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Corn Stover Silage Inoculated with Ferulic Acid Esterase Producing *L. johnsonii*, *L. plantarum*, *L. fermentum*, and *L. brevis* Strains: Fermentative and Nutritional Parameters

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Abstract: Corn stover (CS) is an abundant lignocellulosic by-product of the grain industry. Ferulic acid esterase producing (FAE+)-lactobacilli can potentially improve ensiled forages' nutritive value through the hydrolysis of ferulic acid ester bonds present in cell walls during the fermentation process, but this has not been addressed in CS silage. In this study, we characterized 8 FAE+ lactobacilli regarding their FAE activity and inoculant aptitude: *Lactobacillus (L.) johnsonii* (CRL2237, CRL2238, CRL2240), *L. plantarum* (ETC182, CRL046, CRL2241), *L. fermentum* CRL1446 and *L. brevis* CRL2239. Next, 25% dry matter (DM) CS mini silos were prepared and either not inoculated (UN) or inoculated with each strain (10^5 CFU g fresh matter^{-1}). Compared to UN, DM loss was significantly reduced in CRL046 and CRL2239, and organic matter increased in CRL2241-inoculated silages. Although the rest of the digestibility measures were not improved, in situ acid detergent fiber degradability (ADFD) was increased by the CRL2238 strain when compared to UN. Results in inoculated silages were not correlated with FAE activity quantification or growth/acidification studies in a CS-derived culture broth. This study demonstrates the potential of several FAE+ lactobacilli strains as CS inoculants and encourages further research.

Keywords: silage inoculants; feruloyl esterases; lactic acid bacteria; ruminant nutrition

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1. Introduction

Corn stover (CS) is an abundant lignocellulosic biomass available after cob harvest for human or animal consumption. Its composition depends on many factors—including cultivar, maturity state, and type of harvest—but consists mainly of structural carbohydrates and lignin. The utilization of this by-product differs according to location and technology available, from biofuel production to direct grazing by animals, but it is usually an agricultural waste disposed of by burning [1].

Ruminants' nutrition using high-fiber crop residues constitutes a cost-effective opportunity for some livestock farmers. Although similar when considering modern intensive farms, goat production differs from cattle in most cases, especially in developing countries using extensive or semi-intensive systems [2]. In these smallholdings, which are mostly mixed crop-livestock producers, forage scarcity is frequent during the dry season, inducing an important productive drop and endangering food security [3]. Furthermore, these situations encourage native forests' overgrazing and jeopardize the sustainability of these farms and the surrounding ecosystem [4]. The use of abundant agricultural residues such as CS constitutes a feasible strategy to face this issue but requires further research, especially for its preservation [5–7].

Ensilage is a fermentation method broadly used to preserve feedstock. Bacterial inoculants have been recommended to improve this process, whether to achieve rapid acid production (first-generation inoculants), prolong aerobic stability (second-generation inoculants) or increase the digestibility of the end product (third-generation inoculants) [8]. For CS silage, it is important to enhance the acidification process, as reducing sugars are scarce, and to induce broader digestion of structural carbohydrates, as they constitute the major component [9]. Therefore, it is a frequent practice to apply chemical inoculants when CS is preserved through fermentation.

Ferulic acid esterase (FAE) producing (FAE+) lactobacilli are considered third-generation inoculants for the potential increase in fiber digestibility that they could produce by disrupting cell walls' ester linkages between ferulates and hemicellulose, acting synergistically with xylanases and cellulases [10]. This has been studied in alfalfa [11–13], sorghum [14], barley [15,16], pennisetum [17], and whole plant corn [18–20] silages, using mainly *L. buchneri*, *L. fermentum*, *L. brevis*, and *L. plantarum* strains, obtaining different results. Strain, crop, and assay-dependent effects were detected, and conclusive definitions regarding the extent and profitability of the effects are still lacking [21,22]. Although achieving such conclusions is a difficult objective in silage science as a consequence of multiple variables affecting the outcomes [23], FAE+ inoculants are a relatively new field of research for which information is still scarce. These inoculants can potentially improve ensiled forages' nutritive value through the hydrolysis of ferulic acid ester bonds present in cell walls during the fermentation process, but this has not been addressed in CS silage.

Lactic acid bacteria (LAB) selection to develop new silage inoculants has been addressed by screening methods to reduce the number of strains and therefore enable mini silos studies, which are time and labor consuming [24]. Little is known regarding the efficacy of these proposed assays to predict the outcome in mini silos studies.

Therefore, our objectives in this work were: (i) to quantify and characterize the FAE activity and the potential inoculant aptitude in a CS extract of 8 FAE+ lactobacilli strains; (ii) to evaluate the fermentative and nutritional changes induced by the inoculation of these strains in CS silage; and (iii) to evaluate the possible correlation of results obtained.

2. Materials and Methods

2.1. Lactic Acid Bacteria Strains Used

LAB strains used in this study were obtained from the ETC (Laboratorio de Ecofisiología Tecnológica, CERELA-CONICET) and CRL collection (CERELA-CONICET, Tucumán, Argentina). These were previously identified to the species level by an ARNr 16S sequence analysis and were cultivated from frozen stocks in de Mann, Rogose, and Sharpe broth (MRS, Oxoid™, Basingstoke, Hampshire, UK) at 30 °C for 16 h, three times before every trial was performed. All strains were previously selected for their FAE activity by the screening in agar plate method, according to Donaghy et al. [25]. *L. johnsonii* CRL2240, CRL2237, and CRL2238 were previously coded as ETC150, ETC175, and ETC187 [20], respectively.

2.2. FAE Activity Quantification

FAE activity was quantified through a spectrophotometric method [20,26] using bacterial cell suspensions (Css) in 0.1 M phosphate buffer as an enzymatic solution and 100 µM methyl ferulate (Sigma-Aldrich, St. Louis, MO, USA) as substrate. Reactions were performed at 37 °C—pH 7.0, and the effect of the lower temperature (18 °C) and decreasing pH (6.0, 5.0, or 4.0) on FAE activity was also studied as previously described [20]. Results are expressed as Units of specific FAE activity, which is defined as the amount of Css that hydrolyzes 1 nmol of methyl ferulate per minute per g of cells in a dry weight basis (U g^{-1}).

2.3. Growth in CS Soluble Fraction Medium (CSM)

To evaluate the silage inoculant potential of each strain, a broth medium was prepared using only corn stover (*Zea mays* L., hybrid Bt) aqueous extract (CS Soluble Fraction

Medium, CSM), as previously reported [20]. Inoculated CSM (using Css, 10^4 CFU mL^{-1}) was incubated at 30 °C, and measures of pH were taken at 12, 24, and 48 h, using independent units for each time. Additionally, CFU counts of appropriate dilutions on MRS agar and a measure of remaining reducing sugars by the Somogyi–Nelson method [27] were taken at 48 h.

