COMPARATIVE STUDIES IN VITRO AND IN VIVO TRIALS OF SOME FEED ADDITIVES (GARLIC POWDER AND GARLIC OIL) IN SHEEP RATIONS

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SUMMARY

Feed additives utilization from natural locally resources are an important strategy to increase animal production in developing countries. Many investigations were conducted using nutritional additives at *in vitro* level. While, few *in vivo* studies are available on the effect of such additives on animal and the relationship between the results at *in vitro* and *in vivo* trials. Therefore, garlic cloves (*Allium sativum*) in various combinations, were processed either as a powder or its oil to formulate a supplemented diets for Barki rams. These diets were used to determine *in vivo* digestibility and to record these results in comparison with *in vitro* digestibility procedures. Sixteen Barki rams with an average live weight (40.47±0.99 kg) were allocated into four treatment groups and were individually kept in metabolic cages. The experimental diets were :1) a basal diet without additive (control); 2) a basal diet supplemented with 20g garlic powder/ kg DM; 3) a basal diet supplemented with 40g garlic powder/kg DM; 4) a basal diet supplemented with 2 ml oil/kg DM. *In vitro* dry matter digestibility were strongly correlated (r = 0.77) with their mates by *in vivo* trial. A rumen fermentation pattern, nitrogen utilization and some blood parameters was asses as well. Ruminal pH and volatile fatty acids (VFAs) were not affected by the supplementation of garlic powder and garlic oil in the diets, while ruminal ammonia significantly decreased compared to control diet. Either treated or untreated diets showed no differences of nitrogen (N) intakes, digested and excreted as g /kg BW among the experimental groups. Also, N balance (g /Kg BW) is a similar resulting between the experimental diets. The use of garlic additives in sheep diets improved blood total protein, but decreased triglycerides and urea blood concentration. As conclusion, garlic powder and garlic oil supplementation can be recommended for sheep diets to enhance rumen fermentation and blood parameters however, there is a need for long-term feeding trials and rumen microbial studies should be conducted for better understanding of their impacts in feed metabolism and rumen outputs.

Keywords, *garlic oil; garlic powder; in vitro; in vivo; digestibility; rumen fermentation*

INTRODUCTION

Many researches indicate that *in vitro* of animal feed is highly correlated with *in vivo* digestibility (Lemus *et al.*, 2018; Soutar *et al.*, 2021; Thomson and Ali, 2003; Alvaro Barreto *et al.*, 2023). Adjusting *in vitro* results by the equations generated from the standards (with known *in vivo* digestibility values) allows researchers to compare estimates from different in vitro runs (Mendes *et al.*, 2016). Besides, in vitro technique have some advantages, such as short in duration, less expensive, less labor and material, and not restricted with ethical matter.

On the other hand, the natural feed additives and essential oils have been examined due to their advantages over the antibiotics as growth promoters. For instance, Garlic (*Allium sativum*) has been widely tested as feed additives in *in vitro* level (Booyens *et al.*, 2014; Becker *et al.*, 2012; Arzanlou, 2016 and Ayu *et al.*, 2019). The volatile fatty acids (VFAs) concentration was affected by the extracts of garlic in *in vitro* incubation with buffalo rumen liquor towards more propionate, resulting in decreased acetate to propionate ratio (Patra *et al.*, 2009). In contrast, many studies obtained a decrease proportion of acetate and branched chain fatty acids and increased proportion of butyrate, small peptides and amino acid nitrogen by inclusion of garlic oil in continuous culture system (Busquet *et al.*, 2005 & Busquet *et al.*, 2006). Few *in vivo* studies are available on the effect of garlic oil or powder on nutrient digestibility and nitrogen utilization. Therefore, the current study aimed to compare *in*

vivo to *in vitro* digestibility procedures using different doses of garlic powder and garlic oil to obtain a regression equation that could allow researcher for animal response prediction.

MATERIALS AND METHODS

The present study was conducted at Maryout Research Station, Desert Research Center, 35 km south of Alexanderia, Egypt and were approved by the Animal Ethics and Care Committee of Animal and Poultry Production Division of Desert Research Center, Egypt.

In vitro batch culture technique:

The experimental feed additives are presented in Table 1. In vitro batch culture procedure was applied according to (Szumacher-Strabel *et al.*, 2002).

Table (1): Experimental treatments.

