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Relationship between *In vivo*, *In vitro* Parameters and Chemical Composition to Predict the Nutritive Value of Some Legume Forages

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ABSTRACT

Background: Many *in vivo* and laboratory methods have been used to evaluate ruminant feeds. The objective of this experiment was to determine feed intake, digestibility, kinetics of gas production, *in vitro* organic matter digestibility (OMDv) and metabolizable energy (ME) of *Trifolium alexandrinum* (*T. alexandrinum*) and *Medicago sativa* (*M. sativa*) at different maturity stages through *in vivo* and *in vitro* methods to elaborate predictive equations from chemical constituents.

Methods: *In vivo* assay was carried out with two groups of five rams male kipped in metabolism cage. Samples of forage, refusal and feces were collected and processed for chemical analysis. *In vitro* gaz production technique was performed on forage samples.

Result: The bud and early bloom stage of *M. sativa* recorded the highest digestibility values of the chemical component ($P<0.05$). The OMDv of *M. sativa* was 60.65 and 68.26% ($P<0.01$) for early bloom stage and bud stage, respectively. Crude protein digestibility (CPD) was positively correlated with crude protein rate ($R^2=0.83$, $P<0.05$). The gas production from the insoluble fraction "b" fraction showed a positive correlation with acid detergent fiber (ADF) ($R^2=0.999$, $P<0.001$). However, cumulative gas production at 24 h and 48 h of incubation were negatively correlated ($P<0.05$) with the ADF rate ($R^2= -0.98$ and -0.97, respectively).

Key words: Chemical composition, Digestibility, Green forages, Gas production, Maturity stage, Prediction equations.

INTRODUCTION

Chemical analysis allows to determine fodder chemical constituents which are used to evaluate the nutritive value of forages. But, this is not sufficient or accurate enough. To ensure the evaluation of digestibility of forages and to predict the nutritive value of feeds, *in vivo* and *in vitro* techniques were made available to livestock (Karabulut *et al*., 2007).

Trifolium alexandrinum L. (*T. alexandrinum*) and *Medicago sativa* (*M. sativa*) are popular among livestock farmers as "King and queen of Fodder Crops" and are cultivated on all the continents (Elif and Veysel, 2016; Singh *et al*., 2020). They provide a large amount of soluble protein and enrich the soil by fixing atmospheric nitrogen (Elif and Veysel, 2017). So, they allow an improvement of the productivity and the quality of final products.

The aim of this study was to determine (i) chemical parameters and nutritive value of Tunisian green chopped legume forages (*T. alexandrinum*, *M. sativa*) at different maturity stages, through *in vivo* and *in vitro* methods and (ii) to establish predictive equations of nutritive value parameters using chemical components.

MATERIALS AND METHODS

Forage samples

Trifoluim alexandrinum L. (variety Meskaoui) and *Medicago sativa* L. (variety of Gabes) were cultivated on small plots of 1000 m^2 in the experimental station of the Agronomic Higher Institute of Chott-Meriem (35°56′17″N, 10°33′18″E) during winter and spring 2015.

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These forages were hand-harvested at different maturity stages: Bloom for *T. alexandrinum* and bud, early bloom and seed for *M. sativa*.

In vivo assay

For each trial, two groups of five rams male Black of Thibar with an average age of nine months and an average initial body weight of 33.2 ± 1.62 kg were kept in metabolism cage.

The digestibility test includes a seven day adaptation period, a seven day preliminary period during which ingestion where the animals were fed *ad libitum* and a seven day collection period.

Intake was evaluated daily. Samples of the offered forage, the refusals and feces were collected in plastic bags and were stored at -20°C for further analysis. At the end of each period, those samples were combined homogeneously to determine their chemical composition and to evaluate feed intake and digestibility of dietary nutrients. Urine is collected for each animal in a 10 L plastic container containing 200 mL of a 10% solution of H_2SO_4. At the end of the collection period, the total amount of urine was measured and 20% of the total volume was stored in plastic bottles. At the beginning and at the end of each test, the animals are weighted.

Chemical analysis

Representative samples of forage, refusal and feces were dried at 60°C for 72 h and then grounded at 1 mm screen for chemical analysis composition. Ash, crude protein (CP) and crude fiber (CF) contents were quantified according to AOAC (2000) and acid detergent fiber (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL) were analysed following the procedures described by Van Soest *et al.* (1991).

