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Research article

Inoculation with heterofermentative strains *Lentilactobacillus buchneri* CNCM 40788 and *Lentilactobacillus hilgardii* CNCM I-4785 either alone or combined improves fermentation and aerobic stability of ensiled triticale (X-triticosecale)

Richard Anthony Scuderi[1,*], Pascal Drouin[2], and Emmanuelle Apper[3]

[1] Lallemand Specialties Inc., Milwaukee, WI, USA
[2] Independent researcher, Saint-Jean-sur-Richelieu, QC, Canada
[3] Lallemand SAS, Blagnac, France

* **Correspondence:** Email: rscuderi@lallemand.com; Tel: +1(518)8028034.

Abstract: Triticale, a small-grain forage, was harvested for an ensiling experiment evaluating different silage inoculants. Fresh material (mean dry matter 404.1 g kg^{-1}) was wilted, chopped, and assigned to one of four treatment groups including water only (Control), heterofermentative strains *Lentilactobacillus buchneri* (LB), *Lentilactobacillus hilgardii* (LH), or combination (Combo) of both. Inoculants were applied at 4.0 x 10^5 CFU per g of fresh forage, and the Combo contained both strains at 2.0 x 10^5 CFU per g. Treated forage was packed into 7.57 L mini-silos for openings after 15, 30, and 130 d. Samples were collected at each opening for microbial enumeration of lactic acid bacteria, enterobacteria, yeasts, and moulds. Additional samples were collected for analysis of fermentation profiles, and nutritional analysis following dry matter determination and grinding. Aerobic stability was also evaluated at each opening through a 10-d period. Fermentation profiles were notably affected, including increases in acetic acid (g/kg DM) in LB and Combo treated silage after 15 d which resulted in reduced pH. Aerobic stability was vastly improved through inoculation by prolonging the time to reach 2˚ C above ambient, which was likely a result of decreased yeast counts. Our research validates the use of combined heterofermentative lactic acid bacteria strains on an ensiled small-grain specie, and further agrees with previous findings of prolonged aerobic stability through increases in lactate and acetate in response to co-inoculation.

Keywords: silage inoculants; small grain forage; lactic acid bacteria; silage quality

1. Introduction

Ensiling is an important agricultural practice providing conserved forages for incorporation into livestock rations outside of growing seasons. Additionally, the practice of ensiling small-grain cereal crops is becoming increasingly important in climates where the growth of common forage crops, like corn, is limited due to environmental factors [1]. For instance, triticale (X-triticosecale) has become a popular alternative since it offers larger yields in climates with limited water availability [1]. In addition to environmental flexibility to grow in challenging climates, certain varieties of triticale have been shown to have higher nutritional value than wheat. This has been attributed to an improved protein composition which has higher concentrations of lysine compared to other small-grain cereals [2]. The use of small-grain cereal crops have also been gaining attention as cover-cropping techniques, and triticale has been effective in this application by additional uptake of soil nitrogen and reducing soil erosion [3].

Inoculating forages with lactic acid bacteria (LAB) at the time of harvest is an effective strategy for improving silage fermentation [4]. The use of heterofermentative LAB in inoculant formulations has been shown to also improve aerobic stability of silage during feed out [5]. Improvements in aerobic stability has been linked to the metabolic pathway observed with *Lentilactobacillus buchneri* converting lactate to acetate and 1,2-propanediol [6]. *Lentilactobacillus buchneri* (previous taxonomic identification as *Lactobacillus buchneri* [7]) is one of the most utilized heterofermentative LAB species for ensiling applications, which has been validated in numerous studies across maize, grass, and small-grain silage. Meta-analysis studies have been published on using *L. buchneri* as a silage microbial additive [8], including one that examined its effects across different forage species, which confirmed improvements in aerobic stability through concurrent increases in acetate and propionate [9]. Despite its' success, *L. buchneri* requires substantial fermentation time, often longer than 30 days, to reach the correct ecological niche and initiating his metabolic activities [4].

Alternatively, *Lentilactobacillus hilgardii* (previously *Lactobacillus hilgardii* [7]) has been shown to exhibit earlier heterofermentative activity [10]. Since its' original isolation from ensiled sugar cane that exhibited prolonged aerobic stability [11], the expanded use of *L. hilgardii* in treating silage has resulted in improved silage stability at feed out [12]. Cumulative benefits have also been postulated through the combined use of *L. hilgardii* with *L. buchneri*, and has been evaluated across multiple ensiling experiments [10,13]. This includes a recent studies by Drouin et al. [14] which reported improved fermentation characteristics through the co-inoculation of corn with *L. buchneri* and *L. hilgardii.* This activity was further confirmed through the identification of changes in the microbial populations by 16S sequencing, including a decrease in the average diversity (alpha diversity) of microbial species when silage were treated with both strains. A similar microbial succession paper was published evaluating the co-inoculation of corn with *L. buchneri* and *L. hilgardii* during an aerobic stability challenge in which treated silage retained similar organic acid concentrations and low pH values across the 10 day challenge [15]. Most recently, another succession study was performed on alfalfa silage when treated with *L. bucherni* and *L. hilgardii* that shifted the predominate LAB populations from the *Weisella* genus to *Lactobacillus* genus [16]. As a result from shifting LAB populations in that study, the fermentation profiles of inoculated silage exhibited heterofermentative patterns.

Although the effects of inoculating silage with *L. buchneri* and *L. hilgardii* have been extensively evaluated in corn and grasses, there is limited information on their use in small-grains including triticale. Kleinschmitt and Kung [9] performed a meta-analysis on the effects of *L. buchneri* inoculation on corn, grass, and small-grain silage to examine the effects on fermentation aspects and aerobic stability. However, the analysis was limited to the use of *L. buchneri* and there were no included studies that examined triticale following inoculation. Despite the abundance of information on inoculating corn and grasses using the specific strains studied in the present paper, the differences in nutritional and chemical composition between forage varieties are important considerations while evaluating inoculant types since they can influence fermentation aspects. For example, ensiling small grain forages at earlier stages of growth can inhibit fermentation due to higher buffering capacity and moisture content [17].

Few studies have specifically studied triticale silage fermentation following inoculation. Ozduven et al. [18] reported notable effects during fermentation when triticale was ensiled with a commercial inoculant (containing *Pediococcus acidilactici*, *Lactobacillus plantarum*, *Streptococcus faecium*, and a mixture of enzymes) including rapid pH reduction, higher lactic acid concentrations, and lower ammonia content compared to untreated silage. Another study reported improvements in aerobic stability along with increases in acetic and propionic acids in baled triticale-Hungarian vetch silage treated with *L. bucherni* [19]. Since then, more comprehensive research has investigated the microbial dynamics of triticale silage. Including a microbiome study that investigated the bacterial and fungal communities in triticale silage as well as oat, barley, and an intercropped mixture of all three small-grain varieties [20]. Triticale silage after 90 days had higher relative abundances of the *Lactobacillales* accompanied with decreases across other OTUs, in agreement with other microbiome studies on different forage varieties. Most recently, different LAB strains were isolated from triticale silage and further selected for silage fermentation aspects and probiotic potential [21] with two strains being identified as *Pediococcus pentosaceus* and *Lactobacillus brevis* [21]. These results imply the presence of both homofermentative and heterofermentative LAB strains are responsible for optimal fermentation of triticale silage. Therefore, the application of multiple heterofermentative strains could be similarly advantageous in improving triticale silage quality. Hence, the objective of the current experiment was to expand on the potential of *L. hilgardii* in combining heterofermentative strains from the *L. buchneri* group while inoculating the small-grain cereal forage triticale to evaluate the fermentation profiles and aerobic stability of silage.

