Study of associative effects of date palm leaves mixed with Aristida pungens and Astragalus gombiformis on the aptitudes of ruminal microbiota in small ruminants

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The objectives of this trial were to evaluate interactions between microbial degradation of three substrates: date palm leaves as tannin-rich substrate, Astragalus gombiformis (as nitrogen source) and Aristida pungens (as carbon source). The model included the principal effects of single substrate and mixtures of multiple interactions. The forages were fermented alone or mixed with date palm leaves in various combinations (0, 10, 20, 30, 40 and 50%), in buffered rumen fluid using in vitro gas techniques. Gas production (mmol/g of dry matter) at 3, 6, 9, 24, 48 and 72 h of incubation was measured from all feed combinations. Treatments were carried out in triplicate and data were submitted to analysis of variance using a mixed procedure with repetition considered as the random effect. The interactions between feeds were evaluated on the basis of differences between single and mixed substrates on the different variables measured. The results showed that the included palm leaves reduced gas production when associated with A. pungens (p<0.0001), whereas this effect was less significant with A. gombiformis. This effect was more pronounced at earlier times of incubations. The in vitro organic matter digestibility decreased linearly with the increasing inclusion of date palm leaves in the mixtures. It is concluded that date palm leaves may be a suitable feed supplement for small ruminants browsing A. pungens and A. gombiformis in arid regions. The present results also showed that the inclusion level should be lower than 20% and for a short period in order to minimise tannins effect. The description of the fermentation profile of in vitro gas production showed that Sandoval model was poor and not appropriate for the characteristic varieties of arid and semi-arid areas in Algeria and the multiple regression models revealed a good linear regression for both mixtures.

Key words: Date palm leaves, forage, semi-arid zone, associative effects, rumen, in vitro fermentation.

INTRODUCTION

The problem of forage availability and quality of animal feed are aggravated in arid, semi-arid and tropical regions and because of the scarcity and irregular rainfall that limit the growth of herbaceous species and biomass

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yield in rangelands. Thus, breeding in these regions have
to survive the shortage of insufficient resources for most
parts of the year (Robles et al., 2008; Boufennara et al.,
2012). In semi-arid regions, and in the dry season, crude
protein content of the herbaceous rangeland vegetation
decreases significantly which induces a prolonged period
of under-nutrition of livestock (Yayneshet et al., 2009).
In developing countries where food resources for food
and feed are deficient, only low quality forages, crop
residues and agro-industrial by-products available are
used for feeding ruminants. Under these conditions, the
use of supplementation is inevitable. This pathway
provides to the rumen microorganisms the nutritive
elements necessary for their growth, thus ensuring the
favorable conditions for cellulolysis in the rumen
(Moujahed et al., 2000).
In Algeria, the rangelands represent two-thirds of the
total land widespread, mainly in the arid regions. Some
species may be crucial for grazing ruminants where these
plants contain anti-nutritional secondary compounds
(phenolics and tannins) with potential side effects such as
inhibition of rumen microbial fermentation, as well as
decreased feed digestibility and animal performance (Min
et al., 2003; Waghorn and McNabb, 2003; Mueller-
Harvey, 2006).
In oasis areas, local farmers often offer date-palm
leaves as a supplement for ruminants in spite of their
high tannin content (Arhab et al., 2006). The anti-nutritive
effect of dry leaves of the date palm has been studied in
animal models of ruminants and mono-gastric and it
seems that both reduced digestibility and toxicity may
limit the potential of this plant as a feed supplement. The
nutritional value of date palm crop residues has been
quite extensively studied due to their high availability in
the countries where date production is important
(Alananbeh et al., 2015). Both the energy and protein
values of these by-products are low as compared to that
of cereal straw (Arbouche et al., 2008). However, no
study has been conducted on the associative effect of
palm leaves with other forage on in vitro fermentation.

