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In vitro digestibility and gas production characteristics of *Hedysarum flexuosum* ecotypes from Northwestern Morocco

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- ✓ kinetics of fermentation

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Abstract

This work is aimed to study the *in vitro* digestibility of ten ecotypes of *Hedysarum flexuosum* which are collected from different ecological sites in the northwest of Morocco. *In vitro* incubation of the samples during 72 hours revealed that the gas production of the ecotypes harvested at the vegetative and the budding stage (185.28 and 195.08 mL /g DM respectively) are significantly higher ($P < 0.001$) than that obtained at the blooming (162.90 mL/g DM). The ecotypes have a very variable fermentation profiles. Indeed, the potential gas production varies from 187.74 mL (with E7) to 153.98 mL (with E5). The rate of degradation which varies from 0.128 (E6) to 0.096 h^{-1} (E9) is significantly affected ($P<0.001$) by the fermentation ability of each ecotype. The resulting organic matter digestibility decreases very significantly ($P<0.001$) with the plant growth (77.61% vs 68.63% and 55.91% respectively for the vegetative, the budding and the blooming stage), and the production of microbial biomass drops by 55.57% when passing from the vegetative to the blooming stage (238.09 mg against 105.79 mg respectively). The ecotype E10 showed the highest level of digestibility. But in general, the average OMD varies slightly and insignificantly ($P > 0.05$) from an ecotype to another (from 71.32% for E10 to 65.44% for E3), while the PBM varies significantly within the ecotypes (from 189.97 to 146.91 mg /g DM respectively for E6 and E7; $P<0.001$), indicating a wide variability in dry matter quality of these ecotypes. The results show that this legume constitutes an important nutritional feeding resource, particularly at the vegetative stage. However, the use of this legume at the budding stage presents the advantage of an intermediate quality associated with a relatively higher level of quantitative production (10.50% vs 8.17% of dry matter).

1. Introduction

In Morocco, goat production is subjected to technical, economic and environmental challenges which can be limited by improving the nutritional quality of the basic food ration, and also by the valorization of locally available feeding resources.

In this context, amongst the several fodder legumes growing spontaneously in the North region and proved to be of great agro-economic and nutritional interest, we find the species of *Hedysarum*, distinguished not only by its availability in small areas [1], but also according to several authors; by its high nutritive value and its important role in soil improvement [2,3]. However, and for a better use of these species, it is necessary to well document and gain more knowledge on the nutritive value of the local ecotypes.

Amongst the accurate techniques for predicting the kinetics of fermentation and the digestibility of the forage organic matter with giving results closed to those measured directly on the animal, we find the gas production technique according to Makkar et al. (1995) [4]. Other techniques such as the method of Jones et al. (2000) [5] and the enzymatic digestibility by pepsin cellulase (Aufrère et al., 1989) [6] also gives results well correlated to those obtained by the in vivo digestibility.

2. Materials and methods

2.1. Plant material

Among a survey of 13 ecotypes of Sulla (*Hedysarum flexuosum*) collected from natural pasture land areas in northwestern Morocco (table 1), a selection of 10 ecotypes was cultivated in the experimental station of INRA (Boukhalef-Tangier). The harvest was carried out at the three stages of maturation (vegetative stage, budding and flowering).

Table 1: Origin of collected ecotypes

N° Ecotype	Locality or site	GPS coordinates	Characterization
1	Chrakka	35° 40' 55'' 5° 53' 948''	Late ecotype (at the end of blooming) on steep slope.
2	Chrakka	35° 40' 58'' 5° 53' 954''	Early ecotype (pod apparition).
3	Boughdour	35° 39' 626'' 5° 32' 857''	Ecotype with raised port, long stems, on flat soil, in full blooming.
4	Larbaa dalia	35° 40' 538'' 5° 48' 648''	Ecotype in full blooming, flat soil, raised port.
5	Axe Tetouan Larache	35° 34' 468'' 5° 39' 354''	Sloping soil, eroded, early ecotype, thinner stems.
6	Axe Tetouan Larache	35° 34' 820" 5° 40' 757"	Port slightly erect, very low slope, thicker stems, medium earliness.
7	Barrage 9 april	35° 31' 181" 5° 44' 538"	Slightly crawling port, thin stem, more pronounced flower color.
8	Highway Asilah	35° 22' 822" 6° 04' 287"	Rampant ecotype in a steep slope.
9	Highway Tahaddart	35° 30' 409" 5° 59' 260"	Less pronounced slope, rampant ecotype, premature ended blooming.
10	Highway Tahaddart	35° 36' 558" 5° 57' 690"	Raised port, premature ended blooming, eroded soil, stems of medium sizes.