2. Materials and methods

2.1. Harvest conditions, treatments, mini-silo preparation

Triticale (X-triticosecale) was mechanically harvested at the soft dough stage of maturity from a research parcel at the WH Miner Agricultural Research Institute (Chazy, NY) on June 30th 2014, which had a maximum temperature of 28 °C with no precipitation. The forage was wilted to a mean level of 404.1 g kg^{-1} initial DM content and then chopped to a mean theoretical particle length of 1.9 cm and immediately used for mini-silo preparation. The chopped forage transported by truck to the dairy research facility onsite for packing, which was performed in a controlled setting that was thoroughly cleaned prior to starting the experiment. Delivered forage was allocated into one of the four treatments: water only (Control), *Lentilactobacillus buchneri* NCIMB 40788 at 4.0 x 10^5 CFU per g of fresh forage

(LB), *Lentilactobacillus hilgardii* CNCM-I-4785 at 4.0 x 10^5 CFU per g of fresh forage (LH), and a combination of *Lentilactobacillus buchneri* NCIMB 40788 and *Lentilactobacillus hilgardii* CNCM-I-4785 at an equal ratio of 2.0 x 10^5 CFU per g of fresh forage for each strain (Combo). The treatments were applied by alternating between spraying and mixing of the forage in large clean bins. A total of 72 mini silos were prepared for the four treatments, each with six repetitions across three different opening periods (15, 30, and 130 days of fermentation). Polyethylene buckets with 7.57 L capacity were used as mini silos, which were filled with 4.4 kg of treated forage and sealed with hermetic plastic lids. Mini silos were incubated in a dark room at a temperature set with a mean of 20.5 ℃.

2.2. Sampling

Fresh forage was sampled immediately after inoculation as follows; 200 g was collected for dry matter (DM) analysis (55 ℃ for 48 h), and a 20 g sample was collected for microbial enumeration. At each designated opening (15, 30, and 130 days of fermentation), the silage from each replicate (six per treatment) was thoroughly mixed, and sampled as described. Additionally, a third 200 g sample was collected and immediately frozen for biochemical analysis of the fermentation profiles.

2.3. Microbial enumeration

Upon sampling, the 20 g samples were immediately refrigerated before being transported back to the lab. All samples were analyzed on the day of sampling, and were maintained in the refrigerator prior to analysis. The material was then suspended in 180 mL of a NaCl-Tween 80 buffer and mixed for two one-minute cycles using a Stomacher 400 paddle blender (Seaward, UK), followed by serial dilutions using the same buffer. De Man-Rogosa-Sharpe agar (Oxoid, UK) containing 100 μg L^{-1} of cycloheximide was used to enumerate LAB [22]. Enterobacteria were enumerated on Violet Red Bile Glucose agar (Oxoid, UK), while yeasts and moulds were enumerated using Malt Extract agar (Oxoid, UK) supplemented with 2 g L^{-1} of Rose Bengal (Fisher Scientific) as well as 100 μg L^{-1} streptomycin and 50 μg L^{-1} neomycin (Sigma) [22]. All plates were incubated at 28 ℃.

2.4. Biochemical and nutritional analysis

After drying for DM determination (55 ℃ for 48 h), samples were ground through a 1 mm screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA). Samples were then submitted to Cumberland Valley Analytical Services (Waynesboro, PA) for nutritional analysis using near infrared spectroscopy with calibrations based upon the lab's internal wet chemistry results. Analysis of the fermentation parameters following 15 and 30 days of fermentation were performed in the laboratory. First, 100 g of silage was suspended in 200 mL of milli-Q water and was subject to a brief incubation period under refrigeration (4 ℃). A 45 mL aliquot of extract was centrifuged at 1000 x g for 15 minutes. The supernatant was then filtered through a 25 mm Whatman 540 filter before pH measurement (Mettler Toledo InLab Versatile Pro pH probe) and GC analysis for volatile compounds (lactate, acetate, propionate, butyrate, and ethanol) on a Varian 3800 (Palo Alto, CA, USA) equipped with an FID detector, a 2 m x 2 mm ID glass column filled with 4% Carbowax–80/130 Carbopac B-DA (Supelco, USA). Oven temperature was set at 175 ℃, and the injector was set at 200 ℃. Carrier gas was nitrogen at 15 mL min^{-1}. Hydrogen was used for the FID at 30 mL min^{-1}. For the 130-day opening, the frozen

samples were submitted to Cumberland Valley Analytical Services (Waynesboro, PA) and measured for pH, lactate, acetate, propionate, butyrate, 1,2-propanediol, and ethanol using wet chemistry.

2.5. Aerobic stability assay

Following the sampling procedures from each opening, 500 g of silage was used for a 10-d aerobic stability (AS) analysis. Each sample was placed in a clean plastic bag within a Styrofoam container (inner dimension 6 x 8 x 4 inches) and a temperature probe was placed in the geometric center of the silage (TMC6-HD, Onset, US). The probes were connected to data loggers (UX120, Onset, US) which were programmed to record the temperature every 5 minutes. The 10-d assay was performed in the temperature-controlled room. Following the 10-d assay, the raw temperature data were compiled and used for calculating the time to reach 2 ℃ above the ambient temperature. Additionally, a coefficient of deterioration (summation of the temperature over ambient for all time points over the 2 ℃ above ambient threshold) following AS assay was computed.

2.6. Statistical analysis

Results were analyzed using R version 4.0.4 [www.r-project.org]. Mini-silos for each opening was analyzed separately. All data was checked for normality using Shapiro-Wilk test and further visualized using qq-plots. Since the data was not normally distributed, a non-parametric Kruskal-Wallis test was used for all the parameters (dependent variable~inoculant). Multiple comparisons were performed using a Dunn's post-hoc test. For the microbial enumeration, a $\log_{10}$ transformation was performed and any cell counts that were below detection limit set at $\log_{10}$ 2.0 were replaced with a value equal to one half of the detection limit. Additionally, the AS results for the time to reach 2° C above the ambient temperature was used to perform a Kaplan-Meier estimator analysis in R using the 'survival' and 'survminer' packages [https://cran.r-project.org/package=survminer]. Using parameters from the 130 d opening, a principal component analysis (PCA) was performed in R using 'FactoMineR', 'factoextra', and 'corrplot' packages [23–25]. The following factors were included for the PCA: pH, lactic acid, acetic acid, propionic acid, 1,2-propanediol, ADF, NDF, crude protein, soluble sugars, and ethanol.