Assays 2.2 and 2.3 will be collectively called in vitro characterization studies (IvCS).

2.4. Inoculation of CS Mini Silos

2.4.1. Silage Preparation

The Css of selected strains were evaluated as single silage inoculants when compared to uninoculated silages (UN). Corn (*Zea mays* L.) hybrid NS 7818 (Nidera Seeds, CABA, Argentina) was manually harvested 80 days post-sowing to obtain cobs for human consumption (27°24′17.5″ S, 65°42′55.2″ W La Tipa, Aguilares, Tucumán, ARG, Argentina). After this, 50 plants (harvested at 20 cm from the ground) were chopped using portable equipment (PM-ECO, Metalúrgica Iraloff, Presidencia de la Plaza, Chaco, Argentina) to an average particle size of 5 cm. The obtained CS was separated into three batches of 1 kg per experimental group, and a subsample was preserved for analysis as described in 2.4.2. Batches were sprayed with corresponding Css to obtain a 10^5 CFU g FM^{-1} inoculation rate, or with the same amount of sterile buffer (UN, 5 mL kg FM^{-1}), according to their experimental group (n = 3). From each batch, two mini silos were prepared in vacuum-sealed (15″ vacuum-time, Turbovac MiniJumbo®, Cerveny SAS, Alto Alberdi, Córdoba, Argentina) high-barrier plastic bags (PAB18PtB, Vitopel Argentina SA, Villa del Totoral, Córdoba, Argentina). Silages were incubated in the dark at 24 °C for 300 days. Density was measured by water displacement [28] at the beginning and at the end of the incubation time.

2.4.2. Fermentative, Microbiological, and Nutritional Analysis of Inoculated Silages

Both mini silos from each batch were opened and composited for analysis. Measures of pH, ammonia-N (NH_3-N), organic acids, total phenolic compounds (TPCs), and microbial counts were performed as previously described [20]. Briefly, a 1:10 aqueous extract was used for pH determination and submitted to HPLC analysis to quantify lactic, acetic, propionic, and butiric acids and ethanol. The Folin–Ciocalteu method was employed to measure TPCs. LAB, total mesophilic bacteria (TMB), yeast, and mold counts were performed using a culture of a sterile saline extract (1:10 and serial dilutions) on appropriate selective media (MRS, Plate Count Agar, and Saboreaud Dextrose, respectively).

Nutritional analyses were performed in a reference laboratory of the Instituto Nacional de Tecnología Agropecuaria, according to standardized analytical procedures [29]. Dry matter content (DM at 65 °C, not corrected for volatile compounds loss), organic matter (OM, ash AOAC 942.05), crude protein (CP, 6.25 × Total Nitrogen obtained by Kjeldahl method), neutral detergent fiber (aNDF, measured using a heat stable amylase and expressed inclusive of residual ash using an ANKOM Fiber Analyzer 220®), acid detergent fiber (ADF, using ANKOM Fiber Analyzer 220®), and acid detergent lignin (ADL, using sulfuric acid and ANKOM Fiber Analyzer 220®) were measured. Digestibility measures in vitro were quantified at 48 h of incubation in a Daisy II® incubator (ANKOM Technology, Macedon, NY, USA) [30], using rumen liquor from two donor steers fed a diet of corn grain and lucerne hay. In situ digestibility determinations were performed in two runs at 10 days of incubation in the same donors using Filter Bags F57 (ANKOM Technology, Macedon, NY, USA). Digestion coefficients were calculated as the difference in weight of compositional fraction (aNDF or ADF) before and after ruminal incubation (digestible fractions), divided by the weight of the compositional fraction before ruminal incubation (digestibility coefficient). Based on these studies, DM true digestibility (IVDMD), neutral detergent fiber (NDF) digestibility (NDFD, %aNDF), and digestible NDF (dNDF, %DM) were calculated. Acid detergent fiber (ADF) digestibility (ADFD, %ADF) and digestible

ADF (dADF, %DM) were also measured by in situ studies [31]. Hemicellulose (aNDF-ADF) and cellulose (ADF-ADL) were calculated from the compositional analysis.

2.5. Statistical Analysis

In vitro characterization studies (IvCS) were performed in duplicate in three independent assays (n = 3) and analyzed through an ANOVA procedure using Infostat/L® 2019 for Windows (Universidad Nacional de Córdoba, Córdoba, Argentina). Means were compared by Tukey's test. Graphics were designed using GraphPad Prism® version 9.0 for Windows (GraphPad Software, San Diego, CA, USA). Data obtained in silage analysis (n = 3) was also analyzed through ANOVA followed by Tukey's test ($p < 0.05$). GraphPad Prism® was also used to perform a Principal Component Analysis (PCA) using standardized method, to analyze: (a) data obtained in IvCS assays, (b) variables measured in UN and inoculated silages, and (c) variables measured in IvCS and inoculated silages.

3. Results

3.1. Ferulic Acid Esterase Activity Quantification

Quantification of FAE activity at 37 °C—pH 7.0 is presented in Table 1. *L. johnsonii* CRL2238 showed the highest activity. *L. plantarum* and *L. brevis* strains presented a relatively low enzymatic activity in this trial, showing no detectable differences at varying conditions (Figure 1). For the rest of the strains, a lower temperature had a significant influence on the measured activity, but pH 4.0 was the most inhibiting condition for these enzymes. pH 6.0 was preferred by FAEs of CRL1446, while *L. johnsonii* strains showed no differences between pH 6.0 and 7.0.

Table 1. Strains used, isolation sources, and ferulic acid esterase (FAE) activity quantifications at 37 °C—pH 7.0.

Species	Strain	Isolation Source	FAE Activity [1]
Lactobacillus johnsonii	CRL2240	Goat feces	218 [b]
	CRL2237	Goat feces	211 [b]
	CRL2238	Goat feces	591 [d]
Lactiplantibacillus plantarum	CRL046	Bovine cheese	35 [a]
	CRL2241	Whole plant corn silage	11 [a]
	ETC182	Whole plant corn silage	26 [a]
Limosilactobacillus fermentum	CRL1446	Bovine cheese	330 [c]
Levilactobacillus brevis	CRL2239	Whole plant corn silage	36 [a]
SEM			9.48
p-value			<0.0001

[1] FAE activity expressed as mean U g cells^{-1}. Different superscripts indicate statistically different results ($p < 0.05$) considering the strain variable. SEM: pooled standard error of means (n = 3).

