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RESEARCH ARTICLE

Effects of *Citrus sinensis* Pulp Essential Oil on In Vitro Digestibility Parameters and Methane Production in Small Ruminant

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Abstract

For a better use of *Citrus sinensis* essential as phytobiotics in small ruminants, an experiment was conducted in the Research Unit of Production and Animal Nutrition (RUPROAN) of the University of Dschang between June 2023 and February 2024. Four (4) rations were formulated using *Trypsacum laxum* hay (FTl+HECs0) to which 100, 200 or 300 mg/ Kg DM of *Citrus sinensis* essential oil was added. A non-pregnant adult Djallonke sheep and a Guinea dwarf goat aged of 18 months and 2 years respectively were used as ruminal fluid donors. Results of this study shows that, regardless of the ruminal fluid used, gas production (GP), volatile fatty acid (VFA), metabolizable energy (ME), *in vitro* dry matter digestibility (IVDDM) and *in vitro* organic matter digestibility (IVDOM) significantly ($p<0.05$) decreased with the addition of 100, 200 or 300mg/Kg DM of essential oil compared to the control ration, with the lowest level at 300mg/Kg DM. A contrary trend was observed with microbial weight (MW) and residual nitrogen (NDF-N), whose values increased following the addition of the essential oil. Furthermore, pH, methane percentage and protozoa population values were different ($p<0.05$) between the two species apart from methane percentage for the T0 ration; pH and protozoa population for the T1 ration which were comparable ($p>0.05$). The highest pH and protozoa population values (6.98±0.07and 5.17±0.00 respectively) were obtained with goat ruminal fluid, in contrast to methane percentage, whose highest value (35.88±0.83) was obtained with sheep ruminal fluid. The lowest values for pH, methane percentage and protozoa population (6.77±0.00; 16.11±0.19 and 3.35±0.13 respectively) were obtained with ovine ruminal fluid. Overall, incorporation of *Citrus sinensis* essential oil reduced protozoa population and methane production, and increased residual nitrogen despite adverse effects observed on volatile fatty acids which proportions turned to decrease with essential oil addition.

Keywords: Digestibility, Essential Oil, Methane, Protozoa, Hay.

1. Introduction

Enteric fermentation in ruminants leads to the production of large quantities of methane. Methane is regarded not only as a loss of energy (2-12%) for the animal, but also as a greenhouse gas [1, 2 and 3] that is responsible for increasing global warming. On the one hand, it is wasteful for the animal and, on the other, it causes significant economic losses for the farmer. The search for solutions has therefore been intensified, with a view to identify phytobiotics capable of boosting ruminant productivity without affecting the quality of by-products or consumer health [4]. Furthermore, medicinal plants are a natural source of chemical molecules with antimicrobial properties, making them interesting for modulating

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rumen fermentation activity [5]. However, using the whole plant could mask the effects of secondary metabolites [6]. This has led to the use of medicinal plant extracts and essential oils, concentrates of active compounds capable of improving feed utilisation efficiency and production performance in ruminants [1, 5 and 7].

Essential oils (EO), extracts of aromatic plants obtained by distillation or other extraction processes, are volatile compounds with an oily appearance [1]. They are rich in secondary metabolites: phenols, terpenes, alkaloids, flavonoids and tannins [1]. Studies 5,7 and 8 have shown that the active components of oils have strong and selective antimicrobial activities against a wide range of micro-organisms, including bacteria, protozoa and fungi. Indeed, several authors have reported that essential oils improve fermentation in ruminants [5 and 7]. In addition, the studies of [1 and 3] revealed the anti-protozoal and anti-methanogenic effects of this family of compounds and their ability to reduce methane production. The reduction of methane production is not only beneficial for the animal but also for the environment [9]. Study [10] showed that supplementation with essential oil at a dose of between 100 and 1,200 mg/L could reduce methane production in buffalo by 41%. However, the correlation between protozoa, methane production and variations in fermentation parameters has been the subject of very few studies.

A comparative study of the effect of *Citrus sinensis* essential oil on the *in vitro* digestibility of *Trypsacum laxum* hay and methane production in small ruminants could be interesting in the search for solutions to energy losses in the form of methane and optimisation of the use of feed in ruminants. It is in this context that the following study was initiated with the main objective of contributing to the improvement of knowledge on the valorization of food waste or by-products in the diet of ruminants. More specifically, the aim was to assess the effects of essential oil on *in vitro* digestibility parameters, methane production and protozoan populations in sheep and goat.

2. Material and Methods

2.1 Study Area

This study was conducted between June 2023 and February 2024 in the Animal Production and Nutrition Research Unit (URPRONAN) of the Faculty of Agronomy and Agricultural Sciences (FASA). The FAR-Uds is located at 5°44‘-5°36’ and 5°44‘-5°37’ latitude North and 10°06‘-09°94’ and 10°06‘-09°85’ longitude East, at an altitude of 1,420m. The dry season runs from mid-November to mid-March and the rainy season from mid-March to mid-November corresponds to the crop-growing season. Rainfall varies between 1,500 and 2,000 mm per year. The average annual insolation is 1800 hours, with temperatures ranging from 10°C (July-August) to 25°C (February) and relative humidity varying between 40-97%, alternating between the rainy and dry seasons.

2.2 Biological Material

2.2.1 Animal Material

For this study, a Djallonke ewe and a dwarf non-pregnant adult goat aged 18 and 2 years respectively were reared at FAR and used as ruminal fluid donors. During the two (2) months preceding the digestibility evaluation period, the animals were housed in a building on stilts and received *Trypsacum laxum* hay ad libitum. A month before the beginning of the trial, the animals were dewormed with Oxytetracycline (20%) and Ivermectin (1%) (1ml/10kg weight), a synthetic broad-spectrum anthelmintic active against adult and larval gastrointestinal and pulmonary nematodes.

2.3 Plant Material

2.3.1 Hay Preparation

The *Trypsacum laxum* (T. laxum) used in this study was harvested at the bolting stage. It was chopped by hand with a machete to a size of around 2 to 3 cm. After chopping, the forage dried in the shade, then ground. The resulting hay was stored in jute bags for later use.

2.3.2 Preparation of Orange Zest

Mature oranges of the *Citrus sinensis* variety were purchased from a producer based in the locality of Bafia, Center region of Cameroon and transported to Phytorica laboratory based in the Douala, littoral region of Cameroon and peeled to obtain the fresh pulps which were used for the extraction of the essential oil.

