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INFLUENCE OF EXOGENOUS FIBROLYTIC ENZYMES ON RUMINAL FERMENTATION CHARACTERISTICS AND NUTRIENTS DEGRADABILITY OF DATE SEEDS USING *IN VITRO* GAS PRODUCTION TECHNIQUE

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ABSTRACT: Agro-industrial by-products and crop residues are available in Egypt in large quantities and can play a significant role in the nutrition of ruminants. Using the agricultural by-products in animal nutrition can improve the environmental conditions, economic benefit and the efficiency of agricultural and animal production. The present study was conducted to investigate the effect treatment of date seeds (DS) with preparation of exogenous enzymes (ZADO®) on *in vitro* gas production (GP) and some ruminal fermentation parameters compared with clover hay (CH). Four doses: 0 (control), 6 (low), 12 (medium) and 24 (high) mg/g dry matter of ZADO® as multi-enzyme additive (ENZ) were added with DS inside the incubation tubes. Clover hay (CH) was used as a positive control. By comparing the untreated DS with CH, the values of cumulative GP, predicted organic matter degradability, and crude protein degradability were significantly ($P<0.05$) lowered. However, higher levels of ENZ supplementation (24 mg/ g DM) linearly increased GP at 3, 6, 12, 24, 36, and 48 hr., of incubation. The GP kinetics were insignificantly effected by all levels of ENZ. Also, elevating ENZ concentration resulted in a linear increase in the predicted values of short chain fatty acids (SCFA), Metabolizable energy (ME), Net energy of lactation (NE_L), and organic matter digestibility (OMD), where the high values of indicated predicted parameters were recorded with the high level of ENZ (24 mg/ g) compared with the untreated DS. ZADO® supplementation lead to a linear increase in degradability of dry matter (DMD), crude protein (CPD) and crude fiber (CFD) with a significant enhancement with using the level of 24 mg/g DM. In conclusion, the obtained results suggested that the dietary addition of preparation enzymes (ZADO®), particularly at level of 24 mg/g DM of DS, has potential effect on improving the efficiency of nutrients digestibility of date seed.

Key words: Exogenous enzymes, *In vitro* gas production, feed degradability, date seeds.

INTRODUCTION

In many developing countries, including Egypt, there is a gap between the available feedstuffs and the total required for ruminants feeding. In Egypt, only 4.15 million tons of agricultural by-products out of 33.477 million tons produced are used for feeding ruminants (**Ministry of Agriculture, Egypt, 2006**). The primary factors limiting the utilization of plant by-products in animal feeding are the low crude protein, high crude fiber content, low digestibility, and poor feed palatability (**Abd El-Rahman *et al*., 2014**). The gap between the availability and requirements of animal feeds in Egypt is about nine million tons of dry matter, equivalent to almost four million tons of Total Digestible Nutrients (TDN) per year (**Bendray *et al*., 2006**). Date palm trees are considered the major crop under dry and semi-arid areas in new reclaimed regions in Egypt (**Abd El-Zaher, 2008**). There are 15 million date palm trees in Egypt, which produce about 1.70 million tons of date (almost 21% of the world production estimated at 8 million tons) and 254 thousand tons of seeds, which increased the supply of

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agro-industrial by-products for livestock feeding (**El-Sharabasy and Rizk, 2019**).

Using exogenous fibrolytic enzymes preparation, as a feed additive strategy, improve the production efficiency of ruminants (**Beauchemin *et al*., 2003**). Enzymes improve the fiber degradation in the rumen by acting synergistically with the rumen microflora, thereby increasing their hydrolytic capacity in the rumen (**Morgavi *et al*., 2000; Beauchemin *et al*., 2004**). The enzymatic preparation ZAD® is a biotechnical product made from anaerobic bacteria that convert the polysaccharide into monosaccharide by specific enzymes (**Gado *et al*., 2011**). The enzyme mixture ZADO® contains several enzymes such as endoglucanase, xylanase, α-amylase, and protease activity. It has been used in *in vivo* and *in vitro* studies to improve nutrient digestibility and ruminal fermentation parameters (**Gado *et al*., 2009 and 2011**).

The present work was carried out to study the effect of dietary addition of enzymatic preparation (ZADO®) on ruminal fermentation characteristics and nutrients degradability of date seeds using *in vitro* gas production technique.