3.2. Growth in CS Soluble Fraction Medium (CSM)

Species-specific and strain-specific aptitude to grow in CSM was detected (Table 2). Initial CSM pH was 5.81 ± 0.05. *L. plantarum*-inoculated CSM showed the fastest pH reduction when compared to the rest of the species. Silage-native *L. plantarum* strains (CRL2241 and ETC182) had a higher CFU count than CRL046. CRL2238 reduced pH and remaining RSs more efficiently than the rest of the *L. johnsonii* strains. Obligate heterofermentative LAB, *L. fermentum* CRL1446, and L. brevis CRL2239 showed similar results, but CRL2239-inoculated CSM presented lower remaining RSs.

PCA analysis of IvCs (Figure 2) showed that FAE activity measures were divergent (near 90° angles) with ΔpH-ΔCFU in CSM and with RSs. *L. plantarum* strains were closely clustered for their inoculant aptitude, while *L. johnsonii* CRL2237 and CRL2240 were separated from CRL2238 as for the remaining RSs left in culture media at 48 h.

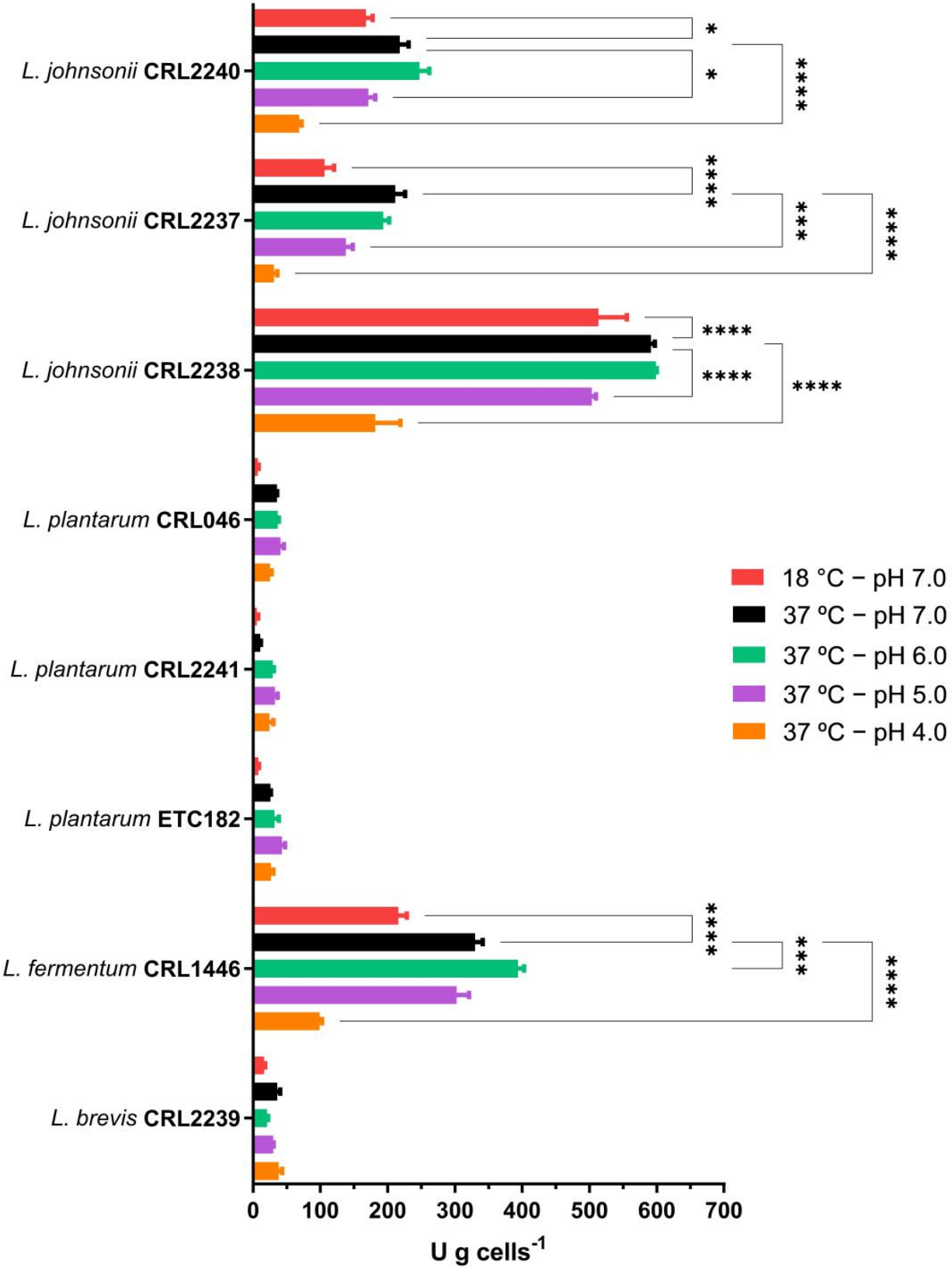


Figure 1. Ferulic acid esterase (FAE) activity quantifications (U g cells^{-1}) for lactic acid bacteria strains at different temperatures (pH 7.0) or pH conditions (at 37 °C). A significant effect of condition on FAE activity for each strain, when compared to 37 °C—pH 7.0, is indicated: (*): $p < 0.05$. (***): $p < 0.001$. (****): $p < 0.0001$.

Table 2. Acidification (ΔpH), growth (ΔCFU mL^{-1}), and remaining reducing sugars of ferulic acid esterase-producing strains when inoculated in Corn Stover Soluble Fraction Medium at 30 °C.

Strain		ΔpH			ΔCFU mL^{-1}	RSs
		12 h	24 h	48 h	48 h	48 h
L. johnsonii	CRL2240	0.18 ^{a}	0.79 ^{a}	2.20 ^{a}	4.7 ^{b}	23 ^{c}
	CRL2237	0.18 ^{a}	0.61 ^{a}	2.39 ^{b}	3.5 ^{a}	24 ^{c}
	CRL2238	0.60 ^{b,c}	1.58 ^{b}	2.80 ^{d}	4.6 ^{b}	7 ^{b}
L. plantarum	CRL046	1.96 ^{e}	2.25 ^{c}	2.99 ^{e}	4.9 ^{b}	4 ^{a,b}
	CRL2241	1.57 ^{d}	2.23 ^{c}	3.00 ^{e}	5.2 ^{c}	1 ^{a}
	ETC182	1.85 ^{e}	2.33 ^{c}	2.98 ^{e}	5.1 ^{c}	2 ^{a,b}
L. fermentum	CRL1446	0.52 ^{b}	1.42 ^{b}	2.46 ^{c}	4.8 ^{b}	20 ^{c}
L. brevis	CRL2239	0.77 ^{c}	1.47 ^{b}	2.38 ^{b,c}	4.7 ^{b}	3 ^{a,b}
SEM		0.04	0.05	0.02	0.04	1.16
***p* value**		<0.0001	<0.0001	<0.0001	<0.0001	<0.01

Means of ΔpH [5.81—pH at × h], remaining reducing sugars (RSs, mmol glucose equivalent L^{-1}), and CFU counts [48 h Log CFU mL^{-1}—initial Log CFU mL^{-1}] are shown. Independent units were used for each incubation period studied. The data were analyzed by means of a one-way ANOVA procedure followed by Tukey's test for each time of incubation and each measure of growth. Superscript letters indicate statistically different results in each column.

