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Effect of combining exogenous fibrolytics enzymes supplementation with alkali and acid pre-treatments on wheat straw hydrolysis and ruminal fermentation

JIHENE JABRI[1], KHALIL ABID[2], HELA YAICH[3], ATEF MALEK[4], JAMEL REKHIS[5] and MOHAMED KAMOUN[6]

National School of Veterinary Medicine Sidi Thabet, University Manouba, 2020 Tunisia

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ABSTRACT

The present study was undertaken to evaluate the efficacy of exogenous fibrolytics enzymes (EFE) to improve the *in vitro* cell wall hydrolysis, ruminal fermentation and digestibility of untreated (WS) and chemically pre-treated wheat straw with NaOH (SWS), urea (UWS), and diluted H_2SO_4 (AWS). An *in vitro* gas production study during 96 h of incubation and an *in vitro* enzymatic hydrolysis during 20 h was conducted. The first EFE was a mixture (1:1, v/v) of cellulase and xylanase (Dyadic complex), applied at increasing doses (1, 2, 5 and 10 µl/g DM). The second EFE (MaxFiber complex) was also applied at increasing dose (0.5, 1, 2 and 4 mg/g DM). The rate and the extent of the *in vitro* gas production (GP) of WS improved with both EFE supplementation especially with the optimal doses D1 and M2. In association with NaOH pre-treatment, the EFE failed to have any effect on wheat straw digestibility. In contrast, the urea pre-treatment seems to have a synergetic effect with EFE on ruminal utilization by accelerating the fermentation process. However, for AWS an antagonist effect was detected with both EFE. Moreover, the *in vitro* enzymic hydrolysis indicated a linear positive effect of EFE on reducing sugar release for almost all substrates and an increase in dry matter losses for only WS and AWS. These results revealed that the positive effect of EFE supplementation effect depended on the type of chemical pre-treatments and it was detected only for WS and UWS.

Key words: Chemical pre-treatments, Exogenous fibrolytics enzymes, *In vitro* fermentation, Wheat straw

High cost of concentrates and high-quality forages is the major restriction that limits livestock production especially for small-scale farming systems. Agricultural by-products are available in large quantities all over the world especially the cereal straws because of the continuous growing crop production according to the FAOSTAT (2018). Hence, it could represent an important feed source for ruminants especially during the winter. Besides, the straw is a complex lignocellulosic biomass with limited amount of available nutrient. So, for years various chemical pretreatments were used to remove cell wall barriers that lock up nutrient (Kim 2018) and to improve the accessibility of ruminal microorganisms and enzymes to the fibrolytic biomass. Whereas, recent studies proved that the use of exogenous fibrolytics enzymes has major potential to optimize the use of fibrous diets by ruminants (Kholif *et al.* 2017). Hence, it was hypothesized that a synergetic effect between chemical pre-treatments and EFE supplementation may improve the ruminal fermentation and the ruminal degradability of wheat straw. Therefore, this study was undertaken to determine the response of *in vitro* ruminal fermentation to chemical pre-treatments associated to increasing levels of EFE by measuring the *in vitro* gas production, the amounts of sugar releases and dry matter losses.

Present address: [1]PhD Scholar (jabrijihene@gmail.com), Faculty of Sciences, Tunisia; [2]PhD Scholar (mr.khalil.abid@gmail.com), [3]Scientist (hela.yaich@gmail.com), [4,5]Senior Scientist (malekatef.9@gmail.com, jrekhis@yahoo.fr), [6]Principal Scientist (Kamoun.mohamed.km@gmail.com), Animal Nutrition Laboratory.

MATERIALS AND METHODS

Substrates preparation: Samples were randomly selected from wheat straw bales, chopped (5 cm) and divided into 4 sub-samples (2 kg). The first sub-sample was left untreated (WS) as a control and the others were subject to 2 alkali and 1 acid pre-treatment. The alkali pre-treatments was performed by 4% NaOH and 4% urea solutions for SWS and UWS, (Dulphy *et al.* 1982, Chermiti *et al.* 1989). From each preparation (WS, SWS, UWS), samples (500 g) were oven dried overnight at 55°C and grounded in a mill to pass through a 1 mm sieve and stored for subsequent analysis. The acid pre-treatment was conducted on ground wheat straw, with about 80% dry matter (DM) content. The 1.8% H_2SO_4 solution was added carefully to straw sample until the DM content comes to 20% (Castro *et al.* 1993) for AWS. Acid pre-treated straw was stored wet at 4°C until further analysis.

