https://doi.org/10.22319/rmcp.v14i2.6188

Article

Nutrient concentrations, *in vitro* digestibility and rumen fermentation of agro-industrial residues of *Cannabis sativa* L. as a potential forage source for ruminants

Elia Esther Araiza-Rosales [a]

Esperanza Herrera-Torres [b]

Francisco Óscar Carrete-Carreón [c]

Rafael Jiménez-Ocampo[d]

Daniel Gómez-Sánchez [e]

Gerardo Antonio Pámanes-Carrasco [f*]

[a] Universidad Juárez del Estado de Durango. CONACYT. Facultad de Medicina Veterinaria y Zootecnia. Carretera Durango-Mezquital km 11.5, Durango, Dgo. México.

[b] Tecnológico Nacional de México, Instituto Tecnológico del Valle del Guadiana. México.

[c] Universidad Juárez del Estado de Durango. Facultad de Medicina Veterinaria y Zootecnia.

[d] Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. México.

[e] Instituto de Investigación del Aprovechamiento de la Cannabis sativa. México.

[f] Universidad Juárez del Estado de Durango. CONACYT. Instituto de Silvicultura e Industria de la Madera. México.

*Corresponding author: gerardo.pamanes@gmail.com

Abstract:

This study aimed to determine the concentration of CP, EE, NSC, fibers, TPC, CT, CBD, THC, *in vitro* digestibility of dry matter and rumen fermentation parameters of agroindustrial

residues of *Cannabis sativa* L. from two extractive processes of cannabinoids, as a potential source of forage in ruminants feeding. The flower of *Cannabis sativa* was exposed to cold-press extraction (CPC) and alcoholic extraction (AEC) process; vegetative residues obtained after extractions were compared to raw flower as a control (RFC) using a completely randomized design and Tukey's test for means comparison. Extractive processes decreased EE, TPC and cannabinoids (CBD and THC). Otherwise, fibers, NSC and digestibility, increased after the extractive processes in CPC and AEC. Similarly, *in vitro* degradability increased after both extractive processes above 120 % as well as latency period. Additionally, protozoa increased with CPC but no changes were observed in AEC. Likewise, no changes were observed in cellulolytic bacteria in CPC and AEC. However, total bacteria were reduced after both extractions. Moreover, N-ammonia in ruminal fermentations decreased with CPC and AEC whereas total volatile fatty acids increased. In addition, gas production increased above 75 % in CPC and AEC; however, no changes were observed in latency period. Furthermore, methane and CO_2 production increased above 80 and 60 %, respectively for CPC and AEC; these augmentations are positively associated with improvements in the ruminal fermentations. In conclusions, the agroindustrial residue of *Cannabis sativa* L. obtained after the analyzed extractive processes may arise as a potential forage source in ruminants feeding.

Keywords: Hemp, Methane, Degradability, Ruminal fermentation, Gas production kinetics.

Received: 06/04/2022

Accepted: 04/11/2022

Introduction

The general acceptance and regulatory outlook concerning to *Cannabis spp*. crops have changed in the past few years. This plant is no longer treated as a solely source of psychotropic agents; biological compounds have been focused on therapeutically approaches successfully[1]. In fact, the cannabidiol (CBD) and tetrahydrocannabidiol (THC) are the main bioactive compounds contained in the plant; these compounds are synthetized mostly in the flower and leafs[2]. Due to the latter, pharmaceutical industry has been attempting in updating and becoming purer the extractive compounds of *Cannabis spp.* for their use in medicines and drugs [1,3]. In fact, some crops of Cannabis sp. have been used as a supplier of hemp for textile industry[4], for production of biofuel[5] and as a component in the automotive industry[6]. In addition, some livestock farmers use seeds as additives in animal feeding[7].

However, the extractive methods of bioactive compounds of the plant generate agricultural residue which may contain minimal concentrations of cannabinoids but considerable contents of fiber, cellulose and hemicellulose; these contents may arise as an important forage source in animal feeding, mainly ruminants. Worldwide information about production and plantations of *Cannabis sp.* is limited due to legal traits. Nevertheless, reports from the USDJ affirmed that the hemp production reached 10,000 t in Mexico in 2006; even though plantations above 31,000 ha were eradicated[8]. Accordingly, an increasing production of *Cannabis sp.* for extraction of cannabinoids with medical purposes may represent a substantial expansion of agricultural residue that may potentially be used as a forage source in ruminants feeding [9]. However, published information about the nutritional value of by-products of hemp in animal nutrition is limited. Furthermore, this study aimed to determine the concentration of crude protein (CP), ether extract (EE), non-structural carbohydrates (NSC), fibers, total phenolic compounds (TPC), condensed tannins (CT), cannabidiol (CBD), delta-9-tetrahydrocannabinol (THC), *in vitro* digestibility of dry matter and rumen fermentation parameters of agro-industrial residues of *Cannabis sativa* L. from two extractive processes of cannabinoids as a potential source of forage in the ruminants feeding.

Material y methods

Study area

This study was carried out in the Faculty of Veterinary Medicine and Husbandry of the Durango State Juarez University, located in Durango, Mexico.

