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ORIGINAL ARTICLE

Enzymatic valorization of alkali-treated chickpea straw and sunflower stalks as high fibrous agricultural wastes for sustainable ruminant nutrition

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Abstract
Chickpea straw (CS) and sunflower stalks (SS) are agricultural wastes with high fibre content and low digestibility. To improve their nutritional value and ruminal digestibility, the effects of NaOH and urea treatments combined with exogenous fibrolytic enzymes (EFE) were investigated. The untreated CS (CCS) and SS (CSS), 4% NaOH treated CS (NCS) and SS (NSS), and 4% urea-treated CS (UCS) and SS (USS) were supplemented by two enzymatic complexes (DCX and MaxFiber) composed mainly of cellulase and xylanase activities at increasing doses: 0, 1, 2, 5, and 10 µL DCX/g DM and 0, 0.5, 1, 2, and 4 mg MaxFiber/g DM. The results of in vitro ruminal fermentation proved that the DCX was more efficient than the MaxFiber complex for both CCS and CSS. Indeed, it improved the rate and the extent of ruminal fermentation, metabolizable energy, organic matter digestibility, and volatile fatty acids (p-value <0.05) by 5 %, 47%, 12%, 12.8%, and 23.8%, respectively, for CCS using 10 µl/g DM and 20.8%, 27.6%, 12.9%, 11.8%, and 22.8%, respectively, for CSS by using 5 µl/g DM. The association between alkali treatments and EFE was depending to the supplemented enzymatic complex, the treated substrate and the alkali treatment. For the CS, the association between alkali and EFE stimulated the ruminal fermentation and improved the digestive use. However, it decreased the efficiency of EFE for SS. Overall, the use of EFE to CS and SS could provide a valuable source of energy from digestible fibre for ruminants.

Keywords Agricultural by-products · Anaerobic fermentation · Digestion · Exogenous fibrolytic enzymes · Ruminants

1 Introduction

In recent years, there has been an increasing focus on sustainable agricultural practices and the efficient utilization of agricultural waste for livestock feed. Chickpea straw and sunflower stalk are abundant agricultural residues that are underutilized and often treated as waste [1]. However, they possess significant potential as fed resources due to their high fibre content, which may provide valuable energy and nutrients for ruminants [2]. To unlock the nutritional value of these lignocellulosic wastes, enzymatic treatment is proposed as a novel approach. Previously, researches have primarily focused on chemical treatments, while enzymatic treatments remain relatively unexplored [3, 4]. The novelty of the current study focuses on the enzymatic valorization of alkali-treated chickpea straw and sunflower stalks for sustainable ruminant nutrition. The chemical treatments could modify the chemical composition and weaken the structural fibre of the treated biomass [5]. And, the exogenous fibrolytic enzymes (EFE) can break down complex carbohydrates present in the lignocellulosic biomasses, into simpler sugars, which can be more easily fermented by the ruminal microorganisms and provide a better source of energy and nutrition for the animal [6]. So, by using specific enzymes and optimizing dosages supplementation and treatment conditions, the nutritional value, the digestive use, and the feed costs could be optimized [7]. However, their use is subject to some challenges, such as the need for an appropriate balance between the type and dose of enzymatic product, the type of substrate being treated, and the desired animal response [8]. This highlights the need to further research to optimize the

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use of exogenous fibrolytic enzymes in agricultural waste management and ruminant nutrition. So, this research aimed to test the hypothesis that the alkali treatments of chickpea straw and sunflower stalk could improve the efficiency of EFE to enhance their nutritional value. The objective was to determine the effect of two different exogenous fibrolytic enzyme complexes at increasing dose levels on the in vitro ruminal fermentation and digestive use parameters of untreated and alkali-treated chickpea straw and sunflower stalk.

2 Material and methods

2.1 Collect and alkali treatments of chickpea straw and sunflower stalks

After chickpea and sunflower seeds harvesting, samples from chickpea straw (CS) and sunflower stalks (SS) were randomly collected from fields located in the northwest region of Tunisia. Then, the samples were manually chopped into small stands of almost 5 cm to facilitate alkali treatments. Once well homogenized, the shopped CS and SS were divided into 9 subsamples of 2 kg each. The first 3 subsamples were kept untreated for CCS and CSS. The second 3 subsamples were subject to NaOH treatment. According to Dulphy et al. [9], the CS and SS samples were pulverized by 4% NaOH solution and left uncovered during 48 h for NCS and NSS. The remaining 3 subsamples were subject to urea treatment for UCS and USS. According to Chermiti et al. [10], the CS and SS were pulverized by 4% urea solution and ensiled in hermetic plastic bags for 2 months to prevent oxygen entrance and ammonia losses. Once all treatments were ready, samples of 500 g from each CS and SS preparations (CCS, NCS, UCS, CSS, NSS, and USS) were dried in a forced air oven overnight at 55 °C until constant weight and then grounded through a 1-mm sieve using a Retsch SK 100 standard, Giessen, Germany, for subsequent analysis.

2.2 Chemical analyses

The untreated and alkali-treated CS and SS were subject to chemical analysis to determine their dry matter (DM, method ID 930.15), ether extract (EE, method ID 920.30), organic matter (OM, method ID 942.05), crude protein (CP, ID 954.01), and crude fibre (CF, ID 962.09) contents according to the methods of the Association of Official Analytical Chemists [11]. The Neutral detergent fibre (NDF, assayed with a heat stable amylase and expressed inclusive of residual ash), acid detergent fibre (ADF, expressed inclusive of residual ash), and acid detergent lignin (ADL, after extraction with sulphuric acid) were determined using the ANKOM fibre analyzer (ANKOM, A2001, New York, NY, USA) in a fibre filter bag (F57-ANKOM Technology Corporation, Macedon, NY, USA) according to Van Soest et al. [12]. The total phosphorus (P) contents were analysed by the molybdovanadate colorimetric method (method ID 965.17) using a spectrophotometer (Shimadzu UV-1201 UV-Vis). The calcium content (Ca) was measured using an atomic absorption spectrophotometer (Varian AA140, Varian, Australia) (method ID 968.08). All chemical analyses were performed in triplicate for each sample ($n = 3$), repeated each time the difference between replication was upper 5%, and presented in Table 1.