2.3.3 Extraction of Essential Oil

The essential oil was extracted at the Phytorica laboratory based in Douala, littoral region of Cameroon using the hydrodistillation method as described by study [11]. This technique consists of placing the plant material in an alembic, then heated with water with 200°C. Intense heat causes the explosion of plant saccules which contain oil and these spread in the water vapor. They will then be channeled in a condenser and cooled to be liquified again. At the end, oil was separates from water and was dried using anhydrous sodium sulphate.

2.4 Analysis of the chemical composition of *Trypsacum laxum*

To analyse the chemical composition of the basic ration, *Trypsacum laxum* (Table 1) was chopped and dried in an oven at 60°C until a constant weight was obtained. A sample of one hundred grams (100g) was taken, ground and analysed at the Laboratory of Nutrition et Animal feeding (LAPRONAN) of the University of Dschang to determine the chemical composition. The determination of dry matter (DM), organic matter (OM), crude fibre (CF) and Crude proteins (CP) in the ration was carried out according to the methods described in [12]. crude fibre (CF) was determined using the method proposed by [13].

Table 1. *Chemical composition of Trypsacum laxum hay*

Constituents	DM	OM (%DM)	CP (%DM)	NDF (%DM)	CF (%DM)	Ash (%DM)	Lipids (%DM)
Quantities	90.11	82	13.12	68.88	29.91	10	3.94

2.5 Experimental Diet

Based on the *Trypsacum laxum* hay (FTl) (T0) previously manufactured and the essential oil, the following treatments were formulated:

- T0: *Trypsacum laxum* hay (Tl) + 0mg of essential oil (Tl+EOCs0) / kg DM: control;

- T1: *Trypsacum laxum* hay (Tl) + 100 mg of orange pulp essential oil/ kg DM (Tl+EOCs100);

- T2: *Trypsacum laxum* hay (Tl)+ 200mg of orange pulp essential oil / kg DM (Tl+EOCs200) ;

- T3: *Trypsacum laxum* hay (Tl) + 300mg orange pulp essential oil / kg DM (Tl+EOCs300).

2.6 In vitro Digestibility

2.6.1 Preparation of Samples and Stock Solution

For each ration and each ruminal liquid, 500 mg of samples were weighed in triplicate using a KERN 770 electric balance with a capacity of 210 g and a sensitivity of 0.001 g. The samples were then placed at the bottom of the digestibility syringes and distributed according to the dose of essential oils and the two sources of ruminal fluid (sheep and goats). Each sample was then covered by the piston of the corresponding syringe, which had previously been sprayed with petroleum jelly to facilitate its movement during gas production. The stock solution was prepared according to the method and procedure described by [14].

2.7 Conditioning And Incubation of Samples and Stock Solution

On the eve of the test, the rations contained in the syringes and the stock solution freshly prepared according to the procedure described above were placed in a Memmert incubator at 39°C overnight. The water bath was also switched on and the temperature controlled by two LAUDA E300 thermostats set at 39°C. The morning before the ruminal fluid was collected, the stock solution was placed in the water bath at 39°C. A continuous stream of CO2 was fed into this solution from a gas bottle, the pressure of which was set at 4 bars. Sodium sulphide (417 mg) and NaOH 6N (0.444 ml) were added to the stock solution.

2.8 Ruminal Fluid Collection and Incubation

Ruminal fluid was collected from the rumen immediately after slaughter and evisceration of an adult ewe or goat in the laboratory. This fluid was immediately filtered under a continuous flow of CO2 from a gas cylinder. To prepare 2100 ml of inoculum, 700 ml of this liquid was taken and introduced into the stock solution, still under a flow of CO2. This mixture (inoculum) was homogenised for 10 minutes using a magnetic stirrer. Forty millilires (40 ml) of the inoculum was withdrawn and injected into each syringe using a Fortuna Optifix precision dispenser, then placed in the water bath at 390°C for incubation.

Incubation lasted 24 hours, during which time gas volumes were recorded at 0, 3, 6, 9, 12, 18 and 24 hours. Gas production was calculated and corrected according to the following formula proposed by [15] and methane values were deduced from these gases using the method described by :

$$GP\ \ (ml/500mg\ MS) = \frac{(V_{24} - V_o - GP_o) \times 200mg \times GP_h}{m \times MS}$$

With:

V24 = Volume of gas read after 24 hours incubation;

V0 = Volume of inoculum in the syringe at the start of incubation;

GP0 = Volume of gas produced by the blank after 24 hours incubation;

GPh = Volume of gas produced by the standard after 24 hours incubation.

2.9 Analysis of Produced Gases

Qualitative analysis of the gases produced was carried out by transferring 30ml of the gas produced into another digestibility syringe, then injecting 4ml of NaOH (10N). Absorption of the CO2 caused the piston to move towards the remaining volume of gas corresponding to the volume of CH4 [1 and 16].

2.10 Measurement of pH

Before stopping the *in vitro* digestibility process, just after transferring the gas, the pH of each syringe was measured using a SANXIN electronic pH meter, model pH5S.

2.11 Enumeration and Counting of Protozoa

Before stoppage of the *in vitro* digestibility process, just after gas transfer, 5 ml of ruminal fluid from each syringe was collected, stained with MSF blue and preserved according to the method described by [17], then taken to the laboratory for enumeration and counting of protozoa.

The enumeration and counting of protozoa were carried out according to the method described by [1]. The sample was placed on a Malassez cell, a thick glass slide with a counting chamber made up of 100 rectangles, 25 of which were subdivided into 20 smaller squares to facilitate counting. The sample to be counted was placed between the slide and the coverslip using a Pasteur pipette, avoiding the formation of air bubbles. The sample was counted on the 25 rectangles subdivided into small squares using a microscope with a ×40 objective. Each sample was counted twice and if the difference between the two results was greater than 10% the count had to be repeated.

The total volume of the cell was equal to 1 µl, i.e. 0.01 µl per rectangle. This allows a quantitative and qualitative assessment of the protozoa.

The number of protozoa was expressed according to the following relationship:

N= n1 x v x n2 x f x 1000

N: number of cells per ml.

n1: number of cells counted.

v: volume of a rectangle = 0.01 µl.

n2: number of rectangles counted= 25.

f: dilution factor.

