SQU Journal for Science, 2020, 25(1), 10-16 DOI:10.24200/squjs.vol25iss1pp10-16
Sultan Qaboos University

Nutrient Content, *in Vitro* Ruminal Fermentation Characteristics and Antimethanogenic Potential of Three Algerian *Asteraceae* Species

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ABSTRACT: The *in vitro* rumen fermentation parameters and the antimethanogenic potential of three *Asteraceae* species: *Chamaemelum nobile*, *Centaurea pulata* and *Chrysanthemum segetum* were determined. Serum bottles containing 200 mg of each plant and 30 ml of the culture medium (artificial saliva plus rumen juice) were incubated for 24 h. After incubation, pH, volatile fatty acid (VFA), ammonia (NH_3) and methane (CH_4) productions were recorded. Methanogens and protozoa were quantified using a Real Time PCR technique (qPCR). Cumulative gas productions, *in vitro* organic matter digestibility and VFA were not significantly affected by the added species when compared to the control ($P > 0.05$). The effects of *Chamaemelum nobile* and *Chrysanthemum segetum* on methane production, NH_3 and acetate to propionate ratio (C2:C3) were similar. The two species were able to modulate rumen fermentation to produce significantly lower CH_4 concentrations (-24.3% and -27.1%, respectively) compared to the control. *C.pulata* produced the highest cumulative gas and stimulated the microbial metabolism with an increase in C2:C3 ratio, NH_3 and methane production ($P < 0.05$). No significant effect of the three species on methanogenic Archaea and protozoa was registered ($P > 0.05$). The three species studied herein show a good potential for mitigating ruminal methane production without any undesirable effects on the main fermentation parameters.

Keywords: Asteraceae; Achaea bacteria; Gas production; Methane; Protozoa and Ruminal fermentation.

المحتوى الغذائي، خصائص تخمر الكرش المخبري و الإمكانات المضادة لإنبعاث الميثان لثلاثة أنواع أستراسيا جزائرية

سيرن أمقران، رابح أرحاب، موفيدا عقون، سيرينا كلابروا، رافييلا توديسكو و فيديريكو انفيسيلي

الملخص: ثلاثة أنواع أسترا سيا : *Chamaemelum nobile*، *Centaurea pulata* و *Chrysanthemum segetum* تم اختيارها عموما لوفرتها في المراعي الطبيعية شرق الجزائر لتقييم إمكاناتها الغذائية و المضادة لإنبعاث الميثان بإستخدام تقنية إنتاج الغاز المخبري. تم حضن زجاجات المصل الحاوية ل 200 مغ من كل نبتة مع 30 مل من الوسط الغذائي (اللعاب الإصطناعي و عصير الكرش) لمدة 24 ساعة. بعد ذالك، تم قياس معدل الحموضة ،الاحماض الدهنية المتطايرة، الأمونيا، إنتاج الغاز والميثان. تم تحديد كمية بكتيريا الكرش المنتجة للميثان و البر وتوزوا بالاعتماد على تقنية PCR الوقت الحقيقي.
لم يتأثر إنتاج الغاز التراكمي، الهضم المخبري للمادة العضوية ، وإنتاج الأحماض الدهنية المتطايرة بشكل ملموس في الأنواع المضافة مقارنة بالمرجع P) (0.05 <.كان تأثير *Chamaemelum nobile* و *Chrysanthemum segetum* *على إنتاج الميثان، الأمونيا وعلى نسبة الخلات/البروبيونات نفسه. كان لنفس النوعين القدرة على تعديل التخمر في الكرش لإنتاج تراكيز من الميثان أقل بكثير مقارنة بالمرجع*(-24,3 و -27,1٪ على التوالي).أنتجت *C.pulata* *كمية أكثر من* الغاز التراكمي و حفزت الأيض الميكروبي مع زيادة في نسبة *الخلات/البروبيونا والأمونيا والميثان* ($P < 0.05$). لم يتم تسجيل أي تأثير للأنواع الثلاثة على البكتيريا المنتجة للميثان والبر وتوزوا ($p> 0.05$). من شأن الأنواع الثلاثة المدروسة هنا أن تظهر إمكانية جيدة لتخفيف إنتاج الميثان في الكرش بدون أي تأثير غيرمرغوب فيه على معظم العوامل التخمرية.