The enzymatic supplementation was carried out with two enzymatic complexes. The first was cellulase: xylanase complex (1:1, v/v) (Dyadic® International, Inc. Jupiter,

Florida) produced by *Trichoderma longibrachiatum* in liquid form. The Dyadic complex contained 22760±152 IU of xylanase, 1160±107 IU of endoglucanase and 113±6.4 IU of exoglucanase. The second enzymatic complex called MaxFiber® (Provita Supplements GmbH, SCHAUMANN) in a powdered form was a crude protein-rich by-product of solid states fermentation (SSF) from five different fungi: *Aspergillus niger, Aspergillus tubingensis, Aspergillus orzyae, Aspergilluss ojae* and *Neurospora intermedia*. It contained 118±6 IU of xylanase, 75±1 IU of endoglucanase and 74±0.3 IU of exoglucanase.

Experiment 1-in vitro fermentation: The technique of *in vitro* batch culture in 100 ml serum bottle was performed (3 runs) as per Gemeda and Hassen (2015). The enzymatic supplementation was performed with 2 different methods according to the enzymes form. The Dyadic complex was diluted by distilled water and then sprayed directly with the adequate dose (D1=1, D2=2, D3=5, D4=10 µl/g DM) onto each wheat straw preparation weighed in advance (200 mg DM) in the incubation bottles. The MaxFiber was mixed with each wheat straw preparation to obtain the suitable enzymatic dose, then, 200 mg from each mix (M1=0.5, M2=1, M3=2 or M4=4 mg/g DM) was weighed into incubation bottles. All treatments were kept at room temperature during 20 h before the *in vitro* incubation. Substrates (WS, SWS, UWS, and AWS) without EFE supplementation were considered as control. Three replications were prepared for each treatment in each run.

The Official Animal Care Committee of the National school of veterinary Medicine Sidi Thabet approved the experimental protocol. Ruminal fluid was collected from 2 cannulated cows before morning feeding, from 4 different sites within the rumen. The ruminal fluid was immediately strained through 4-layers of cheese cloth into a pre-warmed insulated flaskat 39°C.

Anaerobic buffer medium was prepared as per Menke and Steingass (1988) and adjusted to pH 6. The fermentation inoculum was prepared by adding the fresh ruminal fluid to the medium in a ratio of 1:2 (rumen fluid: medium). A quantity equal to 30 ml of inoculum was added to each incubation bottle under continuous flushing with CO_2. The bottles were closed hermetically with a rubber stopper and crimp seal caps and placed in the incubator (39°C) immediately after loading. Bottles containing rumen fluid and buffer medium without substrate were considered as a negative control (blanks).

The GP in each bottle was measured using a pressure transducer connected to a visual display after 2, 4, 6, 8, 12, 24, 48, 72, 96 h of incubation. The gas pressure was converted to gas volume using the following equation:

$$GP[ml]=GPr\times\frac{Vf-Vi}{Patm}$$

where, GPr, recorded gas pressure [bar]; Vf, volume of serum bottle (=117.39 ml), Vi, volume of inoculum added to each bottle and Patm; atmospheric pressure (= 1.01325 bar).

The metabolizable energy (ME) and the *in vitro* organic matter digestibility (DMO) were estimated according to Menke and Steingass (1988) and volatile fatty acids (VFA) as proposed by Getachew *et al.* (1998) for different types of forage. The maximum rate of GP (Rmax) and the time at which the maximum rate of GP is attained (Tmax) were calculated according to Yang *et al.* (2005).