3. Results

The fermentation results across the three openings are listed in Table 1. After 15 d of ensiling, pH was below 4.0 for LB and Combo treated silage, which is likely a result of increased lactate concentrations ($P = 0.007$). Additionally, differences in acetate and propionate were detected at 15 d with higher concentrations in the LB and Combo silage. Following 30 d of ensiling, pH was not impacted by treatment ($P = 0.179$). However, all of the inoculated silage, including LH, produced more acetate and propionate ($P < 0.001$) which also resulted in smaller lactate to acetate ratios ($P < 0.001$). At the 130 d opening, differences in pH were detected with Control and LH-treated silage reaching lower pH levels ($P = 0.006$). All inoculated silage continued to produce more acetate ($P < 0.001$) which lowered the lactate to acetate ratios ($P = 0.003$). Additionally, 1,2-propanediol was detected at higher concentrations in inoculated silage ($P = 0.002$). Ethanol content was lowest in the LB and Combo treated silage after 15 d ($P = 0.015$) and lowest across all three inoculated treatments for the 30 and 130 d openings ($P = 0.006$ and 0.007, respectively).

Table 1. Fermentation profiles of triticale silage after 15, 30, and 130 days of ensiling.

Opening (days)	Treatment	pH	Lactic Acid (g/kg DM)	Acetic Acid (g/kg DM)	Propionic Acid (g/kg DM)	Lactate: Acetate Ratio	Ethanol (g/kg DM)	1,2-Propanediol (g/kg DM)	DM losses (g/kg DM)
15	Control	4.05 a	27.4 bc	7.9 c	0.0 b	3.48 a	9.2 a	NA	12.0
	LB	3.94 b	30.7 ab	14.1 a	1.2 a	2.27 b	5.0 b	NA	12.5
	LH	4.00 a	24.2 c	9.7 b	0.1 b	2.54 ab	8.1 a	NA	11.5
	Combo	3.92 b	42.4 a	15.1 a	3.3 a	2.82 ab	5.0 b	NA	12.0
	P-value	0.004	0.007	<0.001	0.002	0.059	0.015		0.886
30	Control	3.97	29.5	8.6 c	0.0 c	3.43 a	18.7 a	NA	15.5
	LB	3.95	24.6	18.3 a	3.1 a	1.36 c	6.7 b	NA	14.4
	LH	3.92	26.8	12.2 b	0.7 b	2.24 b	7.9 b	NA	19.3
	Combo	3.93	26.6	18.5 a	3.1 a	1.43 c	8.5 b	NA	20.7
	P-value	0.179	0.404	<0.001	<0.001	<0.001	0.006		0.718
130	Control	3.88 b	17.6	7.5 c	0.0	2.37 a	26.4 a	0.3 b	20.7
	LB	3.97 a	14.4	17.3 a	0.2	0.91 c	10.2 b	6.3 a	19.2
	LH	3.89 b	17.5	13.1 b	0.2	1.38 b	9.0 b	4.0 a	18.1
	Combo	4.01 a	14.3	15.0 a	0.1	0.96 bc	8.8 b	5.1 a	18.3
	P-value	0.006	0.266	<0.001	0.053	0.003	0.007	0.002	0.079

[1]Different letters between rows within individual sections are significantly different according to a post-hoc Dunn's test for multiple comparisons at an alpha level of 0.05. NA: Not available/was not measured at selected time point.

Microbial populations were similar across treatments at the time of ensiling (Table 2), followed by increases in LAB in the LB and Combo treated silage after 15 d (P = 0.001). Although not significant compared to the Control, LH treated silage presented nearly one log higher cell counts of LAB compared to non-inoculated silage (Table 2). For the remainder of the trial, inoculated silage had higher cell counts of LAB compared to the Control (P < 0.001). Enterobacteria populations were reduced in LB and Combo treated silage after 15 and 30 d of ensiling (P = 0.002 and 0.009 respectively), whereas LH treated silage presented numeric but not significant reductions (Table 2). The same trend was observed for yeasts for the 15 and 30 d openings in which LB and Combo application reduced total growth (Table 2). After 130 d, yeasts were below the detection limit across all inoculated silage whereas yeasts were still detected in untreated silage (P < 0.001). Finally, mould counts were reduced for LB and Combo at 15 d (P = 0.004) with non-significant reductions also being observed in LH treated silage compared to the Control.

Table 2. Total microbial cell counts of fresh triticale and ensiled material after 15, 30, and 130 days of fermentation.

Opening	Treatment	LAB (log_{10} CFU g^{-1} FM)	Enterobacteria (log_{10} CFU g^{-1} FM)	Yeasts (log_{10} CFU g^{-1} FM)	Moulds (log_{10} CFU g^{-1}FM)
0	Control	4.82	4.91	3.78	3.37
	LB	4.57	4.88	3.80	3.34
	LH	4.89	4.92	3.80	NA [2]
	Combo	5.45	4.87	3.72	NA
	P-value	0.311	0.613	0.414	0.273
15	Control	7.58 b	4.64 a	4.68 a	4.24 a
	LB	9.78 a [1]	3.04 b	3.24 b	3.12 b
	LH	8.52 b	4.46 a	4.51 a	4.19 a
	Combo	9.66 a	3.54 b	3.80 b	2.86 b
	P-value	0.001	0.002	0.001	0.004
30	Control	7.75 c	3.63 a	3.84 a	3.68
	LB	10.33 a	1.40 b	2.11 c	2.25
	LH	9.63 b	3.02 ab	3.51 ab	2.47
	Combo	10.35 a	1.69 b	2.27 bc	2.84
	P-value	<0.001	0.009	0.005	0.127
130	Control	6.36 d	<1	2.13 a	<1
	LB	8.44 a	<1	<1b	<1
	LH	7.61 c	<1	<1b	<1
	Combo	7.95 b	<1	<1b	<1
	P-value	<0.001		<0.001	

[1]Different letters between rows within individual sections are significantly different according to a post-hoc Dunn's test for multiple comparisons at an alpha level of 0.05. NA: [2]Not available/was not measured at selected time point.

In the current experiment, inoculation consistently improved the aerobic stability of triticale silage (Table 3). After 15 d, the time to cross the 2 ℃ above ambient threshold was prolonged for LB and Combo treated silage with greater increases after later openings ($P < 0.001$). Additionally, the maximum temperatures reached during the 10 d assay were lower than the Control silage after 15 d ($P = 0.002$). LB and Combo treated silage also exhibited lower deterioration coefficients compared to the Control after 15 d ($P < 0.001$). Although the time to reach the 2 ℃ above ambient threshold was not significantly prolonged in LH treated silage, the deterioration coefficient was still improved compared to the Control silage after 15 d (Table 2). For the remainder of the trial, all inoculated silage exhibited improved aerobic stability compared to the Control silage.

Table 3. Aerobic stability parameters of triticale silage after 15, 30, and 130 days of ensiling.