Ingredients and feedstuffs

All vegetative material was donated by the IIAC (Institute for Research and Exploitation of Cannabis) located in Durango, Mexico. The flower of *Cannabis sativa* L. was processed by two methods of extraction, which were performed by the IIAC. Briefly, samples of the flower of *Cannabis sativa* L. were cold-pressed for oil obtaining; the process did not exceed 35 ºC and no solvents were used. The obtained residues after cold-pressing were named CPC due to the cold-presses *Cannabis sativa* L. flower cake obtained. On the other hand, other samples of the flower of *Cannabis sativa* L. were exposed to an alcoholic extraction, which was carried out at room temperature for 5 h. Later, the by-product or residue was obtained by filtering the solvent; this treatment was named AEC (alcoholic extraction of *Cannabis sativa*

L. flower cake). Thus, using raw flower of *Cannabis sativa* L. (RFC) as a control, it was compared to agro-industrial residues obtained after extractive processes. Afterwards, samples of agro-industrial wastes of *Cannabis sativa* L. were dried in a forced air oven (Felisa, Model FE-294AD) at 55 ºC for 48 h and were ground in a miller (Thomas Wiley Miller Lab, Model 4) at a 1 mm particle size. Consequently, samples were stored for further analyses.

Chemical composition

Samples were analyzed for dry matter (DM), ether extract (EE), crude protein (CP) and ashes according to standardized procedures(10). Cellulose, hemicellulose, neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were evaluated according to proposed by Van Soest *et al*(11). Non-structural carbohydrates (NSC) were estimated with the following equation:

NSC= 100 – (CP+EE+NDF+A); where NSC= non-structural carbohydrates (% DM); CP= crude protein (% DM); EE= ether extract (% DM); NDF= neutral detergent fiber (% DM); A= ashes (% DM).

In vitro dry matter digestibility (IVDMD) was estimated using the DAISY$^{II®}$ equipment (ANKOM Technology Corp., Macedon, NY) and according to manufacturer procedures(12). Metabolizable energy was calculated according to the following equation(13):

ME = [2.20 + 0.136(GP_{24}) + 0.0057(CP) +0.0029(EE)2]/4.184; where ME= metabolizable energy (MCal/kg); GP_{24}= *in vitro* gas production at 24 h (ml/g); CP= crude protein (g/kg); EE= ether extract (g/kg).

Secondary metabolites

Dry samples for each treatment were exposed to alcoholic extraction (0.5 g dissolved in 45 ml of 70% ethanol-water solution) during overnight. Afterwards, samples were filtered and vacuum-evaporated (at 40 ºC) until total removal of ethanol solution and leave them dry overnight. Yields of concentrated extractions were calculated based on dry matter. Dry samples were stored for further secondary metabolites analyses.

Analysis of condensed tannins (CT)

Briefly, samples for each treatment were diluted (0.5 g dissolved in 45 ml of 70% ethanol-water solution) and let them extract during overnight. Later, 50 µL aliquots were mixed with a 4% solution of vainillina-methanol and concentrated HCl according to Heimler *et al*[14]. Absorbance was measured at 500 nm using catequine as standard. Yield of CT was estimated with the final concentration in solution and yield in dry matter.

Analysis of total phenolic compounds (TPC)

Briefly, samples for each treatment were diluted (0.5 g dissolved in 45 ml of 70% ethanol-water solution) and let them extract during overnight. Total phenolic compounds were estimated through the Folin-Cioucalteau method adapted by Dewanto *et al*[15] using gallic acid as standard and measuring absorbance at 760 nm for every diluted sample. Yield of TPC was estimated with the final concentration in solution and yield in dry matter.

Analysis of cannabinoids

For the detection of cannabinoids (specifically CBD and THC) was used the method of thin layer chromatography (TLC), according to procedures proposed by Novak *et al*[16]. This trial was conducted at the facilities of the IIAC.

In vitro dry matter degradability (IVDMD)

For this analysis, 1 g (DM) of sample from each experimental treatment was placed in nylon bags (F57, ANKOM Technology, Corp., Macedon, NY) into glass modules equipped with electronic transducers for pressure measuring according to manufacturer's procedures (ANKOM, USA) and incubated in triplicate with buffer solutions ($CaCl_2$ 13.2% w/v; $MnCl_2$ 10% w/v; $CoCl_2$ 1% w/v; $FeCl_3$ 8% w/v; $NaHCO_3$ 39% w/v) and ruminal inoculum in a 2:1 ratio, according to Theodorou *et al*[17]. Ruminal inoculum was obtained from two fistulated Angus steers fed with alfalfa hay based diet before the morning feeding; ruminal liquor was extracted from rumen and immediately placed into a thermal container preheated at 39 ºC and then it was transported to the lab for further analysis. The Nylon bags were incubated

individually for each fermentation time (0, 3, 6, 12, 24, 36, 48, 72 and 96 h). Bags were removed from modules and washed until attaining crystalline water. They were placed in the oven at 55 ºC for 48 h. Changes in dry matter were registered and digestibility data was fitted into the Gompertz function for kinetics parameters estimation according to Murillo *et al*[18].

$$Deg = A_d e^{-L_d e^{-(k_d t)}}$$

Where: Deg= dry matter degradability (% DM); A_d= maximum degradability (% DM); k_d= degradability constant rate (h^{-1}); L_d= latency time before degradability begins (h).

