Do FAE-producing microbial inoculants improve fermentation and improve digestibility of stored alfalfa forage?

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Abstract

New silage inoculants contain a bacterial strain that produces ferulic acid esterase (FAE) which may facilitate lignin break down, which may increase the digestibility of the ensiled forage. The objective of this study was to evaluate the efficacy of an FAE-producing microbial inoculant for improving fermentation characteristics, nutritive value, and digestibility of alfalfa or alfalfa-bermudagrass mixtures as silage. This study was conducted at the Coastal Plain Experiment Station in Tifton, GA and the J. Phil Campbell Research and Extension Center (JPC-REC) in Watkinsville, GA on 0.25-acres of previously established 'Bulldog 805' alfalfa (Tifton) and Russell bermudagrass interseeded with 'Bulldog 505' alfalfa (Watkinsville). Forage was harvested twice during the growing season at 10% bloom to simulate differences in lignin content due to growing conditions. Harvested forage was treated with one of three treatments: ferulic acid esterase-producing microbial inoculant (MI+FAE); a heterofermentative microbial forage inoculant (MI); or an untreated water control (CON) before packed into miniature silos to undergo a 60-day fermentation. After fermentation period, forage was analyzed for fermentation characteristics, nutritive value, and digestibility parameters. MI+FAE did not improve fermentation characteristics, nutritive value, or digestibility parameters compared with the MI inoculant, although both MI+FAE and MI generally showed an improvement in fermentation over the control.

Introduction

The production of a high-quality silage is dependent on effective bacterial fermentation. To encourage rapid bacterial fermentation, commercially available microbial inoculants may be applied to the crop at harvest. These products often include *Lactobacillus plantarum*, a homofermentative bacteria that rapidly ferments plant available sugars to produce organic acids (e.g. lactic acid). Other products contain both homofermentative and heterofermentative bacteria (e.g. *L. buchneri*) to promote both rapid fermentation and aerobic stability (Muck et al., 2018; Arriola et al., 2015). The use of effective microbial inoculants can decrease the amount of forage lost to poor fermentation or to spoilage, thus reducing forage storage losses and waste.

New microbial inoculant products incorporate a bacterial strain that produces ferulic acid esterase (FAE). This enzyme can break down the ferulic acid linkages in lignin, releasing the hemicellulose-lignin cross-linkages and increasing the surface area of the hemicellulose and cellulose exposed to microbial digestion, thereby increasing forage digestibility (Cornu et al., 1994; Jung et al.,

2011). Improving forage quality of silage can improve forage digestibility and animal performance and decrease the need for additional animal supplementation when fed.

Research exploring the efficacy of microbial inoculants containing ferulic acid esterase has been inconclusive thus far. Addah et al. (2011) concluded the use of an FAE-containing microbial inoculant may improve feed efficiency and aerobic stability in feedlot steers. These observations were supported by Aboagye et al. (2015), who saw enhanced animal performance in sheep fed forage treated with an FAE-containing product. However, Lynch et al. (2014) observed that FAE-containing products elicited no positive response on fermentation characteristics or nutritive value, even when combined with additional fibrolytic enzymes. Therefore, the objective of this research is to assess the impact of treatment with an FAE-enhanced microbial inoculant when applied to alfalfa or an alfalfa-bermudagrass mixture.

Methods and Materials

Study Sites and Plot Management

This experiment was conducted during the summer of 2018 using previously established 0.2-ha stands of pure-stand of 'Bulldog 805' alfalfa (*Medicago sativa* L.; ALF) located at the Coastal Plains Experiment Station (Tifton, GA) and a mixed stand of 'Bulldog 505' alfalfa and 'Tifton-44' bermudagrass (*Cyondon dactylon* L. Pers.; ABG) located at the J. Phil Campbell Research and Education Center (JPC-REC; Watkinsville, GA). The ALF stand was planted December 2016 using 19-cm row spacing at a rate of 22.4 kg ha^{-1}. The ABG stand had been interseeded with alfalfa in December 2017 using a 35.6-cm row spacing at a seeding rate of 14 kg ha^{-1}.