الكلمات المفتاحية: العائلة المركبة، البكتيريا المنتجة للميثان، إنتاج الغاز، الميثان، البر وتوزوا،الكرش.

1. Introduction

Livestock farming is one of the largest sources of methane emission within the agriculture sector, which accounted for 39% of the sector's total greenhouse gas (GHG) output in 2011, with an increase from 5.0 Gt CO_2 eq yr^{-1} in 2000 to 5.3 Gt CO_2 eq yr^{-1} in 2011. In respect of this fact, and with global demands for milk and meat predicted to double by 2050, global agricultural emissions are expected to increase by 18% and 30% in 2030 and 2050 respectively respectively [1]. Methane (CH_4) emission from enteric fermentation is not only an important GHG associated with environmental problems, but it is also an energetically (2-15% of ingested gross energy) wasteful process. Therefore, reduction in methane emission from ruminants enhances the efficiency of nutrient utilization and the animals' performance, and reduces the impact of CH_4 on global warming and atmospheric pressure.

The *Asteraceae* family is one of the largest families of flowering plants, consisting of approximately 1,600 to 1700 genera and over 24,000 species. Despite the global distribution of *Asteraceae* plants and their potential use as sources of antimicrobial agents [2], their effect as ruminal antimethanogenic agents is very little investigated. In this context, the present study was conducted to determine the *in vitro* fermentation characteristics and to test the antimethanogenic action of three plants belonging to the *Asteraceae* family: *Chamaemelum nobile*, *Chrysanthemum segetum* and *Centaurea Pulata.* These species were selected for their wide variety of medicinal properties (including antibacterial and anti-inflammatory applications), for their prevalent consumption by grazing small ruminants and for their abundance in wild and cultivated habitats in eastern Algeria. Their antimethanogenic activity was evaluated by the survey of *in vitro* methane production, and by methane-forming *Archaea* and protozoa enumeration by QRT-PCR.

2. Materials and methods

2.1 Sample collection and preparation

The aerial parts of *Chamaemelum nobile (CN)*, *Chrysanthemum segetum (CS)* and *Centaurea Pulata (CP)* (commonly named, Roman chamomile, Golden daisy and *black knapweed*, respectively) were harvested between March and June 2012 from a wild population in Ibn Ziad, located in the north-west of Constantine, Algeria (36°22'45" latitude, 6°28'19" longitude). The samples were washed, air dried in an open area and ground to pass through a 1-mm sieve in a Wiley mill (Brabender OHG Duisburg, Germany). The powder was stored in closed jars for chemical analysis and *in vitro* incubation.

2.2 Chemical analysis

Dry matter (DM; method 934.01), ash (ID 942.05) and ether extract (EE; method 920.39C) were determined using AOAC procedures [3]. Nitrogen (N) was determined according to the Kjeldahl method (crude protein CP was calculated as N × 6.25). NDF, ADF and ADL were determined according to the methods of Van Soest *et al*., [4] (Ankom^{200} Technology, Fairport, New York, USA). Hemicelluloses (HC) and cellulose (C) were calculated as NDF – ADF, and ADF – ADL, respectively. Proximate analysis of the control (50% alfalfa hay, 20% ryegrass hay and 30% corn) was also done under the same conditions. All measurements were carried out in triplicate and were presented as the average of three analyses ± standard deviation.

2.3 *In vitro* rumen fermentation

The nutritive value and antimethanogenic potential of the three plants were examined in short term batch incubations using the *in vitro* gas production technique (IVGPT). The trials were conducted in serum bottles (120 ml capacity). Rumen liquor was collected manually from three cows (MW= ± 680 kg) maintained on a standard diet (grass hay: concentrate mixture; 50:50) prior to the morning feeding. The collected inocula were immediately transported to the laboratory in pre-warmed and pre-CO_2-N_2 flushed Thermos flasks, where it was strained through muslin cloth and mixed with a buffer medium in the ratio of 1:2 (V/V) as described by Menke and Steingass [5]. 30 ml of the incubation medium was distributed into each serum bottles containing approximately 200 mg of each plant under a continuous flow of CO_2 to avoid oxygen contamination and maintain anaerobic conditions. The sealed serum bottles were incubated at 39 ± 0.5 °C for 24h in an orbital incubator (STUART, S1500, UK). Under the same conditions, three bottles containing only a buffer-inoculum mixture served as a blank and three flasks containing buffer-inoculum mixture and control diet served as a control.