In vitro gas production and ruminal fermentation parameters

For *in vitro* gas production, 1 g of sample from each treatment previously dried was placed into glass modules equipped with pressure transducer (ANKOM, USA) with 120 mL a mix of buffer solutions ($CaCl_2$ 13.2% w/v; $MnCl_2$ 10% w/v; $CoCl_2$ 1% w/v; $FeCl_3$ 8% w/v; $NaHCO_3$ 39 % w/v) and ruminal inoculum in a 2:1 and were incubated at 39 ºC for 24 h by triplicate according Theodorou *et al*[17]; ruminal inoculum was obtained from two fistulated Angus steers fed with alfalfa hay based diet before the morning feeding; ruminal inoculum was extracted from rumen and immediately placed into a thermal container preheated at 39 ºC and then it was transported to the lab for further analysis. Thus, changes in gas volume were registered at 0, 3, 6, 12, 24, 36, 48, 72 and 96 h and data was fitted into the Gompertz function for kinetics parameters estimation according to Murillo *et al*[18].

$$GP = A_g e^{-L_g e^{-(k_g t)}}$$

Where: GP= gas production (ml); A_g= maximum gas production (ml); k_g= gas production constant rate (h^{-1}); L_g= latency time before gas production begins (h). Meanwhile, two 10 mL aliquots of *in vitro* ruminal fermentation were destined for determination of *in vitro* ruminal parameters after 24 h of fermentation time, were processed with metaphosphoric acid (25% w/v) and sulfuric acid (50% v/v) for volatile fatty acids (VFA) and nitrogen-ammonia ($N\text{-}NH_3$), respectively and according to Galyean[19].

Likewise, the same number of experimental treatments were incubated in glass modules (ANKOM, USA) until 24 h of fermentation time[17]. Once the time was elapsed, modules pressure release valve was opened and released gas was measured for methane and CO_2 compositions according to procedures proposed by Herrera Torres *et al*[20] using the portable gas analyzer GEM5000 (Landtec, USA).

Determination of rumen bacteria and protozoa

This assay was determined by weighing 1 g of sample from each treatment previously dried and placed into glass modules equipped with pressure transducer (ANKOM, USA) with 120 mL a mix of buffer solutions and ruminal inoculum in a ratio 2:1, and were incubated at 39 ºC for 24 h by triplicate[17]; ruminal inoculum was obtained from two Angus steers fed with alfalfa hay based diet. Afterwards, concentrations of total and cellulolytic bacteria were determined according to Dehority[21], protozoa were analyzed according to Ogimoto and Imai[22], whereas fungi were determined according to Joblin[23] for each treatment. Briefly, culture mediums were prepared in sterile Petri dishes with nutritive agar (BD, Bioxon, USA) for total bacteria determinations and nutritive agar (BD, Bioxon, USA) plus carboxymethylcellulose (SIGMA, USA), as a cellulose source for cellulolytic bacteria determinations. Inoculum was obtained from *in vitro* ruminal fermentations of treatments and dilutions were carried out until 10^{-6} was reached. After, dilutions with inoculum were placed in previously labeled Petri dishes and incubated under CO_2 atmosphere at 39 ºC. Total bacteria dishes were incubated for 48 h whereas cellulolytic bacteria dishes were incubated for 72 h. Once the incubation time was elapsed, Petri dishes were opened and microorganisms were measured using the most likely number technique [24]. For fungal determination, culture medium was prepared with PDA agar (3 %) and placed into plates under sterile conditions. Later, 10 mL of *in vitro* ruminal inoculum was mixed with peptone solution (1:9 ratio) and dilutions were prepared with 1 ml of inoculated solution until 10^{-5} was reached and placed in plates previously labeled. Immediately, a sample of each dilution is flushed with CO_2 and incubated under CO_2 atmosphere at 39 ºC for 120 h for identification. For protozoa determination, *in vitro* ruminal inoculum was filtered through four layers of cheese clothes and obtained filtered was placed in a separation funnel during 15 min approximately, until protozoa precipitated (a whitish ring appeared at the bottom of the funnel). Later, 1 mL of the obtained sample with protozoa was mixed with 4 mL of ruminal inoculum (previously incubated at 39 ºC and flushed under CO_2 conditions) in 18 x 150 mm culture tubes and placed one more time in the incubator; this last step was repeated five times for each dilution until 10^{-5} was reached. Consequently, protozoa number was determined through direct quantification in Neubauer chamber using a contrasting microscope (Collegiate 400) and an amplification of 400x.

Statistical design

Obtained data for all agroindustrial residues were analyzed through a completely randomized design using the GLM procedure in SAS[25]. Means comparison was evaluated with the Tukey multiple range test declaring significances at $P<0.05$.