Experiment 2: Enzymic hydrolysis: The study was performed to assess the effect of 2 EFE on reducing sugar release (RS) and dry matter losses (DML) of WS, SWS, UWS and AWS during the preincubation period. As described by Wang *et al.* (2004), in 20 ml glass test tubes, 250 mg of straw preparations were incubated in 10 ml of 0.1 M acetate buffer (pH 6.6). The enzymatic supplementation was performed as described previously in the *in vitro* study. Test tubes without enzymes were considered as controls. All tubes were capped and incubated immediately after enzyme supplementation during 20 h at 24°C with shaking. Three replications were prepared for each treatment in each run. At the end of incubation, the test tubes were immediately placed in boiling water for 15 min to stop the enzymic reaction. Then, all tubes contents were filtered through preweighed Whatman filter paper no.1. The obtained filtrate was analyzed for RS amounts (Nelson 1977) and the filters with retained residues were washed thrice with distilled water, to eliminate soluble fractions, then, dried for 12 h at 105°C, to determine the amounts of DML.

Chemical analysis: Samples of WS, SWS, UWS, AWS were analyzed in triplicate for dry matter (DM) (ID 934.01), ash (ID 942.05) and crude protein (CP) (ID 984.13) according to AOAC (1990). Neutral detergent fiber, acid detergent fiber, and lignin (ADL) were conducted as perVan Soest *et al.* (1991). The fibrolytic activities of exogenous enzymes were assayed in triplicate for endoglucanase, exoglucanase, and xylanase according to Wood and Bhat (1988) and Bailey *et al.* (1992) under pH 6.6 and 39°C to reflect rumen conditions. For the MAXFIBER as a by-product of SSF, an enzyme extraction step is required as described by Tengku Norsalwani *et al.* (2012). So, 10 ml of citrate buffer (pH 6.6) was added to 1 g of MaxFiber product and swirled until it becomes homogeneous. The solid biomass was separated from the suspension by filtration through Whatman filter paper no.1. The extract was used as a source of enzyme preparation.

Statistical analyses: Data from experiment 1 and 2 were analysed as a completely randomized design with three replicates per treatment. The experimental model included the substrate (WS, SWS, UWS and AWS), enzyme treatment (EFE dose) and the interaction between substrate and enzymatic supplementation as fixed effects and replication as the random effect. All data were reanalyzed separately by substrate using the proc MIXED from SAS® Studio 3.6. Polynomial contrasts were used to determine linear and quadratic effects of increasing enzyme doses. As enzyme doses are unequally spaced the Proc IML from SAS® studio was used to generate coefficients for polynomial contrasts. Means were considered significantly different when the P-value is less than 5% and tendencies

were declared at 0.05<P≤0.1. Differences between control (no enzyme) and enzyme doses were detected using the Duncan test (Duncan 1955).

RESULTS AND DISCUSSION

Chemical composition of different wheat straw preparations is presented in Table 1. The alkali pre-treatments decreased the hemicellulose fraction by 40.9% and 23.2% respectively for SWS and UWS. However, the cellulose and the ADL contents were not affected by the alkali pre-treatments. Highest ash content was found in SWS which could be explained by the presence of additional sodium derived from the NaOH (Rai and Mudgal 1996). The CP increased only for UWS because of the addition of urea as a non-protein nitrogen source. The 1.8% diluted H_2SO_4 used in the pre-treatment of wheat straw partially solubilize the ash. As expected, the ruminal fermentation of wheat straw differed widely according to the chemical pre-treatments and subsequently to the dose of EFE.

Increasing doses of exogenous fibrolytics enzymes effect on the *in vitro* fermentation and digestive use parameters are presented in Tables 2, 3. The EFE effect on wheat straw depended on the chemical pretreatment (P<0.05) and to the supplemented dose (P<0.05). So, for the untreated straw (WS), the dyadic and MaxFiber supplementation improved (P<0.01) the rate and the extent of *in vitro* fermentation. Although, in previous studies, there is a general agreement that the EFE addition increased the rate but not the extent of ruminal digestion (Tang *et al.* 2013). But, it is not a rule because the fibrolytics enzymes addition may be influenced by substrate composition, type and level of enzyme and the method of enzyme application (Mao *et al.* 2013). The EFE addition on WS resulted a higher value of organic matter digestibility (OMD) (P<0.001), metabolizable energy (ME) (P<0.001) and volatile fatty acids (VFA) (P<0.001) especially with the optimal dose D1 and M1 accompanied with a linear increase (P<0.001) of the amounts of dry matter losses and reducing sugar release (Table 2, 3). This finding was similar to the study of Freiria *et al*. (2018) on kinetic parameters of *in vitro* ruminal degradation with combinations of exogenous enzymes in ruminant diets. In our study, the highest level of both enzymatic complexes leads to highest RS release but as per Mao *et al.* (2013) the middle enzymes doses were more effective. Thus, the optimal enzyme doses vary according to EFE composition and to cellulase: xylanase ratio (Tirado-González *et al.* 2017). The release of extra amounts of sugar in the fermentation medium could be fermented by ruminal microorganisms. It also proved that the EFE hydrolyze partially the straw cell wall, which could facilate ruminal microorganism attachment to the substrate and elicit an increase of GP and VFA production as proved by Mao *et al.* (2013) for rice straw.

