant); ² Ho = *Lactobacillus plantarum* + *Enterococcus faecium* (homofermentative inoculant). + = added; — = not added; SEM = standard error of the mean. All proportional values are expressed on a dry matter basis unless stated otherwise. CP = crude protein; EE = ether extract; CF = crude fiber; NDF = neutral detergent fiber (aNDFom); ADF = acid detergent fiber (ADFom); ADL = acid detergent lignin; DM = dry matter. a–e: Within each column, treatment means not sharing a common superscript differ significantly ($p < 0.05$).

3.4. Nutritive Value Indices

The effect of genotype variety and inoculant interaction on silage quality and its nutritive value parameters is presented in Table 5. Tarim 92 silages consistently exceeded Bravo, showing higher TDN compared to Bravo's. Tarim 92 also outperformed Bravo in NFC, NEL, and RFV, and all the differences in Tarim 92 and Bravo Silages were significant at $p < 0.001$. The interaction of V $\times$ He $\times$ Ho was significant for HEC ($p < 0.001$), cellulose ($p = 0.003$), total CHO ($p < 0.001$), TDN ($p < 0.001$), NEL ($p < 0.001$), and RFV ($p < 0.001$). In the case of dual inoculation of Tarim 92 silages, it produced the highest TDN, NEL, and RFV (176.25) across all the treatments, indicating superior quality among all treatments. Within Bravo silages, HMF treatment alone yielded the lowest TDN and RFV, whereas these values increased in response to dual inoculation. Dual inoculation also resulted in the lowest HEC values in both Tarim 92 and Bravo silages.

Table 5. Effect of genotype and microbial inoculants on carbohydrate fractions and relative feed value of barley ensiled at early dough stage of maturity.

Item	He ¹	Ho ²	NFC	HEC	Cellulose	Total CHO	TDN	NEL (Mcal kg ⁻¹)	RFV
Variety (V)									
Tarim 92			38.42	21.44	16.58	77.75	74.55	1.76	160.73
Bravo			30.20	21.78	21.99	76.30	67.53	1.61	133.02
Heterofermentative (He)									
Not added			32.74	22.32	20.12	75.37	70.35	1.67	141.75
Added			35.88	20.90	18.45	78.67	71.72	1.70	152.00
Homofermentative (Ho)									
Not added			33.82	21.57	19.31	77.28	70.25	1.67	143.86
Added			34.80	21.65	19.26	76.77	71.82	1.70	149.89
SEM			0.98	0.25	0.63	0.52	0.79	0.02	3.32
Three-way interactions (V × He × Ho)									
Tarim 92	—	—	38.31	21.61 b	17.17 c	78.40 b	74.27 b	1.75 b	158.46 b
Tarim 92	—	+	35.62	21.98 b	18.22 c	72.96 d	72.49 c	1.72 c	150.00 c
Tarim 92	+	—	38.71	21.72 b	15.31 d	79.60 a	74.29 b	1.75 b	158.21 b
Tarim 92	+	+	41.04	20.45 c	15.62 d	80.02 a	77.14 a	1.81 a	176.25 a
Bravo	—	—	29.08	21.40 b	22.07 a	73.49 d	66.11 e	1.59 e	129.22 e
Bravo	—	+	27.95	24.28 a	23.01 a	76.64 c	68.53 d	1.63 d	129.32 e
Bravo	+	—	29.18	21.54 b	22.68 a	77.61 b	66.34 e	1.59 e	129.55 e
Bravo	+	+	34.60	19.90 c	20.22 b	77.45 b	69.13 d	1.65 d	144.00 d
SEM			0.25	0.26	0.26	0.28	0.31	0.01	1.09
<i>p</i> -values									
V			<0.001	0.179	<0.001	<0.001	<0.001	<0.001	<0.001
He			<0.001	<0.001	<0.001	0.079	0.248	0.248	<0.001
Ho			<0.001	<0.001	<0.001	0.728	<0.001	<0.001	<0.001
V × He			0.029	<0.001	0.749	<0.001	<0.001	<0.001	<0.001
V × Ho			<0.001	0.516	<0.001	0.374	0.935	0.935	0.127
He × Ho			<0.001	<0.001	<0.001	<0.001	0.610	0.610	<0.001
V × He × Ho			0.053	<0.001	0.003	<0.001	<0.001	<0.001	<0.001

