Effect of adding cofactors to exogenous fibrolytic enzymes on preingestive hydrolysis, in vitro digestibility, and fermentation of bermudagrass haylage

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ABSTRACT

Our objectives were to examine if adding metal ion cofactors (COF) to exogenous fibrolytic enzymes (EFE) would increase the beneficial effects of the EFE on the preingestive hydrolysis and in vitro digestibility and fermentation of bermudagrass haylage. In experiment 1, 5 COF (Mn^{2+} , Co^{2+} , Fe^{2+} , Ca^{2+} , and Mg^{2+}) were screened to select the best candidates for synergistically enhancing release of water-soluble carbohydrates (WSC) from bermudagrass haylage by 5 EFE. The 5 EFE (1A, 2A, 11C, 13D, and 15D) were sourced from *Trichoderma reesei* and *Aspergillus oryzae* and they were the most effective of 12 EFE at increasing the neutral detergent fiber digestibility of bermudagrass haylage in a previous trial. Adding 1 mM of each of the COF to EFE 2A or 11C synergistically increased release of WSC from bermudagrass haylage, as did adding (1 mM) Fe^{2+} to 1A, Mn^{2+} , Co^{2+} , or Fe^{2+} to 13D, or Co^{2+} or Fe^{2+} to 15D. The greatest release of WSC responses were obtained by adding Mn^{2+} to 11C (38%) or by adding Fe^{2+} to 2A or 13D (10 and 21.9%, respectively). In experiment 2, the effect of increasing the COF dose on in vitro digestibility and fermentation of bermudagrass haylage was examined using the best EFE-COF combinations from experiment 1. Effects of adding increasing doses of these COF on EFE-mediated changes in vitro digestibility depended on the COF-EFE combination. Adding 10 mM Mn^{2+} alone to bermudagrass haylage increased DMD and NDFD by 2.7 and 6.3% and adding 11C alone increased these measures by 6.6 and 15.5%, respectively. However, adding 10 mM Mn^{2+} with 11C resulted in 3.5 and 8.1% increases in DMD and NDFD, respectively, beyond the increases caused by adding 11C alone. Adding Fe^{2+} to 2A had no effects on EFE-mediated digestibility responses, but 2A prevented adverse effects of adding Fe^{2+} alone on DMD and NDFD. In contrast, adding Fe^{2+} to 13D reduced the increases

in DMD and NDFD caused by adding the EFE alone. This study shows that adding COF to EFE can synergistically increase, decrease, or not affect the hydrolytic effects of EFE on bermudagrass haylage cell walls. The outcome depends on the specific EFE-COF combination and the COF dose. More research is required to understand the mechanisms resulting in these outcomes to exploit beneficial effects of COF on EFE.

Key words: fibrolytic enzyme, dairy cattle, bermudagrass, cofactor

INTRODUCTION

Exogenous fibrolytic enzymes (EFE) have been applied to dairy cattle diets to improve animal performance because of their ability to catalyze the depolymerization of forage fiber, which is the main barrier to nutrient availability (Meale et al., 2014). However, the results of EFE application in the literature have been equivocal (Beauchemin and Holtshausen, 2010; Adesogan et al., 2014). This is because the outcome is influenced by numerous factors including the EFE dose (Eun et al., 2007) and activity composition (Eun and Beauchemin, 2007), the prevailing pH and temperature (Arriola et al., 2011), the animal performance level (Schingoethe et al., 1999), the experimental design (Adesogan et al., 2014), and the fraction and proportion of the diet to which the EFE is applied (Krueger et al., 2008; Dean et al., 2013). Despite their well-known effects on the activity of certain enzymes (Voet et al., 2010), effects of metal ion cofactor (COF) addition to EFE on the digestibility of forages are unknown. Metal-activated enzymes and metalloenzymes use COF for structural and catalytical roles, respectively (Voet et al., 2010). Metal-activated enzymes require COF for maximal activity because they improve the conformational stability of the enzymes, but the COF are not direct components of the enzyme reaction mechanism (Glusker, 2011). Consequently, metal-activated enzymes can function without COF at a reduced activity level (Glusker, 2011). Whereas metalloenzymes require COF at their active sites to function because they serve as substrate templates, inducers of free radicals, and

Received September 12, 2014.

Accepted March 20, 2015.

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redox-active COF (Purich, 2011). Among enzymes involved in lignocellulose degradation, only a few, mostly those grouped in the auxiliary activities family, have been identified as metalloenzymes (Harris et al., 2010; CAZy, 2013). Metal ion cofactors such as Mn^{2+} , Co^{2+} , Fe^{2+} , Ca^{2+} , and Mg^{2+} have increased the activity of metal-activated fibrolytic enzymes (BRENDA, 2013), but to our knowledge their effects on EFE used in ruminant nutrition have not been examined.

The productivity of dairy cattle in the southeastern United States is limited by the low digestibility of tropical grasses such as bermudagrass (*Cynodon dactylon*), which is the most widely grown perennial grass in the south (Newman, 2007). Recent research identified 5 EFE that improved the NDF digestibility (NDFD) of bermudagrass haylage and optimized their dose rates (Romero et al., 2015a,b). Whether adding COF to these EFE would synergistically increase their hydrolytic effects is unknown.

The current study is the third of a series of 4 studies aimed at strategic development of EFE that would increase the performance of dairy cows fed tropical forage diets. The first study tested effects of 12 EFE on the preingestive hydrolysis, NDFD, and fermentation of bermudagrass haylage, identified the 5 most-promising EFE, and characterized proteomic differences between the most- and least-effective EFE (Romero et al., 2015a). The second study determined the optimal dose rates of the 5 EFE (Romero et al., 2015b). The final study examined effects of adding the best EFE and dose combination identified in previous studies on the performance of dairy cows (Romero et al., 2014). The objectives of the present study were to examine if adding COF to EFE would increase the beneficial effects of the EFE on the preingestive hydrolysis and in vitro digestibility and fermentation of bermudagrass haylage. The hypothesis was that adding key COF to the EFE would increase their preingestive hydrolytic effects. Furthermore, adding key COF at an appropriate dose would synergistically increase the NDFD and fermentation of EFE-treated bermudagrass haylage.

MATERIALS AND METHODS

Bermudagrass Substrate

An established stand of bermudagrass cultivar Tifton 85 in Alachua County, Florida, was harvested, ensiled, dried, ground, and chemically characterized as described by Romero et al. (2015b). The bermudagrass haylage also contained 0.36 and 0.27% water-soluble calcium and magnesium, and 24.1, 64.3, and 0.17 mg/L of water-soluble iron, manganese, and cobalt, respectively, as determined by inductively coupled plasma

spectrometry (Belicieu et al., 2012) after microwave digestion (CEM Corp., Matthews, NC) at the Dairy One Forage Laboratory, Ithaca, New York.

EFE

Five previously selected (Romero et al., 2015a) commercial and experimental EFE that were evaluated by Romero et al. (2015b) were used. Their enzymatic activities, protein concentrations, densities, forms, mineral concentrations, application rates, and biological sources are listed in Table 1. The EFE application rates were chosen because they had optimized the NDFD of bermudagrass haylage in a previous study (Romero et al., 2015b). The mineral concentrations of the EFE were determined using the methods of Belicieu et al. (2012).

Screening COF for Synergistic Effects on the Hydrolytic Potential of EFE (Experiment 1)

An experiment was carried out to determine the effects that each of 5 COF had on EFE hydrolysis of bermudagrass haylage cell walls before consumption by animals. The quantity of water-soluble carbohydrates (WSC) released during preingestive hydrolysis of bermudagrass haylage was the primary measure of the hydrolytic potential of the EFE and COF. The preingestive hydrolysis procedure described by Krueger and Adesogan (2008) was used except that the EFE were diluted in nanopure water (2 mL) instead of citrate-phosphate buffer to avoid the potential chelating effects of citrate. Metal ion cofactors used were chloride salts of divalent cations of manganese, cobalt, iron, calcium, and magnesium. These COF were chosen because they had increased the activity of fibrolytic enzymes on pure substrates (BRENDA, 2013). Units for COF application rates are given in millimolar based on the convention in enzymology (BRENDA, 2013). Each COF was added to the EFE solution to achieve a final concentration of 1 mM (Lai et al., 2009) and sodium azide was added (0.02% wt/vol) to prevent substrate degradation by microbes (Krueger and Adesogan, 2008). The EFE-COF solution was added to 50-mL tubes containing 0.5 g of bermudagrass haylage and the tubes were incubated for 24 h at 25°C in quadruplicate. For each EFE, 2 blank tubes without substrate were included to correct for contributions from the EFE. After the incubation, 15 mL of nanopure water was added and the suspension was shaken (Eberbach reciprocating shaker, model 6000, Eberbach Corporation, Ann Arbor, MI) for 1 h at 260 oscillations/min to extract water-soluble compounds, filtered through previously dried (60°C for 48 h) and weighed 125-mm Whatman