Treatment	Additive
Control	0
Treatment 2	20g garlic powder / kg DM
Treatment 3	30g garlic powder/ kg DM
Treatment 4	2 ml oil /kg DM

Incubation and sampling procedures:

A mixture of 40 % roughage (alfalfa hay) and 60% concentrate feed mixture (CFM) was used as a substrate. The experimental diets were: 1) a basal diet without additive (control); 2) a basal diet supplemented with 20g garlic powder/ kg DM; 3) a basal diet supplemented with 40g garlic powder/kg DM; 4) a basal diet supplemented with 2 ml oil/kg DM. These doses of additives were recommended by Nassar *et al.* (2017). The concentrate feed mixture consisted of 29 % corn grains, 16 % linseed cake, 30 % wheat bran, 20 % cottonseed meal, 1 % salt, 2% molasses, 2 % premix. Rumen fluid was collected from cannulated animals (rams), which was fed the experimental diets for three weeks, then was mixed and squeezed through 4-layers cheesecloth into a bottle (1 L) with an O_2-free headspace and transported to the laboratory at 39°C where it was used as a source of inoculum. Each diet sample was tested and replicated six times accompanied with blank vessels (no substrate). *In vitro* dry matter digestibility (DMD), digestible crude protein (DCP) and crude fiber digestibility (CFD) determined after terminal of each incubation time by recovery of the undigested fraction according to the methodology of Cieślak *et al.* (2009). *In vitro* DMD, DCP and CFD values from each run were regressed against the *in vivo* values. The slopes of each regression line were compared. Differences between regression equations were also tested.

In vivo trial:

Sixteen Barki rams divided into four groups with an average live weight (40.47±0.99 kg) were individually kept and fed in metabolic cages to determine nutrients digestibility compared to their values in *in vitro* trial. Chemical composition of experimental feed ingredients presented in Table 2. The digestibility trial was extended for three weeks as a preliminary period followed by 7 days as a collection period. All rams were fed on the same diets which were used in *in vitro* trial. Feed offered was calculated to cover the maintenance requirements for adult rams according to Kearl (1982). Garlic powder was mixed with CFM for daily offered. Each ram was taken garlic oil dosage individually. Daily feed offered and refused if any were recorded to estimate the actual feed intake (the real amount) for each group. Total feces were recorded daily. Representative samples of feces about 10% of the total fresh feces weight were taken daily and few drops of sulfuric acid were added to avoid losing nitrogen. At the end of collection period feces samples of each animal was mixed and ground for sampling. Soft water was available free choice during the experimental period. Urine was allowed to drain into bottles containing 5 ml of Sulfuric acid and a slight amount of thymol granules was added to prevent losing nitrogen and fermentations. The volume of urine was recorded daily and a sample of urine represents 10% of

total urine for each animal was taken for proximate analysis. Samples of feed offered, refused, feces and urine were taken and stored during the collection period for analysis. Rumen fluids samples were collected from all animals during the digestibility trail by using a stomach tube at four hours post feeding. The rumen samples were filtered through two layers of cheese-cloth and pH values were recorded immediately by digital pH-meter (WPA CD70) then samples were stored frozen (-18°C) for later analysis.

Table (2): Chemical composition of experimental feed ingredients.

Items	Feed ingredients		
	CM	**Alfalfa hay**	**Control diet**
DM	91.1	91.3	91.4
OM	91.1	87.5	89.6
CP	16.1	15.5	16.4
CF	16.5	31.9	21.2
NDF	33.1	48.3	36.7
ADF	21.4	28.5	23.4
EE	2.2	1.4	1.5
Ash	6.9	13.9	12.8
GE, MJ/Kg DM	17.8	14.4	15.6

CM: concentrate mixture, DM: dry matter, OM: organic matter, CP: crude protein, CF: crude fiber, NDF: neutral detergent, ADF: acid detergent, EE: Ether extract, GE: gross energy.

Sampling and analysis of blood:

Blood samples were taken from all animals group. Blood samples were withdrawn. Then, centrifuged and separated blood serum was stored into a clean dried glass vial at -20 °C for analysis. Biochemical analyses (total proteins, albumin, urea, triglyceride, cholesterol, non-esterified fatty acid) were measured in serum using kits provided by Diamond Company.

Analytical procedures:

The laboratory dry matter (DM) content of feed and feces samples was determined by drying at 105ºC for 24 hours. The crude protein was measured as described by A.O.A.C (2002). Fiber was determined according to Van Soest *et al.* (1991). Quantitative analysis of ammonia concentration was carried out by a modified Nessler's method modified by Szumacher-Strabel *et al.* (2002). Total VFA were determined according to Warner (1964).