¹ He = *Lactobacillus buchneri* (heterofermentative inoculant); ² Ho = *Lactobacillus plantarum* + *Enterococcus faecium* (homofermentative inoculant). + = added; — = not added; SEM = standard error of the mean. All proportional values are expressed on a % dry matter basis unless stated otherwise. NFC = non-fiber carbohydrates; HEC = hemicellulose; Total CHO = total carbohydrates; TDN = total digestible nutrients; NEL = net energy for lactation (Mcal kg⁻¹ DM); RFV = relative feed value. a–e: Within each column, treatment means not sharing a common superscript differ significantly ($p < 0.05$).

3.5. In Vitro Digestibility

The in vitro digestibility of silage, influenced by barley variety and inoculant interaction, is demonstrated in Table 6. Variety, HTF, and HMF inoculants interacted to significantly affect digestible DM, in vitro organic matter digestibility (IVOMD), in vitro neutral detergent fiber digestibility (IVNDFD), and DMI ($p < 0.05$). In contrast, in vitro dry matter digestibility (IVDMD) was significantly influenced by the separate interactions between variety and inoculant treatments (V × He, V × Ho, and He × Ho; $p < 0.001$). Overall, compared to Bravo silages, Tarim 92 silages had greater DMI, digestible DM, IVDMD, and IVOMD ($p < 0.001$). In contrast, Bravo silages had higher IVNDFD values. In Tarim 92 silages, dual inoculation resulted in the maximum intake of DM, digestible DM, IVDMD, and IVOMD. Among all the treatments, the lowest values of IVOMD and IVDMD were achieved by the HTF-alone treatment of Bravo silage. Among all the treatments, only dual inoculation significantly improved the digestibility of Bravo silage, increasing IVOMD and IVDMD and bringing its digestibility parameters closer to those observed in the least effective treatments of Tarim 92 silage. Overall, inoculant effects varied between barley varieties, where Tarim 92 consistently responded better and performed best under dual inoculation, but Bravo required the dual-inoculation treatment to increase digestibility.

Table 6. Effect of genotype and microbial inoculants on in vitro digestibility of barley ensiled at early dough stage of maturity.

Item	He ¹	Ho ²	DM Intake (% BW)	Digestible DM (%)	IVDMD (%)	IVOMD (%)	IVNDFD (%)
Variety (V)							
Tarim 92			2.85	72.73	63.61	62.84	15.53
Bravo			2.51	68.49	60.74	60.41	19.58
Heterofermentative (He)							
Not added			2.60	70.19	62.35	61.43	20.27
Added			2.75	71.02	62.00	61.81	14.84
Homofermentative (Ho)							
Not added			2.64	70.13	61.72	61.24	17.72
Added			2.71	71.08	62.62	62.00	17.39
SEM			0.04	0.48	0.48	0.51	0.89
Three-way interactions (V × He × Ho)							
Tarim 92	—	—	2.82 b	72.56 b	63.60	63.16 b	16.39 b
Tarim 92	—	+	2.71 c	71.48 c	61.44	59.31 d	15.39 b
Tarim 92	+	—	2.81 b	72.57 b	63.63	63.16 b	16.30 b
Tarim 92	+	+	3.06 a	74.29 a	65.76	65.72 a	14.03 c
Bravo	—	—	2.47 d	67.64 e	62.54	61.64 c	24.93 a
Bravo	—	+	2.41 d	69.10 d	61.81	61.61 c	24.38 a
Bravo	+	—	2.47 d	67.77 e	57.13	57.00 e	13.25 c
Bravo	+	+	2.68 c	69.46 d	61.48	61.37 c	15.77 b
SEM			0.01	0.19	0.20	0.19	0.30
<i>p</i> -values							
V			<0.001	<0.001	<0.001	<0.001	<0.001
He			<0.001	0.248	0.268	0.387	<0.001
Ho			<0.001	<0.001	<0.001	<0.001	<0.001
V × He			0.006	<0.001	<0.001	<0.001	<0.001
V × Ho			0.221	0.935	<0.001	<0.001	<0.001
He × Ho			<0.001	0.610	<0.001	<0.001	<0.001
V × He × Ho			0.033	<0.001	0.184	<0.001	<0.001