Table 1. Enzymatic activities measured at 20 and 39°C, protein concentration, form, density, application rate, mineral concentration, and biological source of exogenous fibrolytic enzyme (EFE) preparations used (adapted from Romero et al., 2014b)

Parameter	EFE				
	1A	2A	11C	13D	15D
Activity at 20°C					
Endoglucanase ¹	885	1,654	1,019	100	88
Xylanase ¹	1,313	28,800	1,815	105	3,640
Exoglucanase ¹	0.76	0.59	0.49	0.38	0.36
β-glucosidase ¹	3.4	10.1	3.9	1.0	0.02
Ferulic acid esterase ²	1.14	1.46	2.91	2.35	1.20
Activity at 39°C					
Endoglucanase	1,693	3,624	1,506	286	70
Xylanase	1,276	29,301	1,703	86	6,499
Exoglucanase	1.68	0.84	0.97	0.29	0.29
β-glucosidase	10.1	11.7	12.7	1.9	0.1
Ferulic acid esterase	2.18	1.46	6.30	2.35	2.57
Protein ³	65.3	111.1	81.1	18	28.3
Form	Liquid	Liquid	Liquid	Liquid	Liquid
Density ⁴	1.19	1.20	1.03	1.06	1.11
Application rate ⁵	4.66	4.66	10.4	7.8	7.8
Calcium ⁶	90.6	108.4	83.1	139.3	91.0
Magnesium ⁶	62.1	94.2	42.6	28.4	24.8
Iron ⁶	4.02	4.66	1.40	0.30	0.48
Manganese ⁶	0.65	0.42	0.31	0.07	0.07
Cobalt ⁶	0.033	0.028	0.024	0.026	0.018
Source	<i>Trichoderma reesei</i>	<i>T. reesei</i>	<i>T. reesei</i>	<i>Aspergillus oryzae</i>	<i>A. oryzae</i>

¹μmol of sugar released/min per gram of EFE (Wood and Bhat, 1988).²nmol of ferulic acid released/min per gram of EFE (Lai et al., 2009).³mg/g of EFE (Bradford, 1976).⁴g/mL of EFE.⁵g of EFE/kg of bermudagrass DM.⁶mg/L of EFE (Beliciu et al., 2012).

451 filter paper (Fisher Scientific, Pittsburgh, PA), and filtrate samples were frozen (−20°C). Residues were dried at 60°C for 48 h in a forced-draft oven, weighed, and analyzed sequentially for NDF (Van Soest et al., 1991) and ADF (AOAC International, 2000) using an Ankom 200 Fiber Analyzer (Ankom, Macedon, NY), as described by Krueger and Adesogan (2008). Amylase was used for NDF determination but no sodium sulfite was added. The NDF and ADF results were expressed inclusive of residual ash. Hemicellulose was estimated as the difference between NDF and ADF. Residue and sample weights and DM concentrations were used to calculate DM losses. Filtrate samples were thawed and analyzed for WSC (DuBois et al., 1956) and ferulic (FER) and *p*-coumaric acids (Bio-Rad, 2011) using a Merck Hitachi Elite LaChrome HPLC system (Hitachi, L2400, Tokyo, Japan) and a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA).

Effects of Adding Increasing Doses of COF to EFE on In Vitro Digestibility (Experiment 2)

The most promising EFE-COF combinations selected in experiment 1 (Mn²⁺ + EFE 11C and Fe²⁺ +

EFE 2A or 13D) were evaluated with a 24-h in vitro ruminal digestibility assay. The COF were added to the EFE solution at doses of 0, 0.1, 1, and 10 mM in quadruplicate. The experimental procedure was similar to that for experiment 1 except that after the 24 h of incubation at 25°C, buffered-ruminal fluid (52 mL) was added and the suspension was incubated for 24 h at 39°C (Goering and Van Soest, 1970). The fermentation was stopped by placing the tubes on ice and contents of tubes were filtered through preweighed 125-mm Whatman 451 paper (Fisher Scientific). Filtrate samples were immediately analyzed for pH using an Accumet XL25 pH meter with an automatic temperature correction feature (Fisher Scientific), acidified with 50% H₂SO₄ (1% vol/vol of rumen fluid sample), centrifuged at 8,000 × *g* for 20 min at 4°C and the supernatant was frozen (−20°C). Residues were dried at 60°C for 48 h, weighed, and stored at room temperature for subsequent analysis. No sodium azide was used in the EFE solution and the control treatment consisted of only nanopure water. For each EFE treatment, 2 blank tubes without substrate were included to correct for the fermentation residues resulting directly from the inoculum. The ruminal fluid was representatively collected 3 h after

feeding at 0800 h by aspiration from 2 nonlactating, nonpregnant, ruminally cannulated Holstein cows fed coastal bermudagrass hay ad libitum supplemented with corn (0.5 kg), cottonseed hulls (0.5 kg), soybean meal (1 kg), and a vitamin-mineral mix (37.5 g). The ruminal fluid collection protocol was approved by the University of Florida, Institute of Food and Agricultural Sciences Animal Research Committee. Ruminal fluid was filtered through 4 layers of cheesecloth and flushed with CO₂ before use. All tubes and the artificial saliva were prewarmed at 39°C and artificial saliva was flushed with CO₂ before ruminal fluid addition. The concentrations of iron and manganese in the mixed rumen fluid inoculum from the 2 donor cows were 0.59 and 0.01 mg/L, respectively. These were measured as described by Belicieu et al. (2012). Concentrations of minerals in rumen fluid including contributions from COF are expressed as milligrams per liter based on the convention in animal science studies. Dried residues were analyzed for NDF and ADF, and hemicellulose was calculated as previously described. Residue and original sample weights and their DM and fiber concentrations were used to calculate true DM, NDF, hemicellulose, and ADF digestibility (**DMD**, **NDFD**, **HEMD**, and **ADFD**, respectively; Mertens, 2007). Filtrate samples were analyzed for concentrations of VFA (Muck and Dickerson, 1988) with the HPLC system described previously. Ammonia-N was determined with a Technicon Auto Analyzer (Technicon, Tarrytown, NY) and an adaptation of the Noel and Hambleton (1976) procedure that involved colorimetric N quantification.

Statistical Analyses

Experiment 1 was analyzed as a completely randomized design with a 6 (COF) × 6 (EFE) factorial treatment arrangement and 4 replicates per treatment. The model used to analyze the preingestive hydrolysis data was:

$$Y_{ijk} = \mu + T_i + C_j + TC_{ij} + E_{ijk},$$

where μ = general mean; T_i = effect of EFE i ; C_j = effect of COF j ; TC_{ij} = effect of the EFE i × COF j interaction; and E_{ijk} = experimental error.

The GLM procedure of SAS v.9.1 (SAS Institute, 2003) was used to analyze the data. The objective was to examine effects of COF on specific EFE rather than whether COF addition would make certain EFE better than others. Therefore, Fisher's F-protected least significance difference test was only used to compare COF means within each EFE. Experiment 2 was analyzed as a randomized complete block design with a 2 (EFE) × 4 (COF dose) factorial treatment arrangement and 2

runs each with 4 replicates/treatment. The model used to analyze digestibility and fermentation data was:

$$Y_{ijk} = \mu + T_i + D_j + TD_{ij} + TR_{ik} + DR_{ik} + TDR_{ijk} + E_{ijk},$$

where μ = general mean; T_i = effect of EFE i ; D_j = effect of COF dose j ; R_k = effect of run k ; TD_{ij} = effect of the EFE i × COF dose j interaction; TR_{ik} = effect of the EFE i × run k interaction; DR_{ik} = effect of the COF dose j × run k interaction; TDR_{ijk} = effect of the EFE i × COF dose j × run k interaction; and E_{ijk} = experimental error.

Data from each EFE were analyzed separately with the GLM procedure of SAS. The model included COF dose, EFE, and their interaction. Polynomial contrasts were used to determine dose effects and the Fisher's least significance difference test was used to compare least squares means across doses. The coefficients used in these contrast statements for the unequally spaced COF doses were generated with the IML procedure of SAS. Data were tested for normality using the Shapiro-Wilk test, which revealed that the data were normally distributed. Significance was declared at $P \leq 0.05$.