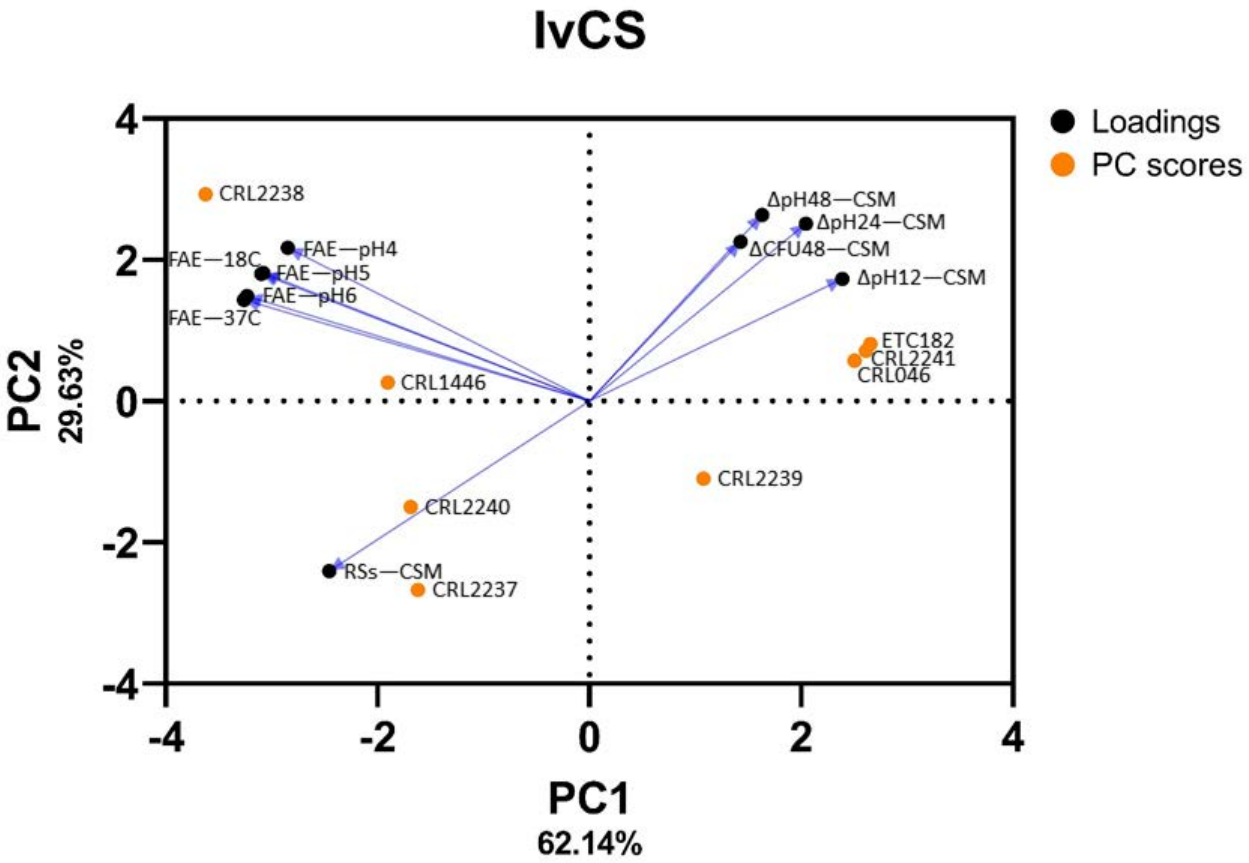


Figure 2. Biplot of Principal Components Analysis for in vitro Characterization Studies measures. FAE—37C: FAE quantification at 37 °C—pH 7.0. FAE—18C: FAE quantification at 18 °C—pH 7.0. FAE—pH4/5/6: FAE activity quantification at 37 °C—pH 4.0,5.0 or 6.0, respectively. pH12/24/48—CSM: ΔpH at 12/24/48 h of incubation in CSM. CFU48h—CSM: ΔLog CFU mL^{-1} at 48 h of incubation in CSM. RSs—CSM: Reducing sugars at 48 h of incubation in CSM.

3.3. Inoculation of Corn Stover Mini Silos

Chemical, Microbiological, and Compositional Analysis of Inoculated Silages

Pre-ensiled CS is characterized in Table 3. The initial and final densities of mini silos were 650 ± 10 and 200 ± 15 kg FM^{-1} $m^{3\ -1}$ (mean ± SD), respectively. No differences between groups were detected for this parameter.

Table 3. Corn stover characteristics before ensiling.

Item	
pH	4.24 ± 0.01
Log CFU g FM^{-1}	
Lactic acid bacteria	6.9 ± 0.01
Total Mesophilic Bacteria	7.4 ± 0.01
Yeast	6.2 ± 0.12
g kg FM^{-1}	
Reducing Sugars	46 ± 2
Dry Matter	250 ± 2
g kg DM^{-1}	
Organic Matter	889 ± 1
Crude Protein	102 ± 2
aNDF	591 ± 3
ADF	327 ± 2
ADL	35 ± 2
IVDMD	683 ± 2

Data shown are Means ± SD. FM: Fresh matter. DM: Dry Matter. aNDF: Neutral detergent fiber. ADF: acid detergent fiber. ADL: Acid detergent lignin. IVDMD: In vitro true dry matter digestibility.

Results for silages fermentative and microbial characterization are presented in Table 4. UN silages had an appropriate pH for forage conservation at the time of opening, although a tendency to lower pH was detected for all inoculants except CRL046. LAB counts were higher in all inoculated silages when compared to UN, except CRL2240 and CRL2239. RSs were higher in CRL2237-inoculated silages when compared to CRL2241. Acetate concentrations tended to be lower by *L. johnsonii* CRL2240 and CRL2238 inoculation, although a significant change in L:A ratio could not be detected. Butyric or propionic acids were not detected in any of the samples.

Table 4. Fermentative and microbiological parameters of uninoculated (UN) and inoculated silages.