¹ He = *Lactobacillus buchneri* (heterofermentative inoculant); ² Ho = *Lactobacillus plantarum* + *Enterococcus faecium* (homofermentative inoculant). + = added; — = not added; SEM = standard error of the mean. All proportional values are expressed on a dry matter basis unless stated otherwise. DM intake = predicted dry matter intake (% body weight); Digestible DM = predicted digestible dry matter (%); IVDMD = in vitro dry matter digestibility; IVOMD = in vitro organic matter digestibility; IVNDFD = in vitro neutral detergent fiber digestibility. a–e: Within each column, treatment means not sharing a common superscript differ significantly ($p < 0.05$).

4. Discussion

The individual and combined effects of variety, homofermentative inoculants (HMF; *Lactobacillus plantarum* and *Enterococcus faecium*), and heterofermentative inoculants (HTE; *Lactobacillus buchneri*) on fermentation characteristics, chemical composition, nutritive profile and in vitro digestibility in a 2 × 2 × 2 factorial arrangement were examined in this study. It significantly indicated the variability of inoculant effects across barley varieties. Without understanding the intrinsic biochemical substrate of the crop, neither the effect of variety on silage outcome nor the inoculant performance can be predicted. The intrinsic biochemistry of silage fermentation, genotypic variation in barley physiology and the functional ecology of homofermentative and heterofermentative lactic acid bacteria are integrated in this study.

4.1. Physical Quality, Fermentation Characteristics, Volatile Fatty Acids, and Nutritional Composition

Fermentation pattern, VFA concentrations, and nutritional composition of barley silages were governed by variety, HMF, and HTF inoculants evident from the significant three-way interactions. These interactions showed that metabolic products translated from microbial inoculants can only be evaluated combined with the intrinsic chemical composition and biochemical compatibility of barley cultivars. Consistent with this notion, mathematical modeling demonstrated that the rate and magnitude of pH decline during ensiling is limited by the forage's buffering capacity and the concentration of WSC available to LAB [16]. A comparison of chemical composition of freshly harvested pre-ensiled barley varieties with that of ensiled barley illustrates the effect of initial chemical composition on the efficacy of inoculants. In addition, although lactic acid was not quantified due to the limitation of GC column, acidification pattern (pH) and Flieg score serve as indicators of lactic acid accumulation.

Greater NFC and lower structural carbohydrate contents in the freshly harvested Tarim 92 barley variety was an ideal source for rapid fermentation and acidification. Consequently, all inoculant treatments in Tarim 92 converged to a pH of 4.11 to 4.30, a characteristic of classic HMF acidification. Furthermore, all Tarim 92 silages were classified in the excellent physical quality category due to their consistent low, stable pH and high Flieg points [18]. It confirms the early lactic acid accumulation accelerated by *L. plantarum* and *E. faecium* that dominated the fermentation process and outperformed the unwanted bacteria (clostridia and enterobacteria) in the first critical aerobic phase of oxygen depletion [8,10,23]. These findings are consistent with the studies of Weinberg and Muck [8] and Li et al. [23] who demonstrated that the pH of silage is more rapidly reduced by HMF inoculants compared to uninoculated silages. In the presence of non-limiting substrate supply (WSC), HMF fermentation driven by *L. plantarum* ensures maximum efficiency of substrate-to-acid conversion [14,24]. This was confirmed by a moderate level of acetic acid concentration in dual-inoculated Tarim 92 silage that reflects abundant utilization of hexose or WSC substrate resulting in efficient initial phase of HMF-driven fermentation that limited the downstream HTF-driven conversion of residual lactic acid pool into acetic acid [15,25]. Tarim 92 maintained high DM during the ensiling period that not only reduced or limited the effluent seepage but also restricted the proteolysis in the pre-acidification phase. Consequently, $\text{NH}_3\text{-N}$ was lower in Tarim 92 silages along with negligible butyric acid concentrations [7,26,27] that indicate the absence of clostridial fermentation. The Tarim 92 barley variety inoculated with HMF and HTF combined showed a synergistic organic acid that resulted in lowest structural carbohydrates (aNFDom, ADFom, and ADL), a modification probably due to partial acid hydrolysis of hemicellulose and cell wall carbohydrates. This might have promoted the potential accessibility to fiber without jeopardizing the nitrogen protection and loss of organic matter to a greater extent [9,13,17].