RESULTS AND DISCUSSION

Experiment 1: Effects of COF Addition on Preingestive Hydrolysis

Applying each COF alone had no effect ($P > 0.05$) on release of WSC (Table 2). However, release of WSC was increased ($P < 0.05$) by applying EFE 1A, 2A, 11C, or 15D alone by 96.3, 238, 83.7, and 35.3%, respectively, but not by applying 13D alone. Effects of COF addition to EFE on release of WSC depended on the EFE-COF combination ($P < 0.001$). Release of WSC was increased ($P < 0.05$) by adding Fe²⁺ to 1A (by 4.83%), by adding Mg²⁺, Ca²⁺, Fe²⁺, Co²⁺, or Mn²⁺ to 2A (by 3.27, 2.64, 9.64, 5.75, and 5.29%, respectively), by adding Mg²⁺, Ca²⁺, Fe²⁺, Co²⁺, or Mn²⁺ to 11C (by 13.18, 14.61, 23.50, 23.21, and 38.4%, respectively), by adding Fe²⁺, Co²⁺, or Mn²⁺ to 13D (by 21.88, 14.06, and 13.02%, respectively), and by adding Fe²⁺ or Co²⁺ to 15D (13.62 and 7.39%, respectively).

Increases in release of WSC due to adding COF to EFE are likely to be the result of the stimulation of metal-activated enzymes rather than metalloenzymes, as almost all fiber-degrading glycoside hydrolyses catalyze acid-base reactions, which do not require the presence of a COF for catalytic activity (Harris et al., 2010). The only cellulolytic enzyme known to require a COF for hydrolysis (metalloenzyme) is polysaccharide monooxygenase, which requires copper and catalyzes

Table 2. Effects of adding metal ion cofactors (COF), to exogenous fibrolytic enzymes (EFE) on preingestive hydrolysis of a 4-wk regrowth of bermudagrass haylage (experiment 1)

Treatment	DM loss (% of DM)	NDF (% of DM)	Hemicellulose (% of DM)	ADF (% of DM)	WSC ¹ (% of DM)	FER ² (µg/g of DM)	<i>p</i> -coumaric acid (µg/g of DM)
No EFE							
No COF	17.3 ^{ab, z}	71.1 ^{a, z}	35.4 ^{a, z}	35.7 ^{bc, z}	1.90 ^{ab, z}	158 ^{a, z}	150 ^{a, y}
Mn ²⁺	17.2 ^a	70.4 ^a	35.4 ^a	35.0 ^a	2.06 ^b	170 ^{ab}	174 ^b
Co ²⁺	17.0 ^a	71.0 ^a	35.3 ^a	35.7 ^c	2.06 ^b	180 ^b	178 ^b
Fe ²⁺	17.8 ^b	70.0 ^a	34.9 ^a	35.1 ^{ab}	1.95 ^{ab}	174 ^b	168 ^b
Ca ²⁺	17.2 ^a	70.3 ^a	34.7 ^a	35.6 ^{ab}	1.99 ^b	183 ^b	152 ^a
Mg ²⁺	17.6 ^{ab}	70.9 ^a	35.2 ^a	35.6 ^{bc}	1.80 ^a	158 ^a	170 ^b
SEM ³	0.21	0.40	0.34	0.20	0.059	5.2	3.8
EFE 1A							
1A	18.4 ^{bc, y}	68.3 ^{a, x}	33.5 ^{a, x}	34.8 ^{ab, y}	3.73 ^{ab, w}	179 ^{b, y}	159 ^{bc, xy}
1A + Mn ²⁺	18.3 ^{bc}	67.8 ^a	33.4 ^a	34.4 ^a	3.77 ^{abc}	194 ^c	165 ^{bc}
1A + Co ²⁺	18.6 ^{cd}	68.5 ^a	33.6 ^a	34.9 ^{ab}	3.88 ^{bc}	182 ^{bc}	166 ^c
1A + Fe ²⁺	19.1 ^{de}	67.3 ^a	32.8 ^a	34.5 ^a	3.91 ^c	177 ^b	156 ^{bc}
1A + Ca ²⁺	19.3 ^e	67.1 ^a	32.6 ^a	34.5 ^a	3.72 ^{ab}	180 ^b	155 ^b
1A + Mg ²⁺	18.0 ^{abc}	68.6 ^a	33.4 ^a	35.2 ^b	3.67 ^a	162 ^a	131 ^a
SEM ³	0.21	0.40	0.34	0.20	0.058	5.2	3.8
EFE 2A							
2A	20.0 ^{a, x}	64.9 ^{a, w}	29.8 ^{a, w}	35.0 ^{ab, y}	6.43 ^{a, v}	367 ^{d, v}	239 ^{d, v}
2A + Mn ²⁺	20.3 ^a	64.9 ^a	29.6 ^a	35.3 ^{ab}	6.77 ^c	334 ^c	195 ^b
2A + Co ²⁺	21.5 ^b	63.5 ^a	28.8 ^a	34.7 ^a	6.80 ^c	393 ^e	225 ^c
2A + Fe ²⁺	21.1 ^b	64.7 ^a	29.0 ^a	35.0 ^{ab}	7.05 ^d	305 ^b	184 ^b
2A + Ca ²⁺	20.6 ^{ab}	64.3 ^a	28.8 ^a	35.5 ^b	6.60 ^b	414 ^f	256 ^e
2A + Mg ²⁺	21.1 ^b	64.0 ^a	28.7 ^a	35.3 ^b	6.64 ^{bc}	271 ^a	156 ^a
SEM ³	0.21	0.40	0.34	0.20	0.058	5.2	3.8
EFE 11C							
11C	19.4 ^{c, x}	68.0 ^{a, x}	33.0 ^{a, x}	35.0 ^{ab, y}	3.49 ^{a, x}	211 ^{b, x}	162 ^{ab, x}
11C + Mn ²⁺	18.6 ^b	67.8 ^a	32.9 ^a	34.9 ^a	4.83 ^d	205 ^{ab}	170 ^{bc}
11C + Co ²⁺	18.6 ^b	68.2 ^a	33.5 ^a	34.7 ^a	4.30 ^c	196 ^a	163 ^{ab}
11C + Fe ²⁺	18.5 ^b	68.8 ^a	33.6 ^a	35.2 ^{ab}	4.31 ^c	192 ^a	156 ^a
11C + Ca ²⁺	18.7 ^b	67.6 ^a	32.9 ^a	34.7 ^a	4.00 ^b	198 ^{ab}	174 ^c
11C + Mg ²⁺	17.8 ^a	68.9 ^a	33.3 ^a	35.6 ^b	3.95 ^b	211 ^b	176 ^c
SEM ³	0.21	0.40	0.34	0.20	0.058	5.2	3.8
EFE 13D							
13D	17.4 ^{abc, z}	70.9 ^{a, z}	35.4 ^{a, z}	35.5 ^{ab, z}	1.92 ^{a, z}	150 ^{a, z}	140 ^{b, z}
13D + Mn ²⁺	17.3 ^{abc}	70.2 ^a	34.8 ^a	35.4 ^{ab}	2.17 ^b	162 ^a	161 ^c
13D + Co ²⁺	16.9 ^a	71.2 ^a	35.5 ^a	35.7 ^b	2.19 ^{bc}	154 ^a	162 ^c
13D + Fe ²⁺	17.4 ^{abc}	69.9 ^a	34.6 ^a	35.4 ^{ab}	2.34 ^c	152 ^a	163 ^c
13D + Ca ²⁺	17.4 ^{abc}	70.3 ^a	35.3 ^a	35.0 ^a	2.01 ^{ab}	148 ^a	183 ^d
13D + Mg ²⁺	17.6 ^{bc}	69.7 ^a	34.4 ^a	35.3 ^{ab}	1.94 ^a	160 ^a	129 ^a
SEM ³	0.21	0.40	0.34	0.20	0.06	5.2	3.8
EFE 15D							
15D	17.4 ^{abc, z}	69.5 ^{a, y}	33.8 ^{a, y}	35.7 ^{ab, z}	2.57 ^{ab, y}	242 ^{d, w}	210 ^{e, w}
15D + Mn ²⁺	17.5 ^{abc}	69.8 ^a	34.6 ^a	35.2 ^a	2.70 ^{bc}	225 ^c	176 ^d
15D + Co ²⁺	17.9 ^{cd}	69.6 ^a	33.8 ^a	35.8 ^b	2.76 ^{cd}	196 ^b	159 ^b
15D + Fe ²⁺	17.9 ^{cd}	69.6 ^a	34.0 ^a	35.6 ^{ab}	2.92 ^d	204 ^b	167 ^{cd}
15D + Ca ²⁺	18.3 ^d	69.4 ^a	33.6 ^a	35.8 ^{ab}	2.62 ^{abc}	222 ^c	160 ^{bc}
15D + Mg ²⁺	18.0 ^{cd}	69.8 ^a	34.4 ^a	35.4 ^{ab}	2.51 ^a	174 ^a	139 ^a
SEM ³	0.21	0.40	0.34	0.20	0.06	5.3	3.8
<i>P</i> -value							
EFE	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
COF	0.005	0.101	0.103	0.047	<0.001	<0.001	<0.001
EFE × COF	<0.001	0.066	0.225	0.014	<0.001	<0.001	<0.001

^{a-c}Within EFE treatments, means in a column with different superscripts differed ($P < 0.05$).^{w-z}For EFE to which no COF were added, means in a column with different superscripts differed ($P < 0.05$).¹WSC = water-soluble carbohydrates.²FER = ferulic acid.³The SEM value is the highest among those for the specific EFE treatment in the column.

oxidation-reduction reactions (Quinlan et al., 2011). This enzyme was identified as one of the main activities in EFE 2A and 11C using isobaric tags for relative

quantification-based quantitative proteomics combined with mass spectrometry (Romero et al., 2015a). To our knowledge, this is the first report of the effects of

COF addition to EFE on release of WSC of forage cell walls, though others have investigated effects of COF addition to fibrolytic enzymes on hydrolysis of pure substrates (Tejirian and Xu, 2010) or steam-pretreated biofuel biomass (Bin and Hongzhang, 2010).