		L. johnsonii			*L. plantarum*			*L. fermentum*	*L. brevis*			
Item	**UN**	**CRL2240**	**CRL2237**	**CRL2238**	**CRL046**	**CRL2241**	**ETC182**	**CRL1446**	**CRL2239**	**SEM**	***p* Value**	
pH	3.63 ^{a,b}	3.58 ^{a}	3.63 ^{a,b}	3.62 ^{a}	3.69 ^{b}	3.59 ^{a}	3.57 ^{a}	3.61 ^{a}	3.62 ^{a}	0.01	***	0.0003
Log CFU g FM^{-1}												
LAB	ND ^{a}	ND ^{a}	3.7 ^{b}	4.1 ^{b}	3.8 ^{b}	3.9 ^{b}	4 ^{b}	4.4 ^{b}	ND ^{a}	0.42	*	0.0171
TMB	5.7 ^{a,b}	5.9 ^{b}	5.3 ^{a,b}	5.6 ^{a,b}	5.8 ^{a,b}	5.4 ^{a,b}	5.8 ^{a,b}	5.7 ^{a,b}	5.1 ^{a}	0.16	*	0.0484
Yeast	ND ^{a}	ND ^{a}	3.6 ^{b}	3.7 ^{b}	ND ^{a}	3.7 ^{b}	3.6 ^{b}	4.3 ^{b}	ND ^{a}	0.33	**	0.0012
g kg DM^{-1}												
RSs	29 ^{a,b}	28 ^{a,b}	36 ^{b}	27 ^{a,b}	23 ^{a,b}	21 ^{a}	23 ^{a,b}	25 ^{a,b}	28 ^{a,b}	3.34	*	0.0289
$N\text{-}NH_3$	1.3	1.0	0.7	0.7	0.9	1.0	0.9	0.7	0.7	0.3	ns	0.8672
Lactate	145	160	147	125	131	158	146	147	128	8.46	t	0.0856
Acetate	31	17	24	16	18	24	19	21	18	2.72	t	0.0642
L:A	5	9	6	8	7	7	8	7	7	0.98	ns	0.2677
Ethanol	4	3	13	5	5	17	17	15	12	4.1	ns	0.1854
Total acids	176	177	171	146	149	182	165	168	141	9.48	ns	0.1298
TPC	48	49	48	49	50	50	50	49	48	0.61	ns	0.2063

FM: fresh matter. LAB: Lactic acid bacteria. TMB: Total mesophilic bacteria. ND: Not detected, inferior to 2.5 (for LAB and TMB) or 2.0 (for yeast) Log CFU g FM^{-1}. DM: dry matter. RSs: Reducing sugars, as mmol glucose equivalent L^{-1}. TPC: total phenolic compounds, as µg Gallic acid equivalent mL^{-1}. All values shown are means (n = 3). SEM: Pooled standard error of means. A different superscript letter in the same row indicates statistically different results ($p < 0.05$). ns (not significant): $p \geq 0.1$; t (tendency): $p < 0.1$. *: $p < 0.05$. **: $p < 0.01$. ***: $p < 0.001$.

The nutritional parameters of silages are shown in Table 5. Compared to UN, DM loss was significantly reduced in CRL046 and CRL2239, and OM increased in CRL2241-inoculated silages. Crude protein was lower in CRL2237 silages when compared to UN. In situ dADF was increased by inoculation of CRL2240, CRL2238, ETC182, and CRL2239. Although a tendency to increase in situ ADFD was also detected for these strains, this tendency was only significantly ($p < 0.05$) modified by CRL2238.

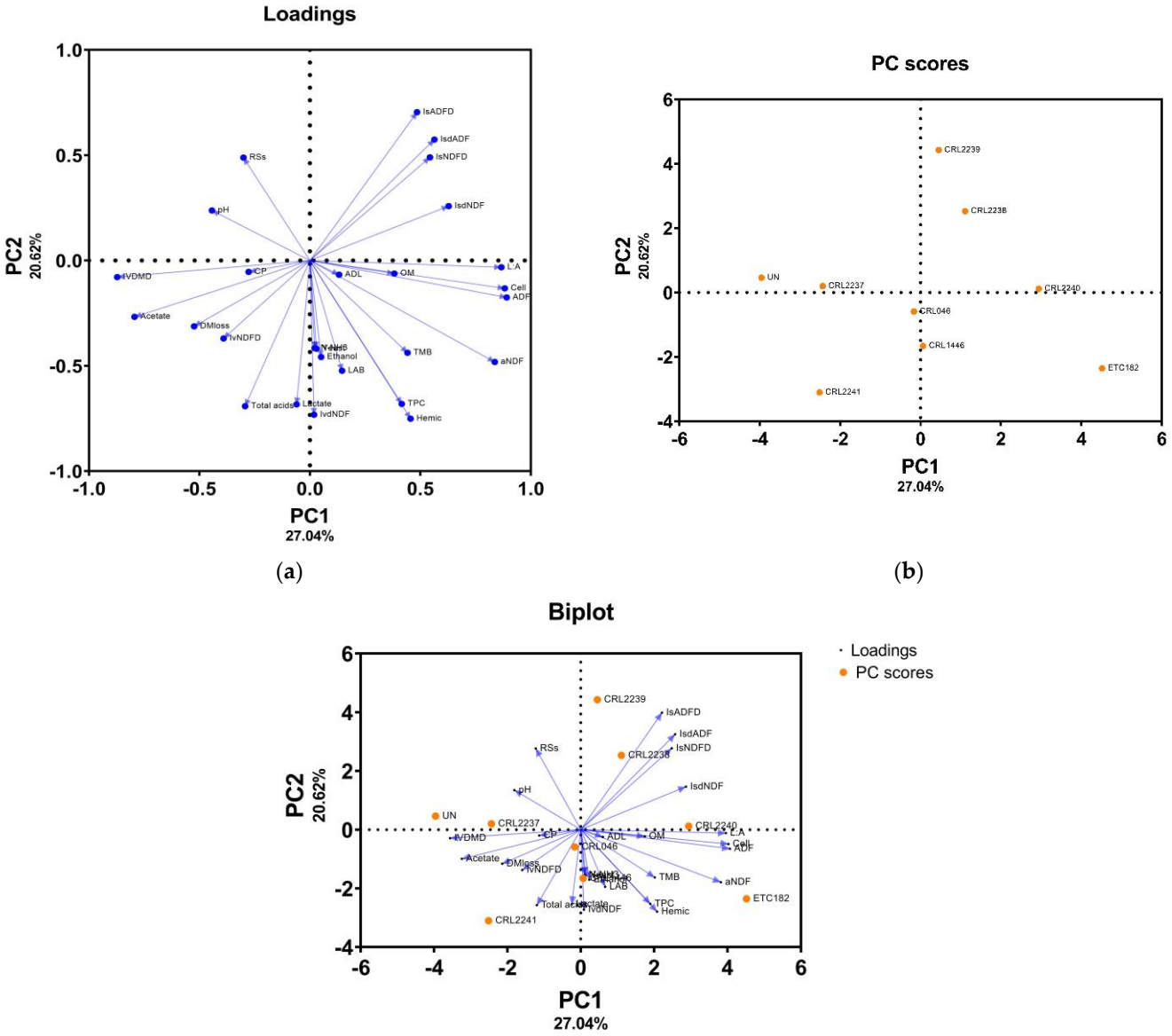
Table 5. Nutritional parameters of uninoculated (UN) and inoculated silages.