MATERIALS AND METHODS

The present study was conducted at the Laboratory of Animal Nutrition, Department of Animal Production, Faculty of Agriculture, Zagazig University, Egypt. Samples of date seeds (DS) and clover hay (CH) were dried at 60°C for 48 hr., in a forced-air oven, finely powdered, and stored in plastic bags for subsequent determination of chemical components and *in vitro* gas study. The sample was analyzed for dry matter (DM), organic matter (OM), crude protein (CP), crude fiber (CF), and ether extract (EE) according to **AOAC (2006)**.

The multi-enzyme feed additive ZADO® a patented (Patent No.: 22155, Cairo, Egypt) commercially available in a powder form, produced from *Ruminococcus flavefaciens*, and manufactured by the Academy of Scientific Research and Technology (Cairo, Egypt). The each gram of multi-enzyme (ENZ) contains 7.1 units of endoglucanase, 2.3 units of xylanase, 61.5 units of α- amylase and 29.2 units of protease activity. For date seeds substrates, four doses of ENZ were included at 0 mg (control), 6 mg (low), 12 mg (medium) and 24 mg (high) per g DM. Clover hay was used without ENZ supplementation as a positive control.

Ruminal fluid was collected from a slaughtered cow, according to **Chaudhry (2008)**. Rumen liquids were transferred in pre-warmed (39°C) isolate flasks and incubated under anaerobic conditions. The rumen fluid was filtered through four layers of cheesecloth and incubated in a water bath at 39°C, and it was saturated with CO_2 until inoculation. Each liter of the buffer solution (MB9 media) contained 2.8 g NaCl, 0.1g $CaCl_2$, 0.1g $MgSO_4.7H_2O$, 2g $KH_2PO_4.H_2O$, and 6 g $Na_2H PO_4$. The pH was adjusted to 6.8, and CO_2 was flushed for 30 minutes to maintain anaerobic conditions. The rumen fluid was added into the buffer solution using a ratio of buffer solution to a rumen fluid of 2:1. Thirty millimeters of mixed ruminal fluid were introduced into glass tubes, containing 200 mg of clover hay (CH) or date seeds (DS) + dose of ZADO® (ENZ), quickly closed with gas release rubber stopper fitted as described by **Tilley and Terry (1963)** with using of tri-way valve connected with a calibrated plastic syringe to collect produced gas and were incubated in a chicer water bath. Total gas production was recorded at 3, 6, 12, 24, 36, 48, and 72 hr., of incubation. Total gas values were corrected from a blank tube. Cumulative gas production data were fitted to the model of **Ørskov and McDonald (1979)**.

At the end of incubation (72 hr.), the contents of three tubes of each treatment were used for determining the degradability of dry matter (DM) with a 30 ml neutral detergent solution and refluxed for 3 hr., at 105°C and filtered through pre-weighed Gooch crucibles. Each sample was dried at 105°C for 3 hr., and the final weight was recorded. The residual DM was estimated according to **Blümmel *et al*. (1997)**. Then it was used to estimate degradability of crude protein (CPD) and crude fiber (CFD) according to **AOAC (2006)**. Another three tubes were used for determining the NH_3-N concentration, total volatile fatty acids (TVFAt's), and protozoa count. The protozoa were counted microscopically, after sample

preparation, according to **Kamra *et al*. (1991)**. Ammonia-N concentration was determined according to the method described by **Conway (1957)**. The total volatile fatty acids (TVFAt's) concentration was determined by the steam distillation method, as described by **Warner (1962).** The evaluation was done by Kjeldahl methods. The partitioning factor (PF) was calculated as the ratio of OM (mg) degradability to gas volume (milliliters in 24 hr.) (**Blümmel *et al*., 1997**). Metabolizable energy (ME, MJ /kg DM) and Net energy of lactation (NE_L, MJ /kg DM) were estimated according to **Menke and Steingass (1988)**.