In both locations, stands were mowed in early May and early July 2018 and forage residue removed in the course of their normal harvest schedule. In early June (8 and 14 June) and early August (7 and 9 August), herbage was harvested from randomly selected areas within the respective fields to a 7.5-cm stubble height when alfalfa reached the early (10%) bloom stage using a flail-type plot harvester (Swift harvester, Swift Machine and Welding, Ltd., Sask., Canada and Gravely harvester, AriensCo, Brillion, WI in Tifton and Watkinsville, respectively) to chop the forage to approximately 2-cm in length. Growth stage determination was estimated based on the procedure from Mueller and Fick (1989).

Soil test results for the ALF and ABG stands in Tifton and Watkinsville, respectively, are presented in Table 1. Both the ALF stand in Tifton and ABG stand in Watkinsville were fertilized during March 2018. In Tifton, the ALF stand was fertilized with 121.7 kg K_2O ha^{-1}, 78.5 kg P_2O_5 ha^{-1} (Mono Ammonium Phosphate, 12-61-0, N-P-K, %; Haifa; Haifa North America, Altamonte Spring, FL), and 3.4 kg B ha^{-1} (10% Liquid Solution; CNI Liquid, CNI AgriMinerals, Albany, GA) and in Watkinsville, ABG stand received 112 kg K_2O ha^{-1}, 44.8 kg P_2O_5 ha^{-1} phosphorus (Mono Ammonium Phosphate, 12-61-0, N-P-K, %; Haifa; Haifa North America, Altamonte Spring, FL); 44.8 kg N ha^{-1} as ammonium sulfate, and 3.36 kg B ha^{-1} (10% Liquid Solution; CNI Liquid, CNI AgriMinerals, Albany, GA).

Beginning in March, both locations were scouted weekly for insects, including: alfalfa weevil [(*Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae)], potato leafhopper [*Empoasca fabae* (Harris) (Hemiptera: Cicadellidae)], three-cornered alfalfa hopper [*Spissistilus festinus* (Say) (Hemiptera:

Membracidae)], fall armyworm [*Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae)], and bermudagrass stem maggot [*Atherigona reversua* (Villaneuve) (Diptera: Muscidae)] (Watkinsville only). In Tifton, lambda cyhalothrin (Lambda-Cy; Nufarm Americas Inc., Burr Ridge, IL) was applied in February 2018 at 34 g a.i. ha^{-1} to control alfalfa weevil. Zeta-cypermethrin (Mustang Maxx; FMC Corporation, Philadelphia, PA) was applied in May and June 2018 at a rate of 28 g a.i. ha^{-1} to control three-cornered alfalfa hopper. Finally, malathion (Malathion 5EC; Drexel Chemical, Memphis, TN) was applied July 2018 to control fall armyworm at 1.4 kg a.i. ha^{-1}. In both locations, zeta-cypermethrin (Mustang Maxx; FMC Corporation, Philadelphia, PA) was applied in July 2018 at a rate of 28 g a.i. ha^{-1} to control three-cornered alfalfa hopper. Pendimethalin (Prowl H2O; BASF Ag Products, Floram Park, NJ) was applied to control annual grass weeds following harvest in June and August at a rate of 1.1 kg a.i. ha^{-1}. No additional applications were made until after the termination of the trial.

Table 1. Soil pH, phosphorus (mg kg^{-1}), potassium (mg kg^{-1}), calcium (mg kg^{-1}), and magnesium (mg kg^{-1}) from topsoil of pure stand or alfalfa-bermudagrass plots harvested in Tifton and Watkinsville, GA and analyzed by the University of Georgia Soil, Plant, and Water laboratory (SPW) in Athens, GA during 2018.

	Tifton	Watkinsville
pH	6.9	6.7
Phosphorus (mg kg^{-1})	50.4	19.0
Potassium (mg kg^{-1})	53.3	48.5
Calcium (mg kg^{-1})	587	617
Magnesium (mg kg^{-1})	80.6	49.0

Forage Preparation and Application of Inoculant Treatments

Harvested forage was mixed and spread onto a 6-m x 12-m tarpaulin to wilt to approximately 60% moisture. Throughout wilting, forage was mixed by hand twice to ensure even wilting. Forage moisture was tested every 30 minutes using the microwave moisture method (Ball et al., 2015). When forage reached 58% moisture, a representative sample was collected, immediately weighed, and dried in a forced air oven at 55°C for three days to confirm forage moisture.