Results and discussion

The chemical composition and dry matter digestibility for treatments are shown in Table 1. The used method for the extraction of cannabinoids affected mainly contents of ash, EE, and fibers (NDF and ADF). In fact, cannabinoids are separated from the flower in an organic phase, which is removed within the EE fraction[3]. As a consequence, there is a reduction of 95 % and 40 % in EE for AEC and CPC treatments after extraction, respectively ($P<0.05$). Otherwise, ash content increased 17 and 8 % in AEC and CPC, respectively when compared to the control (RFC) ($P<0.05$). These augmentations are positively associated with a redistribution of the chemical components after the extractive process; remained values of NDF, ADF and NSC represent higher fractions in the chemical composition while reducing the EE fraction once it was extracted. On the other hand, fiber content in CPC remains similar to the control. Cold press extraction removed more fiber fractions when compared to alcoholic extraction technique. Cold press is one of the most used methods for oil extraction; this method requires less energy than others and it is considered as an ecofriendly process[26]. However, the mechanical force applied in the process may remove higher fiber fractions than any other process. Furthermore, others[27] presented similar contents of fiber in their study. Likewise, CP content was similar among treatments ($P>0.05$). However, it is assumed that a little fraction of soluble protein is removed in the extraction process; the remaining protein may be lower in amount but similar in proportion after redistribution of nutrients after the extraction. Similarly, it was observed this behavior in rapeseeds in cold-press extraction processes[28]. Additionally, another research[27] reported similar concentrations of protein in hempseed cake after cold press extraction. Otherwise, NSC increased in AEC and CPC ($P<0.05$); these augmentations are linked with a dilution effect due to a reduction in fractions of fiber as explained earlier. In fact, Jarrell[29] introduced this term related to plant nutrition studies in the early 80s. Moreover, extractive processes reduced TPC and CT ($P<0.05$). Secondary metabolites as phenolic compounds and condensed tannins are extracted in alcoholic solutions[14]. Secondary metabolites play a very important role in methane mitigation in ruminants. Thus, different mechanism of action was observed directly or indirectly in ruminants' fermentation or ruminants' microorganisms depending on the concentrations of certain metabolites (saponins, condensed tannins, phenolic compounds,

etc.)[30]. Therefore, the importance in evaluating the concentrations of TPC and CT. Fiber fractions (NDF and ADF) increased after alcoholic extraction process (P<0.05). These changes are positively connected to a higher extraction of crude fat in EE. Thus, the more EE is extracted in AEC the higher the fiber compositions are. Additionally, the IVDMD increased about 142 and 97 % in AEC and CPC, respectively when compared to the control (RFC) (P<0.05). Moreover, cannabinoids were also affected by the extractive processes (P<0.05). Both, CBD and THC decreased their concentrations substantially. In this way, concentrations of CBD were reduced 73 and 28 % in AEC and CPC, respectively. Similarly, concentrations of THC decreased 99 and 88 % in AEC and CPC, respectively. Alcoholic extraction is more efficient in the extraction of cannabinoids when compared to a cold-press extraction. Apparently, a polar extraction may be more effective and less expensive when compared to an extractive process using mechanical force, which may remove other nutrients with no interest[26]. The published information regarding to harmless consumption of CBD and THC in animals is very limited. However, Kleinhenz[31] offered industrial hemp to calves as a feed resource. These authors administered 1142 and 120 mg of CBD and THC to calves weighing approximately 215 kg of live weight, respectively; no changes were observed in behavior, feeding intake or chemistry of blood serum (glucose, BUN, creatinine and total protein) in animals. Moreover, THC was not detected in blood plasma; CBD was totally metabolized and was not detected after 48 h. Likewise, Cornette[32] administered 5 mg of CBD/ kg live weight and observed no changes in behavior as well. Hence, according to the latter, AEC presented in this study, can be offered harmlessly until 2 kg per animal per day (animals weighting approximately 215 kg live weight) and no effects on any of the chemical parameters or behavior would be expected. Nevertheless, no information on meat safety and hemp consumption in ruminants is available. In agree to the results given in the present study, lower concentrations of cannabinoids (CBD and THC) and higher concentrations of NSC led to a higher IVDMD and ME. In fact, other study[16] reported antimicrobial activity of cannabinoids, which may affect directly the digestibility. Consequently, IVDMD increased approximately two-fold when compared to control (RFC). In addition, these changes could not be attributed to variations in TPC and CT since their concentrations are similar among AEC and CPC treatments; changes in IVDMD in AEC and CPC could not be correlated to TPC and CT concentrations in both treatments.