***In vitro* assay**

In vitro gas production was carried out using the method described by Menke and Steingass (1988). Rumen fluid was collected from four fistulated Black of Thibar sheep, combined, homogenized, filtered four layers of cheese-cloth and purged with CO_2.The artificial saliva was prepared (Table 1). The inoculum fluid was prepared of rumen fluid and artificial saliva (1:2 v/v). This mixture (pH = 6.9± 0.1) was then kept under CO_2 at 39°C water bath and stirred using a magnetic stirrer.

200 mg of air-dry feedstuffs are incubated in 4 graduated glass syringes, preheated to 39°C, with 30 ml of the prepared mixture. The syringes are closed and the starting volume (V0) is recorded at time t0. Then, they were placed in the incubator. The syringes were gently shaken every 30 minutes during the first 8 to 10 hours of incubation. The volume of gas was recorded after 2, 4, 6, 12, 24, 36, 48, 72 and 96 hours of incubation. Three control syringes in each manipulation were incubated and considered as the blanks.

Table 1: Components of artificial saliva and their concentrations (Menke and Steingass, 1988).

Solution	Reagent	Concentration
Solution A	$CaCl_2$ $2H_2O$	13.2 g (/ 100mL)
	$MnCl_2$ $4H_2O$	10 g
	$CoCl_2$ $6H_2O$	1 g
	FeCl3 $6H_2O$	8 g
Solution B	$NaHCO_3$ 39 g (/ L)	$NaHCO_3$ 39 g (/ L)
Solution C	Na_2HPO_4	5.7 g (/L)
	KH_2PO_4	6.2 g
	$MgSO_4$ $7H_2O$	0.6 g
Solution D	$C_{12}H_6NO_4$	100 mg/ 100 mL
Solution E	NaOH 1N	4 mL (/95mL)
	Na_2S $9H_2O$	625 mg

Total gas values were corrected for the blank incubation. Cumulative gas production data were fitted to the model of Ørskov and McDonald (1979):

$$y = a + b\ (1- e^{-ct})$$

Where

y = Gas produced at the time "t".

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 = The potential gas production (ml).

t = Incubation time (h).

Menke and Steingass (1988) have developed equations to calculate the *in vitro* digestibility of organic matter (OMDv) and the metabolizable energy (ME) of greens:

$$OMDv = 14.51 + 0.8490 \times GP + 0.0653 \times CP + 0.0686 \times Ash$$

Where

GP = 24 h net gas production (ml/200 mg)

ME (kcal) =

$$(1.14 + 0.1439 \times OMDv - 0.0134 \times Ash) \times (1000/4.18)$$

Statistical analysis

The data were analyzed according to the General linear Model (GLM) procedure of the SAS software (2002) studying the effect of a one factor: maturity stage. Significance between individual means was identified using the Student test. Correlations between the *in vivo*, *in vitro* parameters and the chemical composition components were established then the Stepwise procedure was used to establish prediction equations.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of legume forages is shown in Table 2. The CP and CF of *T. alexandrinum* were founded to be 14.22% DM and 21.52% DM, respectively. Its CF content was higher but its CP content was lower than the values founded by Nefzaoui and Chermiti (1989).

The DM and CF content showed a gradual increase throughout the evolution of the maturity stage; the highest value is recorded at the final stage ($P<0.01$). However, the CP content noted a gradual decrease ($P<0.001$). Indeed, the highest CP content was founded at bud stage. The budding stage CP content was higher than the values reported by Nefzaoui and Chermiti (1989) and the tables of INRA (2007). Early bloom stage showed the highest NDF and ADF content ($P<0.05$). Its CF rate was lower than that founded by Nefzaoui and Chermiti (1989) and the tables of INRA (2007). The NDF and ADF contents were relatively

identical with the values of the INRA (2007) and lower than that reported by Hollis *et al.* (2020).

Feed intake, nitrogen balance and nutrient digestibility

T. alexandrinum exhibited the highest value of DM intake, OM intake and intake/$W^{0.75}$ (P<0.001) amounts (Table 3). These results were confirmed by Minson (1990) who mentioned that temperate legumes are ingested in large quantities because they offer less resistance to their reduction. *M. sativa* showed a decrease in DM intake, OM intake and intake/$W^{0.75}$ between the bud and early bloom stage but at the pod stage the situation reversed.

The maturity stage had an effect on the N intake, N balance and retained N (P <0.001). The highest amount of N intake, N balance and retained N has been registered at bud stage of *M. sativa*.