2.2. In vitro digestibility and gas production

This method is aimed to simulate the digestive process in the animal by evaluating the kinetic of gas production which reflects the degree of food fermentation by the inoculum microflora.

Samples were incubated *in vitro* according to the method of Makkar et al. (1995) [4] using ruminal fluid in 100 mL glass graduated syringes. Rumen fluid was obtained from three goats. Samples of 300 mg of dry matter are introduced into the syringes; previously heated at 39 °C before mixing with 30 mL of the rumen-buffer liquid (1:2 v/v). In this mixture, a steady and moderate flow of CO_2 arrived continuously for 15 min. The incubation was carried out in a water bath at 39 °C, and the total gas production volume at 2, 4, 6, 8, 12, 24, 36, 48 and 72h of incubation were estimated by the displacement of the syringe piston. The amounts of gas produced from the fermentation were corrected using blank incubations.

The potential gas production and also the rate of gas production were calculated using the exponential model of Ørskov and Mc Donald (1979) [8]: $GP= a+b*(1- \exp^{(-c*t)})$; where GP represents the cumulative gas production at time t (mL), "a" is the production of gas from the potentially degradable soluble fraction (mL/g DM), "b" the production of gas from the potentially degradable insoluble fraction (mL/g DM), "c" rate of gas production (/h), "a+b" the potential gas production (mL/g DM).

After 72 hours of incubation, the contents of the syringes are moved into nylon filter bags (porosity: 160 µm). The bags containing the indigestible residue were washed with distilled water and then dried at 60 °C for 48 h to estimate the dry matter digestibility. The digestibility of the organic matter was evaluated by incinerating the indigestible dry residue in a muffle furnace (at 550 °C for 12 h).

The production of microbial biomass and the factor of partition were estimated by using the formulas of Blümmel (1997) [9].

These analyzes were carried out at the Animal Nutrition Laboratory belonging to the Animal Production Research Unit which belongs to INRA Tangiers-Morocco.

2.3. Statistical analysis

The obtained results were subjected to variance analysis using the GLM procedure of SAS (version 9.0 for Windows) [13]. The values of the fermentation parameters were calculated using the NLIN procedure of SAS

according to the nonlinear model GP = a+b (1-exp$^{(-ct)}$) of Ørskov and McDonald (1979) [8]. The coefficients of correlation were calculated by the CORR procedure using the same software [13].

3. Results

3.1. Cumulative ruminal gas production

The evolution of gas production generated by the microbial attack on the plant components (figures 1 and 2, tables 2 and 3) provides information about the availability and the importance of *Hedysarum flexuosum* nutrients.

The comparative study between harvesting stages (figure 1 and table 2) shows that the three stages have the same shape of curves. Two phases can be distinguished easily on the cumulative gas production. A fast phase from 2 to 24 h, during which the in vitro gas production is maximum and the slow phase between 24 and 72 hours, where the accumulated GP weakens and tends to stabilize after 48 hours probably due to the depletion of easily fermentable compounds.

From 2 to 8 hours of incubation, the difference in GP between the stages is almost insignificant (P> 0.05). The difference begins to appear at 12 hours of incubation (P = 0.001) with an average GP of 127.79 mL / g DM. After 24 hours of incubation, the variation of GP between the stages becomes very highly significant (P <0.001).

