UNIVERSIDAD POLITÉCNICA DE MADRID

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA,
ALIMENTARIA Y DE BIOSISTEMAS

NUTRITIVE EVALUATION OF OLIVE CAKE AND TOMATO POMACE FOR SMALL RUMINANT FEEDING

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INDEX

Chapter I. INTRODUCCION	1
I.1. Introduction	3
I.2. Olive cake.....	5
I.2.1. Olive cake production	5
I.2.2. Chemical composition and characteristics of olive cake	9
I.2.3. Nutritive value of olive cake	10
I.2.4. Effects of feeding olive cake to meat-producing ruminants	12
I.2.5. Effects of feeding olive cake to dairy ruminants.....	13
I.3. Tomato pomace	16
I.3.1. Tomato pomace production.....	16
I.3.2. Chemical composition and characteristics of tomato pomace	18
I.3.3. Nutritive value of tomato pomace	19
I.3.5. Effects of feeding tomato pomace to dairy ruminants	22
I.4. Other by-products: citrus pulp and dried distillers grains with solubles.....	24
I.4.1. Citrus pulp	25
I.4.2. Dried distillers grains with solubles	27
I.5. Nutritional significance of acid detergent insoluble nitrogen fraction of feeds...	29
I.6. Objectives and working hypothesis	32
Chapter II. METHODOLOGY	35
II.1. Animals and feeding	37
II.2. By-products, feeds and diets	39
II.3. <i>In vitro</i> incubations: measurement of gas production kinetics	43
II.4. <i>In vitro</i> incubations: measurement of fermentation parameters	44
II.5. <i>In vitro</i> intestinal digestibility	45
II.6. Lactation trial	45
II.7. Chemical analyses.....	46
II.8. Calculations and statistical analyses	48
Chapter III. INFLUENCE OF STORAGE TIME AND PROCESSING ON CHEMICAL COMPOSITION AND <i>IN VITRO</i> RUMINAL FERMENTATION OF OLIVE CAKE.....	53
III.1. Introduction	55
III.2. Materials and methods.....	56

III.2.1. Olive cake samples	56
III.2.2. Animals and <i>in vitro</i> incubations.....	57
III.2.3. <i>In vitro</i> degradability	58
III.2.4. Chemical analyses	58
III.2.5. Calculations and statistical analyses.....	59
III.3. Results and discussion.....	60
III.3.1. Chemical composition of olive cake	60
III.3.2. Effects of storage time on <i>in vitro</i> fermentation of olive cake	64
III.3.3. Effects of processing treatment on <i>in vitro</i> fermentation of olive cake.....	70
Chapter IV. VARIABILITY IN THE CHEMICAL COMPOSITION AND <i>IN VITRO</i> RUMINAL FERMENTATION OF OLIVE CAKE BY- PRODUCTS	75
IV.1. Introduction	78
IV.2. Materials and methods	79
IV.2.1. Olive cake samples.....	79
IV.2.2. <i>In vitro</i> incubations.....	79
IV.2.3. Chemical analyses	81
IV.2.4. Calculations and statistical analyses	81
IV.3. Results	82
IV.3.1. Influence of olive cake processing type on chemical composition and <i>in vitro</i> fermentation.....	82
IV.3.2. Estimation of fermentation parameters	83
IV.4. Discussion	90
IV.4.1. Influence of olive cake processing on chemical composition and <i>in vitro</i> fermentation.....	90
IV.4.1. Estimation of fermentation parameters	92
IV.5. Conclusions	93
Chapter V. NUTRITIVE VALUE OF TOMATO POMACE FOR RUMINANTS AND ITS INFLUENCE ON <i>IN VITRO</i> METHANE PRODUCTION	99
V.1. Introduction	102
V.2. Materials and methods.....	103
V.2.1. Tomato pomace samples.....	103
V.2.2. Animals and feeding.....	103

V.2.3. Experimental design	103
V.2.3.1. Experiment 1: <i>in vitro</i> fermentation of TP samples.....	104
V.2.3.2. Experiment 1: <i>in vitro</i> intestinal digestibility of tomato pomace samples	105
V.2.3.3. Experiment 2: <i>in vitro</i> fermentation of diets containing tomato pomace	105
V.2.4. Chemical analyses	107
V.2.5. Calculations and statistical analyses	108
V.3. Results	109
V.3.1. Chemical composition of tomato pomace samples, <i>in vitro</i> fermentation, and intestinal digestibility of crude protein.....	109
V.3.2. <i>In vitro</i> fermentation of diets containing tomato pomace.....	110
V.4. Discussion.....	116
V.4.1. Chemical composition, <i>in vitro</i> fermentation, and <i>in vitro</i> intestinal digestibility of tomato pomace samples	116
V.4.2. <i>In vitro</i> fermentation of diets containing tomato pomace.....	118
V.5. Conclusions	119
Chapter VI. THE ACID DETERGENT INSOLUBLE NITROGEN (ADIN) ANALYSIS OVERESTIMATES THE AMOUNT OF N ASSOCIATED TO ACID DETERGENT FIBRE.....	125
VI.1. Introduction	128
VI.2. Materials and methods	128
VI.2.1. Samples and experimental procedures	128
VI.2.2 Calculations and statistical analyses	130
VI.3. Results	132
VI.4. Discussion	138
VI.5. Conclusions	140
Chapter VII. GENERAL DISCUSSION	143
Chapter VIII. REFERENCES	155
Chapter IX. APPENDIX	181
IX.1. Influence of particle size of crude olive cake on <i>in vitro</i> ruminal fermentation and gas production kinetics	183
IX.1.1. Introduction	185
IX.1.2. Materials and methods	186
IX.1.3. Results and discussion.....	187

IX.1.4. Conclusions	190
IX.2. Effects of agro-industrial by-products supplementation on dairy goats milk characteristics, nutrients utilization, ruminal fermentation and methane production	193
IX.2.1. Introduction	196
IX.2.2. Materials and methods	197
IX.2.3. Results and discussion.....	202
IX.2.4. Conclusions	214
IX.3. Python Script	221

ABBREVIATIONS

Acid detergent fibre	ADF
Acid detergent insoluble nitrogen	ADIN
Acid detergent lignin	ADL
Average gas production rate	AGPR
Body weight	BW
Cetyltrimethylammonium bromide	CTAB
Coefficient of variation	CV
Conjugated linoleic acid	CLA
Control diet	CON
Crude Olive Cake	COC
Crude protein	CP
Cyclone Olive Cake	CYOC
Dried citrus pulp	DCP
Dried distillers grains with solubles	DDGS
Dry matter	DM
Dry matter effective degradability	DMED
Ether extract	EE
European Union	EU
Exhausted Olive Cake	EOC
Experimental diet	BYP
FA methyl esters	FAME
Fatty acids	FA
Fermented organic matter	FOM
Intestinal digestibility of protein	IDCP
Monounsaturated FA	MUFA
Neutral detergent fibre	NDF
Neutral detergent insoluble N	NDIN
Nitrogen	N
Olive Cake	OC
Organic Matter	OM
Polyethylene glycol	PEG
Polyunsaturated FA	PUFA
Potential <i>in vitro</i> DM degradability	DMD120
Potential gas production	PGP
Purine derivative	PD
Saturated fatty acids	SFA
Sunflower	SF
<i>In vitro</i> DM digestibility	DMD

In <i>vitro</i> NDF digestibility	NDFD
Tomato pomace	TP
Total solids	TS
Total soluble polyphenols	TSP
Volatile fatty acids	VFA

FIGURE INDEX

Figure 1. Phases of olive oil extraction and by-product production (Habeed et al., 2017).	7
Figure 2. Prediction of AGPR with a neural network including olive cake (OC) data.	151
Figure 3. Prediction of AGPR with a neural network including olive cake (OC) and tomato pomace (TP) data.....	151
Figure 4. <i>In vitro</i> cumulative gas production of COC samples with different particle size and two forages.....	189

TABLE INDEX

Table 1. Ingredient and chemical composition of the experimental concentrates fed to dairy goats and potential use as human food of concentrate ingredients.	38
Table 2. Samples of olive cake (OC) used in this Thesis representative of those available in Spain for animal feeding were obtained at nine different extraction plants.	40
Table 3. Ingredients and chemical composition of experimental diets containing increased amounts of tomato pomace (TP).	42
Table 4. Chemical composition and <i>in vitro</i> dry matter (DMD) and neutral detergent fibre (NDFD) degradability of olive cake samples obtained after different storage time in ponds and processing.....	62
Table 5. Correlation matrix (Pearson coefficient and P-values in brackets; $n = 21$) of chemical composition of OC samples with gas production parameters, <i>in vitro</i> degradability and fermentation parameters in 24 hr <i>in vitro</i> incubations (only P-values ≤ 0.10 values are shown).	63
Table 6. Effect of processing (PR) and storage time in pond (Time) of olive cake (OC) samples on gas production kinetics parameters.....	65
Table 7. Effect of processing (PR) and storage time in pond (Time) of olive cake (OC) samples on gas production kinetics parameters.....	66
Table 8. Effect of processing (PR) and storage time in the pond (Time) of olive cake (OC) samples on fermentation parameters in 24 hr <i>in vitro</i> ruminal fermentation.	67
Table 9. Effect of processing (PR) and storage time in the pond (Time) of olive cake (OC) samples on fermentation parameters in 24-hr <i>in vitro</i> ruminal fermentation.....	69
Table 10. Chemical composition (g/kg dry matter unless stated otherwise) of olive cake (OC) samples ($n = 42$).	84
Table 11. Influence of olive cake (OC) processing on chemical composition (g/kg dry matter unless stated otherwise).....	85
Table 12. Influence of olive cake (OC) processing on gas production parameters, dry matter effective degradability (DMED) and fermentation parameters in a 24 h <i>in vitro</i>	

fermentation of OC samples.....	86
Table 13. Correlation matrix (Pearson coefficient and p values in brackets; $n = 42$) of the chemical composition of olive cake (OC) samples and storage time with the average gas production rate (AGPR) and fermented organic matter (FOM) of OC samples measured in 24 h <i>in vitro</i> incubations (only $P < 0.05$ values are shown) ¹	88
Table 14. Models for predicting the average gas production rate (AGPR; mL/h) and fermented organic matter (FOM; g/kg OM) after 24 h <i>in vitro</i> incubation of olive cake (OC) samples using OC type (crude (COC), exhausted (EOC) and cyclone (CYOC)) as the fixed effect and either chemical fractions or storage time as covariates ($n = 42$) ¹	89
Table 15. Ingredients and chemical composition of control and experimental diets containing increased amounts of tomato pomace (TP) used in Experiment 2.	106
Table 16. Chemical composition (g/kg dry matter (DM) unless stated otherwise) of tomato pomace samples taken from two processing plants at weekly intervals during the tomato harvesting campaign.....	111
Table 17. Gas production kinetics and fermentation parameters of tomato pomace samples taken from two processing plants at weekly intervals during the tomato harvesting campaign and samples.	112
Table 18. <i>In situ</i> rumen degradability of dry matter (DM) and crude protein (CP) after 16 h of incubation, <i>in vitro</i> intestinal digestibility of CP (IDCP), and total CP digestibility of tomato pomace samples taken from two processing plants at weekly intervals during the tomato harvesting campaign.....	113
Table 19. Correlation matrix (Pearson coefficient and <i>p</i> -values in brackets; $n = 12$) of chemical composition of tomato pomace samples with gas production parameters, dry matter effective degradability (DMED), fermentation parameters measured in 24-h <i>in vitro</i> incubations, <i>in situ</i> rumen degradability of dry matter (DM) and crude protein (CP) after 16 h of incubation, and <i>in vitro</i> intestinal digestibility of CP (only $P < 0.10$ values are shown) ¹	114
Table 20. Gas production parameters and fermentation parameters after 8 and 24 h of <i>in vitro</i> fermentation of diets containing increased amounts of tomato pomace incubated in batch cultures of mixed rumen microorganisms ¹	115
Table 21. Chemical composition (g/kg DM unless otherwise stated) of corn and	

sunflower ^{15}N -enriched samples by using either $^{15}\text{NH}_4\text{NO}_3$ (^{15}NAM) or $\text{NH}_4^{15}\text{NO}_3$ (^{15}NIT) as fertilizers ($n = 3$).	131
Table 22. ^{15}N abundance (atom%) in total nitrogen (N), neutral detergent insoluble N (NDIN) and acid detergent insoluble N (ADIN) of corn and sunflower samples labelled with either $^{15}\text{NH}_4\text{NO}_3$ (^{15}NAM) or $\text{NH}_4^{15}\text{NO}_3$ (^{15}NIT) applied as fertilizers.....	133
Table 23. N contamination of acid detergent insoluble nitrogen (ADIN; g/g) in corn and sunflower samples labelled with either $^{15}\text{NH}_4\text{NO}_3$ (^{15}NAM) or $\text{NH}_4^{15}\text{NO}_3$ (^{15}NIT) applied as fertilizers ¹	134
Table 24. Correlation matrix (values of Pearson coefficient and P values in brackets) of nitrogen (N) contamination of acid detergent insoluble N (ADIN) with fibre fractions in corn and sunflower samples ¹	136
Table 25. Values of acid detergent insoluble N (ADIN) content, ^{15}N -abundance and N-contamination of ADIN, and ADIN corrected for N-contamination from cetyltrimethylammonium bromide (true ADIN) in samples from aerial biomass of corn and sunflower (SF1 and SF2) obtained by using sequential and non-sequential methods of ADFom analysis.	137
Table 26. Chemical composition (g/Kg dry matter) and <i>in vitro</i> dry matter digestibility (IVDMD) of crude olive cake (COC) samples with different particle size and two forages.	188
Table 27. Gas production parameters of crude olive cake (COC) samples with different particle size and two forages ($n = 4$) ¹	188
Table 28. <i>In vitro</i> fermentation parameters after 24 h incubation of crude olive cake (COC) samples with different particle size and two forages with buffered ruminal fluid from sheep ($n = 4$) ¹	191
Table 29. Ingredient and chemical composition of experimental concentrates and potential use as human food of concentrate ingredients.....	199
Table 30. Average values of feed and water intake and apparent digestibility of nutrients in dairy goats fed the experimental diets.	204
Table 31. Average BW and BW change over the trial and values of N and energy utilization in dairy goats fed the experimental diets.....	207

Table 32. Average values of milk yield and composition and feed efficiency in dairy goats fed the experimental diets.	208
Table 33. Average values of fatty acids (FA) composition (g/100 g of identified FA) in milk fat from dairy goats fed the experimental diets.	210
Table 34. Average values of ruminal fermentation parameters, methane emissions, daily urinary excretion of creatinine and purine derivatives and estimated microbial N flow in dairy goats fed the experimental diets.	213

SUMMARY

The use of agroindustrial by-products in animal feeding requires precise information on their nutritional value. However, agroindustrial by-products are heterogeneous and as consequence, values for chemical composition and nutritive parameters are highly variable, and the information related is scarce. For that reason, the objective of this Doctoral Thesis is to evaluate the nutritive value of two by-products that are produced in huge amounts in Spain: olive cake (OC) and tomato pomace (TP). In order to achieve this objective, six Experiments (five *in vitro* and one *in vivo*) were conducted.

The objective of Experiment 1 (Paper 1) was to analyse the influence of storage time and further processing: crude OC (COC), exhausted OC (subjected to a second oil extraction; EOC) and cyclone OC (obtained from a cyclone separator; CYOC) on nutritive value of OC samples. Twelve samples (six COC and six EOC) were obtained monthly from the same pond from 1 to 6 storage months, and nine samples (three COC, three EOC and three CYOC) were obtained monthly from a different pond from 6 to 9 months storage. Chemical composition was analysed, and OC samples were fermented *in vitro* with sheep rumen fluid. Increasing storage time up to 6 months decreased sugars and total soluble polyphenols (TSP) content but increased fibre content in OC. Dry matter effective degradability (DMED) decreased linearly ($P < 0.001$) by 35.9 and 45.5% as storage time augmented from 1 to 6 months for COC and EOC, respectively. COC had lower DMED values than EOC (averaged values 0.255 and 0.294 g/g, respectively). Both potential gas production (PGP) and average gas production rate (AGPR) were lower ($P \leq 0.018$) in COC compared with EOC, which was attributed to the high ether extract (EE) content of COC (≥ 86 g/kg dry matter (DM)). For samples stored longer than 6 months, CYOC had greater ($P < 0.05$) DMED than COC and EOC (averaged values 0.207, 0.164 and 0.164 g/g, respectively). The results indicate that ruminal degradability of OC is reduced with advancing storage time, but only subtle changes were observed during the first two months. CYOC showed greater degradability than COC and EOC, but differences between COC and EOC became negligible after five storage months.

The objective of Experiment 2 (Paper 2) was to determine the variability in the chemical composition and *in vitro* ruminal fermentation of OC by-products. Forty-two

OC samples with different storage times (1–14 months) and processing (25 COC, 9 EOC and 9 CYOC) were fermented *in vitro* with sheep ruminal fluid. EOC samples had a lower EE content than COC and CYOC (15.9, 110 and 157 g/kg DM, respectively), but greater neutral detergent fibre (NDF) (645, 570 and 441 g/kg DM) and acid detergent insoluble nitrogen (ADIN) (9.76, 8.10 and 8.05 g/kg DM) content. EOC had the greatest ($P < 0.05$) AGPR, whereas the greatest fermented organic matter (FOM) was obtained for EOC and CYOC. The best single predictor of the AGPR was total sugars content ($R^2 = 0.898$), whereas NDF was the best one for FOM ($R^2 = 0.767$; $P < 0.001$). Statistical models using storage time as a predictor variable had lower accuracy and R^2 values than those from the chemical composition. In summary, the nutritive value of OC was highly dependent on its processing, but its EE content did not negatively affect ruminal fermentation parameters, which could be estimated from either carbohydrate composition or storage time.

The aim of this Experiment 3 (Paper 5; Appendix) was to estimate *in vitro* the nutritive value of four samples of COC with different particle size obtained during the drying process from the same trommel at different stages of the screening procedure. Particles size was greater than 3 mm (COC3), 2 mm (COC2) and 1 mm (COC1) or smaller than 1 mm (COC0). Alfalfa hay and barley straw were included in the study for comparative purposes. Chemical composition was analysed in all samples. Gas production kinetics was determined in 120 h *in vitro* incubations with sheep rumen fluid as inoculum, and fermentation parameters and *in vitro* dry matter digestibility (DMD) were analysed after 24 h of incubation. COC3 and COC0 samples had lower contents of NDF (415 and 391 g/kg DM), but greater crude protein (CP) (96.6 and 114 g/kg DM) and EE (220 and 242 g/kg DM) contents, than COC2 and COC1 (627 and 624; 42.4 and 48.7; 102 and 124 g/kg DM, respectively). DMD of COC samples ranged from 39.4 to 58.7%, and was lower than that for alfalfa hay (67.5%) for all samples, but greater than that for barley straw (42.4%) for COC0 and COC3 samples. COC3 and COC0 samples had greater ($P < 0.05$) PGP (103 and 66.2 ml/g DM, respectively) and total volatile fatty acids (VFA) production (608 and 600 μmol , respectively) than COC2 and COC1, but values were lower than those for alfalfa hay (202 ml/g DM and 1023 μmol , respectively) and barley straw (198 ml/g DM and 733 μmol). The results indicate that decreasing particle size to <1 mm increased the fermentation potential of COC, but its nutritive value was still slightly lower than that of barley straw. The similar quality

observed for COC3 and COC0 samples was attributed to the high sugar content of COC3 samples.

The objective of Experiment 4 (Paper 3) was to determine the variability in nutritive value for ruminants of TP samples and analyze its effect on *in vitro* fermentation when it was included in a high-concentrate diet. Twelve TP samples were obtained from two processing plants at weekly intervals and analysed for chemical composition, *in vitro* rumen fermentation, and intestinal digestibility. The chemical composition of TP did not differ between processing plants and only slight variations were observed among sampling times. Tomato pomace had a low DM content (< 300 g/kg), a high content of NDF, CP and EE (572, 160, and 82.7 g/kg DM on average, respectively), and was rapidly fermented in the rumen. Protein degradability at 16 h *in situ* incubation was 510 g/kg and *in vitro* intestinal digestibility of CP (IDCP) was low (430-475 g/kg). Replacing soybean meal and barley straw by dried TP increased the *in vitro* fermentation rate and the production of VFA and reduced NH₃-N concentrations without affecting methane. In summary, TP samples showed little variability in nutritive value over sampling time and TP of up to 180 g/kg could be included in high-concentrate diets without negatively affecting rumen fermentation.

The objective of Experiment 5 (Paper 6; Appendix) was to determine the effect of including agroindustrial by-products, such as dried distillers grains with solubles (DDGS), dried citrus pulp (DCP) and EOC in diets for dairy goats on nutrient digestibility, ruminal fermentation, methane production, urinary excretion of purine derivatives (PD) and milk yield and composition. Twelve Murciano-Granadina goats were used in a cross-over design trial with two periods. In each period, six goats received daily a control diet composed of 1 kg of alfalfa hay and 1 kg of high-cereal concentrate and other six goats received a diet (BYP) composed of 1 kg of alfalfa hay and 1 kg of a concentrate including corn DDGS, DCP and EOC in proportions of 180, 180 and 80 g/kg concentrate (as fed basis), respectively. Diet had no effect on total DM intake, but intake of alfalfa hay, CP and EE was greater ($P \leq 0.042$) for the BYP than for control group. There were no differences between diets in nutrients apparent digestibility, with the exception of fat which was greater ($P = 0.002$) for BYP compared with control diet. Although fecal nitrogen (N) tended to be greater ($P = 0.063$) for BYP-diet, there were no differences in N utilization. Compared to control, milk yield tended to be greater ($P = 0.056$) and daily production of milk CP, fat, whey

protein and total solids (TS), as well as milk gross energy, were greater ($P \leq 0.031$) for BYP diet. Diet BYP promoted a healthier fatty acids (FA) profile in milk, as the concentration of C12:0, C14:0 and C16:0 FA was or tended to be lower ($P \leq 0.080$) and that of polyunsaturated FA (PUFA) was greater ($P = 0.001$) in the milk of BYP-fed goats compared with those fed the control diet. Diet had no effect on ruminal parameters (pH, VFA and $\text{NH}_3\text{-N}$ concentrations) and methane emissions, but urinary excretion of total PD tended to be lower ($P = 0.070$) in BYP-fed goats than in those fed the control diet. A mixture of corn DDGS (180 g), DCP (180 g) and EOC (80 g) could replace 44% of cereal grains and protein feeds in the concentrate for dairy goats without compromising nutrient utilization, ruminal fermentation or milk yield, and led to a healthier FA profile in milk.

The objective of Experiment 6 (Paper 4) was to verify lower ^{15}N abundances of ADIN than of total N and neutral detergent insoluble N (NDIN) in different feeds, and to assess the magnitude of ADIN contamination. Samples from one corn crop (aerial biomass, grain and root) and two sunflower (SF) crops (aerial biomass and seeds) were analysed for ^{15}N abundance and content of total N, NDIN and ADIN. Samples had been ^{15}N -labelled by fertilizing with either $^{15}\text{NH}_4\text{NO}_3$ or $\text{NH}_4^{15}\text{NO}_3$, and there were three replicates per type of sample. The ^{15}N -abundance of total N and NDIN was similar ($P > 0.05$) for all samples, whereas that of ADIN was lower ($P < 0.001$ to 0.037) than values for total N and NDIN for all samples, excepting for SF seeds from one crop. Estimates of N-contamination of ADIN from cetyltrimethylammonium bromide (CTAB) were not affected by the fertilizer used to label N ($P \geq 0.207$), and values (g/g) ranged from 0.129 for corn grains to 0.447 for corn aerial biomass. N-contamination of ADIN was positively correlated with NDF, acid detergent fibre (ADF), hemicelluloses and cellulose content ($P < 0.001$; $r = 0.770$ to 0.905 ; $n = 18$ and 22 for corn and SF, respectively), and negatively ($P < 0.001$ to 0.048) with ADIN content (expressed as proportion of ADF). The negative correlations ($P < 0.001$) between N-contamination of ADIN and acid detergent lignin (ADL)/ADF ratio observed for all samples would indicate that N-contamination of ADIN was reduced by cell wall lignification. In conclusion, the results showed that the use of CTAB in the ADF analysis resulted in increased ADIN values, and therefore overestimated the amount of N associated to ADF, questioning the use of ADIN as a parameter indicative of N availability.

RESUMEN

El uso de subproductos agroindustriales en alimentación animal requiere conocer su valor nutritivo. Sin embargo, estos subproductos son heterogéneos y como consecuencia, su composición química y valor nutritivos son muy variables, además de que la información al respecto es escasa. Por esta razón, el objetivo de esta Tesis Doctoral fue evaluar dos subproductos producidos en grandes cantidades en España: orujo de aceituna (OC) y pulpa de tomate (TP). Para lograr este objetivo, se llevaron a cabo seis Experimentos (cinco *in vitro* y uno *in vivo*).

El objetivo del primer experimento (Artículo 1) fue analizar el efecto del tiempo de almacenaje y su posterior procesado: OC crudo (COC), OC extractado (EOC), OC ciclón (CYOC), en el valor nutritivo. Se utilizaron doce muestras (seis COC y seis EOC) obtenidas a intervalos de un mes de la misma balsa con un tiempo de almacenamiento de 1 a 6 meses, y nueve muestras (tres COC, tres EOC y tres CYOC) obtenidas a intervalos de un mes de una balsa diferente con un tiempo de almacenamiento de 6 a 9 meses. Se analizó la composición química y se fermentaron las muestras *in vitro* con líquido ruminal de oveja. El aumento del tiempo de almacenamiento hasta los 6 meses redujo el contenido en azúcares y polifenoles totales solubles (TSP), pero aumentó el contenido en fibra. La degradabilidad efectiva de la materia seca (DMED) se redujo de forma lineal ($P < 0,001$) en un 35,9 y 45,5% al aumentar el tiempo de almacenamiento de 1 a 6 meses en las muestras de COC y EOC, respectivamente. Las muestras de COC tuvieron una menor degradabilidad efectiva de la materia seca (DMED) que las muestras de EOC (valores medios de 0,255 y 0,294 g/g, respectivamente). Tanto la producción potencial de gas (PGP) como el ritmo medio de producción de gas (AGPR) fueron menores ($P \leq 0,018$) en las muestras de COC que en las muestras EOC, y esto se debió al mayor contenido en extracto etéreo (EE) en las muestras de COC (≥ 86 g/kg materia seca (DM)). En las muestras con un tiempo de almacenamiento superior a 6 meses, las muestras CYOC tuvieron mayores ($P < 0,05$) valores de DMED que las muestras COC y EOC (valores medios 0,207, 0,164 y 0,164 g/g, respectivamente). Los resultados indican que la degradabilidad ruminal del OC se reduce al aumentar el tiempo de almacenamiento, pero únicamente se observan pequeños cambios durante los dos primeros meses. El CYOC tuvo mayor degradabilidad ruminal que el COC y EOC, pero las diferencias entre estos dos últimos fueron reducidas a partir de los 5 meses de almacenamiento.

El objetivo del Experimento 2 (Artículo 2) fue estudiar la variabilidad en la composición química y la fermentación ruminal *in vitro* del OC. Para ello se fermentaron *in vitro* con líquido ruminal de ovejas 42 muestras de OC con diferentes tiempos de almacenamiento (1 a 14 meses) y procesado (25 COC, 9 EOC y 9 CYOC). Las muestras de EOC tuvieron menor contenido en EE que las muestras de COC y CYOC (15,9, 110 y 157 g/kg DM, respectivamente), pero mayor contenido en fibra neutro detergente (NDF; 645, 570 y 441 g/kg DM) y nitrógeno insoluble en solución ácido detergente (ADIN; 9,76, 8,10 y 8,05 g/kg DM). Las muestras de EOC tuvieron el mayor ($P < 0,05$) ritmo fraccional de producción de gas (AGPR), mientras que las muestras de EOC y CYOC mostraron la mayor cantidad de materia orgánica fermentada (FOM). La mejor variable para predecir el AGPR fue el contenido en azúcares ($R^2 = 0,898$), mientras que el contenido en NDF fue la mejor variable para predecir la FOM ($R^2 = 0,767$; $P < 0,001$). Los modelos estadísticos que utilizaban el tiempo de almacenamiento como variable predictora tuvieron una menor precisión y menores valores de R^2 en comparación con aquellos que utilizaban la composición química. En resumen, el valor nutritivo del OC depende del procesado, pero su contenido en EE no afectó negativamente a los parámetros de la fermentación ruminal, que podrían ser estimados a partir de su contenido en carbohidratos o del tiempo de almacenamiento.

El tercer Experimento (Artículo 5; Apéndice) se llevó a cabo para estimar *in vitro* el valor nutritivo de cuatro muestras de COC con diferentes tamaños de partícula, que fueron obtenidas durante el proceso de secado en un mismo trommel en diferentes momentos del proceso de tamizado. Los tamaños de partícula fueron: mayor de 3 mm (COC3), 2 mm (COC2) y 1 mm (COC1) o menor de 1 mm (COC0). En este experimento se incluyeron heno de alfalfa y paja de cebada con fines comparativos. Se analizó la composición química en todas las muestras. Los parámetros de producción de gas se obtuvieron mediante una incubación *in vitro* de 120 horas de duración empleado líquido ruminal de oveja como inóculo, y los parámetros fermentativos y la digestibilidad *in vitro* de la materia seca (DMD) se analizaron en una incubación de 24 horas. Las muestras COC3 y COC0 tuvieron menores contenidos de NDF (415 y 391 g/kg DM), pero mayor contenido en proteína bruta (CP; 96,6 y 114 g/kg DM) y EE (220 y 242 g/kg DM) que las muestras COC2 (627, 42,4 y 102 g/kg DM, respectivamente) y COC1 (624, 48,7 y 124 g/kg DM). La DMD de las muestras de COC osciló entre 39,4 y 58,7% y fue menor que la obtenida para el heno de alfalfa (67,5%), pero mayor que la

obtenida para la paja de cebada (42,4%) en las muestras COC0 y COC3. Las muestras COC3 y COC0 tuvieron un mayor ($P < 0,05$) producción potencial de gas (PGP; 103 y 66,2 ml/g DM, respectivamente) y una mayor producción de ácidos grasos volátiles totales (VFA; 608 y 600 μmol , respectivamente) que las muestras COC2 y COC1, pero los valores fueron menores que los del heno de alfalfa (202 ml/g DM y 1023 μmol , respectivamente) y la paja de cebada (198 ml/g DM y 733 μmol). Los resultados sugieren que reducir el tamaño de partícula a menos de 1 mm aumenta la fermentabilidad del COC, pero su valor nutritivo sigue siendo ligeramente inferior al de la paja de cebada. La calidad observada en las muestras COC3, similar a la del COC0 se atribuyó a un mayor contenido en azúcares solubles.

El objetivo del Experimento 4 (Artículo 3) fue analizar la variabilidad en el valor nutritivo para los animales rumiantes de la pulpa de tomate (TP) y analizar su efecto en la fermentación ruminal *in vitro* cuando se incluyó en una dieta con un elevado contenido en concentrado. Se obtuvieron 12 muestras de TP de dos plantas de procesado con intervalos de una semana y se analizó la composición química, la fermentación ruminal *in vitro* y la digestibilidad intestinal. No se observaron diferencias entre las plantas de procesado en la composición química TP y únicamente se observaron ligeras variaciones entre los diferentes tiempos de muestreo. Las muestras de TP tuvieron un reducido contenido en DM (< 300 g/kg), un alto contenido en NDF, CP y EE (572, 160 y 82,7 g/kg DM de media, respectivamente), y fueron rápidamente fermentadas *in vitro*. La degradabilidad ruminal de la proteína, obtenida tras una incubación *in situ* de 16 h, fue 510 g/kg y la digestibilidad intestinal *in vitro* de la CP (IDCP) fue baja, oscilando entre 430 y 475 g/kg. Reemplazar parcialmente la harina de soja y paja de cebada por TP en una dieta rica en concentrado aumentó el ritmo de fermentación *in vitro* y la producción de VFA y redujo la concentración de $\text{NH}_3\text{-N}$ sin afectar a la producción de metano. En resumen, las muestras de TP mostraron poca variabilidad en su valor nutritivo entre los diferentes tiempos de muestreo y podría incluirse TP hasta 180 g/kg en una dieta rica en concentrado sin afectar negativamente a la fermentación ruminal.

El objetivo del Experimento 5 (Artículo 6: Apéndice) fue determinar el efecto de incluir subproductos agroindustriales, en concreto DDGS de maíz (*dried distillers grains with solubles*), pulpa de cítricos desecada (DCP) y EOC, en un concentrado para cabras lecheras en la digestibilidad de los nutrientes, la fermentación ruminal, la

producción de metano, la excreción urinaria de derivados púricos (PD) y la producción y composición de la leche. Para ello, se usaron doce cabras de raza Murciano-Granadina en un diseño experimental cruzado con dos periodos. En cada periodo, seis cabras recibieron una dieta control, compuesta de 1 kg de heno de alfalfa y 1 kg de concentrado basado en cereales, y las otras seis cabras recibieron una dieta (BYP) compuesta de un 1 kg de heno de alfalfa y 1 kg de concentrado con 180, 180 y 80 g/kg (en materia fresca) de DDGS, DCP y EOC, respectivamente. La dieta no afectó a la ingestión de materia seca de concentrado, pero la ingestión de heno de alfalfa, CP y EE fue mayor ($P \leq 0,042$) con la dieta BYP. No se observaron diferencias entre dietas en la digestibilidad aparente de los nutrientes, excepto en la digestibilidad de la grasa que fue mayor ($P = 0,002$) para la dieta BYP. A pesar de que el nitrógeno (N) fecal tendió a ser mayor ($P = 0,063$) con la dieta BYP, no hubo diferencias en la utilización del N. En comparación con la dieta control, la producción de leche de las cabras alimentadas con la dieta BYP tendió a ser mayor ($P = 0,056$) y la producción diaria de CP, grasa, proteína del suero y sólidos totales (TS) en la leche, así como la energía bruta, fue mayor ($P \leq 0,031$) que en las cabras que recibían la dieta control. La dieta BYP favoreció un perfil de ácidos grasos (FA) en la leche potencialmente más saludable, ya que la concentración de C12:0, C14:0 y C16:0 FA fue o tendió a ser menor ($P \leq 0,080$) y el contenido en FA insaturados (PUFA) fue mayor ($P = 0,001$) que con la dieta control. La dieta no tuvo ningún efecto sobre los parámetros ruminales (pH, VFA y $\text{NH}_3\text{-N}$) ni en las emisiones de metano, pero la excreción de PD tendió a ser menor ($P = 0,070$) en las cabras alimentadas con la dieta BYP. Por todo ello, una mezcla de DDGS de maíz (180 g), DCP (180 g) y EOC (80 g) podría reemplazar parcialmente los granos de cereales y las fuentes de proteína de los concentrados de cabras lecheras hasta en un 44% sin afectar a la utilización de los nutrientes, la fermentación ruminal o el rendimiento lechero, y adicionalmente mejoraría el perfil de FA de la leche.