The *in vivo* digestibility of DM, OM, CF, ADF (P <0.05) and CF (P <0.05) were affected by the maturity stage. Indeed, the highest digestibility values of the chemical component were recorded at bud and early bloom stage (Table 3).

The *M. sativa* CPD value was higher than the value reported in the INRA tables (2007). The results of the nitrogen balance followed the same trend as the digestibility of CPD. At the bud stage, the INRA tables (2007) reported values around 52% and 50% for the CFD and ADFD, respectively. The maturity stage has a very sensitive effect on the *in vivo* digestibility of the different chemical components, so the youngest maturity stage is the most digestible. During the plant aging, the cell walls thicken and become encrusted with lignin. This is not only indigestible, but it also constitutes a barrier to the walls digestion's of the forage by the rumen microbes. This is confirmed by the low digestibility of forage fibers in the last maturity stage, which reflects a significant degree of lignification (Jarrige, 1988).

Kinetics of gas production

The Kinetics of gas production of *T. alexandrinum* and *M. sativa*, corrected with blanks are showed in Fig 1. The cumulative gas production profiles of legume forages increased during incubation period. A constant level of gas production was almost reached around 48 h and 72 h of incubation.

The *M. sativa* gas production kinetics curves for the three maturity stages were very close to each other with a slight superiority of the bud stage. At 72 h of incubation, the three curves were intertwined. The GP48 was slightly lower than those reported by Lantcheva *et al.* (1999) and similar to those reported by Karabulut *et al.* (2007).

The GP48 of *T. alexandrinum* was higher than that reported by Sallam *et al.*, 2007 and Nasser *et al.*, 2009).

Table 2: Chemical composition of legume forages.

	DM	Ash	CP	CF	NDF	ADF	ADL
	%	%DM					
Trifolium alexandrinum							
Bloom	20.81^{b}	10.30	14.22^{c}	21.52^{c}	51.02^{ab}	27.70^{c}	6.00
Medicago sativa							
Bud	16.02^{c}	12.90	25.18^{a}	25.86^{b}	48.50^{b}	31.80^{b}	7.28
Early bloom	19.48^{b}	10.90	18.41^{b}	33.45^{a}	53.80^{a}	37.60^{a}	7.14
Seed	24.87^{a}	10.80	16.80^{bc}	35.41^{a}	49.50^{b}	31.60^{b}	6.31
SEM	1.15	1.12	1.16	3.26	3.33	2.12	0.55
P value	0.004	0.446	0.001	<0.001	0.050	0.002	0.83

DM - Dry matter; CP - Crude protein; CF - Crude fiber; NDF - Neutral detergent fiber; ADF - Acid detergent fiber; ADL - Acid detergent lignin; SEM - Standard error of the mean; $^{a, b, c}$ - Means in the row with different superscripts differ significantly (P<0.05)

Table 3: Feed intake, nitrogen balance, retained nitrogen and nutrient digestibility of legume forages.

	DM intake	OM intake	N intake	N Balance	Intake/$W^{0.75}$	Retained N	DMD	OMD	CPD	CFD	ADFD
	g/d				g DM/kg $W^{0.75}$	% ingested N	%				
Trifolium alexandrinum											
Bloom	1342.80^{a}	1211.70^{a}	204.70^{b}	23.70^{b}	96.70^{a}	0.72^{b}	65.41^{b}	68.76^{bc}	74.74^{b}	55.86^{b}	54.26^{b}
Medicago sativa											
Bud	1075.10^{b}	936.20^{b}	277.50^{a}	38.19^{a}	79.70^{b}	0.86^{a}	78.95^{a}	79.37^{a}	87.66^{a}	67.56^{a}	70.47^{a}
Early bloom	842.70^{d}	749.70^{d}	167.20^{c}	20.97^{bc}	63.40^{d}	0.78^{b}	75.64^{a}	75.78^{ab}	82.69^{a}	65.54^{ab}	68.33^{a}
Seed	973,90^{c}	866,70^{c}	116.10^{c}	19.03^{c}	71.50^{c}	0.72^{b}	66.71^{b}	67.32^{c}	74.98^{b}	59.27^{ab}	51.75^{b}
SEM	13.95	12.44	3.03	0.895	2.43	0.022	2.47	2.33	2.02	3.27	3.49
P value	<0.001	<0.001	<0.001	<0.001	<0.001	0.006	0.011	0.019	0.005	0.011	0.011

DM - Dry matter; OM - Organic matter; CP - Crude protein; CF - Crude fiber; NDF - Neutral detergent fiber; ADF - Acid detergent fiber; ADL - Acid detergent lignin; DMD - Dry matter digestibility; OMD - Organic matter digestibility; CPD - Crude protein digestibility; CFD - Crude fiber digestibility; ADFD - Acid detergent fiber digestibility; SEM - Standard error of the mean; $^{a, b, c, d}$ - Means in the row with different superscripts differ significantly (P<0.05).