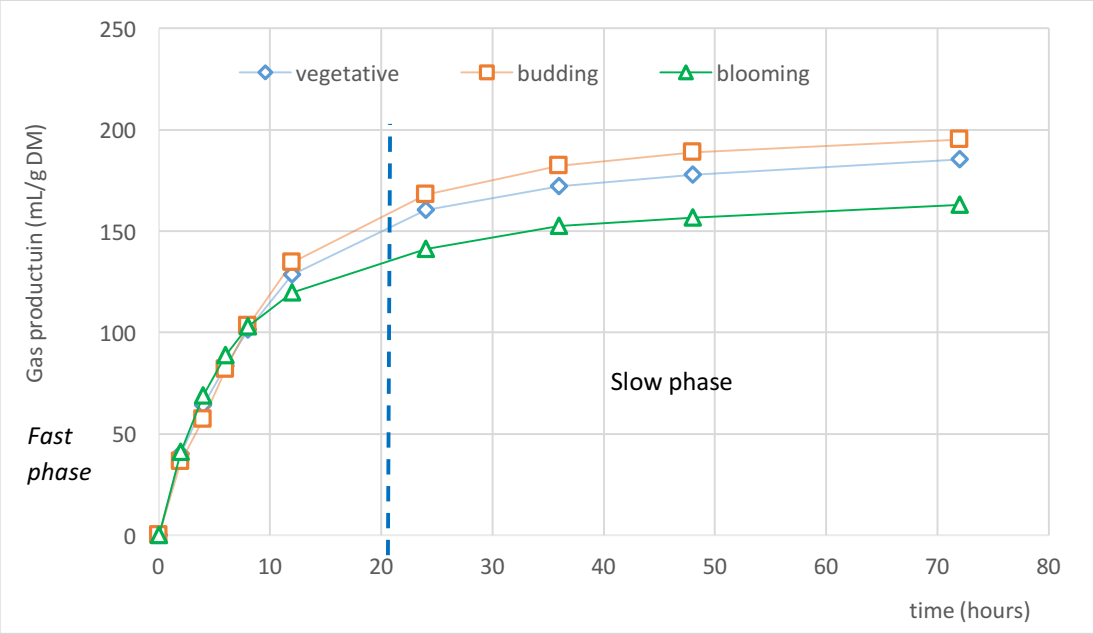

Figure 1: in vitro cumulative gas production of *Hedysarum flexuosum* by stage of growth

After 72 h of incubation, it was noted that the blooming stage had the lowest GP. On the other hand, the budding stage has a significantly higher GP (P<0.001, table 2). The falling down of gas production subsequent to plant growth necessarily implies a decrease in energy and protein content and consequently in digestibility.

3.2. In vitro gas production with the ecotype

From 2 to 24h of incubation, the production of gas is important (figure 2). From 2h to 8h, we observe that there is no significant difference between the ecotypes in terms of gas production (linear trend). The effect of the ecotype begins to be significant after 12 hours of incubation. The difference between the ecotypes becomes more remarkable only after 24h (P<0.001). Indeed, at 72h of incubation we distinguish three ecotypes groups, the first one contains E3, E7, E9 and E10 with GP respectively of 189.42; 194.45; 187.56; and 186.81 mL/g DM (table 3 and figure 2), the ecotype E5 is the one with the lowest value GP (159.96 mL/g DM).

3.3. The in vitro digestibility of dry matter and organic matter

The DMD decreases significantly with the plant growth. The maximum values are recorded in the first stage with an average of 78.82% against 56.46% for the last stage (P<0.001). On the other hand, table 3 shows that the selection of ecotypes constitutes a homogeneous group in terms of DMD. Indeed, this parameter varies from 72.01% for E10 to 65.92% for E2 with an average of 68.65%. This shows that the ecological seed origin has no influence on the dry matter digestibility of the ecotypes.