Singh et al. (1990) reported that activity of exoglucanase II (E.C. 3.2.1.91, which acts on the nonreducing end of cellulose) from *Aspergillus niger* was increased 1.82-, 1.51-, 1.40-, and 1.85-fold when undisclosed doses of Mn^{2+} , Ca^{2+} , Mg^{2+} , or Co^{2+} were added, respectively. Exoglucanases account for up to 80% of the secretome of *Trichoderma reesei* under cellulose-inducing conditions (Glass et al., 2013), and this activity is critical for maximum saccharification. Furthermore, *T. reesei* endoglucanase activity (E.C. 3.2.1.4) on carboxymethyl cellulose was increased by adding undisclosed doses of Mn^{2+} , Fe^{2+} , and Co^{2+} , but inhibited by Ca^{2+} and Mg^{2+} (Saad and Fawzi, 2004). Also, adding 5 mM Mn^{2+} to β -glucosidase (E.C. 3.2.1.21) from *A. oryzae* resulted in a 1.8-fold increase in activity but adding 5 mM Fe^{3+} resulted in a 7.7-fold decrease in activity (Riou et al., 1998). Metal ion cofactor addition also has increased or inhibited the activity of hemicellulolytic enzymes. For instance, John and Schmidt (1988) reported that the activity of β -xylosidase (EC 3.2.1.37) from *Trichoderma viride* was increased by adding 1 mM Fe^{2+} or Mn^{2+} , but inhibited by adding 0.1 mM Ca^{2+} . The EFE tested in the present study contained various fibrolytic activities; hence, it is challenging to identify which specific activities benefited from COF addition. Nevertheless, it is noteworthy that Fe^{2+} addition consistently improved release of WSC by all the EFE. This may be because Fe^{2+} increased the activities of β -xylosidase and endoglucanase, as both of these were likely present in the EFE and both have been increased by Fe^{2+} addition in previous research (John and Schmidt, 1988; Saad and Fawzi, 2004). The greatest increase in release of WSC occurred when Fe^{2+} was added to 11C, perhaps because 11C had one of the lowest iron-to-protein ratios (0.017 vs. 0.030 mg of iron/g of protein) and the lowest iron-to-endoglucanase ratio relative to the other EFE (0.0009 vs. 0.003 mg of iron/mmol of sugar released per minute). Adding Co^{2+} also increased release of WSC by all EFE except 1A. This agrees with previous work that indicated adding Co^{2+} increased exoglucanase (Singh et al., 1990) and endoglucanase activities (Saad and Fawzi, 2004), which determine the rate of cellulose digestion (Zhang and Lynd, 2004). The synergistic increase in release of WSC due to adding Co^{2+} to EFE was greatest for 11C, probably because it had one of the lowest cobalt-to-protein ratios (0.0003 vs. 0.0006 mg of cobalt/g of protein). Adding Mn^{2+} to 2A, 11A, and 13D resulted in 1.05-, 1.38-, and 1.13-fold increases in release of WSC. This

agrees with studies showing that adding Mn^{2+} increased the activity of exoglucanases (Singh et al., 1990), endoglucanases (Saad and Fawzi, 2004), β -glucosidase (Riou et al., 1998), and β -xylosidase (John and Schmidt, 1988). That Mn^{2+} increased the activity of several fibrolytic enzymes explains why its greatest effects were on 11C and 2A, which had among the greatest total cellulolytic activities and lowest manganese-to-protein ratios (0.004 and 0.003 vs. 0.005 mg of manganese/g of protein), respectively. Adding Mg^{2+} or Ca^{2+} only improved release of WSC by 2A and 11C, reflecting their low ratios of magnesium (0.71 and 0.51 vs. 1.03 mg of magnesium/g of protein, respectively) or calcium (0.82 and 1.00 vs. 3.80 mg of calcium/g of protein) to protein relative to the other EFE. Also, because they are macrominerals, the concentrations of these COF in the EFE were at least 10 times greater than those of the other COF, which are microminerals.

Compared with the respective controls, applying each COF alone had no effect ($P > 0.05$) on DM loss or hydrolysis of NDF, ADF, or hemicellulose. The only exception was that applying Mn^{2+} reduced ($P < 0.05$) the ADF concentration by 1.96% but the corresponding increase in WSC concentration was not statistically significant ($P > 0.05$).

Applying EFE 1A, 2A, 11C, or 15D alone increased ($P < 0.05$) NDF hydrolysis by 3.94, 8.72, 4.36, and 2.25%, respectively, whereas applying 13D alone did not. Effects of EFE addition on NDF hydrolysis tended ($P = 0.066$) to be influenced by COF addition. Theoretically, most WSC released during cell wall saccharification by EFE should have been generated by NDF hydrolysis, thus NDF values were expected to decrease as WSC concentrations increased due to saccharification. However, NDF analysis is less precise than many other chemical components of forages, such as WSC, as evidenced by the greater coefficient of variation recommended for NDF assays versus those of other forage chemical components (Galyean, 2010). The variability in the NDF concentrations in our study (SEM = 0.46% units of bermudagrass haylage DM) was identical to the average increase in WSC due to COF addition, thus preventing detection of differences in NDF due to saccharification. In theory, using the Uppsala dietary fiber scheme (Theander, 1995) instead of the NDF scheme to evaluate EFE effects on cell wall saccharification may be more appropriate because it quantifies individual cell wall polysaccharides rather than detergent soluble or insoluble fiber fractions. However, the Uppsala dietary fiber results are less precise than those from the NDF scheme (SD of 3.2 vs. 1.3%, respectively; Mertens, 2003).

Applying EFE 1A, 2A, 11C, or 15D alone reduced ($P < 0.05$) hemicellulose concentration by 5.37, 15.82, 6.78,

and 4.52%, respectively, whereas applying 13D alone did not. Effects of EFE addition on hemicellulose were not influenced by COF addition ($P = 0.225$). In our previous studies, hemicellulose was the fraction mostly hydrolyzed by EFE (Romero et al., 2015a,b). The structure of xylan is more complex than that of cellulose due to its substitutions, which necessitate a greater variety of enzymes with different specificities for its degradation. However, xylan does not form tightly packed crystalline structures as cellulose does and is, thus, more accessible to enzymatic hydrolysis (Saha, 2003).

Applying EFE 1A, 2A, or 11C alone reduced ($P < 0.05$) ADF concentration by 2.52, 1.96, and 1.96%, respectively, whereas applying 13D and 15D alone did not. Effects of COF addition to EFE on ADF hydrolysis depended on the EFE-COF combination ($P < 0.05$), yet adding COF did not affect ADF hydrolysis by any EFE.

Applying EFE 1A, 2A, or 11C alone increased ($P < 0.05$) DM loss by 6.36, 15.6, and 12.14%, respectively, whereas applying 13D or 15D alone did not. Effects of COF addition to EFE on DM loss depended on the EFE-COF combination ($P < 0.001$). Dry matter loss was increased ($P < 0.05$) by adding Ca^{2+} and Fe^{2+} to 1A (4.89 and 3.80%, respectively); Mg^{2+} , Fe^{2+} , and Co^{2+} to 2A (5.50, 5.50, and 7.50%, respectively); Mg^{2+} , Ca^{2+} , Fe^{2+} , Co^{2+} , and Mn^{2+} to 11C (8.25, 3.61, 4.64, 4.12, and 4.12%, respectively). No changes were observed when COF were added to EFE 13D and 15D.