ME (MJ/kg DM) = 0.136 × GP + 0.0057 × CP+ 0.000286×EE^2 + 2.20

NE_L (MJ/kg DM) = 0.096 ×GP + 0.0038 × CP+ 0.000173× EE^2 + 0.54

Where

GP= 24 hr., net gas production (ml/200 mg DM)

CP= Crude protein

EE= Ether extract

In vitro organic matter digestibility (OMD%) was calculated using equation of **Menke *et al*. (1979)** as follows:

OMD (%)= 14.88+0.889GP+ 0.45CP+ 0.0651XA

Where

XA = Ash content (%)

Short chain fatty acids concentrations (SCFAs) were calculated according to **Getachew *et al*. (2002)** as:

SCFA (mmol/200 mg DM)=0.0222 GP − 0.00425

Where: GP is the 24 hr., net gas production (ml/200 mg DM).

Microbial crude protein (MCP) biomass production was estimated according to **Blümmel *et al*. (1997)** as:

MCP (mg/g DM)=mg DMD−(ml gas×2.2 mg/ml)

Where: 2.2 mg/ml is a stoichiometric factor which expresses mg of C, H and O required for the production of SCFA gas associated with production of 1 ml of gas.

The data were analyzed as a completely randomized design and subjected to one-way analysis of variance by the General Linear Model of SPSS 21 statistics software, Chicago, IL (**SPSS, 2014**). Significance between individual means was identified using Dancan Multiple Range Tests (**Dancan, 1955**). Mean differences were considered significant at $p<0.05$ and $p<0.01$. Standard errors of means were calculated from the residual mean square in the analysis of variance. Linear and quadratic polynomial contrasts were used to examine responses of feeds to increasing addition levels of the enzymatic mixture.

RESULTS AND DISCUSSION

Proximate Chemical Analyses

The proximate chemical analyses of date seede (DS) and clover hay (CH) on dry matter basis are presented in Table 1. The value of organic matter (OM) and ether extract (EE) were 97.37% versus 5.68% in DS and 86.3% versus 2.4% in CH, respectively. The crude fiber and crude protein content were lower in DS and valued 16.87% and 7.4% compared to CH wherein valued 26.9% and 15.7%, respectively. The ash content was 2.63% in DS versus 13.70% in CH. Date seed had a higher content of nitrogen free extract (67.42%) compared to 55% for CH. Nutrient contents of the DS which used in this study were within the ranges published by many researchers in Egypt (**Shawket *et al*., 2001; Abou El-Nasar and El-Kerdawy, 2003; Suliman and Moustafa, 2008; Mousa, 2013**).

In vitro GAS PRODUCTION

By comparing untreated DS to CH, the values of cumulative gas production (GP) at 3, 6, 12, 24, 36, and 48 hr., of incubation were significantly ($P<0.05$) reduced (Table 2). No significant differences in GP from the soluble (a), non-soluble fraction (b) and gas production rate (c) between untreated DS and CH. The lower GP for DS could be attributed to its poor feeding value, particularly the fiber content and its structural polysaccharides (**Jalilvand, *et al*., 2008**). There are contradictory between GP and kinetics parameters of DS. Although the GP from DS was very low, there are no significant differences between DS and CH in the fermentation rate (μ, $hr.^{-1}$). Therefore, these results suggest that GP profiles are not necessarily linearly related to degradation or

Table 1. Chemical composition (% on DM basis) of clover hay and date seed

	Clover hay	Date seed
Dry matter	88.6	89.50
Organic matter	86.3	97.37
Ash	13.7	2.63
Ether extract	2.4	5.68
Crude protein	15.7	7.40
Crude fiber	26.9	16.87
Nitrogen free extract	55.0	67.42

Table 2. Gas production of date seed treated by different levels of enzymatic preparation ZADO® compared to clover hay