2.12 Assessment of *in vitro* digestibility of dry matter (IVDDM)

At the end of incubation, the contents of the syringes were emptied into 600 ml beakers. These syringes were rinsed twice with two 15 ml portions of Neutral Detergent Double Solution (NDS) and emptied into these beakers. The samples were boiled over low heat for one hour and filtered through preweighed filter crucibles. The crucibles were dried overnight at 103°C and then weighed. This operation enabled the more or less undegraded substrates to be subtracted. The IVDDM was obtained by the difference between the weight of the incubated substrate and the weight of the undegraded residue after treatment with NDS at the end of incubation using the following formula[18]:

$$\text{IVDDM (\%)} = \frac{Pe - R}{Pe} \times 100$$

where : Pe = sample weight

R = sample weight after incubation.

2.13 Evaluation of *in vitro* digestibility of organic matter (IVDOM) and metabolizable energy (ME)

After 24 h of incubation, the gases produced and corrected by the gases from the control tubes were used to calculate the *in vitro* digestibility of organic matter (IVDOM) using the regression equation of [15]. Metabolizable energy (ME) was calculated using the equation proposed by [19].

$$\text{IVDOM (\%)} = 14.88 + 0.889\ GP + 0.45CP + 0.0651Ash$$

$$\text{ME (MJ/Kg MS)} = 2.20 + 0.136\ GP + 0.057\ CP$$

where :

GP = Quantity of gas produced after 24 hours incubation;

CP = Crude protein of the initial sample,

2.14 Determination of Partitioning Factor (PF) and Volatile Fatty Acids

The cleavage factor (CF), which is the quantity of fermented organic matter that produces 1 ml of gas, was obtained by calculation from the following formula [19]:

$$\text{PF (mg/ml)} = \frac{\text{OMD}}{\text{GP}}$$

where : DOM (mg) = Degraded Organic Matter

GP (ml) = Quantity of Gas produced after 24 hours of incubation

The Volatile Fatty Acids (VFA) were obtained by calculation from the following formulae [19]:

$$\text{VFAs (mmol/ml)} = 0.0239\ GP - 0.0601$$

Where: GP (ml) = Quantity of gas produced after 24 hours incubation.

MM (mg) = OMD – (GP x SF);

SF = stoichiometric factor (2.20 for fodder).

2.15 Statistical Analyzes

Data on *in vitro* digestibility, methane percentage, pH and protozoan population were subjected to one-factor analysis of variance (ANOVA) using the general linear model. Where differences existed between treatments, the means were separated by Duncan's test at the 5% threshold. Data on the *in vitro* digestibility, methane production, pH and protozoa population of the rations were compared between the two species (sheep and goat) using Student's t-test at the 5% threshold. SPSS 20.0 (Statistical Package for Social Sciences) was used for these analyses.

3. Results

3.1 Effect of *Citrus Sinensis* Essential Oil on the *In Vitro* Digestibility of Different Rations Incubated with Goat Ruminal Fluid

Table 2. *Effects of Citrus sinensis essential oil on in vitro digestibility parameters of different rations incubated with goat Ruminal fluid (GRF).*

Rations	GP after$_{24h}$ (ml/500mg)	ME (MJ/ kgDM)	MM (mg)	PF (mg/ml)	VFA (mmol/40ml)	IVDDM (%)	IVDOM (%)	NDF-N
T0	37.21^{a}	8.00^{a}	18.13^{c}	2.35^{c}	0.82^{a}	53.08^{a}	54.52^{a}	0.56^{c}
T1	23.01^{b}	6.07^{b}	71.60^{a}	3.00^{a}	0.48^{b}	52.69^{a}	41.88^{b}	0.81^{a}
T2	20.07^{c}	5.67^{c}	50.66^{b}	2.80^{b}	0.41^{c}	46.15^{b}	39.29^{c}	0.73^{b}
T3	19.50^{d}	5.59^{d}	11.00^{c}	2.33^{c}	0.40^{d}	38.10^{c}	38.78^{d}	0.75^{ab}
SEM	2.17	0.29	7.55	0.08	0.05	1.85	1.93	0.02
p	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

a, b, c and d: Values assigned the same letter on the same column do not differ significantly (p>0.05). T0=FTl+HECs0 (control) = T. laxum hay not associated with essential oil; T1= FTl+HECs100= T. laxum hay + 100mg of Citrus sinensis pulp essential oil / kg DM; T2= FTl+HECs200= T. laxum hay + 200mg of Citrus sinensis pulp essential oil / kg DM; T3=FTl+HECs300= T. laxum hay + 300mg of essential oil from Citrus sinensis pulp / kg DM; SEM = Standard error of the mean; p = Probability. GP = produced gas; ME = metabolizable energy; MM = microbial mass; PF= partitioning factor; VFA = volatile fatty acids; IVDDM = in vitro digestibility of dry matter; IVDOM = in vitro digestibility of organic matter; NDF-N = residual nitrogen.

The effect of *Citrus sinensis* essential oil on the *in vitro* digestibility parameters of the different rations incubated with Goat Ruminal fluid summarised in Table 2 above shows that all the values of the *in vitro* digestibility parameters decreased ($p<0, 05$) following the addition of increasing doses of *Citrus sinensis* essential oil with the exception of MM, PF and NDF-N which increased ($p<0.05$) following the addition of 100mg/kg DM of essential oil and decreased ($p<0.05$) at a higher dose. In fact, the highest values for gases produced (GP), metabolizable energy (ME), volatile fatty acids (VFA), *in vitro* digestibility of dry matter (IVDDM) and *in vitro* digestibility of organic matter (IVDOM) were obtained with the ration without essential oil T0 and the lowest with the ration T3. The highest values for microbial mass (MM), partitioning factor (PF) and residual nitrogen (NDF-N) were obtained with the T1 ration. On the other hand, the lowest values for these parameters were obtained with the T3 ration. The lowest MM and NDF-N were obtained with the T0 ration without essential oil, whereas the lowest FC was obtained with the T3 ration.