El objetivo del Experimento 6 (Artículo 4) fue verificar la existencia de menores abundancias de ^{15}N en la fracción ADIN en comparación con el N total y el N insoluble en solución neutro detergente (NDIN) en diferentes alimentos, así como determinar la magnitud de la posible contaminación nitrogenada del ADIN. Para ello se analizaron muestras de un cultivo de maíz (biomasa aérea, granos y raíces) y de dos cultivos de girasol (SF) (biomasa aérea y semillas), determinándose la abundancia en ^{15}N así como el contenido en N total, NDIN y ADIN. Las muestras fueron marcadas con ^{15}N

mediante la fertilización con $^{15}\text{NH}_4\text{NO}_3$ o $\text{NH}_4^{15}\text{NO}_3$. Se utilizaron tres réplicas para cada tipo de muestra. La abundancia de ^{15}N en el N total y NDIN fue similar ($P > 0,05$) en todas las muestras, mientras que la abundancia en el ADIN fue menor ($P < 0,001$ a $0,037$) en todas las muestras, a excepción de las semillas de SF de uno de los dos cultivos. Las estimaciones de la contaminación con N del ADIN a partir del bromuro de cetiltrimetilamonio (CTAB) no se vieron afectas por el tipo de fertilizante usado para marcar las muestras ($P \geq 0,207$), y los valores (g/g) oscilaron entre 0,129 para los granos de maíz hasta 0,447 para la biomasa aérea del maíz. La contaminación con N del ADIN estuvo correlacionada positivamente con el contenido en NDF, ADF, hemicelulosa y celulosa ($P < 0,001$; $r = 0,770$ a $0,905$; $n = 18$ y 22 para maíz y SF, respectivamente), y negativamente ($P < 0,001$ a $0,048$) con el contenido en ADIN (expresado como proporción de la ADF). La correlación negativa ($P < 0,001$) entre la contaminación con N del ADIN y la relación lignina ácido detergente (ADL)/ADF observada en todas las muestras indicaría que la contaminación del ADIN se reducía al aumentar el nivel de lignificación de la pared celular. En resumen, los resultados indican que el uso de CTAB en el análisis de la ADF aumenta el contenido en ADIN, y por ello sobreestima el contenido de N asociado a la ADF, cuestionando el uso del ADIN como parámetro indicativo de la disponibilidad del N.

Chapter I. INTRODUCCION

I.1. Introduction

Spain is, after Italy, the second largest European fruit and vegetable producer, and the greatest exporter of these products in the European Union (EU). According to the Ministry of Agriculture, Fisheries and Food (MAPA, 2019a), the value of the production of the fruit and vegetable sector in our country is about 14,000 million of euros, and accounts for 47% of total vegetal production and 29% of the agricultural production. The fruit and vegetable sector has also a powerful processing industry that annually generates almost 9 million tons of by-products (skins, bones, pulps, stems, fruits wastes, etc.), as the proportion of wastes can range between 12 and 63% of the processed vegetables or fruits (Alfonso et al., 2010). These by-products usually have high content in water, organic matter (OM) and nutrients, being easily putrescible and causing environmental pollution problems. The generation of these by-products is unavoidable and alternatives for later use are required, such as the extraction of bioactive compounds for human health and food, their use in farm animal nutrition or as fertilizers, or other options (Pascual Vidal, 2015). The use of agroindustrial by-products in animal feeding is currently one of the most valuable alternatives, as it would contribute to reduce feeding costs and the environmental contamination by incorporating into the production chain materials with high pollution potential. The use of agroindustrial by-products in animal feeding would also contribute to sustainability of livestock systems, reducing the carbon footprint of animal products due to their regional/local production (del Prado et al., 2013) and helping to close nutrient cycles locally (Pascual Vidal, 2015). By-products can also contain bioactive compounds that may improve not only animal health but also the quality of animal products, and may also reduce methane emissions contributing to decrease the environmental impact of livestock production (Romero-Huelva et al., 2012).

The use of any unconventional feed, such as agroindustrial by-products, in animal feeding requires precise information on its nutritional value. Matching the nutrient content in the diet to the nutrient requirements of the animals is a crucial point to optimize animal production and the quality of animal products, but also to reduce the environmental impact of livestock production. Therefore, the optimal inclusion of an unconventional feed in the diet requires investigations on its nutritional value and its impact on both the final product (meat, milk, etc.) and the environment.

Ruminant animals can use fibrous by-products more efficiently than non-ruminants, but the nutritive evaluation of their feeds is complex, especially when feeds have highly variable composition, as occurs with some agroindustrial by-products. These difficulties have contributed to the lack of data on the nutritive value of some agroindustrial by-products and data available in the literature are not always representative of those generated in our country. In addition, direct extrapolation of values obtained in one animal species to others is not appropriate, and a specific nutritive evaluation is required for each animal species. Whereas some agroindustrial by-products are well known and have been used successfully in animal feeding for decades (i.e., sugar beet pulp, corn distillery grains, ...), others are less used due to uncertainty regarding their availability, palatability, nutritive value and the risk of toxicity.

There are characteristics of some agroindustrial by-products generated in large amounts in our country that currently limit their practical use in animal feeding, and have also contributed to the limited data available on their nutritive value. One of these characteristics is their heterogeneity, which is due not only to the variability of the original raw material but also to the different processing methods used. As a consequence, values for chemical composition and nutritive parameters found in the literature for a single type of by-product have high coefficients of variation (CV) (Feedipedia, 2019; DePeters et al., 2010; De Blas et al., 2010) that prevent their inclusion in the diets without previous analysis. Another aspect that complicates their routinely use in practical feeding is that some by-products are markedly seasonal, so that their production accumulates in a short period of time and a conservation procedure is required; however, conservation procedures usually increase the price of by-products and frequently makes their use not worthwhile. The practical use of a by-product in animal feeding depends on numerous factors, but its nutrient content, availability, price, and ability to be stored, often determined by its water content, are the main factors.

In ruminants most of digestion occurs in the reticulum-rumen, and therefore the characterization and quantification of final products of rumen fermentation is a crucial point for nutritive evaluation and the formulation of balanced diets. The study of ruminal fermentation *in vivo* requires expensive, laborious and long-term trials. These problems, together with the increased public awareness of animal rights and the need for decreasing the number of fistulated animals used for experimental purposes, have

contributed to the increasing use of *in vitro* techniques for nutritive evaluation (Carro et al., 2009).

The objective of this literature review is to analyze the state of the art on production and nutritive value of two agro-industrial by-products generated in large amounts in Spain (olive cake (OC) and tomato pomace (TP)) and on their effects on animal performance and quality of animal products when they are fed to small ruminants. Additionally, it includes short review on the production and use of citrus pulp and dried distillers grains with solubles (DDGS) on ruminant feeding, as these two by-products were used in one experimental trial of this Doctoral Thesis. Finally, the nutritional significance of the acid detergent insoluble nitrogen (ADIN) fraction of feeds is revised, as the ADIN analysis is used by some ruminant feeding systems as a measure of nitrogen (N) availability. Some agroindustrial by-products are generated in processes involving heating, which can increase their ADIN content (Vanegas et al., 2017), and ADIN is widely accepted as a good measure of heat damage in feeds (NRC, 2001; Goering et al., 1972).

I.2. Olive cake

I.2.1. Olive cake production

Olive oil production is continuously increasing worldwide, but most of the production is concentrated in the Mediterranean countries and Spain, Greece, Italy and Tunisia are the greatest producers (Consejo Oleícola Internacional, 2018). Spain is the world's leading olive oil producer, with an average production of 1.2 millions of tons per season between 2007 and 2013, which was increased by 23% compared to that in the previous decade. This figure represents 60% of the oil produced in the EU and 45% of the total in the world (MAPA, 2018). About 6.5 millions of t of olives were produced in Spain in 2018, being 91% of them used for olive oil production and only 9% consumed as table olives (MAPA, 2018). Olive oil production generates different by-products, such as OC, olive leaves, olive stones, and molasses (Molina-Alcaide and Yáñez-Ruiz, 2008). The generation of OC is later described. Olive leaves are a mixture of leaves and branches which are generated during the harvesting of olives and the cleaning of olive trees, whereas olive stones are obtained by separation from the pulp and are used as energy source. Finally, molasses are the dried and transformed watery waste produced during the oil extraction (Molina-Alcaide and Yáñez-Ruiz, 2008). The

traditional method of oil extraction (Figure 1) consists of a three-phase extraction and in this process 100 kg of olives generates about 55 kg of wastes (Molina-Alcaide and Yáñez-Ruiz, 2008). Two types of wastes are generated in this process: an olive oil mill solid waste with a moisture content of around 40-50%, and huge quantities of wastewater, which is the main pollutant. Several biological, physiochemical and oxidation treatments have been developed to reduce the pollutant effects of the wastewater (Paraskeva and Diamadopoulos 2006). On the other hand, the solid waste still contains small amounts of oil, and normally undergoes a second oil extraction using hexane or other chemical solvents. Afterwards, the solid waste consists primary of lignin and cellulose, and is marketed either as mulch for olive trees or as a solid fuel (Rodríguez et al., 2008; Vlysidis et al., 2004). Currently, the most used olive oil extraction system in Spain is the two-phase system, in which no water is used, less energy is needed, and the wet waste generated contains less oil due to the greater efficiency of the extraction process (Albuquerque et al., 2004). This system decreases the amount of highly polluting wastewater, but generates a high-moisture (50-70% water) solid by-product known as “alperujo” (Centro de Actividades Regionales para la Producción Limpia, 2000). It is estimated that only 20 to 27% of the processed olive turns into oil, while the rest are by-products generated in the process (Awawdeh and Obeidat, 2013). The “alperujo” is a mix of stone pieces, rest of pulp from the olive fruits, and variable amounts of water and oil, and has a high pollution potential. Olive oil production is highly seasonal and great amounts of “alperujo” are generated during the olive oil campaign (Albuquerque et al., 2004). The “alperujo” is sent to the “orujeas” where is stored in large ponds. Subsequently, the “alperujo” is dried and the bones are removed to obtain the crude OC (COC) that is mainly composed of the rest of the pulp and stones, the olive skin, and water (Molina-Alcaide and Yáñez-Ruiz, 2008). It is calculated than in Spain, between 25 and 40 kg of COC are generated per 100 kg of processed olives, accounting for a total production of about 300,000 t per year (De Blas et al., 2010).

Figure 1. Phases of olive oil extraction and by-product production (Habeed et al., 2017).

Chemical composition of COC is highly variable and depends on the content of oil and wastewater. The COC can be subjected to a second chemical extraction, either with hexane or with other authorized food-grade solvents, to extract the residual oil that is marketed as “pomace oil”, and the resulting by-product is called exhausted OC (EOC). In the last part of this process, the solvent is “flushed off” by heating. The chemical composition of EOC differs from that of COC, and is markedly affected by the extraction procedure (De Blas et al., 2010).

The chemical composition of COC is influenced by the chemical composition of the olive fruits and their characteristics, such as the content of skin, pulp and stones, but also by the added water and the amount of oil extracted during the processing to obtain the oil. The edible portion of the olives is mainly composed of water and lipids, which

can range from 14 to 30% depending on the cultivar and the ripening stage (Bianchi, 2003). Olives have a high content of monounsaturated fatty acids (MUFA), especially oleic acid (47-87%) and important amounts of polyunsaturated fatty acids (PUFA), such as linoleic and linolenic acids. On the contrary, the content of sugars is low compared with other fruits (Servili et al., 2016). Phenolic compounds are also present in olives, although the amounts are variable (1-3%), and the most abundant are oleuropein, demethyloleuropein, hydroxytyrosol and verbascoside (Romero et al., 2017; Servili et al., 2016; Blekas et al., 2002).

The oil extraction procedure also affects the chemical composition of COC, as influences the proportion of pulp, stones, water and remaining oil (De Blas et al., 2015; Vlyssides et al., 1998). In addition, the quality of the olive and the extraction efficiency are related with the composition and texture of the olive fruits (Rallo et al., 2018). Another source of variation is the genetic selection programs which are directed towards an increase in the yield of olive oil, and therefore differences in chemical composition between industrial and traditional olive fruits are expected. Soil properties affect water availability, which in turn can affect fruit ripeness (Rallo et al., 2018) and therefore the composition of the generated COC. The type of soil in which olive trees are cultivated also influences the mineral composition of olive fruits (Rallo et al., 2018), as well as climatic conditions. Thus, low temperatures (below -3 °C) have been reported to affect the oxidative processes (Sanzani et al., 2012), decreasing the concentration of most phenolic compounds. On the contrary, high temperatures during the ripening period have a negative effect on fruit weight, oil content, and even fatty acid (FA) composition, as oleic acid decreases (García-Inza et al., 2014). The altitude at which olive trees are cultivated also influences FA composition and phenols content. Strong droughts are reported to affect negatively the fruit size and the pulp/stone ratio, and therefore, irrigation increases the water content, fruit size and the pulp/stone ratio (Rallo et al., 2018). Other factor that can affect the chemical composition of COC is the inclusion of other substances during the processing of olive fruits. The processing olive fruits with high moisture (> 50%) and low non-fat dry matter (around 25%) generates a mixture called “difficult pastes”, that have a negative effect on the extraction process, as they form oil-water emulsions that reduce the efficiency of oil extraction and part of the oil is lost with the residues (Rallo et al., 2018). This negative effect can be minimized by including substances such as talc (hydrated magnesium silicate) that can modify the

composition of COC (Koprivnjak et al., 2016; Canamasas and Ravetti, 2014).

1.2.2. Chemical composition and characteristics of olive cake

In general, COC is characterized by high fibre, polyphenols and fat content, with an average of 54.5 g/kg dry matter (DM) (Molina-Alcaide and Yáñez-Ruiz, 2008), being especially rich in oleic acid (Chiofalo et al., 2004). The content in neutral detergent fibre (NDF) and acid detergent lignin (ADL) is usually high and can reach up to 795 and 320 g/kg DM, respectively. The degradability of the NDF is low, with values that are usually below 30% (De Blas et al., 2010). The crude protein (CP) content is rather low (from 48 to 106 g/kg DM) and it is worth noticing that most CP is bound to the acid detergent fibre (ADF) (Kotsampasi et al., 2017). Removing the stones of the OC can improve its nutritional value, as fibrous components vary largely with the proportion of stones in OC (Molina-Alcaide and Yáñez-Ruiz, 2008) and stones can cause injuries to the digestive epithelium of the animals (De Blas et al., 2010). Yansari et al. (2007) found that destoning and exhausting of olive cake significantly increased CP, N-free extracts and non-fibrous carbohydrates content, and reduced its fat, NDF, ADF and ADL contents.

There are marked differences between types of OC, being the fat content one of the most important. Whereas COC usually has a high fat content with a high proportion of oleic acid (Chiofalo et al., 2004), the EOC obtained after the second oil extraction has lower fat content, but a greater amount of CP bound to the ADF due to the heat treatments that can cause Maillard reactions (Álvarez-Rodríguez et al., 2009). The extraction temperature can also cause a loss of lipophilic antioxidants and other bioactive compounds, such as phenolic compounds (Martín-García et al., 2003; Amro et al., 2002). These compounds are important, as they can improve the meat quality by increasing its oxidative stability when OC is used in the feeding of meat production animals. On the contrary, the drying temperature of the “alperujo” to obtain the COC does not affect the content of bioactive compounds, but can modify the FA profile (Uribe et al., 2013). Some studies have reported differences between COC and EOC in the amount of phenolic compounds (Molina-Alcaide and Yáñez-Ruiz, 2008), but the phenolic content is also affected by the chemical composition of the olive fruit caused by agronomical factors (Romani et al., 1999), the ripening of the fruit (Amiot et al., 1986), climatic conditions (Vinha et al., 2005) and irrigation management (Patumi et al.,

2002), among other factors.

The production of olive by-products is highly seasonal, and in order to use them in animal feeding, an efficient preservation and storage is required. One of the drawbacks of employing COC in animal nutrition is its high moisture content, which causes storage problems and environmental issues (Arco-Pérez et al., 2017). A possible solution to overcome these problems would be drying the COC, but the treatment is expensive. Other solutions include feeding COC fresh, ensiled, or as a component of concentrate pellets or feed blocks, especially in ruminant diets (Panagiotis et al., 2018), and several studies have analysed these options. Ensiling COC is an adequate and cost-effective technology that, additionally, can improve the nutritional quality of the ensiled feed (Meneses et al., 2007). The COC silages can include poultry manure, conventional feeds, urea or alkali (Molina-Alcaide and Yáñez-Ruiz, 2008). Arco-Pérez et al. (2017) showed that silages including oil by-products and sunflower can be included in the diet of dairy goats with no negative effects on rumen fermentation or milk production and increased the nutritional value of the milk. Another option might be the inclusion of COC into feed blocks, which are a simple, low cost and efficient technique for long-term storage of high-moisture by-products, consisting in a solidified mixture of feed ingredients, urea, binder and salt (Ben Salem and Nefzaoui, 2003). Molina-Alcaide et al. (2010) observed that replacing partially the concentrate of dairy goats with feed blocks including COC had no negative effects on nutrient utilization and improved milk quality. In another study, Ben Salem and Znaidi (2008) fed feed blocks containing 44% of COC to lambs and observed no negative effects on growing performance.

1.2.3. Nutritive value of olive cake

All types of OC are characterized by low nutritive value (Sansoucy et al., 1985), as they usually have high content of NDF and ADF (Martín-García et al., 2003; Nefzaoui, 1983) and low degradability of the cell wall (Yansari et al., 2007; Filya et al., 2006). The low degradability can be improved by the treatment with polyethylene glycol (PEG) and NaOH and (Molina-Alcaide and Yáñez-Ruiz, 2008; Molina-Alcaide and Aguilera, 1988). Moreover, OC contains antinutritive compounds, such as polyphenols and tannins, which may decrease both its palatability and digestibility (Makkar, 2003). The low nutritional value of OC makes its use in high-yielding dairy cows inefficient (Chiofalo et al., 2004), but its utilization in dairy ewes and goats is possible, as they

have developed specific mechanisms in order to utilize low-quality feeds (Lanzani et al., 1993). Yáñez-Ruiz et al. (2004a) reported that ruminal environment of goats is more efficient in fermenting diets including OC than that of sheep. Nevertheless, the specific knowledge about the nutritive value of different types of OC, such as the factors influencing its chemical composition and the intestinal digestibility of the rumen undegraded protein, is still scarce.

Martín-García et al. (2003) reported values of effective rumen degradability for CP of OC ranging from 0.38 to 0.44 measured *in situ* in sheep, but the values increased up to 0.51 with PEG treatment. These values, together with the low CP content of OC indicate a low supply of N for ruminal microorganisms. Similarly, degradabilities of OM and ADF are reported to be low, with average values of 0.51 and 0.37, respectively, but these can be improved when OC is treated with ammonia or NaOH (Nefzaoui and Vanbelle, 1986). On the contrary, the rumen degradability of amino acids was reported to be high (0.90 as average), and among them, lysine, phenylalanine, threonine, tyrosine, isoleucine and leucine had the greatest degradability values and cysteine the lowest. (Molina-Alcaide and Yáñez-Ruiz, 2008). Reported values for total and amino acid-N content of the rumen undegraded fraction of OC are 10.8 and 7.30 g/kg DM, accounting for 0.612 and 0.431 of total N, respectively (Martín-García et al., 2003). The most abundant amino acid in this fraction were glutamic acid, alanine and valine, whereas histidine, tyrosine and lysine has the lowest concentrations. The apparent intestinal digestibility of the most abundant and least abundant amino acids were 0.86 and 0.37, respectively (Martín-García et al., 2004).

Studies of rumen fermentation parameters in animals fed OC are limited. Nefzaoui (1985) observed that ruminal concentrations of $\text{NH}_3\text{-N}$ in sheep fed EOC were below the recommended minimum values for an optimal microbial activity (Firkins et al., 2007). However, other studies have obtained better results by introducing OC in concentrate formulation. Thus, Yáñez-Ruiz et al. (2004b) partially replaced alfalfa hay with a concentrate that included OC, and they observed similar nutritive value and digestibility than with a medium quality diet. It has been reported that pelleting OC reduced the OM digestibility, probably due a decrease in rumen retention time caused by the reduction in particle size (Molina-Alcaide and Aguilera, 1988). The digestibility of OC varies depending on the type of OC, but the digestibility of OM and CP are generally low, whereas the digestibility for ether extract (EE) is usually high (0.60-0.90)

regardless of the OC type or the processing method used (Theriez and Bouler, 1970).

I.2.4. Effects of feeding olive cake to meat-producing ruminants

Despite the low nutritive value reported for OC, many studies have investigated the effects of its inclusion in the diet in dairy and meat goats and sheep, with contrasting results. As pointed out by Molina-Alcaide and Yáñez-Ruiz (2008), from a practical standpoint, it is necessary to differentiate between COC and EOC. Tufarelli et al. (2013) reported no negative effect in the productive performance of lambs when they were fed a diet containing 20% of EOC in replacement of oat hay and soybean meal, and Kotsampasi et al. (2017) obtained similar results in lambs fed up to 24% of EOC. Sadeghi et al. (2009) also reported that dietary inclusion of 20% of EOC did not have any negative effect on lamb performance, but increased DM, NDF and CP digestibility. Other studies also showed that including up to 25% of COC in the diet had no detrimental effects on intake and growth of lambs (Awawdeh and Obeidat, 2011; Al-Jassim et al., 1997; Giozelgiannis et al., 1978) and goat kids (Hadjipanayiotou, 1994). In contrast, Mioč et al. (2007) observed that including 30% of COC in the diet of lambs decreased significantly daily gain, final weight, empty carcass weight and dressing percentage of lambs. When feeding high amounts of COC to ruminants it has to be taken into account that high concentrations of unsaturated FA can have negative effects on rumen microbiota, especially on cellulolytic activity (Maia et al., 2007).

The effects of OC on meat quality and FA profile of lambs has been investigated in different studies (Mele et al., 2014; Vera et al., 2009), and the results indicate that OC can increase meat quality. One of the main challenges in the meat sector is the preservation of quality during meat storage and retail display by delaying lipid oxidation, which is the major cause of meat deterioration (Faustman et al., 2010). The oxidative stability is highly dependent on the balance between muscle antioxidant and pro-oxidant components. The antioxidant components include, among others, tocopherols and carotenoids (Descalzo and Sancho, 2008). On the other hand, the PUFA in meat, especially those in the phospholipid fraction of cell membranes, are easily oxidized and some might act as pro-oxidants (Morrissey et al., 1998). The PUFA content in meat is especially interesting, because of their beneficial effects on human health. The intake of antioxidants in the diet can modify both the antioxidant and the pro-oxidant components of the muscle and protect PUFA from oxidation. Generally,

ruminant diets have low content in antioxidants, and thus meat is highly susceptible to oxidative deterioration (Luciano et al., 2012). Feeding concentrates with PUFA sources, such as linseed, is a common practice to increase PUFA concentration in the intramuscular fat. However, feeding PUFA sources without increasing the intake of antioxidants can have negative effects in terms of the oxidative stability of meat (Jacobsen et al., 2008). Compounds present in the OC, especially those in EOC, might influence the oxidative stability of meat. For example, Luciano et al. (2013) investigated the use of stoned COC (24.5% of the diet) combined with linseed in the diet of lambs to enhance the oxidative stability of PUFA-enriched meat. The results of this study demonstrated that the inclusion of COC had no detrimental effect on growth, carcass weight or muscle fat content, and the meat had higher stability to oxidation compared with the meat from lambs fed the diet including linseed without COC.

Several studies have reported changes in meat FA profile when ruminants were fed OC. Kotsampasi et al. (2017) observed a decrease in lauric acid and a slight increase in pentadecanoic acid in the meat of lambs fed a concentrate with 24% EOC as a result of different FA profile in the diet. These authors also observed an increase in α -linoleic acid in the intramuscular fat of the lambs fed the EOC, even though the content of α -linoleic in the concentrate did not differ with those of the control. Oleic, α -linoleic and α -linolenic acid undergo the same biohydrogenation pathways to produce primarily *trans* FA and stearic acid (Wood et al., 2008; Doreau and Ferlay, 1994), and as a consequence of the greater oleic acid content in the EOC concentrate, greater amounts of α -linolenic could have escaped rumen biohydrogenation, as oleic acid was the main substrate for biohydrogenation. Tannins can also influence the concentration of α -linoleic acid in the intramuscular fat of lambs, because they can inhibit the activity of some ruminal microorganisms, hence reducing the ruminal FA biohydrogenation (Vasta et al., 2009).

I.2.5. Effects of feeding olive cake to dairy ruminants

The results on the influence of olive cake feeding on milk yield and composition are also controversial. Many studies (reviewed by Molina-Alcaide and Yáñez-Ruiz, 2008) have reported that the performance of dairy ewes fed concentrates including OC is similar or even better than that of sheep fed concentrates with conventional feed ingredients, but negative effects have been observed in others. For example, Chiofalo et

al. (2004) reported an increase in milk yield when 20% of COC was included in the diet of dairy ewes, Vargas-Bello-Pérez et al. (2013) observed no effect on milk yield by including up to 25% of COC in the diet of dairy ewes, and Molina-Alcaide et al. (2010) found a decrease in milk yield in goats fed a diet containing 12% of COC. Although several factors can be involved in the variable response observed in these studies, differences in the composition of OC can help to explain these results.

The FA profile of ruminants milk is highly dependent on FA composition and fibre content of the diet (Vasta et al., 2008), although dietary PUFA are modified by an intense microbial biohydrogenation activity (Bauman and Griinari, 2003). The composition of the diet can also influence the concentration of fat-soluble micronutrients, such as β -carotene and α -tocopherol (Martin et al., 2004), and of plant secondary metabolites like phenolic compounds (O'Connell and Fox, 2001) in the milk of ruminants. These compounds can help to reduce the photo-oxidation of the milk fat globule membrane, which is the major site of auto-oxidation (O'Connell and Fox, 2001; Lindmark-Mansson and Akesson, 2000). Moreover, the presence of these compounds in the milk can increase its organoleptic and nutritional qualities (Noziere et al., 2006; O'Connell and Fox, 2001). Olive cake contains considerable amounts of plant secondary metabolites, such as different types of polyphenols (Vasta et al., 2008). The intake of these bioactive compounds is considered beneficial for human health as they reinforce the natural defense system, decreasing the formation of free radicals and the detrimental oxidative events in the metabolism (Parr and Bolwell, 2000). Thus, a transfer of these bioactive compounds to the milk is desired. The main antioxidative compounds in milk are enzymes, lactoferrin, vitamins C and E, and carotenoids (Noziere et al., 2006) and, to a lower degree, phenolic compounds (O'Connell and Fox, 2001). O'Connell and Fox (2001) reported that ruminant milk has considerable amounts of phenolic compounds, most of which are of dietary origin. Some studies showed that dietary polyphenolic compounds can modify PUFA rumen biohydrogenation with no negative effect on milk yield and quality (Buccioni et al., 2015; Ferlay et al., 2010). As a consequence, OC can modify milk FA composition by modulating ruminal biohydrogenation (Vasta et al., 2008).

Olive cake is characterized by high MUFA content (mainly oleic acid), and its inclusion in the diet of dairy ewes has been shown to reduce saturated FA (SFA) and increase MUFA content in milk (Vargas-Bello-Pérez et al., 2013; Chiofalo et al., 2004).

Abbeddou et al. (2011) reported that the inclusion of 30% of OC in the diet of dairy ewes decreased the amount of short- and medium-chain SFA in the milk. A decrease in medium-chain SFA (C12:0, C14:0 and C16:0) is considered beneficial for human health, as they have been related to increases in total and low density lipoprotein cholesterol concentrations (Williams, 2000). Both short- and medium-chain SFA are mainly synthesized *de novo* in the mammary gland and this process can be partly inhibited by long-chain FA (either from diet or body fat mobilization) and by *trans* FA from hydrogenated dietary fat or conjugated linoleic acid (CLA) supplements. The oleic acid present in the milk is mainly generated by mammary Δ^9 -desaturation of stearic acid (C18:0), but it can be transferred from the diet to the milk to a certain extent (Chilliard et al., 2007). This would explain the reason for greater concentrations of oleic acid in the milk of ruminants fed diets including OC (Chiofalo et al., 2004). Likewise, greater concentrations of stearic acid in the milk from ruminants fed OC are expected as a result of the greater oleic acid content in the diet. Oleic acid is regarded as a beneficial FA to human health (Dewhurst et al., 2006), whereas stearic acid is considered to have no effect on cholesterol values in the consumers of milk and dairy products. Stearic acid in milk has two main sources: the diet and the complete ruminal biohydrogenation of 18-PUFA (Gomez-Cortes et al., 2008; Chilliard et al., 2007). The biohydrogenation of oleic acid in the rumen generates stearic acid, together with a variety of 18:1 *trans* acids, with the exception of 18:1 *trans* 11. On the contrary, *trans* FA in milk is undesirable, as the intake of these acids is a risk factor for coronary heart disease and diabetes in consumers (Salmeron et al., 2001).

During the olive oil extraction process, more than 98% of total olive polyphenols end up in the wastewater. These polyphenols can be extracted to obtain a product rich in oleuropein-aglycone di-aldehyde and verbascoside (Servili et al., 2011), that can be used in animal nutrition to reduce the ruminal biohydrogenation and modify the FA profile of milk. Capucci et al. (2018) observed that supplementing dairy ewes diets with this olive crude phenolic concentrate and linseed did not have any detrimental effect on milk yield, but increased the content of α -linoleic acid in milk.

In conclusion, large amounts of COC and EOC are produced in our country and their use in ruminant feeding is a viable alternative to recycle them. Replacing conventional feeds by COC and EOC would help to reduce the environmental pollution caused by wastes disposal in the olive industry and contribute to sustainability of

ruminant farms by reducing feeding costs. The inclusion of OC in ruminant diets could also positively influence the quality and antioxidant capacity of meat and improve milk FA profile with no detrimental effects on productivity. However, more information on factors influencing chemical composition and nutritive value of OC is needed to facilitate its use in practical ruminant feeding.

I.3. Tomato pomace

I.3.1. Tomato pomace production

Tomato is one of the most common crop worldwide, and the fruits are rich in lycopene, phenols, organic acids, vitamins and other components (Giovanelli and Paradiso, 2002). According to the FAO statistics (FAO, 2016), tomatoes are mainly produced in China (5.64×10^7 tonnes), America (2.61×10^7 tonnes), India (1.84×10^7 tonnes), Turkey (1.26×10^7 tonnes), Egypt (7.94×10^6 tonnes), Italy (6.44×10^6 tonnes), Iran (Islamic Republic of) (6.37×10^6 tonnes), Spain (4.67×10^6 tonnes), Brazil (4.17×10^6 tonnes), and Mexico (4.05×10^6 tonnes). The total planting area was approximately 4.8×10^6 ha, with a production of approximately 1.8×10^8 tonnes (Lu et al., 2019).

Tomato cultivation currently occupies a total of 54,675 ha in Spain, which represents 15% of the area dedicated to vegetable production, and has an annual production over 4.8 million tons of tomatoes (MAPAMA, 2018). More 60% of total production is consumed fresh and the rest is processed to generate different products, such as paste, juice, sauce, puree and ketchup (Calsamiglia et al., 2016).

Tomato pomace is the by-product of tomato processing, and is made up of a mixture of peels, crushed seeds and small amounts of pulp that remain after processing to produce juices, creams, sauces, etc. (Ventura et al., 2009). Tomato pomace can represent between 3 and 6% of the original fruit weight (Del Valle et al., 2006), and has a high percentage of humidity ($> 70\%$) due to the water added in the final stage of the process to facilitate the exit through the ducts to the storage hoppers (Mendez-Llorente et al., 2014; López-Parra et al., 2011), which difficult its conservation (López-Gallego et al., 2015). Tomato pomace can be dried before being stored or transported, but artificial drying substantially increases its price. For this reason, a large part of the by-product produced is discarded or used fresh for animal feeding in farms located close to the processing plants (Mendez-Llorente et al., 2014; Denek and Can, 2006). As an

alternative, TP can be ensiled and constitutes a cheap source of energy and nutrients. The seasonal production of TP is linked to the tomato harvest period (Feedipedia, 2018), and if the wastes are not used, they not only cause a disposal problem, but also aggravate environmental pollution (Del Valle et al., 2006).

Like most agroindustrial by-products, the chemical composition of TP is highly variable depending on external factors, such as the variety of the crop, maturity of the fruit and growth conditions, and processing practices (Lu et al., 2019). For example, Kiosseoglou and Boskou (1989) reported that the FA profile of TP might differ among different cultivars, and Brodowski and Geisman (1980) observed that amino acid profile in tomato seeds was not influenced by fruit maturity, but the total amino acid content decreased as fruit maturity increased. Herrera et al., (2010) reported differences in fructose and glucose content in tomato peels between harvest years. Even though there are multiple factors that can affect the chemical composition of TP, it seems that two factors can stand out among the others: the final product form and the peeling method applied (Lu et al., 2019). For example, in the production of canned tomatoes, the TP is constituted exclusively of peels. On the other hand, the TP obtained when tomatoes are transformed into homogenized products such as paste, sauce or ketchup, contains a mixture of peels, seeds and a small amount of pulp. In the production of canned tomatoes, the peels are collected directly as TP, whereas in the production of homogenized products, the whole fruit is pressed and the remained material is collected, the seeds accounting for approximately 45% of the resulting TP (Kaur et al., 2005). However, in both cases peeling is an important process influencing TP chemical composition.

There is a lot of information related to the process of tomatoes peeling, and it is currently an active topic of research for food scientists. The traditional methods of peeling include hot lye and steam processes. In the hot lye peeling process, the outermost layer of tomatoes is dissolved and the lye penetrates the exocarp, causing the degradation of the cell wall components and the middle lamella between the epidermal and hypodermal cells, which leads to the stripping of tomato skins under specific friction or pressure washing (Rock et al., 2012). In the steam peeling method, complex thermal-biochemical changes are produced in the epidermis on pectinaceous, polysaccharide and cutin substances. Simultaneously, heating provokes pressure on the tomato body (García and Barrett, 2005), and this separates the peel from the tomato

pulp, making easier to remove the peel. Nonetheless, both processes represent a major environmental burden and other more environmental friendly peeling methods are currently being used, such as infrared, ohmic, ultrasonic and enzymatic peeling methods. Differences in lycopene content have been reported depending on the peeling process (Brown et al., 1970), and when different crushing methods were applied, the resulting tomato peels samples had different particle sizes and content of insoluble and soluble dietary fibre (Wang et al., 2016).