Surprisingly, adding Ca^{2+} , Fe^{2+} , or Co^{2+} alone increased the concentration of FER ($P < 0.05$), though adding Mg^{2+} or Mn^{2+} had no effect ($P > 0.05$). The mechanism by which certain COF increased the FER concentration in the absence of EFE is unknown. Hydration during COF addition may be implicated and the COF may have stimulated endogenous FER esterases in bermudagrass haylage or dead silage bacteria, resulting in the increased FER concentrations. Neither microbial growth nor cross reaction with a reagent can be implicated due to the addition of sodium azide during the incubation and the use of HPLC to quantify FER, respectively. In support of the latter suggestion, Sang et al. (2011) reported that 5 mM Ca^{2+} , Fe^{3+} , and Co^{2+} stimulated ferulic acid esterase activity from an unculturable soil bacterium. Adding EFE 1A, 2A, 11C, or 15D alone increased the concentration of FER ($P < 0.05$), but 13D did not. Effects of COF addition to EFE on release of FER depended on the EFE-COF combination ($P < 0.001$). For instance, adding Mn^{2+} to 1A increased the response but adding Mg^{2+} decreased it. Furthermore, adding Ca^{2+} or Co^{2+} to 2A increased the responses but adding Mg^{2+} , Fe^{2+} or Mn^{2+} decreased them ($P < 0.05$). In addition, adding Fe^{2+} and Co^{2+} to 11C decreased the response, as did adding Mg^{2+} ,

Ca^{2+} , Fe^{2+} , Co^{2+} , and Mn^{2+} to 15D. No change in FER was detected when COF were added to 13D. Therefore, FER release was only increased by adding Mn^{2+} to 1A or by adding Ca^{2+} or Co^{2+} to 2A. No published studies that examined effects of adding Mn^{2+} to *T. reesei* ferulic acid esterase were found, but Kanauchi et al. (2008) reported that Mn^{2+} inhibited ferulic acid esterase from *A. awamori*. The increase in FER due to adding Ca^{2+} to 2A was 1.8 times greater than that detected by adding Ca^{2+} alone, whereas that due to adding Co^{2+} was only 1.2 times greater than that due to adding Co^{2+} alone. Thus, Ca^{2+} was more effective at synergistically increasing the release of FER.

Adding Mg^{2+} , Fe^{2+} , Co^{2+} , or Mn^{2+} alone increased the *p*-coumaric concentration ($P < 0.05$), as did adding 2A, 11C, or 15D alone ($P < 0.05$). However, effects of COF addition to EFE on release of *p*-coumaric depended on the EFE-COF combination ($P < 0.001$). The concentration of *p*-coumaric was decreased ($P < 0.05$) by adding Mg^{2+} to 1A, Mg^{2+} , Fe^{2+} , Co^{2+} , or Mn^{2+} to 2A, Mg^{2+} to 13D, or Mg^{2+} , Ca^{2+} , Fe^{2+} , Co^{2+} , and Mn^{2+} to 15D. In contrast, *p*-coumaric concentration was increased ($P < 0.05$) by adding Ca^{2+} to 2A, Mg^{2+} or Ca^{2+} to 11C, or Ca^{2+} , Fe^{2+} , Co^{2+} , or Mn^{2+} to 13D. Hence, when added to EFE, Ca^{2+} synergistically increased release of *p*-coumaric from the cell wall more consistently than other COF. Yet, McCrae et al. (1994) reported no changes in *Aspergillus awamori* *p*-coumaroyl esterase activity when 20 mM Ca^{2+} was added perhaps because of their high dose and the different EFE source they used.

Experiment 2: Effects of COF Addition to EFE on Digestibility and Fermentation

Effects of adding Mn^{2+} to 11C or Fe^{2+} to EFE 2A or 13D on the main digestibility and ruminal measures are shown below, and effects on additional indices of ruminal fermentation (pH, butyrate, isobutyrate, isovalerate, and ammonia-N) are presented in the Appendix.

Manganese Addition to EFE 11C. Adding Mn^{2+} to 11C gave one of the greatest increases in release of WSC in experiment 1, hence effects of this EFE-COF combination on digestibility and fermentation were explored in experiment 2. Adding EFE 11C alone increased DMD, NDFD, HEMD, and ADFD (Table 3; $P < 0.001$). Increasing the rate of Mn^{2+} application with or without EFE 11C resulted in linear ($P < 0.01$) increases in DMD and NDFD, but the increments were greater when 11C was present (EFE $\times$ Mn^{2+} dose interaction; $P < 0.05$). Adding 10 mM Mn^{2+} to 11C resulted in a 3.5% increase in DMD beyond the 6.6% increase caused by adding 11C alone and a 7.3% increase beyond the 2.7% increase caused by adding 10 mM Mn^{2+} alone.

Likewise, adding 10 mM Mn²⁺ to 11C resulted in a 8.1% increase in NDFD beyond the 15.5% increase caused by adding 11C alone and a 17.2% increase beyond the 6.3% increase caused by adding 10 mM Mn²⁺ alone. Therefore, adding Mn²⁺ synergistically increased the digestibility of bermudagrass haylage by EFE 11C. The increases in DMD and NDFD due to adding 0 versus 10 mM Mn²⁺ are attributable to corresponding increases in ADFD (*P* < 0.001), yet HEMD was unaffected by Mn²⁺ dose. These results may indicate that cellulolytic activities were stimulated by adding Mn²⁺ but xylanolytic activities were not. Manganese increases the activity of exoglucanases, endoglucanases, and β-glucosidases (Singh et al., 1990; Riou et al., 1998; Saad and Fawzi, 2004), which are all involved in cellulose depolymerization. In an early study by Chamberlain and Burroughs (1962), omission of manganese from an in vitro ruminal fluid medium reduced cellulose digestibility. In agreement, Arelovich et al. (2000) reported an increase in in vitro DMD of prairie hay (44.7 vs. 42.0% of DM) due to adding up to 100 mg/L of Mn²⁺ (chloride salt) to ruminal fluid. Martinez and Church (1970) also reported that adding over 100 mg/L of Mn²⁺ (sulfate salt) reduced cellulose digestion by washed suspensions of rumen microbes, but adding 5 to 30 mg/L of Mn²⁺ optimized the digestion. In our study, the manganese concentrations in the buffered-ruminal incubation fluid were 3.16, 3.37, 5.20, and 23.5 mg/L for Mn²⁺ doses of 0, 0.1, 1, and 10 mM in the EFE 11C solution, respectively. Therefore, the 1 and 10 mM doses were within the range reported to stimulate digestion by ruminal bacteria in the previous study. This may explain why adding 10 mM Mn²⁺ to 11C synergistically increased NDFD in the current study. The mixed rumen fluid inoculum had a manganese concentration of 0.01 mg/L, which is below the optimal ranges of 1 to 10 and 0.4 to 11 mg/L suggested by Durand and Kawashima (1980) and Mackie and Therion (1984), respectively. In our study, supplemental manganese from the Goering and Van Soest (1970) medium provided 2.6 mg/L and the bermudagrass substrate provided 0.6 mg/L of in vitro medium. Therefore, the total concentration of manganese was within the optimal range required to optimize fermentation of the in vitro system. Manganese concentrations of 0.25, 0.39, 0.52, and 1.03 mg/L were reported in the rumen fluid of ewes fed orchardgrass hay (Ishida et al., 1988), sheep fed dried grass (Bremner, 1970), cows fed roughage plus concentrate (Nikolic et al., 1978), and steers fed hay with or without added CuSO₄ (Wetzel and Menke, 1978).

Applying 11C alone increased concentrations of acetate and propionate and decreased the acetate-to-propionate ratio (A:P). An EFE × Mn²⁺ dose interaction (*P* = 0.002) was detected for total VFA. Increasing

Table 3. Effects of treating 4-wk regrowth bermudagrass haylage with exogenous fibrolytic enzyme (EFE) 11C with or without increasing doses of Mn²⁺ on in vitro digestibility and fermentation measures (experiment 2)¹

Item	DMD (% of DM)		NDFD (% of NDF)		HEMD (% of hemicellulose)		ADFD (% of ADF)		Total VFA (mM)		Acetate (mM)		Propionate (mM)		A:P	
	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE
Mn ²⁺ (mM)																
0	48.1 ^a	51.3 ^d	35.4 ^a	40.8 ^d	35.1 ^a	40.8 ^b	35.6 ^a	40.9 ^c	60.3 ^{ab}	61.4 ^{abc}	36.0 ^a	37.0 ^b	10.8 ^a	11.6 ^b	3.35 ^c	3.21 ^{ab}
0.1	48.8 ^{ab}	50.7 ^{cd}	36.6 ^{ab}	39.9 ^{cd}	35.2 ^a	39.1 ^b	38.0 ^a	40.6 ^c	62.9 ^c	62.0 ^{abc}	36.3 ^a	37.0 ^b	11.2 ^a	11.2 ^b	3.25 ^{abc}	3.32 ^{bc}
1	48.7 ^{ab}	50.7 ^{cd}	36.3 ^{ab}	39.9 ^{cd}	35.0 ^a	39.5 ^b	37.7 ^a	41.0 ^c	60.9 ^{ab}	62.3 ^{bc}	36.2 ^a	36.7 ^b	10.9 ^a	11.3 ^b	3.31 ^{bc}	3.24 ^{abc}
10	49.4 ^b	53.0 ^e	37.6 ^b	43.7 ^e	34.9 ^a	42.1 ^b	40.4 ^b	45.4 ^d	60.2 ^a	65.1 ^d	36.0 ^a	37.7 ^b	10.8 ^a	12.0 ^b	3.32 ^c	3.15 ^a
SEM ²	0.34	0.34	0.58	0.58	0.74	0.80	0.73	0.73	0.72	0.77	0.59	0.55	0.28	0.28	0.043	0.040
<i>P</i> -value	L**	L**	L**	L**	None	None	None	None	C**	L**	None	None	None	None	None	L*
Contrast ³	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	0.003	0.014	0.014	0.004	0.004	0.009	0.009
EFE	<0.001	<0.001	<0.001	<0.001	0.231	0.15	<0.001	<0.001	0.059	0.059	0.847	0.847	0.692	0.692	0.613	0.613
Mn ²⁺ dose	<0.001	<0.001	<0.001	<0.001	0.048	0.048	0.223	0.223	0.002	0.002	0.748	0.748	0.125	0.125	0.019	0.019
EFE × Mn ²⁺	0.047	0.047	0.048	0.048	0.15	0.15	0.223	0.223	0.002	0.002	0.748	0.748	0.125	0.125	0.019	0.019
dose																