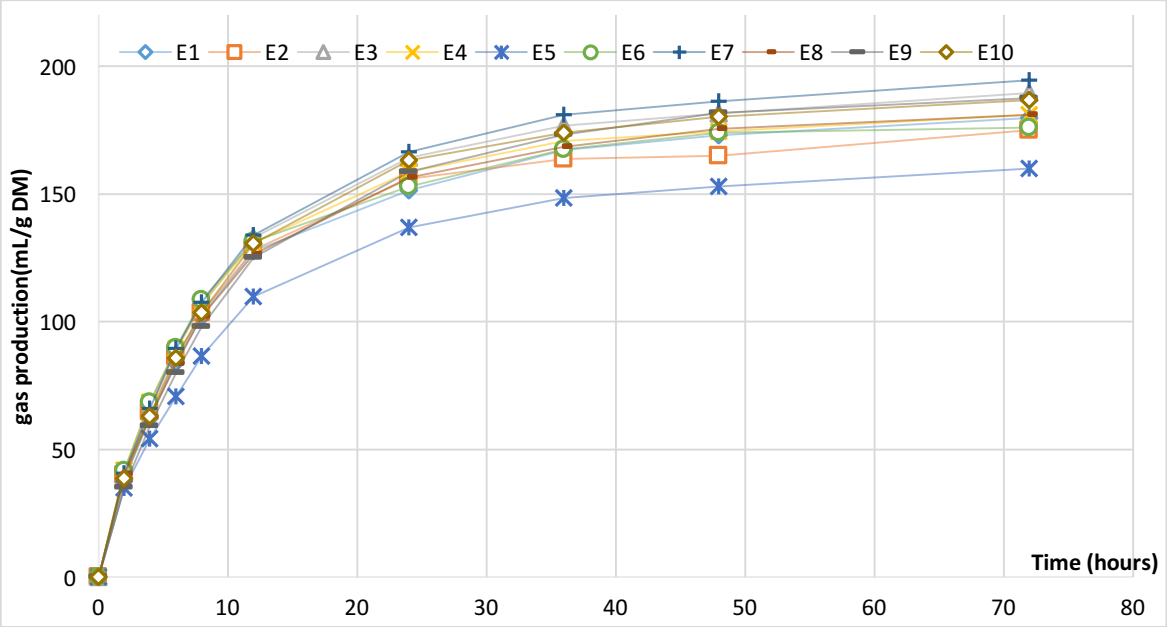

Figure 2: in vitro cumulative gas production of the *Hedysarum flexuosum* ecotypes

Table 2: in vitro gas production and digestibility of *Hedysarum flexuosum* by stage of growth

Stage of growth	GP, 24h	GP, 48h	GP, 72h	DMD (%DM)	OMD (%OM)
vegetative	160.41^{b}	177.77^{b}	185.28^{a}	78.20^{a}	77.61^{a}
Budding	167.95^{a}	188.97^{a}	195.08^{a}	70.13^{b}	68.63^{b}
Blooming	141.07^{c}	156.75^{c}	162.90^{b}	56.46^{c}	55.82^{c}
Average	156.48	174.50	181.09	68.26	67.35
SEM	8.00	9.44	9.52	6.34	6.32
Signification	***	***	***	***	***

GP, Xh: amount of gas produced in mL / g of dry matter after X hours of incubation. **DMD:** In vitro dry matter digestibility, **OMD:** In vitro organic matter digestibility, Values in rows with disparate letters differ significantly (P<0.05), **SEM:** Standard error of the mean, NS: Non-significant, *** p<0.001, ** p<0.01, * p<0.05.

As for the DMD, the ecotype has no significant effect on the digestibility of the organic matter; with a variation from 71.32% for E10 to 65.47% for E2 with an average of 67.32% (table 3). However, the stage of growth influences significantly the OMD (P<0.001). Indeed, the highest OMD was obtained at the vegetative stage with 77.61% against 68.63% and 55.81% respectively for the budding and blooming stage (table 2).

Table 3: In vitro gas production and digestibility of the ecotypes (E1, E2 …E10) of *Hedysarum flexuosum*

Ecotype	GP, 24h	GP, 48h	GP, 72h	DMD (%DM)	OMD (%OM)
E1	151.39^{ab}	173.03^{ab}	179.84^{ab}	67.85^{a}	67.15^{a}
E2	155.94^{a}	165.04^{ab}	175.05^{ab}	65.92^{a}	65.47^{a}
E3	164.08^{a}	181.60^{a}	189.42^{a}	66.12^{a}	65.44^{a}
E4	158.56^{a}	174.54^{ab}	180.88^{ab}	66.99^{a}	66.08a
E5	136.84^{b}	152.86^{b}	159.96^{b}	66.56^{a}	65.32^{a}
E6	152.97^{ab}	174.07^{ab}	176.04^{ab}	70.98^{a}	70.25^{a}
E7	166.52^{a}	186.23^{a}	194.45^{a}	66.73^{a}	65.67^{a}
E8	156.54^{a}	175.44^{ab}	180.88^{ab}	70.09^{a}	68.79^{a}
E9	158.60^{a}	181.81^{a}	187.56^{a}	69.35^{a}	68.00^{a}
E10	163.31^{a}	180.33^{a}	186.81^{a}	72.01^{a}	71.32^{a}
Average	156.48	174.50	181.09	68.26	67.35
SEM	2.66	3.05	3.04	0.69	0.68
Signification	***	***	**	NS	NS