I.3.2. Chemical composition and characteristics of tomato pomace

Tomato pomace is mainly composed of peels and seeds, although it also contains variable amounts of residual pulp. Peels and seeds differ in chemical composition, and therefore chemical composition of TP is markedly influenced by the proportion of each component. Tomato peels are rich in fibre, lycopene and phenols, whereas the seeds are rich in fat and protein. The content of dietary fibre in tomato peels is high, ranging from 62.9 to 88.5%, with 1:5 ratios of soluble dietary fibre/insoluble dietary fibre (Navarro-González et al., 2011). This ratio is much lower than that reported for orange peels (1:1; Talens et al., 2017) and this suggests tomato peels have a low content of pectin and a high content of cellulose. However, pectin content in tomato peels has been reported to be variable (Alancay et al., 2017). Tomato peels are a good source of lycopene, with a concentration that can reach up to 0.288% (Zuorro and Lavecchia, 2013). On the contrary, reported values for lycopene content in tomato seeds are lower (0.013%; Knoblich et al., 2005), although seeds contain relatively high amounts of phenols (0.158%) and of caffeic, procatechoic, vanillic, catechin and gallic acids (Elbadrawy and Sello, 2016), ferulic, sinapic, chlorogenic, *p*-coumaric, and *trans* cinnamic acids (Valdez-Morales et al., 2014), as well as of flavonoids such as quercetin-3-O- β -glucoside, rutin, *iso*-rhamnetin, kaempferol, naringenin, quercetin, apigenin and myricetin. Tomato seeds have low content of dietary fibre and their fat content can range between 17.8 and 24.5% (Mechmeche et al., 2017; Giuffrè and Caposcasale, 2016; Yilmaz et al., 2015). Tomato seed fat is rich in linoleic, oleic, palmitic, stearic and arachidic acids, and has a high proportion of unsaturated FA, reaching as average 80% of total FA. The most abundant FA is linoleic acid (37.6-72.7% of total FA) followed by oleic acid (15.5-29.7%; Yilmaz et al., 2015; Botineştean et al., 2014; Kamazani et al., 2014; Cantarelli et al., 1993). The other main component in tomato seeds is protein, which accounts for 23.6 to 40.9%. Glutamic acid is the most abundant

amino acid (19.4-24.4 %) followed by aspartic acid (8.82-10.3%; Latlief and Knorr, 2010). Compared with cereals protein, tomato seeds have greater levels of lysine (3.4-5.9% vs < 1%; (Mechmeche et al., 2017; Latlief and Knorr, 2010; Lumpkins and Batal, 2005).

As stated before, the chemical composition of TP is highly variable depending on many factors, but the seeds/peel ratio is the major one. Tomato pomace has low DM content, which can vary between 15 and 34% depending on the amount of water added during processing to facilitate the transport of the pulp through the outlet ducts.

Barroso et al. (2017) observed that TP has a moderate nutritional value and reaches, in some cases, protein contents greater than 18% of DM. Composition in essential amino acids of TP is similar to soybean meal protein, but intestinal digestibility of the undegradable fraction might differ (Drouliscos, 1976). In addition, TP has a high content in FND and FAD (as average 58 and 51%, respectively) and an appreciable content (9%) of fat (Calsamiglia et al., 2016). However, its lignin content is high and can exceed 20% of the DM (Feedipedia, 2018; Marcos et al., 2017), which reduces the degradability of the fibre. Tomato pomace contains numerous biologically active substances (lycopene, phenols, flavonoids...), that have antioxidant, hypolipidemic and anticarcinogenic activities (Sánchez-Zapata et al., 2004), being lycopene one of the most abundant. Lycopene is a non-provitamin A carotenoid that is responsible for the red color of tomatoes. The antioxidant effects of lycopene include a considerable reactive oxygen species scavenging activity, which prevents lipid peroxidation and DNA damage and enhances the cellular antioxidant defense systems (Kelkel et al., 2011). Therefore, TP is considered an intermediate energy and protein source for animal feeding, and a valuable source of bioactive compounds such as lycopene, vitamins C and E, phenols, and other carotenoids (Belović et al., 2016).

I.3.3. Nutritive value of tomato pomace

Tomato pomace has a moderate nutritional value, but has some special characteristics that makes it a valuable by-product for ruminant feeding, such as its high CP and EE content. Neutral detergent fibre and ADL content are also high, and are responsible for the moderate OM digestibility values. Average *in vivo* OM digestibility of TP has been reported to be 56% and ruminal OM degradability was 48.0% (Abbeddou et al., 2011, Gasa et al., 1989). Values measured *in situ* for DM

degradability ranged from 38.9 % (Fondevila et al., 1994) to 62.0% (Ben Salem and Znaidi, 2008). A wide range of values of *in situ* ruminal degradability of CP of TP has been reported. Abbeddou et al. (2011) and Salem and Znaidi (2008) reported high values that ranged between 65 and 78%, whereas Fondevila et al. (1994) reported values of 40.6%. Ventura et al. (2009) reported much lower values of CP degradability in tomato fruits wastes (< 20%). These differences are probably related to variability in chemical composition of TP, but might be also due to differences in the *in situ* procedure, that is affected by many factors such as animals, diet, incubation procedure, etc. (Weakley et al., 1983). According to Ventura et al. (2009) the high proportion of protein linked to the ADF, which can reach half of total CP, could explain the low degradability of CP. Several authors have also reported low total tract apparent CP digestibility (Abbeddou et al., 2011; Fondevila et al., 1994), suggesting a low small intestinal digestibility of rumen undegraded TP protein. Finally, the ruminal degradability of TP fibre is low because of the high NDF lignification and the presence of only a small fraction of soluble fibre in the peels. As a consequence, low values of NDF digestibility (36%) have been reported (Gasa et al., 1989). Total phenols in TP can be another reason for the low nutritional values as they might affect negatively the palatability and apparent digestibility of feeds. However, some studies have showed that the inclusion of TP in the diet of sheep (Abbeddou et al., 2011) and beef cattle (Yuangklang et al., 2010) does not affect ruminal fermentation. Metabolizable energy content of TP can range between 8.6 and 9.0 MJ/kg DM (Abbeddou et al., 2011).

The use of TP in animal diets can reduce feeding costs for livestock farmers, and is an opportunity for the producing companies to avoid the cost of disposal (Barroso et al., 2006). Tomato pomace can be a valuable source of energy and nutrients for ruminants with lower price than conventional feed ingredients, but it should be properly preserved (Denek and Can, 2006). Due to storage difficulties, TP is frequently discarded, and therefore only a small volume of the annual production is used directly for feeding (without any treatment), and it is restricted to farms closely located to the processing plants (López-Parra et al., 2011).

I.3.4. Effects of feeding tomato pomace to meat-producing ruminants

Tomato pomace has been included in complete diets for growing lambs up to 20% of diet DM (Fondevila et al., 1994) or 48% of DM in feed blocks (Ben Salem and

Znaidi, 2008) with no negative effects on lambs performance. López-Parra et al. (2011) found that the inclusion of up to 15% of dried TP in the diet of fattening lambs replacing barley did not influence either the growth performance or the quality of the carcass and meat. Likewise, Fondevila et al. (1994) observed no differences by replacing barley grains and part of the soybeans in the diet of fattening lambs with TP. Porte et al. (1993) observed that the inclusion of TP up to 40% of the diet DM did not affect either the daily gain or the conversion rate of beef calves. Therefore, the use of dried TP could be profitable as long as its price was lower than that of barley or other cereals.

Feeding TP to ruminants can improve the quality of the meat because of the high content in both antioxidant compounds and FA beneficial for human health. Inclusion of TP in the diet of lambs has been reported to increase the intake of linoleic and α -linolenic acids, and tended to increase the PUFA, PUFA n-6, α -linolenic acid, linoleic acid, γ -tocopherol and retinol content in meat (Valenti et al., 2018). Both linoleic and α -linolenic acids must be supplied in the diet, but they should be protected from ruminal biohydrogenation in order to increase their content in meat (Bessa et al., 2007). Feeding TP has also been reported to increase the meat quality of rabbits (Peiretti et al., 2013), increasing the proportion of PUFA in the meat FA, especially of linoleic and α -linolenic acids, probably as a result of the greater intake. The increase in linoleic acid intake can modify the PUFA n-6/n-3 ratio to be less beneficial for human health (Wood et al., 2003), but some systematic reviews and meta-analysis have reported that the intake of linoleic acid is negatively related to the risk of coronary heart disease (Farvid et al., 2014). However, in ruminants, high levels of dietary PUFA may inhibit the activity of some rumen bacteria and the biohydrogenation process which favours the accumulation of C18:1 *trans* 10 instead of vaccenic acid as the major C18:1 *trans* isomer in the rumen and, consequently its content in milk and meat (Aldai et al., 2013). Moreover, C18:1 *trans* 10 is considered harmful for human health (Hodgson et al., 1996) and it cannot be converted into rumenic acid by the Δ^9 -desaturase (Griinari et al., 2000).

Dietary secondary compounds, such as phenols and tannins, can affect the FA composition of the meat through their effect on rumen lipid metabolism (Vasta and Luciano, 2011). Therefore, it cannot be excluded that the secondary compounds present in TP could affect the activity of rumen microorganisms. Valenti et al. (2018) reported a decrease in total odd and branched chain FA in meat when TP was fed to lambs, which

have been suggested as potential biomarkers of duodenal flow of microbial biomass (Vlaeminck et al., 2015). Moreover, TP might have a positive effect on meat oxidative stability. Valenti et al. (2018) reported an increase in retinol content in the meat of lambs fed TP, which was attributed either to a higher intake of β -carotene, as it is the direct precursor of retinol in fat tissues (Álvarez et al., 2015), or to a higher intake of lycopene. Even though TP feeding increases the dietary intake of lycopene, an increase in lycopene has not been detected in the meat from pigs (Correia et al., 2017) or lambs (Valenti et al., 2018). In studies performed in mice, it has been reported that lycopene possesses partial pro-vitamin A activity, and thus contributes directly to the synthesis of retinol (Aydemir et al., 2016). Meat oxidative stability can be modified by the balance between oxidizable substrates (e.g., highly unsaturated PUFA) and antioxidant components, either of dietary origin, such as vitamins E and A, carotenoids and others, or endogenous (Bekhit et al., 2013). However, the differences in meat oxidative stability by feeding TP observed by Valenti et al. (2018) were low, although reached the significance level. In another study (Jiang et al., 2015a), lycopene inclusion in lamb diets at levels of 0.05, 0.1, or 0.2 g/kg increased DM intake and average daily gain, and lamb meat tended to be darker and redder as indicated by lower lightness and hue angle values measured at 24 h after slaughtering. In addition, muscle fat content and lipid oxidation values decreased in the lycopene-supplemented groups, while PUFA concentrations increased compared to the control lambs (Jiang et al., 2015a). Lycopene dietary supplementation at 0.2 g/kg also improved the antioxidant status of the lambs and optimizes the plasma lipid profile, by reducing the plasma concentrations of malondialdehyde, total cholesterol, total triglycerides, and low-density lipoprotein cholesterol, and increasing plasma concentrations of vitamin E, total plasma antioxidant capacity and the activities of catalase, glutathione peroxidase and superoxide dismutase (Jiang et al., 2015b).

I.3.5. Effects of feeding tomato pomace to dairy ruminants

Several studies have investigated the effects of feeding TP to dairy cows and sheep. In a palatability test conducted with cows (Hoover and Dennison, 1957), it was observed that TP was as palatable as citrus pulp for cattle when was included up to 30% of the diet. Abbeddou et al. (2015) observed that the inclusion of TP in sheep diets up to 30%, replacing the concentrate, increased milk fat content without affecting either milk production or protein content. Caluya et al. (2003) observed that TP could replace up to

50% of dietary forage due to its high fibre content, regardless of whether it was administered fresh, dried or as silage. Tomato pomace ensiled with 10% wheat straw can be a source of good quality forage for sheep (Denek and Can, 2006). However, feeding high amounts (> 1.5 kg/d) of fresh tomato fruits to adult goats has been reported to cause digestive disturbances (Ventura et al., 2009).

Feeding TP to dairy ewes has been reported to increase milk fat and PUFA content, especially that of linoleic acid and the isomers of linolenic acid, and increase the n-3/n-6 FA ratio (Romano et al., 2010). However, other authors (Zhang et al., 2006) have reported no changes in milk composition of dairy ewes by feeding TP. A fraction of the linoleic and linolenic acids present in the TP can escape the ruminal biohydrogenation and be incorporated into milk fat in the mammary gland (Luna et al., 2008). Likewise, these FA can, to some extent, protect lipids from ruminal biohydrogenation (Ashes et al., 1992). Linoleic, α -linolenic, and γ -linolenic acids are transformed to rumenic, *trans* vaccenic and vaccenic acids by ruminal microbiota (Mosley et al., 2002). Nevertheless, the rumenic acid can be further biohydrogenated to vaccenic acid and then to stearic acid, and both acids can be used for milk FA synthesis. On the other hand, α -linolenic and γ -linolenic acids are hydrogenated to vaccenic acid, which enters the vaccenic acid pool in the mammary gland where is desaturated by $\Delta 9$ -desaturase to rumenic acid (Luna et al., 2008). These could help to explain the reported increases of CLA content in the milk from ruminants fed TP (Romano et al., 2010; Luna et al., 2008; Zhang et al., 2006). Romano et al. (2010) also observed that the milk produced by sheep fed diets with 20.4% of TP contained less cholesterol compared with the milk from sheep fed a control diet.

Antimethanogenic effects have been observed when tomato waste fruits and fresh tomato silage were included in the diet of dairy goats (Arco-Pérez et al., 2013; Romero-Huelva and Molina-Alcaide, 2013; Romero-Huelva et al., 2012). There is currently a great interest on the development of dietary strategies that could reduce methane emissions by ruminants (Martin et al., 2010), as methane is a greenhouse gas and represents an energy loss for the animal that can account up to 12% of the gross energy intake (Johnson and Johnson, 1995). Arco-Pérez et al. (2017) fed goats fresh tomato silage and observed a decrease of 15% in methane production per kilogram of DM ingested, whereas Romero-Huelva et al. (2012) reported a decrease of 39% in methane emission when diets including feed blocks with tomato fruits were fed to dairy goats.

The decrease in methane production might be the result of different processes: a decrease in the OM fermented in the rumen, diverting hydrogen away from methane production by using an alternative hydrogen sink, or inhibition of methanogenic activity and optimization of rumen fermentation (Ben Salem, 2010, Martin et al., 2010). The content of polyphenols or other secondary compounds in tomato by-products might act as natural antimethanogenic compounds. However, different compounds might have variable effects on methanogenesis, as it is speculated that there is a relation between the antimethanogenic effect of plant secondary compounds and their molecular weight together with chemical composition of diets (Vasta et al., 2009; Newbold et al., 2005). The reductions in methane production observed by Romero-Huelva et al. (2012) by feeding tomato waste fruits were accompanied by increased molar proportions of propionate, and the production of this volatile fatty acid (VFA) acts as an alternative hydrogen sink (McAllister and Newbold, 2008). Thus, pyruvate is reduced to propionate in a two-multistep pathway that results in less hydrogen production and hence decreased methane synthesis (Janssen, 2010). The relationships between the reduction in methane emissions and the abundance of methanogens reported in the literature are inconsistent. Moreover, it has been hypothesized that the composition of the *Archaea* community has a more important role in methane synthesis, rather than the abundance of methanogens (Morgavi et al., 2010). The antimethanogenic mechanisms of tomato ingredients remain yet unknown and deserve further research in the future (Arco-Pérez et al., 2017). In addition, to our best knowledge the possible influence of TP on rumen methane production has not yet been tested.

In summary, TP is a seasonal by-product with high-water content that needs to be preserved by drying or ensiling. Feeding ruminants TP is a feasible way to avoid the environmental contamination produced by this by-product, which is rich in protein and fat. However, more data on chemical composition and nutritive value of TP, especially of that produced in Spain, are needed to facilitate its use in ruminant feeding. Finally, the possible antimethanogenic effects of TP need to be assessed.

I.4. Other by-products: citrus pulp and dried distillers grains with solubles

There is a wide range of agroindustrial by-products that are produced in great amounts in Spain, but only citrus pulp and DDGS are reviewed here, as they were included in one diet used in the experiments of this Doctoral Thesis.

I.4.1. Citrus pulp

The genus *Citrus* includes several important fruits (Kale and Adsule, 1995), being the most important on a worldwide basis sweet orange (*C. sinensis*), tangerine (*C. reticulata*), lemon (*C. limon*), grapefruit (*C. paradisi*) and minor citrus genuses that include sour orange (*C. quarantium*), shaddock (*C. grandis*), citron (*C. medica*) and lime (*C. aurantifolia*). According to FAO (2018), in 2017, Brazil, China and India were the greatest producers of oranges (17,459,908, 8,564,425 and 7,647,000 tons, respectively), China, Spain and Turkey were the greatest producers of tangerines and similar (18,026,016, 1,967,018 and 1,550,469 tons), Mexico, China and India of lemons and limes (2,528,174, 2,364,000 and 2,316,876 tons), and China, USA and Vietnam of grapefruit (4,658,672, 633,210 and 568,352 tons).

In 2017, Spain produced 3,357,163 tons of oranges, 1,967,018 tons of mandarin, 923,192 tons of lemons and limes, and 78,752 tons of grapefruits, being the first producer of citrus fruits in the European Union and the sixth worldwide (MAPA, 2018). The main citrus producing areas in Spain are the Comunidad Valenciana, followed by Andalucía and Murcia. The Comunidad Valenciana is the main producer of oranges and tangerines, whereas Murcia is the main producer of lemons and grapefruit (MAPA 2018).

Citrus fruits are principally consumed as fresh fruit or as processed juice. After juice extraction, a residue composed of peels (flavedo and albedo), pulp (juice sac residue), rag (membranes and cores), and seeds is generated. This residue is the main source of citrus by-products that can be used for animal feeding (Ensminger et al., 1990; Sinclair, 1984). The main by-products from citrus processing are the fresh citrus pulp, which is the whole residue after juice extraction, representing between 49 and 69% of the fresh citrus fruit processed (as average, 60-65% peels, 30-35% pulp and 0-10% seeds; Martínez-Pascual and Fernández-Carmona, 1980), the dried citrus pulp (DCP) which is formed by shedding, liming, pressing and drying the peel, pulp and seed residues to reach about 8% moisture, and the citrus meal and fines, which is formed and separated during the drying process. Other citrus by-products are citrus molasses, citrus peel liquor, which is similar to citrus molasses, and citrus activated sludge, as well as the discarded citrus fruits (Madrid et al., 1996).

Citrus pulp, either fresh or dried, is considered a good substitute for cereals in

ruminant diets due to its high energy content, which is similar to that of barley (2.67 Mcal of metabolizable energy/kg; De Blas et al., 2010). In addition, it has a high content of NDF (20% DM) and pectines (10% DM), that are highly fermented in the rumen. Citrus pulp is also a calcium-rich by-product (1-2% DM), although its protein, fat and phosphorus content is low (5-10, 2% and 0.1% DM, respectively). The ash content is generally low (5% DM), as well as ADL content (less than 1% DM), but the content in DCP vary with the processes used (De Blas et al., 2010) and fresh citrus pulp usually has lower calcium content compared to dried pulp (Heuzé et al., 2018; Calsamiglia et al., 2016). Fresh pulp is a more perishable product than DCP due to its greater water content, which makes fresh pulp more difficult to store and transport. The use of fresh citrus pulp is almost exclusive to ruminant animals, and is only justified in areas close to the production center due to the high cost of transportation (De Blas et al., 2010).

Citrus by-products have high digestibility of OM (80-85%) and, unlike cereals whose energy intake is based on starch, the energy provided by citrus pulp is based on soluble carbohydrates and digestible fibre. The carbohydrates in citrus pulp are highly fermentable in the rumen, but they generate greater proportions of acetate and lower of lactate than the fermentation of starch (Wing, 1982), which decreases the risk of acidosis. Therefore, citrus pulp can be used, replacing the cereals, in ruminants fed high-concentrate diets to lower the risk of acidosis. On the other hand, citrus peels and seeds contain limonine that gives a bitter taste to the pulp, so that an excess in the diet can cause a decrease in the intake of animals during adaptation to this by-product. Therefore, levels of less than 20% citrus pulp in the diet and a progressive adaptation are recommended to prevent a reduction in feed intake and productive performance, since it has been observed that higher levels used in lambs can reduce palatability and productivity (Heuzé et al., 2018). In contrast, Bueno et al. (2002) replaced corn grain with up to 69.5% of DCP in the diet of Sannen kids and observed no negative effect on feed intake and daily gain, being the inclusion of 40% of DCP the level that resulted in the best performance of growing kids. Similarly, Martínez-Pascual and Fernández-Carmona (1980) reported no negative effect on fattening lambs performance by feeding up to 30% of DCP.

Regarding milk production, López et al. (2014) replaced 61% of corn in a diet for lactating goats by DCP and observed no negative effect on milk yield, whereas fat milk content increased. The inclusion of citrus by-products in the diet might affect the FA

profile of milk. For example, Ibáñez et al. (2017) included up to 60% of orange pulp in the diet of dairy goats, and reported an increase in the proportions of CLA *trans* 10 *cis* 12, linolenic acid, vaccenic acid, palmitic acid and heptadecanoic acid in the milk compared with a control diet, whereas the proportions of elaidic acid, linoleic acid and CLA *cis* 9 *trans* 11 plus *trans* 9 *cis* 11 decreased. However, the effects of including DCP in the diet of dairy small ruminants can be affected by the rest of feed ingredients, and more studies are needed to clarify the influence of DCP on the production, composition and FA profile of milk.

I.4.2. Dried distillers grains with solubles

Cereal grains can be used as seeds for future crops, as human and animal feeds and to produce biofuel. In 2017, more than 84 thousand tons of biofuel were produced in Spain (MAPA, 2019b). The cereals most used in the generation of biofuels are corn in the USA (excepting some States as Kansas, in which sorghum is the main cereal), wheat in Canada, and barley in the countries of Northern Europe. All these four cereals have been used in our country during the last years. In 2017, Spain produced 4,830,281, 5,785,944, 3,775,645 and 30,138 tons of wheat, barley, corn and sorghum, respectively, being the fifth largest producer of the EU with more than six thousand ha of cereal cultivation area (MAPA, 2019b). Most of the autumn-winter cereals are cultivated in dry land, whereas maize and sorghum are cultivated with irrigation systems. The main cereal regions are Castilla y León, Castilla la Mancha, Aragón and Andalucía (MAPA, 2019b).

The main by-product of the process of distillation of cereal grains in the biofuel industry are the DDGS, which are the whole grain without starch, which is removed during the fermentation process to obtain the ethanol. There are two processes to obtain ethanol from corn, either by wet or dry milling (Kim et al., 2008), and whereas both processes are similar generate different by-products. Basically, the production of ethanol requires a two-stage fermentation of starch. In the first stage, starch is decomposed into glucose and maltose, and in the second one there is a yeast fermentation that converts disaccharides and monosaccharides into ethanol. At the end, solid and liquid phases are generated. The liquid phase is evaporated and condensed into syrup, which is then mixed with the solid phase. The resulting decoction is centrifuged, dried and finally granulated (Pecka-Kielb et al., 2017). Different types of decoctions can be obtained depending on the technology used for ethanol production

(Schingoethe, 2006), being the dried distillers grains and the DDGS the most important. The DDGS are the by-product most widely produced, and it is obtained from wet corn residues mixed with the condensed liquid phase and dried afterwards.

Corn DDGS have high-protein content, which can range from 28 to 36% of DM (De Blas et al., 2010). The DDGS protein is characterized by a low degradability in the rumen, resulting in high content of bypass protein (47-63%) (Schingoethe, 2006). The presence of dead yeast cells improves the amino acid profile and taste of DDGS. The main source of energy in DDGS are fat and NDF. The fat content can range between 8.2 and 11.7% of DM, with unsaturated FA accounting for 80% of the total FA. The DDGS fat is rich in palmitic (13.5%), linoleic (26.0%), and linolenic (55.0%) acids (Pecka-Kielb et al., 2017). The NDF content is also high (40-45%), but the fibre is easily fermentable due to the low ADL content. However, because of the large fragmentation of the decoction, it cannot be considered as a source of physically effective NDF (Pecka-Kielb et al., 2017). In summary, corn DDGS are valuable feed ingredients, rich in protein and moderately high in fat and easily fermentable fibre, which can be used in the feeding of both ruminants and monogastrics (Heuzé et al., 2015).

DDGS are very palatable feeds, especially those which are fed fresh (national origin) to ruminants. However, their inclusion in the diet at high levels can affect negatively the fibrolytic activity in the rumen due to their high content of unsaturated fat (De Blas et al., 2010). In mixed diets, DDGS can replace much of concentrate feeds (such as soybean meal) and some cereal grains. One advantage of the use of DDGS as cereal replacers is that they tend to reduce the incidence and severity of acidosis, laminitis and fatty liver caused by an excess of fermentation of starch in the rumen (Heuzé et al., 2015), as energy in DDGS comes from fat and NDF.

Most studies analyzing the effects of including DDGS in the diet of dairy animals have been conducted in cows. Schingoethe et al. (2009) reviewed this topic and concluded that diets containing 20% of DDGS supported similar or greater milk production compared with those composed of traditional feeds, but with diets containing more than 30% of DDGS gut fill may limit DM intake. In addition, when feeding DDGS as forage replacers, an adequate effective fibre supply is needed to avoid milk fat depression. The inclusion of DDGS in the diet can also modify milk composition. Sasikala-Appukuttan et al. (2008) and Anderson et al. (2006) reported that

including up to 20% of DDGS in the diet of dairy cattle increased the CLA concentration in milk, without having any negative effects on milk production. The effect of DDGS in the FA profile of milk can be result of the high fat content of the DDGS which might modify ruminal biohydrogenation. In another study, Kurokawa et al. (2012) reported that feeding DDGS to dairy cattle decreased milk protein content, but increased fat, lactose and total solids (TS) contents in milk as well as milk yield.

The number of studies conducted to assess the effects of feeding DDGS to small ruminants is limited. In sheep, the inclusion of 10-20% of DDGS in the diet had no negative effect on milk production but slightly decreased protein, TS and fat content (Wertz-Lutz et al., 2007). In goats, only Cais-Sokolińska et al. (2015) have evaluated the effect of feeding DDGS, but the study was focused on milk FA profile and fermentation process and aroma profile of the resulting kefir, and no information on milk yield, composition and quality is available. In this study, the inclusion of DDGS in the diet increased the PUFA content in the kefir produced from the milk of DDGS-fed goats (Cais-Sokolińska et al., 2015). Positive results were also observed when DDGS were fed to meat producing small ruminants. Thus, including corn DDGS in the diet of fattening lambs resulted in greater average daily gain and lower feeding costs per kg of gain compared to a conventional diet (Heuzé et al., 2015). In another study, the inclusion of 20% of DDGS replacing a rapeseed grain mixture did not adversely affect either the average daily gain or carcass characteristics of fattening lambs (McKeown et al., 2010). Finally, a decrease in methane production by feeding up to 30% of DDGS has been observed in dairy ewes and cows (Pecka-Kielb et al., 2015; Benchaar et al., 2013), which has been related to the lower acetate/propionate ratio caused by the reduced fibre degradation as a consequence of the high fat content of DDGS.

In conclusion, DDGS are a good energy and protein source, that have shown positive results in dairy cows feeding, but no information on DDGS effects on the yield, composition and quality of goat's milk is available.

I.5. Nutritional significance of acid detergent insoluble nitrogen fraction of feeds

The fibrous fraction of feeds, which includes carbohydrates (mainly cellulose, hemicellulose and pectins) and other compounds such lignin, is considered to be the

least digestible portion (Van Soest et al., 1991). However, ruminants can degrade the fibrous fraction of feeds more efficiently than monogastric animals, and pure hemicellulose and cellulose are completely degradable in the rumen. However, in laboratorial analysis, fibre is usually determined according to the content of hemicellulose, cellulose, lignin and other minor compounds. The most widely used fibre analysis in the field of ruminant nutrition is the Van Soest detergent system (Van Soest et al., 1991; Van Soest, 1966), which not only quantifies the amount of cell wall (NDF analysis), but also measures the amounts of hemicellulose, cellulose and lignin (Van Soest and McQueen, 1973). The proportion of cell wall is measured as NDF after treating the feed with neutral detergent solution, although this fraction does not include the pectins that are soluble in neutral detergent solution. The ADF fraction is obtained after treating the feed with acid detergent solution, and it includes lignin, cellulose, silica and insoluble forms of N, but not hemicellulose. Therefore, when both analyses are performed sequentially, the content of hemicelluloses can be calculated by difference. Because pectins are soluble in neutral detergent solution but insoluble in acid detergent solution, the pectin fraction of feeds can be determined by difference after performing the ADF analysis both directly and sequentially after NDF analysis.

During laboratory analysis, ADF fraction is the residue remaining after boiling a feed sample for 1 h in acid detergent solution, which consists of sulfuric acid and cetyltrimethylammonium bromide (CTAB). The ADIN fraction is the N content of the ADF residue, i.e. the amount of N that is insoluble in acid detergent solution (Firkins et al., 1984), and therefore a positive relation between ADF and ADIN might exist. However, Machacek and Kononoff (2009) reported a poor relationship between these two variables. In forages, the ADIN content has been closely related to N digestibility, as higher contents of ADIN turned into lower forage digestibility (Yu and Thomas, 1976; Goering et al., 1972; Van Soest, 1965). On the other hand, poor relationships between ADIN content and N indigestibility have been reported for non-forage plant protein sources that have been heat treated (Weiss et al., 1989; Nakamura et al., 1994). However, the ADIN analysis is widely accepted as a good measure of heat damage in forages (NRC, 2001; Goering et al., 1972).

In ruminant nutrition, the use of protein sources with low ruminal degradation increases the feed protein reaching the small intestine and can reduce N excretion to the environment, and it is important to make accurate estimations of both rumen

degradability and total tract digestibility of CP. Some feeding systems for ruminants use the ADIN analysis as a measure of N availability for the host animal. For example, the UK Metabolizable Protein System (Webster, 1987) assumes that the ADIN fraction is both undegradable in the rumen and indigestible in the small intestine, and therefore the amount of digestible rumen undegraded protein in a feed is predicted from its ADIN content. The Cornell Net Carbohydrate and Protein System also assumes that the ADIN fraction is undegradable, and it contains heat-damaged proteins (i.e. Maillard reaction products) and the proteins associated with lignin and tannins (Sniffen et al., 1992).

Several authors reported that total tract N digestibility decreases when concentration of ADIN in the feeds increases (Van Soest, 1994; Weiss et al., 1986; Thomas et al., 1982; Yu and Thomas, 1976). However, all these results were reported for forages, whereas in other feeds the relationship between ADIN and N digestibility is not clear. Nakamura et al. (1994) reported that ADIN was digestible to some extent (up to 58%) in heat-damaged corn gluten meal and DDGS, which suggest that suppositions about ADIN indigestibility are not totally accurate. Machacek and Kononoff (2009) stated that ADIN might not be a good indicator of unavailable protein, however a negative relationship between ADIN content and total tract N digestibility exists and it might differ from the one-to-one negative relationship reported in forages. In the study of Nakamura et al. (1994) the ADIN absorbed could not be used by growth lambs and cattle, and the NRC (2001) suggests that ADIN could be a useful indicator of non-usable N but may not be useful for estimating the digestibility of the rumen undegradable protein.

Reported levels of ADIN in feeds range from 0.4% of total N in corn gluten feed and cottonseed meal to 31% of total N for DDGS, forages and some concentrates, either wet or dried (Vargas-Bello-Perez et al., 2008; Kleinschmit et al., 2007; NRC, 2001; Edionwe and Owen, 1989; Weiss et al., 1989; Clark et al., 1987; Pena et al., 1986), but the proportion can be even greater in heat-damaged feeds (NRC, 2001). Heat damage might cause the creation of Maillard products that are formed during the thermal processing of feed ingredients and/or diets. These compounds result from the covalent bond formed between a free reactive NH_2 group of an amino acid and the carbonyl group of a reducing sugar (Friedman, 1996), and they are indigestible (Broderick et al., 2000). The formation of Maillard compounds might be quite common in agroindustrial by-products, as they go through many processes involving heat and moisture treatment.

For example, traditional methods for tomato peeling utilize hot lye or steam processes (Lu et al., 2019) and in the olive oil industry, heat is used to remove the solvents used during the second oil extraction of OC to obtain olive pomace oil. Therefore, agroindustrial by-products may have high ADIN contents. For example, Molina-Alcaide and Yáñez-Ruiz (2008) reported that ADIN content can reach up to a 70% of total N in OC samples, and high ADIN (16-26% of total N) values have also been reported to TP (Rahbarpour et al., 2012; Weiss et al., 1997; Fondevila et al., 1994). The ADIN analysis would be of special interest in agroindustrial by-products to evaluate their N availability, as many of them are originated in processes involving heating. Nevertheless, the use of ADIN to accurately determine N digestibility requires further investigation, as there is no consensus regarding that topic.

In addition, several studies (Vanegas et al., 2016; Guevara-González et al., 2015; Arroyo and González, 2013) have reported that ADIN degradation in the rumen was greater than that observed for the neutral detergent insoluble N (NDIN) fraction. Thus, Vanegas et al. (2016) observed lower ^{15}N abundance in the ADIN than in total N and the NDIN of three different feeds, which was attributed to a N contamination of the ADF residue with CTAB, a N-containing reagent used in the ADF analysis. However, this hypothesis needs to be confirmed, as it would imply an overestimation of ADIN content in feeds.

I.6. Objectives and working hypothesis

The general objective of this Doctoral Thesis is to obtain information on chemical composition and nutritional value for small ruminants of two agroindustrial by-products produced in high amounts in Spain: olive cake (OC) and tomato pomace (TP). Our hypothesis was that processing would influence both chemical composition and nutritional value of these by-products, and therefore several factors related to processing are evaluated. In addition, it was hypothesized that the inclusion of agricultural by-products in the diet of dairy goats would improve the yield and quality of the milk. The general objective was divided in the following partial objectives:

The first objective was to assess the influence of several factors (storage time, processing and particle size) on chemical composition and *in vitro* ruminal fermentation of OC samples obtained at different Spanish extraction plants. In addition, the possibility to estimate the *in vitro* ruminal degradation of OC from chemical

composition was assessed. This was the objective of the first and second papers (Experiments 1 and 2) entitled “Influence of storage time and processing on chemical composition and *in vitro* ruminal fermentation of olive cake” and “Variability in the chemical composition and *in vitro* ruminal fermentation of olive cake by-products” and a fifth paper (Experiment 3) which is included in the Appendix.