Table 1. Chemical composition of untreated and chemically pretreated wheat straw

Item	Chemical composition (g/kg DM)			
	WS	SWS	UWS	AWS
DM	890±0.28	291±1.4	790±0.28	200±0.32
Ash	55±0.7	111±0.14	66±0.15	18±0.2
NDF	739±0.2	655±1.4	756±0.39	702±0.17
ADF	469±0.08	496±1.7	548±0.54	462±0.34
ADL	51±0.05	53±0.15	57±0.17	52±0.19
Cellulose	418±0.12	443±0.3	491±0.22	410±0.2
Hemicellulose	271±0.03	159±0.25	208±0.19	240±0.1
CP	32±0.49	36±0.12	141±0.14	101±0.1

WS, untreated wheat straw; SWS, NaOH pretreated wheat straw, UWS, urea pretreated wheat straw; AWS, acid pretreated wheat straw; DM, dry matter; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein.

The alkali pre-treatments of wheat straw resulted in higher gas production at all incubation times but the EFE effect varied because of the modification of the chemical composition and the cell wall properties of the straw (Table 1). In fact, for UWS, the dyadic decreased (P<0.05) the ½ times B and Tmax (Tables 2 and 3) and the optimal dose of MaxFiber to M 0.5=0.5 mg/g DM that improved (P<0.05) the rate and the extent of UWS fermentation. Thus, we suggest that there is a synergetic effect between urea pre-treatment and EFE supplementation. In the same context, Eun *et al.* (2006) suggested a synergetic effect between NH_3 pre-treatment and exogenous xylanases addition. So, we can hypothesize that the extra N source delivered by NH_3 or urea stimulate the EFE effect. The detailed mechanism by which urea pre-treatment improved the efficacy of EFE remains unexplained. However, it's known that urea pre-treatment increases rumen NH_3-N content, thereby, the total ruminal bacteria and particularly, rumen fibrolytic microbes (Vinh *et al*. 2011). Coupled with the fact that alkali pre-treatment disrupts the cell wall by cleaving ester bonds between lignin, cellulose, and hemicellulose and reduce the physical enmeshment of cellulose. It causes the swelling of the cell wall and the decreasing of the crystallinity of cellulose (Sun 2013) and thus makes the substrate more susceptible to the physical and hydrolytic effect of endogenous and exogenous fibrolytic enzyme.

For SWS, both EFE had no effects on the fermentation and the digestive use parameters. So, the ability of NaOH pre-treatment to remove partially some cell wall barriers could be insufficient to create a synergy with EFE. This finding supports the previous idea, that the addition of extra N source for ruminal microorganism is the major factor that stimulates the EFE to improve the digestibility of wheat straw. Instead, Wang *et al*. (2004) found that applying EFE increased the GP, the rate and the extent of dry matter digestion of NaOH pre-treated wheat straw. The reason for the different response is not clear, but there is a possibility that the hydroxide pre-treatment requires a certain level of enzyme activity. Thus, the NaOH pre-treatment alone was more effective than EFE for wheat straw.