GP, Xh: amount of gas produced in mL / g of dry matter after X hours of incubation, **DMD:** In vitro dry matter digestibility, **OMD:** In vitro organic matter digestibility, $^{a,b,c,...}$ **:**Values in rows with disparate letters differ significantly (P<0.05), **SEM:** Standard error of the mean, **NS:** Non-significant. *** p<0.001, ** p<0.01, * p<0.05.

The effect of interaction between growth stage and ecotype on digestibility (figure 3) is very significant (P <0.001). The OMD is remarkably higher with E6, E8, and E10 harvested at the vegetative stage (85.34%, 80.29% and 75.38% respectively, figure 3).

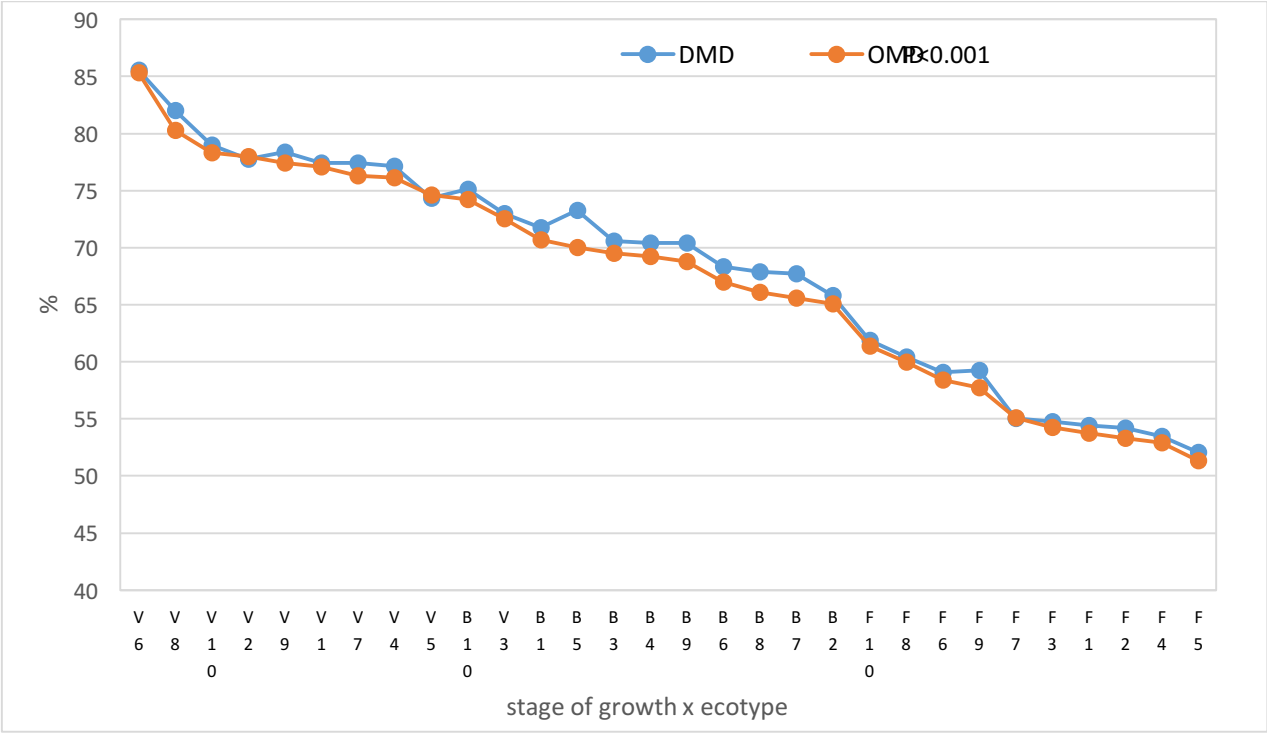

Figure 3: Effect of interaction between the stage of growth (V: vegetative, B: budding and F: blooming) and ecotype (1, 2…10) on in vitro digestibility of *Hedysarum flexuosum*