The second objective was to analyze the variability in the nutritive value of TP samples collected from two processing plants during the tomato campaign and to assess the effects of including increasing doses of TP in a diet for fattening lambs on *in vitro* fermentation and methane production. This objective was addressed in the third paper (Experiment 4) entitled “Nutritive value of tomato pomace for ruminants and its influence on *in vitro* methane production”.

The third objective was to assess the effects of replacing conventional ingredients in the diet of dairy goats with a mixture of by-products (DDGS, DCP and EOC) on feed intake and nutrient digestibility, milk yield and composition, urinary excretion of PD and methane emissions. This objective was addressed in the sixth paper (Experiment 5) titled “Effects of including a mixture of agroindustrial by-products in the diet of dairy goats on nutrients utilization, ruminal fermentation, methane production and milk yield and composition”, that is included in the Appendix.

The fourth objective was to verify the lower ^{15}N abundance in the ADIN fraction compared with both total N and NDIN fractions, and to assess the possible ADIN contamination and the factors influencing it. This was the objective of the fourth paper (Experiment 6) entitled “The acid detergent insoluble nitrogen (ADIN) analysis overestimates the amount of N associated to acid detergent fibre”.

Chapter II. METHODOLOGY

II.1. Animals and feeding

Four adult rumen-fistulated Lacaune sheep (63.5 ± 2.05 kg of body weight (BW)) were used as donors of ruminal fluid for the *in vitro* Experiments (1, 2, 3 and 4) and to conduct the *in situ* incubations (Experiment 4). Sheep were individually housed and had free access to fresh water over the trials. Animals were fed twice daily (8:00 and 18:00) a 2:1 grass hay:concentrate diet at a rate of 45 g DM/kg BW^{0.75}. The diet contained 114, 365, and 160 g of CP, NDF, and ADF per kg DM, respectively, and was formulated to meet maintenance requirements of the experimental animals (NRC, 2007). Sheep were managed in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of 1st February on the protection of animals used for experimentation or other scientific purposes) and all experimental procedures were approved by the Institutional Animal Care and Use Committee of the Comunidad Autónoma de Madrid (PROEX 035/17).

For the *in vivo* study described in the Appendix (Experiment 5; Paper 6), twelve Murciano-Granadina dairy goats producing 1642 ± 157.7 kg milk/day, with 50.8 ± 1.85 kg of BW, and 9.8 ± 1.17 days in milk were divided into two homogeneous groups based on BW and milk yield at the beginning of the Experiment. Goats were placed in individual boxes with free access to fresh water, and each group was randomly assigned to one of the two experimental diets. Animals were handled in accordance with the Spanish guidelines for experimental animal protection, and all experimental procedures were approved by the Ethic Committee for Animal Experimentation of the Spanish Research Council and the Junta de Andalucía (approval number 29/08/2016/145).

Two diets were formulated to meet the N and energy requirements of Murciano-Granadina goats producing daily 2 kg of milk (Aguilera et al., 1990; Prieto et al., 1990). The control diet (CON) was based on alfalfa hay and a high-cereal concentrate in a 50:50 ratio. The experimental (BYP) diet was also based on a 50:50 ratio alfalfa hay and concentrate, in which 44% of conventional ingredients (corn, barley, soybean meal, palmiste meal and wheat bran) was replaced with a mixture of 180, 180 and 80 g of corn DDGS, DCP and EOC per kg concentrate, respectively (fresh matter basis). Diets were supplied twice a day at 09:00 and 16:00 over the trial. The ingredients of both concentrates, and chemical composition and FA profile of feeds of both diets is shown in Table 1.

Table 1. Ingredient and chemical composition of the experimental concentrates fed to dairy goats and potential use as human food of concentrate ingredients.

Item	Concentrate	
	CON	BYP
Ingredients, g/kg as fed		
Corn	330	268
Barley	200	-
Wheat	100	100
Soybean meal	122	102
Palm meal	88	-
Colza meal	25	25
Wheat bran	100	30
Corn DDGS	-	180
Dry citrus pulp	-	180
Exhausted olive cake	-	80
Calcium soap	12.0	12.0
CaCO ₃	10.0	10.0
NaHCO ₃	8.0	8.0
NaCl	3.0	3.0
Vitamin-mineral mixture	2.0	2.0
Chemical composition, g/kg as fed basis		
Dry matter	894	883
Organic matter	843	824
Crude protein	160	174
Neutral detergent fibre	205	208
Acid detergent fibre	75.9	85.7
Acid detergent lignin	17.4	23.4
Ether extract	28.2	47.0
Total soluble polyphenols	1.95	6.05
Fatty acids, g/100 g total fatty acids		
C12:0	7.01	0.282
C14:0	2.78	0.371
C14:1 <i>cis</i> 9	ND ¹	0.001
C16:0	21.6	20.6
C16:1 <i>cis</i> 9	0.169	0.001
C18:0	2.78	2.76
C18:1 <i>trans</i> 9	0.077	0.004
C18:1 <i>trans</i> 11	0.080	0.002
C18:1 <i>cis</i> 9	26.6	34.5
C18:1 <i>cis</i> 11	0.828	0.875
C18:2 <i>trans</i> 9, <i>trans</i> 12	0.004	0.009
C18:2 <i>cis</i> 9, <i>cis</i> 12	34.1	36.7
C20:0	0.343	0.427
Potential use of ingredients as human food (%) ²	64.4	38.7

¹ ND: not detected.

² Calculated according to Wilkinson (2011)

II.2. By-products, feeds and diets

For the first and second Experiments (Papers 1 and 2), a total of 42 samples of OC representative of those available in Spain for animal feeding were obtained at nine different extraction plants located in the South of Spain during the 2015/16 and 2016/17 olive oil campaigns (Table 2). Samples were classified according to processing type (COC, EOC and cyclone OC (CYOC)), and storage time in the open-air ponds (from one to 14 months). Twenty-five samples of COC were obtained by drying the “alperujo” and all of them were pelleted, whereas nine EOC samples were generated by subjecting the dried “alperujo” to oil extraction using hexane and drying the exhausted cake, and eight CYOC samples came from the particles collected from the air in cyclone decanters during the “alperujo” drying process. Processing of all OC samples were performed at the extraction plants using the habitual procedures in each of them. Storage time of the “alperujo” in the ponds was three months or lower for 10 samples (four COC, three EOC and three CYOC), between four and six months for 16 samples (11 COC, three EOC and two CYOC), and seven months or longer for 16 samples (10 COC, two EOC and four CYOC). All samples were ground to pass a 1 mm screen before chemical analyses and *in vitro* incubations.

Twenty-one samples from the batch of OC samples described above were used independently (Experiment 1; Paper 1) conducted to assess the influence of storage time on chemical composition and *in vitro* ruminal fermentation of COC, EOC and CYOC samples. The samples used are described as follows: six COC (AC1 to AC6) and six EOC (AE1 to AE6) samples obtained at monthly intervals from a single pond (A) from December (one month storage time) to May (six month storage time). The rest of the samples were obtained from a different pond (B) at monthly intervals from May (six months storage) to July (eight months storage). Samples were either COC (BC6 to BC8), EOC (BE6 to BE8) or CYOC (BCY6 to BCY8). All samples were ground (1 mm screen) before analysis of chemical composition and *in vitro* incubations.

In the third experiment (Paper 5; Appendix), four samples of COC from the same trommel at different stages of the screening process with different particle size were studied. The samples used are described as follow: bigger than 3 (COC3), 2 (COC2) and 1 mm (COC1), or smaller than 1 mm (COC0). Additionally, alfalfa hay and barley straw samples were used with comparative purposes.

Table 2. Samples of olive cake (OC) used in this Thesis representative of those available in Spain for animal feeding were obtained at nine different extraction plants.

Sample	"Orujera"	Storage time (months)	OC Type
AE1	Puente Genil	1	Exhausted OC
AE2	Puente Genil	2	Exhausted OC
AE3	Puente Genil	3	Exhausted OC
AE4	Puente Genil	4	Exhausted OC
AE5	Puente Genil	5	Exhausted OC
AE6	Puente Genil	6	Exhausted OC
AC1	Puente Genil	1	Crude OC
AC2	Puente Genil	2	Crude OC
AC3	Puente Genil	3	Crude OC
AC4	Puente Genil	4	Crude OC
AC5	Puente Genil	5	Crude OC
AC6	Puente Genil	6	Crude OC
BC6	Puente Genil	6	Crude OC
BC7	Puente Genil	7	Crude OC
BC8	Puente Genil	8	Crude OC
BCY6	Puente Genil	6	Cyclone OC
BCY7	Puente Genil	7	Cyclone OC
BCY8	Puente Genil	8	Cyclone OC
BE6	Puente Genil	6	Exhausted OC
BE7	Puente Genil	7	Exhausted OC
BE8	Puente Genil	8	Exhausted OC
CC112	Puente Genil	12	Crude OC
CC212	Puente Genil	12	Crude OC
CC312	Puente Genil	12	Crude OC
DC6	Linares	6	Crude OC
DCY6	Linares	6	Cyclone OC
DC7	Linares	7	Crude OC
DCY7	Linares	7	Cyclone OC
CC7	Puente Genil	7	Crude OC
CC8	Puente Genil	8	Crude OC
DC6	Palenciana	6	Crude OC
EC6	Marchena	6	Crude OC
FC6	Baena	6	Crude OC
GC6	Bogarre	6	Crude OC
HC6	Cabra	6	Crude OC
IC6	Pedro Abad	6	Crude OC
DC8	Palenciana	8	Crude OC
DCY8	Palenciana	8	Cyclone OC
ECY11	Marchena	1	Cyclone OC
EC11	Marchena	1	Crude OC
EC21	Marchena	1	Crude OC
ECY21	Marchena	1	Cyclone OC

In the fourth Experiment (Paper 3), 12 samples of TP were obtained from two of the biggest Spanish tomato processing plants (Tomates del Guadiana Soc. Coop., Badajoz, Spain, and PRONAT, Badajoz, Spain) during the 2016 tomato harvesting campaign. The tomato campaign in Spain is usually concentrated in August and September. Samples (4-5 kg) were obtained weekly at each plant on the 12th, 19th, and 26th August and the 2nd, 9th, and 16th of September, frozen (-20 °C) and lyophilized. In all cases, samples were the final tomato-processing disposal. All samples were ground at 2 mm, and subsamples were ground at 1 mm before chemical analyses and *in vitro* incubations.

Additionally, in this Experiment, four diets were used to analyze the effects of including increasing amounts of dried TP in a high-concentrate diet for fattening lambs. A control diet and three experimental diets were formulated by replacing different amounts of barley straw, soybean meal, and wheat bran in the control diet with 60, 120, or 180 g per kg (fresh matter basis) of a dried TP sample. A composited sample of the 12 TP samples analysed previously was used, and it contained 967, 163, 572, 446, and 95.2 g of OM, CP, NDF, ADF and EE per kg DM, respectively. All diets were formulated to have similar CP and NDF content to the control diet and therefore TP replaced fibrous (barley straw and wheat bran) and protein (soybean meal) conventional ingredients. The ingredients and chemical compositions of the four experimental diets are given in Table 3. Subsamples of the diets were ground at 1 mm before *in vitro* incubations.

Eighteen corn and 22 sunflower (SF) ^{15}N -enriched samples were used in the sixth Experiment (Paper 4), which was conducted to verify the lower ^{15}N abundance in ADIN compared with both total N and NDIN, and to assess the possible ADIN contamination and the factors influencing it. Samples were obtained from previous agricultural experiments aimed at measuring the effects of nitrification inhibitors on N_2O losses in different crops, and the results have been reported by Guardia et al. (2018). Briefly, corn (*Zea mays* L. FAO class 600) samples were obtained in 2015 from six microplots (1×1 m; three microplots/treatment) which received either $^{15}\text{NH}_4\text{NO}_3$ (^{15}NAM) or $\text{NH}_4^{15}\text{NO}_3$ (^{15}NIT); both 98 atom% ^{15}N ; Campro Scientific GmbH, Berlin, Germany) to be labelled. Both ^{15}NAM and ^{15}NIT were applied as a top- dressing at a rate of 90 kg total N/ha. At harvest, plants were cut by hand at the soil level, and then the aerial biomass (stem, leaves and cob) was separated from grain. In addition, roots

situated in the middle of each microplot were sampled and washed with water to separate roots from soil. In total, there were 18 corn samples (six microplots (three ^{15}N AM and three ^{15}N IT) $\times$ three plant fractions).

Table 3. Ingredients and chemical composition of experimental diets containing increased amounts of tomato pomace (TP).

Item	Control	TP6	TP12	TP18
Ingredient (g / kg fresh matter)				
Barley	315	315	315	315
Corn	252	275	284	285
Wheat	130	130	130	130
Barley straw	120	85.0	35.0	20.0
Soybean meal 46%	110	96.0	66.0	60.0
Wheat bran	48.1	20.0	40.0	-
Tomato pomace	-	60.0	120	180
Calcium soap	15.0	15.0	-	-
Calcium carbonate	5.0	5.0	5.0	5.0
Mineral/vitamin premix	5.0	5.0	5.0	5.0
Chemical composition ¹				
Dry matter	903	903	902	905
Organic matter	945	957	969	970
Crude protein	145	145	145	145
Neutral detergent fibre	245	242	245	250
Acid detergent fibre	100	107	111	126
Ether extract	44.8	46.2	46.3	52.2
Non structural carbohydrates	510	524	533	523

¹ Calculated from analysed composition of individual feed ingredients. All chemical fractions are expressed as g/kg dry matter, excepting dry matter (g/kg fresh matter). Non-structural carbohydrates were calculated by difference (organic matter – crude protein – neutral detergent fibre – ether extract).

Sunflower (*Helianthus annuus* L., P64LL62 hybrid Pioneer) samples were obtained in 2016 using two different schemas of ^{15}N fertilization: either the residual ^{15}N from the previous corn crops (SF1) or by applying either ^{15}N AM or ^{15}N IT (both 10 atom% ^{15}N ; Campro Scientific GmbH, Berlin, Germany) as a top-dressing at a rate of 68 kg total N/ha to parcels which had not been previously ^{15}N -fertilized (SF2). Sunflower plants were cut as previously described and seeds were separated from the aerial biomass (stem+leaves+flower capitula or head). Unfortunately, one replicate for

¹⁵NAM-SF2 treatment was lost, and therefore a total of 22 SF samples were available.

II.3. *In vitro* incubations: measurement of gas production kinetics

For the determination of gas productions kinetics of samples of OC, TP and diets containing TP, four replicates per sample were obtained by using the ruminal fluid from each of the four rumen-fistulated sheep as inoculum. Ruminal contents of each sheep were obtained before the morning feeding, strained through four layers of cheesecloth into previously warmed thermal flasks, and immediately transported to the laboratory. The fluid of each sheep was independently mixed with pre-warmed (39 °C) culture medium (Goering and Van Soest, 1970; without trypticase) in a proportion 1:4 under CO₂ flushing. The medium of Goering and Van Soest (1970) was modified by replacing the (NH₄)HCO₃ with NaHCO₃ and excluding the trypticase to avoid N supply. Samples (200 mg of DM) of each feed were accurately weighed into 60 ml vials. Vials were filled up with 20 ml of the rumen fluid—culture medium mixture using a Watson–Marlow 520UIP31 peristaltic pump (Watson–Marlow Fluid Technology Group, Cornwall, UK), sealed with rubber stoppers, and incubated at 39 °C for 120 h. Gas production was measured at 3, 6, 9, 12, 15, 22, 26, 31, 36, 48, 58, 72, 96 and 120 h using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments S.L, Barcelona, Spain) and a plastic syringe and the gas produced at each sampling time was released to prevent gas accumulation. Two blanks (vials without sample) per inoculum were also incubated to correct gas production values for endogenous production.

The potential *in vitro* DM degradability (DMD120) was determined by weighing 300 mg of each sample (three bags per sample) into polyester bags of 30 µm pore size (Ankom Corp #57, Ankom Technology Corp., Fairport, NY, USA) and incubating the bags in a 1:4 mixture of ruminal fluid and the incubation medium of Goering and Van Soest (1970) in an Ankom Daisy II incubator at 39 °C under continuous rotation for 120 h. A mixture of equal parts of the ruminal fluid from each sheep was used as inoculum. After 120 h, bags were washed with cold water, dried at 60 °C for 48 h, and weighed to calculate DMD120.

The *in vitro* NDF and DM degradability (NDFD and DMD, respectively) in the first Experiment (Paper 1) were determined by weighing 300 mg of OC samples in polyester bags (30 µm pore size; Ankom Corp #57, Ankom Technology Corp., Fairport, NY, USA), which were incubated with buffered ruminal fluid in an Ankom Daisy II

incubator (Ankom Technology Corp., Fairport, NY, USA) at 39 °C under continuous rotation. Ruminant fluid was obtained from each sheep as described before and equally mixed before conducting the incubations. After 48 h, bags were washed with cold water, dried at 60 °C for 48 h and weighed to calculate the DMD. Bags were then extracted with boiling neutral detergent solution (Van Soest et al., 1991) for 1 h using an Ankom 220 Fibre Analyser unit (Ankom Technology Corp., Fairport, NY, USA), dried at 60 °C for 48 h and weighed to calculate the NDFD.

II.4. *In vitro* incubations: measurement of fermentation parameters

Samples of OC, TP and diets including TP were fermented *in vitro* for 24 h using the methodology described before to determine the main fermentation parameters, with some modifications in the study with TP samples. Gas production was measured, vials were then uncapped, their content was homogenized and the pH was measured using a Crison Basic 20 pH meter (Crison Instruments, Barcelona, Spain). For determining VFA and NH₃-N concentrations, 3 ml of the content of each vial was mixed with 3 ml of HCl 0.5 N, and samples were stored at -20 °C until analyses. Vials were then uncapped, their content was homogenized, the pH was immediately measured (Crison Basic 20 pH-meter, Crison Instruments, Barcelona, Spain), and 3 ml of the vials' contents were mixed with 3 ml of 0.5 M HCl. Samples were frozen (-20 °C) until VFA and NH₃-N analyses.

In the study with TP (Experiment 4; Paper 3) samples, the amount of sample was increased to get enough gas for the analysis of methane concentration; therefore, 400 mg of each diet was fermented with 40 ml of the ruminal fluid and culture medium mixture in 120 ml vials. Gas production was measured and a gas sample (10 ml) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for analysis of methane concentration. Additionally, after 8 h of incubation, gas production was measured and the gas was sampled for methane analyses as described. Immediately, 1 ml of each vial content was taken using an insulin syringe, mixed with 1 mL of 0.5 M HCl, and stored at -20 °C for VFA and NH₃-N analyses. After 24 h, the gas produced and the pH of the vials' contents were measured and samples for methane, VFA and NH₃-N analyses were taken as before described.

II.5. *In vitro* intestinal digestibility

The determination of the *in vitro* intestinal digestibility of protein (IDCP) in the fourth Experiment with TP samples followed the three-step procedure described by Gargallo et al. (2006) and was performed as detailed by Belverdy et al. (2019). Briefly, 3 g of each TP sample (2 mm size) was weighed into nylon bags (7×14 cm; 46 µm pore size) and incubated in the rumen of each sheep immediately before the afternoon feeding on two different days. After 16 h of *in situ* incubation, bags were washed, frozen (-20 °C, 48 h) to facilitate the detachment of particle-associated bacteria, thawed, washed three times with cold water (5 min each) in a turbine washing machine, frozen again (-20 °C), lyophilized, and weighed. Finally, bag residues were analysed for N content to calculate the *in situ* degradability of CP. Bag residues were pooled by TP sample and sheep and 0.5 g of each were weighed in duplicate into nylon bags (5×5 cm; 46 µm pore size). Bags were incubated into an Ankom Daisy II incubation bottles (Ankom Technology Corp., Fairport, NY, USA) containing 2 l of a 0.1 N HCl solution (pH 1.9; 39 °C) with 1 g per l of pepsin (P-7000, Sigma, St. Louis, MO, USA) under constant rotation at 39 °C for 1 h. Bags were then rinsed with tap water and incubated in a pancreatin solution (0.5 M KH₂PO₄ buffer, pH 7.75, 50 ppm of thymol and 3 g per l of pancreatin; P-7545, Sigma, St. Louis, MO, USA) at 39 °C for 24 h under constant rotation. Finally, bags were rinsed with tap water, dried at 40 °C for 72 h, and weighed. Residues of the *in vitro* incubation were pooled by sheep and TP sample and analysed for N concentration to calculate IDCP values.

II.6. Lactation trial

The trial with dairy goats (Experiment 5 and Paper 6; Appendix) was conducted as a cross-over design with two periods of 37 days each, comprising 21 days of adaptation to diets, and 16 days for measurements and sampling. Over the trial, animals were milked once a day in the morning in a 1x10 stall milking parlour (DeLaval, Madrid, Spain), and were weighted at the beginning and end of each experimental period. On day 22 of each experimental period, animals were moved to individual metabolism crates and after three-day period of adaptation, total feces and urine voided by each animal were quantitatively collected for five days. Urine was collected into buckets containing 10% HCl (vol/vol) to keep the pH below 3 and avoid N losses. Aliquots (10%) of urine were taken daily for density measurement, and immediately

frozen until analyses of N content, energy and PD and creatinine concentrations. Samples (20%) of total fecal output were collected each day for digestibility determination and stored at -20°C until chemical analyses. Samples of feces and urine were pooled for each goat to form composite samples.

On day 30 goats were moved to floor pens, and on day 31 approximately 20 ml of ruminal fluid was collected from each animal before feeding using a stomach tube attached to a vacuum pump. The fluid was strained through four layers of cheesecloth, the pH was immediately measured, and 3 ml of ruminal fluid were mixed with 3 ml 0.5 M HCl and stored frozen until $\text{NH}_3\text{-N}$ and VFA analyses. From day 32 to 37, four goats from each dietary treatment were sequentially placed in four individual open circuit respiration chambers (one goat per chamber; two days of measurement) to measure individual methane emissions as described by Romero-Huelva and Molina-Alcaide (2013). Short methane measurement interruptions (10 min) occurred daily at 09:00 h for diet supply, milking, and chamber floor cleaning, but they had little effect on daily methane emissions as the corresponding exhaust duct was not sampled during this period. Fluxes were determined twice per day and averaged to calculate the 24-hour emissions.

Individual feed and water intake were measured over the trial, but only values measured during the sampling period were used for the statistical analysis. During the sampling period, alfalfa hay and concentrate refusals from each animal were weighted daily, pooled, and stored at -20°C until analyses. Each day of the sampling period, milk yield was recorded, milk density was measured, and aliquots were stored at -30°C without preservatives for chemical analyses.

II.7. Chemical analyses

All samples used in the *in vitro* and *in vivo* studies were ground to pass a 1 mm screen before chemical analyses. DM (ID 934.01), ash (ID 048.13) and EE (ID 920.39) contents were determined according to the AOAC International (2005) methods. Total N content was measured using a Leco FP258 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI, USA). Analyses of NDF (Van Soest et al., 1991), ADF and ADL (Robertson and Van Soest, 1981) were carried out sequentially using an Ankom 220 Fibre Analyser unit (Ankom Technology Corp., Fairport, NY, USA). The NDF analyses were performed with α -amylase and without sodium sulphite, and NDF, ADF and ADL

values were expressed exclusive of residual ash. The amount of ADIN was determined by analyzing the N content in the residue obtained after the treatment of the sample with acid detergent solution. Total sugars and total soluble polyphenols (TSP) were analysed by colorimetric methods following the anthrone method (Yemm and Willis, 1954) and the Folin–Ciocalteu assay (Singleton and Rossi, 1965), respectively, using an Epoch spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA).

Concentrations of $\text{NH}_3\text{-N}$ in vial contents in the *in vitro* incubations were determined by the phenol-hypochlorite method as described by Weatherburn (1967), and those of VFA were conducted by gas chromatography as described by García-Martínez et al. (2005). All analyses were performed in duplicate.

In the study with dairy goats (Experiment 5), energy content of diet ingredients, refusals, and of feces and urine was determined by using an adiabatic calorimeter (model 1356, Parr Instruments Co., Moline, IL, USA). The analyses of VFA and $\text{NH}_3\text{-N}$ concentrations in ruminal fluid were performed, respectively by gas chromatography as described by Romero-Huelva and Molina-Alcaide (2013) and by a colorimetric method as proposed by Weatherburn (1967). Total solids in milk were determined by lyophilization of milk samples, and total N content was analysed by the Dumas method. Non protein-N was analysed in samples of milk filtrates after precipitation with 12% (wt/vol) trichloroacetic acid solution. Non casein-N was determined in milk filtrates after precipitation with 10% (wt/vol) acetic acid at a pH of 4.1 (Recio et al., 1997). Values of N in milk were converted to the corresponding protein by multiplying by 6.38. The milk fat content was measured following the Gerber method (Pearson, 1976). Extraction of total FA in samples of alfalfa hay and concentrates was performed following the procedure of Folch et al. (1957), and the FA were methylated according to Kramer and Zhou (2001), with slight modifications, as a double methylation was carried out, using first NaOH/methanol, at 50 °C for 15 min, followed by HCl/methanol at 50 °C for 1 h to obtain the FA methyl esters (FAME). For milk FA composition analysis, FA were extracted and transesterified to FAME as described by Abecia et al. (2012). The FAME were separated and quantified using a gas chromatograph (Model Focus GC, Thermo Scientific, Milan, Italy) equipped with a flame-ionization detector and a 100 m fused silica capillary column (0.25 mm i.d., 0.2 µm film thickness; SP-Supelco, Bellefonte, PA) and helium as the carrier gas. Total FAME profile in a 1 µl sample volume at a split ratio of 1:50 was determined using a temperature gradient program

(Shingfield et al., 2003). Nonadecanoic acid (C19:0; Sigma-Aldrich-N5252) was used as an internal standard, and FAME peaks were identified by comparing their retention times with those of standards (47885 U and O5632: Sigma Aldrich, Madrid, Spain; 90-1093, Larodan Fine Chemicals, Stockholm, Sweden). Results are expressed as percentage of total FAME identified.

Methane concentrations were measured continuously in the air stream in each duct using a gas analyzer ADC MGA3000 (Spurling Works, Herts, UK) as detailed by Romero-Huelva et al. (2017). The concentration of creatinine and PD (PD; allantoin, uric acid, xanthine and hypoxanthine) in urine were determined by HPLC following the method described by Balcells et al. (1992) and the equipment detailed by Romero-Huelva et al. (2017).

In the samples used in the sixth Experiment, analysis of ^{15}N abundance, NDIN, ADIN and CTAB was performed by combustion using a Thermo 1112 Flash HT elemental analyzer hyphenated interfaced to a Thermo Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

II.8. Calculations and statistical analyses

Data of gas production obtained for samples of OC, TP and diets including TP were fitted with time using the exponential model $\text{Gas} = \text{PGP} \times (1 - e^{(-c \times (t - \text{Lag}))})$, where PGP is the potential gas production, c is the fractional rate of gas production, Lag is the time before starting gas production, and t is the time of gas measurement. Gas production parameters were estimated using the NLIN procedure of SAS (2017) by an iterative least squares procedure. The average gas production rate (AGPR) was calculated as $\text{AGPR} = \text{PGP} \times c \div [2 \times (\ln 2 + c \times \text{Lag})]$ and it was defined as the rate between the incubation start and the time at which half PGP is reached. The DM effective degradability (DMED) was estimated as $\text{DMED} = [(\text{DMD120} \times c) \div (c + \text{Kp})] \times e^{(-c \times \text{Lag})}$, using a rumen particulate outflow (Kp) of 0.042 per h. Additionally, the amount of fermented OM (FOM) in the Experiments 2, 3 and 4, was calculated from acetate, propionate, and butyrate production in each vial, as described by Demeyer (1991).

In the study on the effects of storage time on the chemical composition and *in vitro* fermentation of OC (Experiment 1 and Paper 1) gas production and fermentation

parameters were analysed separately for each group of OC samples (either stored up to six months or from six to eight months). Data were analysed as a mixed model with repeated measures using the PROC MIXED of SAS (2017), in which the effects of the treatment and time were considered fixed and the effect of the inoculum was random. Orthogonal polynomial contrasts were used to test for linear and quadratic effects (only for samples stored until six months) of storage time. Relationships between chemical composition of OC samples and either gas production or fermentation parameters were assessed by correlation analysis using the PROC CORR of SAS (2017).

Data on OC chemical composition in the second Experiment (Paper 2) were analysed using the PROC GLM of SAS (2017) with OC type as the main effect. Data on fermentation parameters were analysed as a mixed model using the PROC MIXED of SAS (2017), where the effect of OC type (COC, EOC and CYOC) was considered fixed and inoculum was considered a random effect. In both models, when effects were significant, means were compared by using the Tukey's test. Pearson correlation coefficients among the chemical composition of OC samples, gas production or fermentation parameters, and storage time of the "alperujo" were assessed using the PROC CORR of SAS (2017). Models to predict the AGPR and FOM were developed using the PROC GLM of SAS, including OC type (COC, EOC and CYOC) as the fixed effect and chemical fractions as linear and quadratic covariates. The interaction between OC type and the chemical composition was also studied. Slopes of the models for different OC types were compared by Student's test. In addition, the accuracy of predicting the AGPR and FOM from OC type and the duration (months) of the "alperujo" storage in ponds as a covariate was evaluated using the same model.

In the Experiment 3 (Paper 5; Appendix), data were analysed as a mixed model using the PROC MIXED of SAS (2017) and comparison of means was performed by the Tukey's test.

In the study with TP samples (Experiment 4 and Paper 3), chemical composition data were analysed using the PROC GLM of SAS (2017) with sampling time as the main effect. In vitro fermentation data from TP samples were analysed as a mixed model with repeated measurements using the PROC MIXED of SAS, in which the effect of sampling time (one to six weeks) was considered fixed and that of the inoculum was considered random. Data from the diets including TP were analysed as a

mixed model, with the effect of the diet being fixed and that of the inoculum being random. Non-orthogonal polynomial contrasts were used to test for linear and quadratic effects of TP inclusion. Relationships between chemical composition of the TP samples and either gas production or fermentation parameters were assessed by linear regression using the PROC CORR of SAS (2017).

In the study with dairy goats (Experiment 5 and Paper 6; Appendix I), N and energy balances were calculated as the difference between the amount of N and energy ingested and that lost in urine, feces and milk, and the energy lost as methane (0.890 MJ/mol of methane; Newbold et al., 2007). Milk protein-N content was calculated as the difference between total and non protein-N in milk, whereas the casein-N concentration in milk was calculated as the difference between total N and non casein-N. The amount of whey-N in milk was calculated as the difference between milk protein and casein-N. Milk lactose was calculated as the difference between the amount of TS and that of protein, fat, and total ash in milk. Data from this study were analysed as a mixed model using the PROC MIXED of SAS (2017), in which the effects of diet, period and their interaction were considered fixed and that of goat was considered random.

Finally, in the sixth Experiment (Paper 4), the possible contamination of ADIN with N from the CTAB during the ADF analysis was calculated by using Pearson's square method as: $(\text{averaged } ^{15}\text{N abundance in total N and NDIN} - ^{15}\text{N abundance in ADIN}) / (\text{averaged } ^{15}\text{N abundance in total N and NDIN} - ^{15}\text{N abundance in CTAB})$, in which all ^{15}N abundances were expressed as atom%. The average ^{15}N abundance in total N and NDIN was used in the calculations because of the lack of differences between both values. The ^{15}N abundance of CTAB was measured (0.3660 atom%) and used in the calculations. ^{15}N abundance values were analysed independently for each feed sample as a mixed model by the PROC MIXED of SAS (SAS, 2017).

In all studies, effects were declared significant at $P < 0.05$, and P-values between 0.05 and 0.10 were considered as a trend. When a significant effect of sampling time was detected, means were compared by Tukey's test.

Additionally, in General Discussion, another method to predict nutritive value of all the samples (OC and TP) used in this Doctoral Thesis was used. This method was based on Neural Networks. Neural Network was a net including two hidden layers with

four nodes each and a RELU function. Data introduced in the net was standardized. To test the accuracy of the model, four samples of OC and one sample of TP was randomly selected and excluded of the pool of training data. The Neural Network predictive power was then tested in these samples to determine the accuracy of the model. For this, Keras package was used, and the Python script can be found in the Appendix of this Doctoral Thesis.

Chapter III. INFLUENCE OF STORAGE TIME AND PROCESSING ON CHEMICAL COMPOSITION AND *IN VITRO* RUMINAL FERMENTATION OF OLIVE CAKE

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Abstract: Olive oil extraction generates olive cake (OC) that could be used in ruminant feeding. However, the chemical composition of OC is affected by multiple factors, being therefore highly variable. The objective of this study was to analyse the influence of storage time and further processing: crude, exhausted (subjected to a second oil extraction) and cyclone (obtained from a cyclone separator) on nutritive value of OC samples. Twelve samples (six crude and six exhausted) were obtained monthly from the same pond from 1 to 6 storage months, and nine samples (three crude, three exhausted and three cyclone) were obtained monthly from a different pond from 6 to 9 months storage. Chemical composition was analysed, and OC samples were fermented *in vitro* with sheep rumen fluid. Increasing storage time up to 6 months decreased sugars and total soluble polyphenols content but increased fibre content in OC. Dry matter effective degradability (DMED) decreased linearly ($P < 0.001$) by 35.9 and 45.5% as storage time augmented from 1 to 6 months for crude and exhausted OC, respectively. Crude OC had lower DMED values than exhausted OC (averaged values 0.255 and 0.294 g/g, respectively). Both potential production and rate of gas production were lower ($P \leq 0.018$) in crude compared with exhausted OC, which was attributed to the high fat content of crude OC (≥ 86 g/kg dry matter). For samples stored longer than 6 months, cyclone had greater ($P < 0.05$) DMED than crude and exhausted OC (averaged values 0.207, 0.164 and 0.164 g/g, respectively). The results indicate that ruminal degradability of OC is reduced with advancing storage time, but only subtle changes were observed during the first two months. Cyclone showed greater degradability than crude and exhausted OC, but differences between crude and exhausted OC became negligible after five storage months.