2.3 Exogenous fibrolytic enzymes and their enzymatic activities

The obtained CS and SS preparations (CCS, NCS, UCS, CSS, NSS, and USS) were supplemented by two different

Table 1 Chemical composition (% dry matter, unless otherwise stated) of untreated (CCS, CSS), NaOH (NCS, NSS), and urea (UCS, USS)-treated chickpea straw and sunflower stalk (n=3)

	Chickpea straw			Sunflower stalk		
	CCS	NCS	UCS	CSS	NSS	USS
Dry matter*	92.4^{a}	32.6^{c}	76.1^{b}	90.5^{a}	33.1^{c}	71.4^{b}
Ash	4.7^{c}	8.6^{a}	5.2^{b}	8.6^{b}	15.6^{a}	8.5^{b}
Crude protein	3.7^{b}	3.6^{b}	16.9^{a}	5.7^{b}	4.59^{b}	17.5^{a}
Ether extract	0.7	1.2	0.8	0.7	0.6	0.8
NDF	56.9^{a}	46.6^{b}	51.6^{a}	63.4^{a}	63.8^{a}	55.0^{b}
ADF	35.5	28.6	33.4	47.1	45.2	40.4
ADL	7.3^{a}	4.6^{b}	5.2^{b}	11.1^{a}	10.2^{a}	8.2^{b}
Hemicellulose	21.4^{a}	18.1^{b}	18.6^{b}	16.3	18.6	14.6
Cellulose	28.2^{a}	24.2^{b}	28.2^{a}	36.3^{a}	29.3^{b}	32.2^{a}
Calcium	0.4^{b}	0.6^{a}	0.4^{b}	0.5	0.6	0.6
Phosphorus	0.047^{b}	0.068^{a}	0.056^{a}	0.07^{a}	0.074^{a}	0.037^{b}

*The dry matter was expressed as % fresh matter of chickpea straw and sunflower stalk preparations; ^{a,b,c} means within a row with different superscripts differ significantly (p-value < 0.05).

xylanase to cellulase enzymatic complexes. The first was a mixture (1:1, *v/v*) of two commercial products in liquid form which are Cellulase PLUS and Xylanase PLUS (DCX), produced by the fermentation of non-genetically modified *Trichoderma longibrachiatum*, and are composed primarily of endo-1,4-β-D-xylanase (E.C. 3.2.1.8) and endoglucanase (EC 3.2.1.4), in addition to other side additional activities such as pectinase, mannanase, amylase, and protease. The DCX was supplemented at increasing dose levels as recommended by Jabri et al. [13]: 0, 1, 2, 5, and 10 µl/g DM. The supplementation was performed by diluting the DCX complex with distilled water (10-fold) and directly sprayed onto the grounded CS and SS preparations with the appropriate dose/g DM. The second enzymatic complex is a commercial protein rich by-product in powdered form, obtained from solid-state fermentation of *Aspergillus* strains and *Neurospora intermedia*, contained xylanase, endoglucanase, and exoglucanase activities. The MaxFiber was also supplemented at increasing dose levels according to the manufacturer instructions: 0, 0.5, 1, 2, and 4 mg/g DM. Both enzymatic complexes (DCX and MaxFiber) were assessed in triplicate in each of three runs (n=9) for the xylanase (EC 3.2.1.8, Endo-β-1,4-xylanase), exoglucanase (EC 3.2.1.91, Exo-β-1,4-glucanase), and endoglucanase (EC 3.2.1.4, Endo-β1,4-glucanase) activities according the methods of Wood and Bhat [14] and Bailey et al. [15] (Table 2).

Table 2 Fibrolytic activities of the studied enzymatic complexes (n = 9)

Enzymatic activities**	DCX*	MaxFiber
Xylanase	2573 ± 131.3	5118 ± 6.2
Endoglucanase	1554 ± 76.4	75 ± 1.1
Exoglucanase	160 ± 10.2	74 ± 0.3
Ratio***	1.50	0.75

*DCX (50% cellulase-PLUS et 50% xylanase-PLUS), **xylanase, endoglucanase, and exoglucanase activities are expressed as µmol of xylose and glucose, respectively, released by 1 ml of undiluted enzyme per minute (IU). ***Ratio of fibrolytic activities "xylanase to cellulase"

2.4 In vitro ruminal fermentation

The in vitro ruminal fermentation using batch culture technique according to Theodorou et al. [16] was used in this study according the ruminal fermentation workflow (Fig. 1). To collect the fresh ruminal fluid, two cannulated non lactating cows (600–650 kg body weight) were fed a stable diet composed of oat hay ad libitum and 2 kg commercial concentrate formulated for dairy cows (Alfa® 7 standard) with free access to water and mineral/vitamin licks to meet the nutritional requirements as recommended by INRA [17]. The ruminal fluid was collected before morning feeding in prewarmed insulated flasks, from different sites within the rumen via electric pump, and then immediately transferred to the lab and strained through 4 layers of cheesecloth under anaerobic conditions. The fermentation inoculum was prepared by mixing the freshly collected ruminal fluid and the anaerobic buffer medium (pH=6) prepared in advance as described by Menke and Steingass [18] in a ratio of 1:2 (ruminal fluid: buffer medium). Samples of dry 200 ± 10 mg DM ground CCS, NCS, UCS, CSS, NSS, and USS were weighed in the fermentation bottles in triplicate each and then supplemented with the corresponding EFE dose level 20 h before the in vitro incubation as recommended by Beauchemin et al. [19]; then all fermentation bottles were filled with 30 ml of fermentation inoculum, immediately sealed with a butyl rubber stopper and an aluminium crimp cap, and incubated at 39 °C water bath for 96 h. All in vitro ruminal fermentation preparation steps were performed