		Supplemented doses of ZADO®					P value		
	CH.	**0 mg/g^{-1}**	**6 mg/g^{-1}**	**12 mg/g^{-1}**	**24 mg/g^{-1}**	**SEM.**	**T**	**L**	**Q**
Gas production, ml/g DM									
3 hr.	55.00^{a}	33.75^{b}	39.38^{b}	55.83^{a}	57.50^{a}	2.70	0.002	0.000	0.457
6 hr.	70.00^{a}	44.75^{b}	52.00^{b}	71.42^{a}	73.75^{a}	3.31	0.003	0.000	0.479
12 hr.	97.50^{a}	65.00^{c}	74.38^{bc}	86.67^{ab}	93.33^{a}	3.19	0.004	0.000	0.730
24 hr.	122.50^{a}	85.38^{b}	87.75^{b}	104.67^{ab}	115.83^{a}	3.93	0.006	0.001	0.574
36 hr.	141.25^{a}	98.88^{c}	103.50^{bc}	114.75^{ab}	127.58^{a}	3.97	0.008	0.004	0.557
48 hr.	147.00^{a}	112.00^{b}	112.25^{b}	124.00^{ab}	135.92^{ab}	3.95	0.046	0.019	0.470
72 hr.	157.00	125.25	123.00	143.83	145.92	5.01	0.280	0.099	0.963
Estimated kinetic parameters model									
a	37.52^{ab}	24.52^{b}	25.04^{b}	47.42^{a}	41.59^{a}	2.66	0.003	0.001	0.314
b	120.91	129.25	101.40	182.10	104.82	23.18	0.795	0.959	0.538
c	0.05	0.05	0.04	0.04	0.05	0.00	0.851	0.966	0.372
a+b	157.79	148.39	254.67	140.26	133.83	22.92	0.497	0.520	0.349

a = the gas production from the immediately soluble fraction (ml); b = the gas production from the insoluble fraction (ml); c = the gas production rate constant for the insoluble fraction b (h); a+b = potential gas production (ml); a,b,c Different superscripts following means among enzymes in the row indicate differences at $P<0.05$; SEM = standard error of means; Probability of main effects of treatment (T), linear (L), and quadratic (Q).

fermentation of feedstuffs according to the results of the kinetics of GP parameters (**Sallam *et al*., 2007**).

As presented in Table 2, the higher levels of ENZ linearly increased (P<0.05) GP at 3, 6, 12, 24, 36, and 48 hr., of incubation and tended (P=0.099) to linearly increase GP at 72 hr., of incubation without quadratic effects. The GP kinetics were insignificantly affected by ENZ application with different levels expect GP from the soluble fraction (a), which was linearly (P=0.001) increased with increasing ENZ levels. The increased GP with increasing ENZ levels are in harmony with previous studies **(Nsereko *et al*., 2002; Eun *et al*., 2006; López *et a*l., 2013**). Improving GP by ENZ may be due to stimulate the fermentation by its content of *Ruminococcus flavefaciens*. Also, increased GP may be a reflection of an increase in bacterial numbers, and hence, the hydrolytic capacity of the ruminal fluid. This view agree with previous hypotheses which suggested that exogenous ENZ increased fibrolytic activity due to increased numbers of ruminal microbes (**Colombatto *et al.*, 2003**), and increased bacterial attachment and synergistic effects with hydrolysis of ruminal microorganisms. Enzyme addition tended to affect asymptotic GP, but had no effect on rate of GP, which is consistent with previous studies using other types of enzymes (**Jalilvand *et al.*, 2008**).

Predicted Parameters

As shown in Table 3, no significant differences in predicted SCFA, ME, NEL, MCP, and PF, but a significant decrease in OMD (%) was noted in the untreated DS compared to the CH. The lower of OMD (%) of DS predicted from GP might be due to the lower total gas production, crude protein, and ash contents, which was most evident during the first 24 hr., of incubation (**Menke *et al*., 1979**). However, the addition of ENZ resulted in a linear increase (P<0.001) in the predicted value of SCFA, ME, NE_L, and OMD with increasing the ENZ concentration. High values was detected in the substrate supplemented with ENZ at 24 mg/g compared with untreated DS. These results agreed with **Gado *et al.* (2009) and Salem *et al.* (2013)** who reported that supplementation of enzymes increased total SCFA concentrations. Also these results agreed with **Omar *et al*. (2009)**, who concluded that supplementation of enzymes to steer rations improved digestibility and rumen SCFA concentrations. The enhanced predicted values of DS with the ENZ addition might be due to increased ruminal fermentation (**Nsereko *et al*., 2002**), enhanced attachment and colonization to the plant cell wall material by rumen microorganisms (**Wang *et al*., 2001; Nsereko *et al*., 2002**) and/or by synergism between ruminal enzymes and the enzymes of the ENZ as the most likely mode of action of the enzyme (**Morgavi *et al*., 2001**). Similarly, **Beauchemin *et al.* (2003)** showed that additional ENZ increased the digestible energy intake of the animal when the fed diet was rich in fiber and low in energy.

