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The Effect of Ultrasound Processing and Exogenous Cellulase Supplementation on the Ruminal Degradability of Palm Date Seeds

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Abstract: The objective of this study was to evaluate the effects of sonication and enzyme supplements on rumen degradability of date palm seeds (DPS). The effect of alkali-assisted sonication and the addition of cellulase enzyme supplements (40 endoglucanase units/g dry matter) derived from *Aspergillus niger* or *Trichoderma longibrachiatum* on DPS ruminal degradability were evaluated under *in vitro* conditions. The *in vitro* ruminal degradability of neutral detergent fiber (NDF) and organic matter (OM) for the unsonicated seeds was not affected ($p>0.10$) by enzyme supplements. On the other hand, alkali-assisted sonication increased the *in vitro* rumen degradability of seeds' NDF and OM and the addition of *Trichoderma longibrachiatum* cellulase enzyme to sonicated seeds slightly but significantly increased ($p<0.01$) their *in vitro* NDF and OM degradability. The results indicate that sonication along with alkali pretreatment could improve the NDF and OM degradability of DPS in rumen. The pretreatment combined effect of ultrasound and *Trichoderma longibrachiatum* cellulase enzyme could be beneficial in increasing the inclusion rate of DPS in ruminant animals' diets.

Keywords: Date Palm Seeds, Cellulase Supplement, Ultrasonication, Alkali Assisted-Sonication, *In vitro* Degradability

Introduction

The date palm (*Phoenix dactylifera*) seeds (DPS) represent about 10-15% of date weight. The use of DPS in animal feed is the most common practice, mainly in the rural areas in the Middle East. Date palm seeds are high in cellulose (24-46%), hemicellulose, (7-28%) and lignin (7-26%) and therefore their rumen degradability is very low (22.7%, Boufennara *et al*., 2016). The low rumen degradability of the low quality feeds is mainly due to the crystal structure of cellulose and the covalent linkages between structural carbohydrates and lignin (Jackson, 1977). Additionally, the acetyl groups in hemicellulose are another important barrier for the enzymatic hydrolysis of fibers (Kong *et al*., 1992). Chemical pretreatments such as dilute acid, ammonia, sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$) and urea and physical pretreatments such as grinding and milling, steaming, irradiation, temperature and pressure have been used to improve the nutritional value of low quality feeds (Haddad *et al*., 1998; Hamed and Elimam, 2010; Sarnklong *et al*., 2010).

Biological pretreatments such as enzyme and microorganism supplementation have some advantages such as low energy and chemical use and being safe and eco-friendly method for lignin removal. However, microorganism supplementation is a slow and inefficient degradation processes (Ma *et al*., 2011). Fibrolytic enzymes used in animal feed industry are mostly of fungal or bacterial origin and their addition to ruminants diets have been shown to improve the utilization of low quality forages (Salem *et al*., 2013; Rojo *et al*., 2015; Tirado-González *et al*., 2018). Sonication (ultrasound) may also used to improve the rumen degradability of low quality forages (Bussemaker and Zhang, 2013; Liyakathali *et al*., 2016; Aboragah *et al*., 2019). The ultrasonic waves from sonication produce pressure differences in the solution medium breaking the carbohydrate substrate complex and exposing larger surface area of feeds to enzymes attachment (Bussemaker and Zhang, 2013). The main objective of this study was to evaluate the rumen degradability of DPS under alkali-assisted sonication and cellulolytic enzyme supplements.

Materials and Methods

Experimental Design, Incubation Procedure and Laboratory Analysis

Date palm seeds were brought from cultivars in Saudi Arabia. The samples were soaked in boiling water to remove any non-seed debris and then dried in the oven at 70°C for 48 h (Precision Scientific Co, 70147 series, Chicago, IL). Samples were then milled to 2 mm (Thomas – Wiley, Model 4, Philadelphia, PA) and stored at room temperature until use.