Keywords: crude olive cake, exhausted olive cake, gas production, *in vitro* ruminal fermentation, storage time

III.1. Introduction

Olive oil production is constantly increasing worldwide, but it is still concentrated in the Mediterranean area, with Spain, Greece, Italy and Tunisia being the highest producers and producing 1.27, 0.429, 0.346 and 0.280 millions of tons in the 2017/18 campaign (Consejo Oleícola Internacional, 2018). Spain is the largest producer and contributes to about 45% of total world production (MAPA, 2018). The two-phase system, that is currently used in 90% of Spanish olive mills to obtain olive oil, generates

annually more than 4.3×10^6 t of a high-moisture waste called “alperujo” (Callejo et al., 2015). The alperujo is a thick sludge that contains pieces of stone, pulp from the olive fruits and variable amounts of vegetable water and oil (Molina-Alcaide and Yáñez-Ruiz, 2008), and is a major eco- logical issue due to its high organic load, low pH and high oxygen demand (Dermeche et al., 2013). As large volumes of alperujo are generated in a brief period of olive oil production, usually November to February in the Mediterranean countries, the environmental problem is further aggravated (Alburquerque et al., 2004). The alperujo is sent to extraction plants (“orujeiras”) where it is stored in large ponds, sometimes for several months, before being further processed. Processing can consist in removing the stones and drying the residue off, thus generating a by-product called olive cake (OC), which can be pelleted and used in animal feeding (crude OC). Alternatively, OC can be subjected to a chemical extraction, using hexane or other authorized food-grade solvents to obtain pomace olive oil, and this process generates “exhausted OC” as a by-product. Exhausted OC is currently mostly used to produce biofuel but could also be used in ruminant feeding.

The use of OC in ruminant feeding would increase its economic value and contribute to alleviate the environmental problem, but its chemical composition and nutritive value are highly variable (Molina-Alcaide and Yáñez-Ruiz, 2008), being influenced by different factors such as the olive cultivar, ripeness of the fruit, climate and agronomic conditions, storage conditions and processing techniques (Dermeche et al., 2013). In the practice, storing time of OC is variable, as processing of alperujo depends on the priorities of the ex- traction plant. However, to the best of our knowledge, there is no information on the influence of storage time on nutritive value of OC for ruminants. Therefore, the objective of this study was to assess chemical composition and *in vitro* ruminal fermentation of crude and exhausted OC samples obtained at different storage times. In addition, the relationships between chemical composition and *in vitro* fermentation parameters were studied.

III.2. Materials and methods

III.2.1. Olive cake samples

A total of 21 samples of OC were obtained at an extraction plant located at Puente Genil (Córdoba, Spain). The first group of 12 samples were obtained at monthly intervals from a single pond (A) from December (1 month storage) to May (6 months

storage). Ponds were located in south Spain under Mediterranean-Continental weather conditions, characterized by mild winters and hot summers and low annual rains mainly produced in autumn and spring. Samples were either dried and pelletized (crude OC; AC1 to AC6) or dried and subjected to a second oil extraction using hexane (exhausted OC; AE1 to AE6). The rest of the samples were obtained from a different pond (B) at monthly intervals from May (6 months storage) to July (8 months storage). Samples were dried and pelletized (BC6 to BC8), dried and extracted with hexane (BE6 to BE8) or obtained from a cyclone separator after the drying process (BCY6 to BCY8). The cyclone was used as an air pollution control device, as it removed particulate matter from the dirty air resulting from alperujo drying. All samples were ground (1 mm screen) before analysis of chemical composition and *in vitro* incubations.

III.2.2. Animals and *in vitro* incubations

Ruminal fluid used as inoculum was obtained from four rumen-cannulated Lacaune sheep (64.7 ± 2.10 kg body weight), which were managed according to the protocols approved by the Institutional Animal Care and Use Committee of the Comunidad Autónoma de Madrid (PROEX 035/17). Sheep were fed grass hay and concentrate in 2:1 proportion twice daily (8:00 and 18:00 hr) at a rate of 45 g dry matter (DM)/kg live weight^{0.75} and had free access to fresh water over the trial.

Two different *in vitro* incubations were performed using the same methodology. Two hundred mg of DM of each sample was weighed into 60 ml serum vials. Ruminal contents of each sheep were obtained immediately before the morning feeding, strained through four layers of cheesecloth into previously warmed thermal flasks, and immediately transported to the laboratory. The fluid of each sheep was independently mixed with pre-warmed (39 °C) culture medium of Goering and Van Soest (1970) (no trypticase added) in a proportion 1:4 under continuous CO₂ flushing. Vials were pre-warmed (39 °C) prior to the addition of 20 ml of the mixture, sealed with rubber stoppers and placed in an incubator at 39 °C.

The first incubation trial was conducted to determine gas production kinetics, and vials were incubated for 120 hr. Gas production was measured at 3, 6, 9, 12, 15, 22, 26, 31, 36, 48, 58, 72, 96 and 120 hr using a pressure transducer (Widereager Wide Range Pressure Meter, Sper Scientific LTD) and a plastic syringe, and the gas produced at each measurement time was released. Vials without OC samples (blanks; two per

inoculum) were also incubated to correct for endogenous gas production. The second incubation trial analysed the main fermentation parameters. Vials were incubated for 24 hr before measuring gas production as before described. Vials were then uncapped, the pH was measured immediately (Crison Basic 20 pH meter; Crison Instruments) and 3 ml of vials content were mixed with 3 ml of HCl 0.5 N. The obtained mixtures were stored at -20 °C until volatile fatty acid (VFA) and NH₃-N analyses were performed. Within each trial, incubations were performed in four different days, and each day the rumen fluid from one sheep was used as inoculum to obtain four replicates per experimental treatment.

III.2.3. *In vitro* degradability

The *in vitro* DM and neutral detergent fibre (NDF) degradabilities (DMD and NDFD, respectively) were determined by weighing 300 mg of samples in polyester bags (30 µm pore size; Ankom Corp #57, Ankom Technology Corp) which were incubated with buffered ruminal fluid in an Ankom Daisy II incubator (Ankom Technology Corp) at 39 °C under continuous rotation. Ruminal fluid was obtained from each sheep as described before and equally mixed before conducting the incubations. After 48 hr, bags were washed with cold water, dried at 60 °C for 48 hr and weighed to calculate the DMD. Bags were then extracted with boiling neutral detergent solution (Van Soest et al., 1991) for 1 hr using an Ankom220 Fibre Analyser unit (Ankom Technology Corp), dried at 60°C for 48 hr and weighed to calculate the NDFD. Finally, potential *in vitro* DM degradability (DMD120) was determined as described before with the exception that incubation lasted for 120 hr (Vanegas et al., 2017). After 120 hr, bags were washed with cold water, dried at 60 °C for 48 hr and weighed to calculate the DMD120. In all cases, three bags were used for each sample.

III.2.4. Chemical analyses

Dry matter (ID 934.01) and ash (ID 048.13) contents were determined according to the AOAC International methods (AOAC, 2005). Total N content was measured using a Leco FP258 Nitrogen Analyzer (Leco Corporation). Ether extract (EE) content was measured by extraction with petroleum ether (AOAC Method 945.16) using an Ankom XT10 extractor (Ankom Technology Corp).

Neutral detergent fibre analyses were carried out as described by Van Soest et al.

(1991) and those of acid detergent fibre (ADF) and acid detergent lignin (ADL) followed the procedures of Robertson and Van Soest (1981), results were expressed exclusive of residual ash. Acid detergent insoluble nitrogen (ADIN) was measured using a Leco FP258 Nitrogen Analyzer (Leco Corporation) after ADF extraction. Sugars content was determined in defatted samples by the Anthrone method as described by Yemm and Willis (1954) using an Epoch spectrophotometer (BioTek Instruments Inc). Total soluble polyphenols (TSP) were determined by the Folin-Ciocalteu assay (Singleton and Rossi, 1965) using an Epoch spectrophotometer (BioTek Instruments Inc).

Samples of vials content were defrosted, centrifuged ($13,000 \times g$, 20 min, 4 °C) and the supernatant was used to analyse $\text{NH}_3\text{-N}$ concentrations by a modified colorimetric method (Weatherburn, 1967). For VFA analyses, 0.8 ml of the supernatant were added to 0.5 ml of deproteinizing solution (20 g/l metaphosphoric acid and 0.6 g/l crotonic acid) and the mixture was kept at 4 °C for 24 hr. Samples were centrifuged again under the same conditions listed above, and the supernatant was transferred to vials for determination of VFA by gas chromatography (García-Martínez et al., 2005).

III.2.5. Calculations and statistical analyses

Gas production data were fitted with time using the exponential model:

$$Gas = PGP (1 - e^{(-c(t-lag))})$$

where PGP is the asymptotic gas production (ml), c is the fractional rate of gas production (h^{-1}), lag is the time before gas production begins (hr), and t is the time of gas measurement. The parameters PGP, c and lag were estimated using the NLIN procedure of SAS (version 9.2; SAS Inst. Inc) by an iterative least squares procedure. The average gas production rate (AGPR; ml gas/hr) was calculated as

$$AGPR = \frac{PGP * c}{[2(\ln 2 + c * lag)]}$$

and was defined as the average gas production rate between the start of the incubation and the time when half of the PGP was reached. Finally, the DMED was estimated assuming a rumen particulate outflow (K_p) of 0.04 per hour according to the equation proposed by France et al. (2000):

$$DMED = \frac{DMD_{120} * c}{c + Kp} * e^{(-c*lag)}$$

Gas production and fermentation parameters were analysed separately for each group of OC samples (pond A or B). Data were analysed as a mixed model with repeated measures using the PROC MIXED of SAS, in which the effects of the treatment and time were considered fixed and the effect of the inoculum was random. Orthogonal polynomial contrasts were used to test for linear and quadratic effects (only for samples from pond A) of storage time. Significance was declared at $P \leq 0.05$. Relationships between chemical composition of OC samples and either gas production or fermentation parameters were assessed by correlation analysis using the PROC CORR of SAS.

III.3. Results and discussion

III.3.1. Chemical composition of olive cake

Proportions of chemical fractions in OC samples (Table 4) were within the range reported in previous studies (Molina-Alcaide and Yáñez-Ruiz, 2008; Albuquerque et al., 2004; Molina-Alcaide and Nefzaoui, 1996). Increasing storage time of OC samples from 1 to 6 months resulted in augmented NDF, ADF and ADL contents, thus reflecting the greater resistance of cell wall components to biodegradation compared to other chemical fractions. As expected, for both groups of samples EE content was greater in crude OC samples compared with exhausted OC due to the second oil extraction. There was no clear effect of storage time on N content for any group of OC samples, but in accordance with previous studies (reviewed by Molina-Alcaide and Yáñez-Ruiz, 2008) a great proportion of N (39.1%–72.1%) was bound to acid detergent fibre, indicating low N availability. Sugars and TSP contents clearly decreased as storage time increased from 1 to 6 months, but changes from 6 to 8 storage months were less marked. Albuquerque et al. (2006) also observed that TSP content of OC decreased linearly over a 12-month composting period, and Alhamad et al. (2017) reported similar results through a 2-month fermentation period. As discussed by Albuquerque et al. (2006), enzymes contained in OC (i.e., phenol-oxidase and β -glucosidase) may remove phenols by oxidizing low-molecular weight phenolic compounds, which can be polymerized to produce less soluble substances (Greco et al., 1999). In addition, several microbial

groups can contribute to phenol degradation (Martínez et al., 1993), and therefore both enzymatic and microbial processes can lead to decreased TSP content as storage time progresses.

Both DMD and NDFD decreased as storage time augmented from 1 to 6 months, but the drop was more pronounced in exhausted OC (dropped by 47.0% for DMD and by 45.6% for NDFD) than in crude OC (43.1% for DMD and 14.7% for NDFD). Decreases in DMD and NDFD from 6 to 8 storage months were less pronounced (26.4 and 38.5, 30.0, and 16.9, and 21.4 and 11.2% for crude, exhausted and cyclone samples, respectively). The reduction in DMD with advancing storage time is consistent with the changes in chemical composition. In fact, DMD values were negatively correlated with NDF ($P = 0.021$) and ADF ($P = 0.045$) content and positively correlated ($P < 0.001$) with sugars content (Table 5).

Table 4. Chemical composition and *in vitro* dry matter (DMD) and neutral detergent fibre (NDFD) degradability of olive cake samples obtained after different storage time in ponds and processing.

Pond	Sample	Storage time (months)	Processing ¹	Dry matter (g/kg)	g/kg dry matter (DM) unless otherwise stated										
					Organic matter	Neutral detergent fibre	Acid detergent fibre	Acid detergent lignin	Nitrogen (N)	ADIN	EE	Total sugars	TSP	DMD	NDFD
A	AC1	1	Crude	924	931	445	325	172	13.8	7.6	104	202	12.2	0.483	0.218
	AC2	2		898	925	494	365	191	15.5	9.6	118	106	11.5	0.438	0.214
	AC3	3		906	925	513	366	182	15.6	8.3	86	80	12.4	0.439	0.213
	AC4	4		888	928	617	438	209	16.2	9.3	118	33	9.6	0.328	0.212
	AC5	5		921	948	652	453	204	14.9	7.0	103	33	7.7	0.304	0.185
	AC6	6		862	934	706	501	250	16.5	11.9	100	34	6.4	0.275	0.186
	AE1	1	Exhausted	869	923	528	385	209	16.5	10.4	5	197	26	0.568	0.329
	AE2	2		899	919	538	387	204	16	10.4	27	106	29.2	0.556	0.335
	AE3	3		898	925	559	398	197	15.8	8.0	22	80	25.3	0.494	0.222
	AE4	4		887	916	661	469	223	17.8	9.7	32	47	22.8	0.398	0.218
	AE5	5		908	935	751	511	233	17.3	7.8	22	33	16.7	0.307	0.208
	AE6	6		873	934	766	533	261	17.4	11	5	22	15.4	0.301	0.179
B	BC7	6	Crude	924	912	495	355	184	18.1	8.3	131	11	14.8	0.337	0.122
	BC8	7		932	941	574	389	197	13.3	5.2	111	8	9.3	0.256	0.081
	BC9	8		924	905	554	389	200	17.6	7.3	145	10	10.6	0.248	0.075
	BE6	6	Exhausted	860	894	606	434	234	23.5	12.2	4	15	11.6	0.36	0.160
	BE7	7		903	920	697	471	214	17.4	9.1	21	8	10.4	0.256	0.100
	BE8	8		873	908	695	494	248	18.7	9.2	5	12	8.61	0.252	0.133
	BCY6	6	Cyclone	930	901	424	306	177	21.1	9.4	148	14	16	0.398	0.17
	BCY7	7		944	910	426	291	136	20.2	9.1	172	16	14.7	0.381	0.145
	BCY8	8		917	887	454	321	190	22.2	10.8	195	11	11.8	0.313	0.151

¹ Crude samples were dried and pelletized; Exhausted samples were dried and subjected to an extraction using hexane; Cyclone samples were obtained from a cyclone separator after drying the crude samples

Table 5. Correlation matrix (Pearson coefficient and P-values in brackets; $n = 21$) of chemical composition of OC samples with gas production parameters, *in vitro* degradability and fermentation parameters in 24 hr *in vitro* incubations (only P-values ≤ 0.10 values are shown).

	Neutral detergent fibre	Acid detergent fibre	Acid detergent lignin	Nitrogen	Ether extract	Sugars	Total soluble polyphenols
Gas production parameters							
PGP	-0.52 (0.015)	-0.47 (0.031)	-0.43 (0.050)	-	-	0.87 (<0.001)	0.70 (<0.001)
<i>c</i>	-	0.39 (0.079)	0.40 (0.076)	-0.45 (0.044)	-0.58 (0.006)	0.59 (0.005)	-
<i>Lag</i>	-	-	-	-	-	0.50 (0.020)	-
AGPR	-	-	-	-0.43 (0.055)	-	0.90 (<0.001)	0.66 (0.001)
DMED	-0.34 (0.021)	-0.44 (0.045)	-	-	-	0.82 (<0.001)	0.77 (<0.001)
<i>In vitro</i> degradability							
DMD	-0.50 (0.021)	-0.44 (0.045)	-	-	-	0.82 (<0.001)	0.77 (<0.001)
NDFD	-	-	-	-	-0.38 (0.088)	0.75 (<0.001)	0.70 (<0.001)
<i>In vitro</i> fermentation parameters							
Gas	-0.45 (0.038)	-0.44 (0.030)	-	-	-	0.89 (<0.001)	0.68 (<0.001)
pH	0.39 (0.082)	-	-	0.45 (0.043)	-	-0.89 (<0.001)	-0.66 (0.001)
NH ₃ -N	-	-	-	-	-	-0.53 (0.014)	-0.69 (<0.001)
Total VFA	-0.60 (0.004)	-0.56 (0.009)	-0.52 (0.015)	-	-	0.84 (<0.001)	0.65 (0.001)
Acetate	-0.58 (0.006)	-0.54 (0.011)	-0.51 (0.018)	-	-	0.85 (<0.001)	0.64 (0.002)
Propionate	-0.56 (0.009)	-0.51 (0.019)	-0.47 (0.033)	-	-	0.86 (<0.001)	0.67 (0.001)
Butyrate	-0.68 (<0.001)	-0.66 (0.001)	-0.61 (0.004)	-	-	0.77 (<0.001)	0.68 (<0.001)
Minor VFA ¹	-0.40 (0.074)	-0.46 (0.037)	-0.47 (0.033)	0.39 (0.080)	0.52 (0.016)	-0.48 (0.027)	-0.42 (0.059)

¹ Calculated as the sum of isobutyrate, isovalerate and valerate.

III.3.2. Effects of storage time on *in vitro* fermentation of olive cake

Values of PGP and AGPR were linearly reduced ($P < 0.001$) by increasing storage time from 1 to 6 months (Table 6), whereas c and Lag values decreased and tended to decrease quadratically ($P < 0.001$ and 0.075 , respectively). Similarly, in the samples stored from 6 to 8 months (Table 7) values of PGP, c and AGPR were reduced linearly ($P \leq 0.007$) with advancing storage time, but Lag was unaffected ($P > 0.05$).

The linear decrease ($P < 0.001$) of PGP and AGPR values with increasing storage time from 1 to 6 months (Table 6) is in accordance with the changes observed in chemical composition of OC samples. In fact, PGP was negatively correlated ($P \leq 0.050$) with NDF, ADF and ADL content, and positively ($P < 0.001$) with sugars and TSP content (Table 5). Both NDF and ADF are less degradable than other fractions (i.e., N, sugars,...), and therefore their fermentation produces lower amounts of gas. In contrast, sugars are rapidly and extensively fermented by rumen microorganisms, and in agreement with our results positive correlations between sugars content and both rate and extent of gas production have been reported for other feedstuffs (Piquer et al., 2009). Polyphenols are secondary compounds that can reduce ruminal degradation of fibre by decreasing the attachment of microbes to feed particles (Theodorou et al., 2006), and negative correlations between TSP content and DM degradability are frequently reported (Molina-Alcaide et al., 2017). The positive relationship ($P \leq 0.001$) between TSP content and PGP, AGPR, DMED, DMD and NDFD seem to be the consequence of the similar changes produced in all these variables as storage time augmented than to a direct effect of TSP on samples degradation.

Values of DMED decreased linearly ($P < 0.001$) with advancing time over the first 6 months of storage, with reductions being less marked for crude (regression slope -0.026 ± 0.0050) than for exhausted OC (-0.041 ± 0.0053). Increasing storage time from 6 to 8 months also reduced linearly DMED ($P < 0.001$). The low values of DMED of OC samples, ranging from 0.130 to 0.382 g/g, agree well with the results from other studies reviewed by Molina-Alcaide and Yáñez-Ruiz (2008), who reported an average value of 0.27 for *in vitro* DM apparent digestibility.

Table 6. Effect of processing (PR) and storage time in pond (Time) of olive cake (OC) samples on gas production kinetics parameters.

Item ¹	PR ²	Storage time (months) Time						SEM _{PR} ³	SEM _{Time} ⁴	P-value		
										PR	Time	
		1	2	3	4	5	6				Linear	Quadratic
PGP (ml/g DM)	ACOC	119	97.7	95.1	72.7	60.5	51.7	2.93	3.45	<0.001	<0.001	0.262
	AEOC	122	122	99.8	86.2	69.3	50.7					
<i>c</i> (h ⁻¹)	ACOC	0.057	0.060	0.063	0.050	0.042	0.067	0.0028	0.0031	0.018	0.493	<0.001
	AEOC	0.065	0.059	0.058	0.054	0.054	0.068					
<i>Lag</i> (hr)	ACOC	0.54	0.34	0.68	0.00	0.00	0.45	0.170	0.209	0.839	0.557	0.075
	AEOC	0.31	0.35	0.46	0.00	0.00	0.74					
AGPR (ml/hr)	ACOC	4.62	4.09	4.00	2.58	1.82	2.38	0.066	0.104	<0.001	<0.001	0.011
	AEOC	5.47	5.03	3.98	3.32	2.69	2.33					
DMED (%)	ACOC	0.320	0.283	0.291	0.242	0.186	0.205	0.0037	0.0064	<0.001	<0.001	0.868
	AEOC	0.382	0.376	0.311	0.283	0.201	0.208					

¹AGRP, gas production rate until it has reached half of the PGP; *c*, fractional rate of gas production; DMED, effective degradability of dry matter calculated for a rumen passage rate of 0.04 per hour; *Lag*, time until the production of gas begins; PGP, potential gas production; ²ACOC, crude olive cake (samples AC1 to AC6); AEOC, exhausted olive cake (samples AE1 to AE6); ³ standard error of the mean for processing effect; ⁴ standard error of the mean for time effect.

Table 7. Effect of processing (PR) and storage time in pond (Time) of olive cake (OC) samples on gas production kinetics parameters.

Item ¹	PR ²	Storage time (months)			SEM _{PR} ³	SEM _{Time} ⁴	P-value	
		6	7	8			PR	Time ^a
PGP (ml/g DM)	BCOC ^a	68.5	51.5	50.2	2.91	2.91	<0.001	<0.001
	BEOC ^a	63.2	48.5	40.9				
	BCYO ^b	72.7	79.8	65.8				
<i>c</i> (h ⁻¹)	BCOC ^a	0.033	0.037	0.026	0.0024	0.0024	<0.001	0.007
	BEOC ^b	0.044	0.041	0.045				
	BCYO ^a	0.039	0.036	0.026				
<i>Lag</i> (hr)	BCOC	0.00	0.00	0.00	0.066	0.066	0.123	0.244
	BEOC	0.00	0.00	0.00				
	BCYO	0.33	0.18	0.00				
AGPR (ml/hr)	BCOC ^a	1.61	1.36	0.95	0.118	0.118	<0.001	<0.001
	BEOC ^b	2.04	1.44	1.32				
	BCYO ^b	1.99	2.05	1.21				
DMED (g/g)	BCOC ^a	0.207	0.155	0.130	0.0065	0.0065	<0.001	<0.001
	BEOC ^a	0.197	0.148	0.147				
	BCYO ^b	0.255	0.223	0.144				

¹AGRP, gas production rate until it has reached half of the PGP; *c*, fractional rate of gas production; DMED, effective degradability of dry matter calculated for a rumen passage rate of 0.04 per hour; *Lag*, time until the production of gas begins; PGP, potential gas production; ² BCOC, crude olive cake (samples BCOC6 to BCOC8); BCYOC, obtained from a cyclone separator after drying the OC (samples BCYOC6 to BCYOC8); BEOC, exhausted olive cake (samples BEOC6 to BEOC8); ³ standard error of the mean for processing effect; ⁴ standard error of the mean for time effect.

^{a, b} Within each parameter, different superscripts indicate differences among treatments ($P < 0.05$).

^a Linear effect of time.

Table 8. Effect of processing (PR) and storage time in the pond (Time) of olive cake (OC) samples on fermentation parameters in 24 hr *in vitro* ruminal fermentation.

Item	PR ¹	Storage time (months) Time						SEM _{PR} ²	SEM _{Time} ³	P-value		
		1	2	3	4	5	6			PR	Time	
											Linear	Quadratic
Gas (ml/g dry matter (DM))	ACOC	92.5	72.5	73.0	56.0	47.5	43.0	1.55	2.40	<0.001	<0.001	0.074
	AEOC	98.5	96.0	71.0	66.0	52.0	47.0					
pH	ACOC	6.78	6.87	6.90	6.97	7.04	7.08	0.010	0.014	0.004	<0.001	0.274
	AEOC	6.77	6.79	6.89	6.90	7.00	7.09					
Volatile fatty acids (VFA; mmol/g DM)												
Total	ACOC	4.54	4.06	3.98	3.38	3.08	2.39	0.254	0.259	<0.001	<0.001	0.211
	AEOC	4.82	4.51	3.74	3.66	3.24	2.74					
Acetate	ACOC	2.89	2.57	2.52	2.10	1.92	1.40	0.148	0.152	<0.001	<0.001	0.079
	AEOC	3.05	2.85	2.34	2.30	2.03	1.65					
Propionate	ACOC	1.02	0.90	0.88	0.72	0.63	0.54	0.059	0.062	0.001	<0.001	0.640
	AEOC	1.10	1.02	0.83	0.81	0.69	0.61					
Butyrate	ACOC	0.46	0.42	0.40	0.37	0.34	0.29	0.043	0.044	0.005	<0.001	0.891
	AEOC	0.50	0.48	0.41	0.39	0.36	0.32					
Minor VFA ⁴	ACOC	0.17	0.18	0.18	0.19	0.18	0.16	0.020	0.020	0.008	0.117	0.017
	AEOC	0.18	0.17	0.17	0.17	0.17	0.17					
Acetate/Propionate (mol/ mol)	ACOC	2.83	2.87	2.86	2.92	3.07	2.61	0.115	0.125	0.444	0.151	0.004
	AEOC	2.77	2.79	2.84	2.85	2.93	2.70					
NH ₃ -N (mg/l)	ACOC	217	199	202	229	238	201	23.4	25.2	0.002	0.127	0.812
	AEOC	170	178	180	165	199	206					

¹ ACOC, crude olive cake (samples AC1 to AC6); AEOC, exhausted olive cake (samples AE1 to AE6); ² standard error of the mean for processing effect; ³ standard error of the mean for time effect. ⁴ Calculated as the sum of isobutyrate, isovalerate and valerate.

The fermentation parameters determined after "24 hr" incubation confirmed the negative effect of storage time on ruminal degradation of OC (Tables 8 and 9). Increasing storage time linearly increased pH values ($P < 0.001$) and decreased gas and total VFA production ($P < 0.001$) for both groups of samples. Total VFA production for 6-month stored OC samples was only 0.53 and 0.57 of that observed for crude and exhausted OC samples stored for 1 month, which is in accordance with the observed decreases in DMED. Acetate, propionate and butyrate productions were linearly decreased ($P < 0.001$) with advancing time, with changes being similar for both crude and exhausted OC samples. The reduction in VFA production with advancing storage time was lower after 6 months than that observed during the first storage months, as acetate, propionate and butyrate productions for 8-month stored samples were 0.89, 0.82 and 0.95 of the productions for 6-month stored samples, respectively (values averaged across processing treatments). Acetate/propionate ratio remained relatively unchanged during the first five months of storage but showed the lowest value ($P < 0.001$) at month 6 due to reduced acetate production. In contrast, acetate/propionate ratio increased linearly ($P < 0.001$) from 6 to 8 storage months due to reductions in propionate proportions (21.6, 21.0 and 19.9/100 mol at 6, 7 and 8 months, respectively; averages for processing treatments), as acetate proportions remained unchanged over this period (61.4, 61.3 and 61.4/100 mol). Production of total and main individual VFA were negatively correlated ($P \leq 0.033$) with NDF, ADF and ADF contents and positively correlated ($P \leq 0.002$) with sugars content, reflecting the lower fermentability of fibre fractions compared with sugars. The observed trend ($P = 0.080$) to a positive correlation between N content and minor VFA production possibly reflects the deamination of branched amino acids which produces isobutyrate, valerate and isovalerate (Wallace and Cotta, 1988), as the incubation medium only contained non-protein N.

There was no effect of storage time ($P \geq 0.127$) on $\text{NH}_3\text{-N}$ concentrations for any group of samples. Although most samples had low N content, the use of an N-enriched medium explains that $\text{NH}_3\text{-N}$ concentrations were always above the level limiting *in vitro* ruminal microbial growth (Satter and Slyter, 1974).

Table 9. Effect of processing (PR) and storage time in the pond (Time) of olive cake (OC) samples on fermentation parameters in 24-hr *in vitro* ruminal fermentation.

Item	PR ¹	Storage time (months)			SEM _{PR} ²	SEM _{Time} ³	P-value	
		6	7	8			PR	Time ^a
Gas (ml/g dry matter (DM))	BCOC ^a	51.5	40.5	38.0	1.70	1.57	0.001	<0.001
	BEOC ^a	49.0	42.5	39.5				
	BCYO ^b	51.5	40.5	38.0				
pH	BCOC ^{ab}	7.03	7.09	7.10	0.017	0.017	0.011	<0.001
	BEOC ^b	7.06	7.09	7.11				
	BCYO ^a	7.00	7.02	7.09				
Volatile fatty acids (VFA; mmol/g DM)								
Total	BCOC ^a	3.28	2.95	2.90	0.219	0.219	<0.001	<0.001
	BEOC ^a	3.28	3.17	3.00				
	BCYO ^b	3.55	3.69	3.11				
Acetate	BCOC ^a	2.01	1.81	1.78	0.129	0.129	<0.001	<0.001
	BEOC ^a	2.01	1.96	1.85				
	BCYO ^b	2.19	2.25	1.90				
Propionate	BCOC ^a	0.71	0.61	0.58	0.035	0.035	<0.001	<0.001
	BEOC ^a	0.70	0.65	0.58				
	BCYO ^b	0.78	0.82	0.64				
Butyrate	BCOC ^a	0.38	0.35	0.35	0.047	0.047	0.003	0.069
	BEOC ^a	0.38	0.37	0.36				
	BCYO ^b	0.39	0.41	0.38				
Minor VFA ⁴	BCOC ^a	0.19	0.18	0.19	0.022	0.022	0.002	0.043
	BEOC ^b	0.19	0.20	0.20				
	BCYO ^b	0.19	0.20	0.20				
Acetate/Propionate(mol/ mol)	BCOC ^b	2.82	2.99	3.10	0.140	0.140	<0.001	<0.001
	BEOC ^b	2.89	3.03	3.19				
	BCYO ^a	2.80	2.74	2.97				
NH3-N (mg/l)	BCOC	249	197	217	26.8	26.8	0.787	0.671
	BEOC	199	262	228				
	BCYO	234	249	215				

¹ BCOC, crude olive cake (samples BCOC6 to BCOC8); BCYOC, obtained from a cyclone separator after drying the OC (samples BCYOC6 to BCYOC8); BEOC, exhausted olive cake (samples BEOC6 to BEOC8); ² standard error of the mean for processing effect; ³ standard error of the mean for time effect. ⁴ Calculated as the sum of isobutyrate, isovalerate and valerate. ^{a-b} Within each parameter, different superscripts indicate differences among treatments (P < 0.05).

III.3.3. Effects of processing treatment on *in vitro* fermentation of olive cake

In the first group of OC samples, all gas production parameters excepting *Lag* were greater ($P \leq 0.018$) in exhausted compared with crude OC samples (Table 6). This was attributed to the high EE content of crude OC samples, as unsaturated fatty acids resulting from the triglyceride hydrolysis are toxic to many ruminal bacteria, but especially for fibrolytic bacteria (Maia et al., 2007). All exhausted OC samples had EE contents below the maximal level of 60 g/kg DM usually recommended to avoid reductions in fibre digestibility (National Research Council, 2001), but EE content was greater in all crude OC samples (≥ 86 g/kg DM). Similarly, for OC samples stored from 6 to 8 months values of *c* and AGPR were greater ($P \leq 0.013$) for exhausted than for crude OC samples, with no differences ($P > 0.05$) in PGP and *Lag* (Table 7). Cyclone OC samples had greater ($P < 0.001$) PGP and DMED than both crude and exhausted OC samples, which is consistent with the lower NDF content of cyclone OC samples (Table 4). The greater ($P < 0.001$) values of DMED and VFA production of cyclone OC samples compared with both crude and exhausted OC samples would indicate that the high EE content of cyclone OC samples (≥ 148 g/kg DM) did not negatively affect their ruminal fermentation. Although fatty acid profile of OC samples was not measured, it is possible that fat composition changed during storage due to oxidation and microbial activity (Ramos-Hinojosa and Ruiz-Méndez, 2004) and the resulting fatty acids would be less toxic to ruminal microorganisms. The lack of differences in DMED between crude and exhausted OC samples taken at 6, 7 and 8 storage months is in agreement with the similar DMED values observed for crude and exhausted OC samples taken at 5 and 6 months of storage (Tables 6 and 7). These results indicate that differences in the nutritive value between crude and exhausted OC are reduced as storage time advanced.

In conclusion, the nutritive value of olive cake was reduced with advancing storage time and values of ruminal degradability at 6-months storage decreased by 0.64 and 0.54 of values measured after one-month storage for crude and exhausted olive cake samples, respectively. This was mainly due to the loss of the easily fermentable fractions produced during storage, but the results indicate that there were only subtle changes in OC samples during the first two months of storage. Exhausted OC samples showed greater ruminal degradability values than crude OC samples over the first five

months of storage, but differences disappeared from 6 months storage. Ruminal degradability of cyclone OC samples was 1.3 times greater than that of crude and exhausted OC samples obtained at the same storage time.

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Chapter IV. VARIABILITY IN THE CHEMICAL COMPOSITION AND *IN VITRO* RUMINAL FERMENTATION OF OLIVE CAKE BY-PRODUCTS

Marcos, C. N., García-Rebollar, P., De Blas, C., and Carro, M. D. (2019). Variability in the Chemical Composition and In Vitro Ruminal Fermentation of Olive Cake By-Products. *Animals* **9**, 109-120. doi: 10.3390/ani9030109

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Simple Summary: Olive cake is a by-product of oil production that can be used in ruminant feeding, but its composition can vary with multiple factors. The objective of this study was to determine the variability in the chemical composition and *in vitro* ruminal fermentation of 42 olive cake samples to evaluate their nutritive value for ruminants. The results showed that the chemical composition of olive cake is highly variable and is markedly affected by processing type and its storage time before processing. Cyclone olive cake had greater nutritive value for ruminants than both crude and exhausted olive cake. All olive cake types were poorly degraded *in vitro* and, therefore, their nutritive value is highly dependent on their ether extract content. Statistical models using olive cake processing type, and either the chemical composition or storage time before processing as covariates, can be used in the practice to predict *in vitro* ruminal fermentation variables of olive cake samples. The obtained information may contribute to increase the use of olive cake in ruminant feeding.