In contrast, freshly harvested Bravo barley forage had greater contents of structural carbohydrates and lower concentration of fermentable carbohydrates. Bravo silages demonstrated a fermentation pattern primarily governed by substrate-limiting constraints due to higher proportion of structural carbohydrates and lower availability of readily fermentable substrates required for rapid lactic acid production during ensiling. Inoculation with the HMF inoculant showed a complete failure of the acidification process since fermentable carbohydrates were quickly exhausted, resulting in failure of continuous fermentative activity of HMF bacteria [13,26] thereby exhibiting elevated pH, low Flieg points, and peak $\text{NH}_3\text{-N}$. A high concentration of $\text{NH}_3\text{-N}$ indicates long-term secondary fermentation and excessive proteolysis [7,26] due to insufficient acidification that promotes the breakdown of proteins into $\text{NH}_3\text{-N}$ due to microbial degradation and plant proteases [27,28]. Although CP was consistently high in Bravo inoculated with HMF alone, this might be an artifact caused

by DM loss and nutrient instead of protein preservation [13,26]. VFA analysis further assisted in explaining the alternative fermentation pathways in Bravo silage treatments. Although total VFA concentration was greater in Bravo silages than Tarım 92 silages, pH was consistently greater in Bravo silages than in their Tarım 92 counterparts. Among Bravo silages, the HTF-only group showed relatively better pH reduction and Flieg point than the HMF-only counterpart that might be attributed to the coenzyme B12-dependent metabolic pathway that converts lactic acid into acetic acid and 1,2-propanediol, thus providing secondary metabolites with antifungal and acidifying properties, with lesser dependence on WSC [11,15,25,29]. However, HMF + HTF inoculation in Bravo was unable to yield appropriate preservation evident from greater concentrations of acetic acid along with total VFAs in association with consistent butyric acid and high $\text{NH}_3\text{-N}$. Although acetic acid favors aerobic stability, persistent butyric acid in HTF-only and HMF + HTF-inoculated Bravo is a clear indication of secondary clostridial fermentation arising due to insufficient early acidification [26,30] that yields loss of DM and declines the palatability.

Additionally, a comprehensive assessment of CP, $\text{NH}_3\text{-N}$, and VFA concentrations indicates the inability of combined inoculation of HMF and HTF to preserve the protein quality in Bravo cultivar since rumen microbiota have poor utilization of $\text{NH}_3\text{-N}$ [31]. Propionic acid and caproic acid were consistently low among the treatment. On the contrary, isobutyric acid, a branched chain VFA produced from microbial degradation of leucine, valine, and isoleucine, was greater in Tarım 92 silages despite greater $\text{NH}_3\text{-N}$ content in Bravo silages. It implies that $\text{NH}_3\text{-N}$ was primarily driven by degradation via plant proteases as opposed to microbial degradation [26,32].

4.2. Feed Value Indices

The integral feed quality parameters that govern the ruminant energy supply were determined by the specific combination of genotype and inoculant treatment but not by either factor independently. This was demonstrated by the significant $V \times \text{He} \times \text{Ho}$ interaction for TDN, NEL, RFV, HEC, cellulose, and total CHO. These findings indicate that barley silage show genotype-dependent inoculant responses and the performance outcomes observed in one variety may not be transferable to another variety.