Opening (days)	Treatment	AS losses (%)	AS Time to reach 2 °C[2]	AS Maximum Temperature (°C)	Coefficient of AS deterioration[3]
15	Control	NA	39.16 b	41.21 a	1347.29 a
	LB	NA	157.51 a[1]	27.04 b	222.84 c
	LH	NA	51.34 b	38.60 a	1161.45 b
	Combo	NA	154.62 a	26.59 b	340.01 c
	P-value		<0.001	0.002	<0.001
30	Control	NA	54.28 c	39.63 a	1333.30 a
	LB	NA	209.33 ab	25.98 c	65.47 c
	LH	NA	150.22 b	31.30 b	342.13 b
	Combo	NA	217.57 a	24.33 c	28.75 c
	P-value		<0.001	0.002	<0.001
130	Control	35.3 a	155.92 a	26.26 a	309.12 a
	LB	5.8 c	240.00 b	20.11 b	0.00 b
	LH	8.4 bc	223.65 b	20.96 b	0.67 b
	Combo	10.2 b	240.00 b	20.13 b	0.00 b
	P-value	0.003	<0.001	0.005	0.001

[1]Different letters between rows within individual sections are significantly different according to a post-hoc Dunn's test for multiple comparisons at an alpha level of 0.05. [2]*P*-value obtained from Kaplan-Meier estimator analysis (survival probability). [3]Calculation of the area under the curve for the time (in hours) spent above the 2 °C threshold. NA: Not available / was not measured at selected time point.

Nutritional analysis using near infrared spectroscopy detected some changes in fiber content in the present study. Although there were differences between the treatments in the fresh material, they were small and did not result in differences at later timepoints (higher in Combo treated silage compared to Control and LB treated silage). Despite the lack of differences in NDF during ensiling, ADF content in LH treated silage was lower (11.8 g/kg DM difference) compared to LB treated silage after 130 d of ensiling (Table 4; $P = 0.040$). No additional reduction in fiber content was identified. As expected, inoculation significantly increased the utilization of soluble sugars. Significant reductions in soluble sugars were detected in the LB and Combo treated silage after 15 and 30 d of ensiling ($P < 0.001$). Inoculation did not have any impact on additional nutrients in the experiment (Table 4).

A principal component analysis was performed using the following ten variables: lactate, acetate, propionate, 1,2-propanediol, ethanol, pH, soluble sugars, crude protein, NDF, and ADF. The parameters were correlated to respective fermentation patterns in which the treated silage were similarly grouped (Figure 1). The first dimension explained 41.9% of the variation, while the second dimension explained 17.2%. Propionic acid, acetic acid, 1,2-propanediol were positively grouped with inoculated silage (dimension 1, Figure 1), which contrasts with the negative correlation with lactic acid and ethanol (dimension 3, Figure 1).

Table 4. Nutritional composition of fresh triticale and ensiled material after 15, 30, and 130 days of fermentation.

Opening	Treatment	DM (g/kg)	ADF (g/kg DM)	NDF (g/kg DM)	Crude Protein (g/kg DM)	Soluble Protein (g/kg DM)	Soluble Sugars (g/kg DM)	Non-Fiber Carbohydrates (g/kg DM)	Non-Structural Carbohydrates (g/kg DM)
0	Control	404.1	372.5	559.0 b	85.2	33.2	126.8	253.7	126.8
	LB	395.9	376.0	555.8 b	86.5	34.0	133.8	237.8	133.8
	LH	403.9	377.8	567.8 ab	86.3	49.7	127.5	222.7	127.5
	Combo	405.3	375.0	573.3 a	84.3	34.0	126.2	233.5	126.2
	P-value	0.523	0.618	0.017	0.913	0.626	0.627	0.056	0.627
15	Control	378.4	392.7	577.7	95.3	55.0	62.3 a	NA	NA
	LB	380.4	386.8	571.8	92.2	51.7	32.5 b	NA	NA
	LH	382.4	388.2	573.0	93.8	53.5	57.7 a	NA	NA
	Combo	382.3	388.5	578.2	90.0	50.8	34.7 b	NA	NA
	P-value	0.787	0.916	0.915	0.069	0.167	< 0.001		
30	Control	387.9	387.8	583.8	96.8	56.5	53.8 a	NA	NA
	LB	387.7	391.3	576.5	95.3	54.7	20.5 c	NA	NA
	LH	390.7	373.2	561.4	90.0	52.0	40.6 a	NA	NA
	Combo	386.7	389.8	573.2	93.2	53.0	30.0 b	NA	NA
	P-value	0.883	0.055	0.425	0.131	0.056	< 0.001		
130	Control	368.9	403.0 ab [1]	581.7	94.3	60.8	31.8	210.7	61.8
	LB	372.3	404.2 a	570.7	93.0	59.0	25.5	217.5	48.7
	LH	374.2	392.3 b	565.0	93.7	60.2	21.7	228.8	65.5
	Combo	373.6	396.0 ab	569.3	93.3	62.0	21.0	222.8	46.5
	P-value	0.717	0.040	0.125	0.785	0.142	0.068	0.599	0.227

[1]Different letters between rows within individual sections are significantly different according to a post-hoc Dunn's test for multiple comparisons at an alpha level of 0.05. [2]P adjusted values were not significant between treatments. NA: Not available / was not measured at selected time point.

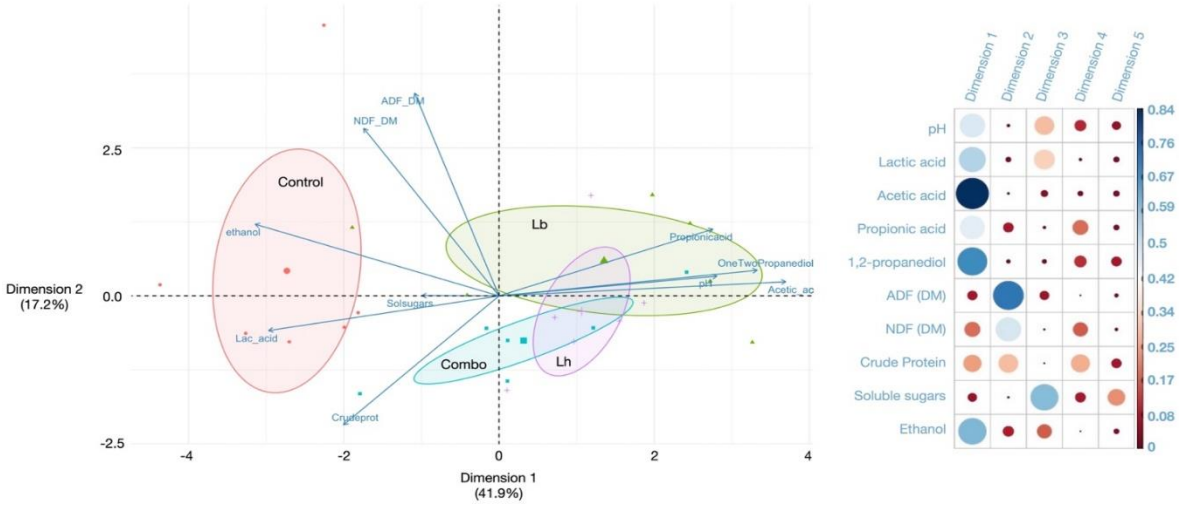


Figure 1. Principal component analysis and correlation plot of selected fermentation and nutritional parameters of ensiled triticale after 130 d.