2.4 Fermentation parameters

Gas pressure accumulated in the headspace of each bottle was recorded, following the reading pressure technique (RPT) as described by Theodorou *et al*., [6], using a manual pressure transducer (Cole and Parmer Instrument Co, Illinois, USA). Total volume of gas (related to incubated organic matter, ml/g) was corrected by subtracting total gas produced in the blank from total gas produced by the tested flasks. Methane concentration was estimated using a gas chromatograph (GC-17 A, Shimadzu, Japan) equipped with a Porapack Q column (80/100mesh), TCD (Thermal

Conductivity Detector) and FID (Flame Ionization Detector) using a certified gas standard mixture of 50% CH_4 and 50% CO_2. Gas and methane production were recorded at 3, 6, 9, 12 and 24h post-inoculation.

After 24h of incubation, the pH of each media culture was recorded (Hanna Instruments, Inc., Woonsocket, RT, USA). Individual volatile fatty acids were determined by gas chromatography equipped with packed 15% SP-1220/1% H_3PO_4 on a 100/120 column. 1ml from each bottle was mixed with 1ml oxalic acid (0.06 M), mixed uniformly then centrifuged (at 12,000 g and 4 °C for 10 min). Supernatant was collected into appropriate GC vials for VFA analysis. The ammonia was determined by the Kjeldahl procedure. For determination of digestibility, the incubation content of each flask was filtered through pre-weighed sintered glass crucibles (Schott Duran, Mainz, Germany, porosity # 2) under vacuum. The residues were dried (105 °C for 24h), ashed (550 °C over night) and reweighed. The weight difference between incubated OM and the undegraded residue represents the true organic matter digestibility (IVOMD, %). The partitioning factor (PF) and microbial biomass yield (MBY) were calculated according to Makkar [7] and Blûmmel *et al*., [8] equations. The relative (R) antimethanogenic effect was evaluated as mentionned by López *et al*., [9].

2.5 Quantitative Real Time PCR for methanogens and protozoa quantification

Total rumen genomic DNA was extracted from fermentation liquor using the Fast DNA Spin Kit for soil (MP Biomedicals, Heidelberg, Germany) according to the manufacturer's instructions. For each sample, 1.5 ml aliquot taken from each serum bottle was centrifuged at 12,000 g for 5 min and the supernatant was removed before DNA extraction. Total microbial DNA isolated in duplicate was purified using the silica-based spin^{TM} filter method, and stored at -20 °C until the analysis. Nucleic acid concentration was measured by spectrophotometer (Nanodrop 2000c, Thermo Scientific, German) and evaluated by separating 2 µl of each sample on agarose gel in 1x Tris-Borate-EDTA buffer (0.8%, W/V). The primer sets for total bacteria were the following: Forward: 5′-GTGSTGCAYGGYTGTCGTCA-3′ and R: 5′-ACGTCRTCCMCACCTTCCTC-3′ [10]. The primer sets for quantification of methanogenic Archaea were targeted against the methyl coenzyme-M reductase (mcrA) gene: Forward: 5'-TTCGGTGGATCDCARAGRGC-3' was designed to target the conserved amino acid sequence FGGSQR, while the reverse primer 5'-GBARGTCGWAWCCGTAGAATCC-3' targeted the GFYGYDL conserved amino acid sequence [11]. Assays were set up using the SYBR® Green PCR Master Mix (Applied Biosystems), 300 nM forward and reverse primers, DNA template (100 ng), and water to 25 µl, under the following conditions: one cycle of 50 °C for 2 min and 95 °C for 2 min for initial denaturation, 40 cycles at 95 °C for 15 seconds and 60 °C for 1 min for primer annealing and product elongation. The relative quantification of methanogenic Archaea was expressed as a proportion of total rumen bacterial 16S rDNA according to the equation of Denman and McSweeney [12]: Relative quantification = $2^{-(Ct\ target\ -\ Ct\ total\ bacteria)}$. For ciliate Protozoa enumeration, the primers were targeted against 18S rDNA gene: F: 5'-GAGCTAATACATGCTAAGGC-3'and R: 5'CCTCACTACAATCGAGATTTAAGG-3' [13]. 1.2 µl of DNA template was used in 30µl, which included 15 µl of SYBR® Green PCR Master Mix (Applied Biosystems),) and 400 nM of each primer. Cycling conditions were: 50 °C for 2 min and 95 °C for 8 min, followed by 40 cycles of 95 °C for 15 secondes, 55 °C for 30 s, and 72 °C for 30s, with a final step of 72 °C for 5 min [14]. The qPCR assays were performed on a 7300 Real-Time PCR System (Applied Biosystems). Each qPCR was done in triplicate. A negative control without the template DNA was used in every qPCR assay.