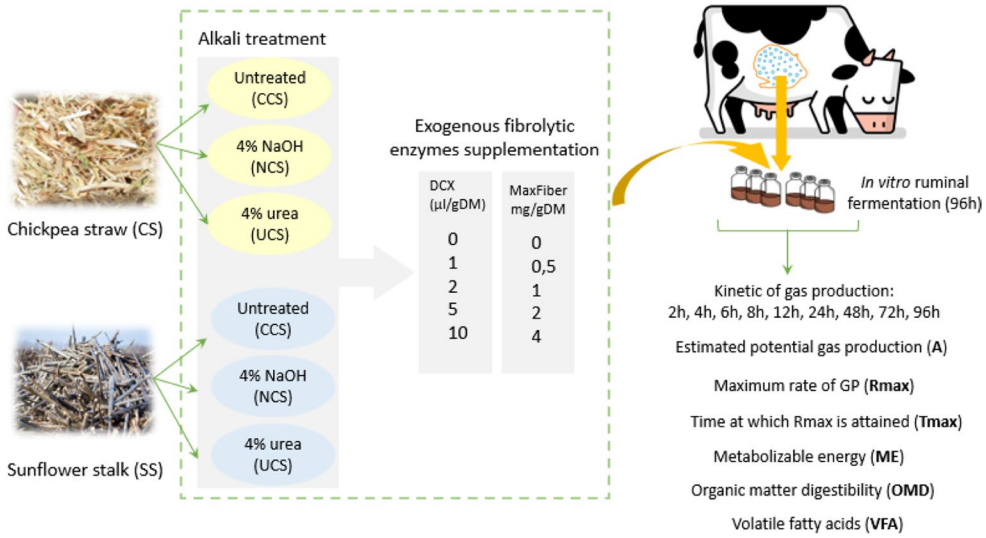

Fig. 1 Experimental pipeline for the in vitro ruminal fermentation of untreated and alkali-treated chickpea straw and sunflower stalk supplemented with two different enzymatic complexes at increasing dose levels

under continuous flushing with CO_2 at 39 °C water bath. To ensure results accuracy, negative control bottles containing inoculum fermentation without substrate and positive control bottles containing substrate without enzymatic supplementation (0 µl DCX/g DM and 0 mg MaxFiber/g DM) were used in six replications each. The in vitro ruminal fermentation run was repeated three times with the same procedure (n=9). The incubation was repeated each time the difference of gas production (GP) in positive control bottles was larger than 5% between runs. The GP was measured for each bottle after 2, 4, 6, 8, 12, 24, 48, 72, and 96 h of incubation by inserting a 23-gauge (0.6 mm) needle attached to a pressure transducer connected to a visual display. After each measurement, the transducer was removed, leaving the needle in place to permit venting.

2.5 Calculations and statistical analysis

The measured gas pressures for each bottle were converted to gas volume using the following equation:

$$GP\ (ml) = GPr \times \frac{Vf - Vi}{Patm} \tag{1}$$

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

Subsequent to the GP measurement, the metabolizable energy (ME), organic matter digestibility (OMD), and volatile fatty acids (VFA) were determined according to Menke and Steingass [18] and Getachew et al. [20] prediction models:

$$\begin{aligned}\text{ME (MJ/kg DM)} = {} & 2.2 + (0.136 \times \text{GP24h}) \\ & + (0.057 \times \text{CP}) + \left(0.00286 \times \text{EE}^2\right)\end{aligned} \tag{2}$$

$$\begin{aligned}\text{OMD (\%)} = {} & 14.88 + \left(0.889 \times \text{GP}_{24\text{h}}\right) \\ & + (0.45 \times \text{CP}) + (0.0651 \times \text{Ash})\end{aligned} \tag{3}$$

$$\text{VFA (mmol/200 mg DM)} = 0.00425 + \left(0.0222 \times \text{PG}_{24\text{h}}\right) \tag{4}$$

The measured GP kinetics were fitted using the residual least square method of the reduced generalized gradient algorithm of the solver function in Microsoft Excel software according to Groot et al. [21] model:

$$GP\ [ml] = \frac{A}{\left[1 + \left({}^{B}/_{t}\right)^{C}\right]} \tag{5}$$

where A is the estimated potential GP (ml/g DM); B is the required time to produce ½ A (h); C is the curve sharpness.

The parameters maximum rate of GP (Rmax) and the time at which Rmax is attained (Tmax) were calculated according to Yang et al. [22] as Eqs. (6) and (7):

$$Rmax\ \left[ml/h\right] = AB^{C}C\left[\frac{Tmax^{(-C-1)}}{\left(1 + B^{C} \times Tmax^{-C}\right)^{2}}\right] \tag{6}$$

$$Tmax\ [h] = B\left[\frac{C-1}{C+1}\right]^{1/c} \tag{7}$$

All collected data were analysed as a completely randomized design and were conducted using the GLM procedure of SAS Studio (3.6) (2017) according the following statistical model Yijk = µ + Di + Tj + (Di * Tj) + εijk, where Yijk is an individual observation for each dependent variable, μ is the overall mean, Di is the fixed effect of the supplemented EFE dose rate, Tj is the fixed effect of chemical treatment, (Di * Tj) is the interaction between the chemical treatment and the EFE dose rate, and εijk is the residual error.

The mean values of each sample were used as the experimental unit. The polynomial contrasts (linear and quadratic effects) of increasing dose levels supplementation were determined. As the studied dose levels are unequally spaced, the Proc IML from SAS® studio (3.6) (2017) was used to generate coefficients for polynomial contrasts.

The differences between dose levels means were assessed using the multivariate Duncan test [23]. Means were considered significantly different at p-value less or equal to 5% and tendencies were declared at $0.05<p\text{-value}<0.1$.

3 Results and discussion

3.1 Chemical composition of untreated and alkali-treated chickpea straw and sunflower stalks

The chemical composition of the studied by-products CCS and CSS revealed that both are high fibrous lignified biomasses containing 56.9% and 63.4% NDF, 35.5% and 47.1% ADF, and 7.3% and 11.1% ADL, respectively. So, the cellulose content varied between 28.2 and 36%, the hemicellulose between 21.4 and 16.3%, and the ADL between 7.3 and 11.1% for CCS and CSS, respectively (Table 1). These results were different to those previously reported by de Souza et al. [24], Durmaz and Ates [25], and Maheri-Sis et al. [26]. These differences could be related to the plant species, genetics, and growth conditions. It is noteworthy that different factors could interact with each other's causing high variability in plant's chemical composition. Comparing to cereal straw, which is a commonly used by-product

for livestock feeding [27], both CS and SS presented an interesting nutritive value richer in CP by 3.7% and 5.7%, respectively, against 3.2% for wheat straw [27], which could significantly contribute to ruminant feeding.