Table 1: Chemical composition and *in vitro* digestibility of agro-industrial residues of *Cannabis sativa L*

Variable	RFC	AEC	CPC	*P*	SEM
		%, DM			
Ash	11.5±0.25^{b}	13.5±0.25^{a}	12.5±0.04^{ab}	0.001	1.33
CP	21.2±0.25^{a}	20.9±0.29^{a}	20.2±0.0.28^{a}	0.10	0.12
EE	12.3±0.06^{a}	0.5±0.04^{b}	7.5±0.0^{b}	<0.001	0.08
NDF	27.9±0.39^{b}	32.2±0.33^{a}	28.0±0.86^{b}	0.003	0.58
ADF	16.5±0.02^{b}	18.9±0.69^{a}	15.5±0.19^{b}	0.015	1.47
ADL	2.2±0.15^{b}	2.5±0.08^{a}	2.3±0.006^{ab}	0.002	0.07
HEM	11.5±0.31^{b}	13.3±2.23^{a}	12.4±0.28^{a}	<0.001	1.06
CEL	14.4±0.14^{a}	16.4±0.36^{a}	13.1±0.11^{a}	0.009	1.24
NSC	26.5± 0.16^{b}	32.7± 0.82^{a}	31.1± 1.0^{a}	<0.001	0.77
TPC, mg/g DM	15.7±0.10^{a}	14.8±0.08^{b}	15.4±0.05^{b}	<0.001	0.06
CT, mg/g DM	6.7±0.09^{a}	4.6±0.16^{c}	5.4±0.05^{b}	<0.001	0.09
IVDMD, %	23.9±1.11^{c}	57.9±1.58^{a}	47.2±2.74^{b}	<0.001	1.58
ME, Mcal	1.4 ± 0.04^{c}	1.9±0.07^{a}	1.6 ±0.06^{b}	<0.001	0.05
CBD, g/kg	1.8± 0.01^{a}	0.5± 0.003^{c}	1.3± 0.006^{b}	<0.001	0.03
THC, g/kg	10.9± 0.07^{a}	0.08± 0.001^{c}	1.4± 0.007^{b}	<0.001	0.001

RFC= raw flower *Cannabis sativa L.*; AEC= alcoholic extracted *Cannabis sativa L.* flower residue; CPC= cold-pressed *Cannabis sativa L.* flower residue; SEM= standard error of the difference among means; CP= crude protein; EE= ether extract; NDF= neutral detergent fiber; ADF= acid detergent fiber; ADL= acid detergent lignin; HEM= hemicellulose; CEL= cellulose; NSC= non structural carbohydrates; TPC= total phenolic compounds; CT= condensed tannins; IVDMD= *in vitro* dry matter digestibility; ME= metabolizable energy; CBD= cannabinol; THC= tetrahydrocannabinol.

^{abc} Means with different letters in the same row indicate differences (*P*<0.05).

In vitro ruminal degradability for treatments is presented in Table 2. Extractive processes affected degradability; AEC and CPC increased maximum degradability (A_d parameter) above of 120 % in both treatments when compared to control (*P*<0.05). Likewise, extractive processes increased degradability specific rate (k_d parameter) about 120 % for both treatments whereas latency period (L_d parameter) increased 140 % for both treatments (*P*<0.05). Augmentations in the kinetics parameters may be positively associated with a reduction in the cannabinoids content and changes in the NSC; this effects were observed earlier in the IVDMD. Higher values in A_d, k_d and L_d are presented in the treatment with lower values of cannabinoids (AEC). According to this, Semwogerere *et al*[33] reported that digestibility of hempseed cake is lower than the digestibility of canola meal and soybean meal. In fact, it was affirmed that hempseed cake increased the rumen retention time which is comparable with the fermentation time in *in vitro* assays[27]; this effect was observed in this study. Actually, AEC and CPC presented higher values in degradability specific rate (k_d parameter) which led to reach the asymptotic value (A_d) in a shorter time; RFC would need

more time to reach A_d which is consistent with previous studies[27]. Otherwise, more time is necessary for microorganisms to begin degradation of substrate as observed in values of latency time (L_d parameter) for AEC and CPC. The latter may be associated with changes in microorganisms' populations (protozoa and bacteria) which will be discussed later.

Table 2: *In vitro* ruminal degradability parameters of agroindustrial residues of *Cannabis sativa L.* after two extractive methods

Parameter	RFC	AEC	CPC	*P*	SEM
A_d, %	24.1±0.29^c	57.6±0.01^a	54.6±0.81^b	<0.001	0.502
L_d, h	1.9±0.01^b	4.7±0.04^a	4.6±0.16^a	0.004	0.096
k_d, %/h	0.14±0.005^b	0.31±0.005^a	0.32±0.000^a	<0.001	0.002

RFC= Raw flower *Cannabis sativa L.*; AEC= alcoholic extracted *Cannabis sativa L.* flower residue; CPC= cold-pressed *Cannabis sativa L.* flower residue; SEM= standard error of the difference among means; A_d: maximum degradability; k_d= specific rate of degradability; L_d= latency period before the degradation begins; (lag phase).

^{abc} Means with different letters in the same row indicate differences (P<0.05).

The population of microorganisms after ruminal fermentation of different residues is shown in Table 3. Protozoa increased after the extractive process in CPC when compared to control (P<0.05); no changes were observed in AEC (P>0.05). Contrariwise, no traces of fungi were observed in the control (RFC); nevertheless, fungi increased after the extractive process (P<0.05). Otherwise, total bacteria were reduced after the extractive process in both treatments (P<0.05). However, no changes were registered in cellulolytic bacteria (P>0.05). Novak *et al*[16] affirmed that extracts of hemp exhibited antimicrobial activity in most bacterial habitats from human and animal but these changes were not observed in fungi; conversely, extracts from hemp showed antifungal activity in *in vitro* assays reported previously[34]. Additionally, reductions in total bacteria are associated with an increase in the protozoa populations. This is in accordance with others[35], who claim that the bacteria predation is caused mainly by protozoa activity.