Two types of commercial exogenous cellulase enzyme supplements (BIO-CAT, Inc; Troy, Virginia) were used in the current study. The first cellulase enzyme is derived from fungus species (*Aspergillus niger* cellulase) and the second enzyme is derived from fungus species [*Trichoderma longibrachiatum* (formerly *Trichoderma reesei*) cellulase]. Enzymes were applied at a rate of 40 endoglucanase units/g of substrate DM. Both enzymes were powdered and their endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91) activities were determined before use (Colombatto and Beauchemin, 2003; Giraldo *et al*., 2008). The enzyme treatments were selected based on the results obtained in previous *in vitro* studies (Giraldo *et al*., 2008).

Treatments in the experiment were run in quadruplicate. For the sonication treatment, 14 g of the ground DPS were placed into a 400 ml metal reactor containing 168 ml of 4% NaOH accordance to previous research that showed the optimum NaOH level for feeds delignification is 4% (Mowat and Ololade, 1970; Lesoing *et al*., 1980) with a 12:1 feed to solution ratio (Zhang *et al*., 2008). Samples in the reactor were then sonicated for 5 min using an ultrasonic processor (Ultrasonic Processor FS-900N, Hanchen Instrument, China) with 900 W and 20 kHz. A 13-mm diameter ultrasonic probe was introduced into the reactor with the probe tip immersed into the center of the reactor 50 mm away from the reactor's bottom. At the end of sonication, the DPS in the reactor were transferred into 50 mL falcon tubes and then centrifuged at 2000 × g for 3 min (Centra - GP8 (OM3121 model, Needham Heights MA, international equipment company). The supernatant was discarded and the process was repeated until all the DPS in the reactor were transferred. The collected seeds were then stored at -20°C for 24 h and then freeze-dried for 72 h. To evaluate rumen degradability, 2.5 g of the sonicated and unsonicated ground DPS were placed in Dacron bags (5×10 cm, 50±10 μm pore size; R 510, Ankom Technology). Rumen fluid for the batch cultures was collected from a lactating Holstein cow fed a total mixed ration (60:40; forage:concentrate). Dacron bags were then incubated in 250 ml jars containing 120 ml of chemical buffer and 30 mL of rumen fluid according to Goering and Van Soest (1970). Each jar also contained two grams of a diet that consisted of alfalfa hay (500 g/kg), ground corn (300 g/kg), soybean meal (150 g/kg) and soy hull (50 g/kg). Enzymes were added to jars at the rate of 40 endoglucanase units/g of diet dry matter (DM). Jars were then incubated in a water bath at 39°C for 24 h according to Goering and Van Soest (1970). To maintain the environment anaerobic, each jar was gassed with carbon dioxide for 30 s before sealing. After 24 h, the Dacron bags were removed from the jars and washed in cold water several times to remove any feed or rumen residues. Bags were then dried at 70°C for 24 h and OM and NDF degradability were measured.

Dry matter and ash were analyzed using the AOAC established methods (AOAC, 2000). Neutral detergent fiber was analyzed by ANKOM 200 (ANKOM Technology, Macedon, NY) according to the method described by Van Soest *et al*. (1991).

Statistical Analysis

Treatments were arranged in factorial design to test the effects of sonication, enzymes and their interactions on DPS rumen degradation. All data were subjected to analysis of variance using the General Linear Models (GLM) procedure (JMP Version Pro, 14.1 SAS Institute Inc. 2018). The statistical model was:

$$yij = \mu + Di + Ej + DEij + eij$$

Where:
Yij = The observation
μ = The overall mean
Di = The seeds (i = sonication, unsonication)
Ej = The enzymes (j = with, without)
DEij = The interaction between seeds and enzymes and
eij = The residual error

The Tukey test was used for comparison among the means and significance at $p<0.01$.

Results

The effect of sonication and two types of cellulase supplements on *in vitro* ruminal degradability of DPS is presented in Table 1. The average *in vitro* ruminal degradability of seeds NDF and OM for the unsonicated seeds were 26.4% and 23.4%, respectively and both were not affected ($p>0.10$) by enzyme supplements. On the other hand, the alkali-assisted sonication increased the *in vitro* rumen degradability of seeds NDF and OM to 72.2% and 67.6%, respectively and the addition of *Trichoderma longibrachiatum* cellulase to sonicated seeds significantly ($p<0.01$) increased the seeds *in vitro* NDF and OM degradability to 77.0% and 71.9%, respectively.