Abstract: The objective of this study was to determine the variability in the chemical composition and *in vitro* ruminal fermentation of olive cake (OC) by-products. Forty-two OC samples with different storage times (1–14 months) and processing (25 crude (COC), 9 exhausted (EOC) and 9 cyclone (CYOC)) were fermented *in vitro* with sheep ruminal fluid. Exhausted OC samples had a lower ether extract content than COC and CYOC (15.9, 110 and 157 g/kg dry matter (DM), respectively), but greater neutral detergent fiber (NDF; 645, 570 and 441 g/kg DM) and acid insoluble nitrogen (9.76, 8.10 and 8.05 g/kg DM) content. Exhausted OC had the greatest ($P < 0.05$) average gas production rate (AGPR), whereas the greatest fermented organic matter (FOM) was obtained for EOC and CYOC. The best single predictor of the AGPR was total sugars content ($R^2 = 0.898$), whereas NDF was the best one for FOM ($R^2 = 0.767$; $P < 0.001$). Statistical models using storage time as a predictor variable had lower accuracy and R^2 values than those from the chemical composition. In summary, the nutritive value of OC was highly dependent on its processing, but its ether extract content did not negatively affect ruminal fermentation parameters, which could be estimated from either carbohydrate composition or storage time.

Keywords: olive cake; storage time; processing; *in vitro* ruminal fermentation; prediction models

IV.1. Introduction

The use of agroindustrial by-products in animal feeding is increasing worldwide, as it is a viable solution to value wastes and minimize environmental pollution from food industries (Romero-Huelva et al., 2017). However, the chemical composition of these ingredients is highly variable and frequently only limited data on their nutritive value are available, thus limiting their use as regular ingredients in animal rations.

The Mediterranean area concentrates most of world olive oil production, with Spain being the world's leading producer (FAO, 2018). The system of olive oil extraction most widely used in Spain generates a wet solid-waste (500–700 g moisture/kg) called “alperujo”, which is stored in open-air ponds before being processed to obtain different types of olive cake (OC). After removal of olive stones, the alperujo is dried to produce crude olive cake (COC), which can be treated with solvents to extract the residual oil (pomace olive oil), obtaining the exhausted olive cake (EOC). In order to clean the air used to dry the alperujo before being emitted to the atmosphere, the OC particles floating in the air are collected from cyclone decanters generating cyclone OC (CYOC). Crude OC is the by-product more frequently used for ruminant feeding, whereas EOC is mostly used as biomass fuel for electricity generation, and small amounts of CYOC are marketed as high-quality COC. The chemical composition of OC by-products can be influenced by multiple factors such as the olive cultivar, the ripeness of the fruit, the alperujo storage conditions and the processing techniques (Dermeche et al., 2013; Molina-Alcaide y Yáñez-Ruiz, 2008). The amount of data on the chemical composition of different types of OC is variable (Heuzé et al., 2015), but to the best of our knowledge there is no information on the composition of CYOC. Moreover, the existing information on the dry matter ruminal degradability of OC is scarce, although available data indicate low values with a wide range of variation (from 8% to 42%) (Molina-Alcaide y Yáñez-Ruiz, 2008). Therefore, the first objective of this study was to analyse the variability in the chemical composition and *in vitro* ruminal fermentation of different types of OC to evaluate their potential nutritive value for ruminants. A previous study (Marcos et al., 2019) showed a marked influence of the storage time of the alperujo on the chemical composition and *in vitro* fermentation of OC and, therefore, samples of OC stored for different times were obtained at various extraction plants. The samples used in the study of Marcos et al. (2019) were included

in the present study to increase the sample size. In addition, statistical models were developed to estimate the fermentation characteristics of OC from processing type and either the chemical composition or storage time of the alperujo in the ponds.

IV.2. Materials and methods

IV.2.1. Olive cake samples

In order to obtain OC samples representative of those available in Spain for animal feeding, a total of 42 samples of OC were obtained at nine different extraction plants located in the South of Spain during the 2015/16 and 2016/17 olive oil campaigns. Samples were classified according to processing type (COC, EOC and CYOC) and storage time in the open-air ponds (from 1 to 14 months). Twenty-five samples of COC were obtained by drying the alperujo and all of them were pelleted, whereas 9 EOC samples were generated by subjecting the dried alperujo to oil extraction using hexane and drying the exhausted cake, and 8 CYOC samples came from the particles collected from the air in cyclone decanters during the alperujo drying process. Processing of all OC samples were performed at the extraction plants using the habitual procedures in each of them. Storage time of the alperujo in the ponds was 3 months or lower for 10 samples (4 COC, 3 EOC and 3 CYOC), between 4 and 6 months for 16 samples (11 COC, 3 EOC and 2 CYOC), and 7 months or longer for 16 samples (10 COC, 2 EOC and 4 CYOC). All samples were ground to pass a 1 mm screen before chemical analyses and *in vitro* incubations.

IV.2.2. *In vitro* incubations

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Comunidad Autónoma de Madrid (PROEX 035/17). Four Lacaune sheep (64.7 ± 2.10 kg body weight), each provided with a permanent cannula in the rumen, were used as donors of ruminal fluid for the *in vitro* incubations. Sheep were individually housed, had access to fresh water over the trial, and were fed a diet composed of grass hay and concentrate in a 2:1 ratio at a rate of 45 g dry matter (DM)/kg body weight^{0.75}. Diet was fed in two equal meals at 08:00 and 18:00 h. The diet contained 112, 350 and 166 g of crude protein, neutral detergent fiber (NDF) and acid detergent fiber (ADF) per kg DM, respectively.

Two *in vitro* trials were conducted in different days using the same methodology to determine gas production kinetics and the main ruminal fermentation parameters, respectively. In each incubation trial, four replicates per OC sample were obtained by conducting the incubations on four different days and each day the ruminal fluid from one sheep was used as the inoculum. Ruminal contents of each sheep were obtained before the morning feeding, strained through four layers of cheesecloth and immediately transported to the laboratory in thermal flasks. The fluid was mixed with the culture medium (Goering and Van Soest, 1970) (without trypticase; 39 °C) in a 1:4 ratio under CO₂ flushing. Samples (200 mg of DM) of each OC were weighed into 60-mL vials before the addition of 20 mL of the mixture of ruminal fluid and culture medium. Vials were then sealed with rubber stoppers and incubated at 39 °C. In the first trial, to determine gas production kinetics, vials were incubated for 120 h, and gas production was measured at 3, 6, 9, 12, 15, 22, 26, 31, 36, 48, 58, 72, 96 and 120 h using a pressure transducer (Widereager Wide Range Pressure Meter, Sper Scientific LTD, Scottsdale, AZ, USA) and a plastic syringe. The gas produced at each measurement time was released to prevent gas accumulation. Blanks (vials without a sample; two per inoculum) were also incubated to correct values of gas production for endogenous gas production. The second incubation trial was conducted to analyze the main fermentation parameters after 24 h of incubation. Gas production was measured as described before. Vials were then uncapped, their content was homogenized and the pH was measured using a Crison Basic 20 pH meter (Crison Instruments, Barcelona, Spain). For determining volatile fatty acid (VFA) and NH₃-N concentrations, 3 mL of the content of each vial was mixed with 3 mL of HCl 0.5 N, and samples were stored at -20 °C until analyses.

In order to calculate DM effective degradability (DMED), the potential *in vitro* DM degradability (DMD120) was determined by weighing, in triplicate, 300 mg of each OC sample into polyester bags (30-µm pore size; Ankom Corp #57, Ankom Technology Corp., Fairport, NY, USA). Bags were incubated with a 1:4 ratio of ruminal fluid (mixture of the fluid from the 4 sheep) to incubation medium (Goering and Van Soest, 1970) in an Ankom Daisy II incubator (Ankom Technology Corp., Fairport, NY, USA) at 39 °C and under continuous rotation. After 120 h, bags were washed with cold water, dried at 60 °C for 48 h, and weighted to calculate DMD120.

IV.2.3. Chemical analyses

The proximate composition of OC samples was analyzed using the procedures of the Association of Official Agricultural Chemists (AOAC, 2005) for dry matter (ID 934.01), ash (ID 048.13) and ether extract (EE; ID 945.16). Concentrations of NDF, ADF and acid detergent lignin (ADL) were determined following the procedures of Van Soest et al. (1991) and Robertson and Van Soest (1981), respectively, using an ANKOM220 Fiber Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA). Sodium sulphite was used in the sequential analysis of NDF, ADF and ADL, and results were expressed exclusive of residual ash. Nitrogen was measured by the Dumas combustion method employing a Leco FP258 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI, USA). Acid detergent insoluble nitrogen (ADIN) was determined by analyzing the N content in the residue obtained after treatment with acid detergent solution. Total sugars and total soluble polyphenols (TSP) were analyzed by colorimetric methods following the anthrone method (Yemm and Willis, 1954) and the Folin–Ciocalteu assay (Singleton and Rossi, 1965), respectively, using an Epoch spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA).

Concentrations of $\text{NH}_3\text{-N}$ in vial contents after 24 h of fermentation were determined by the phenol-hypochlorite method as described by Weatherburn (1967), and those of VFA were conducted by gas chromatography as described by García-Martínez et al. (2005). All analyses were performed in duplicate.

IV.2.4. Calculations and statistical analyses

Data of gas production were fitted with time using the model: $\text{Gas} = \text{PGP} (1 - e^{(-c(t - \text{lag}))})$, where PGP is the potential gas production (mL), c is the fractional rate of gas production (h^{-1}), lag is the initial delay in the onset of gas production (h), and t is the time of gas measurement. Gas production parameters were estimated using the NLIN procedure of SAS (SAS, 2017) by an iterative least squares procedure. The average gas production rate (AGPR; mL gas/h) was defined as the average gas production rate between the start of the incubation and the time when half of the PGP was reached, and was calculated as $\text{AGPR} = \text{PGP} \cdot c / [2 (\ln 2 + c \text{ lag})]$. The DMED was estimated for a rumen particulate outflow (K_p) of 0.042 per h from gas production parameters and DMD_{120} as: $\text{DMED} = [(\text{DMD}_{120} \times c) / (c + K_p)] e^{(-c \times \text{lag})}$. This rumen

particulate outflow corresponds to a rumen retention time of 24 h. Finally, the amount of fermented organic matter (FOM) was calculated from acetate, propionate and butyrate production in each vial as described by Demeyer (1991).

The chemical composition data of OC samples was analyzed using the PROC GLM of SAS (SAS, 2017) with OC type as the main effect. Data on fermentation parameters were analyzed as a mixed model using the PROC MIXED of SAS (SAS, 2017), where the effect of OC type (COC, EOC and CYOC) was considered fixed and inoculum was considered a random effect. In both models, when effects were significant, means were compared by using the Tukey's test. Pearson correlation coefficients among the chemical composition of OC samples, gas production or fermentation parameters, and storage time of the alperujo were assessed using the PROC CORR of SAS (SAS, 2017). Models to predict the AGPR and FOM were developed using the PROC GLM of SAS, including OC type (COC, EOC and CYOC) as the fixed effect and chemical fractions as linear and quadratic covariates. The interaction between OC type and the chemical composition was also studied. Slopes of the models for different OC types were compared by Student's test. In addition, the accuracy of predicting the AGPR and FOM from OC type and the duration (months) of the alperujo storage in ponds as a covariate was evaluated using the same model.

IV.3. Results

IV.3.1. Influence of olive cake processing type on chemical composition and *in vitro* fermentation

The chemical composition of OC samples (Table 10) was highly variable. There were great differences in EE content (coefficient of variation (CV) of 53%), but TSP and sugars were the most variable fractions, with CV values ranging from 44.1% to 126%. For each of the three OC types, the most variable fractions were also sugars (CV $\geq$ 107%) and TSP (CV $\geq$ 41.1%), whereas CV values for the rest of the chemical fractions were lower than 24%, with the exception of EE for EOC (69.7%). Crude OC and EOC groups showed similar CV values for most components analyzed, and CYOC had greater CV values for ADL and ADIN compared to COC and EOC.

As shown in Table 11, CYOC had greater ($P < 0.05$) DM, N and EE content

than COC, but lower organic matter (OM), NDF, ADF and ADL content. Compared with COC, EOC had lower EE but greater NDF, ADIN and TSP content. Total sugars were the only fraction analyzed in which no differences among OC types were observed.

Samples of COC had lower ($P < 0.05$) PGP, AGPR and DMED values than EOC, whereas CYOC showed intermediate values of PGP and DMED (Table 12). After 24 h of incubation, neither total VFA production nor molar proportions of individual VFA differed among OC types, with the exception of minor VFA proportions that were lower ($P = 0.003$) for EOC compared with COC samples. Samples of EOC also had lower $\text{NH}_3\text{-N}$ concentrations than both COC and CYOC. Calculated FOM values were greater for EOC and CYOC samples than for COC.

IV.3.2. Estimation of fermentation parameters

The average gas production rate and FOM were selected for developing prediction models as they reflect the fermentation rate and extent of rumen fermentation, respectively. Table 13 shows the correlation coefficients between the chemical composition, fermentation parameters (AGPR and FOM) and storage time of the alperujo. Sugars and TSP were the chemical fractions more closely correlated with the AGPR ($r = 0.90$ and 0.69 , respectively; $P < 0.001$), whereas NDF, total sugars and TSP content were highly correlated ($r = -0.68$, 0.82 and 0.78 , respectively; $P < 0.001$) with FOM. Storage time showed negative correlations ($r \geq -0.74$; $P < 0.001$) with total sugars and TSP content, and both fermentation parameters.

Table 10. Chemical composition (g/kg dry matter unless stated otherwise) of olive cake (OC) samples ($n = 42$).

Item	Mean	CV	OC Type (Range of Values and CV) ¹		
			Crude OC ($n = 25$)	Exhausted OC ($n = 9$)	Cyclone OC ($n = 8$)
Dry matter (g/kg)	906	2.77	862–946 [2.49]	860–908 [1.95]	914–952 [1.43]
Organic matter	913	1.98	873–948 [2.03]	894–935 [1.38]	885–910 [0.91]
Neutral detergent fiber	561	17.8	402–721 [13.4]	528–766 [14.0]	367–528 [13.5]
Acid detergent fiber	409	17.2	298–501 [12.6]	385–533 [12.2]	256–374 [13.5]
Acid detergent lignin	201	14.6	170–250 [10.0]	197–261 [9.41]	123–190 [15.4]
Nitrogen (N)	16.7	14.1	13.1–18.6 [10.7]	15.8–23.5 [13.0]	12.5–22.2 [17.5]
Acid detergent insoluble N	8.45	19.8	5.20–11.9 [18.4]	7.85–12.2 [14.4]	4.70–10.8 [23.9]
Ether extract	99	53.1	73.3–145 [21.3]	4.00–32.0 [69.7]	108–195 [20.0]
Total sugars	43.2	123	7.10–202 [126]	8.00–197 [107]	6.50–149 [120]
Total soluble polyphenols	14.5	44.8	5.30–29.1 [42.0]	8.60–29.2 [41.1]	9.70–29.2 [41.9]

¹ Minimum and maximum values and coefficients of variation (CV; %). Crude OC: samples were dried and pelletized; Exhausted OC: samples were dried and subjected to a second oil extraction using hexane; Cyclone OC: samples were obtained from cyclone decanters during drying COC. n = number of samples. Values between brackets are CV (%).

Table 11. Influence of olive cake (OC) processing on chemical composition (g/kg dry matter unless stated otherwise).

Item	OC Type ¹			SEM ²	P-Value
	Crude OC (<i>n</i> = 25)	Exhausted OC (<i>n</i> = 9)	Cyclone OC (<i>n</i> = 8)		
Dry matter (g/kg)	904 ^a	886 ^a	934 ^b	6.1	<0.001
Organic matter	916 ^b	919 ^b	895 ^a	4.9	0.004
Neutral detergent fiber	570 ^b	645 ^c	441 ^a	23.4	<0.001
Acid detergent fiber	409 ^b	454 ^b	309 ^a	15.4	<0.001
Acid detergent lignin	205 ^b	225 ^b	163 ^a	6.5	<0.001
Nitrogen (N)	15.8 ^a	17.8 ^b	18.0 ^b	0.65	0.013
Acid detergent insoluble N	8.10 ^a	9.76 ^b	8.05 ^a	0.473	0.025
Ether extract ³	110 ^b	15.9 ^a	157 ^c	7.35	<0.001
Total sugars ³	33.9	57.8	43.9	11.0	0.244
Total soluble polyphenols ³	12.4 ^a	18.4 ^b	16.7 ^{ab}	1.93	0.031

^{a, b} Within each chemical fraction, mean values with different superscripts differ ($P < 0.05$). ¹ Crude OC: samples were dried and pelletized; Exhausted OC: samples were dried and subjected to a second oil extraction using hexane; Cyclone OC: samples were obtained from cyclone decanters during drying COC. *n* = number of samples. ² SEM = Standard error of the mean. ³ the Kruskal–Wallis test was used to compare these variables, as they were not normally distributed.

Table 12. Influence of olive cake (OC) processing on gas production parameters, dry matter effective degradability (DMED) and fermentation parameters in a 24 h *in vitro* fermentation of OC samples.

Item	OC Type ¹			SEM ²	P-Value
	Crude OC (<i>n</i> = 25)	Exhausted OC (<i>n</i> = 9)	Cyclone OC (<i>n</i> = 8)		
Gas production parameters ²					
PGP (mL/g dry matter)	64.5 ^a	78.1 ^b	74.4 ^{ab}	3.67	0.008
<i>c</i> (h ⁻¹)	0.048 ^b	0.054 ^b	0.040 ^a	0.0022	<0.001
<i>Lag</i> (h)	0.16	0.21	0.33	0.091	0.208
AGPR (mL/h)	2.23 ^a	3.07 ^b	2.13 ^a	0.182	<0.001
DMED (g/kg DM)	208 ^a	245 ^b	217 ^{ab}	10.1	0.025
Fermentation parameters					
Total volatile fatty acids (VFA; mmol/g DM)	3.35	3.57	3.56	0.09	0.067
Molar proportions of VFA (mol/100 mol)					
Acetate (Ac)	61.8	62.1	62.2	0.24	0.351
Propionate (Pr)	21.4	21.7	21.6	0.24	0.642
Butyrate	11.0	11.1	10.7	0.15	0.263
Minor VFA ³	5.79 ^b	5.16 ^a	5.46 ^{ab}	0.15	0.003
Ac/Pr (mol/mol)	2.92	2.89	2.91	0.036	0.854
NH3–N (mg/L)	222 ^b	207 ^a	222 ^b	6.3	0.004
FOM (g/kg organic matter) ⁴	311 ^a	333 ^b	338 ^b	8.6	0.025

^{a,b} Within each parameter, mean values with different superscripts differ ($P < 0.05$). ¹Crude OC: samples were dried and pelletized; Exhausted OC: samples were dried and subjected to a second oil extraction using hexane; Cyclone OC: samples were obtained from cyclone decanters during drying COC. *n* = number of samples. PGP: potential gas production; *c*: fractional rate of gas production; *Lag*: time until the production of gas begins; AGPR: the average gas production rate until half of the PGP has been reached; FOM: fermented organic matter, DMED was calculated for a rumen passage rate of 0.042 per h. ³ Calculated as the sum of isobutyrate, isovalerate and valerate. ⁴ Fermented organic matter calculated from VFA production as described by Demeyer (1991).

The prediction equations to estimate the AGPR and FOM from OC type and chemical composition are shown in Table 14. The total sugars content was the best covariate to predict the AGPR, as the models including TSP content or any other chemical fractions as predictor variables had greater residual standard deviation (RSD) values (i.e., 0.68 mL/h for TSP content; equations not shown). The sugars content had a positive linear effect ($P < 0.001$) and a quadratic negative effect ($P < 0.001$) on the AGPR. Moreover, an OC type effect was detected ($P < 0.001$) on the intercept term, which had the lowest value in the CYOC model and the greatest in the EOC model.

The NDF content was the best predictor variable of FOM, as models including either sugars or TSP content had greater RSD values (32.1 and 35.0 g/kg OM, respectively; equations not shown) than the model using NDF (27.2 g/kg OM). The NDF content had the same negative effect on FOM values for all OC types, as no OC type–NDF interaction was detected on the slope of the model. The type of OC affected ($P = 0.031$) the intercept term, which had the lowest value in the CYOC model and the greatest in the EOC model.

The storage time was closely correlated ($P < 0.001$) with sugars and TSP content (Table 13) and, therefore, prediction models of fermentation parameters including storage time were developed separately from those involving chemical analyses. As shown in Table 14, the prediction equations of the AGPR and FOM with OC type and storage time as a covariate had lower R^2 and greater RSD values than those using OC type and chemical fractions. A linear and quadratic effect of storage time on the AGPR and FOM was observed, but the interaction between OC type and storage time ($P < 0.001$) on the slope of the AGPR model indicates that the effect of storage time differed among OC types. For a fixed increment of time, the AGPR increased in the order $CYOC \leq COC < EOC$. In contrast, neither the intercept nor the slope of the model for FOM prediction was affected by OC processing type.

Table 13. Correlation matrix (Pearson coefficient and p values in brackets; $n = 42$) of the chemical composition of olive cake (OC) samples and storage time with the average gas production rate (AGPR) and fermented organic matter (FOM) of OC samples measured in 24 h *in vitro* incubations (only $P < 0.05$ values are shown)¹.

Item	Neutral detergent fiber	Acid detergent fiber	Acid detergent lignin	N	Acid detergent insoluble N	Ether extract	Total sugars	Total soluble polyphenols	Storage Time
Neutral detergent fiber	-	0.99 (<0.001)	0.90 (<0.001)	-	0.43 (0.004)	-0.61 (0.004)	-0.43 (0.004)	-0.47 (0.002)	0.39 (0.010)
Acid detergent fiber	-	-	0.93 (<0.001)	-	0.44 (0.003)	-0.63 (<0.001)	-0.40 (0.009)	-0.44 (0.003)	0.36 (0.018)
Acid detergent lignin	-	-	-	-	0.49 (0.001)	-0.61 (<0.001)	-0.34 (0.027)	-0.41 (0.007)	0.36 (0.018)
N	-	-	-	-	0.58 (<0.001)	-	-0.33 (0.035)	-	-
Acid detergent insoluble N	-	-	-	-	-	-0.42 (0.006)	-	-	-
Ether extract	-	-	-	-	-	-	-	-	-
Total sugars	-	-	-	-	-	-	-	0.60 (<0.001)	-0.75 (<0.001)
Total soluble polyphenols	-	-	-	-	-	-	-	-	-0.74 (<0.001)
<i>Fermentable parameters</i>									
AGPR	-0.35 (0.024)	-0.31 (0.048)	-	-0.32 (0.039)	-	-	0.90 (<0.001)	0.69 (<0.001)	-0.76 (<0.001)
FOM	-0.68 (<0.001)	-0.64 (<0.001)	-0.58 (<0.001)	-	-	-	0.82 (<0.001)	0.78 (<0.001)	-0.75 (<0.001)

¹ AGPR: the average gas production rate until it reached half of PGP; fermented organic matter (FOM) was calculated from volatile fatty acids production as described by Demeyer (1991).

Table 14. Models for predicting the average gas production rate (AGPR; mL/h) and fermented organic matter (FOM; g/kg OM) after 24 h *in vitro* incubation of olive cake (OC) samples using OC type (crude (COC), exhausted (EOC) and cyclone (CYOC)) as the fixed effect and either chemical fractions or storage time as covariates ($n = 42$)¹.

Covariate	Parameter ³	Prediction Equation ⁴	RSD ⁵	P-Value ⁶				R^2
				p_{OC}	p_L	$p_{OC * L}$	p_Q	
Chemical fractions (g/kg dry matter)	AGPR (mL/h)	(1.54 (±0.169) + A) + 0.039 (±0.0040) Total sugars - 0.00010 (±0.000021) Total sugars ² A values: -0.393 (±0.1601), 0 and -0.645 (±0.1982) for COC, EOC and CYOC, respectively	0.41	<0.001	<0.001	-	<0.001	0.898
Chemical fractions (g/kg dry matter)	FOM (g/kg organic matter)	(743 (±39.1) + A) - 0.64 (±0.059) Neutral detergent fiber A values: -69.1 (±11.84), 0 and -124 (±18.2) for COC, EOC and CYOC, respectively (6.55 (±0.480) + A) + B * Time + 0.0355 (±0.00907) Time ²	27.2	0.031	<0.001	-	-	0.767
Storage time (months)	AGPR (mL/h)	A values: -1.65 (±0.553), 0 and -2.56 (±0.663) for COC, EOC and CYOC, respectively B values: -0.71 (±0.122), -0.95 (±0.120) and -0.58 (±0.109) for COC, EOC and CYOC, respectively	0.55	0.002	-	<0.001	<0.001	0.795
Storage time (months)	FOM (g/kg organic matter)	432 (±17.4) - 27.2 (±5.91) * Time + 1.07 (±0.481) Time ²	35.7	<0.001	-	-	0.019	0.618

¹ COC samples were dried and pelletized; EOC samples were dried and subjected to an extraction using hexane; CYOC samples were obtained from a cyclone separator after drying the crude samples. ³ AGPR: the average gas production rate until it reached half of PGP; FOM: fermented organic matter calculated from volatile fatty acids production as described by Demeyer (1991). ⁴ Values in parentheses are standard errors. ⁵ Residual standard deviation. ⁶ p_{OC} : effect of OC type; p_L : linear effect of covariate; p_Q : quadratic effect of covariate; $p_{OC * L}$: OC type–covariate interaction effect

IV.4. Discussion

IV.4.1. Influence of olive cake processing on chemical composition and *in vitro* fermentation

The chemical composition of COC and EOC samples was in agreement with values reported by others (Heuzé et al., 2015; Molina-Alcaide and Yáñez-Ruiz, 2008; Albuquerque et al., 2004), and both OC types showed high variability. Factors such as the olive cultivar, ripeness of the fruit, agronomic conditions, storage time in open-air ponds and climatic conditions influence the chemical composition of the alperujo (Dermeche et al., 2013) and, therefore, the composition of the OC obtained. As expected, EE content was lower in EOC than in COC due to the second oil extraction and this caused an increase in the remaining chemical components, although only NDF showed significant differences. The degree of lignification of NDF and ADF fractions (36% and 50%, respectively; calculated as $(\text{lignin/NDF}) \times 100$) was similar between OC types reflecting no changes due to processing. All OC samples had low N content (≤ 23.5 g/kg DM), and in accordance with others (Heuzé et al., 2015; Molina-Alcaide and Yáñez-Ruiz, 2008) ADIN was a high proportion of total N (51.3, 54.8 and 44.7% for COC, EOC and CYOC, respectively), indicating low N availability. However, $\text{NH}_3\text{-N}$ concentrations in the *in vitro* incubations were above the level limiting *in vitro* ruminal microbial growth (Satter and Slyter, 1974) for all OC samples due to the use of an N-enriched medium. The greater ADIN concentrations in EOC compared with COC and CYOC samples were attributed to the second heat treatment applied to remove the hexane solvent used in the extraction of pomace olive oil from COC, as heating may lead to the formation of indigestible compounds via Maillard reactions between sugar aldehyde groups and free amino groups (Broderick et al., 2000).

To the best of our knowledge, there was no previous information on the chemical composition of CYOC in the literature. The lower NDF and ADF content of CYOC than in COC and EOC may reflect the smaller amount of olive stone fragments, as CYOC is composed of the lighter pomace particles floating in the air collected during drying the alperujo. The lower NDF, ADF and ADL content of CYOC compared with both COC and EOC also supports this hypothesis, as hemicellulose, cellulose and lignin are the main components of olive stones (Rodríguez et al., 2008). In

addition, CYOC had the greatest EE content and, therefore, it is expected to have greater energy content for ruminants than COC and EOC.

In agreement with the results reported by others (reviewed by Molina-Alcaide and Yáñez-Ruiz, (2008)) the ruminal degradability of OC was low, but there was a wide variability in DMED values which ranged from 99.2 to 378 g/kg DM. For example, Álvarez-Rodríguez et al. (2009) observed that only 120 g/kg DM of a sample of EOC disappeared from the rumen after 72 h of incubation, whereas this value was 370 g/kg DM for a COC sample, and a slightly greater *in situ* value (420 g/kg DM) was reported by Martín-García et al. (2003) for a COC sample. Variations in DMED could be partly explained by the chemical composition of OC, as DMED was positively correlated with total sugars ($P < 0.001$) and negatively with NDF, ADF and ADL ($P \leq 0.024$; values not shown) content.

Both the unchanged VFA production and VFA profile in all OC groups indicate a similar fermentation pattern. Unsaturated free fatty acids resulting from the triglyceride hydrolysis are toxic to fibrolytic bacteria (Maia et al., 2007), but the similar acetate and butyrate proportions observed in the three OC groups does not indicate a negative effect of the high EE content of some COC and CYOC samples on fiber fermentation. A maximal level of 60 g of fat per kg of DM is usually recommended in the diet of ruminants to avoid reductions in fiber digestibility (NRC, 2001); other studies revealed no negative effect of greater EE levels both *in vivo* (Toral et al., 2010) and *in vitro* (Gómez-Cortés et al., 2008). Finally, the lack of correlation between EE and any fermentation parameter supports the idea that there was no negative effect of the high EE content on ruminal fermentation.

The hydrolysis of olive oil lipids has been reported to be much lower than that of more unsaturated oils such as linseed oil (68% vs. 95% at 24-h incubation, respectively) (Garton et al., 1961), thus liberating less fatty acids. In addition, the high lignification degree of the NDF in all OC types ($\geq 34.9\%$) indicates low NDF degradability and this may help to explain the lack of an apparent negative effect of EE content on total VFA production from COC and CYOC samples. In contrast, the lower the AGPR observed for COC and CYOC compared with EOC might indicate a reduced fermentation. However, gas production data from samples with high EE

content should be interpreted with caution, as no gas arises from EE fermentation in the rumen, and total gas production can even be reduced by fat supplementation due to decreased CH₄ production (Danielsson et al., 2017).

IV.4.1. Estimation of fermentation parameters

The average gas production rate was influenced by the OC processing type and the intercept of the model was greater for EOC than for COC and CYOC, possibly due to the differences in their EE content, and, therefore, in the amount of potentially fermentable dry matter, as no gas is formed from fat fermentation. The positive linear effect of sugars content in the prediction equations of the AGPR reflects that sugars contribute to increased gas production in the first hours of incubation, when sugars are rapidly and extensively fermented by rumen microorganisms. In fact, sugars/EE ratio was greater in EOC than in COC and CYOC samples (6.8, 0.31 and 0.28, respectively). However, there was also a negative quadratic effect of sugars content that might be due to the positive relationship observed between sugars and TSP content in OC samples. The presence of TSP in feeds can reduce feed ruminal degradability (Theodorou et al., 2006), and negative correlations between TSP content and DM degradability are frequently reported (Molina-Alcaide et al., 2017).

The significant effect of OC type on the intercept of the FOM model indicates differences between OC types, with COC and CYOC having lower FOM values than EOC for samples with similar NDF content. This might be related to the greater EE content in COC and CYOC, as EE was not related to NDF content and EE does not lead to VFA production (only low VFA amounts are generated from glycerol fermentation (Van Soest, 1994)). The lack of influence of OC type on the slope of the FOM prediction model reflects similar changes in FOM with increases in NDF for all OC types, as clearly shown by the similar slopes observed in the linear models developed individually for each OC group ($r = -0.622$, -0.648 and -0.690 for COC, EOC and CYOC samples, respectively). This is consistent with the similar lignification degree of the NDF in all OC types (36.0%, 34.9% and 37.0% for COC, EOC and CYOC, respectively), as lignin is one of the major factors limiting NDF degradability.

Models involving OC type and storage time as predictor variables were also developed, as this would be a cheap and rapid form to estimate fermentation parameters.

Differences in intercept values on the AGPR model were related to the differences in EE content among OC types that were not correlated with changes in the covariate of the model. The negative effect of storage time on the slope is in accordance with the observed changes in the chemical composition of OC over the storage period. Sugars content in OC samples clearly decreased as storage time in the pond augmented ($r = -0.769$; $P < 0.001$; $n = 42$), whereas NDF content, a less fermentable fraction, increased with advancing storage time ($r = 0.395$; $P = 0.010$; $n = 42$).

The significant interaction between OC type and storage time on the slope observed in the AGPR model reflects a different timely evolution for each OC type. The greater slope of the EOC model compared with those for COC and CYOC samples may reflect the greater increases in NDF fraction with time (167 g/kg DM from months 1 to 8) in EOC compared with those in COC and CYOC (109 and 87 g/kg DM, respectively), because NDF fermentability is lower than in other fractions (i.e., sugars, starch, etc.). In contrast, in the prediction of FOM, there was no effect of OC type on the intercept, which is consistent with the lack of correlation between EE and storage time. The negative effect of storage time on the slope of the FOM model reflected the decrease in total sugars and, therefore, in OM fermentation as the alperujo storage time advanced. Finally, the quadratic effect of storage time in all models confirms the results of Marcos et al. (2019), who observed that the loss of the nutritive value of OC was more pronounced during the first months of storage and almost imperceptible from 6 storage months.

Although the models using OC type and storage time as predictor variables had lower R^2 and greater RSD values than those involving OC type and the chemical composition, it has to be taken into account that analysis of the chemical composition is expensive and time consuming. The possibility of predicting *in vitro* fermentation parameters from OC type and storage time could be a cheap and rapid alternative.

IV.5. Conclusions

The results show that the chemical composition of OC by-products is highly variable, markedly affected by the processing type and storage time of the alperujo before processing. However, all samples had high lignification of both NDF and ADF fractions and low N content, with ADIN being greater than 44% of total N. Cyclone

OC had the lowest NDF and the greatest EE content, as well as an intermediate sugars content, which indicates that it would have greater nutritive value for ruminants than COC and EOC. All OC types were poorly degraded *in vitro* and, therefore, their nutritive value for ruminants is highly dependent on their EE content. Models using OC processing type, and either the chemical composition or the alperujo storage time before processing as covariates, were useful to predict the *in vitro* ruminal fermentation variables of OC.

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Chapter V. NUTRITIVE VALUE OF TOMATO POMACE FOR RUMINANTS AND ITS INFLUENCE ON *IN VITRO* METHANE PRODUCTION

Marcos, C. N., De Evan, T., Molina-Alcaide, E., and Carro, M. D. (2019). Nutritive Value of Tomato Pomace for Ruminants and Its Influence on In Vitro Methane Production. *Animals* **9**, 343-358. doi: 10.3390/ani9060343

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Simple Summary: Reutilization of agroindustrial by-products in animal feeding could contribute to the reduction of environmental problems associated with their accumulation, but it is necessary to assess their nutritive value. Tomato pomace (TP) is a by-product of the tomato industry that could be used in ruminant feeding, but data on its nutritive value are limited. The aim of this work was to analyze the chemical composition, *in vitro* rumen fermentation, and intestinal digestibility of 12 TP samples obtained from two processing plants at different times during the tomato campaign and to assess the *in vitro* fermentation of diets including increased TP amounts. The chemical composition of TP showed little variability. Samples of TP had high fiber, protein, and fat content and were rapidly fermented in the rumen, but the *in vitro* intestinal digestibility of the protein was low. The *in vitro* results provide useful information for including TP in ruminant diets, indicating that amounts of TP up to 180 g/kg could be included in a diet for fattening ruminants without negatively affecting rumen fermentation, but these results should be confirmed *in vivo*.