A subsample of forage was placed onto one of three additional tarpaulins of the same size, each corresponding to one of three inoculant treatments applied in an aqueous solution in deionized water: 1) a conventional, commercially available non-FAE-producing microbial inoculant (MI), 2) a FAE-producing microbial inoculant (MI+FAE), and 3) a similarly applied quantity of deionized water as a control (CON). The MI treatment was Pioneer 11G22 (Pioneer DuPont, Johnston, IA) to provide 1.1 x 10^{11} cfu g^{-1} of *L. plantarum* and *L. buchneri*. The MI+FAE treatment was Pioneer 11AFT (pure-stand alfalfa) or Pioneer 11GFT (alfalfa-bermudagrass mixture) in accordance with company recommendations for crop differences and both products provided 1.1 x 10^{11} cfu g^{-1} and 1.3 x 10^{11} cfu g^{-1} of *L. plantarum* and *L. buchneri* of the LN4017 strain which produces FAE. These application rates are consistent with the manufacturer recommended rates.

The inoculant treatments were applied using one of three 3.8-L garden sprayers (ISO 14001 Home and Garden Sprayer, Chapin; Batavia, NY) assigned to each treatment that are identical except for the contents. Pre-weighed powdered inoculant was added to 3.8 L of deionized water and thoroughly mixed. All tarpaulins and sprayer tanks were color coded and numbered to correspond

with their associated treatments to prevent cross-contamination. Forage was sprayed thoroughly to ensure coverage with the liquid inoculant treatment.

Once treated, the forage was immediately packed into miniature silos for storage so that fermentation may proceed. Miniature silos were constructed from 76.2-cm polyvinyl chloride (PVC) tubing and sealed on each end using rubber end caps. The bottom of the silo was packed with a small layer (~2.5 cm) of the chopped and treated alfalfa, then covered with a small layer of plastic before the mini-silo was filled with treated alfalfa and compacted to a density of 0.20-0.24 kg DM/L (12-15 lbs DM/ft^3) until 3 cm from the top. Forage dry matter densities in each silo were held constant by weighing the same amount of forage into each miniature silo. There were differences in mass, however, between the pure-stand alfalfa and the alfalfa-bermudagrass mixture. After filling, a second layer of plastic was placed on the packed alfalfa. Before the silo was sealed an additional 2.5-cm layer of treated alfalfa was packed into the silo to prevent air leakage. To provide a consistent moisture amount within a block, a complete set of the 3 treatments (CON, MI, and MI+FAE) was treated and packed into the mini-silos before the process was replicated. Thus, the experimental design was a 2 x 3 factorial, with two forage types and three inoculant treatments in a randomized complete block design with five replications.

After packing, silos were kept outdoors in ambient air conditions but under cover. Carbon dioxide was manually released daily from every silo for the first 21 days post-harvest, and silos were monitored, and pressure released as necessary for the duration of the trial.

Forage Sampling and Analysis

After a 60-day fermentation period, the miniature silos were opened, and forage was collected for analysis. The top and bottom 20-cm of each silo was discarded, and samples were obtained from the center ca 30-cm. Forage was placed into a quart-sized bag and immediately frozen. A portion of the sample was sent to a commercial laboratory (Cumberland Valley Analytical Services, Waynesboro, PA) for nutritive value analysis including: dry matter, moisture, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin, starch, and ash and a fermentation profile, including: pH, total volatile fatty acids (VFA), lactic acid, acetic acid, propionic acid, butyric acid, and Ammonia N. Calibration statistics for nutritive value NIR haylage equations were as follows: NDF, SEC = 0.811, R^2 = 0.993; SECV = 0.826; ADF, SEC = 0.770, R^2 = 0.972; SECV = 0.794; CP, SEC = 0.519, R^2 = 0.988; SECV = 0.529; where SEC = standard error of calibration and SECV = standard error of validation, in g kg^{-1} on a DM basis.