The alkali treatment of CS and SS modified their chemical composition as presented in Table 1. The urea treatment improved the solubilization of hemicellulose and ADL contents by 28.7% and 13.1%, respectively, of UCS as compared to the CCS. As for the SS, it caused lignin and cellulose solubilization by 26.1% and 10.5%, respectively, as compared to the CSS. The NaOH treatment caused a significant solubilization of hemicellulose, cellulose, and ADL especially for the NCS which their contents decreased by 15.8%, 14.8%, and 37.0% respectively (Table 1). Generally, the use of NaOH and urea treatments for agricultural by-product led to an increase of cellulose, hemicellulose, and lignin solubility [28]. However, the efficacy of alkali treatments could vary depending on a number of factors, including the substrate properties, the alkali concentration and its mode of action, the pH of the treatment environment, the temperature, and the duration of the treatment [29]. Indeed, the NaOH is a strong alkali that can dissolve hemicellulose, cellulose, and lignin through depolymerization [30], whereas the urea is an organic compound that can also dissolve lignocellulosic content by breaking the hydrogen bonds between the polysaccharides in anaerobic conditions [31]. The ash content improved significantly after NaOH treatment by 82.9% for NCS and 81.4% for NSS, which could be attributed to the residual NaOH [32]. Moreover, the urea treatment improved significantly the crude protein content for both UCS and USS (Table 1). During the urea treatment, the pH and temperature increased, creating a favourable environment for microorganisms' growth and protein synthesis from the urea non-protein nitrogen [33, 34].

3.2 Effect of EFE supplementation on in vitro ruminal fermentation of untreated and alkali-treated CS and SS

Both studied EFE complexes (DCX and MaxFiber) supplied xylanase, endoglucanase, and exoglucanase activities under ruminal conditions (pH= 6.6, T°= 39 °C) with a xylanase to cellulase ratio equal to 1.5 and 0.75, respectively, as presented in Table 2.

As presented in Figs. 2 and 3, Tables 4 and 6, the in vitro ruminal fermentation results proved that the fermentation profile, the extent and the rate of GP, and the digestive use parameters of CCS and CSS are comparable to cereal straw [27] and higher than some industrial by-products like sesame seed coats [35] and olive cake [36, 37]. Indeed, the CCS and CSS could be an interesting source of nutrients, such as fibre, which is an important diet component for ruminant as it promotes rumen health and function, and it is essential for maintaining optimal animal performances. However, the physical structure of the sunflower stalks can be abrasive to the animal's mouth and may cause dental issues if not properly processed before inclusion in the diet. So, both chickpea straw and sunflower stalk could be included in ruminant diet; however, it's important to consider the nutritional value and the physical properties of the biomass, as well as the animal's requirements. Indeed, properly balancing ruminant's diet with other feedstuffs, applying adequate processing techniques such as chopping, grinding or ensiling, and adjusting the inclusion levels can help to optimize the digestive use of these agricultural by-products by ruminants.

The NaOH and urea treatments of CS and SS caused variable effects on the in vitro ruminal fermentation due the modifications of their chemical composition. Indeed, for the NCS, the NaOH treatment improved slightly the rate of GP and the digestive use parameters by 3.0% (Rmax), 4.0% (ME), 5.6% (OMD), and 7.1% (VFA) as compared to CCS. As the urea treatment improved the rate and the extent of chickpea straw by 8.2% (A) and 31.8% (Rmax), then the estimated digestive use parameters by 26.0% (ME), 28.5% (OMD), and 16.6% (VFA), as compared to the CCS. The NaOH and urea treatments improved slightly the ruminal fermentation of CS by promoting the hemicellulose and lignin hydrolysis, making the cellulose more accessible to ruminal microorganisms, which can lead to faster and more efficient fermentation of CS as compared to wheat straw [37]. On the other hand, for the SS, both studied alkali treatments decreased the ruminal fermentation profile, and all studied parameters of in vitro ruminal GP and digestive use as compared to the CSS (Fig. 1, Tables 3 and 4). This finding could be attributed to the initial high content of lignin which is more resistant to ruminal fermentation. The NaOH and urea treatments can break down these components, but they can also make them less accessible to rumen microorganisms, decreasing the fermentation efficiency as found by Moradi et al. [38] for pistachio by-products. So, the NaOH and urea treatments efficiency is dependent to the substrate initial lignocellulosic matrix.

The EFE supplementation effects at increasing doses on untreated and alkali-treated CS and SS are depicted in Figs. 1 and 2 and Tables 3, 4, 5, and 6. During this study, the considered optimal dose was the minimum dose required to obtain the greatest significant improvement for the studied fermentation parameters as suggested by Eun et al. [39]. The effect of both studied EFE enzymatic complexes depended significantly to the supplemented dose level and the alkali treatment for most studied parameters as the GP kinetic (Tables 3 and 5) and the in vitro ruminal fermentation and the digestive use parameters (Tables 4 and 6).

Both studied EFE complexes (DCX and MaxFiber) exerted quadratic effects at increasing doses on the GP kinetic, the in vitro ruminal fermentation, and digestive use parameters of