3.2 Effects of *Citrus sinensis* essential Oil on the pH, Percentage of Methane and Protozoa Population of Different *Trypsacum Laxum* Hay-Based Rations in Goats

Table 3. *Response of pH, methane production and protozoa population to increasing doses of Citrus sinensis essential oil added to Trypsacum laxum hay in goats.*

Rations	pH	Methane(%)	Protozoa (Log$_{10}$ Cells/ml)
T0	6.88	33.05^{a}	5.17^{a}
T1	6.98	23.88^{b}	3.48^{b}

T2	6.94	20.41[c]	3.41[b]
T3	6.95	17.50[d]	3.40[b]
SEM	0.02	1.76	0.22
p	0.22	0.00	0.00

a, b, c and d: Values assigned the same letter on the same column do not differ significantly (p>0.05).T0= Tl+EOCs0 (control) = T. laxum hay not associated with essential oil; T1= FTl+HECs100= T. laxum hay + 100mg of Citrus sinensis pulp essential oil / kg DM; T2=FTl+HECs200mg= T. laxum hay + 200mg of Citrus sinensis pulp essential oil / kg DM; T3= FTl+HECs 300= T. laxum hay + 300mg of essential oil from Citrus sinensis pulp / kg DM; SEM = Standard error of the mean; p = Probability.

This table shows that the addition of increasing doses of *Citrus sinensis* essential oil reduced (p<0.05) methane production and the protozoan population in goats. However, an opposite trend was observed with pH, where values were comparable (p>0.05) following the addition of *Citrus sinensis* essential oil. The highest methane percentage (33.05) was obtained with the control ration without EO (T0) and the lowest (17.50) with the T3 ration. The same trend was observed for the protozoan population.

3.3 Effect of *Citrus Sinensis* Essential Oil on the *In Vitro* Digestibility of Different Rations Incubated With Sheep Ruminal Fluid

Table 4. *Effects of Citrus sinensis essential oil on in vitro digestibility parameters of different rations incubated with Sheep Ruminal Fluid (SRf).*

Rations	GP after 24h (ml/500mg)	ME (MJ/ kgMS)	MM (mg)	PF (mg/ ml)	VFA (mmol/40ml)	IVDDM (%)	IVDOM M(%)	NDF-N
T0	31.36[a]	7.21[a]	49.10[ab]	2.66[b]	0.68[a]	55.31[a]	49.31[a]	0.36[c]
T1	10.13[d]	4.32[d]	24.20[c]	2.58[b]	0.18[d]	32.03[d]	30.43[d]	0.63[b]
T2	15.51[b]	5.05[b]	54.03[a]	2.94[a]	0.31[b]	42.18[b]	35.24[b]	0.65[b]
T3	12.37[c]	4.63[c]	46 .86[d]	2.89[a]	0.23[c]	38.51[c]	32.44[c]	0.80[a]
SEM	2.50	0.34	3.56	0.04	0.05	2.57	2.22	0.04
p	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

a, b, c and d: Values assigned the same letter on the same column do not differ significantly (p>0.05). T0=FTl+HECs0 (control) = T. laxum hay not associated with essential oil; T1= FTl+HECs100= T. laxum hay + 100mg of Citrus sinensis pulp essential oil / kg DM; T2= FTl+HECs200= T. laxum hay + 200mg of Citrus sinensis pulp essential oil / kg DM; T3=FTl+HECs300= T. laxum hay + 300mg of essential oil from Citrus sinensis pulp / kg DM; SEM = Standard error of the mean; p = Probability. GP = produced gas; ME = metabolizable energy; MM = microbial mass; PF= partitioning factor; VFA = volatile fatty acids; IVDDM = in vitro digestibility of dry matter; IVDOM = in vitro digestibility of organic matter; NDF-N = residual nitrogen.

The values of the *in vitro* digestibility parameters decreased (p<0.05) following the addition of variable doses of *Citrus sinensis* essential oil to *Trypsacum laxum* hay in sheep (Table 4). The highest values for gas production (GP), metabolizable energy (ME), volatile fatty acids (VFA), *in vitro* digestibility of dry matter (IVDDM) and *in vitro* digestibility of organic matter (IVDOM) were obtained with the control ration without E0 (T0), while the lowest values were obtained with the T1 ration. With regard to microbial mass (MM) and partitioning factor (PF), the highest values were obtained with the T2 ration and the lowest with the T1 ration. For residual nitrogen (NDF-N), the highest and lowest values were obtained with rations T3 and T0 respectively.

3.4 Effect of *Citrus sinensis* essential oil on pH, % Methane and Protozoa Population of Different Rations Incubated with Sheep Ruminal Fluid

Table 5. *Response of pH, methane production and protozoan population to the addition of increasing doses of Citrus sinensis essential oil to Trypsacum laxum hay in sheep (Ovine)*

Rations	pH	Methane%	Protozoa (Log_{10} Cells/ml)
T0	6.77[c]	35.88[a]	5.31[a]
T1	6.87[a]	16.11[d]	3.35[c]
T2	6.81[b]	26.33[b]	3.97[b]
T3	6.86[a]	22.77[c]	4.07[b]
ESM	0.01	2.15	0.21
p	0.00	0.00	0.00

a, b, c and d: Values assigned the same letter on the same column do not differ significantly (p>0.05).T0= FTl+HECs0 (control) = T. laxum hay not associated with essential oil; T1= FTl+HECs100= T. laxum hay + 100mg of Citrus sinensis pulp essential oil / kg DM; T2=FTl+HECs200mg= T. laxum hay + 200mg of Citrus sinensis pulp essential oil / kg DM; T3= FTl+HECs 300= T. laxum hay + 300mg of essential oil from Citrus sinensis pulp / kg DM; SEM = Standard error of the mean; p = Probability.

The addition of *Citrus sinensis* essential oil reduced (p<0.05) methane production and the protozoan population in sheep (Table 5). The opposite trend was observed with pH, which increased (p<0.05) with the addition of variable doses of *Citrus sinensis* essential oil. The highest methane percentage (35.88) was obtained with the (T0) ration and the lowest (16.11) with the T1 ration. The same trend was observed with the protozoan population. With regard to pH, the highest value (6.87) was obtained with the T1 ration and the lowest with the control ration without EO (T0).