Approximately 25 g of each of the samples was freeze-dried (VirTis FreezeMobile 12ES; SP Industries, Warminster, PA) and ground to pass through a 6-mm Wiley Mill screen (Thomas Scientific, Swedesboro, NJ). The 6-mm grind size was selected due to concerns that a 1-mm screen would not detect differences in digestibility between the MI and MI+FAE products (Addah et al., 2010). Following grinding, samples were subjected to in-vitro dry matter digestibility (IVDMD) and ruminal digestion kinetics in the rumen microbiology lab at the University of Georgia. To do this, 0.6-g of each freeze-dried forage sample was weighed into a heat-sealed nylon bag in triplicate (n = 3 for each forage) (F57 Ankom Fiber Filter Bag; Ankom Technology, Macedon, NY) and placed into an *in vitro* fermentation system using mixed ruminal microorganisms based on the procedure of Callaway et al. (1997). Fiber bags were placed into individual 125-mL serum glass bottles and 100-mL of mixed ruminal media was added to each bottle. Media was comprised of 33% ruminal fluid obtained from

dairy steers at the University of Georgia Teaching Dairy (Athens, GA; AUP #: A2018 10-023-Y1-A0) and 67% anoxic media (Cotta and Russell, 1982) maintained at pH 6.5. Fiber bags were fully submerged in the mixed ruminal fluid and gas was released and measured via syringe throughout. Samples were maintained in a water bath (Blue M Constant Temperature Bath, Blue M Electric Company; Blue Island, Illinois) at 39°C for 48 hours. Following a 48-h incubation, samples were removed, placed on ice to halt fermentation, rinsed in deionized water, placed in a forced air oven at 55°C for 48 h, and weighed to determine IVDMD.

Forage analysis to determine NDF and ADF disappearance was conducted using an Ankom Fiber Analyzer (Model A2000, Ankom Technology; Macedon, NY). Additionally, immediately following the 48-h incubation, ruminal fluid was measured for pH (Accument AB150; Fisher Scientific, Waltham, MA) and an aliquot of ruminal fluid was collected for VFA and NH_3 analyses (Callaway et al., 1997). A 0.5-mL ruminal fluid subsample was analyzed for VFA by gas chromatography (Shimadzu GC-2010 Plus; Shimadzu Corp., Kyoto, Japan) using a flame ionization detector and a capillary column (Zebron ZB-FFAP GC Cap. Column 30m x 0.32 mm x 0.25 μ; Phenomenex Inc., Torrance, CA). The column was initially set to 110°C, and gradually increased to 200°C. Injector and detector temperatures were set to 250 and 350°C, respectively (Lourenco et al., 2016). Ammonia nitrogen concentrations were measured using the meta-phosphoric acid-2 ethyl butyrate method as described by Lourenco et al. (2016) using spectrophotometry at 625 nm (GENESYS 30 Visible Spectrophotometer; ThermoFisher Scientific, Waltham, MA).

Statistical Analysis

The experiment was analyzed using the PROC MIXED model procedure in SAS 9.4 (Cary, NC). Inoculant treatment, harvest time, and their interactions were considered fixed effects within each forage type (pure-stand alfalfa or alfalfa-bermudagrass mixture) and replication was considered the random effect. Mean separation was by Tukey's honest significant difference (HSD) test, with differences considered significant at $P \leq 0.05$ and tendency at $0.05 < P < 0.10$.

Project Objectives and Corresponding Results

Project Objectives

Assess the effects of applying an FAE-enhanced microbial inoculant compared to a conventional inoculant on alfalfa or alfalfa-bermudagrass silage in terms of:

1. fermentation characteristics,
2. forage nutritive value,
3. dry matter digestibility, and
4. ruminal fatty acid profiles

Project Results

Relative to a comparable conventional microbial inoculant, using the evaluated FAE-producing inoculants on pure- and mixed-stand alfalfa, each harvested at two time points during the growing season, did not result in any significant improvement in:

1. fermentation characteristics,
2. forage nutritive value,
3. dry matter digestibility, or
4. ruminal fatty acid profiles

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Results and Discussion

Environmental Data

Monthly precipitation and average maximum and minimum temperatures during the 2018 growing season and historical climate data from March through August for both study sites were acquired from the University of Georgia's Automated Environmental Monitoring Network (UGA-AEMN, 2018) located at each location (Table 2). Monthly average maximum and minimum temperatures were slightly greater in Tifton, GA than in Watkinsville, GA, which is typical for the two locations.

Table 2. Monthly rainfall (cm) and average maximum and minimum monthly temperature (°C), in comparison to 100-year average from March through November 2016-2018 at the University of Georgia Coastal Plain Experiment Station in Tifton, GA and the J. Phil Campbell Research and Extension Center in Watkinsville, GA.