3.4. Estimated fermentation parameters

The amount of microbial biomass produced in mg provides information on the fodder ability to provide essential nutrients (protein and energy) for the proliferation of rumen microorganisms. According to table 5, the highest PBM were obtained with the ecotypes E6, E9, E10 and E2 (189.97, 189.59, 188.61 and 183.26 mg respectively) and the lowest value was recorded for E7 (146.91 mg). Indeed, the ecotype factor has a highly significant effect (P <0.001) on the variation of this parameter.

For the factor of partition, which refers to the actually degraded organic matter (mg), relative to the amount of gas produced during incubation, there is a highly significant influence of the ecotype on this parameter (P <0.01, table 5). As for PBM, the growth of the plant has a negative effect on the FP (P <0.001, table 4).

Table 4: Estimated fermentation parameters with the stage of plant growth

Stage	PBM (mg)	FP (mg/mL)	a (mL/g DM)	b (mL/g DM)	c (h^{-1})	a+b (mL/g DM)
Vegetative	238.09^{a}	3.52^{a}	3.69^{a}	176.13^{b}	0.1383^{a}	179.82^{b}
Budding	167.80^{b}	3.14^{b}	0.81^{c}	188.85^{a}	0.1024^{b}	189.66^{a}
Blooming	105.79^{c}	2.90^{b}	3.31^{b}	148.55^{c}	0.0948^{b}	151.86^{c}
Average	170.65	3.19	2.60	171.18	0.1118	173.78
SEM	38.22	0.18	0.90	11.89	0.0134	11.32
Signification	***	***	***	***	**	***

PBM: production of microbial biomass, **FP**: the factor of partition, **a:** the production of gas from the potentially degradable soluble fraction, **b**: the production of gas from the potentially degradable insoluble fraction, **c**: rate of gas production, **a+b:** the potential gas production, **SEM**: Standard error of the mean, **NS:** Non-significant. *** p<0.001, ** p<0.01, * p<0.05.

The gas production rate decreased significantly (P<0.01) from the vegetative stage (0.1383 h^{-1}) to the blooming (0.0948 h^{-1}) with an average of 0.1188 h^{-1} (table 4). This parameter also varies significantly between ecotypes (P <0.05, table 5), with a variation from 0.1276 to 0.0961 h^{-1} recorded for E6 and E9 respectively.

As for the production of gas from the potentially degradable fraction, there is also a significant difference between the stages of growth. Indeed, we noted that the lowest gas production of this fraction was recorded at the budding stage (0.81 mL / g DM). On the other hand, this stage registered the highest value of gas production concerning the insoluble fraction (188.85 mL/ g DM). Therefore, a relatively higher gas production potential of 189.66 mL / g DM was observed at the budding stage in comparison to lower values noted at the vegetative and the blooming stages (179.82 and 151.86 mL respectively, table 4).

Table 5: Fermentation parameters of *Hedysarum flexuosum* ecotypes

Ecotype	PBM (mg)	FP (mg/mL)	a (mL/g DM)	b (mL/g DM)	c (h^{-1})	a+b (mL/g DM)
E1	180.70^{ab}	3.20^{ab}	3.19^{ab}	169.70^{c}	0.1125^{ab}	172.89^{e}
E2	183.26^{a}	3.27^{ab}	2.69^{b}	165.17^{e}	0.1247^{a}	167.86^{g}
E3	158.63^{abc}	3.01^{b}	3.61^{ab}	179.27^{b}	0.1121^{ab}	182.88^{b}
E4	148.03^{bc}	3.11^{b}	3.87^{ab}	170.79^{c}	0.1219^{a}	174.66^{d}
E5	149.55^{bc}	3.75^{a}	3.71^{ab}	150.27^{f}	0.1032^{ab}	153.98^{h}
E6	189.97^{a}	3.44^{ab}	3.50^{ab}	167.29^{d}	0.1276^{a}	170.78^{f}
E7	146.91^{c}	2.91^{b}	3.74^{ab}	184.00^{a}	0.1080^{ab}	187.74^{a}
E8	170.36^{abc}	3.13^{ab}	4.00^{a}	171.40^{c}	0.1096^{ab}	175.40^{d}
E9	189.59^{a}	3.06^{b}	2.75^{b}	180.21^{b}	0.0961^{b}	182.96^{b}
E10	188.61^{a}	2.98^{b}	2.68^{b}	178.64^{b}	0.1091^{ab}	181.33^{c}
Average	170.56	3.19	3.37	171.67	0.1125	175.05
SEM	18.19	0.08	0.16	3.08	0.0031	3.06
Signification	***	**	**	***	*	***