The influence of different feed ingredients on rumen
microbial activity can be variable and contradictory. For
instance, increasing the level of cereal grain supple-
mentation reduced ruminal fermentation of fiber (Mould
et al., 1983a), whereas supplementation of alfalfa hay with
corn stalks resulted in positive associative effects on N
utilization that caused reduced intake of corn stalk (Wang
et al., 2008). Rations formulated for ruminants are
generally a mixture of individual feeds, and its net (NE) or
metabolizable (ME) energy value is generally calculated
by adding up the energy values of the individual feeds in
it, on the hypothesis that the NE or ME value of individual
feeds will be unchanged when they are fed in combination
with other feeds. This assumption may not be true, as
some published reports indicated that a level of
association exists among feeds in rations (Dixon and
Stockdale, 1999; Franci et al., 1997; Haddad, 2000; Hong
et al., 2001, 2002; Mould et al., 1983a, b; Wang et al.,
2008). However, while associative effects are often
discussed in ruminant nutrition, at least theoretically, they
are seldom taken into account in feed formulation. While
the meaning of an associative effect is clear (the sum of
the parts being less, or more, than the combination of the
parts), reasons for an associative effect are not clear. On
the other hand, a true associative effect does not relate to
correction of a known nutrient deficiency, such as
fermentable N, but to unknown (or obscure) nutrient
interactions.

In 1979, Menke et al. proposed an estimation of the
energy value of feedstuffs from their in vitro gas
production associated with chemical parameters. The gas
test (HFT or Hohenhimer Futterwert Test) method is
based on the assumption that the accumulated 24 h gas
production by a substrate, incubated in a syringe with
rumen liquor and a nutritive solution, is proportional to the
amount of digestible carbohydrates, and thus highly
correlated to the energy value of feedstuffs or to the in
vivo organic matter digestibility (OMD).

The objectives of this study were to (i) investigate the
associative effects on fermentation abilities of ruminal
microbiota of sheep and gas production of plant leaves of
the date palm and characteristics of two perennial plants
in arid zones: Astragalus gombiformis and Aristida
pungens (Stipagrostis pungens) mixed with different
combinations and (ii) establish an interaction between
the main components of different substrates.

MATERIALS AND METHODS

Forage material

Forages were collected from El Oued, located in South-East area of
Algeria. The climate of this region is arid with a mean annual rainfall
of 75 mm, and average temperature of 1°C in January and 43°C in
July.

The studied forages consisted of two native species of North
Africa: A. gombiformis (Foulet el Ibel), rich in protein (Arhab, 2006),
A. pungens (Stipagrostis pungens) (drinn) rich in fibres (Arhab,
2006) and a by-product of the date palm: the leaves, rich in tannins
(Arhab, 2006). These plants were harvested at a mature stage
while date palm leaves were harvested at senescence. About six to
ten specimens of each plant species were sampled to obtain a
representative sample of the plant biomass.

The aerial part of the plants, leaves, thin branches (young stems)
and some flowers (when existing) were clipped with scissors and
taken immediately to the laboratory where the samples from the
different specimens were pooled and dried in an circulating air oven
at 60°C for 48 h. The samples were then coarsely ground in a
laboratory using a chopper and were then ground again and passed
through a 1 mm screen; the latter are mixed with the leaves of date
palm at different percentages (0, 10, 20, 30, 40, 50 and 100%).

Animal material

The experiment was conducted on three Texel (origin of these
sheep Texel island (breed of sheep), aged 12 months and an average live weight of 82 kg, deemed healthy by veterinary control, castrated and fitted with ruminal fistula. The animals were housed in individual pens. These subjects received a daily ration of 1200 g of oat vetch hay in two equal meals (8:00 am and 4:00 pm) with free access to water. Two weeks adaptation period were planned to allow a good adaptation of ruminal microbiota.

**Analysis methods**

**Chemical analysis**

The determination of the dry matter (DM) and organic matter of samples was conducted following the methods of AOAC (1999, 942.05 method ID). The analysis of the compounds of the plant cell wall was performed as described by Van Soest et al. (1991). The crude protein content of plants on one hand and content of crude protein associated with the fraction of neutral detergent fiber (NDF neutral detergent fiber) on the other were determined by the Kjeldahl method.