	Rainfall ---cm---		Avg. Max Temp. ---C---		Avg. Min Temp. ---C---	
Month	**2018**	**100-yr avg**	**2018**	**100-yr avg**	**2018**	**100-yr avg**
			Tifton, GA			
March	8.6	12.2	19.9	21.2	7.5	8.2
April	7.0	9.9	23.4	25.4	10.9	12.1
May	17.6	8.2	30.0	29.3	18.9	16.5
June	15.0	11.7	32.2	32.0	21.7	20.2
July	14.9	13.8	31.8	32.8	22.4	21.5
August	24.2	12.4	32.4	32.7	22.1	21.3
			Watkinsville, GA			
March	10.8	13.4	16.3	18.2	4.7	4.6
April	14.7	10.3	20.9	23.2	7.7	8.8
May	15.2	10.3	28.3	27.1	17.0	13.5
June	21.3	9.9	31.3	30.6	20.0	17.9
July	9.3	11.4	30.9	32.0	21.0	19.9
August	10.6	10.1	30.8	31.4	20.2	19.5

Average maximum temperatures in 2018 were slightly below the 100-year average during March, April, July, and August and slightly above the 100-year average during May and June at both study sites. During May, the monthly average minimum temperature was above the 100-year average, but otherwise temperatures were comparable. Precipitation in both locations was well above the 100-year average. In Tifton, precipitation was almost double the normal monthly average in May and August. In Watkinsville, precipitation was slightly above average in April and May, and more than double than average in June.

Nutritive Value

Chemical composition of ALF and ABG forage treatments are presented in Table 3. Compositions of crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin, total digestible nutrients (TDN), ash, and calcium (Ca) were different ($P < 0.01$) between ALF and ABG forages. Concentrations of CP, ADF, lignin, and Ca were higher in ALF than ABG, which is to be expected based on differences in stand composition. It should be noted that Ca levels can affect

the buffering capacity of a forage and prevent the fermentation from lowering the pH as much as desired. This may have participated in the fermentation profile differences between the forage types, but would not be expected to affect the efficacy of the microbial inoculant treatments.

Due to the differences in stand type between ALF and ABG forages (pure-stand alfalfa vs grass-legume mix), ALF and ABG were treated with different strains of the FAE-producing inoculant based on recommendations of the manufacturer. Although the FAE-producing bacterial strain is present in both inoculants, other bacterial species that differ between the two products could inhibit the efficacy of FAE production in one product or the other (Muck et al., 2018). Therefore, results of this study are presented separately for each forage.

For the ALF silage, chemical composition was affected by the main effect of harvest, but not by the inoculant treatments or their interaction with the other factors. Crude protein, NDF, ADF, lignin, ethanol soluble carbohydrates (ESC), TDN, and ash were affected by harvest ($P < 0.01$), but not inoculant treatment. Crude protein and TDN concentrations were higher in the August harvest than in June while NDF, ADF, and lignin were lower. Pre-ensiling moisture and starch were not affected by inoculant, harvest, or their interaction.

For the ABG silage, chemical composition was affected by the main effects of harvest, inoculant treatment, and their interactions. Pre-ensiling moisture, CP, lignin, ESC, starch, and Ca were all affected by harvest ($P < 0.01$) and NDF, ADF, and TDN had a tendency ($P < 0.1$) to be affected harvest. Crude protein, ADF, lignin, and Ca concentrations were all greater in the June harvest compared with August, which likely indicates a greater proportion of alfalfa present in the stand during that harvest.

Levels of ADF and ESC in the ABG forage treatment were influenced by inoculant treatment ($P = 0.04$ and $P < 0.01$ for ADF and ESC, respectively). Acid detergent fiber concentration was higher ($P = 0.04$) in MI than CON, and MI+FAE tended to be higher than CON ($P = 0.09$); MI and MI+FAE were not different (378.6, 389.2, and 387.8 g kg^{-1} for CON, MI, and MI+FAE, respectively). Additionally, ESC was higher ($P < 0.01$) in CON than either the MI or MI+FAE inoculant (23.1 vs 16.0 vs 16.7 g kg^{-1} for CON, MI, and MI+FAE, respectively). Greater ESC post-fermentation suggests MI+FAE and MI- treated forages may have undergone a more extensive degree of fermentation than the untreated control. Guo et al. (2013) observed the same trend, where grass silage treated with a homo- and heterofermentative inoculant combination had lower NSC concentrations following a 60-day ensiling period compared with the untreated forage. Addah et al. (2011) also observed lower WSC and starch in post-fermentation samples treated with an FAE inoculant than in untreated forage; however, this comparison was made between untreated forage and an FAE product, therefore no conclusions can be drawn regarding the use of an FAE product and a similar combination inoculant with the FAE-producing capacity.