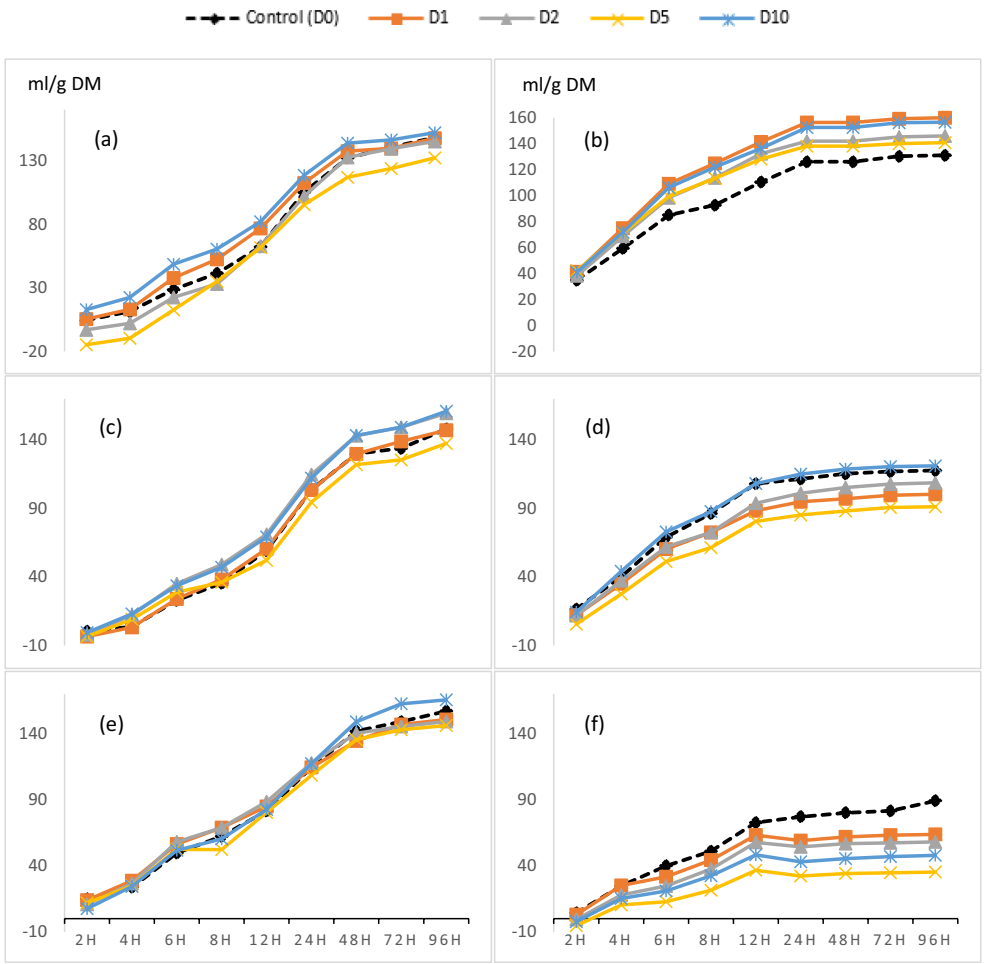

Fig. 2 Effect of DCX enzymatic complex at increasing dose levels on ruminal gas production kinetics (ml/g DM) during 96 h of in vitro ruminal fermentation of untreated (**a**, **b**), NaOH (**c**, **d**), and urea (**e**, **f**)-treated chickpea straw and sunflower stalks (n=9)

CCS and CSS. The same tendencies were recorded by Yang et al. [40] using EFE derived from *Trichoderma reesei* on ruminal degradability of faba bean silage. This means that as the EFE dosage increased, the fermentation efficiency of the substrate also increased until it reaches optimal improvement, after which further increase in enzyme dosage results in a decrease in fermentation efficiency. However, Souza et al. [41] recorded linear effects of the supplemented EFE on the in vitro ruminal degradation, gas production, and fermentative profile of maize silage and sugarcane silage, whereas Arriola et al. [7] found that the EFE could be ineffective on ruminant's digestibility. Accordingly, it is important to note that the effect of EFE supplementation depend on the type of the supplemented substrate, the enzyme source, the supplemented fibrolytic activity, and the xylanase to cellulase ratio [13]. For both studied by-products (CCS and CSS), the effect of EFE supplementation was variable depending on the type of used enzymatic complex. Indeed, for the CS, the optimal improvements (p-value <0.05) were recorded by supplementing the DCX complex at the optimal dose D10 at 5 %, 47%, and 31% for A, Rmax, and Tmax, respectively, as compared to the CCS control (D0). Accordingly, the digestive use parameters improved (p-value <0.05) by 12% (ME), 12.8% (OMD), and 23.8% (VFA) as compared to the control D0. As for the CSS, the optimal improvements were recorded by the optimal dose D5= 5 µl/g DM of DCX by 20.8% (A), 27.6% (Rmax), 12.9% (ME), 11.8% (OMD), and 22.8% (VFA). The MaxFiber complex improved only the in vitro ruminal fermentation parameters by 11.6% and 28.7%, respectively, for A and Rmax by the dose M1=1 mg/g DM. Therefore, we may conclude that the DCX complex was more effective as it improved (p-value <0.05) the rate and the extent of GP and then the estimated digestive use parameters of CCS and CSS by their optimal doses D10= 10 µl/g DM and D5= 5 µl/g DM, respectively, proving the presence of enzyme-substrate specificity [42].

Fibrolytic enzymes could modify the cell wall structure of some forages by hydrolyzing polysaccharides bonds and breaking down the cell wall into smaller, more readily soluble molecules [6], which prepares the cell wall to ruminal microorganism attachment and provides endogenous