Statistical analysis:

The obtained data were statistically analyzed according to SAS (1999) using the following model;

$$Yij = \mu + Ti + eij$$

Whers; Yij = experimental observation, μ = overall mean, Ti = effect of treatment, eij = experimental error. Separation between means was carried out by using Duncun Multiple Range test (1955).

RESULTS AND DISSCUTION

In vivo vs in vitro nutrient digestibility:

Regression analysis of the average of all *in vitro* six runs of each diet sample compared to the average of their mate's values of *in vivo* trial are presented in Fig 1, 2 and 3. The slope showed that dry matter digestibility (DMD) for *in vitro* (test tube) vs. *in vivo* (by the animal) trails had a good agreement, and were good correlated (r = 0.77). In other words, 58% of the changes of *in vitro* DMD could explain by the values of *in vivo* DMD .

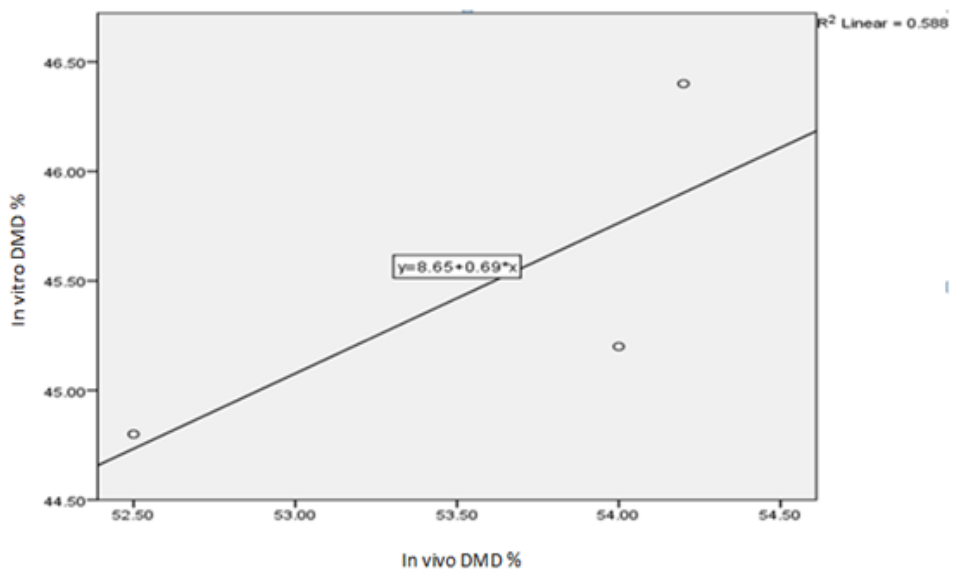

Figure 1. Regression analysis of *in vivo* vs. *in vitro* DMD digestibility

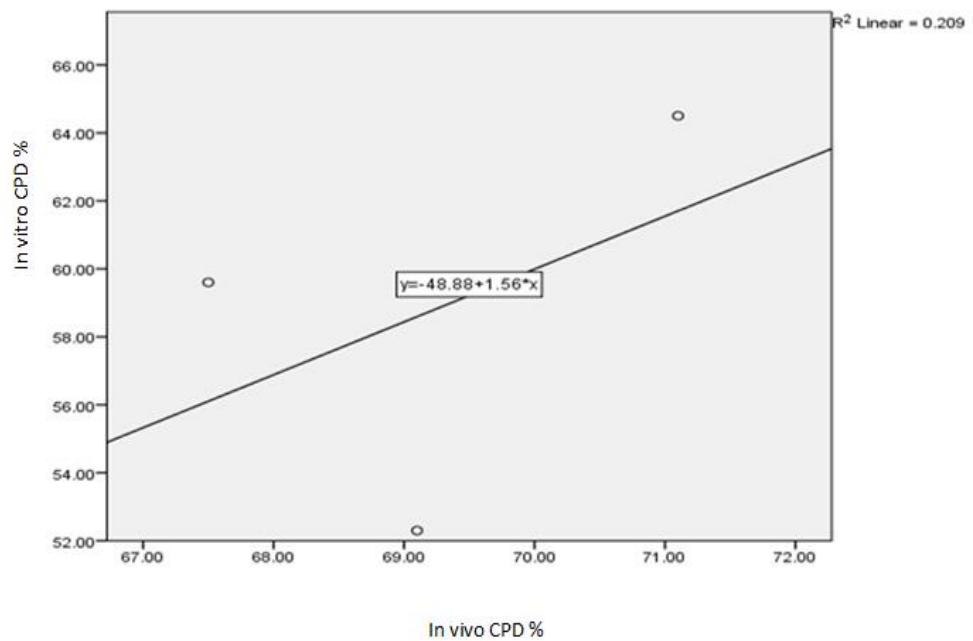

Figure (2): Regression analysis of *in vivo* vs. *in vitro* CP digestibility.