PBM: production of microbial biomass, **FP**: the factor of partition, **a:** the production of gas from the potentially degradable soluble fraction, **b**: the production of gas from the potentially degradable insoluble fraction, **c**: rate of gas production, **a+b:** the potential gas production, **SEM:** Standard error of the mean, **NS**: Non-significant. *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

The ecotypes collection presents a very large heterogeneity ($P<0.001$) in terms of production of microbial biomass PBM, the factor of partition FP, rate of gas production c (h^{-1}), the production of gas from the potentially degradable soluble and insoluble fraction a and b. This variation assumes that the origin of the ecotype is a major factor of variation in nutrient content of *H. flexuosum,* knowing that digestibility (OMD) is influenced only by the stage of development (tables 2 and 3).

4. Discussion

The production of gas and the *in vitro* digestibility are directly related to the chemical composition of the food, especially to the content of indigestible fibers, proteins, and condensed tannins. A higher concentration of digestible and fermentable components will lead to high levels of gas production.

4.1. Digestibility and cumulative gas production

This test has proved the effect of the ecotype and the stage of development on the *in vitro* digestibility and fermentation parameters of *H. flexuosum*. Cumulative gas production and OMD decreased remarkably ($P<0.001$) when passing from the vegetative to the blooming stage. Obviously, the stage of maturation affects the digestibility negatively, mainly because of the relatively high concentrations of cell walls and lignin.

Overall, gas production is characterized by a fast phase from 2 to 24h, in this interval no significant difference ($P>0.05$) is observed during the first eight hours of incubation, either between the three stages or between the ten ecotypes of *H. flexuosum*. After 24 hours, the variation is very important ($P<0.001$) and it increases progressively during the stationary phase (between 24 and 72 hours) and the fermentation slows down enormously and tends towards stability, with an amount of gas produced of only 15.73% compared with the first phase. This difference indicates that the major part of the degradable fraction of the plant is exhausted during the first 24h of incubation, consequently proving the good level of the plant degradability during this phase.

According to Pitt et al. (1999) [10], the GP is directly related to digestibility and energy content. Indeed, because of their high GP, the ecotypes E3, E7, E9 and E10 can be considered to be more degradable. On the other hand, OMD varies slightly between ecotypes (71.32% for E10 to 65.67% for E2).

The correlation between GP 72h and OMD is low ($r=0.45$; $P=0.0125$; $N=30$). Knowing that the protein content varies considerably between the ecotypes and the harvesting stages of this legume and consequently the

quantity of ammoniac (NH_3). Menke and Steingass (1988) [7] reported that the amount of the *in vitro* gas production is reduced by the formation of ammonium bicarbonate ($NH_4 HCO_3$) when NH_3 is released from protein degradation, which justifies the weak correlation between gas production and digestibility.