3. Statistical analysis

The data were analyzed using one way ANOVA in Statistical Package for the Social Sciences (IBM SPSS Statistics, version 17.0.0.3, 2009). The minimum significant difference was generated from Tukey's test as the basis of the multiple comparisons among means. The magnitude of correlation between variables was done using Pearson's multiple comparison tests.

4. Results and discussion

4.1 Chemical composition of the *Asteraceae* species and control

The proximate analysis of the three species and the control are reported in Table 1. The main constituents of the three tested plants are structural carbohydrates (between 234 and 470 g/kg DM) and nonfibrous carbohydrates (ranging from 282 to 500.9 g/kg DM). The three species are also characterised by their high ash content (between 108 and 139 g/kg DM). According to Guimaràtes *et al*., [15], carbohydrates were the most abundant macronutrients followed by crude protein in *C. nobile*, and fructose was the most abundant sugar, followed by glucose and sucrose. The crude protein (CP) content varied between the species samples, being particularly high for *C. pulata* (101 g/kg DM) and *C. nobile* (94.8 g/kg DM) comparatively to *C. segetum* (50.5 g/kg DM), which could give them wide nutritional benefits to supplement poor quality nitrogen deficient feedstuffs nitrogen-poor feedstuffs. Regarding lipid content, *C. pulata* and *C. segetum* showed half the oil content (47.9 and 49.3 g/kg DM, respectively) compared to *C. nobile* (84.1 g/kg DM). Furthermore, the analysis of insoluble dietary fibre content (cellulose, hemicelluloses and lignin) showed that cellulose was the most abundant fraction (373, 239 and 192 g/kg DM for *C. segetum*, *C. nobile* and *C.pulata*, respectively), whereas lignin was less abundant fraction, being two times superior for *C. nobile* (80 g/kg DM) than for

C. segetum and *C. pulata* (49 and 36.7 g/kg DM, respectively). Chang *et al*., [16] reported similar data for lignin content but a much lower content of cellulose (101 g/kg DM) in *Chrysanthemum coronarim* (42 g/kg DM). However, our results were not far from those reported for similar species, other *Asteraceae* species and other Mediterranean shrubs [15, 17, 18]. The differences observed between authors were probably due to the geographical location, season, and maturity stage of plants sampled.

Table 1. Chemical composition (g kg^{-1} DM) of the *Asteraceae* species and control diet (Means ± SD).

Substrates	**Control diet**	***C. pulata***	***C. nobile***	***C. segetum***
DM	**908 ± 0.57**	936 ± 0.14	939 ± 5.23	932 ± 0.71
Ash	**60.9 ± 0.28**	115 ± 1.56	139 ± 3.82	108 ± 2.55
CP	**90.5 ± 0.14**	101 ± 0.21	94.8 ±0.21	50.5 ± 3.54
EE	**17.9 ± 0.22**	47.9 ± 1.20	84.1 ± 1.70	49.3 ± 0.92
NDF	**426 ± 0.71**	234 ± 0.07	399 ± 5.59	470 ± 5.09
ADF	**329 ± 0.35**	229 ± 5.59	319 ± 7.28	422 ± 0.64
ADL	**61.6 ± 0.21**	36.7 ± 2.69	80 ± 1.20	49 ± 2.69
Cellulose (C)	**267 ± 0.71**	192 ± 2.90	239 ± 6.08	373 ± 2.05
Hemicelluloses (HC)	**97.5 ± 0.49**	5.3 ± 5.66	79.8 ± 12.8	47.2 ± 5.73
NFC	**404 ± 0.57**	500.9 ± 0.07	282 ± 3.68	321 ± 10.2
DM= dry matter, CP= crude protein, EE= ether extract, NDF=Neutral detergent fiber, ADF= Acid detergent fiber, ADL=Acid detergent lignin, NFC= Non Fibrous Carbohydrates: [100 – (% NDF + % CP + % EE + % Ash)].				