Greater mean TDN, NEL, and RFV were exhibited by Tarım 92 silages compared to Bravo silages. This demonstrates the strong influence of lower ADF concentration within NRC-derived prediction equations [33]. These findings are comparable to the values of high-quality barley silages reported by Bikel et al. [2] and Borowiec et al. [5]. Tarım 92 silage treated with dual inoculation exhibited the higher range of nutritive values. This might be attributed to the pronounced reduction in ADF under dual-inoculation treatment. Although RFV systems were primarily developed for hay crops, the observed RFV still indicates exceptionally high predicted forage quality for a cereal silage system.

The dual inoculation with HMF and HTF inoculants produced non-additive effects on carbohydrate composition independent of variety. This was indicated by the significant $\text{He} \times \text{Ho}$ interaction for NFC, HEC, cellulose, and total CHO. The lowest HEC values in both Tarım 92 silage and Bravo silage were yielded by the dual-inoculated silage and the highest value was observed in the Bravo silage treated with HMF only. This reduction is most likely due to enhanced structural carbohydrate solubilization under combined organic acid production [18]. This is due to genotype-dependent variation in barley cell wall fractions. The importance of substrate composition in determining inoculant response is usually emphasized [17].

This study clearly demonstrates the dependence of achievable forage quality on the strong genotype and the greater importance of inoculant treatment in the lower performing Bravo genotype. This is indicated by the RFV hierarchy across treatments as Tarım

92 dual inoculant treatment > Tarim 92 control > Tarim 92 HTF treatment > Tarim 92 HMF treatment > Bravo dual inoculant treatment > Bravo HTF treatment > Bravo HMF treatment $\approx$ Bravo control. Among all the treatments of Bravo silages, RFV was improved by only dual-inoculation treatment compared to the control. Bravo silage treated with only HMF inoculants failed to enhance the predicted forage value. This indicates that HMF inoculation without HTF inoculation was not sufficient to improve the nutritive quality of Bravo silages. Therefore, the digestible fraction of NDF and the attainable forage value in barley silages is limited by the genotype-dependent variation in lignification and cell wall structure [17,34].

4.3. In Vitro Digestibility

In vitro digestibility provided the direct evidence of practical consequences arising from genotype $\times$ inoculant interactions, with the V $\times$ He $\times$ Ho interaction significant for IVOMD and IVNDFD, along with highly significant two-way interactions governing IVDMD and DMI. These findings translate the fermentation biochemistry and compositional dynamics into biologically relevant nutritional outcomes, demonstrating that the consequences of poor application of inoculant are manifested not only in fermentation quality, but also in the predicted availability of digestible nutrients to the animal.

In comparison with Bravo silage, Tarim 92 silage showed significantly higher IVDMD, IVOMD, DDM, and DMI, as well as lower IVNDFD, suggesting lower structural carbohydrate concentration and better fermentation quality. An essential principle of ruminant nutrition is the inverse relationship between ADF concentration and forage digestibility. This is attributed to the increased cell wall lignification that limits the microbial access to digestible polysaccharides. Therefore, it limits both DDM and DMI potential in cereal forages [34]. Tarim 92 silage exhibited lower mean ADF concentrations that probably contributed to the varietal advantage in IVDMD observed in this study. This directly connected compositional differences to the nutritional outcomes that are biologically significant.

The lowest IVOMD and IVDMS values were recorded for Bravo silage treated with only HTF inoculants, and fall even below the value of uninoculated Bravo silage. This indicates a detrimental nutritional outcome under commercial conditions. This response was likely due to the incompletely acidified acetic acid which dominated the fermentation profile of the Bravo silage treated with only HTF inoculants. This may result in the reduction in overall digestibility since selective loss of readily digestible cell constituents may have been promoted while leaving structurally resistant fiber fractions proportionally enriched. During early fermentation, elevated pH may have allowed continued plant enzymatic activity because plant proteases remain active until sufficiently low pH is achieved [26]. This probably contributed to the degradation of soluble nutritive fractions and further decrease in IVOMD and IVDMD.