3.5 Comparative Study of the Variation in *In Vitro* Digestibility Parameters of Different Rations According to the Source of Ruminal Fluid

The comparative study of the effect of the source of ruminal liquid on the *in vitro* digestibility parameters of *Trypsacum laxum* hay in combination with *Citrus sinensis* essential oil (Table 5) shows that the values of microbial mass (MM) for the T2 ration, IVDDM and residual nitrogen (NDF-N) for the T3 ration did not vary (p>0.05) according to the source of ruminal liquid. The values of produced gases (GP), metabolizable energy (ME), volatile fatty acids (VFA), *in vitro* dry matter digestibility (IVDMD) and *in vitro* organic matter digestibility (IVD0M) of the control ration (T0) were higher (p<0.05) with goat ruminal fluid than those obtained with sheep ruminal fluid, in which the values of these same parameters were lower (p<0.05) for the same ration. MM, PF and NDF-N values were higher (p<0.05) in goats on the T1 ration than in sheep on either ration. On the other hand, the lowest MM and PF values were obtained with the T3 and T0 rations for NDF-N in goats. Contrary to goats, the lowest values were obtained with the T1 ration for MM and PF and the T0 ration for NDF-N respectively in sheep. In general, sheep showed the lowest values of GP, ME, MM, PF, VFA, IVDDM and IVD0M with the T1 ration after 24h of incubation, unlike goats in which they were obtained with the T3 ration. Irrespective of ruminal fluid, the lowest values of NDF-N were obtained with the T0 ration. The comparative study of the variation of the *in vitro* digestibility parameters of the different rations according to the source of ruminal liquid revealed that the goat gave higher values of the digestibility parameters than the sheep.

Table 6. *Comparative study of the variation in* in vitro *digestibility parameters of different rations according to the source of ruminal fluid*

Rations	Ruminal fluid	GP after 24h (ml/500mg)	ME (MJ/kg DM)	MM (mg)	PF (mg/ml)	VFA (mmol/40ml)	IVDDM (%)	IVDOM (%)	NDF-N
T0	Goat	37.21±0.24^{a}	8.00±0.03^{a}	18.13±0.11^{b}	2.35±0.00^{b}	0.82±0.00^{a}	53.08±0.24^{b}	54.52±0.25^{a}	0.56±0.03^{a}
	Sheep	31.36±0.49^{b}	7.213±0.06^{b}	49.10±4.25^{a}	2.66±0.03^{a}	0.68±0.01^{b}	55.31±1.31^{a}	49.31±0.40^{b}	0.36±0.02^{b}
	p	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00
T1	Goat	23.01±0.04^{a}	6.07±0.00^{a}	71.60±10.8^{a}	3.00±0.12^{a}	0.48±0.00^{a}	52.69±1.95^{a}	41.88±0.01^{a}	0.81±0.02^{a}
	Sheep	10.13±0.26^{b}	4.32±0.04^{b}	24.20±5.24^{b}	2.58±0.08^{b}	0.18±0.00^{b}	32.03±0.83^{b}	30.43±0.26^{b}	0.63±0.01^{b}
	p	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0,00
T2	Goat	20.07±0.24^{a}	5.67±0.03^{a}	50.66±5.21^{a}	2.80±0.06^{b}	0.41±0.00^{a}	46.15±0.72^{a}	39.29±0.25^{a}	0.73±0.02^{a}
	Sheep	15.51±0.01^{b}	5.05±0.00^{b}	54.03±1.58^{a}	2.94±0.01^{a}	0.31±0.00^{b}	42.18±0.78^{b}	35.24±0.02^{b}	0.65±0.02^{b}
	p	0.00	0.00	0.34	0.02	0.00	0.00	0.00	0.01
T3	Goat	19.50±0.31^{a}	5,59±0,04^{a}	11.00±0.00^{b}	2.33±0.00^{b}	0.40±0.00^{a}	38.10±0.20^{a}	38.78±0.31^{a}	0.75±0.04^{a}
	Sheep	12.37±0.02^{b}	4,63±0,00^{b}	46.86±1.61^{a}	2.89±0.02^{a}	0.23±0.00^{b}	38.51±0.23^{a}	32.44±0.06^{b}	0.80±0.04^{a}
	p	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.24

a and b: Values assigned the same letter on the same column do not differ significantly (p>0.05). T0: FTl+HECs0 (control) = T. laxum hay not associated with essential oil; T1: FTl+HECs100= T. laxum hay + 100mg of Citrus sinensis pulp essential oil / kg DM; T2: FTl+HECs200= T. laxum hay + 200mg of Citrus sinensis pulp essential oil / kg DM; T3=FTl+HECs300= T. laxum hay + 300mg of essential oil from Citrus sinensis pulp / kg DM; SEM = Standard error of the mean; P = Probability. GP = produced gas; EM = metabolizable energy; MM = microbial mass; PF= partitioning factor; VFA = volatile fatty acids; DIVMS = in vitro digestibility of dry matter; DIVMO = in vitro digestibility of organic matter; NDF-N = residual nitrogen

3.6 Effects of Different Doses of *Citrus Sinensis* Essential Oil on PH, Methane Percentage and Protozoa Population According to the Source of Ruminal Fluid

A study of the effect of variation in pH, methane production and protozoa population as a function of ruminal fluid source showed that pH, methane production and protozoa population values varied ($p<0.05$) between the two species apart from the from the pH and protozoa population values for the T1 ration, which were close ($p>0.05$).

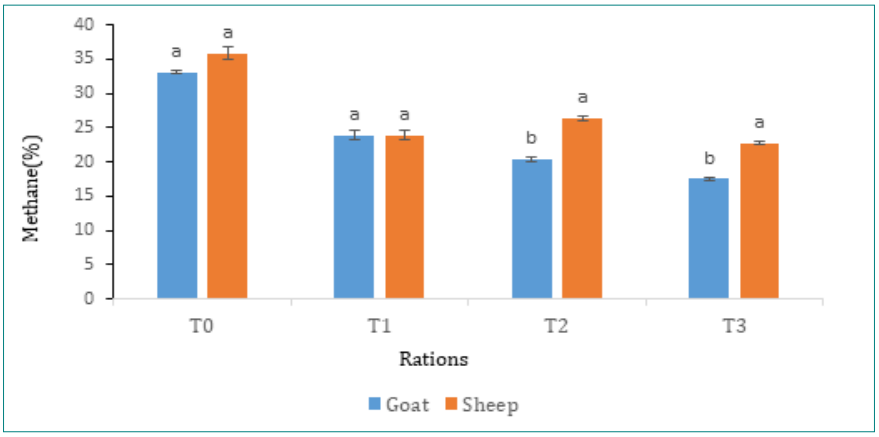

Figure 1. *Comparative study of the effect of* Citrus sinensis *essential oil on the variation in methane percentage between goat and sheep*