^{a-c}Means with different superscripts within a measure differed (*P* ≤ 0.05).
¹Digestibility of DM (DMD), NDF (NDFD), hemicellulose (HEMD), and ADF (ADFD), acetate-to-propionate ratio (A:P).
²The SEM value is the highest among those for the specific EFE treatment in the column.
³Linear (L), cubic (C); None = *P* > 0.05).
P* ≤ 0.05; *P* ≤ 0.01.

the Mn^{2+} dose in the absence of 11C resulted in a cubic total VFA response ($P < 0.01$), in which only the 0.1 mM dose increased total VFA concentration compared with 0 mM (4.31%; $P < 0.05$). The reasons behind this cubic response are unclear. However, when EFE 11C was present, total VFA concentration increased linearly ($P < 0.01$) with increasing Mn^{2+} dose. Biswas and Purnendu (2012) and Durand and Kawashima (1980) also reported that total VFA concentration was increased by adding manganese to the ruminal incubation fluid used to ferment rice straw- and prairie hay-based diets, respectively. In our study, the 10 mM Mn^{2+} dose along with EFE 11C increased ($P < 0.05$) total VFA concentration by 6.1% relative to the value achieved by adding 11C alone in agreement with corresponding synergistic increases in DMD and NDFD. Therefore, adding 10 mM Mn^{2+} to 11C may synergistically increase energy supply to cows.

Effects of 11C on the A:P ratio were influenced by adding Mn^{2+} ($P < 0.02$). In the absence of 11C, no Mn^{2+} dose effects on A:P ratio were evident, but in the presence of 11C increasing the Mn^{2+} dose linearly decreased the A:P ratio ($P < 0.05$). Therefore, adding Mn^{2+} to 11C would likely increase the potential beneficial effects of 11C on ruminal energy supply (from gluconeogenic substrates). Adding increasing doses of Mn^{2+} to 11C resulted in cubic fluctuations ($P < 0.01$) in concentrations of isovalerate and valerate ($\text{EFE} \times \text{Mn}^{2+}$ dose interaction; $P < 0.05$).

Iron Addition to EFE 2A or 13D. Adding Fe^{2+} to EFE 2A or 13D resulted in some of the greatest increases in release of WSC in experiment 1; hence, effects of these EFE-COF combinations on digestibility and fermentation were explored in experiment 2. Adding EFE 2A or 13D alone increased DMD, NDFD, and ADFD and 2A also increased HEMD (Tables 4 and 5; $P < 0.001$). Increasing the rate of Fe^{2+} application linearly decreased ($P < 0.01$) all digestibility measures in the absence of 2A, but had no effect in the presence of 2A ($\text{EFE} \times \text{Fe}^{2+}$ dose interaction; $P < 0.05$). Therefore, 2A retained its hydrolytic effect in the presence of Fe^{2+} and prevented adverse effects of adding 10 mM Fe^{2+} alone on DMD (−6.03%), NDFD (−14.41%), and HEMD (−14.24%). This suggests that certain EFE can be used to reduce adverse effects of Fe^{2+} toxicity on the digestion of feeds. Harrison et al. (1992) reported that ferric and ferrous cations consistently inhibited the DM digestion of a forage-based TMR by up to 36% when added to ruminal fluid cultures at concentrations of 100 to 1,000 mg/L. The toxicity of heavy metals, such as iron in anaerobic environments is attributable to the disruption of enzymatic activity and structural binding of the metals with thiol and other groups on protein molecules or by replacing naturally occurring

metals in enzyme prosthetic groups (Chen et al., 2008). More research is required to understand and exploit the beneficial effects of adding fungal enzymes to rumen fluid media with high iron concentrations.

Increasing the rate of Fe^{2+} application linearly decreased ($P < 0.01$) DMD, NDFD, and ADFD in the presence or absence of 13D, but only decreased HEMD in the absence of the EFE. Therefore, adding 10 mM Fe^{2+} to 13D prevented the beneficial effects of adding 13D alone on DMD and NDFD ($\text{EFE} \times \text{Fe}^{2+}$ dose interaction; $P < 0.05$). Harrison et al. (1992) found that adding 100 mg/L of Fe^{2+} (chloride salt) to the ruminal incubation fluid reduced (−10.18%) the DMD of a fescue-based TMR compared with adding no Fe^{2+} . Martinez and Church (1970) reported that adding up to 50 mg/L of Fe^{3+} (chloride salt) had no toxic or negative effects on cellulose digestion by washed suspensions of rumen microbes, and adding 2 to 5 mg/L of Fe^{3+} increased the response. In our study, adding 0, 0.1, 1, and 10 mM Fe^{2+} to 2A or 13D resulted in mean iron concentrations in the incubation fluid of 1.86, 2.07, 3.93, and 22.55 mg/L, respectively. Therefore, the results of the present study seem to indicate that a lower ruminal fluid iron concentration (22.5 mg/L) than that indicated by Martinez and Church (1970; 50 mg/L) can cause iron toxicity in ruminal in vitro incubations. Different toxicity outcomes among the latter study and ours may be because washed microbe suspensions were used in the latter study instead of the whole rumen fluid used in the current experiment. However, the lower iron doses used in our study (0.1 to 1 mM) did not decrease digestibility and the doses resulted in ruminal fluid iron concentrations that were within the optimal range recommended for optimizing microbial metabolism by Durand and Kawashima (1980).

The mixed rumen fluid inoculum had a concentration of 0.59 mg/L of iron, which is similar to the value observed in steers fed orchardgrass hay (0.5 mg/L; Ishida et al., 1988), but below the optimal range of 1 to 10 mg/L suggested by Durand and Kawashima (1980) and the value observed by Nikolic et al. (1978) in cows fed undisclosed roughage plus concentrate (8.17 mg/L). In the present study, supplemental iron from the Goering and Van Soest (1970) medium provided 1.5 mg/L and the bermudagrass substrate provided 0.2 mg/L of in vitro medium. Therefore, the iron concentration in the in vitro system was within the optimal range.

No $\text{EFE} \times \text{Fe}^{2+}$ dose interaction was detected ($P > 0.05$) for ADFD due to adding Fe^{2+} to 2A or 13D. Yet, unlike lower doses, adding 10 mM Fe^{2+} decreased ADFD. Therefore, it seems that EFE 2A and 13D were more effective at preventing adverse effects of Fe^{2+} addition on digestion of hemicellulose than on ADF digestion. Riou et al. (1998) reported that a β -glucosidase from

Table 4. Effects of treating 4-wk regrowth bermudagrass haylage with exogenous fibrolytic enzyme (EFE) 2A with or without increasing doses of Fe²⁺ on in vitro digestibility and fermentation measures (experiment 2)¹

Item	DMD (% of DM)		NDFD (% of NDF)		HEMD (% of hemicellulose)		ADFD (% of ADF)		Total VFA (mM)		Acetate (mM)		Propionate (mM)		A:P	
	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE
Fe ²⁺ (mM)																
0	48.1 ^b	50.6 ^c	35.4 ^b	39.7 ^c	35.1 ^b	39.6 ^c	35.6 ^b	40.6 ^d	60.3 ^{bc}	64.9 ^d	36.0 ^c	37.6 ^d	10.8 ^{bc}	11.9 ^d	3.35 ^{ef}	3.16 ^c
0.1	48.4 ^b	50.8 ^c	35.9 ^{bc}	40.0 ^c	34.6 ^b	39.3 ^c	36.2 ^b	40.8 ^d	57.5 ^b	60.9 ^c	34.3 ^b	35.6 ^c	10.1 ^{ab}	10.8 ^{bc}	3.40 ^f	3.30 ^{de}
1	48.0 ^b	50.5 ^c	35.1 ^b	39.4 ^c	33.6 ^b	38.3 ^c	36.6 ^b	41.2 ^d	59.1 ^{bc}	59.3 ^{bc}	34.8 ^b	35.2 ^e	10.7 ^{bc}	10.5 ^b	3.25 ^d	3.36 ^{ef}
10	45.2 ^a	50.2 ^c	30.3 ^a	39.0 ^c	30.1 ^a	38.7 ^c	32.4 ^a	39.9 ^c	50.8 ^a	59.1 ^{bc}	28.7 ^a	31.5 ^d	9.5 ^a	11.5 ^{cd}	3.02 ^b	2.79 ^a
SEM ²	0.43	0.40	0.74	0.68	0.66	0.66	0.87	0.87	1.10	1.10	0.69	0.64	0.30	0.28	0.033	0.031
P-value																
Contrast ³	L**	None	L**	None	L**	None	L**	None	C*	C*	C*	L**	C*	C*	Q*	C*
EFE	<0.001		<0.001		<0.001		<0.001		<0.001		0.002		<0.001		<0.001	
Fe ²⁺ dose	<0.001		<0.001		<0.001		0.009		<0.001		<0.001		0.008		<0.001	
EFE × Fe ²⁺ dose	0.004		0.004		0.007		0.232		0.004		0.316		0.003		<0.001	

^{a–f}Means with different superscripts within a measure differed ($P \leq 0.05$).