For AWS, highest amounts of RS were recorded which

Table 2. Effect of increasing doses (D1,1; D2, 2; D3, 3; D4, 4 µl/g DM) of Dyadic enzyme on the *in vitro* gas production parameters; estimated energy utilization and enzymic hydrolysis of untreated and chemically pretreated wheat straw (n=9)

	Gas production parameter					GP-96 h	Fermentation profile			Hydrolysis	
	A	B	C	R_{MAX}	T_{MAX}		ME	OMD	VFA	RS	DML
Untreated wheat straw (WS)											
Control	147.7^{b}	10	1.8	9.2^{b}	4.9^{a}	147.7^{b}	5.9^{c}	398^{c}	0.57^{c}	2.1^{c}	118.3^{b}
D1	187.5^{a}	8.2	1.8	14.2^{a}	4.1^{ab}	187.5^{a}	7.2^{ab}	480^{ab}	0.78^{ab}	2.5^{c}	166^{ab}
D2	176^{ab}	8.4	1.7	13.1^{a}	3.8^{b}	171.5^{ab}	7.3^{a}	489^{a}	0.8^{a}	4.9^{c}	204.4^{a}
D3	197.2^{a}	9.9	1.7	12.5^{ab}	4.4^{ab}	197.2^{a}	6.9^{ab}	463^{ab}	0.74^{ab}	9.1^{b}	179.9^{a}
D4	163^{ab}	9.4	1.8	10.8^{ab}	4.5^{ab}	171.5^{ab}	6.5^{bc}	438^{bc}	0.67^{bc}	16.9^{a}	182.4^{a}
Linear	NS	NS	NS	NS	NS	NS	NS	NS	NS	***	**
Quadratic	***	NS	NS	***	*	*	***	***	***	NS	NS
NaOH pretreated wheat straw (SWS)											
Control	176.3	11.4	2.3	10.6	7.3	176.3	6.6	445	0.69	3.4^{c}	202.4
D1	166.7	11.1	2.5	11.1	7.8	166.7	6.6	445	0.69	7.3^{b}	195.5
D2	183.5	11	2.5	12.2	7.8	183.5	7.1	474	0.76	8.1^{b}	204.4
D3	158.2	11.5	2.4	9.9	7.8	158.2	6.7	426	0.64	10.7^{b}	210
D4	163.1	10.5	2.3	11.2	7.1	163.1	6.9	463	0.74	23.9^{a}	224
Linear	NS	NS	NS	NS	NS	NS	NS	NS	NS	***	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Urea pretreated straw (UWS)											
Control	178	11.4^{a}	2.5^{a}	11.3	8^{a}	178	6.9	461	0.73	1.8^{c}	128.8
D1	182.6	10.5^{ab}	2.1^{b}	11.6	6.4^{b}	182.6	7.3	488	0.80	11.6^{b}	129
D2	181	10.5^{b}	2.1^{b}	11.5	6.4^{b}	181	6.9	463	0.74	13.1^{ab}	124.7
D3	187.4	10.7^{ab}	2.2^{b}	11.8	6.8^{b}	187.4	7.1	475	0.77	16.1^{a}	106.8
D4	180.3	10.3^{b}	2.1^{b}	11.7	6.5^{b}	180.3	6.93	464	0.74	13.5^{ab}	147.5
Linear	NS	*	NS	NS	*	NS	NS	NS	NS	***	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS
Acid pretreated straw (AWS)											
Control	180.3^{a}	5.6	1.2	22.3	0.8	174.3	6.9^{a}	493^{a}	0.73^{a}	72.1^{b}	58.9^{b}
D1	164^{ab}	5.5	1.2	20.8	0.7	156	6.5^{ab}	464^{ab}	0.66^{ab}	102.6^{a}	115.9^{a}
D2	168^{ab}	6.1	1.2	18.8	0.8	161.7	6.6^{ab}	471^{ab}	0.68^{ab}	105.7^{a}	119.2^{a}
D3	154.5^{b}	6.2	1.2	17.5	0.9	141.3	6.2^{b}	448^{b}	0.62^{b}	98.4^{a}	122.6^{a}
D4	151.2^{b}	5.4	1.2	19.7	0.6	146	6.2^{b}	444^{b}	0.61^{b}	102.7^{a}	141.1^{a}
Linear	**	NS	NS	NS	NS	NS	**	**	*	***	***
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	*
SEM	11.57	1.2	0.28	2.34	1.58	10.34	0.34	1.67	0.04	4.5	1.9
EFE doses	NS	NS	NS	NS	NS	NS	NS	NS	NS	***	***
Chemical Treatment	***	***	***	***	***	*	***	*	NS	***	***
EFE doses × Chemical Treatment	***	NS	NS	***	NS	NS	NS	NS	NS	NS	NS