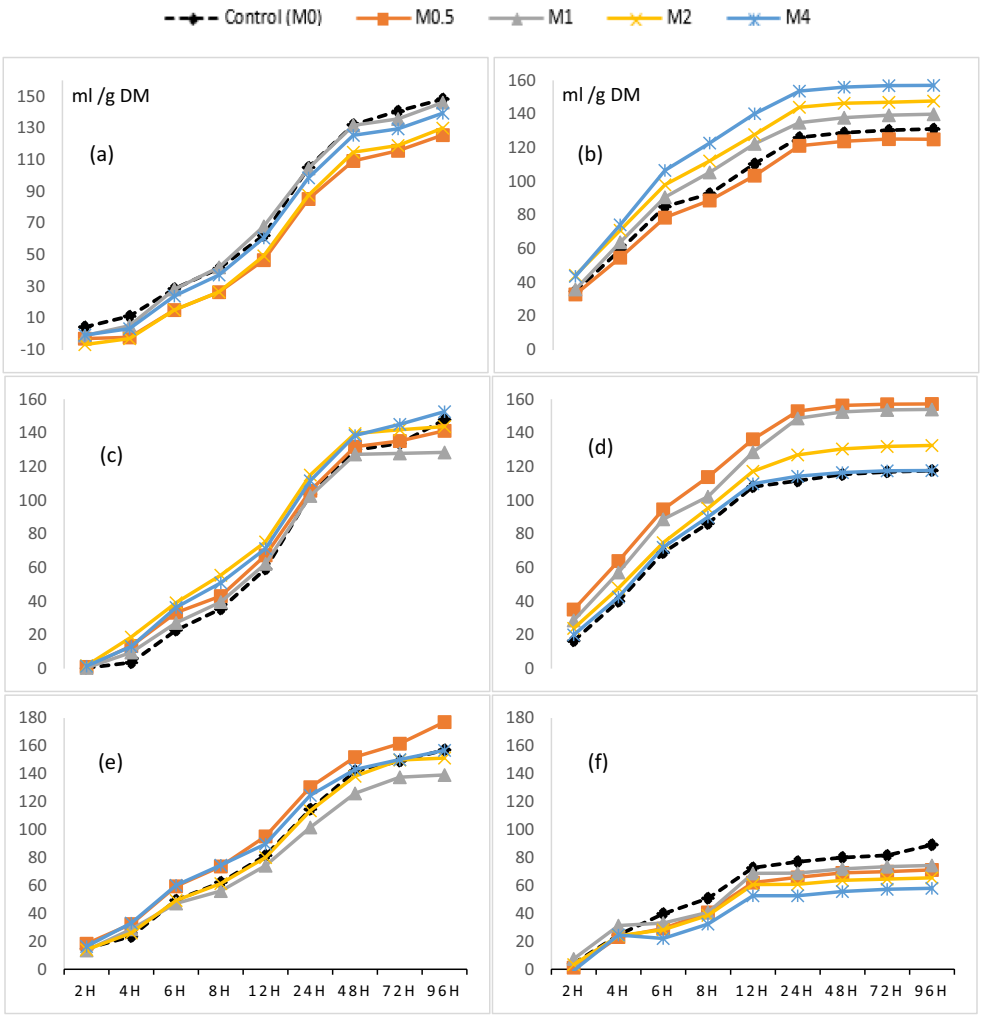

Fig. 3 Effect of MaxFiber enzymatic complex at increasing dose rates on ruminal gas production kinetics (ml/g DM) during 96 h of in vitro ruminal fermentation of untreated (**a**, **b**), NaOH (**c**, **d**), and urea (**e**, **f**)-treated chickpea straw and sunflower stalk ($n = 9$)

Table 3 The effect of DCX increasing dose levels, the chemical treatment, and the interaction between them on the in vitro ruminal gas production kinetics

	Chickpea straw									Sunflower stalk								
	2 h	4 h	6 h	8 h	12 h	24 h	48 h	72 h	96 h	2 h	4 h	6 h	8 h	12 h	24 h	48 h	72 h	96 h
D	NS	NS	NS	**	**	**	**	**	NS	NS	NS	NS	NS	.	*	*	*	*
T	**	**	***	**	**	*	.	*	NS	NS	***	***	***	***	***	***	***	***
D×T	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	.	NS	*	*	*	*	*

D, enzymatic increasing dose rate effect; T, alkali treatment effect; D×T, interaction between the enzymatic dose rate and the chemical treatment. ***p-value <0.001; **p-value <0.01; *p-value <0.05; p-value <0.1; *NS* not significant

enzymes greater access to the cell wall matrix [42–45]. These modifications facilitate the extraction of valuable compounds such as sugars and other biomolecules from the plant material, which could help to improve the nutritional value of agricultural wastes for livestock [46]. Consequently, ruminant's performances could improve. In fact, Jabri et al. [4] support these results by recording improvement of lamb's average daily gain and nutrient digestibility of wheat straw using the same DCX enzymatic complex. Also, Romero et al. [47] proved that the supplementation of total mixed dairy cattle with EFE increased the DM intake and milk yield.

Table 4 Effect of DCX supplementation at increasing dose levels (D1=1; D2=2; D5=5, and D10=10 µl/g DM) on the in vitro ruminal fermentation and the digestive use parameters of untreated (CCS, CSS), NaOH (NCS, NSS), and urea (UCS, USS)-treated chickpea straw and sunflower stalk during 96 h of in vitro ruminal fermentation (n=9)

	Chickpea straw						Sunflower stalk					
	A	Rmax	Tmax	ME	OMD	VFA	A	Rmax	Tmax	ME	OMD	VFA
Untreated												
Control (D0)	145.2^{b}	6.6^{b}	6.8^{ab}	5.0^{b}	33.6^{b}	0.42^{b}	135.6^{c}	19.5^{c}	2.0^{a}	5.8^{c}	41.4^{c}	0.57^{c}
D1	146.3^{b}	6.5^{b}	6.4^{ab}	5.5^{ab}	35.6^{ab}	0.49^{ab}	154.6^{b}	23.4^{a}	1.7^{bc}	6.3^{b}	44.9^{b}	0.66^{b}
D2	144.8^{b}	6.5^{b}	8.6^{ab}	5.2^{ab}	35.0^{ab}	0.44^{ab}	140.2^{bc}	22.3^{ab}	1.5^{c}	6.0^{b}	42.5^{b}	0.60^{b}
D5	125.4^{c}	6.1^{b}	9.3^{a}	5.0^{b}	33.8^{b}	0.42^{b}	163.9^{a}	24.9^{a}	2.2^{a}	6.5^{a}	46.3^{a}	0.70^{a}
D10	151.9^{a}	9.7^{a}	4.7^{b}	5.6^{a}	37.9^{a}	0.52^{a}	153.1^{b}	23.2^{ab}	1.2^{ab}	6.2^{b}	44.3^{b}	0.65^{b}
Linear	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Quadratic	*	*	*	*	*	*	*	*	*	*	*	*
NaOH treated												
Control (D0)	147.5^{b}	6.8^{b}	7.8	5.2^{b}	35.5^{b}	0.45^{b}	126.9	17.8	2.9	5.4	39.1	0.52
D1	146.8^{b}	6.4^{b}	8.0	5.2^{b}	35.5^{b}	0.45^{b}	130.2	17.4	3.0	5.6	40.3	0.55
D2	159.4^{a}	7.3^{a}	6.4	5.5^{a}	37.5^{a}	0.50^{a}	122.6	16.4	3.0	5.4	38.8	0.52
D5	137.4^{b}	5.7^{c}	7.1	4.9^{b}	33.9^{b}	0.40^{b}	127.1	15.6	3.0	5.5	39.6	0.53
D10	160.8^{a}	7.2^{ab}	6.4	5.5^{a}	37^{a}	0.50^{a}	123.4	16.8	2.9	5.4	38.9	0.52
Linear	NS	NS	NS	NS	NS	NS	NS	.	.	NS	NS	NS
Quadratic	*	*	NS	*	*	*	NS	NS	NS	NS	NS	NS
Urea treated												
Control (D0)	157.2^{b}	8.7^{b}	3.5^{a}	6.3	43.2	0.49	78.6^{a}	10.5^{a}	3.9	4.3^{a}	36.5^{a}	0.32^{a}
D1	150.9^{b}	8.8^{b}	3.3^{b}	6.3	43.2	0.50	61.4^{b}	7.51^{b}	4.3	3.8^{b}	33.4^{b}	0.24^{b}
D2	149.3^{b}	8.6^{b}	3.6^{a}	6.4	43.8	0.51	63.4^{b}	7.69^{b}	4.1	3.9^{b}	33.8^{b}	0.25^{b}
D5	146.2^{b}	9.5^{a}	3.4^{b}	6.1	42.2	0.47	59.6^{b}	7.38^{b}	4.3	3.8^{b}	33.1^{b}	0.24^{b}
D10	165.7^{a}	10^{a}	2.8^{c}	6.3	43.7	0.51	61.2^{b}	8.61^{b}	3.4	3.8^{b}	33.4^{b}	0.24^{b}
Linear	*	*	**	NS	NS	NS	*	.	NS	*	*	*
Quadratic	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
SEM	7.90	1.12	1.41	2.20	0.32	0.03	22.40	3.71	0.62	2.71	0.63	0.10
D	*	*	.	*	*	*	*	NS	NS	NS	NS	NS
T	NS	**	***	***	***	***	***	***	***	***	***	***
D × T	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