4. Discussion

The use of microbial inoculants, including those containing heterofermentative LAB strains, has become a well-accepted practice in forage management systems. Inoculant usage is not only useful in reducing nutrient losses during ensiling, but the addition of effective heterofermentative strains is also advantageous in improving aerobic stability at feed out [4]. The heterofermentative activity has been thoroughly investigated in different ensiling studies across different forage types [5,13,26]. *L. buchneri* is a well-studied heterofermentative LAB specie, and its' confirmed metabolic activities have been documented to improve silage stability by way of increased aerobic stability and decreased DM losses [4,6,9]. The inclusion of the heterofermentative specie *L. hilgardii* in silage has also been advantageous in improving aerobic stability [12] and the efficacy of combining *L. hilgardii* with *L. buchneri* has been validated in corn, grass, and high-moisture corn silage as well [10,13,15]. However, this combination has yet to be evaluated in small grain silages to the best of our knowledge. The justification of combining *Lb* and *Lh* is based upon their metabolic differences, with an earlier onset of heterofermentative activity by *Lh* [10] even if the understanding of the mode of action underlying the synergic and additive effects reported elsewhere still requires further investigation. The combination of the two strains not only increase aerobic stability through the production of organic acids that have greater antifungal activity, but also help with an earlier onset of heterofermentation by *Lh* [4,10,13]. In the current experiment, the quality of ensiled triticale that was treated with three different microbial inoculants was evaluated by examining the fermentation parameters, nutritional analysis, and aerobic stability aspects after 15, 30, and 130 days of ensiling. By inoculating the triticale with either LB, LH, or Combo, there were apparent differences in the fermentation profiles for each of the three openings compared to no-inoculation treatment. Additionally, aerobic stability was improved with the inclusion of these heterofermentative strains. Such results could confer to changes in silage quality.

The fermentation profiles were in agreement with the heterofermentative patterns expected in inoculated silage. Indeed, acetic acid content was significantly higher in treated silage across all three openings compared to the Control. These results are indicative of the heterofermentative LAB activity, which is also supported by the lactate to acetate ratios, significantly lower in inoculated silage after 30 and 130 d opening. Considering the impact of a strong acid like lactate (pKa = 3.86) on pH, the increase in lactate helps explain the differences in pH between treatments at 15 d [27], in which LB- and combo-inoculated silage harbored lower pH than the other two treatments. Furthermore, some studies have reported more lactate production from *Lh* compared to *Lb* which could explain the differences in pH [28]. There were no changes in pH at the 30 d opening, which corroborates the lack of changes in lactate content between treatments.

The accumulation of ethanol can partially explain DM losses during fermentation, which has been observed in ensiled sugar cane [29]. LAB can also produce ethanol following the degradation of pyruvate using the formate lyase pathway [30]. Additionally other microbial species can produce ethanol under anaerobic ensiling conditions including yeasts, enterobacteria, and sometimes clostridia [30]. In the current experiment, ethanol content was consistently higher in the Control silage across all three openings, although concentrations for LH-inoculated silage were similar to the Control after 15 d. Despite a lack of differences in DM losses, the treated silage followed a biological trend toward lower DM losses after 130 d compared to untreated silage (P = 0.079). The lid system used on the mini silos may have prevented losses of volatile compounds, which would minimize differences in recording

losses between treatments. Furthermore, acetic acid content can also partially explain DM losses during ensiling. Considering DM losses were largely unaffected in this experiment, losses as a result from higher acetate concentrations in treated silage does not seem likely in this experiment. Furthermore, DM losses as a result from acetate volatilization in treated silage are lower compared to the DM losses from untreated silage during aerobic deterioration. Despite the higher counts of enterobacteria and yeasts observed in the untreated silage after ensiling, definitive conclusions cannot be made on which microorganisms were responsible for the higher ethanol concentrations in the study.

As expected, LAB inoculation in the experiment resulted in an increase of LAB counts (CFU/g) whereas lower in counts of enterobacteria, yeast and moulds were observed in treated silage. Higher LAB counts have been observed in other studies that have utilized similar inoculation rates, and a recent meta-analysis confirmed a trend towards increased LAB counts when corn silage was inoculated [31]. However, the same authors did not observe this trend in inoculated alfalfa silage [32]. Additionally, the LAB counts for untreated triticale silage was similar to a study by Dunière et al. [20]. In our study, there were higher counts of enterobacteria in the Control silage compared to LB and the Combo after 15 and 30 d of fermentation. Elevated counts of enterobacteria in silage are problematic due to their negative impact on dry matter losses following their fermentation capabilities, as well as the pathogenic potential from some species. They are facultative anaerobes that can compete with LAB during the early stages of ensiling [33]. Since they generally do not tolerate acidic environments (>4.5), a decline of the enterobacteria counts can be indicative of proper silage fermentation and good forage management practices [33,34].

Yeast counts were comparable to enterobacteria counts which were lowest in LB and Combo treated silage across all openings by nearly one log. The decrease in yeast counts in *L. buchneri* treated silage has been consistently reported [9]. Similar reductions in yeast counts have also been published for *L. hilgardii* in whole-plant corn and high moisture corn silage [13,35] as well as sugarcane silage [28]. In another trial testing triticale forage, Duniere and others reported yeast counts around 6 $\log_{10}$ CFU/g DM in untreated silage after 90 days of fermentation [20]. Considering the DM content and pH reported in that experiment are similar to our results, the reduction of yeasts in this study as a result from inoculation are notable from the standpoint of improving the microbial composition. The same decreasing trend was observed for moulds in which LB and Combo treated silage had lower counts after 15 and 30 d of ensiling. All these results are in agreement with the fermentation profiles of inoculated silage, in which higher concentrations of acetic and propionic acids should result in lower counts of enterobacteria, yeasts, and moulds, as those two products may exhibit antimicrobial properties [4,36].

Aerobic stability was significantly improved when using silage inoculants in the experiment. These improvements in AS were further pronounced on the temperature profiles, which corresponds to the difference of the ambient and the mean of the silage temperatures for individual treatments (Supplementary Figure 1). Between the four treatments, the Combo maintained a lower temperature for the first five days compared to LB. LH treated silage exhibited improved aerobic stability compared to Control after 30 d of fermentation, which is consistent with the fermentation and microbial results. Although the AS results for LH after 30 d indicated lower stability parameters compared to LB and Combo treated silage, the fluctuations in temperature were far less pronounced and remained below the 2 ℃ for a prolonged period compared to the Control silage (150.2 h vs 54.3 h; Supplementary Figure 1). These results align with other studies reporting improvements in aerobic stability when silage were treated with LB and LH. One example comes from a recent study reporting improvements

in aerobic stability after 30 days of ensiling as a result of treating sorghum silage with the same strains either separately or in combination [26].