All determinations were conducted in triplicate and expressed as a percentage of DM. Phenolics were determined by addition of two or three drops of ferric chloride (FeCl3) in 1 ml of the methanol extract that was diluted in 50% of distilled water. The change in color indicated the presence of phenolic compounds as follows: no change indicates absence of phenolic compounds, dark blue indicates presence of phenols or hydrolysable tannins, and dark green indicates presence of condensed tannins. The method followed was Folin-Ciocalteau as described in Julkunen-Titto (1985).

**Quantitative analysis of phenolic compounds**

Total phenols (TP) and total condensed tannins (TCT) was dosed separately.

**Total Phenols (TP):** The phenolic compounds analysis was carried out in three replicates. The dried plant material (200 mg) was extracted with acetone (10 ml, 70% v/v), then the solution was subjected, at ambient temperature for 20 min, to ultrasonic treatment. The content was centrifuged (4°C, 10 min and 3000 g) and stored in ice for analysis, then the centrifugate was treated as described above. The total phenols were estimated using Folin Ciocalteu reaction (Makkar et al., 1993). Tannic acid was used to perform calibration curve. TP was quantified as tannic acid equivalents and expressed as tannic acid eqg/kg DM.

**Tanins:** For the total condensed tannins (TCT), 0.5 ml of extract was treated with n-Butanol HCl (3 ml, 95%) in the presence of ferric ammonium sulfate (0.1 ml). The reactants were heated for 1 h in a boiling water bath. Absorbance was read at 550 nm. TCT were expressed as leucocyanidin using the equation:

\[
\text{TCT} = \frac{A\times 78.26x}{(\text{weight of sample on DM})}
\]

where: A is the absorbance at 550 nm assuming that E1% efficiency, 1 cm, 550 nm is 460 leucocyanidin (Porter et al., 1986). Total tannins (TT) were determined as the difference in total phenolic compounds (measured by Folin-Ciocalteau reagent) before and after treatment with insoluble polyvinylpyrrolidone (Makkar et al., 1993).

**In vitro study**

The *in vitro* fermentation inoculum was obtained from the filtered rumen fluid taken from three sheep. This juice was mixed to obtain a homogeneous inoculum. Ruminal fluid was collected 1 h before the morning meal, placed in a container preheated to 39°C and saturated with CO2. This container was sealed immediately and transported to the laboratory for further analysis within 2 h of collection (Menke et al., 1979).

Sheep rumen fluid was mixed well, then filtered through four layers of muslin and bubbled with CO2 at 39°C. All the handling was carried out under a constant stream of CO2. The activity of ruminal microbiota requires an anaerobic and the solution was bubbled with a continuous flow of CO2, resulting in the reduction of artificial saliva indicated by the color change from pink to transparent.

**In vitro fermentation of forages by rumen microbiota**

The technique followed was that of Theodorou et al. (1994). *In vitro* gas production technique is a simulation of food degradation by the rumen microflora. At the end of fermentation, the gas is measured using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL). It is a simple and inexpensive technique, based on the measurement of gas production.

**In vitro gas production**

The method of Theodorou et al. (1994) was used for gas production. Four hundred milligrams of each feed were weighed into bottles of 125 ml serum and incubated in a water bath at 39°C with 15 ml of ruminal fluid and 25 ml of artificial saliva (Van Soest, 1994). Monitoring fermentation kinetics was performed by measuring the gas pressure produced at different incubation hours: 3, 6, 9, 24, 48, and 72 h.

**Statistical analysis**

The evolution of gas production was followed according to the incubation time and the percentage of *A. pungens* or *A. gomphiformis* in nutrient mixtures. A factorial ANOVA (the factorial ANOVA is based on design S <A*B> treating the effects of two crossed factors. The groups are assumed to be independent in each of the conditions defined by the intersection of two factors. The sources of variation to take into account are the factors A (time) and B (percentage) and optionally AB (time*percentage) interaction) was performed to find out the effects of the incubation time, the percentage of added plants and their mutual interaction on gas production. A fermentation profile model from the literature, based on Levenberg-Marquardt algorithm (Levenberg, 1944; Marquardt, 1963) which is an improvement of the Gauss-Newton method for the resolution of nonlinear least-squares regression problems, was tested. Then, a multiple regression was performed to determine the equation giving the best estimate of gas production based on the independent variables and their interaction. Statistical analyses were conducted with Statistica 10, Statsoft Inc, Tulsa, USA.