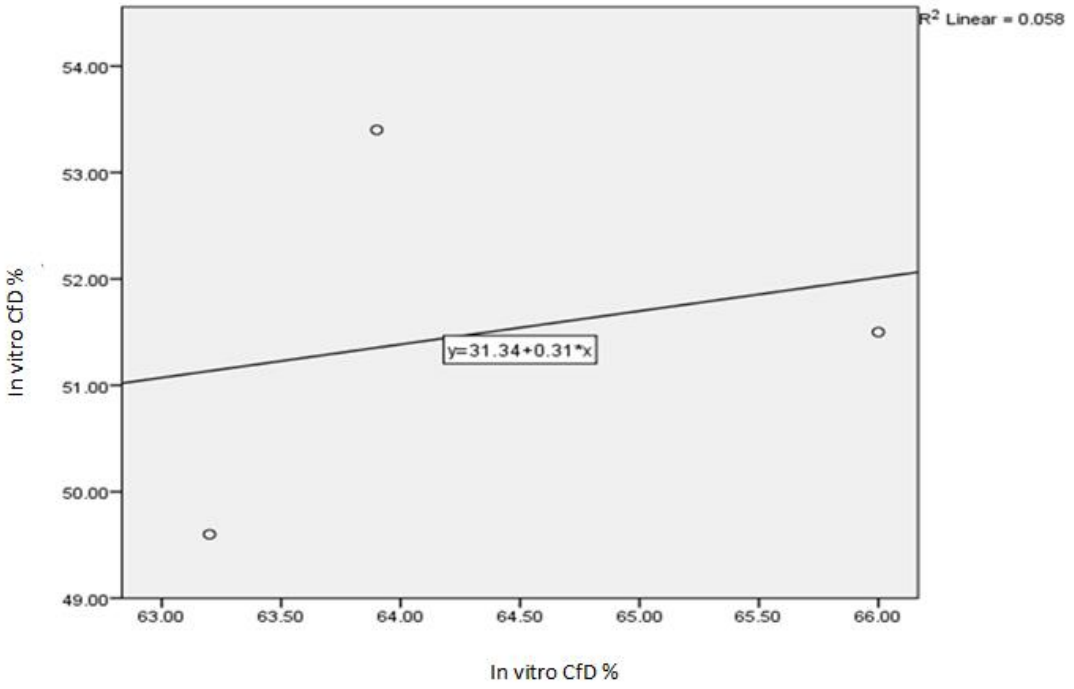

Figure (3): Regression analysis of *in vivo* vs. *in vitro* CF digestibility.

A highly correlation (fig 1.) between DMD values for in vitro and in vivo trails was agreed with Geisert *et al.* (2007), who found that in vitro DMD of forages is highly correlated with their mates of in vivo digestibility experiment for five hay samples. This result allows researchers to adjust the in vitro DMD to in vivo DMD, which allows for more accurate ration formulation and animal response prediction. On the other hand, a lower correlation was obtained in fig 2 and fig 3. among the values of CP and CF digestibility for different diets. This difference between the runs indicated the need for standards to adjust *in vitro* values in order make comparisons with *in vivo* nutrients digestibility (CF and CP) and between different animal diets. In contrast, Mendes *et al.* (2016) recorded that the method of *in vitro* digestibility of proteins showed better correlation with *in vivo* method, and should preferably be used to predict the true digestibility. Our current study suggests that the variation in dose and diet composition appeared to be the important difference in the nonconformity of result between previous studies. In general, a positive relationship was observed in Fig 1, 2 and 3 that could reflect a prediction of *in vivo* nutrients digestibility by *in vitro* approach. However, the differences between *in vitro* values could be attributed to differences in technicians and the handling of rumen fluid prior placing in the tubes.