		L. johnsonii			*L. plantarum*			*L. fermentum*	*L. brevis*			
Item	**UN**	**CRL2240**	**CRL2237**	**CRL2238**	**CRL046**	**CRL2241**	**ETC182**	**CRL1446**	**CRL2239**	**SEM**	***p*-Value**	
DM loss, *g kg FM*$^{-1}$	44 ^{b}	35 ^{a,b}	46 ^{b}	42 ^{b}	25 ^{a}	45 ^{b}	32 ^{a,b}	37 ^{a,b}	23 ^{a}	0.29	***	0.0001
ADIN, *g kg total N*$^{-1}$	124	116	103	104	103	119	94	98	97	12	ns	0.5404
g kg DM^{-1}												
Organic matter	885 ^{a}	902 ^{a,b}	902 ^{a,b}	900 ^{a,b}	901 ^{a,b}	905 ^{b}	900 ^{a,b}	896 ^{a,b}	902 ^{a,b}	4	*	0.0451
Crude protein	100 ^{b}	90 ^{a,b}	84 ^{a}	87 ^{a,b}	88 ^{a,b}	88 ^{a,b}	88 ^{a,b}	98 ^{a,b}	90 ^{a,b}	4	*	0.0462
aNDF	606	625	610	612	617	610	640	622	608	11	ns	0.5782
ADF	358	376	366	364	363	361	380	366	366	7	ns	0.5198
ADL	40	44	38	38	39	42	39	42	42	2	ns	0.6194
Hemicellulose	248	249	245	248	254	250	259	255	242	5	ns	0.4327
Cellulose	317	332	327	326	324	318	341	324	324	6	ns	0.2582
In vitro digestibility												
IVDMD	657	633	658	648	655	655	635	641	640	8	ns	0.2462
dNDF, *g kg DM*$^{-1}$	263	257	268	260	272	265	274	262	248	7	ns	0.2527
NDFD, *% aNDF*	43	41	44	43	44	43	43	42	41	0.9	t	0.0953
In situ digestibility												
dNDF, *g kg DM*$^{-1}$	165	167	167	169	164	137	185	165	167	13	ns	0.5996
NDFD, *% aNDF*	27	27	27	28	26	22	29	27	28	1.8	ns	0.4784
dADF, *g kg DM*$^{-1}$	17 ^{a}	29 ^{b}	14 ^{a}	46 ^{b}	15 ^{a}	14 ^{a}	27 ^{b}	14 ^{a}	34 ^{b}	6	**	0.0072
ADFD, *% ADF*	5 ^{a}	8 ^{a,b}	4 ^{a}	13 ^{b}	4 ^{a}	4 ^{a}	7 ^{a,b}	4 ^{a}	10 ^{a,b}	1.5	**	0.0064

DM: Dry matter. ADIN: Acid detergent insoluble nitrogen. aNDF: neutral detergent fiber, expressed inclusive of residual ash. ADF: acid detergent fiber. ADL: Acid detergent lignin. IVDMD: In vitro dry matter true digestibility. dNDF: Digestible aNDF. NDFD: aNDF digestibility. dADF: Digestible ADF. ADFD: ADF digestibility. All values shown are means ($n = 3$). SEM: Pooled standard error of means. Different superscript letter in the same row indicates statistically different results ($p < 0.05$). ns (not significant): $p \geq 0.1$. t (tendency): $p < 0.1$. (*): $p < 0.05$. (**): $p < 0.01$. (***): $p < 0.001$.

PCA analysis for silage variables found moderate significance, as interpreted through the cumulative percentage of variance (Figure 3). PC1 was mainly formed by fiber composition (aNDF, ADF), digestibility (IVDMD), and fermentative parameters (acetate and L:A ratio), while PC2, in turn, was formed by hemicellulose, IvdNDF, IsADFD and lactate, total acids, and TPC content (Supplementary Table S1). Analysis of correlated variables in silages (Supplementary Table S2) indicated that ADF content was negatively correlated with pH and positively correlated with the L:A ratio. Furthermore, in situ dADF and ADFD were negatively correlated with acetate concentration. TPC content was higher in samples with lower RSs and higher hemicellulose (Figure 3a). Allocation of experimental groups according to their PC scores (Figure 3b,c) moderately identified three clusters: (i) *L. johnsonii* CRL2237 is the closest to the UN group, (ii) *L. plantarum* and *L. fermentum* inoculated silages allocate negatively to PC2, and (iii) *L. johnsonii* CRL2238 and CRL2240, and *L. brevis* CRL2239, are positively correlated with CP1 and CP2.

PCA of IvCS assays and parameters of inoculated silages revealed a PC1 mainly influenced by FAE activity (positively) and growth in CSM (negatively) measures (Figure 4a, Supplementary Tables S3 and S4). Visual observation of PC Scores (Figure 4b,c) separated *L. plantarum* from the rest of the strains across the PC1 axis. Correlation analysis showed that higher acidification and growing ability in CSM were correlated ($p < 0.05$) with lower RSs and higher TPC in mini silos. The measure of FAE-specific activity was not relevant to predict any outcome in silages of the present trial. No other quality indicator could be predicted through these IvCS assays.



(**a**) (**b**)

(**c**)

Figure 3. (**a**) Loadings plot, (**b**) PC scores, and (**c**) Biplot graph of Principal Component Analysis for fermentative, microbiological, and nutritional measures of uninoculated (UN) and inoculated silages. LAB: Lactic acid bacteria. TMB: Total mesophilic bacteria. DM: dry matter. OM: Organic matter. CP: crude protein. RSs: Reducing sugars. L:A: Lactic: acetic ratio. TPC: total phenolic compounds. DM: Dry matter. ADIN: Acid detergent insoluble nitrogen. aNDF: neutral detergent fiber, expressed inclusive of residual ash. ADF: acid detergent fiber. ADL: Acid detergent lignin. Cell: Cellulose. Hemic: Hemicellulose. IvDMD: In vitro dry matter true digestibility. Is/IvdNDF: In situ/in vitro digestible aNDF, respectively. Is/IvNDFD: In situ/in vitro aNDF digestibility, respectively. IsdADF: In situ digestible ADF. IsADFD: In situ ADF digestibility.