Abstract: The objective of this study was to determine the variability in nutritive value for ruminants of tomato pomace (TP) samples and analyze its effect on *in vitro* fermentation when it was included in a high-concentrate diet. Twelve TP samples were obtained from two processing plants at weekly intervals and analyzed for chemical composition, *in vitro* rumen fermentation, and intestinal digestibility. The chemical composition of TP did not differ between processing plants and only slight variations were observed among sampling times. Tomato pomace had a low dry matter content (<300 g/kg), a high content of neutral detergent fiber, crude protein, and ether extract (572, 160, and 82.7 g/kg dry matter on average, respectively), and was rapidly fermented in the rumen. Protein degradability at 16 h *in situ* incubation was 510 g/kg and *in vitro* intestinal digestibility of protein was low (430–475 g/kg). Replacing soybean meal and barley straw by dried TP increased the *in vitro* fermentation rate and the production of volatile fatty acids and reduced NH₃-N concentrations without affecting CH₄. In summary, TP samples showed little variability in nutritive value over sampling time and TP of up to 180 g/kg could be included in high-concentrate diets without negatively affecting rumen fermentation.

Keywords: tomato pomace; *in vitro* rumen fermentation; methane; rumen degradability; intestinal digestibility

V.1. Introduction

The world production of processing tomato has increased up to 37.8×10^6 tons in 2017 according to estimates of the World Processing Tomato Council (WPTC, 2019). In the European Union, Italy and Spain are the greatest tomato producers, accounting for 4.7×10^6 and 2.8×10^6 tons of tomatoes for processing in 2017, respectively. Tomato pomace (TP) is a by-product of the tomato industry that consists mainly of peels, seeds, and small quantities of pulp that remain after the production of juice, paste, sauce, and other processed products, representing between 5% and 10% of the original fruits (Fondevila et al., 1994). The high water content of TP (usually greater than 75%) due to the water added in the last step of the industrial process is the most important limitation for its further utilization in animal feeding, as fresh TP spoils very quickly (Caluya, 2000); therefore, the use of fresh TP for animal feeding is generally restricted to farms closely located to the processing plants (Denek and Can, 2006), also limited because high-moisture feeds reduce feed intake in ruminants. Tomato pomace can be preserved either by drying or by ensiling with cereal straw (Denek and Can, 2006), but these procedures increase their price. The use of TP in animal feeding would contribute to the reduction of environmental problems caused by their accumulation, mainly related to bad odor and pests breeding in accumulations of spoiled TP (Caluya et al., 2003). In addition, TP can improve the quality of animal products by increasing the content of fatty acids beneficial to human health (Arcos-Pérez et al., 2017; Vasta et al., 2008). However, the nutritive value of TP depends on many factors, such as cultivars, season, or processing, among others (Del Valle et al., 2006), but current knowledge on its variability is limited.

Previous studies have shown that the inclusion of tomato fruit wastes in the diet of goats reduced CH₄ emissions (Romero-Huelva and Molina-Alcaide, 2013; Romero-Huelva et al., 2012) and this antimethanogenic effect has also been observed *in vitro* (Romero-Huelva et al., 2013). The antimethanogenic mechanisms of tomato fruit wastes are still unknown (Romero-Huelva and Molina-Alcaide, 2013), but the presence of bioactive substances affecting the rumen methanogenic archaea cannot be ignored. However, to our best knowledge, the possible influence of TP on rumen CH₄ production has not yet been tested. Therefore, the first objective of this study was to analyze the variability in the nutritive value of TP samples collected from two processing plants

during the tomato campaign. The second objective was to assess the effects of including increasing doses of TP in a diet for fattening lambs on *in vitro* fermentation and CH₄ production.

V.2. Materials and methods

V.2.1. Tomato pomace samples

Twelve samples of TP were obtained from two of the biggest Spanish tomato processing plants (Tomates del Guadiana Soc. Coop., Badajoz, Spain, and PRONAT, Badajoz, Spain) during the 2016 tomato harvesting campaign. The tomato campaign in Spain is usually concentrated in August and September. Samples (4–5 kg) were obtained weekly at each plant on the 12th, 19th, and 26th August and the 2nd, 9th, and 16th of September, frozen (–20 °C) and lyophilized. In all cases, samples were the final tomato-processing disposal. All samples were ground at 2 mm, and subsamples were ground at 1 mm before chemical analyses and *in vitro* incubations.

V.2.2. Animals and feeding

Four adult rumen-fistulated Lacaune sheep (63.5 ± 2.05 kg body weight (BW)) were individually housed and had free access to fresh water over the trial. Animals were fed twice daily (8:00 and 18:00) a 2:1 grass hay:concentrate diet at a rate of 45 g dry matter (DM)/kg BW^{0.75}. The diet contained 114, 365, and 160 g of crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) per kg DM, respectively, and was formulated to meet maintenance requirements of the experimental animals (NRC, 2007). Sheep were managed in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of 1st February on the protection of animals used for experimentation or other scientific purposes) and all experimental procedures were approved by the Institutional Animal Care and Use Committee of the Comunidad Autónoma de Madrid (PROEX 035/17).

V.2.3. Experimental design

The study comprises two experiments. The *in vitro* rumen fermentation and the intestinal digestibility of protein (IDCP) of the TP samples were measured in Experiment 1, whereas Experiment 2 was designed to analyze the effects of including

dried TP in the diet on *in vitro* rumen fermentation, with special emphasis on CH₄ production.

V.2.3.1. Experiment 1: *in vitro* fermentation of TP samples

Two *in vitro* trials were conducted on different days using the same methodology and in each of them there were four replicates per TP sample by using the ruminal fluid from each sheep as inoculum.

The first trial was carried out to determine the gas production kinetics of samples and the second one to assess the main fermentation parameters and CH₄ production. Ruminal contents of each sheep were obtained before the morning feeding, strained through four layers of cheesecloth into previously warmed thermal flasks, and immediately transported to the laboratory. The fluid of each sheep was independently mixed with pre-warmed (39 °C) culture medium (Goering and Van Soest (1970); without trypticase) in a proportion 1:4 under CO₂ flushing. The medium of Goering and Van Soest (1970) was modified by replacing the (NH₄)HCO₃ with NaHCO₃ and excluding the trypticase to avoid N supply. Samples (200 mg of DM) of each feed were accurately weighed into 60-mL vials. Vials were filled up with 20 mL of the rumen fluid—culture medium mixture using a Watson–Marlow 520UIP31 peristaltic pump (Watson–Marlow Fluid Technology Group, Cornwall, UK), sealed with rubber stoppers, and incubated at 39 °C for 120 h. Gas production was measured at 3, 6, 9, 12, 15, 22, 26, 31, 36, 48, 58, 72, 96, and 120 h using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a plastic syringe and the gas produced at each sampling time was released to prevent gas accumulation. Two blanks (vials without sample) per inoculum were also incubated in this trial to correct gas production values for endogenous production. The potential *in vitro* DM degradability (PDMD) was determined by weighing 300 mg of each feed (3 bags per feed) into polyester bags of 30 µm pore size (Ankom Corp #57, Ankom Technology Corp., Fairport, NY, USA) and incubating the bags in a 1:4 mixture of ruminal fluid and the incubation medium of Goering and Van Soest (1970) in an Ankom Daisy II incubator at 39 °C under continuous rotation for 120 h. A mixture of equal parts of the ruminal fluid from each sheep was used as inoculum. After 120 h, bags were washed with cold water, dried at 60 °C for 48 h, and weighed to calculate PDMD.

In the second *in vitro* trial, vials were incubated at 39 °C for 24 h as described before and samples to analyze the main fermentation parameters were taken. Gas production was measured and a gas sample (10 mL) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for analysis of CH₄ concentration. Vials were then uncapped, their content was homogenized, the pH was immediately measured (Crison Basic 20 pH-meter, Crisson Instruments, Barcelona, Spain), and 3 mL of the vials' contents were mixed with 3 mL of 0.5 M HCl. Samples were frozen (−20 °C) until volatile fatty acid (VFA) and NH₃-N analyses.

V.2.3.2. Experiment 1: *in vitro* intestinal digestibility of tomato pomace samples

The determination of the IDCP of TP samples followed the three-step procedure described by Gargallo et al. (2006) and was performed as detailed by Belverdy et al. (2019). Briefly, 3 g of each TP sample (2 mm size) was weighed into nylon bags (7 × 14 cm; 46 µm pore size) and incubated in the rumen of each sheep immediately before the afternoon feeding on 2 different days. After 16 h of *in situ* incubation, bags were washed, frozen (−20 °C, 48 h) to facilitate the detachment of particle-associated bacteria, thawed, washed 3 times with cold water (5 min each) in a turbine washing machine, frozen again (−20 °C), lyophilized, and weighed. Finally, bag residues were analyzed for N content to calculate the *in situ* degradability of CP. Bag residues were pooled by TP sample and sheep and 0.5 g of each were weighed in duplicate into nylon bags (5 × 5 cm; 46 µm pore size). Bags were incubated into Daisy II incubation bottles (ANKOM Tech. Co., Fairport, NY, USA) containing 2 liters of a 0.1 N HCl solution (pH 1.9; 39 °C) with 1 g per l of pepsin (P-7000, Sigma, St. Louis, MO, USA) under constant rotation at 39 °C for 1 h. Bags were then rinsed with tap water and incubated in a pancreatin solution (0.5 M KH₂PO₄ buffer, pH 7.75, 50 ppm of thymol and 3 g per l of pancreatin; P-7545, Sigma, St. Louis, MO, USA) at 39 °C for 24 h under constant rotation. Finally, bags were rinsed with tap water, dried at 40 °C for 72 h, and weighed. Residues of the *in vitro* incubation were pooled by sheep and TP sample and analyzed for N concentration to calculate IDCP values.

V.2.3.3. Experiment 2: *in vitro* fermentation of diets containing tomato pomace

The objective of this experiment was to analyze the effects of including increasing amounts of dried TP in a high-concentrate diet for fattening lambs (control

diet) on *in vitro* rumen fermentation. Three additional diets were formulated by replacing different amounts of barley straw, soybean meal, and wheat bran in the control diet with 60, 120, or 180 g per kg (fresh matter basis) of a dried TP sample. A composited sample of the TP samples tested in Experiment 1 was used and it contained 967, 163, 572, 446, and 95.2 g of organic matter (OM), CP, NDF, ADF and ether extract (EE) per kg DM, respectively. All diets were formulated to have similar CP and NDF content to the control diet and therefore TP replaced fibrous (barley straw and wheat bran) and protein (soybean meal) conventional ingredients. The ingredients and chemical compositions of the four experimental diets are given in Table 15.

Table 15. Ingredients and chemical composition of control and experimental diets containing increased amounts of tomato pomace (TP) used in Experiment 2.

Item	Control	TP6	TP12	TP18
Ingredient (g/kg fresh matter)				
Barley	315	315	315	315
Corn	252	275	284	285
Wheat	130	130	130	130
Barley Straw	120	85	35	20
Soybean meal 46%	110	96	66	60
Wheat bran	48.1	20	40	-
Tomato pomace	-	60	120	180
Calcium soap	15	15	-	-
Calcium carbonate	5	5	5	5
Mineral/vitamine premix	5	5	5	5
Chemical composition ¹				
Dry matter	903	903	902	905
Organic matter	945	957	969	970
Crude protein	145	145	145	145
Neutral detergent fiber	245	242	245	250
Acid detergent fiber	100	107	111	126
Ether extract	44.8	46.2	46.3	52.2
Non structural carbohydrates	510	524	533	523

¹ Calculated from analyzed composition of individual feed ingredients. All chemical fractions are expressed as g/kg dry matter, except dry matter (g/kg fresh matter). Non-structural carbohydrates were calculated as the difference between organic matter and the sum of crude protein, neutral detergent fiber and ether extract.

All diets were incubated *in vitro* for 24 h following the methodology described in Experiment 1, with the exception that the amount of sample was increased to get enough gas for the analysis of CH₄ concentration; therefore, 400 mg of each diet was fermented with 40 mL of the ruminal fluid and culture medium mixture in 120-mL vials. After 8 h of incubation, gas production was measured and the gas was sampled for CH₄ analyses as described in Experiment 1. Immediately, 1 mL of each vial content was taken using an insulin syringe, mixed with 1 mL of 0.5 M HCl, and stored at -20 °C for VFA and NH₃-N analyses. After 24 h, the gas produced and the pH of the vials' contents were measured and samples for CH₄, VFA and NH₃-N analyses were taken. Four replicates per diet were obtained by using the ruminal fluid from each sheep independently as inoculum.

V.2.4. Chemical analyses

Chemical composition of TP samples and feed ingredients of experimental diets were analyzed using the Association of Official Analytical Chemists (AOAC, 2005) procedures for DM (ID 934.01), ash (ID 048.13), and EE (ID 945.16). Concentrations of NDF, ADF, and lignin were determined following the procedures of Van Soest et al. (1991) and Robertson and Van Soest (1981), respectively, using an ANKOM220 Fiber Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA). Sodium sulphite and α -amylase were used in the sequential analysis of NDF, ADF, and lignin and ash-free values are reported. Nitrogen was measured by the Dumas combustion method employing a Leco FP258 N Analyzer (Leco Corporation, St. Joseph, MI, USA) and the amount of acid detergent insoluble N (ADIN) was determined by analyzing the N content in the residue obtained after the treatment of the sample with acid detergent solution. Total sugars and total soluble polyphenols (TSP) were analyzed by colorimetric methods following the anthrone method (Yemm and Willis, 1954) and the Folin–Ciocalteu assay (Singleton and Rossi, 1965), respectively, using an Epoch spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Concentrations of NH₃-N were determined by the phenol-hypochlorite method (Weatherburn, 1967) and those of VFA and CH₄ by gas chromatography as described by García-Martínez et al. (2005) and Martínez et al. (2010), respectively. All analyses were performed in duplicate.

V.2.5. Calculations and statistical analyses

Data of gas production were fitted with time using the exponential model $\text{Gas} = \text{PGP} \times (1 - e^{(-c \times (t - \text{Lag}))})$, where PGP is the asymptotic gas production, c is the fractional rate of gas production, Lag is the time before starting gas production, and t is the time of gas measurement. Gas production parameters were estimated using the NLIN procedure of SAS (SAS, 2017) by an iterative least squares procedure. The average gas production rate (AGPR) was calculated as $\text{AGPR} = \text{PGP} \times c \div [2 \times (\ln 2 + c \times \text{Lag})]$ and it was defined as the rate between the incubation start and the time at which half PGP is reached. The DM effective degradability (DMED) was estimated as $\text{DMED} = [(\text{PDMD} \times c) \div (c + \text{Kp})] \times e^{(-c \times \text{Lag})}$, using a rumen particulate outflow (Kp) of 0.042 per h. Finally, the amount of fermented OM (FOM) was calculated from acetate, propionate, and butyrate production in each vial, as described by Demeyer (1991).

As there were no differences ($P \geq 0.191$) in the chemical composition of the TP samples between the 2 processing plants, chemical composition data were analyzed using the PROC GLM of SAS (SAS Institute, Cary, NC, USA) (SAS, 2017) with sampling time as the main effect. Data from Experiment 1 were analyzed as a mixed model with repeated measurements using the PROC MIXED of SAS, in which the effect of sampling time (1 to 6 weeks) was considered fixed and that of the inoculum was considered random. Data from Experiment 2 were analyzed as a mixed model, with the effect of the diet being fixed and that of the inoculum being random. Non-orthogonal polynomial contrasts were used to test for linear and quadratic effects of TP inclusion. Significance was declared at $P < 0.05$, whereas $P < 0.10$ values were considered as a trend. When a significant effect of sampling time was detected, means were compared by Tukey's test. Relationships between chemical composition of the TP samples and either gas production or fermentation parameters were assessed by linear regression using the PROC CORR of SAS (SAS, 2017).

V.3. Results

V.3.1. Chemical composition of tomato pomace samples, *in vitro* fermentation, and intestinal digestibility of crude protein

Chemical composition of TP (Table 16) was relatively unchanged over the sampling period and only lignin and EE contents varied significantly ($P \leq 0.017$) with time; in general, lignin increased with advancing time, whereas EE decreased. Both NDF and ADF contents tended ($P = 0.096$ and 0.063 , respectively) to be greater in the last weeks of sampling compared with the first sampling week. Dry matter, total sugars, and TSP were the most variable fractions, with coefficients of variation (CV) of 21.9%, 20.1%, and 25.1%, respectively (results not shown), whereas the rest of the analyzed fractions showed a CV lower than 10%, except ADIN and EE (15.4% and 18.1%, respectively).

As shown in Table 17, there were differences ($P \leq 0.043$) among TP samples taken at different times in all gas production and *in vitro* fermentation parameters, except for the fractional gas production rate, *Lag*, NH_3 concentrations, and CH_4 production, although a trend ($P \leq 0.095$) was detected for lag and CH_4 production. Fermentation data indicated that TP samples taken at the end of the campaign were less degradable than the rest of samples.

Values of 16-h *in situ* degradability of DM ranged from 488 to 540 g/kg, and decreased ($P = 0.005$) as sampling time advanced (Table 18). In contrast, 16 h *in situ* degradability of CP, IDCP, and total CP digestibility values did not change ($P \geq 0.190$) over the sampling period.

Table 19 shows the correlation coefficients between chemical composition and gas production parameters (PGP and AGPR), DMED, some fermentation parameters, *in situ* rumen degradability of DM and CP, and IDCP of TP samples. Organic matter was the chemical fraction more closely correlated ($r \geq 0.860$; $P < 0.001$) with PGP, AGPR, and total VFA production; ADF was the only fraction significantly correlated ($P = 0.030$) with CH_4 production. *In situ* DM degradability showed negative correlations ($r \geq -0.699$; $P \leq 0.011$) with NDF, ADF, and lignin, whereas *in situ* CP degradability was negatively correlated with ADF ($r = -0.724$; $P = 0.008$) and positively with CP content

($r = 0.796$; $P = 0.002$). The IDCP was negatively affected by TSP content ($r = -0.744$; $P = 0.006$), and total CP digestibility was positively correlated with CP content ($r = 0.790$; $P = 0.002$).

V.3.2. *In vitro* fermentation of diets containing tomato pomace

There were no differences ($P \leq 0.175$) among diets (Table 20) in PGP, *Lag*, AGPR, and DMED values, but the fractional rate of gas production was linearly ($P = 0.016$) and quadratically ($P = 0.015$) augmented with increasing TP amounts in the diet. Total VFA production at 8 h of incubation was not affected ($P \geq 0.161$) by the inclusion of TP, but after 24 h of incubation a linear increase ($P = 0.029$) in total VFA production was observed. At 8 h of incubation, the VFA profile was modified by TP inclusion and there was an increase in molar proportion of acetate (quadratic; $P = 0.038$) and propionate (linear; $P = 0.007$) and a linear decrease in the proportion of butyrate ($P = 0.009$) and minor VFA (calculated as the sum of isobutyrate, isovalerate, and valerate; $P = 0.002$) as TP increased. However, after 24 h of incubation, only a linear increase ($P = 0.002$) in propionate proportion and a linear decrease ($P = 0.002$) in the proportion of minor VFA were observed. As a consequence, acetate/propionate ratios decreased linearly at both incubation times ($P = 0.039$ and 0.007 at 8 and 24 h, respectively). Concentrations of $\text{NH}_3\text{-N}$ decreased linearly at 8 h ($P = 0.049$) with increasing amounts of TP in the diet and a trend ($P = 0.066$) was detected after 24 h of incubation. Including TP in the diet did not affect CH_4 production at any time ($P \geq 0.194$), but a linear reduction ($P = 0.047$) of CH_4/VFA ratio was observed at 8 h of incubation; however, this effect disappeared after 24 h of incubation.

Table 16. Chemical composition (g/kg dry matter (DM) unless stated otherwise) of tomato pomace samples taken from two processing plants at weekly intervals during the tomato harvesting campaign.

Item	Sampling Time (Weeks)						Average	SEM ¹	P-Value
	1	2	3	4	5	6			
DM (g/kg)	260	277	244	276	231	291	263	36.2	0.841
Organic matter	961	965	957	962	963	970	963	3.5	0.35
Crude protein	173	150	160	158	159	161	160	8.4	0.623
Acid detergent insoluble N (ADIN)	2.22	1.58	2.07	2.08	2.12	2.24	2.05	0.146	0.126
Neutral detergent fiber	541	574	565	589	576	584	572	9.5	0.096
Acid detergent fiber	408	442	434	466	442	442	439	9.4	0.063
Acid detergent lignin	217 ^a	244 ^{ab}	229 ^{ab}	259 ^b	246 ^{ab}	270 ^b	244	7.2	0.017
Ether extract	107 ^c	101 ^{bc}	76.5 ^{ab}	69.7 ^a	81.9 ^{ab}	84.4 ^{ab}	82.7	5.36	0.015
Total sugars	123	124	134	108	113	127	122	15.4	0.856
Total soluble polyphenols	4.05	4.36	3.42	3.40	3.05	2.55	3.47	0.408	0.137
Potential DM degradability	595	574	614	583	595	519	580	27	0.323

^{abc} Within each variable, mean values with different superscripts differ ($P < 0.05$). ¹ SEM = standard error of the mean.

Table 17. Gas production kinetics and fermentation parameters of tomato pomace samples taken from two processing plants at weekly intervals during the tomato harvesting campaign and samples.

Item	Sampling Time (Weeks)						Average	SEM ¹	P-Value
	1	2	3	4	5	6			
Gas production parameters ²									
PGP (mL/g dry matter (DM))	199 ^a	201 ^{ab}	226 ^b	211 ^{ab}	210 ^{ab}	187 ^a	206	5.9	0.001
<i>c</i> (%/h)	7.28	7.17	6.97	7.04	7.17	6.88	7.09	0.181	0.316
<i>Lag</i> (h)	2.33	2.63	2.33	2.50	2.42	2.48	2.45	0.130	0.064
AGPR (mL/h)	8.51 ^{ab}	8.32 ^{ab}	9.39 ^b	8.81 ^b	8.73 ^b	7.48 ^a	8.54	0.262	0.001
DMED (g/kg)	324 ^c	305 ^{bc}	332 ^c	313 ^b	320 ^c	276 ^a	312	7.3	<0.001
Fermentation parameters ³									
pH	6.81 ^{ab}	6.80 ^{ab}	6.76 ^b	6.75 ^b	6.79 ^{ab}	6.83 ^a	6.79	0.015	0.004
Total volatile fatty acids (VFA; mmol/g DM)	6.21 ^{ab}	6.34 ^b	6.52 ^b	6.37 ^b	6.41 ^b	5.78 ^a	6.27	0.250	0.004
Molar proportions (mol/100 mol)									
Acetate	66.8 ^{ab}	65.9 ^a	66.3 ^{ab}	66.3 ^{ab}	67.0 ^b	66.8 ^{ab}	66.5	0.23	0.007
Propionate	23.0 ^{ab}	23.5 ^{ab}	23.6 ^b	23.5 ^{ab}	23.0 ^{ab}	22.7 ^a	23.2	0.21	0.014
Butyrate	7.17	7.41	7.44	7.36	7.23	7.37	7.33	0.068	0.043
Minor VFA	2.98 ^{ab}	3.14 ^b	2.64 ^a	2.82 ^{ab}	2.78 ^{ab}	3.10 ^b	2.91	0.105	0.011
Acetate/propionate (mol/mol)	2.91 ^{ab}	2.80 ^a	2.81 ^{ab}	2.83 ^{ab}	2.93 ^{ab}	2.95 ^b	2.87	0.035	0.009
NH ₃ (mg/L)	173	158	155	156	159	155	159	8.2	0.608
CH ₄ (mL/g DM)	65.2 ^{ab}	63.8 ^b	75.0 ^b	76.3 ^a	65.0 ^{ab}	61.8 ^a	67.9	4.02	0.060
CH ₄ /VFA (mL/mmol)	10.1	9.33	10.3	10.6	9.91	10.6	10.1	0.346	0.112

^{ab} Within each parameter, mean values with different superscripts differ ($P < 0.05$). ¹ SEM: Standard error of the mean. ² PGP: Asymptotic gas production (mL/g DM); *c*: Fractional rate of gas production (%/h); *Lag*: Time before starting gas production; AGPR: Average gas production rate; DMED: Dry matter effective degradability calculated for a rumen particulate outflow of 4%/h. ³ Minor VFA: Calculated as the sum of isobutyrate, isovalerate and valerate.

Table 18. *In situ* rumen degradability of dry matter (DM) and crude protein (CP) after 16 h of incubation, *in vitro* intestinal digestibility of CP (IDCP), and total CP digestibility of tomato pomace samples taken from two processing plants at weekly intervals during the tomato harvesting campaign.

Item	Sampling Time (Weeks)						Average	SEM ¹	P-Value
	1	2	3	4	5	6			
<i>In situ</i> degradability of DM (g/kg)	540 ^b	523 ^{ab}	530 ^{ab}	488 ^a	492 ^a	489 ^a	510	11.0	0.005
<i>In situ</i> degradability of CP (g/kg)	563	515	492	511	502	478	510	26.6	0.344
IDCP (g/kg)	450	430	467	466	475	467	459	19.0	0.593
Total CP digestibility (g/kg)	761	730	726	739	741	722	737	10.7	0.190

^{ab} Within each parameter, mean values with different superscripts differ (P < 0.05). ¹ SEM: Standard error of the mean

Table 19. Correlation matrix (Pearson coefficient and P-values in brackets; $n = 12$) of chemical composition of tomato pomace samples with gas production parameters, dry matter effective degradability (DMED), fermentation parameters measured in 24-h *in vitro* incubations, *in situ* rumen degradability of dry matter (DM) and crude protein (CP) after 16 h of incubation, and *in vitro* intestinal digestibility of CP (only $P < 0.10$ values are shown) ¹.

Item	OM ²	NDF	ADF	Lignin	Lignin/NDF	N	EE	TSP
Gas production parameters ³								
PGP	0.860 (<0.001)		0.552 (0.063)		-0.590 (0.044)			
AGPR	-0.892 (<0.001)			-0.646 (0.023)				
DMED	-0.684 (0.014)			-0.770 (0.003)	-0.741 (0.006)			
Fermentation parameters								
Total volatile fatty acid (VFA) production	-0.857 (<0.001)		0.549 (0.064)		-0.613 (0.034)			
CH ₄	-0.789 (0.002)		0.617 (0.033)					
<i>In situ</i> degradability and <i>in vitro</i> intestinal digestibility								
<i>In situ</i> rumen DM degradability		-0.838 (0.001)	-0.726 (0.008)	-0.699 (0.011)			0.600 (0.039)	0.559 (0.059)
<i>In situ</i> rumen CP degradability			-0.724 (0.008)			0.796 (0.002)		
<i>In vitro</i> intestinal digestibility of CP			0.568 (0.054)				-0.511 (0.090)	-0.744 (0.006)
Total digestibility of CP			-0.516 (0.086)			0.790 (0.002)		

¹ Total sugars and acid detergent insoluble N (ADIN) were not correlated to any of the analyzed parameters. ² OM: Organic matter; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; EE: Ether extract; TSP: Total soluble polyphenols. ³ PGP: Potential gas production (mL/g dry matter); AGPR: Average gas production rate until it has reached half of PGP (mL/h); DMED: Calculated for a rumen particulate outflow of 4% per h (%).

Table 20. Gas production parameters and fermentation parameters after 8 and 24 h of *in vitro* fermentation of diets containing increased amounts of tomato pomace incubated in batch cultures of mixed rumen microorganisms ¹.

Item and Incubation Time	Control	TP6	TP12	TP18	SEM ¹	P-Value	
						Lineal	Quadratic
Gas production parameters ²							
PGP (mL/g dry matter (DM))	336	334	326	334	3.67	0.453	0.229
<i>c</i> (%/h)	4.17 ^a	4.62 ^b	4.73 ^b	4.58 ^b	0.121	0.016	0.015
<i>Lag</i> (h)	1.63	1.42	2.03	1.90	0.30	0.325	0.894
AGPR mL/h	9.16	10.1	9.74	9.76	0.37	0.416	0.236
DMED (%)	33.4	35.4	34.1	33.3	0.73	0.983	0.175
Fermentation parameters at 8 h incubation ³							
Total VFA (mmol/g DM)	3.47	3.44	3.52	3.62	0.075	0.161	0.430
Molar proportions (mol/100 mol)							
Acetate (Ac)	57.7 ^a	58.3 ^b	58.1 ^b	58.2 ^b	0.12	0.059	0.038
Propionate (Pr)	23.9 ^a	23.9 ^a	24.4 ^b	24.4 ^b	0.13	0.007	0.971
Butyrate	15.0 ^b	14.6 ^a	14.4 ^a	14.4 ^a	0.09	0.009	0.053
Minor VFA	3.37 ^c	3.18 ^b	3.08 ^a	3.04 ^a	0.043	0.002	0.095
Ac/Pr (mol/mol)	2.46 ^{ac}	2.48 ^{ac}	2.42 ^{ab}	2.42 ^a	0.022	0.039	0.405
NH ₃ -N (mg/L)	185 ^b	182 ^{ab}	181 ^{ab}	178 ^a	2.1	0.049	0.815
CH ₄ (mL/g DM)	18.5	18.0	18.0	18.4	0.36	0.901	0.296
CH ₄ /VFA (mL/mmol)	5.34 ^b	5.24 ^{ab}	5.16 ^{ab}	5.09 ^a	0.082	0.047	0.836
FOM (mg/g)	332	328	334	345	7.2	0.178	0.325
Fermentation parameters at 24 h incubation ³							
pH	6.71 ^c	6.71 ^c	6.67 ^b	6.63 ^a	0.011	0.003	0.222
Total VFA (mmol/g DM)	6.82 ^a	6.84 ^a	6.91 ^{ab}	6.99 ^b	0.05	0.029	0.61
Molar proportions (mol/100 mol)							
Acetate (Ac)	58.6	58.8	58.4	58.5	0.07	0.150	0.569
Propionate (Pr)	19.4 ^a	19.6 ^a	20.1 ^b	20.1 ^b	0.14	0.002	0.594
Butyrate	17.5	17.4	17.5	17.5	0.1	0.705	0.576
Minor VFA	4.40 ^b	4.14 ^b	4.10 ^b	3.96 ^a	0.071	0.002	0.421
Ac/Pr (mol/mol)	3.08 ^b	3.05 ^b	2.96 ^a	2.96 ^a	0.022	0.007	0.465
NH ₃ -N (mg/L)	284 ^b	270 ^b	271 ^b	269 ^a	4.8	0.066	0.215
CH ₄ (mL/g dry matter)	43.1	41.8	41.0	42.4	1.38	0.658	0.371
CH ₄ /VFA (mL/mmol)	6.33	6.11	5.93	6.05	0.213	0.312	0.450
FOM (mg/g)	660 ^a	661 ^a	668 ^{ab}	680 ^b	4.3	0.007	0.293

^{abc} Within each parameter, mean values with different superscripts differ ($P < 0.05$). ¹ Control, TP6, TP12, and TP18 diets contained 0, 60, 120, and 180 g of tomato pomace per kg, respectively (fresh matter basis). ² PGP: Asymptotic gas production; *c*: Fractional rate of gas production; *Lag*: Time before starting gas production; AGPR: Average gas production rate; DMED: Dry matter effective degradability calculated for a rumen particulate outflow of 4%/h. ³ VFA: Volatile fatty acids; minor VFA were calculated as the sum of isobutyrate, isovalerate, and valerate; FOM: Fermented organic matter calculated from acetate, propionate, and butyrate production as described by Demeyer (1991).

V.4. Discussion

V.4.1. Chemical composition, *in vitro* fermentation, and *in vitro* intestinal digestibility of tomato pomace samples

Chemical composition of TP was within the range of values previously reported (Bakshi et al., 2016; Abbeddou et al., 2011; Denek and Can, 2006), although other studies (Fondevila et al., 1994; Gasa et al., 1988) reported lower lignin and greater EE values. Tomato pomace is a fibrous by-product with a relatively high content of CP and EE. As reported by others (Hetzroni et al., 2011; Del Valle et al., 2006; Abdel-Rahman, 1982), most of fiber and CP comes from the peel fraction of TP, whereas seeds contain most of ash and EE; therefore the relative proportions of peels and seeds in TP can help to explain the observed differences in lignin and EE due to sampling time in the present study, as well as variations in chemical composition among studies (Hetzroni et al., 2011). In addition, chemical composition of tomato fruits has been reported to be highly variable, depending on factors such as cultivars, crop conditions, origin, etc. (Del Valle et al., 2006), which also contributes to variability in TP composition. Additional factors, such as tomato industrial processing characteristics, can increase variability. However, in the present study, there were no differences in any chemical fraction between the TP samples from the two processing plants, which might be due to a similar processing type in both of them. Finally, the application of high temperatures during tomato processing may result in the formation of indigestible compounds via the Maillard reactions between sugar aldehyde groups and free amino groups (Broderick et al., 2000) that resulted in high ADIN values (16%–26%) reported in some studies (Rahbarpour et al., 2012; Weiss et al., 1997; Fondevila et al., 1994). However, ADIN concentrations in our study were relatively low, ranging from 6.6% to 8.8% of total N. Differences in the ADIN content could be attributed to variability in tomato fruits or processing (Del Valle et al., 2006), but also to different processing temperatures.

Despite the relatively low variability in TP chemical composition observed in our study, there were some differences between sampling times in PGP, AGPR, and DMED, with samples taken at week 6 having lower values than those taken at week 2. Abbeddou et al. (2011) pointed out that CP and non-structural carbohydrates of TP are easy and rapidly degraded, whereas NDF has low degradability. The low *Lag* values ($\leq$

2.63 h) agree with this observation. These results indicate that TP was rapidly fermented *in vitro*.

Values of total VFA production agree well with gas production parameters, as TP samples taken at week 6 had lower values than the rest of the samples, except those taken at week 1. Despite the fact that there were significant effects of sampling period on molar proportions of all individual VFA, changes were of minor importance and the VFA profile remained relatively constant over the sampling period.