Table 1: The effect of sonication and cellulase enzyme supplements on the *in vitro* ruminal degradability of date palm seeds

Treatments		Rumen degradation (%)	
Alkali-assisted sonication[1]	Enzyme[2]	OM	NDF
-	-	23.41[d]	26.44[c]
-	*Aspergillus niger* cellulase	22.85[d]	25.75[c]
-	*Trichoderma longibrachiatum* cellulase	23.24[d]	25.88[c]
+	-	67.56[b]	72.38[b]
+	*Aspergillus niger* cellulase	65.80[c]	72.09[b]
+	*Trichoderma longibrachiatum* cellulase	71.94[a]	77.00[a]
MSE		0.507	0.763
Main effects			
Seeds		0.01	0.01
Enzymes		0.10	0.11
Seeds x enzymes		0.01	0.01

[1+] Seeds sonicationin 4% sodium hydroxide solution for 5 min
[2]40 endoglucanase unit/g dry matter
OM- organic matter; NDF-neutral detergent fiber
[abcd]mean with different letters within the same column are significantly different ($p<0.01$)

Discussion

Previous studies (Mowat and Ololade, 1970; Lesoing *et al*., 1980) showed that the *in vitro* rumen degradability of feeds progressively increased as the concentration of NaOH increased up to 4% with no further increase thereafter. Additionally, Nikolić *et al*. (2010) investigated the effects of sonication time (0.5, 1, 3, 5, 10, 20 and 30 min) on glucose yield from corn meal and reported the yield of glucose increased after 5 min on the sonication and the additional increase in sonication time (10, 20 and 30 min) did not further increase glucose yield. Unpublished data from our laboratory also looked at the effects of sonication time (5, 10, 15 and 30 min) and power (600, 700 and 900 W) on rumen fiber degradation and showed the highest increase in fiber degradability was seen after 5 min of sonication at 900 W.

Fibrolytic enzymes in ruminant rations have the potential to improve the nutritional value of low quality forages and improve feed efficiency (Salem *et al*., 2013; Rojo *et al*., 2015; Tirado-González *et al*., 2018). Fibrolytic enzyme supplementation to ruminant diet improves the hydrolytic capacity of the rumen due to enhanced attachment by rumen microorganisms (Nsereko *et al*., 2002; Beauchemin *et al*., 2003), creation a stable enzyme-feed complex (Kung *et al*., 2000) and/or alteration in cellulose structure (Giraldo *et al*., 2007). In current study, the lack of effect of the enzymes supplement on the measured *in vitro* rumen OM and NDF degradability of unsonicated PDS suggested that neither of them were able to breakdown the crystalline complex of cellulose and the covalent linkages between the structural carbohydrates and lignin. Previously, Krueger and Adesogan (2008) reported pretreatment of bahia grass hay with the combination of ferulic acid esterase, cellulase and xylanase did not increase DM digestibility after 24 or 96 h of incubation in rumen fluid. Lynch *et al*. (2014) also reported no changes in the ruminal DM degradability of alfalfa silages inoculated with fibrolytic enzymes. However, Kholif *et al*. (2015) reported the pretreatment of DPS with commercial fibrolytic enzymes increased DM and OM digestibility *in vitro*. Abid *et al*. (2019) also reported a slight improvements in the OM digestiblity of DPS when supplemented with commercial fibrolytic enzymes. The effect of fibrolytic enzyme supplementation is influenced by many factors such as diet composition, pH, temparature, type of enzyme, specific enzyme activity, enzyme stability, application method and amount of enzyme supplementation (Meale *et al*., 2014). It should be noted, in general, the treatment of feed with fibrolytic enzymes stimulate the initial phases of feed degradation, but the effects reduce as incubation time increases. Previously, Nsereko *et al*. (2000) and Colombatto *et al*. (2003) observed that the treatment with fibrolytic enzymes can increase the rate of digestion without increasing the extent of digestion.