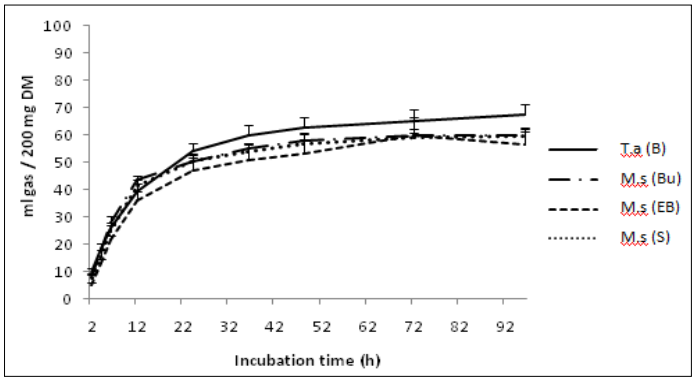

Fig 1: Kinetics of gas production of legume forages.
T.a (B) - *Trifolium alexandrinum* (Bloom); M.s (Bu) - *Medicago sativa* (Bud); M.s (EB) -*Medicago sativa* (Early Bloom); M.s (S) - *Medicago sativa* (Seed).

Table 4: Estimated kinetics parameters model, *in vitro*organic matter digestibility and energy content of legume forages.

	a ml	b ml	c ml/h	a+b ml	OMDv %	ME kcal/ kgMS
Trifolium alexandrinum						
Bloom	2.18^{a}	63.50	0.073^{b}	65.68	61.62^{b}	2063.90^{b}
Medicago sativa						
Bud	-3.58^{b}	61.83	0.11^{a}	58.24	68.26^{a}	2209.25^{a}
Early bloom	-4.47^{b}	59.02	0.09^{ab}	54.55	60.65^{b}	2011.39^{b}
Seed	-5.50^{b}	61.71	0.11^{a}	56.21	61.43^{b}	2041.26^{b}
SEM	1.298	2.443	0.007	2.718	1.207	37.13
P value	0.012	0.647	0.020	0.080	0.007	0.023

SEM - Standard error of the mean; a - The gas production from the immediately soluble fraction; b - The gas production from the insoluble fraction; c - The gas production rate constant for the insoluble fraction b; a+b - Potential gas production; OMDv - *In vitro* digestibility of organic matter; ME - Metabolizable energy. $^{a, b}$ - Means in the row with different superscripts differ significantly (P<0.05).

Estimated kinetic parameters model, *in vitro* organic matter digestibility and energy content

« a » of *T. alexandrinum* showed an average value of 2.18 mL. It was higher than the values reported by Borba *et al.* (2001) and Nasser *et al.* (2009). All *M. sativa* "a" values were negative (P<0.05) (Table 4). Karabulut *et al.* (2007) reported a positive value of « a ». The absence of production means the incapacity of microorganism to develop in the medium (Rakotoarison, 2005) so there is a lag time preceding the start of the fermentations (Sallam *et al.*, 2007; Elif and Veysel, 2017).

« b » ranged from 59.02 to 63.50 (P>0.05). « a+b » ranged from 54.55 to 65.68 and followed almost the same trend of «b» (Table 4). The « b » value of *T. alexandrinum* was higher than the values reported by Borba *et al.* (2001) and by Nasser *et al.* (2009). The « b » and the « a+b» of *M. sativa* was lower than the value reported by Karabulut *et al.* (2007).

« c » of *T. alexandrinum* was equivalent to 0.073 mL / h (P<0.05) and was lower than values reported by Borba *et al.* (2001) and Nasser *et al.* (2009). The rate of gas production was similar between the three maturity stages of *M. sativa*. It was higher than that founded by Borba *et al.* (2001) but lower than that noted by Karabulut *et al.* (2007). The high and fast gas production recorded for these legume forages means the presence of immediately and easily fermentable substances in this forage.