A cautious interpretation is needed for the elevated IVNDFD value observed in the Bravo silage treated with only the HMF inoculant and uninoculated Bravo silage compared to others. IVNDFD reflects the proportion of NDF disappearing during ruminal simulation. The poorly fermented Bravo silages exhibited greater IVNDF values which may indicate the increased accessibility of structurally less preserved fiber fractions to microbial and physical degradation during incubation [28,34]. The dual-inoculated silages exhibited lower IVNDFD values which may reflect altered structural carbohydrate composition associated with improved fermentation dynamics and reduced proportions of readily degradable fiber components. Therefore, IVNDFD responses should be interpreted along with overall fermentation quality and total digestible indices.

Among all the treatments of Tarim 92 silage, dual-inoculated silage exhibited exceptionally high predicted nutritive value for a whole-crop barley silage having the highest

IVOMD, DDM, and DMI. Dual inoculation also showed pronounced reductions in ADF and ADL values that were because of the role of lignification and structural carbohydrate concentration in limiting ruminal fiber digestibility and overall forage value [22,34].

Overall, three conclusions can be interpreted from the in vitro digestibility data. First, genotype selection emerged as the dominant determinant of silage digestibility and predicted nutritive value across all treatments. Second, among both varieties, dual-inoculation treatment consistently exhibited the highest digestibility and energy value responses. Third, using HTF-alone treatment in varieties with greater structural carbohydrate content and limited fermentable substrate availability can reduce digestibility to the levels lower than uninoculated silage, which highlights the importance of modifying inoculant strategies according to the specific barley genotype under commercial production conditions.

5. Conclusions

Our study demonstrated that barley variety governs the efficacy of microbial inoculants that is largely constrained by the inherent chemical composition as well as the substrate availability in terms of fermentable carbohydrates. Ensiling of Tarım 92, a barley variety characterized by higher non-fiber and lower structural carbohydrates at harvest, with combined homofermentative (*L. plantarum* and *E. faecium*) and heterofermentative (*L. buchneri*) inoculants yields high-quality silage with optimal nutrients and in vitro digestibility. In contrast, ensiling Bravo (low non-fiber and greater structural carbohydrates) failed to produce even a good quality silage evident from poor protein preservation, high pH, poorer volatile fatty acids, and poor in vitro digestibility. From a practical management viewpoint, barley varieties with low structural carbohydrates and greater fermentable carbohydrates can be ensiled with combined inoculation of homofermentative and heterofermentative inoculants, whereas barley cultivars with high structural carbohydrates and lower fermentable carbohydrates require optimization of harvest maturity to secure adequate levels of water-soluble carbohydrates for efficient fermentation.

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Abbreviations

The following abbreviations are used in this manuscript:

LAB	Lactic acid bacteria
HMF	Homofermentative
HTF	Heterofermentative

VFA	Volatile fatty acid
DLG	Deutsche Landwirtschafts Gesellschaft
NH ₃ -N	Ammonia nitrogen
DM	Dry matter
CP	Crude protein
EE	Ether extract
CF	Crude fiber
aNDFom	Ash-free neutral detergent fiber after amylase treatment
ADFom	Ash-free acid detergent fiber
ADL	Acid detergent lignin
NFC	Non-fiber carbohydrates
DMI	Dry matter intake
RFV	Relative feed value
Total CHO	Total carbohydrates
TDN	Total digestible nutrients
HEC	Hemicellulose
OM	Organic matter
IVOMD	In vitro organic matter digestibility
IVDMD	In vitro organic matter digestibility
IVNDFD	In vitro neutral detergent fiber digestibility
WSC	Water-soluble carbohydrates