¹Digestibility of DM (DMD), NDF (NDFD), hemicellulose (HEMD), and ADF (ADFD), acetate to propionate ratio (A:P).

²The SEM value is the highest among those for the specific EFE treatment in the column.

³Linear (L), quadratic (Q), cubic (C); None = $P > 0.05$.

* $P \leq 0.05$; ** $P \leq 0.01$.

Table 5. Effects of treating 4-wk regrowth bermudagrass haylage with exogenous fibrolytic enzyme (EFE) 13D with or without increasing doses of Fe²⁺ on in vitro digestibility and fermentation measures (experiment 2)¹

Item	DMD (% of DM)		NDFD (% of NDF)		HEMD (% of HEM)		ADFD (% of ADF)		Total VFA (mM)		Acetate (mM)		Propionate (mM)		A:P	
	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE
Fe ²⁺ (mM)																
0	48.1 ^b	49.8 ^d	35.4 ^b	38.3 ^d	35.1 ^{bcd}	37.0 ^d	35.6 ^b	39.7 ^d	60.3 ^{bc}	61.0 ^c	36.0 ^d	35.6 ^{cd}	10.8 ^{bc}	10.8 ^c	3.35 ^{cd}	3.28 ^c
0.1	48.4 ^{bc}	49.7 ^d	35.9 ^{bc}	38.1 ^d	34.6 ^{bc}	37.3 ^d	36.2 ^b	39.0 ^d	57.5 ^b	60.8 ^c	34.3 ^c	35.5 ^{cd}	10.1 ^{ab}	10.9 ^c	3.40 ^d	3.28 ^c
1	48.0 ^b	49.3 ^{cd}	35.1 ^b	37.4 ^{cd}	33.6 ^b	36.8 ^d	36.6 ^b	36.7 ^d	59.1 ^{bc}	61.7 ^c	34.8 ^{cd}	35.8 ^{cd}	10.7 ^{bc}	11.1 ^{cd}	3.25 ^c	3.25 ^c
10	45.2 ^a	48.4 ^{bc}	30.3 ^a	35.9 ^{bc}	30.1 ^a	36.3 ^{cd}	32.4 ^a	36.3 ^c	50.8 ^a	59.5 ^{bc}	28.7 ^a	32.3 ^b	9.5 ^a	11.6 ^d	3.02 ^b	2.78 ^a
SEM ²	0.40	0.40	0.69	0.69	0.72	0.66	0.88	0.88	1.14	1.05	0.58	0.53	0.29	0.26	0.043	0.039
P-value																
Contrast ³	L**	L**	L**	L**	L**	None	L**	Q*	C*	None	C*	L**	C*	None	Q*	L**
EFE	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	
Fe ²⁺ dose	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001		0.345		<0.001	
EFE × Fe ²⁺ dose	0.043		0.047		0.014		0.077		0.003		0.005		0.002		0.038	

^{a–f}Means with different superscripts within a measure differed ($P \leq 0.05$).

¹Digestibility of DM (DMD), NDF (NDFD), hemicellulose (HEMD), and ADF (ADFD), acetate to propionate ratio (A:P).

²The SEM value is the highest among those for the specific EFE treatment in the column.

³Linear (L), quadratic (Q), cubic (C); None = $P > 0.05$.

* $P \leq 0.05$; ** $P \leq 0.01$.

A. oryzae was inhibited strongly by 5 mM Fe^{3+} , which could partly explain the inhibition of ADF digestion by high concentrations of iron in our study. In general, 13D was more susceptible than EFE 2A to the deleterious effects on digestibility of high iron concentrations.

Adding increasing doses of Fe^{2+} alone decreased A:P ($P < 0.05$, quadratic) as well as total VFA, acetate, and propionate concentrations ($P < 0.05$, cubic), whereas adding 2A alone increased total VFA, acetate, and propionate concentrations and decreased the A:P ratio ($P < 0.05$). Adding as little as 0.1 mM Fe^{2+} to 2A prevented the increase in total VFA caused by treatment with 2A alone, but no further decrease was detected as the Fe^{2+} dose increased (EFE $\times$ Fe^{2+} dose interaction; $P = 0.004$). Increasing the dose of the Fe^{2+} added to 2A resulted in cubic effects ($P < 0.05$) on propionate and A:P ratio. The reasons for the cubic responses are unclear. Harrison et al. (1992) reported that adding 100, 200, and 500 mg/L of Fe^{2+} increased total VFA concentration, but no further increase occurred when 1,000 mg/L were added. However, in their experiment, ferrous sulfate was used rather than the ferrous chloride used in our study. The form of the iron likely determines the outcome, as it affects its solubility. The sulfate in ferrous sulfate would be rapidly metabolized to sulfide in the rumen, forming a black precipitate, which complexes with iron and makes it less soluble and accessible to rumen microbes, whereas ferrous chloride is more soluble and consequently more toxic (Harrison et al., 1992).

Adding increasing doses of Fe^{2+} to 13D did not affect total VFA concentration (EFE $\times$ Fe^{2+} dose interaction; $P < 0.01$), but linearly decreased the acetate concentration and the A:P ratio ($P < 0.05$). Adding 10 mM Fe^{2+} to 13D increased propionate concentration relative to adding 13D alone, but lower doses did not (EFE $\times$ Fe^{2+} dose interaction; $P < 0.01$).

In summary, adding Fe^{2+} to 2A or 13D is not recommended because though they increased release of WSC, they did not influence and in fact mostly decreased beneficial effects of the EFE on measures of digestibility and fermentation in the current study. Even the slight increase in propionate concentration and the decrease in A:P ratio due to adding 10 mM Fe^{2+} to 13D are likely outweighed by corresponding decreases in DMD, NDFD, and ADFD.

CONCLUSIONS

Adding 1 mM each of the COF to EFE 2A or 11C synergistically increased release of WSC of bermudagrass haylage, as did adding 1 mM Fe^{2+} to 1A, Mn^{2+} , Co^{2+} , and Fe^{2+} to 13D, or Co^{2+} or Fe^{2+} to 15D. The greatest release of WSC was obtained by adding Mn^{2+}

to 11C (38%) or by adding Fe^{2+} to 2A or 13D (10 and 21.9%, respectively). Effects of adding increasing doses of these COF on EFE-mediated changes in vitro digestibility depended on the COF and EFE. Adding 10 mM Mn^{2+} to EFE 11C resulted in a 8.1% increase in NDFD beyond the 15.5% increase caused by adding EFE 11C alone and a 17.2% increase beyond the 6.3% increase caused by adding 10 mM Mn^{2+} alone. Whereas adding Fe^{2+} to 2A had no effects on EFE-mediated digestibility responses but 2A prevented adverse effects of adding Fe^{2+} alone on DMD and NDFD. In contrast, adding Fe^{2+} to 13D reduced the increases in NDFD caused by adding the EFE alone. This study shows that adding COF to EFE can synergistically increase, decrease, or not affect the hydrolytic effects of EFE on forage cell walls depending on the specific EFE-COF combination. More work is required to understand the mechanisms resulting in these outcomes to exploit beneficial effects of COF on EFE.

ACKNOWLEDGMENTS

We gratefully acknowledge the statistical advice of Mihai Giurcanu of the Statistical Consulting Unit at the Institute of Food and Agricultural Sciences, University of Florida, as well as funding for the project by a USDA-Tropical Subtropical Agricultural Research grant.