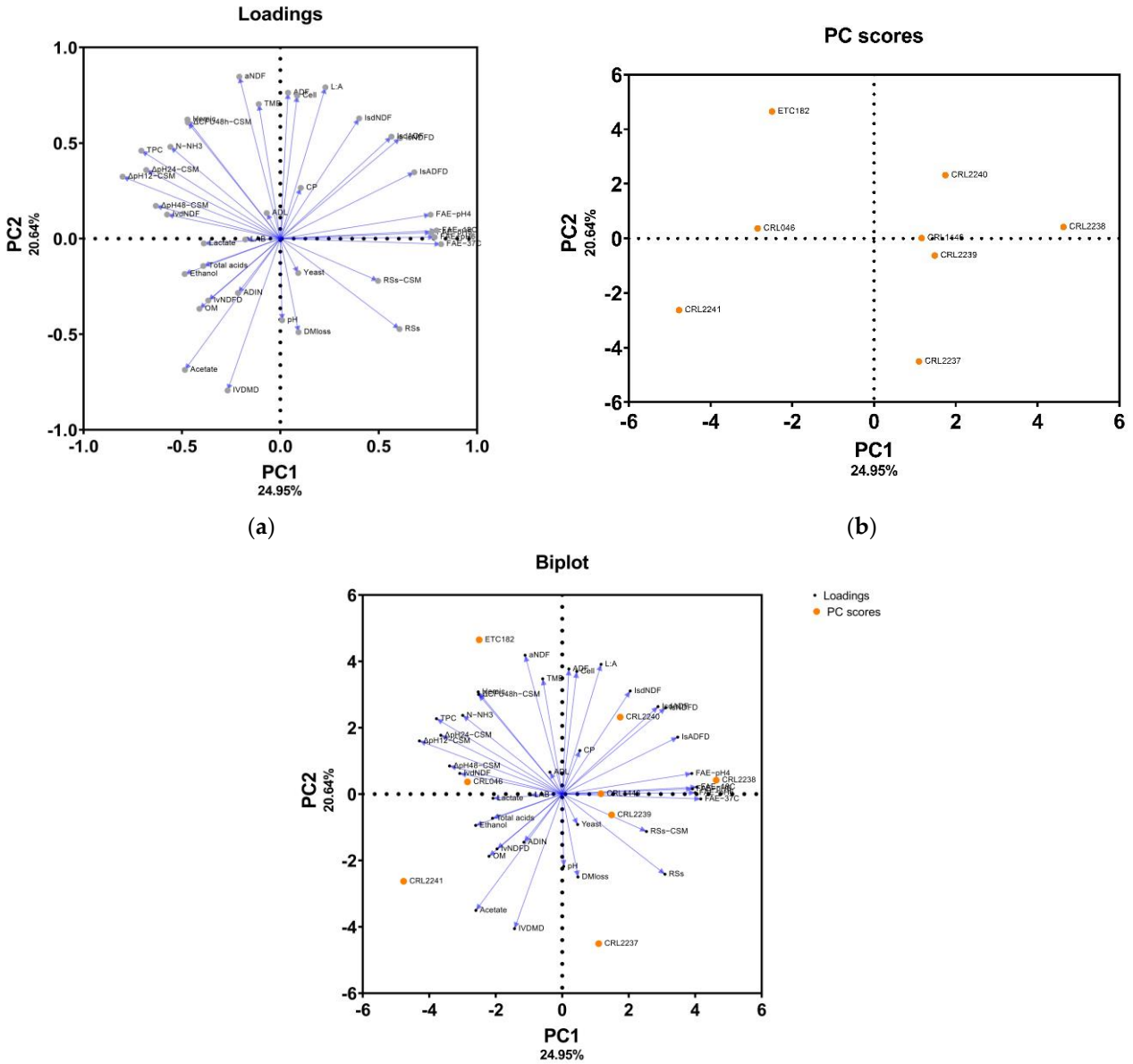


Figure 4. (**a**) Loadings plot, (**b**) PC scores, and (**c**) Biplot graph of Principal Component Analysis for fermentative, microbiological, and nutritional measures of inoculated silages, measures of growth in Corn Stover Soluble Fraction Medium (CSM), and FAE activity quantification. LAB: Lactic acid bacteria. TMB: Total mesophilic bacteria. DM: dry matter. OM: Organic matter. CP: crude protein. RSs: Reducing sugars. L:A: Lactate: acetate ratio. TPC: total phenolic compounds. DMloss: Dry matter loss. ADIN: Acid detergent insoluble nitrogen. aNDF: neutral detergent fiber, expressed inclusive of residual ash. ADF: acid detergent fiber. ADL: Acid detergent lignin. Cell: Cellulose. Hemic: Hemicellulose. IvDMD: In vitro dry matter true digestibility. Is/IvdNDF: In situ/in vitro digestible aNDF, respectively. Is/IvNDFD: In situ/in vitro aNDF digestibility, respectively. IsdADF: In situ digestible ADF. IsADFD: In situ ADF digestibility. FAE—37C: FAE quantification at 37 °C—pH 7.0. FAE—18C: FAE quantification at 18 °C—pH 7.0. FAE—pH4/5/6: FAE activity quantification at 37 °C—pH 4.0, 5.0 or 6.0, respectively. pH12/24/48—CSM: ΔpH at 12/24/48 h of incubation in CSM. CFU48h—CSM: ΔLog CFU mL^{-1} at 48 h of incubation in CSM. RSs—CSM: Reducing sugars at 48 h of incubation in CSM.

4. Discussion

Characterization of LAB's FAEs showed that the highest specific activity was observed in *L. johnsonii* and *L. fermentum* strains, while *L. plantarum* and *L. brevis* strains showed the lowest values. This has been proven to be a strain-specific trait [32]. The optimal pH for *L. johnsonii* and *L. fermentum* strains is consistent with previously reported for these or closely related species [33]. A drastic reduction of hydrolysis under pH 5.0 was also observed in other studies [13]. Although this spectrophotometric method was useful for identifying differences in enzymatic activity at different conditions for medium-to-high conversion rates-strains [32,34], it was not able to detect differences in low conversion ones. A probable cause is the cellular allocation of enzymes in different LAB species: several authors have reported essentially intracellular location of FAE activity for *L. johnsonii* closely related species [35] or *L. fermentum* [25], while successful characterization of *L. plantarum* and *L. brevis* activity was performed using supernatants-based enzyme preparations [13].