Table 3: Microorganisms populations in *in vitro* ruminal fermentation of agroindustrial residues of *Cannabis sativa L.* after two extractive methods

Microorganisms	RFC	AEC	CPC	P	SEM
Protozoa, mL^{-1} x10^4	7.3±0.07^b	15.8±0.30^{ab}	26.6±0.37^a	0.007	0.22
Cellulolytic bacteria, mL^{-1} x10^5	142.1±2.88^a	127.6±14.77^a	115.6±7.21^a	0.232	7.84
Total bacteria, mL^{-1} x10^5	187.2±20.20^a	100.6±6.74^b	115.2±6.92^b	0.006	10.57
Fungi, CFU	ND	7.2±0.57^a	6.3±1.15^a	0.001	0.60

RFC= raw flower *Cannabis sativa L.*; AEC= alcoholic extracted *Cannabis sativa L.* flower residue; CPC= cold-pressed *Cannabis sativa L.* flower residue; CFU: colony forming units; ND= non-detected; SEM= standard error of the difference among means..

^{abc} Means with different letters in the same row indicate statistical differences (P<0.05).

Ruminal fermentation parameters and gas production kinetics are shown in Table 4. No changes were observed in pH (P>0.05). Nevertheless, extractive processes in AEC and CPC affected concentrations of N-ammonia and TVFA (P<0.05). A reduction in the N-ammonia is positively associated with a defaunation of ruminal bacteria due to the presence of protozoa. In fact, Wang *et al* [36] reported a reduction in the protozoa populations when using hempseed oil in ruminal fermentations. Thus, it is expected that RFC promote a reduction of protozoa and an increase in bacteria populations; the latter encourages an increase in the N-ammonia. Furthermore, a reduction in the deamination of protein would be expected as a consequence of an increase of bacteria which would lead to a reduction in the N-ammonia[37]. These asseverations were observed in this study. Protozoa increased with both treatments and a reduction of total bacteria is observed (P<0.05). Additionally, protozoa play an important role in the synthesis of some volatile fatty acids. As a matter of fact, some others[38] affirmed that the ability of protozoa to digest fatty acids could divert more carbon towards volatile fatty acids synthesis; these changes were observed in the present study in AEC and CPC with the higher protozoa populations. Regarding to gas production kinetics, A_g increased above 87 and 77 % in AEC and CPC, respectively (P<0.05). These changes suggest a better nutrients utilization of microorganisms which led to a higher fermentation process and a higher gas production; hence, it is expected a higher gas production rate. However, no changes were observed in latency period (L_g) and gas production specific rate (k_g) (P>0.05) but similar trends to those in degradability are observed.

On the other hand, methane production increased 81 and 97 % in AEC and CPC, respectively (P>0.05). Likewise, CO_2 increased about 60 % in both treatments when compared to control (P<0.05). Apparently, the reduction of cannabinoids and the increase in the NSC led to an augmentation of the fermentation process, which increased methane production. Whereas, the CH_4: CO_2 ratio increased 18 % in CPC when was compared to control (P<0.05). This ratio indicates the volume of methane produced divided into the volume of CO_2 present in

the process; higher values of this variable suggest that more methane is being synthetized through the CO_2 reduction pathway[18]. Thus, these changes in CH_4: CO_2 ratio may suggest that more methane is being synthetized through the CO_2 pathway and that the increases in methane production are not only associated to an improvement in the ruminal fermentation and an extension of the total gas production. In fact, the presence of cannabinoids may result as inhibitors in methanogenesis through the CO_2 reduction pathway since lower values of CH_4: CO_2 ratio are presented with higher values of cannabinoids. The higher the cannabinoids are the lower the protozoa; methane synthesis and protozoa number is positively associated[39]. Thus, if there is a reduction in protozoa a reduction in methane would expected as well. An increase in the protozoa may affect the bacteria including the methanogens. Regarding to the latter, it was reported[33] that hempseed oil is more efficient in the inhibition of methanogens since it has more terpenes, polyphenols and lignin. Similarly, Embaby *et al*[40] found a 10 % reduction in methane production when using hempseed oil and compared to corn oil. However, Patra *et al*[41] affirmed that reductions in methane production are associated to the presence of polyunsaturated fatty acids (PUFA) than the presence of cannabinoids; these fatty acids were not evaluated in this study. According to the findings in this study, raw flower in RFC inhibits not only degradability but methane synthesis and protozoa populations as well. Nevertheless, these affections are highly associated with the nutrients utilization from microorganisms which may lead to a better ruminal fermentation process as exposed earlier in this study.