**RESULTS AND DISCUSSION**

The chemical composition of forages

The chemical composition of the substrates is shown in Table 1. There was a change in all nutrient elements of forages. The highest fiber content was recorded in *A. pungens* (*Stipagrostis pungens*) (509.4 g/kg DM),
followed by A. gombiformis (445.2 g/kg DM) and the leaves of dry palms (422.1 g/kg DM). The highest crude protein content was noted in A. gombiformis (125 g/kg DM) followed by palm leaves (45.9 g/kg DM). Regarding phenolic compounds (total phenols, TT and TCT) were 61.8, 49.1 and 36.2 g/kg DM for dry palm leaves, by Astragalus and A. pungens, respectively. The change in the mineral and organic elements concentration for the studied plants was strongly associated with the type of soil, climate, stage of maturity and harvest season genotypic characteristics, and the factors affecting the nutritional properties of forages (Arhab, 2007; Bahman et al., 1997; Pascual et al., 2000; Genin et al., 2004; Ramirez et al., 2004; Ammar et al., 2004).

Table 1. Chemical composition of simple fodder (in g/kg DM).

<table>
<thead>
<tr>
<th>Forages</th>
<th>DM</th>
<th>CP</th>
<th>NDF</th>
<th>ADF</th>
<th>ADL</th>
<th>TP</th>
<th>TT</th>
<th>TCT</th>
<th>PPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. gombiformis</td>
<td>551.7</td>
<td>125.0</td>
<td>614.9</td>
<td>445.2</td>
<td>78.1</td>
<td>34.0</td>
<td>21.3</td>
<td>4.0</td>
<td>ND</td>
</tr>
<tr>
<td>A. pungens</td>
<td>797.3</td>
<td>53.6</td>
<td>794.7</td>
<td>509.4</td>
<td>84.4</td>
<td>2.4</td>
<td>1.6</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>Date palm leaves</td>
<td>896.3</td>
<td>59.5</td>
<td>586.1</td>
<td>422.1</td>
<td>97.1</td>
<td>61.8</td>
<td>49.1</td>
<td>36.2</td>
<td>55.5</td>
</tr>
</tbody>
</table>

DM, Dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, lignin determined by solubilisation of cellulose with sulphuric acid; TP, total phenols; TT, total tannins; TCT, total condensed tannins; PPC, protein precipitating capacity.

For the mixture of the leaves of date palms with palm leaves increased the DM level especially the level of tannins (TT and TCT) of mixtures and reduced the levels of fiber and crude protein in proportion to percentages (Tables 2 and 3). This can be explained by the ability of tannins to form complexes with proteins and therefore the fall of the levels of proteins (Dalzell and Kerven, 1998). Furthermore, it was found that for less percentage of mixtures (10-20%), TCT and TT levels were low, 7.22-10.44 and 24.08-26 86 g/kg DM, respectively, while the level of total nitrogen contents was higher (118.5 and 111.9 g/kg DM) in the palm leaves mixture and A. gombiformis as compared to those obtained in the leaves of date palm only.

For the mixture of date palm leaves and A. pungens, it was noted that at the same percentage of incorporation, the level of the nitrogen material was low as compared to the first mixture (54.2-54.8 g/kg DM) while the tannins levels were similar for both mixtures. According to a research carried out by Paterson et al. (1996), forages which have MAT contents below 70 mg/g DM require nitrogen supplementation to improve their ingestion by ruminants.

**Gas production**

The gas production of mixture of the date palm leaves with A. pungens (S. pungens) or A. gombiformis at different percentages of fermentation for individual substrates and mixtures are presented in Figures 1 and 2. The results of factorial ANOVA showed that adding palm leaves decreased gas production when they were associated with A. pungens, with significant effects (P < 0.0001), whereas, this effect was significant but less pronounced with A. gombiformis. For the mixture of the leaves of date palms with palm A. pungens, a highly significant effect of time (F=1341.6, p<0.0001), percentage (F=23.64, p<0.0001) and interaction of time with percentage (F=7.28, p<0.0001) was found. For the mixture of the leaves of date palms with A. gombiformis, there was a highly significant effect of time (F=323.5, p<0.0001), percentage (F=10.84, p<0.0001) while no effect of interaction of time with percentage (F=0.78, p=0.785>0.05) was found.