The OMDv showed the highest value (P <0.01) at the earliest maturity stage (Bud stage of *M. sativa*) (Table 4). For the calculated ME, the same trend was observed than that of OMDv (P<0.05).The OMDv of *M. sativa* was lower than that reported by Karabulut *et al.* (2007). The OMDv of *T. alexandrinum* was higher than that reported by Nasser *et al.* (2009) and Sallam *et al.* (2007).

The ME of *M. sativa* was lower than that founded by Kamalak *et al.* (2005b) and Karabulut *et al.* (2007).

Correlation among *in vivo*, *in vitro* parameters and chemical composition

Only, CPD was positively correlated with CP rate (P<0.05) (Table 5). It was also predicted by CP content in the French system but with better precision (Andriew *et al.*, 1979).

A few *in vitro* parameters have been found to have high and significant correlations with ADF content. "b" was positively correlated with ADF (P<0.001). GP24 and GP48 were negatively correlated (P<0.05) with the ADF rate. OMDv and ME were positively correlated with CP and negatively correlated with fiber content (Table 5). This result is consistent with the findings of Parissi *et al.* (2005), Kamalak *et al.* (2005a) and Karabulut *et al.* (2007).

Prediction of *in vivo* and *in vitro* parameters among chemical composition

The best prediction of CPD was obtained with CP (R^2=0.69, P<0.01). The ADF was the best variable predictor since its R^2 varies from 0.96 (P<0.05) for GP24 to 0.999 (P<0.001) for "b" (Table 6). This result is in agreement with the findings of Karabulut *et al.* (2007).

Table 5: Correlation coefficient among feed intake, nutrient digestibility, estimated kinetic parameters, cumulative gas production, *in vitro* organic matter digestibility, metabolizable energy and chemical constituents of legume forages.

	CP	CF	NDF	ADF
DM intake	0.23	-0.43	0.15	-0.43
Intake/W0.75	0.27	-0.44	0.15	-0.42
DMD	0.34	0.33	0.29	0.63
OMD	0.11	0.14	0.12	0.44
CPD	0.83*	0.23	0.42	0.59
CFD	0.13	0.39	0.20	0.59
ADFD	0.32	0.11	0.18	0.53
a	-0.47	-0.89	-0.03	-0.70
b	-0.23	-0.70	-0.69	0.999***
c	0.65	0.63	-0.46	0.30
a+b	-0.42	-0.89	-0.28	-0.87
GP24	-0.41	-0.75	-0.51	-0.98*
GP48	-0.30	-0.84	-0.53	-0.97*
GP96	-0.47	-0.82	-0.34	-0.92
OMDv	0.89	-0.39	-0.70	-0.19
ME	0.82	-0.49	-0.75	-0.32

CP - Crude protein; CF - Crude fiber; NDF - Neutral detergent fiber; ADF - Acid detergent fiber; DMD - Dry matter digestibility; OMD - Organic matter digestibility; CPD - Crude protein digestibility; CFD - Crude fiber digestibility; ADFD - Acid detergent fiber digestibility; a - The gas production from the immediately soluble fraction; b - The gas production from the insoluble fraction; c - The gas production rate constant for the insoluble fraction b; a+b - Potential gas production; GP24 - Cumulative gas production after 24h of incubation; GP48 - cumulative gas production after 48h of incubation; GP96 - cumulative gas production after 96h of incubation; OMDv - *In vitro* digestibility of organic matter; ME - Metabolizable energy; * p<0.05; *** p<0.001.

Table 6: *In vivo* and *in vitro* parameter prediction equations based on chemical composition.

Y variable	Equation	R^2
CPD	0.675 + 0.709 × CP	0.69**
b	76.131 - 44.967 × ADF	0.99***
GP24	48.991 - 47.088 × ADF	0.96*
GP48	52.221 - 61.020 × ADF	0.94*

CP - Crude protein; ADF - Acid detergent fiber; CPD - Crude protein digestibility; b - The gas production from the insoluble fraction; GP24 - Cumulative gas production after 24h of incubation; GP48 - Cumulative gas production after 48h of incubation; *p<0.05; ***p<0.001

CONCLUSION

It may be concluded that the maturity stage affected DM, CP, CF, NDF, ADF content, feed intake, *in vivo* digestibility, estimated parameters such as "a", "c" fractions, OMDv and the ME. The ADF content had a determinent effect on the prediction of some gas production parameters. Therefore, further studies are needed to evaluate the nutritive value of other Tunisian green forages, hays and by-products to be more informative about ruminant nutrition.

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