In vitro Rumen Fermentation Parameters and Feed Degradability

The values of NH_3-N, protozoal count, TVFA, DMD, and CFD were not significantly different between untreated DS and CH. A significant (P<0.05) increase in pH, and a significant reduction in CPD, were observed in the untreated DS compared to CH (Table 4). The higher pH value for untreated DS possibly reflected the differences in the available soluble carbohydrate of the substrates for fermentation, as well as their ammonia-N concentration with buffering capacity (**Arriola and Adesogan, 2013**).

Incubation of DS with ENZ lead to a linear increase in DMD, CFD, and CPD with a significant (P<0.05) effect at the level of 24 mg/g (Table 4). The positive effect of enzymes mixture on *in vitro* degradation of DS are consistent with those reported by **Gado *et al*. (2013)** who has detected a significant increase in DM and CF degradation of rice straw treated by ENZ. These improvements in the feed degradation could be linked to the fact that ENZ might enhance rumen enzyme activity (**Hristov *et al*., 2008**) due to increments of soluble carbohydrates released from undigested feed particles, which provides additional energy for microbial growth and shortening the lag time for microbial colonization (**Sutton *et al*., 2002**). Additionally, it could participate in the increase of the soluble fraction that would be expected if feeds are directly treated with enzymes because

Table 3. Predictive value of date seed treated by different levels of enzymatic preparation ZADO® compared to clover hay

		supplemented doses of ZADO®					P value		
	CH.	0 g/kg^{-1}	6 g/kg^{-1}	12 g/kg^{-1}	24 g/kg^{-1}	SEM.	T	L	Q
Predictive value									
SCFA mmol	0.43^{ab}	0.33^{b}	0.34^{b}	0.45^{ab}	0.49^{a}	0.019	0.003	0.000	0.558
ME(MJ/kg DM)	4.94^{ab}	4.29^{b}	4.35^{b}	5.02^{ab}	5.31^{a}	0.114	0.003	0.000	0.558
NE_L(MJ/kg DM)	2.47^{ab}	2.01^{b}	2.06^{b}	2.52^{ab}	2.73^{a}	0.080	0.003	0.000	0.558
OMD%	40.17^{a}	30.49^{b}	30.91^{b}	35.24^{ab}	37.14^{a}	0.807	0.000	0.000	0.558
MCP (mg/g DM)	384.85	372.18	411.29	408.48	437.08	8.24	0.087	0.011	0.796
PF (mg TDOM/ml gas)	2.31	2.16	2.08	1.75	1.66	0.074	0.025	0.001	0.969

a,b,c Different superscripts following means among enzymes in the row indicate differences at P<0.05.
SEM = standard error of means; Probability of main effects of treatment (T), linear (L), and quadratic (Q).
OMD = in vitro organic matter digestibility; ME = metabolizable energy; NE_L = net energy lactation;
PF = the partitioning factor at 72 h of incubation; SCFA = short chain fatty acids; MCP= microbial crude protein production.

Table 4. Fermentation parameters and nutrient degradability of date seed treated by different levels of enzymatic preparation ZADO® compared to clover hay

		supplemented doses of ZADO®					P value		
	CH.	0 g/kg^{-1}	6 g/kg^{-1}	12 g/kg^{-1}	24 g/kg^{-1}	SEM.	T	L	Q
Fermentation parameter									
pH	6.25^{c}	6.36^{a}	6.36^{a}	6.33^{ab}	6.29^{bc}	0.01	0.000	0.131	0.000
Ammonia-N	34.30	26.37	28.00	28.23	28.00	1.56	0.851	0.781	0.520
TVFA	95.00	101.00	150.67	132.33	138.00	7.60	0.132	0.071	0.218
Protozoa (×10^{3}cell/mL)	210.00	174.33	193.33	193.33	206.67	6.17	0.49	0.134	0.833
Nutrient degradability									
DMD	43.88^{ab}	41.12^{b}	44.75^{ab}	44.89^{ab}	48.45^{a}	0.812	0.033	0.002	0.949
CFD	35.41^{b}	35.43^{b}	37.28^{b}	37.15^{b}	47.68^{a}	1.56	0.019	0.007	0.093
CPD	59.37^{a}	40.89^{c}	48.39^{bc}	51.49^{ab}	53.43^{ab}	1.86	0.008	0.000	0.119