The alkali agents such as NaOH, $Ca(OH)_2$ or ammonia can increase break down the ester bonds that link lignin with the plant cell wall (Behera *et al*., 2014). In general, the elimination of main parts of lignin in poor quality feeds allows the rumen microorganisms to attach more easily to the structural carbohydrates, increasing microbial colonisation and therefore enhancing fiber degradation. Alkali pretreatment can also reduce the strength of intermolecular hydrogen bonds in cellulose fibrils, resulting in swelling of the cellulose (Jackson, 1977). The increased swelling leads to a reduction in degree of polymerization and crystallinity of the cellulose, thereby increasing substrate surface area (Behera *et al*., 2014). The increases in measured *in vitro* NDF and OM degradability of DPS under the alkali-assisted sonication may improved the access of fibrolytic enzymes to cellulose and hemicelluloses. Previously, it was reported alkali-assisted sonication increased

delignification, removal of uronic acid substitutions and acetyl groups in hemicelluloses and broken down the crystalline complex of cellulose of low quality forages (Sun and Tomkinson, 2002; Zhang *et al*., 2008; Soontornchaiboon *et al*., 2016). The increases in measured *in vitro* NDF and OM degradability of DPS under the alkali-assisted sonication may also be due to the removal of condensed tannin in DPS. Previously, it was shown that sonication pretreatment resulted in degradation of phenolic compounds (Svitelska *et al*., 2004).

Proper intensity ultrasonic waves can enhance the dissolution of substrate (Bochu *et al*., 2003) thus the catalysis effect of enzymes improves (Delgado-Povedano and De Castro, 2015). Dalagnol *et al*. (2017) reported a 17% increase in the catalytic efficiency of cellulase with sonication possibly by increasing the reaction stability. In an earlier study, Gadalkar and Rathod (2017) observed an increase of 1.2 fold in the yield of ferulic acid with ultrasound assisted enzymatic pretreatment. The pretreatment combined effect of ultrasound and enzyme enhanced the hydrolysis of sugarcane bagasse (Gasparotto *et al*., 2015). Easson *et al*. (2011) reported sonication increased fermentation and the rate of hydrolysis compared to enzyme alone. The observed decrease in the measured *in vitro* OM degradability of sonicated PDS with the *Aspergillus niger* cellulase supplement may be due to its lower adsorption to the substrate. The adsorption of enzyme onto the substrate is essential for an effective hydrolysis (Guo *et al*., 2014). Haven and Jørgensen (2013) reported the β-glucosidase from *Trichoderma longibrachiatum* cellulases adsorbed on lignin or on biomass but not the β-glucosidase from *Aspergillus niger*. During the cellulose digestion, cellobiohydrolase and endoglucanase degrade mainly more complex substrates including glucan and xylan polysaccharides whereas β-glucosidases mostly break down short gluco-oligosaccharides (Zhang *et al*., 2006). *Trichoderma longibrachiatum* cellulase mainly promotes to cellobiohydrolase and endoglucanase activity and *Aspergillus niger* cellulase to β-glucosidase activity (Sternberg *et al*., 1977). The increases in the measured *in vitro* NDF and OM degradability of sonicated DPS supplemented with *Trichoderma longibrachiatum* cellulase suggested these combined pretreatments showed beneficial effect on further improving rumen degradation of DPS.

Conclusion

The current study demonstrates that sonication along with alkali treatment can improve the nutritional and feeding values for low quality feeds, such as DPS. The effectiveness of the fibrolytic enzyme supplementation under alkali-assisted sonication can be influenced by cellulase enzyme type. The addition of cellulase enzyme derived *Trichoderma longibrachiatum* along with alkali-assisted sonication increased the measured *in vitro* NDF and OM degradability of DPS. The results indicated that alkali-assisted sonication pretreatment along with enzyme supplementation could improve the rumen degradability of DPS and therefore increase their inclusion rate in ruminant animals' diets. Future studies should examine the effects of feeding sonicated DPS with cellulase enzyme derived *Trichoderma longibrachiatum* on milk and meat quality and animal performance for ruminant animals.

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Author's Contributions

Ahmed Aboragah: Conduction of study, analysis and interpretation of data, drafting of manuscript.

Mohammed G. Embaby: Laboratory analsyis.

Mevlüt Günal: Drafting of manuscript.

Amer A. AbuGhazaleh: Experimental design, interpretation of data and drafting of manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

Disclosure Statement

Th eauthors warrant that there are no conflicts of interests among authors and between authors and other people, institutions or organizations.

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