Fermentation Characteristics

Similar to nutritive value parameters, fermentation characteristics were analyzed separately by forage treatment to account for possible differences in the MI+FAE formulations used for each forage. Data are presented for inoculant treatments in each forage and across harvests in Table 4.

Table 3. Forage moisture (%) and chemical compositions (g kg^{-1}) of pure-stand alfalfa (ALF) and alfalfa-bermudagrass mixture (ABG) harvested in June and August 2018 as measured by commercial laboratory following a 60-day ensile and fermentation period.

	Month	Forage Treatment		SEM[1]	P-Value		
		ALF	ABG		Forage	Harvest	Forage*Harv
Moisture (%)	June	74.0	65.3	2.08	< 0.01	< 0.01	< 0.01
	August	74.0	55.4	2.08			
CP[2] (g kg^{-1})	June	179	150	2.73	< 0.01	0.29	< 0.01
	August	192	141	2.73			
NDF (g kg^{-1})	June	517	592	7.58	< 0.01	< 0.01	< 0.01
	August	464	605	7.58			
ADF (g kg^{-1})	June	448	389	5.81	< 0.01	< 0.01	< 0.01
	August	410	382	5.81			
TDN[3] (g kg^{-1})	June	537	567	4.4	< 0.01	< 0.01	< 0.01
	August	569	572	4.39			
Lignin (g kg^{-1})	June	111	73	2.29	< 0.01	< 0.01	0.04
	August	100	69	2.29			
ESC (g kg^{-1})	June	4.5	13.9	1.73	< 0.01	< 0.01	0.03
	August	10.5	24.3	1.47			
NSC (g kg^{-1})	June	6.6	27.5	2.59	< 0.01	< 0.01	< 0.01
	August	15.5	47.9	2.43			
Starch (g kg^{-1})	June	3.25	14.7	1.37	< 0.01	< 0.01	< 0.01
	August	6.29	23.7	1.34			
Ca (g kg^{-1})	June	15.6	7.7	0.24	< 0.01	< 0.01	0.04
	August	15.2	6.6	0.24			
Ash (g kg^{-1})	June	102	87	1.54	< 0.01	0.16	< 0.01
	August	109	84	1.53			

[1]Standard error of means (SEM) calculated at $P < 0.05$.
[2]Crude Protein (CP) = 6.25 x %N
[3]Total Digestible Nutrients (TDN) = (NFC x 0.98) + (CP x 0.87) + (FA x 0.97 x 2.25) + [NDFn x (NDFDp /100)] – 10.

Fermentation characteristics of ALF were not affected by harvest, treatment, or their interactions with the exception of propionic acid concentrations. Propionic acid was higher ($P = 0.02$) in MI+FAE than CON, with MI not different from either (3.0, 5.0, and 7.2 g kg^{-1} in CON, MI, and MI+FAE, respectively).

The pH and total VFA of ABG were also not influenced by harvest, inoculant, or their interactions, however the concentrations of individual acids assessed were affected. Unlike the ALF forage, propionic acid was higher ($P = 0.01$) in CON than MI+FAE (0.6 and 0.1 g kg^{-1} for CON and MI+FAE), while the MI treatment was intermediate and not different from either CON or MI+FAE

Table 4. Fermentation characteristics of pure-stand alfalfa (ALF) or alfalfa-bermudagrass mixture (ABG) harvested treated with either an untreated control (CON), microbial inoculant (MI), or microbial inoculant containing ferulic-acid esterase (MI+FAE) following a 60-day ensile and fermentation period as measured by commercial laboratory.