Indeed, there is an increase in the amount of gas generated as a function of time. The cumulative volume
The incorporation of dry palms in the diet of ruminants can affect the fermentation process in the rumen. Tannins, for example, have been shown to inhibit the degradation of cell wall components such as cellulose and hemicelluloses, thereby reducing methane production. The presence of condensed tannins (TT) has been found to be particularly effective in inhibiting methane production, with inhibition rates of up to 25% observed in the early hours of incubation with mixture levels of 15 to 25%. This effectively proves that the incorporation of dry palms inhibited A. pungens degradation by ruminal microbiota. This can be explained by the chemical composition of dry palms in tannins. Indeed, effects of this are well established on fermentation activity. According to McSweeney et al. (2001), tannins can directly influence the ruminal microbiota and enzyme activity; in fact, condensed tannins exert an inhibitory action on growth of rumen microorganisms. Furthermore, Makkar and Becker (2009) suggested that TT have an influence on the reduction of methane production.

Unlike the association of palms-drinn, palms did not
Figure 1. Gas production based on percentage of date palm leaves and *A. pungens* (*S. pungens*) at different times.

Figure 2. Gas production based on percentage of date palm and *A. gombiformis* at different times.

affect the fermentation of *A. gombiformis*. This may be explained by the fact that dry palms, rich in tannins, do not affect the proteolytic enzymes (specificity of action and structure), or that the chemical composition of *A. gombiformis* limits the effects of palm leaves on the fermentation parameters. *A. gombiformis* is rich in
nitrogen matter, it generates the production of biomass. Indeed, the degradation of crude protein of *A. gombiformis* generates biomass production. Liu et al. (2002) demonstrated in an *in vitro* system, that an amount of nitrogen is sufficient to sustain microbial growth. In addition, it has been shown that the nitrogen can make only a small contribution to gas production. According to McSweeney (2001), the nitrogen digestibility in ruminants reported favorable responses when providing as supplement. Wang et al. (2008) had shown that supplementation with nitrogen increase digestibility of forage.

Our results are consistent with those reported by Aregheore et al. (2000), Long et al. (1999) and Khazaal et al. (1993). These authors report that the contribution of the total nitrogenous matter (MTA) on gas production is not a significant influencing factor. This suggests an intensification and stimulation of rumen fermentation activity of the microbiota when the latter is in the presence of a food rich in nitrogen and energy (Tendonkeng, 2004; Getachew et al., 2000 and Florence et al., 1999).

Otherwise, supplementation with greater amounts of energy-rich feeds with a source of protein could reduce the time taken to finish cattle for market and increase profitability. Numerous reports in the literature indicate substantial increases in live weight (LW) gain by supplementing cattle consuming low digestibility forages with energy and protein supplements (Hennessy and Morrison, 1982; Lee et al., 1987; Hennessy et al., 1995).

A positive effect on associative gas production has been reported when the leaves of forage trees were mixed with concentrate diets (Sandoval-Castro et al., 2002) and when the straw was mixed with tree leaves (Liu et al., 2002).

### Modeling of gas production

The model of Sandoval-Castro (2000) was used for the description of the fermentation profile of the *in vitro* gas production. Therefore, the authors tried to stimulate the development of gas production according to the equation: \( \text{Gas Prod} = a + b \left(1 - e^{-kt}\right) \) based on the percentage of *A. pungens* or *A. gombiformis*. The estimation method is that of Levenberg-Marquardt where a and b were two coefficients to be determined, k the hourly rate of gas production (%.t) and t the time. The results are summarized in Table 4. The coefficients determination \( R^2 \) were always >0.90. Negative values of “a” (Orskov and Ryle, 1990) were noted. According to several authors, a negative value of “a” is the result of the existence of a latent phase during which microorganisms attach and colonize food particles before any damage is detectable (Ahmed and El-Hag, 2004). This could also indicate the difficulty of ruminal microbiota to degrade these substrates and the need for an adjustment period so that it begins this degradation.