Compared to other legumes, Gasmi-Boubaker et al. (2012) [3] reported higher fermentation performances. In fact, *Hedysarum coronarium* recorded the highest GP at 48h with 325 mL (65 mL /200 mg DM), followed by *Vicia sativa, Pisum sativum* and *Medicago truncatula* (311, 245 and 228 mL /g DM respectively). These results exceed our maximum value (195.08 mL /g DM recorded for budding after 72 h of incubation). However, the same authors reported a digestibility that varied between 78.10% (*Vicia sativa*) and 67.60% (*Medicago truncatula*). The species *Hedysarum coronarium* has an intermediate digestibility of 74.60%. Generally, these results and those reported by Aufrère et al. (2008) [11] for Common Sainfoin (*Onobrychis viciifolia*) (71.20% and 68.20% for the vegetative stage and the beginning of flowering respectively), are similar to those recorded for our ecotypes at the vegetative and budding stages (77.61% and 68.63% respectively). The difference in GP may also be related to the variation in the content of condensed tannins that reduce the *in vitro* gas production (Tan et al., 2011) [17], depending on the species and also on the quality and the proportion of rumen liquid-buffer mixture used.

4.2. Estimated fermentation parameters

According to the obtained results, microbial biomass production is correlated with OMD ($r = 0.91$; $P<0.001$; $N = 30$) but not with the GP ($r = 0.31$; $P>0.05$; $N = 30$), While Essafi et al. (2005) [12] reported a significant and inversely proportional relationship between PBM and GP ($r = -0.77$) for *Atriplex halimus*. Concerning our selection of ecotypes, PBM varies significantly with the ecological origin of the seed (from 189.97 mg with E6 to 146.91 mg with E7). But in this case, the evolution of this parameter such as for the OMD is defined in relation with the stages of development. Indeed, there is a considerable decrease of -55.57% for the PBM from the vegetative stage to the flowering (238.09 against 105.79 mg respectively). Knowing that in the data analysis, the fermentation was mainly considered to be of acetic nature and that PBM is influenced by the chemical composition and especially by the tannins content that limits the *in vitro* gas production [4]. In fact, the E6 ecotype is the one with the highest level of total phenols (2.26%) yet it is characterized by a relatively low GP, on the other hand, the lowest Tannins content was recorded with E3 (0.32%), but this ecotype has a relatively high GP. This confirms that phenolic compounds have a remarkable influence on GP and on digestibility.

Furthermore, among the possible reasons for the decrease in microbial production could be the complexation of tannins with nutrients consequently decreasing their availability by the attack of microorganisms [14]. In fact, this is reflected on the efficiency of microbial biomass (PF), which varies significantly between ecotypes ($P <0.05$) and follows slightly the evolution of OMD ($r = 0.53$; $P<0.05$; $N = 30$). Also, FP is gently correlated with PBM ($r = 0.54$; $P<0.05$; $N = 30$) and there is also no significant and positive relationship between FP and the GP evolution.

In addition, the gas production rate that partly explain the intake capacity of the forage and globally control the transmission rate in the rumen [15], varies significantly between ecotypes and evolves in the same way as the OMD within the vegetative cycle of the plant. Azuhnwi et al. (2012) [16] also reported a decrease of the kinetic of degradation with the stage of harvest for the Common Sainfoin, with recorded values (0.100 and 0.092 h^{-1} for the 1st and 2nd harvest respectively) that are approximately comparable to the results for the budding and flowering stages of Sulla (0,102 and 0,095 h^{-1} respectively).

Concerning the gas production potential, the same as for the GP, it is linked to the degradability and especially to the quality of the food. The ecotypes E7, E9, E3 and E10 recorded the highest fermentation performances (187.74, 182.96, 182.88, 181.33 mL /g DM respectively). This shows that the studied ecotypes do not provide a comparable concentration of nutrients to the rumen microflora.

Conclusion

The ten selected ecotypes of *Hedysarum flexuosum* are all characterized by a high digestibility which is affected only by the stage of growth. However, the differences in fermentation performance reveal the existence of a significant variation in the nutritional quality of the selected ecotypes, which is related to the chemical composition of the plant, mainly the condensed tannins concentration.

Fermentation performance and digestibility are better when ecotypes are harvested at the early stage (vegetative). However, the budding stage is characterized by an intermediate digestibility, but has the advantage

of providing more forage biomass than the vegetative stage (10.50 vs 8.17% of dry matter respectively). For this stage, the ecotype E10, E1 and E5 recorded the highest OMD respectively.

Sulla genotypes from northwestern Morocco are a very promising feeding resources for animal husbandry, particularly the E10 ecotype (Tahadart origin), is characterized by a relatively higher OMD for the three harvesting stages and an interesting fermentation profile.

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