Estimated potential gas production (A, ml/g DM), time of incubation at which the half of A has been produced (B, h), sharpness of the curve (c), the maximum rate of GP (Rmax, ml/h), time at which Rmax is attained (Tmax, h). GP-96 h, gas production at 96 h after incubation. Metabolizable energy (ME, MJ/kg DM), organic matter digestibility (ME, g/kg), volatile fatty acids (VFA, mmol/200 mg DM), reducing sugar releases (RS, mg/g DM), dry matter losses (DML, g/ kg).[a;b;c] means with different superscripts differ significantly (P<0.05). SEM, standard error of the mean. P<0.1, *P<0.05, **P<0.01, ***P<0.001, NS not significant.

could explain the improvement of the *in vitro* fermentation of wheat straw compared to WS because diluted H_2SO_4 pre-treatment was commonly used to disrupt lignin-carbohydrate matrix, and hence to facilitate enzymatic hydrolysis (Zhu *et al*. 2009). However, the EFE supplementation affected negatively the straw fermentation especially with the Dyadic supplementation. So, a linear decrease of potential gas production (P<0.01) and all digestive use parameters (P<0.01) was detected despite the highest amounts of released reducing sugar (P<0.0001), thus, it seems like the H_2SO_4 pre-treatment obstructs enzymes effect. The antagonist mechanism created between EFE and the acid pre-treatment remains unexplained but many researchers confirmed that the acid pre-treatment coupled with relatively high temperature lead to the formation of ruminal fermentation inhibitory compounds such as furfural and 5-hydroxymethylfurfural from free sugar released in the medium (Hsu *et al.* 2010).

These compounds inhibit the activity of ruminal microbes and free enzymes but at some levels, rumen microbes can metabolize and resist to furfural (Castro *et al*. 1994). Since the presence of EFE, which increased the amount of RS, it can stimulate probably the formation of high levels of furfural and 5-hydroxymethylfurfural that

Table 3. Effect of increasing doses (M1=0.5; M2=1; M3=2 and M4=4 mg/g DM) of MaxFiber enzyme on *in vitro* gas production parameters; estimated energy utilization and enzymic hydrolysis of untreated and chemically pretreated wheat straw (n=9)