After 130 d of ensiling, all of the inoculated silage had markedly improved AS compared to Control silage, which likely resulted in lower fresh matter losses during the assay (35.3 g/kg losses in Control vs. a mean fresh matter losses of 8.13 g/kg for the inoculated treatments). The same trends were observed in the maximum temperatures reached during AS following inoculation in which the LB and Combo significantly reduced the values across all openings, while LH had lower maximum temperature after 30 d of ensiling only. This implies further control of the silage even when stability was comprised, in which the extent of heating was significantly reduced.

In the study, a coefficient of AS deterioration was calculated as an additional parameter to interpret the aerobic stability results. This was computed by taking the sum of the temperature over ambient (silage temperature minus ambient temperature) for all recordings above the 2 ℃ threshold. This coefficient extends the potential of area under the temperature profile curve with the recorded temperature, and corresponds to the area between the temperature profile and the 2 ℃ above ambient threshold. Doing so provides comparison potential between treatments having similar time to reach AS level but with differing responses during instability. As expected, Control silage had a much higher coefficient of AS deterioration across all three openings. Silage treated with LB and Combo had significantly lower AS deterioration coefficients. Similar to other variables measured, LH had a lower coefficient of AS deterioration after 30 and 130 d of ensiling compared to Control silage. Additionally, significant improvement was observed after 15 d for LH compared to Control. Despite being higher compared to LB and the Combo, this result differs from what was observed in other parameters including time to reach 2℃ after 15 d. Hence, the coefficient of AS deterioration provides a detailed perspective for the duration of the 10-d assay.

While the production of mixed organic acids from heterofermentation is one of the most probable explanations that substantiate these improvements in aerobic stability, the exact mechanism is still not fully understood. The production of acetic acid as well as 1,2-propanediol, which can be converted into propionic acid, are end-products of lactate degradation that provide effective antifungal effects as strong acids [4,6]. There was an increase in 1,2-propanediol content after 130 d of ensiling across the inoculated silage, but that compound was not measured at the earlier openings (mean of 5.1 g/kg DM in the inoculated silage vs. 0.3 g/kg in Control). The concentration of propionic acid was decreased across all treatments after 130 d of ensiling, but was higher in inoculated silage after 30 d. Therefore, the connection between 1,2-propanediol and propionic acid is not clear in the study. Despite this, the differences observed in other fermentation parameters could sufficiently explain the stability of the inoculated silage across the openings. This is especially true considering the elevated acetic acid concentrations. This explanation is further supported by the decreased counts of enterobacteria, yeasts, and moulds in the inoculated silage compared to the Control.

The heterofermentative metabolism of *Lb* has been well documented, although, one of the limitations of this specie in silage inoculants is the prolonged time needed for heterofermentation to initiate [4]. Therefore, examining other strains like *Lh* that can initiate heterofermentation earlier is an advantage that can benefit producers when time is a constraint. In a study by Reis and others evaluating *Lh* in corn silage, increases in acetate and 1,2-propanediol were observed in *Lh* treated silage as early as 19 days compared to untreated silage [12]. Although aerobic stability was not evaluated at the 19 day opening in that study, the fermentation results were similar to other *Lh* treated silage in different studies that exhibited improved aerobic stability. Furthermore, Reis et al., measured aerobic stability

in their study after 103 days and reported improvements when corn was treated with *Lh* [12]. In some instances, *Lh* has been shown to produce higher concentrations of lactate. Since the accumulation of lactate will ultimately induce stress responses in LAB, one could postulate a faster accumulation of lactate by *Lh* activity could possibly initiate lactate degradation sooner. Although not significant, numeric increases in lactate production by *Lh* alone after 10 days was reported in a study using high moisture corn [13]. Additionally, *Lh* treated silage after 10 days had reduced yeast counts and increased aerobic stability. However, the small increases in lactate in that study were only numerically higher when compared to untreated and *Lb* treated silage [13]. Therefore, the possibility of initiating lactate degradation activity by way of increased lactate concentrations does not seem to fully explain the responses observed in silage treated with *Lh*.

Another possible explanation is the production of secondary metabolites with antifungal properties. The production of phenyllactic acid and 4-hydroxy-phenyllactic acid by *Lh* has been documented, and both compounds have known antifungal properties [37]. Unfortunately, the production of both compounds were not measured in this study. Additionally, some LAB species have been reported to produce antimicrobial proteins (bacteriocins), which in theory could potentially control or inhibit the onset of microbial species responsible for aerobic deterioration [38].Therefore, an effect of *Lh*, in combination with *Lb* on the microbial diversity of LAB in silage could be another explanatory avenue to explore [14]. Through the increased use of culture-independent approaches to characterize the bacterial and fungal communities during ensiling, studies are reporting shifts in diversity as a response to inoculation [13,15,16]. While there is a clear effect of improving aerobic stability by reducing lactate-assimilating microorganisms including yeasts when inoculating silage with *Lh*, further research is needed to fully explain the underlying mechanism. Differential changes in fermentation aspects is likely part of that explanation, but other secondary metabolites cannot be ruled out in explaining such improvements.