TVFA = total volatile fatty acid; DMD = dry matter degradability; CFD= crude fiber degradability; CPD= crude protein degradability.
a,b,c Different superscripts following means among enzymes in the row indicate differences at P<0.05. SEM = standard error of means; Probability of main effects of treatment (T), linear (L), and quadratic (Q).

this treatment has been shown to start fiber degradation and to reduce the NDF content of different feeds (**Giraldo *et al*., 2008**). The release of sugars from feeds arises at least partially from the solubilization of NDF and ADF (**Hristov *et al*., 2008**). This is consistent with increased soluble fraction and rate of in situ digestion (**Hristov *et al*., 2008**).

In conclusion, the chemical analyses showed that of CP, CF, and ash are low but OM, EE, and NFE are high in DS compared with CH. The lower nutritional quality of DS resulted in a decrease in the *in vitro* GP as well as OM and CP degradability. However, the addition of ENZ at 24 mg/g DM of DS improved gas production, SCFA, energy content, and the degradation of DM, CP, and CF compared with the untreated DS. However, further *in vivo* trials are recommended to be carried to reassess the obtained results.

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تأثير الإنزيمات الخارجيه المحللة للألياف على خصائص التخمر فى الكرش وهضم العناصر الغذائية لنوى التمر باستخدام تقنية إنتاج الغاز معملياً

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تلعب مخلفات التصنيع الزراعية ومخلفات المحاصيل والتى تتوفر بكميات كبيرة دوراً هاماً في سد جزء من العجز الغذائى للحيوانات المجترة، حيث يؤدي الاستخدام الأمثل لهذة المخلفات في تغذية الحيوان إلى تحسين الظروف البيئية والعائد الاقتصادى وكفاءة الإنتاج الزراعي والحيواني، أجريت الدراسة الحالية لمعرفة تأثير إضافة مستحضر مخلوط الإنزيمات الخارجية لنوى التمر على إنتاج الغازات معملياً وبعض قياسات التخمر فى الكرش مقارنة مع دريس البرسيم، تمت إضافة أربع مستويات من مخلوط الانزيمات: صفر (الكنترول)، ٦ ملجم (منخفض) ، ١٢ ملجم (متوسط) و ٢٤ ملجم (عالي)/ جم ماده جافه لعينات نوى التمر فى أنابيب التحضين، تم استخدام عينات من دريس البرسيم للمقارنة مع نوى التمر غير المعامل، عند مقارنة عينات نوى التمر غيرالمعاملة بمستحضر مخلوط الإنزيمات مع دريس البرسيم، كان هناك إنخفاضاً معنوياً فى إنتاج الغاز التراكمى، معامل هضم الماده العضوية المتوقع، معامل هضم البروتين الخام، ومع ذلك، أدت اضافة المستويات العالية من الانزيم (٢٤ ملجم/جم) إلى زيادة خطيه فى إنتاج الغاز بعد ٣ و ٦ و ١٢ و ٢٤ و ٣٦ و ٤٨ ساعة من التحضين، لم تتأثر ديناميكية إنتاج الغاز بشكل معنوى نتيجة للمعاملة بالمستويات المختلفه للمستحضر الانزيمى، كما أدى رفع تركيز المستحضر الانزيمى إلى زيادة خطية في القيم المتوقعة لـلأحماض الدهنية قصيرة السلسة والطاقة الممثلة والطاقة الصافية وهضم المادة العضوية لنوى التمر، حيث سجلت أعلى القيم مع المستوى الاعلى من المستحضر الانزيمى (٢٤ ملجم/جم) مقارنةً بـنوى التمرغير المعامل، أدت إضافة المستحضر الانزيمى ZADO® إلى زيادة خطية في معاملات هضم المادة الجافة والالياف الخام والبروتين الخام، كان التحسن معنوياً عند مستوى ٢٤ ملجم/جم مادة جافة، إجمالاً، تشير نتائج الدراسة الحاليه إلى أن اضافة المستحضر الانزيمى ZADO® بمستوى ٢٤ ملجم/جم ماده جافه أدى الى تحسين هضم المركبات الغذائيه فى نوى التمر.

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