However, a moderate correlation was obtained in fig 2. among the different values of crude protein digested (CPD) by animals or at *in vitro* levels (r = 0.48). Concern crude fiber digestibility, a weak correlation (r=0.24) was recorded between in vitro and in vivo CF digestibility for different diets (fig 3).

Rumen fermentation:

The effects of the experimental diets on some rumen fermentation parameters are shown in Table 3. Either ruminal pH levels or TVFAs (meq/100 ml) concentration were not significantly affected by the different additives. However, ammonia nitrogen concentration was significantly ($P<0.05$) decreased by garlic both powder or oil additives compared with the control group, the lowest value of ammonia nitrogen was recorded for rams fed diets enriched with garlic oil then for rams that fed diets enriched with 2% and 3% garlic powder in descending order.

Table (3): Rumen fermentation parameters of Barki rams fed diets enriched with garlic powder and garlic oil.

	Experimental rations					
Variable	**Control**	**Control +2% of garlic powder**	**Control +3% of garlic powder**	**Control +2 ml of garlic oil**	**SEM**	**P value**
pH	7.1	6.6	6.8	6.6	1.65	0.325
TVFA (meq/100 ml)	6.2	6.3	6.1	6.2	0.16	0.819
NH3-N (mg/100ml)	21.6^{a}	20.9^{ab}	19.7^{b}	18.1^{b}	2.31	0.208

a,b Means having different superscripts within the same row differed significantly (P<0.05), otherwise no significant differences were detected.

It is noticed that pH value was not affected by additives and within the normal range, which reflect the microbial digestion of fiber and protein (Sahli *et al.,* 2018 and Nassar 2020). The obtained results were in harmony with the finding of Kholif *et al.* (2012) who found no effect of adding garlic or ginger essential oils on ruminal pH of lactating goats. Similar results were obtained by Nassar *et al.* (2017) who reported that either ruminal pH levels or VFA concentration were not significantly affected by including garlic powder or garlic oil in lactating ewes diets. On the other hand, this result is not consistent with finding of Onder Canbolat *et al.* (2021) who reported that the rumen PH increased significantly (P<0.05) with supplementation of garlic oil in ram diets.

With regard to the reduction of ammonia nitrogen concentration by garlic powder and garlic oil compared with the control group, this result is consistent with finding of Klevenhusen *et al.* (2011) who also reported that supplementation of garlic oil significantly decreased the ammonia concentration. While, Chaves *et al.* (2008) reported that the rumen ammonia was not affected by supplementation of garlic oil in growing lambs diets. The difference between two experiments is mainly associated with dose level of garlic oil supplementation. Other studies (Blanch *et al.,* 2016 and Mbiriri *et al.,* 2017) suggested that reducing rumen ammonia nitrogen due to garlic additives could be explain by the limitations on microorganisms involved in the degradation of proteins in rumen. It could be obtained that supplementation of garlic powder and oil additives can increase the efficiency of protein utilization in the rumen decreasing the degradability of protein.

Nitrogen balance:

Nitrogen intake (NI), N-excreted and N-balance by rams fed different experimental diets are presented in Table 4. Data showed that outputs of N in feces, urine, and digested N (g /kg BW) were not affected by different additives. However, its relative percentage (fecal N, urinary N and digested N %) to N intake were significantly (P<0.05) different across the experimental groups, the control group showed the highest total nitrogen execrated values (42.22 %) while group fed diet enriched with garlic oil recorded the lowest total nitrogen execrated values (37.02%). Comparable values were obtained for both groups fed diets enriched with 2% and 3% of garlic powder (35.37 and 35.76%, respectively). Either treated or untreated diets showed no significant differences of N intakes, digested and excreted as g /kg BW among the experimental groups resulting in a similar N balance (g /Kg BW) between groups fed diets enriched with garlic additives and control.

As total dry matter intake (TDMI) was not significantly differed among the experimental groups, N intakes (g/kg BW) were nearly similar without any impacts of garlic additives for all groups. Besides, N in feces, urine, and digested N (g /kg BW) followed the same trend of N intakes.

Few studies are available on the effect of garlic oil or powder on N-balance. For instance, Benchaar *et al.* (2008) found no change in N-retention when cows were fed 2.0 and 0.75g/d of the essential oils including thymol, eugenol, vanillin, guaiacol, limonene, respectively. This result was agreed with, Kewan *et al.* (2021) for rams fed diets enriched with 40g garlic powder/head/day. While the combined of garlic powder with yeast in rams diet exhibited 15.2% increase of N balance above the control group.