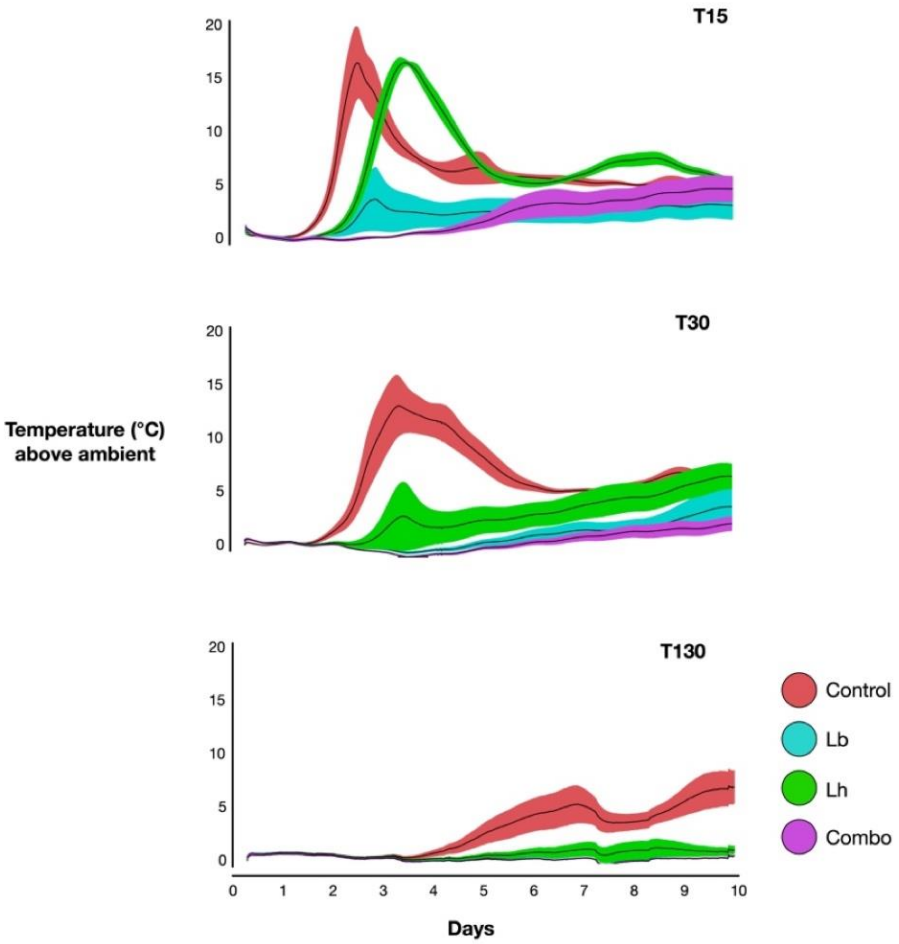
Soluble sugars were rapidly depleted during ensiling as expected, and even more so in LB and Combo treated silage after 15 and 30 d of ensiling. This has been consistently reported across different meta-analyses [9,31], and can be best explained by the rapid conversion of available sugars into organic acids by effective LAB strains. The fiber content expressed as neutral detergent fiber (NDF) was not affected by treatment following ensiling, although a small reduction in acid detergent fiber (ADF) was observed in LH treated silage after 130 d. While these differences are minimal and could be explained by differences in DM content, decreases in fiber content have been reported in inoculated silage including triticale[18]. Although minimal, this could suggest differences in metabolic capabilities between *Lentilactobacilli* species. Similarly, decreases in ADF were reported when corn silage were inoculated with LAB [31], and reductions in both ADF and NDF were also reported in inoculated grass silage [32]. Further research and application of expansive methods for evaluating changes in fiber degradation are necessary to obtain a better understanding of any fibrolytic activities mediated by LAB.

The principal component analysis was used to identify trends in the fermentation parameters and nutrition aspects across treated silage from the 130 d samples. The correlations between fermentation end products further confirm the heterofermentation activity specific to inoculants of the *L. buchneri* group carried out by the selected strains in the individual treatments (LB, LH) as well as the Combo. As expected, soluble sugars were also inversely correlated to the inoculated silage implying their rapid utilization for fermentation [39]. Ethanol was also inversely correlated to the inoculated silage, which provides some support towards the notion of these strains preferring metabolic pathways that produce

more acetate and less ethanol during the degradation of lactate (Oude Elferink et al., 2001). In contrast, the control silage was correlated with ethanol and lactate. These results not only suggest homofermentative type activity, but it could also be a result of activity from enterobacteria and yeasts, or a combination of both [33].

5. Conclusions

Inoculation of triticale silage improved fermentation aspects, including rapid acidification and increased concentrations of organic acids, which further translated to decreased counts of enterobacteria, yeasts, and moulds. Subsequent improvements in aerobic stability were also observed when triticale silage was inoculated with heterofermentative LAB strains individually or combined. Although further investigation is necessary to understand the exact mode of action when combing strains, this experiment validates the use of *L. buchneri* and *L. hilgardii* combination as an inoculant for treating triticale, which would allow for improved silage quality for producers.



Supplementary Figure 1. Temperature profiles (expressed in °C above ambient temperature) of the triticale silage during the 10 day aerobic stability assay for the 15, 30, and 130 day openings. Control: red, Lb: blue, Lh: green, Combo: purple.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

Both Richard Anthony Scuderi and Emmanuelle Apper are employed by Lallemand Animal Nutrition (part of Lallemand Specialties Inc in the USA, and Lallemand SAS in France respectively). However, their affiliation did not impede their ability to follow journal guidelines or remain impartial during the preparation of this manuscript.

References

1. Salmon DF, Mergoum M, Gomez-Macpherson H (2004) Triticale production and management. *Triticale improvement and production,* Rome, Food and Agriculture Organization of the United Nations, 27–32.
2. McGoverin CM, Snyders F, Muller N, et al. (2011) A review of triticale uses and the effect of growth environment on grain quality. *J Sci Food Agric* 91: 1155–1165. https://doi.org/10.1002/jsfa.4338
3. Ayalew H, Kumssa TT, Butler TJ, et al. (2018) Triticale improvement for forage and cover crop uses in the Southern Great Plains of the United States. *Front Plant Sci* 9: 1130. https://doi.org/10.3389/fpls.2018.01130
4. Muck RE, Nadeau EMG, McAllister TA, et al. (2018) Silage review: Recent advances and future uses of silage additives. *J Dairy Sci* 101: 3980–4000. https://doi.org/10.3168/jds.2017-13839
5. Driehuis F, Elferink SJWHO, Spoelstra SF (1999) Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. *J Appl Microbiol* 87: 583–594. https://doi.org/10.1046/j.1365-2672.1999.00856.x
6. Oude Elferink SJWH, Krooneman J, Gottschal JC, et al. (2001) Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. *Appl Environ Microbiol* 67: 125–132. https://doi.org/10.1128/AEM.67.1.125-132.2001
7. Zheng J, Wittouck S, Salvetti E, et al. (2020) A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. *Int J Syst Evol Microbiol* 70: 2782–2858. https://doi.org/10.1099/ijsem.0.004107