References

1. Josephides, C. Analysis of adaptation of barley, triticale, durum and bread wheat under Mediterranean conditions. *Euphytica* **1992**, *65*, 1–8. [\[CrossRef\]](#)
2. Bikel, D.; Ben-Meir, Y.A.; Shaani, Y.; Solomon, R.; Richker, I.; Portnik, Y.; Jacoby, S.; Miron, J.; Ben-David, R. Nutritive value for high-yielding lactating cows of barley silage and hay as a substitute for wheat silage and hay in low-roughage diets. *Anim. Feed. Sci. Technol.* **2020**, *265*, 114498. [\[CrossRef\]](#)
3. Polan, C.; Starling, T.; Huber, J.; Miller, C.; Sandy, R. Yields, compositions, and nutritive evaluation of barley silages at three stages of maturity for lactating cows. *J. Dairy Sci.* **1968**, *51*, 1801–1805. [\[CrossRef\]](#)
4. Hargreaves, A.; Hill, J.; Leaver, J. Effect of stage of growth on the chemical composition, nutritive value and ensilability of whole-crop barley. *Anim. Feed. Sci. Technol.* **2009**, *152*, 50–61. [\[CrossRef\]](#)
5. Borowiec, F.; Furgał, K.; Kamiński, J.; Zając, T. Nutritive value of silage made of whole barley crop harvested at various stages of maturity. *J. Anim. Feed. Sci.* **1998**, *7*, 45–54. [\[CrossRef\]](#)
6. Ali, M.F.; Tahir, M. An overview on the factors affecting water-soluble carbohydrates concentration during ensiling of silage. *J. Plant Environ.* **2021**, *3*, 63–80. [\[CrossRef\]](#)
7. Driehuis, F.; Elferink, S.O. The impact of the quality of silage on animal health and food safety: A review. *Vet. Q.* **2000**, *22*, 212–216. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Weinberg, Z.G.; Muck, R. New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiol. Rev.* **1996**, *19*, 53–68. [\[CrossRef\]](#)
9. Amanullah, S.; Kim, D.; Lee, H.; Joo, Y.; Kim, S.; Kim, S. Effects of microbial additives on chemical composition and fermentation characteristics of barley silage. *Asian Australas. J. Anim. Sci.* **2014**, *27*, 511. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Addah, W.; Baah, J.; Groenewegen, P.; Okine, E.; McAllister, T. Comparison of the fermentation characteristics, aerobic stability and nutritive value of barley and corn silages ensiled with or without a mixed bacterial inoculant. *Can. J. Anim. Sci.* **2011**, *91*, 133–146. [\[CrossRef\]](#)
11. Taylor, C.; Ranjit, N.; Mills, J.; Neylon, J.; Kung, L., Jr. The effect of treating whole-plant barley with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for dairy cows. *J. Dairy Sci.* **2002**, *85*, 1793–1800. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Ahsan, U. Effect of single or combined homo-and heterofermentative silage additives on the quality, nutritive value, and in vitro digestibility of ensiled wheat harvested at early dough stage of maturity. *Mehmet Akif Ersoy Univ. J. Health Sci. Inst.* **2023**, *11*, 267–274. [\[CrossRef\]](#)
13. Muck, R.E. Silage microbiology and its control through additives. *Rev. Bras. Zootec.* **2010**, *39*, 183–191. [\[CrossRef\]](#)
14. Bolsen, K.; Ashbell, G.; Weinberg, Z. Silage fermentation and silage additives-Review. *Asian Australas. J. Anim. Sci.* **1996**, *9*, 483–494. [\[CrossRef\]](#)