However, the results of N balance in current study contrast with the finding of Amagase *et al.* (2006) who recorded improving the N balance in sheep fed hay supplemented with garlic leaf as result of the antioxidant effects for the bioactive components of garlic. Besides, Ahmed *et al.* (2014) observed that the low level of eucalyptus essential oil (10 ml/d) improved N balance in sheep. Our current study suggests that, studding of

garlic oils or garlic powder effects on rumen bacteria population could help very will to explain their impact of rumen fermentation out puts and N pathway as well.

Table (4): Nitrogen utilization by Barki rams fed diets enriched with garlic powder and garlic oil.

Variable	Experimental rations					
	Control	**Control +2% of garlic powder**	**Control +3% of garlic powder**	**Control + 2 ml of garlic oil**	**SEM**	**P value**
TDMI, kg	741	714	706	723	15.86	0.808
Nitrogen intake						
g/kg BW	0.51	0.49	0.48	0.50	0.024	< 0.001
Fecal nitrogen						
g/kg BW	0.18	0.14	0.15	0.16	--	--
%of intake	35.00^{a}	29.00^{b}	31.00^{b}	33.00^{ab}	3.05	0.824
Digested nitrogen						
g/kg BW	0.33	0.35	0.33	0.33	-	-
%of intake	65.00^{b}	71.00^{a}	69.00^{a}	67.00^{ab}	3.78	0.014
Urine nitrogen						
g/kg BW	0.04	0.03	0.02	0.02	-	-
%of intake	7.22^{a}	6.37^{a}	4.76^{b}	4.02^{b}	3.68	0.021
Total nitrogen excretion						
g/kg BW	0.22	0.17	0.17	0.18	0.020	< 0.001
%of intake	42.22^{a}	35.37^{b}	35.76^{b}	37.02^{b}	2.46	0.741
Nitrogen balance						
g/kg BW	0.30	0.32	0.31	0.31	--	--
%of intake	57.78^{a}	64.63^{b}	64.24^{b}	62.98^{b}	8.98	0.945

a,b Means having different superscripts within the same row differed significantly (P<0.05), otherwise no significant differences were detected.

Blood parameters:

Data in Table 5. obtained that most blood parameters were affected by different additives. Total protein and albumin concentration significantly increased with increasing level of garlic powder supplementation and with diet included garlic oil. Whereas supplementation decreased blood urea, triglyceride and cholesterol concentration compared to control ration. Blood glucose values was not significantly affected by treatments and was within the normal rang for adult rams. Non esterified fatty acids was slightly increased with garlic powder addition and reached the highest values for those fed diets with garlic oil.

The blood glucose level recorded in the study (Table 5) was lower than that determined by Chaves *et al.* (2008) but was higher than that reported by other studies Anassori *et al.* (2011) and Blanch *et al.* (2016). Researchers explained the decrease in blood glucose levels with the addition of garlic additives to the decrease in the concentration of propionic acid (involved in the synthesis of blood glucose), which is not determined in the current study. While Total proteins (P<0.05) increased by supplementation compared to control diet as result of the improvement of ruminal microbial protein synthesis which increased blood albumin. Besides, the reduction of blood urea concentration by garlic additives was mainly attributed to the rumen ammonia nitrogen inhibition as showing antimicrobial effect (Table 3). The obtained results in the current experiment are consistent with finding of Anassori *et al.* (2011).

The decrease in blood triglyceride level due to garlic powder and garlic oil addition can be explained by the decrease in the blood glucose level used as an energy source in animals and the increase in the rate of esterified fatty acid. While Reduction of blood cholesterol levels could be attributed to the organ sulfide compound in garlic oil which limited the enzyme responsible for cholesterol synthesis Nassar (2020).

Table (5): Blood serum parameters of Barki rams fed diets enriched with garlic powder and garlic oil.