4.2 *In vitro* gas and methane productions, and true digestibility of the *Asteraceae* species

In addition to the broader potential of using plants as feed additives in rumen nutrition illustrated by the accomplishment of the FC Framework 5 project "Rumen up", several screening assays have been reported in literature, where a large number of plant species (more than 500 species) have been examined in *in vitro* batch cultures to study their potential to enhance the fermentation pathway and decrease methanogenesis. For instance, García-González *et al*., [19] have examined more than 150 herbs and species for their potential to enhance ruminal fermentation and decrease CH_4 production; these authors identified *Rheum officinale* (rhizomes and roots), *Frangula alnus* (bark) and *Allium sativum* (bulb) as the most efficient in decreasing methanogenesis (more than 20%). In another experiment, the same authors examined *Frangula alnus* and *Rheum officinale* in a rumen-simulating fermenter (Rusitec). They concluded that the milled rhizomes of *Rheum* spp. were the most effective in methane abatement without any notable effect on rumen fermentation pattern. Similarly, Durmic *et al*., [20] assessed 128 Australian woody perennial plants for their potential to enhance fermentation pathways in the rumen, and reported that CH_4 production was reduced with the plant species *Cullen australasicum, Enchy-laena tomentosa, Eremophila longifolia, Maireana astrotricha* and *Templetonia retusa*. This favourable effect has been attributed as most likely due to the presence of plant secondary metabolites. In our experiment, the three plants species were chosen because *Chrysanthemum segetum* and *Centaurea pulata* have not been yet investigated regarding their effect on ruminal fermentation pattern and methanogenesis, while a few reports have examined *Chamaemelum nobile* as an additive to reduce methane production *in vitro*.

Cumulative gas production (CGP), recorded for the three species after 24h of incubation, was not significantly affected, compared to the control ($P > 0.05$, tab.2). A low gas production was recorded for *C. segetum* (108 ml/g IOM) and a high one was noted for *C. pulata* (118.1 ml/g IOM). This latter species stimulates methane production. However, *C. segetum* and *C. nobile* produced less methane than the control ($P < 1\%$; 30.1 and 31.1 ml/g IOM, respectively). Methane concentration in the gas was decreased by greater than 20% when incubating *C. nobile* and *C. segetum in vitro* in a ruminal fluid buffer mixture. This reduction was -24.3% and -27.1% for *C. nobile* and *C. segetum*, respectively. In the case of these two species, methane represented approximately 27% of the gas pool. Furthermore, its production for *C. pulata* was 8 units higher than *C. nobile* and *C. segetum*. The same results were reported by Kulivand and Kafilzadeh [17]. These authors have studied eight different grasses collected from Kermanshah (Iran) for their chemical composition, kinetic parameters and antimethanogenic effect and have remarked that *Chamaemelum nobile* is the best grass in terms of nutritive value and antimethanogenic effect (more than 20%). However, our results are inconsistent with those obtained by Garcia-Gonzalez *et al*., [19], who did not observe any noticeable effect of *Chamaemelum nobile* flowers on *in vitro* methane production and other fermentation parameters.

The reasons for such inconsistencies are not clear, but we hypothesize that the effect of the phenolic compounds contained in plants prevails over that of the fibre content. Therefore, CH_4 production was affected due to the effect of these active plant compounds on the metabolic process involved in methanogenesis, either by reducing available metabolic H_2 and redirecting it to other sinks and thus limiting the substrate supply for methanogenesis, or by directly inhibiting the enzymes of microbes associated with methanogenesis.

Table 2. *In vitro* gas and methane productions and true organic matter digestibility of the three species and control.