^{a,b,c} means within a row with different superscripts differ significantly ($p < 0.05$). D, enzymatic increasing dose rate effect; T, alkali treatment effect; D×T, interaction between the enzymatic dose rate and the chemical treatment. ***p-value <0.001; **p-value <0.01; *p-value <0.05; p-value <0.1; *NS* not significant. *SEM*, standard error of the mean; *A*, potential GP (ml/g DM); *Rmax*, maximum rate of GP (ml/h); Tmax is the required time at which Rmax is attained; *ME*, metabolizable energy (MJ/kg DM); *OMD*, organic matter digestibility (%); *VFA*, volatile fatty acid (mmol/200 mg DM)

Table 5 The effect of MaxFiber increasing dose levels, the chemical treatment and the interaction between them on the in vitro ruminal gas production kinetics

	Chickpea straw									Sunflower stalk								
	2 h	4 h	6 h	8 h	12 h	24 h	48 h	72 h	96 h	2h	4 h	6 h	8 h	12 h	24 h	48 h	72 h	96 h
D	***	NS	NS	.	**	**	*	*	*	NS	NS	NS	NS	*	*	*	*	*
T	NS	***	***	***	***	***	***	***	*	***	***	***	***	***	***	***	***	***
D×T	NS	*	**	**	***	**	**	*	NS	**	**	**	*	**	**	**	**	**

D, enzymatic increasing dose rate effect; T, alkali treatment effect; D×T, interaction between the enzymatic dose rate and the chemical treatment. ***p-value <0.001; **p-value <0.01; *p-value <0.05; *NS* not significant

The effects of combining NaOH or urea treatments with EFE supplementation on ruminal fermentation varied depending on the treated substrate and the specific enzymatic complex. Indeed, for NCS, the DCX supplementation improved (p-value< 0.05) the rate and extent of in vitro ruminal fermentation by 8.1% (A) and 7.3% (Rmax)

Table 6 Effect of MaxFiber supplementation at increasing dose levels (M0.5=0.5; M1=1; M2=2; and M4=4 mg/g DM) on the in vitro ruminal fermentation and the digestive use parameters of untreated (CCS, CSS), NaOH (NCS, NSS) and urea (UCS, USS)-treated chickpea straw and sunflower stalk during 96 h of in vitro ruminal fermentation (n=9)

	Chickpea straw						Sunflower stalk					
	A	Rmax	Tmax	ME	OMD	VFA	A	Rmax	Tmax	ME	OMD	VFA
Untreated												
Control (M0)	145.2^{a}	6.6^{ab}	6.8^{b}	5.3^{a}	35.6^{a}	0.46^{a}	135.6^{b}	19.5^{b}	2.0^{a}	5.8	41.4	0.57
M0.5	123.6^{c}	5.1^{c}	9.5^{a}	4.7^{b}	32.0^{b}	0.37^{b}	139.1^{b}	21.5^{b}	1.3^{c}	5.9	42.0	0.59
M1	144.4^{ab}	6.8^{a}	7.2^{b}	5.3^{a}	35.4^{a}	0.46^{a}	151.4^{a}	25.1^{a}	1.6^{bc}	6.2	44.1	0.64
M2	127.2^{b}	5.4^{bc}	9.7^{a}	4.8^{b}	32.4^{b}	0.38^{b}	138.1^{b}	22.3^{ab}	1.6^{bc}	5.9	41.9	0.59
M4	138.1^{abc}	6.2^{abc}	7.8^{b}	5.1^{ab}	34.4^{ab}	0.43^{ab}	131.5^{b}	20.3^{b}	1.7^{b}	5.7	40.7	0.56
Linear	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Quadratic	*	*	**	.	.	.	*	*	**	NS	NS	NS
NaOH treated												
Control (M0)	147.5^{b}	6.8^{c}	7.8^{a}	5.2^{b}	35.5^{b}	0.45^{b}	126.9	17.8^{a}	2.9^{b}	5.5	39.1^{a}	0.52^{a}
M0.5	141.3^{b}	$_{7.0}^{b}$	6.4^{ab}	5.3^{b}	35.8^{b}	0.46^{b}	138.3	15.7^{b}	2.4^{b}	5.5	39.7^{a}	0.53^{a}
M1	128.5^{c}	7.2^{b}	7.4^{a}	5.2^{b}	35.3^{b}	0.45^{b}	126.7	16.0^{ab}	2.9^{b}	5.5	39.5^{a}	0.53^{a}
M2	143.7^{ab}	7.4^{b}	5.6^{b}	5.5^{a}	37.5^{a}	0.50^{a}	123.6	16.3^{ab}	3.1^{a}	5.5	39.1^{a}	0.52^{a}
M4	152.8^{a}	8.5^{a}	5.9^{b}	5.4^{a}	36.9^{a}	0.49^{a}	122.7	14.5^{b}	3.0^{a}	5.2	37.3^{b}	0.47^{b}
Linear	*	*	*	*	*	*	NS	NS	NS	NS	NS	NS
Quadratic	NS	NS	NS	NS	NS	NS	NS	*	.	NS	.	.
Urea treated												
Control (M0)	157.2^{ab}	8.7^{b}	3.5^{a}	6.3^{b}	43.2^{b}	0.50^{b}	78.6^{a}	10.5^{a}	3.9^{c}	4.3^{a}	36.5^{a}	0.32^{a}
M0.5	177.1^{a}	10.6^{a}	2.5^{b}	6.7^{a}	46.1^{a}	0.57^{a}	77.3^{a}	9.1^{b}	4.4^{b}	4.2^{a}	36.0^{a}	0.31^{a}
M1	139.2^{b}	8.3^{b}	3.0^{a}	5.9^{c}	41.0^{c}	0.44^{c}	49.2^{b}	5.6^{c}	5.0^{a}	3.5^{b}	31.4^{b}	0.20^{b}
M2	151.4^{ab}	8.7^{b}	3.6^{a}	6.2^{bc}	43.1^{bc}	0.50^{bc}	49.9^{b}	6.1^{c}	5.6^{a}	3.5^{b}	31.6^{b}	0.20^{b}
M4	156.8^{ab}	9.6^{a}	2.7^{b}	6.5^{ab}	$_{45.0}^{ab}$	0.49^{ab}	53.3^{b}	6.5^{c}	4.8^{b}	3.6^{b}	32.1^{b}	0.20^{b}
Linear	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Quadratic	*	*	*	*	*	*	*	***	*	*	*	*
SEM	9.60	0.91	1.42	2.60	0.30	0.03	11.51	3.73	0.85	2.50	0.51	0.09
D	*	NS	NS	**	**	**	*	*	NS	*	*	*
T	*	***	***	***	***	***	***	***	***	***	***	***
D × T	NS	**	*	**	**	**	.	*	NS	.	.	.