Variable	Experimental rations				SEM	P value
	Control	Control +2% of garlic powder	Control +3% of garlic powder	Control + 2 ml of garlic oil		
Glucose, mg/100 mL	81.2	76	76.3	74.2	0.60	0.604
Total protein	7.57^{c}	8.19^{ab}	7.99^{b}	8.55^{a}	0.74	< 0.001
Albumin(g/dl)	5.21^{c}	5.98^{ab}	5.35^{b}	6.04^{a}	2.23	0.021
Globulin(g/dl)	2.36	2.21	2.64	2.51		0.030
Urea, mg/100 mL	15.4^{a}	14.2^{a}	14^{a}	11.9^{b}	3.21	< 0.001
Triglyceride, mg/100 ml	26.2^{a}	24.1^{b}	25.7^{ab}	22.3^{c}	6.45	0.014
Cholesterol, mg/100 mL	63.4^{a}	61.2^{b}	60.4^{b}	58.2^{b}	4.52	--
Non-esterified fatty acids µmol/L	231	239	240	242	11.0	0.424

^{a,b,c} *Means having different superscripts within the same row differed significantly (P<0.05), otherwise no significant differences were detected.*

CONCLUSION

It could be concluded that nutrients digestibility of diets enriched with garlic additives could be determined by *in vitro* techniques, which showed highly dry matter correlation with *in vivo* method, and could be used to predict the true digestibility. The effect of garlic additives as powder or oil on rumen fermentation outputs still inconsistent with literature and more studies should conduct on rumen microorganisms response for better explanation of their effects. The use of garlic additives in the feed of sheep is also safe and could improve blood total protein, decrease triglycerides and urea blood concentration, however, before recommending it for practical application, there is a need for long-term feeding trials and economic study should be consider.

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دراسات مقارنة بين التجارب المعملية والحقلية لبعض إضافات الأعلاف (مسحوق الثوم وزيت الثوم) في علائق الأغنام

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تعد الاستفاده من إضافات الأعلاف من المصادر الطبيعية المحلية استراتيجية هامة لزيادة الإنتاج الحيواني في البلدان النامية. وقد استخدمت الاضافات الغذائية فى العديد من التجارب المعملية. في حين أن التجارب التى درست تاثير هذه الاضافات على الحيوان ودراسة العلاقة بين النتائج المعملية والحقلية لا تزال محدودة. لذلك تم عمل توليفات مختلفة من الثوم (*Allium sativum* ، إما مسحوق الثوم أو زيت الثوم لعمل علائق مدعمه للكباش البرقى. واستخدمت هذه االعلائق لتقدير قابليتها للهضم من قبل الحيوان وتسجيل هذه النتائج ومقارنتها بنظيرتها معمليا. كما قدرت تخمرات الكرش والاستفادة من النيتروجين وكذلك بعض مقاييس الدم.

تم توزيع ستة عشر كبش برقي بمتوسط وزن حي (40.47 ± 0.99 كجم) في أربع مجموعات حيث وضعت فرديا في صناديق الهضم. كانت العلائق التجريبية هي: 1) نظام غذائي أساسي بدون إضافات (مجموعة الضابطة)؛ 2) نظام غذائي أساسي مدعم بـ 20 جرام من مسحوق الثوم / كجم مادة جافة؛ 3) نظام غذائي أساسي مدعم بـ 40 جرام من مسحوق الثوم / كجم مادة جافة؛ 4) نظام غذائي أساسي مدعم بـ 2 مل من الزيت / كجم مادة جافة. ارتبط هضم المادة الجافة معمليا ارتباطًا وثيقًا (r = 0.77) بنظيرتها على الحيوان. لم يتأثر الرقم الهيدروجيني للكرش والاحماض الدهنية التيارة بإضافة مسحوق الثوم وزيت الثوم في العلائق الغذائية بينما انخفضت الأمونيا في الكرش بشكل كبير مقارنة بالمجموعه الضابطة. لم تظهر العلائق المدعمه او العليقة الضابطه أي اختلافات في النيتروجين الماكول، المهضوم والمفرز كجم/كجم من وزن الجسم بين المجموعات التجريبية مما أدى إلى ميزان نيتروجين متماثل (غم/كغم من وزن الجسم) بين المجموعات التي تغذت على العلائق التجريبية. أدى استخدام إضافات الثوم في علف الأغنام إلى تحسين البروتين الكلي في الدم وتقليل تركيز الدهون الثلاثية واليوريا في الدم.

ونتيجة لذلك، يمكن التوصية بمكملات مسحوق الثوم وزيت الثوم في علف الأغنام لتحسين تخمرات الكرش ومعايير الدم، ومع ذلك، هناك حاجة إلى تجارب غذائية طويلة الأمد ويجب إجراء دراسات ميكروبية على الكرش لفهم أفضل لتأثيراتها في عملية التمثيل الغذائي للأعلاف ومخرجات الكرش.