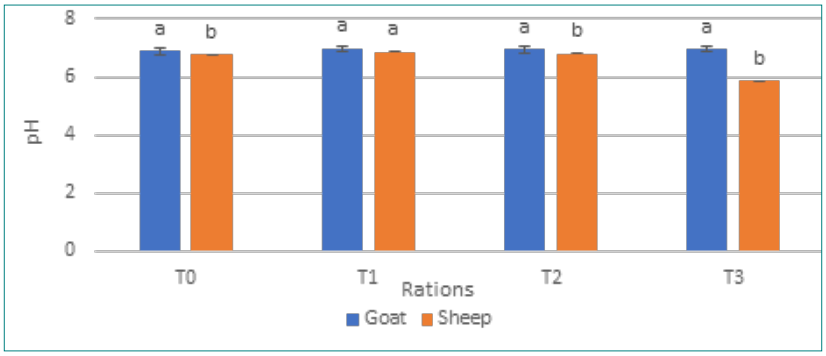

Figure 2. *Comparative study of the effect of Citrus sinensis essential oil on the variation in between goat and sheep*

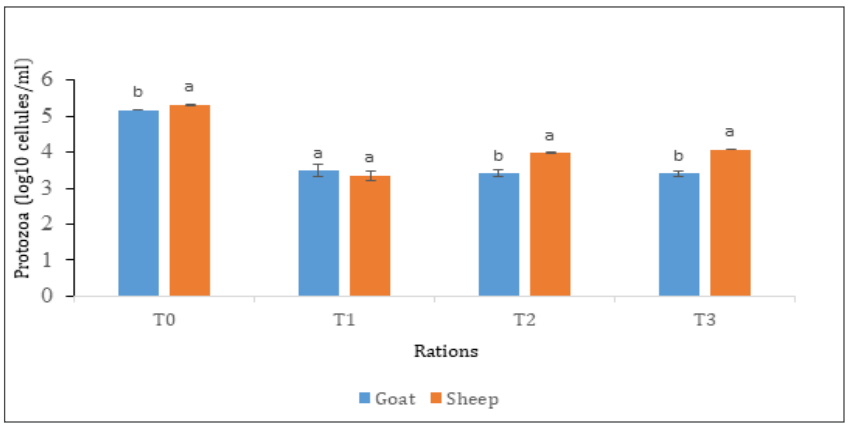

Figure 3. *Comparative study of the effect of* Citrus sinensis *essential oil on the variation in the number of protozoa between goat and sheep*

In general, the highest values for pH (Figure 2) and protozoa number (Figure 3) (6.98±0.07 and 5.17±0.00 respectively) were obtained with goat ruminal fluid; in contrast, the highest value for methane percentage (Figure 1) (35.88±0.83) was obtained with sheep. The lowest values for pH, percentage of methane and number of protozoa (6.77±0.00, 16.11±0.19 and 3.35±0.13) were obtained with sheep ruminal fluid.

4. Discussion

The comparative study of the digestibility of *Trypsacum laxum* hay associated with the essential oil of *Citrus sinensis* according to the source of the ruminal fluid revealed that, whatever the ruminal fluid considered, the production of gases (GP) after 24 hours incubation, volatile fatty acid (VFA), metabolizable energy (ME), *in vitro* digestibility of

dry matter (IVDDM) as well as *in vitro* digestibility of organic matter (IVDOM) significantly decreased with the addition of 100, 200 or 300 mg of essential oil of *Citrus sinensis pulps* / kg DM to *Trypsacum laxum* hay compared to the control ration with the lowest level at 300 mg /kg DM. On the other hand, an opposite trend was observed with the Partitioning factors (PF), microbial mass (MM) and residual nitrogen (NDF-N), the values of which increased following the addition of essential oil. The addition of increasing doses of orange essential oil led to a considerable reduction in GP, IVDOM, IVDDM and ME. The low values obtained from this study compared to others could be explained by differences in the composition of the rations used. Indeed [10] reported that increasing the level of orange essential oil in the reaction medium reduced the efficiency of hydrogen use for the synthesis of VFAs and methane. The low gas production of rations incubated with different levels of the essential oil of *Citrus sinenesis* in both species would also be due to the more pronounced antimicrobial activity at high doses of active compounds such as limonene against gram-positive and gram-negative bacteria [20].

The lowering of digestibility parameters would be the work of metabolites of the essential oil having selective antimicrobial effects on fermentative microorganisms like rumen protozoa, thus leading to a lowering of fiber and protein metabolism and to an increase in certain specific populations of rumen microorganisms translated by an increase in the microbial mass in the treatments compared to the control. These results agree with those of [10] who recorded a reduction in GP, ME, IVDDM and IVDOM when they used doses of between 100 and 1200ml of orange essential oils in the sheep's ration.

The reduction in produced gases and the number of protozoa opposed to the increase in MM and residual nitrogen (NDF-N) observed in this study could be explained by the selective inhibition of protozoa and proteolytic microorganisms. This suggests that certain beneficial bacteria as well as rumen protozoa would be very sensitive to limonene, even at low doses. According to [21], essential oils and secondary metabolites of plants have highly specific activity, which increases the possibility that these components are used to neutralize methanogens at low doses. These results are in agreement with those of [21], who reported a reduction in the digestibility of dry matter with increasing doses of essential oil. Throughout fermentation, microbial mass increased significantly with lower gas production in goats, which is in agreement with the work of [12], who reported that high gas production resulted of a low microbial mass.

This study revealed a reduction in methane production following the addition of increasing levels of essential oil. Indeed, like all phytobiotics, essential oils reduce methane production by directly inhibiting methanogens and hydrogen-producing bacteria such as *Lachnospira multiparus, R. albus* and *R. flavefaciens* as well as protozoa [22]. These observations are in agreement with [23] who reported a decrease in methane production with increasing doses of essential oils of various plants in cattle. In addition, the percentages of methane obtained in the work of [23] following the addition of essential oils (clove, eucalyptus, garlic, oregano and peppermint) at 1g/L were between 34.4%, 17.6%, 42.3%, 87% and 25.7% respectively; which were close to the 46% and 53% (16 and 17% methane) reduction obtained in the present study with 100 and 300 mg of essential oil per Kg/DM in sheep and goats respectively. Indeed, these results suggest that the protozoa of sheep would be more sensitive to low doses of essential oil than those of goat which would be more sensitive to higher doses.