8. Oliveira AS, Weinberg ZG, Ogunade IM, et al. (2017) Meta-analysis of effects of inoculation with homofermentative and facultative heterofermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. *J Dairy Sci* 100: 4587–4603. https://doi.org/10.3168/jds.2016-11815
9. Kleinschmit DH, Kung L (2006) A meta-analysis of the effects of *Lactobacillus buchneri* on the fermentation and aerobic stability of corn and grass and small-grain silages. *J Dairy Sci* 89: 4005–4013. https://doi.org/10.3168/jds.S0022-0302(06)72444-4
10. Ferrero F, Piano S, Tabacco E, et al. (2019) Effects of conservation period and *Lactobacillus hilgardii* inoculum on the fermentation profile and aerobic stability of whole corn and sorghum silages: Effects of *Lactobacillus hilgardii* on corn silage. *J Sci Food Agric* 99: 2530–2540. https://doi.org/10.1002/jsfa.9463
11. Carvalho BF, Ávila CLS, Miguel MGCP, et al. (2015) Aerobic stability of sugar-cane silage inoculated with tropical strains of lactic acid bacteria. *Grass Forage Sci* 70: 308–323. https://doi.org/10.1111/gfs.12117
12. Reis CB, de Oliveira dos Santos A, Carvalho BF, et al. (2018) Wild *Lactobacillus hilgardii* (CCMA 0170) strain modifies the fermentation profile and aerobic stability of corn silage. *J Appl Anim Res* 46: 632–638. https://doi.org/10.1080/09712119.2017.1371609
13. Silva EB, Smith ML, Savage RM, et al. (2020) Effects of *Lactobacillus hilgardii* 4785 and *Lactobacillus buchneri* 40788 on the bacterial community, fermentation and aerobic stability of high-moisture corn silage. *J Appl Microbiol* 130: 1481–1493. https://doi.org/10.1111/jam.14892
14. Drouin P, Tremblay J, Chaucheyras-Durand F (2019) Dynamic succession of microbiota during ensiling of whole plant corn following inoculation with *Lactobacillus buchneri* and *Lactobacillus hilgardii* Alone or in Combination. *Microorganisms* 7: 595. https://doi.org/10.3390/microorganisms7120595
15. Drouin P, Tremblay J, Renaud J, et al. (2020) Microbiota succession during aerobic stability of maize silage inoculated with *Lentilactobacillus buchneri* NCIMB 40788 and *Lentilactobacillus hilgardii* CNCM-I-4785. *Microbiol Open* 10: e1153. https://doi.org/10.1002/mbo3.1153
16. Drouin P, Tremblay J, da Silva ÉB, et al. (2022) Changes to the microbiome of alfalfa during the growing season and after ensiling with *Lentilactobacillus buchneri* and *Lentilactobacillus hilgardii* inoculant. *J Appl Microbiol* 133: 2331–2347. https://doi.org/10.1111/jam.15641
17. Bergen WG, Byrem TM, Grant AL (1991) Ensiling characteristics of whole-crop small grains harvested at milk and dough stages. *J Anim Sci* 69: 1766. https://doi.org/10.2527/1991.6941766x
18. Ozduven ML, Kursun Onal Z, Koc F (2009) The effects of bacterial inoculants and/or enzymes on the fermentation, aerobic stability and in vitro dry and organic matter digestibility characteristics of triticale silages. *Kafkas Univ Vet Fak Derg* 16: 751–756. https://doi.org/10.9775/kvfd.2009.1628
19. Keles G, Demirci U (2011) The effect of homofermentative and heterofermentative lactic acid bacteria on conservation characteristics of baled triticale–Hungarian vetch silage and lamb performance. *Anim Feed Sci Technol* 164: 21–28. https://doi.org/10.1016/j.anifeedsci.2010.11.017
20. Duniere L, Xu S, Long J, et al. (2017) Bacterial and fungal core microbiomes associated with small grain silages during ensiling and aerobic spoilage. *BMC Microbiol* 17: 50. https://doi.org/10.1186/s12866-017-0947-0

21. Soundharrajan I, Kim D, Kuppusamy P, et al. (2019) Probiotic and *Triticale* silage fermentation potential of *Pediococcus pentosaceus* and *Lactobacillus brevis* and their impacts on pathogenic bacteria. *Microorganisms* 7: 318. https://doi.org/10.3390/microorganisms7090318
22. Drouin P, Ferrero F (2020) Testing selectivity of bacterial and fungal culture media compared to original silage samples using next generation sequencing. *J Microbiol Methods* 179: 106088. https://doi.org/10.1016/j.mimet.2020.106088
23. Kassambara A, Fabian M (2020) Factoextra: Extract and visualize the results of Mulitvariate Data Analyses. Available from: http://www.sthda.com/english/rpkgs/factoextra.
24. Lê S, Josse J, Husson F (2008) FactoMineR: An R package for multivariate analysis. *J Stat Soft* 25: 1–18. https://doi.org/10.18637/jss.v025.i01
25. Wei T S (2021) R Package 'corrplot': Visualization of a correlation matrix. Available from: https://github.com/taiyun/corrplot.
26. Arriola KG, Vyas D, Kim D, et al. (2021) Effect of *Lactobacillus hilgardii*, *Lactobacillus buchneri*, or their combination on the fermentation and nutritive value of sorghum silage and corn silage. *J Dairy Sci* 104: 9664–9675. https://doi.org/10.3168/jds.2020-19512
27. Kung L, Shaver RD, Grant RJ, et al. (2018) Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *J Dairy Sci* 101: 4020–4033. https://doi.org/10.3168/jds.2017-13909
28. Santos WP, Ávila CLS, Pereira MN, et al. (2017) Effect of the inoculation of sugarcane silage with and on feeding behavior and milk yield of dairy cows. *J Anim Sci* 95: 4613–4622. https://doi.org/10.2527/jas.2017.1526
29. Pedroso A de F, Nussio LG, Loures DRS, et al. (2008) Fermentation, losses, and aerobic stability of sugarcane silages treated with chemical or bacterial additives. *Sci Agric (Piracicaba, Braz)* 65: 589–594. https://doi.org/10.1590/S0103-90162008000600004
30. Rooke JA, Hatfield RD (2003) Biochemistry of ensiling. In: Buxton DR, Muck RE, Harrison JH (Eds.), *Agronomy Monographs,* Madison, WI, USA, American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, 95–139.
31. Blajman JE, Páez RB, Vinderola CG, et al. (2018) A meta-analysis on the effectiveness of homofermentative and heterofermentative lactic acid bacteria for corn silage. *J Appl Microbiol* 125: 1655–1669. https://doi.org/10.1111/jam.14084
32. Blajman JE, Vinderola G, Páez RB, et al. (2020) The role of homofermentative and heterofermentative lactic acid bacteria for alfalfa silage: A meta-analysis. *J Agric Sci* 158: 107–118. https://doi.org/10.1017/S0021859620000386
33. Pahlow G, Muck RE, Driehuis F, et al. (2003) Microbiology of ensiling. In: Buxton DR, Muck RE, Harrison JH (Eds.), *Agronomy Monographs,* Madison, WI, USA, American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, 31–93.
34. Driehuis F, Wilkinson JM, Jiang Y, et al. (2018) Silage review: Animal and human health risks from silage. *J Dairy Sci* 101: 4093–4110. https://doi.org/10.3168/jds.2017-13836
35. Ferrero F, Tabacco E, Borreani G (2021) *Lentilactobacillus hilgardii* inoculum, dry matter contents at harvest and length of conservation affect fermentation characteristics and aerobic stability of corn silage. *Front Microbiol* 12: 675563. https://doi.org/10.3389/fmicb.2021.675563
36. Ávila CLS, Carvalho BF, Pinto JC, et al. (2014) The use of *Lactobacillus* species as starter cultures for enhancing the quality of sugar cane silage. *J Dairy Sci* 97: 940–951. https://doi.org/10.3168/jds.2013-6987

37. Valerio F, Lavermicocca P, Pascale M, et al. (2004) Production of phenyllactic acid by lactic acid bacteria: An approach to the selection of strains contributing to food quality and preservation. *FEMS Microbiol Lett* 233: 289–295. https://doi.org/10.1111/j.1574-6968.2004.tb09494.x
38. Pessione E (2012) Lactic acid bacteria contribution to gut microbiota complexity: Lights and shadows. *Front Cell Inf Microbio* 2: 00086. https://doi.org/10.3389/fcimb.2012.00086
39. Junges D, Schmidt P, Novinski CO, et al. (2013) Additive containing homo and heterolactic bacteria on the fermentation quality of maize silage. *Acta Sci Anim Sci* 35: 371–377. https://doi.org/10.4025/actascianimsci.v35i4.18833

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