^{a,b,c} means within a row with different superscripts differ significantly ($p < 0.05$). D, enzymatic increasing dose rate effect; T, alkali treatment effect; D×T, interaction between the enzymatic dose rate and the chemical treatment. ***p-value <0.001; **p-value <0.01; *p-value <0.05; p-value <0.1; *NS* not significant. *SEM*, standard error of the mean; *A*, potential GP (ml/g DM); *Rmax*, maximum rate of GP (ml/h); Tmax is the required time at which Rmax is attained; *ME*, metabolizable energy (MJ/kg DM); *OMD*, organic matter digestibility (%); *VFA*, volatile fatty acid (mmol/200 mg DM)

and estimated digestive use parameters by 5.7% (ME), 5.6% (OMD), and 11.1% (VFA) using lowest DCX dose D2 = 2 µl/g DM. As for the UCS, the highest dose D10 improved linearly the in vitro ruminal fermentation parameters A and Rmax by 5.4% and 15% without affecting the ME, DMO, and VFA (Table 4). As for the MaxFiber effect, it seems to be stimulated by the alkali treatments of CS. Indeed, for the NCS, improvements by 3.6% for A, 25% for Rmax, 4% for ME, 4% for DMO, and 9% for VFA were recorded using the M4 dose. As for the UCS, the highest improvements were recorded by using the lowest MaxFiber dose M0.5, by 12.6%, 21.8%, 6.8%, 6.7%, and 14% for A, Rmax, ME, DMO, and VFA, respectively (Table 6). So, as compared to the chemically untreated by-products, the MaxFiber complex seems to be more effective on alkali-treated chickpea straw since the NaOH and urea treatment modified the structure and chemical composition of the substrate, making it more susceptible to exogenous enzyme hydrolysis. Furthermore, the NaOH treatment decreased the DCX optimal dose form D10 to D2 which could have economic benefits by reducing the cost of the enzymatic treatment.

On the other hand, for the SS, and as compared to the untreated SS, the alkali treatments decreased the efficacy of both studied EFE (DCX and MaxFiber). Indeed, the association between EFE complexes and NaOH or urea treatments had no significant improvements in the GP kinetics

throughout the 96 h of incubation and on the estimated fermentation and digestive use parameters of sunflower stalks and may cause detrimental effects when combined with urea treatment. This finding was similar to those reported by Jabri et al. [48] for sunflower head by-products using the same enzymatic complexes. Depending on the biochemical composition and the polysaccharide matrix of the treated substrate, the alkali treatment could modify the cell wall structure [49] by breaking down the lignocellulosic biomass which may generate by-products such as lignin-derived phenolic compounds (e.g. vanillyl alcohol, coniferyl alcohol, and sinapyl alcohol) and decrease the efficiency of enzymatic hydrolysis [50]. Generally, to optimize the efficiency of EFE and the yield of fermentable sugars from plant biomass, it is crucial to optimize the conditions of the alkali treatment process. These findings emphasize the importance of enzyme-substrate specificity to determine the effectiveness of EFE treatments on the ruminal fermentation of agricultural wastes.

4 Conclusion

The obtained results from this study proved that both chickpea straw and sunflower stalk could be included in ruminant diet, but it's important to consider their nutritional value and their physical properties, as well as the animal's requirements. The supplementation by two EFE composed mainly of cellulase and xylanase activities improved the rate and the extent of in vitro ruminal fermentation and the digestive use parameters of both studied by-products, proving that lignified agricultural waste could be valorised in ruminant nutrition providing digestible source of fibre. The association between alkali treatments (NaOH and urea) and EFE exerted variable effects depending on the type of used enzymatic complex, the treated substrate and the alkali treatment. For chickpea straw, the association between alkali and EFE stimulated the ruminal fermentation and improved the digestive use. However, the alkali treatment of sunflower stalk decreased the efficiency of both studied EFE. Thus, it is essential to emphasize the specificity of the enzyme-substrate interaction to ensure the effectiveness of exogenous fibrolytic enzymes on the digestive utilization of agricultural by-products. However, further research is needed to explore the presence of potential anti-nutritional factors and validate the in vitro findings through in vivo trials, which will contribute to a comprehensive understanding of the nutritional value, limitations, and practical application of these feed resources in sustainable ruminant nutrition.

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Data availability The datasets and materials used during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval The article does not contain any studies with human participants. It also does not perform experiments directly on animals. So, this experience does not need ethics statement.

Consent to participate All the authors of this article are consented to participate.

Competing interests The authors declare no competing interests.

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