Forage		Inoculant Treatment			SEM[1]	P-Value
		CON	MI	MI+FAE		
ALF	pH	5.10	5.16	5.29	0.11	0.22
	Total VFA (g kg^{-1})	95.2	100.8	114.7	7.98	0.14
	Lactic Acid (g kg^{-1})	30.0	25.6	16.6	7.32	0.21
	Lactic:Total VFA	30.5	25.7	15.7	7.11	0.19
	Acetic Acid (g kg^{-1})	31.4	40.5	38.4	4.09	0.11
	Propionic Acid (g kg^{-1})	3.0^{b}	5.0^{ab}	7.2^{a}	0.93	0.02
ABG	pH	4.74	4.72	4.71	0.06	0.86
	Total VFA (g kg^{-1})	57.3	56.1	58.9	4.92	0.92
	Lactic Acid (g kg^{-1})	28.5^{a}	15.9^{b}	17.0^{b}	3.63	0.02
	Lactic:Total VFA	49.8^{a}	28.1^{b}	27.6^{b}	4.33	< 0.01
	Acetic Acid (g kg^{-1})	28.8^{b}	40.2^{a}	41.9^{b}	3.87	0.08
	Propionic Acid (g kg^{-1})	0.58^{a}	0.34^{ab}	0.16^{b}	0.08	0.01

[1]Standard error of means (SEM) and means without common superscript within the same row are considered different at $P < 0.05$.

(0.3 g kg^{-1}). Addah et al. (2011) found no differences in propionic acid between forages treated with or without an FAE-producing microbial inoculant. Further, the extremely low values of propionic acid in the ABG suggest these differences have few practical implications.

Lactic acid of ABG was higher in CON than either MI or MI+FAE (28.5 vs 15.9 vs 17.0 g kg^{-1}). The lactic:VFA ratio was also higher in the CON than MI or MI+FAE (49.8 vs 28.1 vs 27.6). Similar trends were observed by Addah et al. (2011) who found greater lactic acid production in an untreated control compared that treated with an FAE product. Further, Guo et al. (2013) also recorded a decrease in the lactic:VFA ratio in forages treated with a combination of *L. plantarum* and *L. buchneri* compared with forages not treated with an inoculant. Conversely, Lynch et al. (2014) found that FAE-treated forage was higher in lactic:VFA compared with an untreated control.

Inoculant treatments also tended to affect ($P = 0.08$) the concentrations of acetic acid in ABG. The MI and MI+FAE treatments produced greater acetic acid than the control (40.2 and 41.9 vs. 28.8 g kg^{-1} for MI, MI+FAE, and CON, respectively), and were not different from one another. It should be noted that although not significant, in the ALF treatment, lactic acid concentration was higher ($P = 0.21$) and acetic acid concentration was lower ($P = 0.11$) in CON (30.0 and 31.36 g kg^{-1} for lactic and acetic acids) than MI (25.6 and 40.5 g kg^{-1}) or MI+FAE (16.6 and 38.4 g kg^{-1}). The high concentrations of acetic acid in the MI and MI+FAE are likely because of the inclusion of the heterofermentative bacteria, *L. buchneri*, which produces high levels of acetic acid (Kung et al., 2003; Adesogan et al., 2014). Because the heterofermentative bacteria use lactic acid as a substrate to produce acetic acid, the *L. buchneri* in both MI and MI+FAE are likely the cause of both the low lactic and elevated acetic acid concentrations in treated forages.

In-Vitro Dry Matter Digestibility and Gas Production

Inoculant treatment did not affect IVDMD, gas production, NDF disappearance, or ADF disappearance of either forage (Table 5). The difference in IVDMD among the three inoculants was less than 3.5% in ALF and less than 2.5% in ABG. Additionally, neither IVDMD or gas production was influenced by harvest or the interaction of treatment and harvest. Aboagye et al. (2015) and Addah et al. (2011) reported improved animal performance and feed efficiency through the use of an FAE-containing product, but they also did not observe a significant improvement in IVDMD.

Rumen Fluid pH and Volatile Fatty Acid Profile

Inoculant treatment did not affect ruminal pH, total VFA, individual volatile fatty acids that were measured, the acetate:propionate ratio, or ammonia production of ALF (Table 5). Harvest influenced acetate concentrations and ammonia production and butyrate was influenced by the interaction of inoculant and harvest. Acetate concentration was higher ($P = 0.05$) in ALF harvested in June compared with August (43.7 vs 40.1 *m*M), but NH_3 production was higher ($P < 0.01$) in August-harvested forage (50.1 vs 47.2 *m*M for August and June, respectively).