Substrates	Control	*C. pulata*	*C. nobile*	*C. segetum*	*S.E.M*	*Prob.*
OMCV (mmol/g IOM)	**5.17^{a}± 0.08**	5.27^{a}± 0.19	5.04^{a}± 0.22	4.85^{a}± 0.51	0.06	0.13
OMCV (ml/g IOM)	**116^{a}±1.99**	118.1^{a}±4.35	113.1^{a}±5.09	108^{a}±11.5	1.47	0.13
CH_4 (mmol/g IOM)	**1.85^{b}± 0.10**	1.87^{b}± 0.05	1.38^{a}± 0.17	1.34^{a}± 0.11	0.05	< 0.0001
CH_4 (ml/g IOM)	**41.4^{b}± 2.28**	42^{b}± 1.20	31.1^{a}± 3.83	30.1^{a}± 2.55	1.26	< 0.0001
CH_4 (mmol/mmol gas)	**35.7^{b}± 2.40**	35.6^{b}± 1.87	27.6^{a}± 3.94	27.8^{a}± 2.75	0.99	< 0.0001
R´ (%)	-	1.74^{b}± 6.38	-24.3^{b}± 12.3	-27.1^{a}±6.91	3.74	< 0.0001
IVOMD (%)	**44.3^{ab}± 0.80**	48.1^{b}± 1.64	42.8^{a}± 5.43	41.7^{a}± 3.89	0.02	< 0.0001
PF (mg/ml)	**4.89^{c}± 0.13**	4.42^{b}± 0.11	4.07^{a}± 0.19	4.48^{b}± 0.11	0.07	< 0.0001
MBY (mg)	**244.5^{ab}± 6.94**	257^{c}± 11.64	206.5^{a}± 8.72	226^{b}± 6.90		0.01

OMCV = cumulative gas production related to incubated organic matter, CH_4= methane related to incubated OM, IVOMD = *in vitro* true organic matter digestibility, R'= effect of plant on methane production, PF = partitioning factor, MBY = microbial biomass yield, S.E.M.= standard errors of means, Prob.= probability. Means with different superscripts within the same line being significantly different (P < 0.05).

The nature, activity and concentration of these secondary moieties have been reported to influence the antimethanogenic activity of various plants additives differently [7, 21, 22]. Thus, the reduction in methane production for *C. nobile* and *C. segetum* is probably due to the use of the accumulated hydrogen by another metabolic pathway. In this case, it is probably the propionate production pathway because the concentration of this fatty acid is high for both species compared to the control (25.7 and 31.7%, respectively).

Very limited data are available on the effect of *C. segetum, C. nobile and C. pulata* phytochemical compounds on rumen fermentation and methanogenesis, Amokrane *et al*., [23] reported the presence of substantial amounts of polyphenols in *C. segetum and C. nobile* extracts (207.3 and 99.4 g/kg DM, respectively), represented mainly by flavonoids, followed by condensed tannins. Hence, this information could indicate that flavonoids contained in the two species may be responsible for the decrease of methane production observed *in vitro* in our study. The sesquiterpenelactones and flavonoids are the major constituents of *Centaurea* species [24]. The compound or combination of compounds responsible for these effects should be identified using advanced chemical tools to confirm this hypothesis.

C. pulata produced the highest cumulative gas and was also characterized by its high IVOMD (48.1%) compared to the control (44.3%) and the two other species (p <1%, tab. 2). This indicates that this species is highly fermentable. In addition, this species stimulates ruminal microbiota growth because the microbial biomass yield (MBY) was also significantly increased compared to the control (5.1%) and to the other species (19.7% and 12% for *C. nobile* and *C. segetum*, respectively). Despite the PF at 24h of the tested plant significantly decreased as compared to the control, it remained in the theoretical range (2.75 and 4.41) reported by Blümmel *et al*.**,** [8] for a good microbial synthesis.

It is widely recognized that feedstuffs with higher gas production and IVDMD tend to have higher CH_4 production per gram DM incubated [25]. In our study, the higher CH_4 production with *C.pulata* was not expected as its high soluble carbohydrate content (500.9 g/kg DM) suggests the promotion of propionate production. Our results were inconsistent with those of Chaves *et al*., [26] who indicated in their study that diet quality affected CH_4 production in an *in vitro* study where low concentrations of non-fibrous carbohydrates in both legumes and grasses contributed to a low IVDMD and consequently high CH_4 production per gram digested DM. Hence, a positive correlation between NFC concentrations and CH_4 production was observed (0.796, P < 0.05, results not presented). However, no correlation was registered between NDF content and CH_4 production (-0.513, P < 0.05). As speculated above, the presence of secondary metabolites would likely be responsible for these discrepancies. Due to its nutritional particularity (especially high CP and ash content) and its high digestibility, *C. pulata* could have a complementary role for animal feeding and grazing.