Despite the values of negative “a” and high coefficients of determination \( R^2 >0.90 \), the convergence remained mediocre. Accordingly, this theoretical model remains poor, inflexible and not suitable for varieties of arid and semi-arid areas. This mathematical model does not apply to plants of Saharan zones. That is why the authors proposed the model of multiple regression (Figures 3 and 4) with a relationship type Gas Production = a + b t + cP + dP t for the two mixtures where a, b, c and d are constant coefficients to be determined, t the time (h) and P the percentage of *A. pungens* or *A. gombiformis*. The analysis showed a good regression with multiple \( r = 0.896 \), with highly significant effects of t and interaction Pt (p<0.0001), but with non sensible effect of the percentage (p=0.365). Gas Production = 2.332 + 0.045t − 0.395P + 0.0453Pt for mixtures of leaves palm/A. pungens. For the palm mixture with *A. gombiformis*, a good regression with multiple \( r = 0.799 \), with significance of t (p<0.001), but with no sensible effect of P or interaction Pt (p=0.41 and p=0.10 respectively) was found. The equation was Gas Production = 2.723 + 0.0548t + 0.518P + 0.0233Pt. So, the regression model can be considered as available.

### Table 4. Results of the Levenberg-Marquardt estimation method with convergence criteria at 10^-6.

<table>
<thead>
<tr>
<th>Mixture (% Drin.)</th>
<th>a</th>
<th>b</th>
<th>R^2</th>
<th>Mixture (% A. gombiformis)</th>
<th>a</th>
<th>b</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-3.765</td>
<td>8.551</td>
<td>0.961</td>
<td>0</td>
<td>-4.154</td>
<td>9.234</td>
<td>0.961</td>
</tr>
<tr>
<td>50</td>
<td>-2.896</td>
<td>7.958</td>
<td>0.937</td>
<td>50</td>
<td>-7.490</td>
<td>13.924</td>
<td>0.973</td>
</tr>
<tr>
<td>60</td>
<td>-3.702</td>
<td>9.162</td>
<td>0.945</td>
<td>60</td>
<td>-7.066</td>
<td>13.402</td>
<td>0.973</td>
</tr>
<tr>
<td>70</td>
<td>-3.276</td>
<td>8.764</td>
<td>0.936</td>
<td>70</td>
<td>-7.054</td>
<td>13.864</td>
<td>0.970</td>
</tr>
<tr>
<td>80</td>
<td>-3.512</td>
<td>9.351</td>
<td>0.936</td>
<td>80</td>
<td>-8.031</td>
<td>14.941</td>
<td>0.973</td>
</tr>
<tr>
<td>90</td>
<td>-2.432</td>
<td>7.935</td>
<td>0.905</td>
<td>90</td>
<td>-4.953</td>
<td>10.984</td>
<td>0.948</td>
</tr>
<tr>
<td>100</td>
<td>-4.499</td>
<td>10.932</td>
<td>0.945</td>
<td>100</td>
<td>-7.418</td>
<td>14.236</td>
<td>0.965</td>
</tr>
</tbody>
</table>

a and b are the two coefficients of \((\text{Gas prod})=a+b(1-e^{kt})\), where k is the hourly rate of the gas production (%.t), t the time (h), R^2 is the coefficient of determination.
This study has shown that associative effects on in vitro gas production are consistently higher when the leaves of the date palm were incubated with *A. gombiformis* than with *A. pungens* (*S. pungens*). In addition, palm leaves can be a food supplement suitable for small ruminants browsing *A. gombiformis* or *A. pungens* (*S. pungens*) in arid regions. The present study results also demonstrated that the percentages of the incorporated palm leaves had to be less than 20% and for short periods in order to minimize the effect of tannins. The description of the fermentation profile of in vitro gas production revealed that Sandoval (2000) model is poor and therefore not appropriate for the characteristics of varieties of arid and semi-arid areas in Algeria, whereas the model of multiple regression has revealed a good linear regression for the two types of mixtures.

**Conflict of Interests**

The authors have not declared any conflict of interests.

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