	Gas production Parameters					GP-96h	Fermentation Profile			Hydrolysis	
	A	B	C	R_{MAX}	T_{MAX}		ME	OMD	VFA	RS	DML
Untreated wheat straw (WS)											
Control	147.7^{b}	10^{a}	1.87	9.2^{b}	4.9^{ab}	147.7	5.9^{b}	398^{b}	0.57^{b}	2.1^{c}	118.4^{b}
M1	169.3^{b}	9.5^{ab}	2.03	12^{ab}	5.2^{ab}	169.3	7.0^{a}	470^{a}	0.75^{a}	2.9^{bc}	114.4^{b}
M2	193^{a}	9.5^{ab}	2.09	13.8^{a}	5.6^{a}	193.1	7.3^{a}	487^{a}	0.79^{a}	3.1^{bc}	140^{a}
M3	162^{b}	8.8^{ab}	1.89	11.9^{ab}	4.6^{ab}	162.1	6.5^{ab}	436^{ab}	0.67^{ab}	3.8^{ab}	149.4^{a}
M4	197.4^{a}	8^{b}	1.71	15.5^{a}	4.1^{b}	197.4	7.1^{a}	475^{a}	0.77^{a}	4.8^{a}	159^{a}
Linear	**	*	NS	**	*	NS	*	*	*	***	***
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
NaOH pretreated wheat straw (SWS)											
Control	176.3	11.4	2.3	10.6	7.3	176.3	6.6	445	0.69	3.4^{c}	199.5
M1	173.3	10.6	2.3	11.9	7.1	173.3	6.8	456	0.72	3.3^{c}	222.3
M2	193.6	10.3	2.4	13.6	7.1	193.7	7.4	493	0.81	5^{b}	198.3
M3	167.4	10.7	2.4	11.6	7.4	167.4	7.1	448	0.70	5.7^{ab}	200.1
M4	182.6	10.3	2.3	11.4	6.9	182.6	7.1	475	0.76	6.6^{a}	209.6
Linear	NS	NS	NS	NS	NS	NS	NS	NS	NS	***	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Urea pretreated wheat straw (UWS)											
Control	178^{ab}	11.5^{a}	2.45	11.3^{bc}	8	178^{ab}	6.9^{ab}	461^{ab}	0.73^{ab}	1.8^{d}	128.9^{a}
M1	195.7^{a}	10.6^{b}	2.36	13.1^{a}	7.2	195.7^{a}	7.4^{a}	493^{a}	0.81^{a}	4.5^{c}	117^{b}
M2	170.4^{b}	10.9^{ab}	2.31	11^{c}	7.3	170.4^{b}	6.7^{b}	448^{b}	0.7^{b}	4.5^{c}	119.6^{b}
M3	195.9^{a}	10.6^{b}	2.27	12.9^{a}	7	196^{a}	7.3^{a}	492^{a}	0.81^{a}	6.8^{b}	119.4^{b}
M4	192.7^{a}	10.7^{ab}	2.25	12.4^{ab}	7	192.7^{a}	7.3^{a}	486^{a}	0.79^{a}	16.8^{a}	120^{b}
Linear	*	.	NS	.	NS	*	NS	NS	NS	***	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	**
Acid pretreated wheat straw (AWS)											
Control	180.3	5.56	1.2	22.31	0.76	174.3	6.9	493	0.68	72.1	58.9^{b}
M1	163.6	5.95	1.15	19.69	0.61	157.3	6.6	461	0.66	75.6	102^{a}
M2	173.6	5.75	1.19	20.75	0.74	167.7	6.8	482	0.71	79.6	76.5^{ab}
M3	168.9	5.7	1.16	20.93	0.63	162.8	6.6	473	0.68	83.6	96.5^{a}
M4	173.5	5.56	1.2	21.16	0.76	168	6.8	464	0.66	81.5	91.6^{a}
Linear	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
SEM	12.2	1.15	0.26	2.25	1.43	8.41	0.39	2.1	0.05	1.9	0.9
EFE doses	NS	**	NS	*	NS	NS	NS	NS	NS	***	***
Chemicaltreatment	NS	**	***	***	***	*	NS	NS	NS	***	***
EFE doses × Chemical treatment	NS	NS	NS	NS	NS	.	NS	NS	NS	***	**

Estimated potential gas production (A, ml/g DM), time of incubation at which the half of A has been produced (B, h), sharpness of the curve (c), the maximum rate of GP (Rmax, ml/h), time at which Rmax is attained (Tmax, h). GP-96 h, gas production at 96 h after incubation. metabolizable energy (ME, MJ/Kg DM), organic matter digestibility (ME, g/kg), volatile fatty acids (VFA, mmol/200mg DM), reducing sugar releases (RS, mg/g DM), dry matter losses (DML, g/ kg). ^{a,b,c}means with different superscripts differ significantly (P<0.05). SEM, standard error of the mean, P<0.1, *P <0.05, **P <0.01, *** P <0.001 and NS Not significant.

become toxic to rumen microbes, which may explain the negative linear effect on straw fermentation with the Dyadic mixture. The production of furfural compounds means a loss of fermentable sugar and inhibition of microbial and enzymes activity, consequently the decrease of *in vitro* ruminal fermentation.

As a conclusion, the EFE supplementation improved the rate and the extent of ruminal digestion of untreated wheat straw. The combined effect of EFE and chemical pre-treatments had different responses, in fact, with NaOH pre-treatment, the EFE failed to have any effect on wheat straw digestibility. In contrast, the urea pre-treatment seems to have a synergetic effect with EFE on the ruminal utilization of wheat straw. On the other hand, the pre-treatment with H_2SO_4 had a negative effect on the *in vitro* digestibility with all EFE used doses. The combination of chemical pre-treatments and EFE supplementation promotes the hydrolysis of cell wall component by improving the release of reducing sugar. Further research is required to investigate the optimum conditions and factors that optimize ruminant utilization of fibrolytics enzymes supplementation on chemically pre-treated by-products.

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