4.3 *In vitro* fermentation parameters and *Archaea* bacteria and protozoa quantification of the three species and the control

Ammonia production and acetate to propionate ratio were similar for *C. nobile* and *C. segetum* (p > 0.05). For both parameters, the highest values were recorded for *C. pulata* (p <1%, tab.3). The same table shows that *C. pulata* has the highest concentration of total VFA (5.15 mmol./g OM) compared to the control (5.04 mmol./g OM), *C. nobile* (4.85 mmol./g OM) and *C. segetum* (4.76 mmol./g OM) although a significant slight decrease in pH was registered for *C. pulata*. The values are in the optimum range for methane production (7.0-7.2), gas production (6.6-7.6) and all rumen microbiota development [27]. Although there was no significant effect of the three species on methanogenic *Archaea* bacteria and protozoa (tab. 3, P > 0.05), the methanogenesis (methane production) declined for *C. nobile* and *C. segetum*. In our study no correlation was observed between methane production and methanogens count. However, methane production and the number of protozoa were strongly correlated (0.566, P < 0.01). Kamra *et al*., [21] have confirmed that in the presence of 5 mM of bromoethanesulphonic acid (BES), methanogenesis was completely

inhibited while the number of methanogens assessed by real time PCR was not affected. In addition, Zhou *et al*., [28] have reported using PCR-DGGE analysis that the activity of individual species rather than the total number of methanogens has the greatest effect on CH_4 production.

Table 3. *In vitro* fermentation parameters recorded after 24h of incubation (pH, volatile fatty acids and ammonia productions) of the *Asteraceae* species and control.

Substrates	**Control**	***C. pulata***	***C. nobile***	***C. segetum***	***S.E.M***	***Prob.***
***In vitro* fermentation parameters**						
pH	**7.08^{b} ± 0.01**	6.98^{a} ± 0.06	7.07^{b} ± 0.07	7.06^{ab} ± 0.02	0.02	0.01
N-NH_3 (mg/l)	**14.6^{b} ± 0.40**	14.8^{b} ± 0.73	13^{a} ± 0.88	12.9^{a} ± 0.87	0.23	< 0.0001
VFAt (mmol/g OM)	**5.04^{ab} ± 0.05**	5.15^{b} ± 0.09	4.85^{ab} ± 0.32	4.76^{a} ± 0.24	0.05	0.01
Acetate (mmol.)	**3.09^{ab} ± 0.10**	3.34^{c} ± 0.12	2.51^{a} ± 0.69	2.59^{a} ± 0.37	0.10	0.005
Propionate (mmol.)	**1.01^{a} ±0.06**	1.04^{a} ± 0.03	1.33^{b} ± 0.21	1.27^{b} ± 0.08	0.03	< 0.0001
Butyrate (mmol.)	**0.53^{a} ± 0.09**	0.61^{a} ± 0.06	0.61^{a} ± 0.05	0.64^{a} ± 0.08	0.16	0.14
A : P ratio	**3.05^{b} ± 0.26**	3.20^{b} ± 0.21	2.04^{a} ± 0.23	1.91^{a} ± 0.19	0.14	< 0.0001
***Archaea* bacteria and protozoa quantification**						
Archaea bacteria ($\times 10^5$ cell/ml)	**8.47^{a}**	7.51^{a}	7.59^{a}	9.03^{a}		0.247
Ciliate protozoa ($\times 10^3$ cell/ml)	**3.85^{a}**	4.10^{a}	3.11^{a}	3.32^{a}		0.054

VFA = total volatile fatty acids, NH_3 = ammonia production, A: P = acetate and propionate ratio, S.E.M. = standard error of the mean, Prob. = probability, means with different superscripts within the same line being significantly different ($P < 0.05$).

5. Conclusion

This is the first report on the effect of *C. pulata* and *C.segetum* species on ruminal fermentation pattern and CH_4 production *in vitro*. In addition to its nutritional diversity, *C.pulata* behaved similarly to the control; thus, it could have a supplementary role for animal feeding and grazing. *C.nobile* and *C.segetum* were able to successfully modulate rumen fermentation characteristics, and offer potential as antimethanogenic agents without compromising forage digestibility. Consequently pasturing of these two species may be a potent strategy to decrease CH_4 emissions in Algeria. The discrepancy encountered between chemical composition and methane production from *in vitro* trials suggests further assessment of these species regarding their secondary metabolites contents, although, in most studies, the specific PSM responsible for their effects on CH_4 have not been identified.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgment

The authors acknowledge CRBt and DMPVA for financial support.

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Received 20 February 2019
Accepted 5 December 2019