In this study, the increase in pH following the reduction in the number of protozoa would be the cause of the increase in the residual nitrogen level observed. The inclusion of the essential oil would have led to an increase in pH, which suggests that the limonene contained in the essential oil of *Citrus sinensis* would have inhibited the activity of the protozoa, hence the reduction in fermentation parameters. On the other hand, an increase in the number of protozoa would be favorable to protein degradation [24] with an increase in methane production. However, a reduction in the number of protozoa would lead to efficient use of proteins and energy consumed, which would result in an improvement in the productivity of small ruminants [4].

According to [25], the major component of orange essential oil (limonene) which is from the terpene family would have the ability to stimulate the production of short-chain fatty acids as final fermentation products, which could be responsible of lowering ruminal pH; thus leading to the inhibition of the growth of pathogenic microorganisms. On the other hand, an opposite effect was observed in the present study. The addition of orange essential oil, rather have induced a reduction in the production of VFA followed by an increase in ruminal pH.

The effect of the essential oil on the total concentration of VFAs could depend on factors such as the type of substrate used and the conditions of the fermentation medium. The present study revealed a reduction of VFAs with an increase in pH value. The effect of the essential oil on the total concentration of VFAs could depend on factors such as the type of substrate used, doses of additives, type of active components of the additive and the conditions of the fermentation medium. This variability of results would be due to the synergistic effect between limonene and the other components of orange essential oil as reported by [26]. According to these authors, the antimicrobial properties of orange essential oil could be attributed to its composition and that there would be a synergy of action between the components [27]. Limonene, the major component of orange essential oil, has a selective and beneficial activity against rumen microorganisms while the other minor components in the oil could inhibit pathogenic bacteria in the rumen [27]. Indeed, [28] attributed the increase in pH following the reduction in the total concentration of VFAs (reflecting a reduction in the fermentation of rations) to the antibacterial activity of phenolic compounds. This supposes that the phenolic compounds of essential oils could have harmful effects on microbial fermentations at higher doses [8]. Given that the production of VFAs represents the main source of energy for ruminants, a reduction in the latter could have harmful consequences if this occurred in real conditions in animals [8]. The opposite effects observed in the activity of different essential oils could be explained by the nature of the active compounds of the essential oil used which would have played a capital role in the fermentation processes [5]. Indeed, study [20] showed that a concentration of 500 mg/l of eugenol did not affect the digestibility and production of VFA. On the other hand, the same authors reported that a concentration of 500 mg/l thymol significantly reduced the digestibility and production of VFA. This suggests that certain beneficial bacteria as well as rumen protozoa would be very sensitive to limonene, even at low doses.

The low values of digestibility parameters observed in this study compared to those reported by [29] with *Trypsacum laxum* hay in sheep and goat could be explained by the level of sophistication of the fermentation processes as well as the individual variabilities of the microbial populations of ruminal fluid donor animals. In addition, depending on the source of the ruminal fluid, differences between certain values of digestibility parameters depending on the level of essential oil were observed. This is contradictory to the observations of [5] who obtained comparable *in vitro* digestibility results with the rumen fluids of sheep and goat incubated with the same ration.

5. Conclusion

- At the end of this study on the effect of *Citrus sinensis* essential oil on *in vitro* digestibility parameters and methane production in sheep and goat, it appears that:
- The addition of the essential oil led to a reduction in methane production whatever the source of the ruminal fluid. The essential oil addition led to a reduction in number of protozoa
- Incorporation of oil at 100mg/Kg DM could be beneficial in goat unlike sheep in which the best incorporation rate would be at 200mg/Kg DM for a best development of microbial population in the rumen.

Incorporation of oil at 100mg/Kg DM could be beneficial in goats unlike sheep in which the best incorporation rate would be at 200mg/Kg DM for a best development of microbial population in the rumen.

Although the results are satisfactory at doses 100 and 200 mg/Kg DM, it would be desirable to carry out the study of ingestion and in vivo digestibility of *Trypsacum laxum* hay in combination with orange essential oil in small ruminants.

6. References

1. Khenaka K., 2011. Effet de diverses plantes médicinales et de leurs huiles essentielles sur la méthanogenèse ruminale chez l'ovin. Mémoire de master. 81p
2. Medjekal S., Bodas R., Bousseboua H and López S., 2017 Evaluation of three medicinal plants for methane production potential, fiber digestion and rumen fermentation *in vitro*. *Energy Procedia*, 119, 632–641. www.elsevier.com/locate/procedia
3. Jiménez-Ocampo R., Montoya-Flores M. D., Pamanes-Carrasco G., Herrera-Torres E., Arango J., Estarrón M., Aguilar-Pérez C F., Araiza-Rosales E E., Guerrero-Cervantes M and Ku-Vera J. C., 2022. Impact of orange essential oil on enteric methane emissions of heifers fed bermudagrass hay. *Veterinary Sciences*, 9,863-910. Doi: 10.3389/fvets.2022.863910
4. Dorantes-Iturbide G., Orzuna-Orzuna J. F., Lara-

Bueno A., Miranda-Romero L. A., Mendoza-Martínez G. D and Hernández-García P. A., 2022. Essential Oils as a Dietary Additive for Small Ruminants: A Meta-Analysis on Performance, Rumen Parameters, Serum Metabolites and Product Quality. *Veterinary Science,* 9(9), 475; https://doi.org/10.3390/vetsci9090475

5. Mekuiko W. H., Tendonkeng F., Ngoula F., Miégoué E., Lemoufouet J., Fongang Z. B., Chounna A., Mouchili M and Pamo T. E., 2018. Effect of essential oil of Rhizomes of *Zingiber officinale* on the *in vitro* digestibility of *Pennisetum clandestinum* hay in ruminants. *International journal of current innovation research*, 4(1), 984-989. DOI:10.24327/IJCIR

6. Garcia-Gonzalez R., Lopez S., Fernandez M and Gonzalez J S., 2008. Dose-response effects of *Rheum officinale* root and *Frangula alnus* bark on ruminal methane production *in vitro*. *Animal Feed Science and Technology*, 145, 319–334