None of the response variables in ABG were affected by inoculant treatment, harvest date, or their interaction (Table 5). To date, no other studies have looked at the effect of an FAE-producing microbial inoculant on gas production, rumen fluid pH, or VFA or ammonia production.

Conclusions

The use of microbial inoculants to improve fermentation and reduce forage losses through spoilage is promising, although research evaluating the use of microbial inoculants that include an FAE-producing bacterial strain have been inconclusive. In this study, silage made from pure- and mixed-stand alfalfa harvested at two time points during the growing season were generally improved by microbial inoculant addition in terms of fermentation profile, forage nutritive value, digestibility,

Table 5. *In-vitro* dry matter digestibility (IVDMD), rumen pH, gas production, acetate, propionate, butyrate, total volatile fatty acids (VFA), the ratio of acetate to propionate (A:P), and ammonia production as measured by gas chromatography at the University of Georgia ruminant nutrition laboratory from the alfalfa and alfalfa-bermudagrass mixture treated with either an untreated control (CON), microbial inoculant (MI), or microbial inoculant containing ferulic-acid esterase (MI+FAE) harvested in June and August 2018 following a 60-d ensile and fermentation and subjected to a 48-hr incubation and ruminal fermentation.

Alfalfa

	Inoculant					Harvest			
	CON	MI	MI+FAE	SEM[1]	P	June	August	SEM	P
IVDMD[2], (%)	53.5	51.7	50.0	1.52	0.22	50.7	52.7	1.31	0.22
Ruminal pH	6.61	6.63	6.62	0.01	0.31	6.62	6.62	0.004	0.32
Gas Production (mL g aDMD^{-1})	321	313	313	6.1	0.59	322	309	4.9	0.08
Acetate, (*m*M)	41.6	42.0	42.1	1.69	0.97	43.7	40.1	1.46	0.05
Propionate, (*m*M)	10.0	9.9	9.7	0.43	0.86	10.3	9.5	0.37	0.09
Butyrate, (*m*M)	7.3	7.4	8.0	0.28	0.08	7.5	7.6	0.26	0.92
Total VFAs, (*m*M)	64.9	64.9	65.8	2.38	0.93	67.2	63.2	2.06	0.10
A:P	4.1	4.3	4.3	0.06	0.10	4.3	4.2	0.05	0.48
NH_3	48.8	48.1	49.1	0.59	0.46	47.2[b]	50.1[a]	0.48	< 0.01

Alfalfa-Bermudagrass Mixture

	Inoculant					Harvest			
	CON	MI	MI+FAE	SEM	P	June	August	SEM	P
IVDMD, (%)	45.8	43.7	45.4	1.01	0.34	44.2	45.7	0.81	0.26
Ruminal pH	6.61	6.59	6.60	0.01	0.66	6.60	6.61	0.01	0.50
Gas Production (mL g aDMD^{-1})	362	367	367	7.4	0.84	358	372	6.1	0.11
Acetate, (*m*M)	41.4	39.7	42.4	1.78	0.58	42.8	39.5	1.44	0.14
Propionate, (*m*M)	10.4	10.1	10.8	0.46	0.62	10.9	9.9	0.38	0.08
Butyrate, (*m*M)	7.0	6.7	7.0	0.24	0.50	7.0	6.8	0.20	0.33
Total VFAs, (*m*M)	64.3	61.9	65.8	2.56	0.56	66.3	61.7	2.09	0.14
A:P	4.0	3.9	3.9	0.05	0.53	3.9	4.0	0.05	0.30
NH_3	48.1	48.6	49.9	0.70	0.27	48.8	49.0	0.52	0.87

[1] Standard error of means (SEM) and letters without common superscript within row represent differences at $P < 0.05$.

[2] *In-vitro* dry matter digestibility (IVDMD) (%) was calculated following a 48-hr incubation and fermentation period.

but not ruminal fatty acid profiles. However, the FAE-producing inoculants did not perform better than the comparable non-FAE producing microbial inoculants. Based on our results, the FAE-producing inoculant appears unlikely to improve fermentation, nutritive value, or forage digestibility compared with a similar microbial inoculant product without the capacity for FAE production.

Acknowledgments

Funding for this study was provided by the U.S. Alfalfa Farmer Research Initiative of the National Alfalfa & Forage Alliance.

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