IvCS of potential silage inoculants are frequently interpreted through PCA in order to obtain a set of strains that share several desired characteristics [36,37]. In our study, both specific enzymatic quantification and substrate adaptation was assessed, showing that these characteristics were divergent in studied strains: better inoculant aptitude was found in low FAE producers. It has been previously stated that the ability of a FAE+ LAB to dominate the fermentation process is more important than its level of FAE activity [31]. Regarding this aspect, *L. plantarum* and *L. brevis* have been reported as dominant CS-silage species [38,39], which is consistent with our results in CSM.

Mini silo preparation was designed to imitate frequent in-field conditions and possibilities of small- to medium-scale goat production systems [6,40,41]. Packing density was selected based on that obtained in 30 kg-CS bag mini silos manufactured by goat producers in our local area (data unpublished), which is also similar to that reported in small holders' surveys for whole plant maize silage [42,43]. When compared to similar CS previously reported, characterization of fresh CS indicated a higher aNDF and ADF and much lower CP content (298 or 463 vs. 102 g kg DM^{-1}) [4,44]. Ensiling CS in these conditions, without the addition of other common fermentation enhancers such as urea or molasses, can be considered a challenging objective for its low RSs content, even when compared to other CS [4,45]. Nevertheless, excellent fermentative qualities were observed in UN silages according to pH, lactate, NH_3-N, and ADIN values [46,47]. DM loss in the UN group is similar to that reported in a similar CS mini-silo trial after 45 days of ensiling [44]. Digestibility measures of the pre-ensiled CS and UN groups showed relatively high values [45], which can therefore be considered difficult to improve.

Nutrients preservation through ensiling, measured as DM and OM loss reduction, was significantly enhanced by *L. brevis* CRL2239, *L. plantarum* CRL046 (both approximately 50% of DM loss observed in UN group) and CRL2241 (2% improvement in OM content) inoculation. Fermentation dominance can be interpreted through these parameters and end-product alterations, such as the acetate reduction observed in silages inoculated with homolactic *L. johnsonii* CRL2238 and CRL2240. These are the first reported strains of this species used as silage inoculants. Acetate reduction is a generally desired effect, especially in high moisture silage [47].

A PCA to evaluate silage variations induced by different inoculants can be a useful tool to interpret a large set of variables. A previous report on grass silage found a similar cumulative percentage of variation explained by PC1 and PC2 [48]. In this study, PCA analysis revealed that *L. johnsonii* CRL2237 inoculation was not successful in inducing important variations when compared to indigenous LAB present in UN silages, which is consistent with observed growth/acidification parameters in CSM for this strain.

In a previous publication, we evaluated the potential ruminant probiotics *L. johnsonii* CRL2237, CRL2238, and CRL2240 considering the hypothesis that certain selection studies can predict the ability of a strain as a silage inoculant, obtaining unconvincing results [20]. In the present work, we proved this hypothesis against different LAB species and strains, finding that IvCS observations were also not able to predict important aspects such as

DM or OM preservation under these conditions. If a selection process was performed according to data obtained in IvCS, *L. brevis* CRL2239 and *L. johnsonii* CRL2240 should have been discarded (as a result of PCA interpretation), but they exerted desirable effects in CS silages. Nevertheless, the remaining RSs of silages were significantly correlated with ΔpH, ΔCFU, and RSs in CSM. RSs is a determining parameter for aerobic stability, for which this observation should be considered in further investigations. As it was previously discussed [49,50], a systematic and effective approach for screening silage inoculants has yet to be developed. Still, ensilage fermentation is a complex process affected by numerous factors (crop, ambient conditions, ensiling method, etc.), and it is likely that any in vitro approach can effectively reproduce it. Selection procedures using crop-based culture media [24,50,51], detection of antagonistic activities [37], and enzymatic characterizations [13] constitute a rational approach to finding the most promising inoculant strains.

Several strategies have been tested to improve CS silage digestibility, obtaining different results [4,44,45,52,53]. In a previous trial, *L. johnsonii* CRL2240 and *L. fermentum* CRL1446 single inoculation could induce lower ADF content and higher IVDMD in whole plant corn silage [20], but these effects were not observed in the present work, probably due to forage composition: for instance, corn grains contribute with fermentable substrates and contain a high degree of ferulic esters. Still, in situ dADF and ADFD were improved by CRL2240, CRL2238, ETC182, and CRL2239 strains when compared to UN. Digestibility alterations in ADF were reported in two similar studies using FAE+ LAB: an increase in 48 h in situ ADFD in ryegrass silages inoculated with *L. buchneri* or *L. reuteri* strains [31] and a reduction of in vitro 24 h ADFD for a combined FAE+-LAB-fibrolytic enzyme inoculant in whole plant corn silage [19]. Although values obtained for in vitro and in situ digestibility measures are usually expected to be correlated, this was not detected for corn silage samples by Raffrenato et al. [54] nor in this assay. The differences observed were similar to those reported by DeFeo et al. for barley samples [55].

Among digestibility measures, NDFD is considered to be a major influence on animal DM intake and milk yield in intensive systems [56]. There is growing but inconsistent evidence that FAE activity can especially influence this parameter [14,31]. The mean in vitro value obtained in this study is similar to previously reported for CS silages [4,44]. A tendency to slightly lower in vitro NDFD for CRL2240 and CRL2239 silages could be observed in the present work, which can be the result of the degradation of the more readily fermentable fiber [57]. An *L. plantarum* strain could efficiently increase IVDMD and NDFD in CS ensiled, which presented lower control silage-IVDMD than observed in the present study (588 vs. 657 g kg DM^{-1}) [44]. Gao et al. reported a positive effect in IVDMD for cellulase or LAB + cellulase-treated, but not for only LAB-treated CS silages [45]. It has been stated that FAEs inclusion in forages has a limited effect if not accompanied by xylanases or cellulases, for it has been studied in several trials [11,19,57–59].

5. Conclusions

This is the first report of FAE+ lactobacilli as inoculants for CS silage. Evidence of inoculant aptitude was observed in fermentative and nutritional parameters studied in CS silages for several strains, including DM and OM preservation and ADF digestibility. Limitations of IvCS for the selection of silage inoculants were analyzed, as scarce correlations were observed. Mixed FAE+ LAB-inoculation, combined inoculation with fibrolytic enzymes, optimization of inoculation rates, and application to different varieties of CS are to be investigated in order to develop an effective third-generation CS silage inoculant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9040331/s1, Table S1: Principal Component Analysis of silage variables: correlation-probability matrix; Table S2: Principal Component Analysis of silage variables: correlations between the Principal Components and the original variables; Table S3: Principal Component Analysis of silage-IvCS variables: Correlation-probability matrix; Table S4: Principal Component Analysis of silage-IvCS variables: correlations between the Principal Components and the original variables.

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