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APPENDIX

Effects of COF Addition to EFE on Additional Fermentation Indices in Experiment 2

Manganese Addition to EFE 11C. Applying 11C alone increased the butyrate and valerate concentrations and decreased the pH (Table A1). Adding increasing doses of Mn^{2+} alone decreased ammonia-N ($P < 0.01$, linear) and valerate ($P < 0.05$, quadratic) concentrations and resulted in cubic fluctuations in isobutyrate concentrations. However, in the presence of EFE 11C, no changes in isobutyrate or ammonia-N were observed, but cubic fluctuations occurred for isovalerate and valerate (EFE $\times$ Mn^{2+} dose interaction; $P < 0.05$).

Iron Addition to EFE 2A and 13D. Adding 2A alone increased butyrate, isobutyrate, isovalerate, and pH and decreased the ammonia-N concentration (Table A2), and 13D alone increased isobutyrate concentration and decreased ammonia-N (Table A3; $P < 0.05$). Adding increasing doses of Fe^{2+} alone linearly increased pH ($P < 0.01$) and decreased butyrate, isovalerate, and ammonia-N concentrations ($P < 0.05$, cubic). Increasing the dose of the Fe^{2+} added to 2A resulted in no changes on pH, linear increases ($P < 0.01$) in valerate, a quadratic decrease ($P < 0.01$) in isobutyrate, and cubic effects ($P < 0.05$) on butyrate and ammonia-N concentrations (EFE $\times$ Fe^{2+} dose interaction; $P < 0.05$). Adding increasing doses of Fe^{2+} to 13D did not affect isovalerate and decreased the butyrate concentration (linear, $P < 0.05$) as well as the ammonia-N concentration (quadratic, $P < 0.01$; EFE $\times$ Fe^{2+} dose interaction; $P < 0.05$).

Table A1. Effects of treating 4-wk regrowth bermudagrass haylage with exogenous fibrolytic enzyme (EFE) 11C with or without increasing doses of Mn^{2+} on in vitro fermentation measures (experiment 2)

Item	Butyrate (mM)		Isobutyrate (mM)		Isovalerate (mM)		Valerate (mM)		Ammonia-N (mg/dL)		pH	
	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE	–EFE	+EFE
Mn^{2+} (mM)												
0	5.27 ^a	5.63 ^b	2.45 ^a	2.69 ^{ab}	2.73 ^{bc}	2.70 ^{bc}	3.10 ^{bc}	3.58 ^e	48.4 ^{bc}	47.7 ^{bc}	7.02 ^b	6.96 ^a
0.1	5.46 ^a	5.51 ^b	3.22 ^c	2.52 ^a	2.56 ^{abc}	2.28 ^a	2.90 ^{ab}	3.09 ^{abc}	47.4 ^{bc}	48.9 ^c	7.07 ^b	6.99 ^a
1	5.53 ^a	5.58 ^b	2.67 ^{ab}	2.80 ^{ab}	2.32 ^a	2.62 ^{abc}	3.27 ^{cd}	3.30 ^{de}	48.1 ^{bc}	48.2 ^{bc}	7.06 ^b	7.01 ^a
10	5.34 ^a	5.71 ^b	2.43 ^a	3.00 ^{bc}	2.76 ^c	2.38 ^{ab}	2.83 ^a	3.40 ^{de}	45.2 ^a	47.2 ^b	7.06 ^b	7.00 ^a
SEM ¹	0.103	0.095	0.150	0.150	0.132	0.132	0.097	0.103	0.69	0.47	0.019	0.019
P-value	None	None	C**	None	None	C**	Q*	C**	L**	None	None	None
Contrast ²	0.004		0.562		0.276		<0.001		0.04		<0.001	
EFE			0.249		0.113		0.003		<0.001		0.068	
Mn^{2+} dose	0.726		0.001		0.043		0.025		0.02		0.802	
EFE $\times$ Mn^{2+} dose	0.151											

^{a–c}Means with different superscripts within a measure differed ($P \leq 0.05$).

¹The SEM value is the highest among those for the specific EFE treatment in the column.

²Linear (L), quadratic (Q), cubic (C); None = $P > 0.05$.

* $P \leq 0.05$; ** $P \leq 0.01$.

Table A2. Effects of treating 4-wk regrowth bermudagrass haylage with exogenous fibrolytic enzyme (EFE) 2A with or without increasing doses of Fe²⁺ on in vitro fermentation measures (experiment 2)

Item	Butyrate (mM)		Isobutyrate (mM)		Isovalerate (mM)		Valerate (mM)		Ammonia-N (mg/dL)		pH	
	-EFE	+EFE	-EFE	+EFE	-EFE	+EFE	-EFE	+EFE	-EFE	+EFE	-EFE	+EFE
Fe ²⁺ (mM)												
0	5.27 ^a	6.06 ^d	2.45 ^a	3.28 ^c	2.73 ^b	2.96 ^d	3.10 ^{abc}	3.15 ^{bc}	48.4 ^d	45.9 ^c	7.02 ^{ab}	7.10 ^{cd}
0.1	5.15 ^a	5.55 ^b	2.48 ^a	2.81 ^{ab}	2.31 ^a	2.61 ^c	3.21 ^{cd}	3.22 ^{cd}	46.1 ^c	47.5 ^d	7.0 ^a	7.12 ^d
1	5.55 ^b	5.59 ^b	2.92 ^{abc}	2.49 ^a	2.38 ^a	2.41 ^c	2.87 ^a	3.17 ^{bcd}	45.4 ^c	47.6 ^d	7.08 ^{bcd}	7.03 ^{abc}
10	4.66 ^c	5.50 ^b	2.67 ^a	3.23 ^{bc}	2.28 ^a	2.60 ^c	2.98 ^{ab}	3.39 ^d	44.0 ^b	42.8 ^a	7.14 ^d	7.12 ^d
SEM ¹	0.046	0.046	0.167	0.155	0.062	0.062	0.084	0.078	0.364	0.364	0.021	0.025
P-value	C**	C**	None	Q**	C**	C**	None	L**	C**	C*	L**	None
Contrast ²	<0.001		0.006		<0.001		0.001		0.854		0.048	
EFE	<0.001		0.195		<0.001		0.084		<0.001		0.016	
Fe ²⁺ dose	<0.001		0.002		0.062		0.041		<0.001		0.003	
EFE × Fe ²⁺ dose												

^{a-d}Means with different superscripts within a measure differed ($P \leq 0.05$).

¹The SEM value is the highest among those for the specific EFE treatment in the column.

²Linear (L), quadratic (Q), cubic (C); None = $P > 0.05$.

* $P \leq 0.05$; ** $P \leq 0.01$.

Table A3. Effects of treating 4-wk regrowth bermudagrass haylage with exogenous fibrolytic enzyme (EFE) 13D with or without increasing doses of Fe²⁺ on in vitro fermentation measures (experiment 2)

Item	Butyrate (mM)		Isobutyrate (mM)		Isovalerate (mM)		Valerate (mM)		Ammonia-N (mg/dL)		pH	
	-EFE	+EFE	-EFE	+EFE	-EFE	+EFE	-EFE	+EFE	-EFE	+EFE	-EFE	+EFE
Fe ²⁺ (mM)												
0	5.27 ^{bc}	5.41 ^{cd}	2.45 ^a	3.03 ^b	2.73 ^b	2.84 ^{bc}	3.10	3.18	48.4 ^d	45.9 ^c	7.02 ^{ab}	7.02 ^a
0.1	5.15 ^b	5.43 ^{de}	2.48 ^a	2.94 ^b	2.31 ^a	3.05 ^c	3.21	3.07	46.1 ^c	45.7 ^c	7.0 ^a	7.05 ^{ab}
1	5.55 ^{de}	5.32 ^{bc}	2.92 ^a	3.22 ^b	2.38 ^a	2.89 ^{bc}	2.87	3.20	45.4 ^{bc}	44.4 ^{ab}	7.08 ^{bc}	7.13 ^{cd}
10	4.66 ^a	5.60 ^e	2.67 ^a	3.48 ^b	2.28 ^a	3.05 ^c	2.98	2.97	44.0 ^a	43.6 ^a	7.14 ^d	7.24 ^e
SEM ¹	0.061	0.068	0.179	0.179	0.10	0.10	0.095	0.095	0.36	0.36	0.021	0.021
P-value	C**	L*	None	None	C**	None	None	None	C**	Q*	L**	Q**
Contrast ²	<0.001		<0.001		<0.001		0.281		<0.001		0.001	
EFE	<0.001		0.055		0.388		0.195		<0.001		<0.001	
Fe ²⁺ dose	<0.001		0.51		0.002		0.076		0.018		0.115	
EFE × Fe ²⁺ dose												

^{a-e}Means with different superscripts within a measure differed ($P \leq 0.05$).

¹The SEM value is the highest among those for the specific EFE treatment in the column.

²Linear (L), quadratic (Q), cubic (C); None = $P > 0.05$.

* $P \leq 0.05$; ** $P \leq 0.01$.