7. Ribeiro A. D. B., Ferraz Junior M.V.C., Polizel D. M., Miszura A. A., Barroso J. P. R., Cunha A. R., Souza T. T., Ferreira E. M., Susin I and Pires A. V., 2020. Effect of thyme essential oil on rumen parameters, nutrient digestibility, and nitrogen balance in wethers fed high concentrate diets. *Arquatic Brasilian Medecine Veterinary Zootechny,* 72(2), 573-580. http://dx.doi.org/10.1590/1678-4162-11322

8. Benchaar C., Chaves A. V., Fraser G. R., Wang Y. Beauchemin K. A and Mc Allister T. A., 2007. Effects of essential oils and their components on *in vitro* rumen microbial fermentation. *Canadian Journal of Animal Science*, 87, 413–419. DOI: 10.4141/CJAS07012

9. Gerber P. J., Steinfeld H. Henderson B. Mottet A., Opio C., Dijkman J. Falcucci A and Tempio G., 2013. Tackling Climate Change through Livestock – A Global Assessment of Emissions and Mitigation Opportunities. *Food and Agriculture Organization of the United Nations* (FAO), Rome E-ISBN 978-92-5-107921-8

10. Kamalak A., Atalay A. I., Ozkan C. O., Tatliyer A and Kaya E., 2011. Effect of essential orange (*Citrus sinensis L*) oil on rumen microbial fermentation using *in vitro* gas production technique. *Journal of Animal and Plant Science*, 21,764–9.

11. Wang L and Waller C., 2006. Recent advanced in extraction of nutraceuticals from plants. Trends in *Food Science and technology*, 17(6), 300-312

12. A.O.A.C (Association of Official Analytical Chemists)., 1990. Association of Official Analytical Chemists. *Official Methods of Analysis* (13th eds). Washington D.C.

13. Van Soest J. P., Robertson J. B and Lewis B. A., 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74,3583-3597

14. Menke K. H., Raab L., Salewski A., Steingass H., Fritz D and Schneider W., 1979. The estimation of digestibility and metabolizable energy content of ruminant feedstuffs from gas production when they are incubated with rumen liquor. *Journal of Agricultural Science*, 93, 217-222

15. Menke K. H and Steingass H., 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Animal Research and Development*, 28, 7-55

16. Babayemi O. J., Hamzat R. A., Bamikole M. A., Anurudu N. F., Olomola O. O., 2006. Preliminary Studies on Spent Tea Leaf: *In vitro* Gas Production as Affected by Chemical Composition and Secondary Metabolites. *Pakistan Journal of Nutrition*, 5(5), 497–500.

17. Ogimoto K and Imai S ., 1981. Atlas of Rumen Microbiology. *Japan Science Society Press*, 231p

18. Van Soest J. P and Robertson J. B. 1985. Laboratory manual for animal Science. Cornelle University. New York, USA.

19. Makkar H. P. S., 2002. Application of the *in vitro* method in the evaluation of feed resources, and enhancement of nutritional value of tannin-rich tree/browse leaves and agro-industrial by-products. In**:** Development and field evaluation of Animal Feed supplementation packages. Proceeding of the final review meeting of an IAEA Technical Co-operation Regional AFRA Project organized by the Joint FAO/IAEA Division of Nuclear Techniques in *Food and Agriculture and held in Cairo*, Egypt, 25-29, 23-40.

20. Castillejos L., Calsamiglia S and Ferret A., 2006. Effect of Essential Oil Active Compounds on Rumen Microbial Fermentation and Nutrient Flow in *In Vitro* Systems. *Journal of Dairy Sciences*, 89,2649-2658

21. Beauchemin K. A and McGinn S. M., 2006. Methane emissions from beef cattle: Effects of fumaric acid, essential oil, and canola oil. *Journal of Animal Science*, 84(6), 1489- 1496. DOI: 10.2527/2006.8461489x.

22. Cobellis G., Trabalza-Marinucci M., Marcotullio M. C and Yu Z., 2016. Evaluation of different essential oils in modulating methane and ammonia production, rumen fermentation, and rumen bacteria *in vitro*. *Animal Feed Science and Technology,* 215, 25–36. http://dx.doi.org/10.1016/j.anifeedsci.2016.02.008

23. Patra A. K and Yu Z., 2012. Effects of Essential Oils on Methane Production and Fermentation by,

and Abundance and Diversity of, Rumen Microbial Populations. *Apply Environmental Microbiology*, 78(12), 4271. DOI: 10.1128/AEM.00309-12

24. Newbold C. J., De La Fuente G., Belanche A., Ramos-Morales E and McEwan N. R., 2015. The Role of Ciliate Protozoa in the Rumen. *Front Microbioly*, 6, 1313.

25. Souza C. S., Vieites F. M., Justino L. R., Lima M. F., Chaves A. S., Minafra C. S. and Lima C. A. R., 2021. Orange essential oil in the diet of broilers: performance, organ biometrics, bone characteristics, and intestinal morphometry. *Revista Brasileira de Zootechnia,* 50, e20200097. https://doi.org/10.37496/rbz5020200097.

26. Guimarães A. C., Meireles L. M., Lemos M. F., Guimarães M. C. C., Endringer D C., Fronza M and Scherer R., 2019. Antibacterial activity of terpenes and terpenoids present in essential oils. *Molecules,* 24, 2471. https://doi.org/10.3390/ molecules24132471

27. Ambrosio C. M. S., Ikeda N. Y., Miano A. C., Saldaña E., Moreno A. M., Stashenko E., Contreras-Castillo C. J and Gloria E M., 2019. Unraveling the selective antibacterial activity and chemical composition of *citrus* essential oils. *Scientific Reports,* 9:17719

28. Busquet M., Calsamiglia S., Ferret A and Kamel C., 2006. Plant extracts affect *in vitro* rumen microbial fermentation. *Journal of Dairy Sciences*, 89,761–771

29. Mboko A. V., Matumuini F. N. E., Touré A., Zougou G T., Tendonkeng F., Miégoué E., Allogho M. O., Pamo E. T and Benoît B., 2020. *In vitro* digestibility of *Echinochloa crus pavonis*, *Pennisetum purpureum* and *Trypsacum laxum* in dry season in southeastern Gabon. *American Journal of Engineering Research,* e-ISSN: 2320-0847 p-ISSN: 2320-0936. 9(3), 321-326 www.ajer.org