15. Holzer, M.; Mayrhuber, E.; Danner, H.; Braun, R. The role of *Lactobacillus buchneri* in forage preservation. *Trends Biotechnol.* **2003**, *21*, 282–287. [CrossRef] [PubMed]
16. Pitt, R.; Leibensperger, R. The effectiveness of silage inoculants: A systems approach. *Agric. Syst.* **1987**, *25*, 27–49. [CrossRef]
17. Nair, J.; Christensen, D.; Yu, P.; Beattie, A.D.; McAllister, T.; Damiran, D.; Preston, N.; Fuhr, L.; McKinnon, J.J. A nutritional evaluation of common barley varieties grown for silage by beef and dairy producers in western Canada. *Can. J. Anim. Sci.* **2016**, *96*, 598–608. [CrossRef]
18. Dong, Z.; Yuan, X.; Wen, A.; Desta, S.T.; Shao, T. Effects of calcium propionate on the fermentation quality and aerobic stability of alfalfa silage. *Asian-Australas. J. Anim. Sci.* **2017**, *30*, 1278. [CrossRef] [PubMed]
19. Meeske, R.; Van der Merwe, G.; Greyling, J.; Cruywagen, C. The effect of the addition of a lactic acid bacterial inoculant to maize at ensiling on silage composition, silage intake, milk production and milk composition. *S. Afr. J. Anim. Sci.* **2002**, *32*, 263–270.
20. Association of Official Analytical Chemists. *Official Methods of Analysis*, 17th ed.; Association of Official Analytical Chemists: Gaithersburg, MD, USA, 2000.
21. Calik, A.; Ceylan, A.; Ekim, B.; Adabi, S.G.; Dilber, F.; Bayraktaroglu, A.G.; Tekinay, T.; Özen, D.; Sacakli, P. The effect of intra-amniotic and posthatch dietary synbiotic administration on the performance, intestinal histomorphology, cecal microbial population, and short-chain fatty acid composition of broiler chickens. *Poult. Sci.* **2017**, *96*, 169–183. [CrossRef] [PubMed]
22. Horrocks, R.D.; Valentine, J.F. *Harvested Forages*; Academic Press: Cambridge, MA, USA, 1999.
23. Li, Y.; Da Silva, E.; Li, J.; Kung, L., Jr. Effect of homo-fermentative lactic acid bacteria inoculants on fermentation characteristics and bacterial and fungal communities in alfalfa silage. *Fermentation* **2022**, *8*, 621. [CrossRef]
24. Stephen, J.M.; Saleh, A.M. Homofermentative *Lactobacilli* isolated from organic sources exhibit potential ability of lactic acid production. *Front. Microbiol.* **2023**, *14*, 1297036. [CrossRef] [PubMed]
25. Weinberg, Z.G.; Ashbell, G.; Hen, Y.; Azrieli, A.; Szakacs, G.; Filya, I. Ensiling whole-crop wheat and corn in large containers with *Lactobacillus plantarum* and *Lactobacillus buchneri*. *J. Ind. Microbiol. Biotechnol.* **2002**, *28*, 7–11. [CrossRef] [PubMed]
26. McDonald, P.; Henderson, A.; Heron, S. *The Biochemistry of Silage*, 2nd ed.; Chalcombe Publications: Marlow, UK, 1991.
27. Givens, D.; Rulquin, H. Utilisation by ruminants of nitrogen compounds in silage-based diets. *Anim. Feed. Sci. Technol.* **2004**, *114*, 1–18. [CrossRef]
28. Huhtanen, P.; Jaakkola, S.; Nousiainen, J. An overview of silage research in Finland: From ensiling innovation to advances in dairy cow feeding. *Agric. Food Sci.* **2013**, *22*, 35–56. [CrossRef]
29. Eurofins Agro. Acetic Acid. Available online: <https://www.eurofins-agro.com/en/acetic-acid> (accessed on 3 August 2026).
30. Eurofins Agro. Butyric Acid. Available online: <https://www.eurofins-agro.com/en-gb/butyric-acid-and-butyric-acid-bacteria> (accessed on 3 August 2026).
31. Atasoglu, C.; Newbold, C.J.; Wallace, R.J. Incorporation of [¹⁵N] ammonia by the cellulolytic ruminal bacteria *Fibrobacter succinogenes* BL2, *Ruminococcus albus* SY3, and *Ruminococcus flavefaciens* 17. *Appl. Environ. Microbiol.* **2001**, *67*, 2819–2822. [CrossRef] [PubMed]
32. Apajalahti, J.; Vienola, K.; Raatikainen, K.; Holder, V.; Moran, C.A. Conversion of branched-chain amino acids to corresponding isoacids—an in vitro tool for estimating ruminal protein degradability. *Front. Vet. Sci.* **2019**, *6*, 311. [CrossRef] [PubMed]
33. National Research Council. *Nutrient Requirements of Dairy Cattle: 2001*; National Academies Press: Washington, DC, USA, 2001.
34. Van Soest, P.J. *Nutritional Ecology of the Ruminant*; Cornell University Press: Ithaca, NY, USA, 1994.

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