Table 4: Ruminal fermentation parameters and gas production kinetics of agroindustrial residues of *Cannabis sativa L.* after two extractive methods

Variable	RFC	AEC	CPC	P	SEM
pH	6.8±0.11[a]	6.9±0.10[a]	6.8±0.12[a]	0.251	0.13
$N\text{-}NH_3$, mg/dL	15.2±0.08[a]	10.9±0.11[c]	13.8±0.11[b]	<0.001	0.29
TVFA, mM	0.11±0.007[b]	0.16±0.003[a]	0.16±0.008[a]	<0.001	0.05
A_g, ml/g DM	41.82±8.89[b]	78.46±5.13[a]	74.27±2.08[a]	<0.009	4.93
L_g, h	2.6±0.64[a]	3.6±0.35[a]	3.24±0.37[a]	0.384	0.15
k_g, %/h	0.09±0.05[a]	0.22±0.01[a]	0.24±0.02[a]	0.063	0.02
CH_4, ml/g DM	3.7±0.15[b]	6.7±0.29[a]	7.3±0.20[a]	<0.001	0.22
CO_2, ml/g DM	32.5±2.470[b]	52.8±0.89[a]	52.3±1.52[a]	<0.001	1.75
CH_4/CO_2 ratio	0.11±0.004[b]	0.12±0.003[ab]	0.13±0.005[a]	0.023	0.004

RFC= Raw flower *Cannabis sativa L.*; AEC= alcoholic extracted *Cannabis sativa L.* flower residue; CPC= cold-pressed *Cannabis sativa L.* flower residue; SEM= standard error of the difference among means; $N\text{-}NH_3$: N-ammonia; TVFA= total volatile fatty acids; A_g: maximum gas production; k_g= specific rate of gas production; L_g= latency period before the gas production begins (lag phase).

[abc] Means with different letters in the same row indicate differences (P<0.05).

Conclusions and implications

Both residues obtained after the two extractive processes offer acceptable nutritional properties in animal feeding. Residues obtained after the alcoholic extraction offered a better nutrient utilization of microorganisms present in the ruminal fermentation which led to increase in IVDMD and fermentation parameters. Therefore, the agroindustrial residue of *Cannabis sativa L.* obtained after the extractive processes may arise as a potential forage source in ruminants feeding. However, more *in vitro* and *in vivo* assays using these agro-industrial residues as a part of a ration are highly recommended, considering potential secondary effects of cannabinoids on animal health and food safety.

Acknowledgments

The authors would like to thank to the Durango State Council of Science and Technology for its support through the grant #2020-123. Authors would like to thank also to the Institute for Research and Exploitation of Cannabis for the availability in vegetative material and the facilities used in some analyses.

Literature cited:

1. Jenatová A, Franková A, Tlustos P, Hamouz K, Bozik M, Kloucek P. Yield and cannabinoids contents in different cannabis (*Cannabis sativa* L.) genotypes for medical use. Ind Crops Prod 2018;(112):363-367.

2. Suero-García C, Martín-Banderas L, Holgado MA. Efecto neuroprotector de los cannabinoides en las enfermedades neurodegenerativas. Ars Pharm 2015;56(2):77-87.

3. León CJJ. Editorial: El aceite del cannabis. Rev Soc Quím Perú 2017;83(3):261-263.

4. Ebskamp MJ. Engineering flax and hemp for an alternative to cotton. Trends Biotechnol 2002;20(6):229-230.

5. Small E, Marcus D. Hemp: A new crop with new uses for North America. In: Janick J, Whipkey A. editors. Trends in new crops and new uses. ASHS Press, Alexandria, VA. 2002:284–326.

6. Hernández-Díaz D, Villar-Ribeira R, Julián F, Tarres Q, Espinach FX, Delgado-Aguilar M. Topography of the interfacial shear strength and the mean intrinsic tensile strength of hemp fibers as a reinforcement of polypropylene. Mater 2020;13(1012):1-16.

7. Bailoni L, Bacchin E, Trocino A, Arango S. Hemp (*Cannabis sativa* L.) Seed and co-products inclusion in diets for dairy ruminants: A review. Anim 2021;(11)856.

8. USDJ (National drug threat assessment 2008: Marijuana) United States Department of Justice. https://www.justice.gov/archive/ndic/pubs25/25921/marijuan.htm Accessed march 29, 2022.

9. Kleinhenz MD, Magnin G, Ensley AM, Griffin JJ, Goeser J, Lynch E, PAS, Coetzee JF. Nutrient concentrations, digestibility, and cannabinoid concentrations of industrial hemp plant components. Appl Anim Sci 2020;36(4):489-494.

10. AOAC (Association of Official Analytical Chemists). Official Methods of Analysis. 2019.

11. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci 1991;74(10): 3583-3597.

12. ANKOM (Gas production system operator's manual). ANKOM Technology, USA. 2015.

13. Menke KH, Steingass H. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Anim Res Dev 1988;28(1):7-55.

14. Heimler D, Isolani L, Vignolini P, Tombelli S, Romani A. Polyphenol content and antioxidative activity in some species of freshly consumed salads. J Agric Food Chem 2007;(55):1724-1729.

15. Dewanto V, Wu X, Adom KK, Liu RA. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J Agric Food Chem 2002;(42):3010-301.

16. Novak J, Zitterl-Eglseer K, Deans SG, Franz CM. Essential oils of different cultivars of *Cannabis sativa* L. and their antimicrobial activity. Flavour Fragr J 2001;(16):259–262.

17. Theodorou MK, Williams BA, Dhanoa MS, McAllan AB, France J. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim Feed Sci Technol 1994;(48):185-197.

18. Murillo OM, Herrera TE, Corral LA, Pámanes CG. Effect of inclusion of graded level of water hyacinth on *in vitro* gas production kinetics and chemical composition of alfalfa hay based beef cattle diets. Indian J Animal Res 2018;52(8):1298-1303.

19. Galyean ML. Laboratory Procedures in Animal Nutrition Research. 13th ed. Lubbock: USA; 2010.

20. Herrera-Torres E, Murillo-Ortiz M, Méndez J, Araiza-Rosales E, Reyes Jáquez D, Pámanes-Carrasco G. *In vitro* methane production and *in situ* degradability of prickly pear pretreated with yeast cultures. Trop Subtrop Agroecosystems 2021;(24):100.

21. Dehority BA. Rumen microbiology. Nottingham: Nottingham University Press; 2003:372.

22. Ogimoto K, Imai S. Atlas of Rumen Microbiology. Japan Sci Soc Press. Tokyo 1981:231.

23. Joblin KN. Isolation, enumeration and maintenance of rumen anaerobic fungi in roll tubes. Appl Environ Microbiol 1981(30):27-37.

24. Harrigan WF, McCance EM. Métodos de laboratorio en microbiología de los alimentos y productos lácteos. España: Ed. Academia. León, 1979;(32-35):361-366.

25. SAS. SAS User´s Guide (Release 9.1.3). Cary NY, USA. SAS Inst.Inc. 2003.

26. Çakaloğlu B, Özyurt VH, Ötles S. Cold press in oil extraction: A review. Ukr J Food 2018;7(4):640-654.

27. Hessle A, Eriksson M, Nadeau E, Turner T, Johansson B. Cold-pressed hempseed cake as a protein feed for growing cattle. Acta Agric Scand Anim Sci 2008;(58):136–145.

28. Östbring K, Malmqvist E, Nilsson K, Rosenlind I, Rayner M. The effects of oil extraction methods on recovery yield and emulsifying properties of proteins from rapeseed meal and press cake. Foods 2020;9(19):2-14.

29. Jarrell WM, Beverly RB. The dilution effect in plant nutrition studies. Adv Agron 1981;(34):197-224.

30. Ku-Vera JC, Jiménez-Ocampo R, Valencia-Salazar Sara S, Montoya-Flores MD, Molina-Botero IC, *et al*. Role of secondary plant metabolites on enteric methane mitigation in ruminants. Front Vet Sci 2020;7:1-14.

31. Kleinhenz MD. Plasma concentrations of eleven cannabinods in cattle following oral administration of industrial hemp. Appl Anim Sci 2020;36(4):489-494.

32. Cornette HE. Pharmacokinetics of single feeding of cannabidiol in cattle: A pilot study. Honors College Thesis, Murray State University Honors College, 2022.

33. Semwogerere F, Chenaimoyo LF, Katiyatiya1 OC, Chikwanhal MC, Cletos M. Bioavailability and bioefficacy of hemp by-Products in ruminant meat production and preservation: A Review. Front Vet Sci 2020;(7).

34. Ali EMM, Almagboul AZI, Khogali SME, Gergeir UMA. Antimicrobial activity of *Cannabis sativa* L. J Chinese Med 2012;3(6):1–4.

35. Belanche A, de la Fuente G, Pinloche E, Newbold CJ, Balcells J. Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis. J Anim Sci 2012;(90):3924–3936.

36. Wang S, Kreuzer M, Braun U, Schwarm A. Effect of unconventional oilseeds (safflower, poppy, hemp, camelina) on in vitro ruminal methane production and fermentation. J Sci Food Agric 2017;(97):3864–3870.

37. Getahun D, Alemneh T, Akeberegn D, Getabalew M, Sewdie D. Urea metabolism and recycling in ruminants. Biomed J Sci Tech Res 2019;20(1):14790–14796.

38. Karnati SKR, Sylvester JT, Ribiero DM, Gilligan LE, Firkins JL. Investigating unsaturated fat, monensin, or bromoethanesulfonate in continuous cultures retaining ruminal protozoa. I. Fermentation, biohydrogenation, and microbial protein synthesis. J Dairy Sci 2009;(92):3849-3860.

39. Guyader J, Eugéne M, Noziére P, Morgavi DP, Doreau M, Martin C. Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. Animal 2014;8(11):1816-1825.

40. Embaby MG, Günal M, Abughazaleh A. Effect of unconventional oils on *in vitro* rumen methane production and fermentation. Cienc Investig Agrar 2019(46):276–285.

41. Patra A, Park T, Kim M, Yu Z. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. J Anim Sci Biotechnol 2017;(8):13.