The main fatty acids present in TP are oleic and linoleic acids (Romano et al., 2010) and the unsaturated fatty acids which are produced by the hydrolysis of triglycerides are toxic to the fibrolytic bacteria (Maia et al., 2007). However, the lack of correlation ($P \geq 0.339$) between EE content and any gas production or *in vitro* fermentation parameter indicates that EE had no negative effect on TP fermentation, despite the fact that its concentrations were greater than the maximal level of 60 g of fat per kg of DM recommended in the diet of ruminants to avoid reductions in fiber digestibility (NRC, 2001). There were some differences between samples in CH₄ production, but these differences disappeared when the CH₄/total VFA ratio was calculated.

Although OM content showed low variability among TP samples, it was the chemical fraction more closely related to gas production parameters and VFA and CH₄ production, with the correlation being negative; this might be explained because of the close relationship between OM and the lignin/NDF ratio ($r = 0.830$; $P < 0.001$), as lignification is one of the main factors limiting NDF degradability (Van Soest, 1994). In fact, the lignin/NDF ratio was negatively correlated with PGP, DMED, and total VFA, indicating that NDF lignification was a major factor involved in the differences observed in fermentation parameters.

Values of 16 h *in situ* rumen DM degradability were similar to those reported by Abbeddou et al. (2011) and Gasa et al. (1988) after 16 and 24 h of ruminal incubation, respectively (476 and 471 g/kg), but greater than the 389 g/kg reported by Fondevila et al. (1994) after 24 h of incubation in the rumen. Values of *in situ* degradability of CP were greater than the 406 g/kg reported by Fondevila et al. (1994) for 24 h of incubation, but lower than the 649 g/kg reported by Abbeddou et al. (2011) for 16 h of

incubation. Differences among studies are probably due to variability in TP chemical composition, but may be also related to the performance of the *in situ* procedure which is influenced by many factors, such as animals, diet, incubation procedure, etc. (Yuangklang et al., 2010). *In situ* degradability of both DM and CP was negatively correlated with ADF content, reflecting the low degradability of this fraction, which is the main constituent of fiber in TP; this might also restrict the access of microorganisms to protein.

The values of IDCP revealed that only nearly half of the CP undegraded in the rumen can be digested in the intestine, and total CP digestibility reached 737 g/kg as average. These values agree with previous observations reporting lower protein digestibility in TP compared with protein concentrates such as soybean meal (Yuangklang et al., 2010; Fondevila et al., 1994). The positive correlation of IDCP with ADF fraction is in accordance with the observation that intestinal digestibility of CP increases as rumen degradability of CP decreases (González et al., 2018); this would explain the fact that ADF was negatively correlated with *in situ* CP degradability and positively with IDCP. The negative correlation between IDCP and TSP content may indicate that TSP formed less digestible complexes with dietary proteins.

V.4.2. *In vitro* fermentation of diets containing tomato pomace

The increased fractional rates of gas production indicate that diets including TP were fermented more rapidly than the control diet, which is in agreement with the increased VFA productions observed at 24 h of incubation, although only TP18 diet showed significant differences with the control diet. These results are consistent with the lower amounts of barley straw in the TP-diets than in the control diet, as barley straw is slowly degraded in the rumen and greater VFA productions and FOM were observed for the TP-diets after 24 h of incubation. Including TP in the diet also changed the VFA profile. Our results are in agreement with those of Soto et al. (2015), who observed that *in vitro* molar proportion of acetic acid increased and butyric and minor VFA proportions decreased when tomato fruit wastes were included in the diet. Similarly, Arco-Pérez et al. (2017) reported an increase in propionate proportions and a decrease in the acetate/propionate ratio in the rumen of goats receiving tomato silage compared with those fed a control diet.

The reduced $\text{NH}_3\text{-N}$ concentrations observed with increasing TP levels could be due to the lower degradation of TP protein, as $\text{NH}_3\text{-N}$ is one of the main final products of protein degradation in the rumen. The decreased proportions of minor VFA as the level of TP in the diet increased are consistent with this hypothesis, but $\text{NH}_3\text{-N}$ concentrations were adequate for rumen microorganism growth in all diets (Satter and Slyter, 1974). Tomato pomace replaced increased amounts of soybean meal in the experimental diets and rumen degradability of TP has been reported to be lower than that of soybean meal (Fondevila et al., 1994), which would indicate greater bypass protein in TP than in soybean meal. According to Drouliscos (1976), the essential amino acids profile in TP protein is similar to that in soybean meal, but the low values of IDCP observed in our study indicate that about half of the protein reaching the small intestine may not be digested, whereas intestinal digestibility of soybean meal is greater than 90% (Satter and Slyter, 1974). In agreement with these results, Yuangklang et al. (2010) and Fondevila et al. (1994) reported a significant decrease in CP digestibility when replacing soybean meal by TP in the diet of lambs and beef cattle, respectively.

Although some studies have shown that the inclusion of tomato fruit wastes in the diet reduced CH_4 emission both in goats (Arcos-Pérez et al., 2017; Romero-Huelva and Molina-Alcaide, 2013; Romero-Huelva et al., 2012) and in *in vitro* fermentations (Romero-Huelva et al., 2013), no changes in CH_4 production were observed in our study. The anti-methanogenic mechanisms of tomato fruit ingredients remain yet unknown (Arcos-Pérez et al., 2017), but either the extraction of the bioactive compounds during the industrial processing of tomato or their destruction by the heat applied in the process might help to explain the lack of anti-methanogenic properties of TP. The reduction of the CH_4 /total VFA ratio observed in TP-diets after 8 h of incubation could be due to the fermentation of soluble and rapidly degraded fractions, which also contributed to greater propionate proportions.

V.5. Conclusions

Tomato pomace is a high-moisture, fibrous by-product, but its dry matter contains a relatively high content of CP and EE and it is rapidly fermented in the rumen. Both the chemical composition and *in vitro* rumen fermentation of TP samples obtained from two processing plants at different times over the tomato campaign presented little

variability. Crude protein of TP had low degradability in the rumen (average 510 g/kg) indicating a high bypass fraction, but the *in vitro* intestinal digestibility of the undegraded CP was low (average 459 g/kg). *In vitro* results indicated that dried TP could be included up to 180 g/kg in a high-concentrate diet without negative effects on rumen fermentation. However, TP did not show the anti-methanogenic properties previously reported for fresh tomato wastes, which was attributed to chemical changes produced during tomato processing.

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C.N.M. did the statistical analysis and wrote the draft; M.D.C. and E.M.-A. provided advice and critically reviewed the manuscript. All authors revised and approved the final manuscript.

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Chapter VI. THE ACID DETERGENT INSOLUBLE NITROGEN (ADIN) ANALYSIS OVERESTIMATES THE AMOUNT OF N ASSOCIATED TO ACID DETERGENT FIBRE

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Abstract: The acid detergent insoluble nitrogen (ADIN) fraction of feeds is usually assumed to be indigestible, and some feeding systems for ruminants use the ADIN analysis as a measure of nitrogen (N) availability. In a preliminary study conducted with 3 different feeds, we observed lower ^{15}N abundance in ADIN compared with that in both total N and neutral detergent insoluble N (NDIN). Based on these results, we hypothesized that ADIN analysis overestimates the amount of N associated to acid detergent fibre (ADFom) due to N-contamination from cetyltrimethylammonium bromide (CTAB), a reagent used in the ADFom analysis. The objective of this work was to verify lower ^{15}N abundances of ADIN than of total N and NDIN in different feeds, and to assess the magnitude of ADIN contamination. Samples from one corn crop (aerial biomass, grain and root) and two sunflower crops (SF; aerial biomass and seeds) were analysed for ^{15}N abundance and content of total N, NDIN and ADIN. Samples had been ^{15}N -labelled by fertilizing with either $^{15}\text{NH}_4\text{NO}_3$ or $\text{NH}_4^{15}\text{NO}_3$, and there were three replicates per type of sample. The ^{15}N -abundance of total N and NDIN was similar ($P > 0.05$) for all samples, whereas that of ADIN was lower ($P < 0.001$ to 0.037) than values for total N and NDIN for all samples, excepting for SF seeds from one crop. Estimates of N-contamination of ADIN from CTAB were not affected by the fertilizer used to label N ($P \geq 0.207$), and values (g/g) ranged from 0.129 for corn grains to 0.447 for corn aerial biomass. N-contamination of ADIN was positively correlated with neutral detergent fibre (aNDFom), ADFom, hemicelluloses and cellulose content ($P < 0.001$; $r = 0.770$ to 0.905 ; $n = 18$ and 22 for corn and SF, respectively), and negatively ($P < 0.001$ to 0.048) with ADIN content (expressed as proportion of ADFom). The negative correlations ($P < 0.001$) between N-contamination of ADIN and lignin (Lignin (sa))/ADFom ratio observed for all samples would indicate that N-contamination of ADIN was reduced by cell wall lignification. In conclusion, the results showed that the use of CTAB in the ADFom analysis resulted in increased ADIN values, and therefore overestimated the amount of N associated to ADFom, questioning the use of ADIN as a parameter indicative of N availability. The possibility of replacing CTAB by a N-free reagent in the ADFom analysis should be explored.

Keywords: Acid detergent insoluble nitrogen; ADFom; neutral detergent insoluble nitrogen; ^{15}N ; N contamination; overestimation

VI.1. Introduction

The acid detergent insoluble nitrogen (ADIN) analysis is widely accepted as a good measure of heat damage in forages (NRC, 2001; Goering et al., 1972), but poor relationships between ADIN and nitrogen (N) indigestibility have been reported for non-forage plant protein sources that have been heat treated (Nakamura et al., 1994; Weiss et al., 1989). Despite this, some feeding systems for ruminants use the ADIN analysis as a measure of N availability. For example, in the Cornell Net Carbohydrate Protein System (Sniffen et al., 1992) the fraction C of crude protein (CP) is determined chemically as the ADIN and is considered to be undegradable. In the UK Metabolizable Protein System (Webster, 1987), the ADIN fraction is assumed to be both undegradable in the rumen and indigestible in the small intestine, and therefore the amount of digestible rumen undegraded protein in a feed is predicted from its ADIN content. However, several studies have demonstrated partial ADIN degradation in the rumen (Machacek and Kononoff, 2009; McNiven et al., 2002; Nakamura et al., 1994). Recent studies by our group (Vanegas et al., 2016; Guevara-González et al., 2015; Arroyo and González, 2013) showed a rapid initial degradation of ADIN, which was markedly greater than that observed for neutral detergent insoluble N (NDIN). Using ^{15}N -enriched feeds to analyze cell wall protein degradation, Vanegas et al. (2016) observed lower ^{15}N abundance in ADIN than in total N and NDIN of three feeds, but the low number of samples available in their study precluded further investigations to explain these results. We hypothesized that the lower ^{15}N abundance of ADIN might be due to contamination from cetyltrimethylammonium bromide (CTAB), a N-containing reagent used in the ADFom analysis. To test our hypothesis, ^{15}N -enriched samples of different plants were obtained from a previous agricultural study. The objective of this work was to verify the lower ^{15}N abundance in ADIN compared with both total N and NDIN, and to assess the possible ADIN contamination and the factors influencing it.

VI.2. Materials and methods

VI.2.1. Samples and experimental procedures

The ^{15}N -enriched samples used in this study were obtained in previous agricultural experiments aimed at measuring the effects of nitrification inhibitors on

N₂O losses in different crops (Guardia et al., 2018). Briefly, corn (*Zea mays* L. FAO class 600) samples were obtained in 2015 from 6 microplots (1 × 1 m; 3 microplots / treatment) which received either ¹⁵NH₄NO₃ (¹⁵NAM) or NH₄¹⁵NO₃ (¹⁵NIT); both 98 atom% ¹⁵N; Campro Scientific GmbH, Berlin, Germany) to be labelled. Both ¹⁵NAM and ¹⁵NIT were applied as a top-dressing at a rate of 90 kg total N / ha. At harvest, plants were cut by hand at the soil level, and then the aerial biomass (stem, leaves and cob) was separated from grain. In addition, roots situated in the middle of each microplot were sampled and washed with water to separate roots from soil. In total there were 18 corn samples (6 microplots (3 ¹⁵NAM and 3 ¹⁵NIT) × 3 plant fractions). Sunflower (*Helianthus annuus* L., P64LL62 hybrid Pioneer) samples were obtained in 2016 using two different schemas of ¹⁵N fertilization: either the residual ¹⁵N from the previous corn crops (SF1) or by applying either ¹⁵NAM or ¹⁵NIT (both 10 atom% ¹⁵N; Campro Scientific GmbH, Berlin, Germany) as a top-dressing at a rate of 68 kg total N/ha to parcels which had not been previously ¹⁵N-fertilized (SF2). Sunflower plants were cut as previously described and seeds were separated from the aerial biomass (stem + leaves + flower capitula or head). Unfortunately, one replicate for ¹⁵NAM-SF2 treatment was lost, and therefore a total of 22 SF samples were available.

All samples were ground to pass a 1 mm screen before chemical analyses. Dry matter (ID 934.01), ash (ID 048.13) and ether extract (ID 920.39; analysed only in SF seed samples) contents were determined according to the AOAC International (2005) methods. Total N content was measured using a Leco FP258 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI, USA). Analyses of neutral detergent fibre (aNDFom) (Van Soest et al., 1991), acid detergent fibre (ADFom; sequential analysis) and acid soluble lignin (lignin (sa)) (Robertson and Van Soest, 1981) were carried out sequentially using an ANKOM220 Fibre Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA). The aNDFom analyses were performed with alpha-amylase and without sodium sulphite, and aNDFom, ADFom and lignin (sa) values were expressed exclusive of residual ash. In addition, non-sequential ADFom analyses were performed in all samples of aerial biomass from ¹⁵NIT treatment in order to compare N-contamination in both ADFom procedures (sequential and direct). Hemicellulose, cellulose and pectin contents were estimated as aNDFom - ADFom, ADFom - lignin (sa), and non-sequential ADFom - sequential ADFom, respectively. Samples of SF seeds were

defatted with diethyl ether employing an ANKOM XT10 extractor unit (ANKOM Technology Corporation, Fairport, NY, USA) before fibre analyses. Chemical composition of feed samples is given in Table 21.

Feed samples and residues in the ANKOM bags used in aNDFom and ADFom analysis were ground ($< 100 \mu\text{m}$) with a ball mill, and total N content was analysed as before described. Analysis of ^{15}N abundance in feed samples, NDIN, ADIN and CTAB was performed by combustion using a Thermo 1112 Flash HT elemental analyzer hyphenated interfaced to a Thermo Delta V Advantage isotope ratio mass spectrometer (all Thermo Fisher Scientific, Bremen, Germany).

VI.2.2 Calculations and statistical analyses

The possible contamination of ADIN with N from the CTAB during the ADFom analysis was calculated by using Pearson's square method as: $(\text{averaged } ^{15}\text{N} \text{ abundance in total N and NDIN} - ^{15}\text{N} \text{ abundance in ADIN}) / (\text{averaged } ^{15}\text{N} \text{ abundance in total N and NDIN} - ^{15}\text{N} \text{ abundance in CTAB})$, in which all ^{15}N abundances were expressed as atom%. The average ^{15}N abundance in total N and NDIN was used in the calculations because of the lack of differences between both values. The ^{15}N abundance of CTAB was measured (0.3660 atom%) and used in the calculations.

^{15}N abundance values were analysed independently for each feed sample as a mixed model by the PROC MIXED of SAS (SAS Inst. Inc., Cary, NC, USA). Effects of nitrogenous fraction, fertilizer and nitrogenous fraction x fertilizer interaction were included in the model as fixed effects, whereas microplot was considered as a random effect. The sums of squares were further partitioned by orthogonal contrasts to analyze differences among the three nitrogenous fractions. The contrasts were distributed as follows, C1: total N vs. NDIN, and C2: total N and NDIN vs. ADIN. Values of N-contamination of ADIN estimated in samples labelled either with ^{15}N AM or ^{15}N IT were compared by a one-way ANOVA. The same analysis was used to compare the values of ^{15}N abundance and N-contamination of ADIN obtained by using either sequential or non-sequential ADFom analyses. Significance was declared at $P \leq 0.05$, whereas $P < 0.10$ values were considered to be a trend. Relationships between N contamination in ADIN and chemical composition of feed samples were investigated by linear regression using the PROC CORR of SAS.

Table 21. Chemical composition (g/kg DM unless otherwise stated) of corn and sunflower ^{15}N -enriched samples by using either $^{15}\text{NH}_4\text{NO}_3$ (^{15}NAM) or $\text{NH}_4^{15}\text{NO}_3$ (^{15}NIT) as fertilizers ($n = 3$).

Sample	Fertilizer	Organic matter	Nitrogen (N)	Ether extract ²	aNDFom	ADFom	Lignin (sa)	Neutral detergent insoluble N (NDIN)		Acid detergent insoluble N (ADIN)	
								g/kg aNDFom	g/kg N	g/kg ADFom	g/kg N
Corn											
Aerial Biomass ¹	¹⁵ NAM	943	7.4	—	750	383	25.6	3.6	364	2.3	11.6
Grain	¹⁵ NAM	986	16.4	—	136	15	4.9	23.1	192	9.2	8.6
Roots	¹⁵ NAM	822	7.6	—	726	428	67.9	5.8	547	5.6	316
Aerial Biomass ¹	¹⁵ NIT	944	10.2	—	721	360	23.9	4.8	337	2.2	76.7
Grain	¹⁵ NIT	986	17.8	—	131	15	4.8	20.6	152	8.8	7.4
Roots	¹⁵ NIT	831	8.2	—	635	380	72.6	5.9	458	6.1	283
Sunflower 1											
Aerial Biomass ²	¹⁵ NAM	842	16.0	—	476	289	55.4	8.1	241	4.4	80.2
Seeds	¹⁵ NAM	972	33.2	421	277	174	54.6	14.9	72.0	6.5	19.6
Aerial Biomass ²	¹⁵ NIT	863	15.9	—	478	294	58.4	7.5	227	4.4	80.9
Seeds	¹⁵ NIT	972	34.6	399	289	184	58.5	15.3	76.7	6.2	19.7
Sunflower 2											
Aerial Biomass ²	¹⁵ NAM	866	12.9	—	446	275	53.4	8.2	284	4.4	94.7
Seeds	¹⁵ NAM	959	37.8	317	303	178	57.5	18.5	101	6.0	19.3
Aerial Biomass ²	¹⁵ NIT	868	13.5	—	464	288	52.5	7.8	270	4.4	94.8
Seeds	¹⁵ NIT	963	40.1	337	293	173	61.0	16.6	80.3	6.0	17.1

¹ Ether extract was determined only in sunflower seeds.

² Non sequential ADF values for ^{15}NIT aerial biomass samples were 422, 367 and 360 g/Kg DM for corn, SF1 and SF2, respectively

VI.3. Results

Content of N and aNDFom in samples was variable and ranged from 7.4 to 40.1 and from 131 to 750 g/kg DM, respectively (Table 21). There were also marked differences among samples in NDIN and ADIN content, which varied from 72 to 547 and from 7.4 to 316 g/kg N, respectively.

As shown in Table 22, there were no differences ($P \geq 0.31$) in ^{15}N abundance between total N and NDIN fractions for any group of samples, but ^{15}N abundance of ADIN was lower ($P < 0.001$ to 0.02) than the average value of total N and NDIN for all samples excepting SF1 seeds ($P = 0.17$). Fertilization treatment x N fraction interactions ($P < 0.001$ to 0.04) were detected for all corn and SF2 samples. The ^{15}N abundances in total N, NDIN and ADIN were greater ($P < 0.001$) for ^{15}NIT compared with ^{15}NAM treatment for all corn and SF2 samples, whereas for SF1 samples differences did not reach the significance level ($P \geq 0.14$).

Estimates of N-contamination were not affected ($P = 0.21$ to 0.99; Table 23) by the ^{15}N abundance in feeds, which was determined by the compound used as fertilizer. Values of N-contamination of ADIN varied widely among samples, with aerial biomass samples having greater values (> 0.375 g/g) than both grain and seed samples (< 0.226 g/g) for both corn and SF.

Table 22. ^{15}N abundance (atom%) in total nitrogen (N), neutral detergent insoluble N (NDIN) and acid detergent insoluble N (ADIN) of corn and sunflower samples labelled with either $^{15}\text{NH}_4\text{NO}_3$ (^{15}NAM) or $\text{NH}_4^{15}\text{NO}_3$ (^{15}NIT) applied as fertilizers.

Sample ²	Fertilizer						SEM	P =			
	¹⁵ NAM			¹⁵ NIT				Fertilizer	N-fraction ¹		Fertilizer x N fraction
	Total N	NDIN	ADIN	Total N	NDIN	ADIN			C1	C2	
Corn											
Aerial biomass	4.68	4.68	2.70	11.90	11.71	6.82	0.166	< 0.001	0.578	< 0.001	< 0.001
Grain	6.25	6.16	5.44	13.92	13.81	12.1	0.129	< 0.001	0.455	< 0.001	< 0.001
Root	5.55	5.57	3.96	11.36	11.33	7.79	0.388	< 0.001	0.997	< 0.001	0.040
Sunflower 1											
Aerial biomass	1.33	1.33	0.94	2.28	2.29	1.56	0.15	0.310	0.980	0.017	0.960
Seed	1.74	1.71	1.46	2.66	2.62	2.10	0.209	0.140	0.861	0.173	0.920
Sunflower 2											
Aerial biomass	1.12	1.10	0.80	2.66	2.64	1.52	0.061	< 0.001	0.757	< 0.001	0.003
Seed	1.39	1.40	1.19	3.32	3.24	2.65	0.030	< 0.001	0.314	< 0.001	0.030

¹ C1: Total N vs. NDIN; C2: Total N and NDIN vs. ADIN.

² A total of 18 samples of corn, 12 samples of sunflower 1, and 10 samples of sunflower 2

Table 23. N contamination of acid detergent insoluble nitrogen (ADIN; g/g) in corn and sunflower samples labelled with either $^{15}\text{NH}_4\text{NO}_3$ (^{15}NAM) or $\text{NH}_4^{15}\text{NO}_3$ (^{15}NIT) applied as fertilizers¹.

Sample	Fertilizer		SEM	P =
	¹⁵ NAM	¹⁵ NIT		
Corn				
Aerial biomass	0.456	0.437	0.0371	0.730
Grain	0.129	0.128	0.0426	0.990
Root	0.310	0.327	0.0451	0.800
Sunflower 1				
Aerial biomass	0.376	0.400	0.0673	0.820
Seed	0.202	0.225	0.0265	0.570
Sunflower 2				
Aerial biomass	0.414	0.493	0.0308	0.210
Seed	0.198	0.217	0.0093	0.300

¹ Calculated by Pearson's square method as (average ^{15}N abundance in total N and NDIN - ^{15}N abundance in ADIN) / (average ^{15}N abundance in total N and NDIN - ^{15}N abundance in CTAB). All ^{15}N abundances expressed as atom%.

When chemical composition on DM basis were used (values in Table 21), N-contamination of ADIN was positively correlated with aNDFom, ADFom, hemicelluloses and cellulose content ($P < 0.001$; $r = 0.77$ to 0.91 ; $n = 18$ and 22 for corn and SF, respectively) and negatively ($P < 0.001$ to 0.05) with content of NDIN and ADIN (expressed as proportion of aNDFom and ADFom, respectively), for both corn and SF samples with the exception of NDIN for SF samples ($P = 0.44$). The N-contamination of ADIN was also negatively correlated ($P < 0.001$) with lignin (sa)/ADFom ratio for all samples, and with lignin (sa) content and lignin (sa)/aNDFom ratio for SF samples ($P < 0.001$ to 0.03). When fibre fractions of SF-seeds were expressed on defatted seed DM basis, no correlation was observed between N-contamination of ADIN and aNDFom, ADFom and hemicelluloses content (Table 24), but the negative correlation between N-contamination of ADIN and lignin (sa) content was stronger than that detected when chemical composition was expressed on DM basis ($P = 0.03$ and < 0.001 , respectively).

As shown in Table 25, lower ADIN values were obtained by sequential ADFom

analysis compared with the non-sequential procedure for all tested samples ($P \leq 0.02$). However, the influence of the method of ADFom analysis on ^{15}N abundance and N-contamination of ADIN varied among samples. For corn samples, the non-sequential ADFom analysis resulted in 43.3% lower ^{15}N abundance of ADIN than the sequential method, but differences did not reach the significance level ($P = 0.09$). N-contamination tended to be greater ($P = 0.08$) for the non-sequential compared with the sequential ADFom analysis, but there were no differences between methods ($P = 0.65$) in the amount of true ADIN estimated as ADIN corrected for N-contamination from CTAB. In contrast, for SF samples no differences were detected either in ^{15}N abundance or in N-contamination of ADIN, excepting a trend ($P = 0.09$) to greater N-contamination in non-sequential compared with sequential ADFom procedure for SF1 sample. However, the amount of true ADIN was greater ($P \leq 0.01$) for the non-sequential compared with the sequential ADFom analysis for both SF1 and SF2 samples.

Table 24. Correlation matrix (values of Pearson coefficient and P values in brackets) of nitrogen (N) contamination of acid detergent insoluble N (ADIN) with fibre fractions in corn and sunflower samples¹.

Sample	aNDFom	ADFom	Lignin (sa)	Hemicelluloses	Cellulose	Acid detergent insoluble N (NDIN)	ADIN	Lignin (sa)/aNDFom	Lignin (sa)/ADFom
Corn (n = 18)	0.864 (< 0.001)	0.807 (< 0.001)	0.253 (0.310)	0.905 (< 0.001)	0.875 (< 0.001)	-0.873 (< 0.001)	-0.903 (< 0.001)	-0.192 (0.450)	-0.925 (< 0.001)
Sunflower (n = 22) ²									
A	0.829 (< 0.001)	0.843 (< 0.001)	-0.467 (0.030)	0.770 (< 0.001)	0.867 (< 0.001)	-0.172 (0.440)	-0.426 (0.050)	-0.856 (< 0.001)	-0.864 (< 0.001)
B	0.138 (0.540)	0.140 (0.530)	-0.798 (< 0.001)	0.035 (0.880)	0.686 (< 0.001)	-0.172 (0.440)	-0.426 (0.050)	-0.870 (< 0.001)	-0.888 (< 0.001)

¹ Data expressed as g/kg DM, excepting NDIN and ADIN that are expressed as g/kg of NDF and ADF, respectively.

² A: using chemical composition data shown in Table 21; B: using chemical composition data of defatted seeds.

Table 25. Values of acid detergent insoluble N (ADIN) content, ^{15}N -abundance and N-contamination of ADIN, and ADIN corrected for N-contamination from cetyltrimethylammonium bromide (true ADIN) in samples from aerial biomass of corn and sunflower (SF1 and SF2) obtained by using sequential and non-sequential methods of ADFom analysis.

Item	Corn ($n = 3$)				Sunflower 1 ($n = 3$)				Sunflower 2 ($n = 3$)			
	Sequential ADFom	Non-sequential ADFom	SEM	P =	Sequential ADFom	Non-sequential ADFom	SEM	P =	Sequential ADFom	Non-sequential ADFom	SEM	P =
ADIN, g/kg ADF	2.17	3.33	0.096	0.010	4.37	6.83	0.111	0.004	4.27	6.81	0.227	0.020
ADIN, atom% ^{15}N - abundance of	6.82	3.87	0.658	0.090	1.53	1.38	0.038	0.110	1.56	1.53	0.283	0.290
N-contamination of ADIN, g/g	0.437	0.685	0.0555	0.080	0.400	0.471	0.0167	0.090	0.493	0.491	0.0126	0.530
True ADIN, g/kg ADF	1.22	1.09	0.183	0.650	2.66	3.63	0.074	0.010	2.22	3.48	0.100	0.010

VI.4. Discussion

Samples with variable chemical composition and a wide range of ^{15}N abundance were used to test the hypothesis that ADIN might be contaminated with N from CTAB during the ADFom analysis. Chemical composition of samples was in the range reported in the literature (NRC, 2001). The lower ^{15}N abundance in ^{15}N AM samples was mainly attributed to the fact that ammonia-N is not as readily utilized by the plants as nitrate-N and it can be partially lost to the atmosphere.

The lack of differences in ^{15}N abundance between total N and NDIN fractions agrees with the results of Rodríguez and González (2006), who observed either similar values or only subtle differences ($< 0.9\%$) between the natural ^{15}N abundance of the whole feed and that of the insoluble fraction (obtained by washing 3 times for 5 min in a turbine washing machine) in 14 feeds commonly used in ruminant feeding. These results would indicate a homogeneous distribution of ^{15}N atoms within each plant tissue. The decrease in ^{15}N abundance of ADIN compared with the average ^{15}N abundance of total N and NDIN ranged from 12.7% for corn grain to 42.6% for SF2 aerial biomass (both labelled with ^{15}NIT). Lower ^{15}N abundances of ADIN than of total N and NDIN have also been reported by Vanegas et al. (2016) in 3 single samples (sunflower seed, wheat grain and wheat straw). These results might indicate a N- contamination with N from CTAB (0.3660 atom% ^{15}N abundance) during the ADFom analysis, as CTAB is the only N-containing chemical used during the ADFom procedure. N-contamination values for SF1 and SF2 seeds were similar ($P = 0.86$; averaged values 0.213 and 0.208 g/g, respectively), and they are in agreement with the 0.192 value calculated from the results by Vanegas et al. (2016) for another single sample of SF seeds.

The observed relationships between ADIN contamination and chemical composition of samples showed that N-contamination of ADIN increased with increasing structural carbohydrate content of feeds, but was reduced by ADFom lignification. The negative correlation between N-contamination of ADIN and ADIN content is justified by the fact that N-contamination was expressed as proportion of ADIN, and therefore N-contamination was greater for feeds with lower N content. The fact that no significant correlation was observed between N-contamination of ADIN and aNDFom, ADFom and hemicelluloses content of SF samples when fibre fractions of SF seeds were expressed on defatted seed DM basis can be explained by the similar content

of SF aerial biomass and seeds samples in aNDFom (477 vs. 482 g / kg DM for SF1, and 457 vs. 442 g / kg DM for SF2, respectively), ADFom (292 vs. 307 g / kg DM, and 283 vs. 260 g / kg DM) and hemicelluloses (185 vs. 176 g / kg DM, and 174 vs. 182 g / kg DM) when seeds data were expressed on defatted basis. The stronger correlation between N-contamination of ADIN and lignin (sa) content observed when chemical composition was expressed on defatted DM basis compared with that when expressed on DM basis ($P = < 0.001$ and 0.03 , respectively) reinforces the idea of the negative influence of lignin (sa) content on N-contamination of ADIN.

The N-contamination of ADIN during the ADFom analysis can help to explain previous results reporting high digestibility values of ADIN in non-forage protein feeds (Machacek and Kononoff, 2009; McNiven et al., 2002; Weiss et al., 1989). Previous studies by our group (Vanegas et al., 2016; Arroyo and González, 2013) also showed a rapid initial rate of ADIN degradation and high ruminal degradability values (close to those observed for NDIN) for high-fibre low-protein forages (poor-quality Italian ryegrass hay and wheat straw, respectively). The ADIN overestimation observed in the present study is consistent with these results, because it seems probable that N-contamination of ADIN will be greater in feeds than in faeces or incubation residues analysed to determine digestibility or ruminal degradability of ADIN, which usually have greater lignin content than feeds.

In agreement with our results on aerial biomass samples, others have reported lower ADIN values by sequential ADFom analysis compared with the non-sequential one (Arroyo and González, 2013; Cassida et al., 2007; Demarquilly et al., 1989). Differences between both procedures have been mainly attributed to the removal by the treatment with neutral detergent of feed components (mainly pectins) that are not removed by the treatment with acid detergent solution. However, the influence on ^{15}N abundance and N-contamination of ADIN varied among samples (Table 25), which might be related to their pectin content and characteristics. Despite the low number of corn aerial biomass samples, a positive correlation ($P = 0.005$; $r = 0.999$; $n = 3$) was observed between the increase in N-contamination in the non-sequential ADFom analysis compared with the sequential procedure and the content in pectins, resulting in a N-contamination increase of 1.04 percentage points per g of pectin. This might indicate an additional retention of N from CTAB in the pectins in the non-sequential analysis, but further analysis with a large number of samples are required to confirm this

hypothesis.

The overestimation of ADIN content by N-contamination from CTAB might have also other implications, as it would cause an underestimation of the microbial contamination in the ADFom incubation residues obtained in the *in situ* method when this method was performed combined with ^{15}N infusion as in some previous studies (Guevara-González et al., 2015; Arroyo and González, 2013). Finally, it is worth to notice that N associated to fibre fractions obtained by other methods of fibre analysis might be contaminated if N-containing reagents are used in these procedures, but the analysis of ^{15}N abundance in ^{15}N -enriched samples would be a suitable method to test this hypothesis. Determining ^{15}N abundance allows estimating N-contamination, but does not inform about the whole compound absorbed from CTAB by the ADFom. The potential importance of ADFom overestimations supports the need of additional studies on contamination from CTAB and of replacing the CTAB by a N-free reagent in the ADFom analysis.

VI.5. Conclusions

The results of this study showed that the use of CTAB in the ADFom analysis overestimated the amount of N associated to ADFom. The magnitude of the N-contamination varied with the feed tested, as it was greater for feeds with high structural carbohydrates content and was reduced with high ADFom lignification. Compared with the sequential ADFom analysis, non-sequential ADFom analysis resulted in increased ADIN content for all tested samples. The amount of ADIN corrected for N-contamination from CTAB was greater for the non-sequential analysis compared with the sequential analysis for sunflower samples, but no differences were detected for corn samples. The overestimation of ADIN showed in this study for both ADFom procedures questions the use of ADIN as a parameter indicative of N availability of feeds. The possibility of replacing CTAB by a N-free reagent in the ADFom analysis should be explored.

Conflict of interest: The authors declare that they have no conflict of interest.

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Chapter VII. GENERAL DISCUSSION

Spain is one of the largest producers of fruit and vegetables of the European Union (MAPA, 2019a). As a consequence, a large volume of agroindustrial by-products are generated in our country. However, these by-products can be used in animal feeding, which is one of the most valuable options as it reduces the environmental impact related to the production of large quantities of such products. Utilization of agroindustrial by-products in animal feeding could also contribute to improve the sustainability of livestock systems (Pascual Vidal, 2015; del Prado et al., 2013). In addition, by-products also can contain bioactive compounds that might improve the quality of animal products (Romero-Huelva et al., 2012). Nevertheless, utilization of agroindustrial by-products in animal feeding requires proper knowledge on their nutritional value. Therefore, the objective of this Doctoral Thesis was to evaluate the nutritive value of two by-products that are produced in huge amounts in Spain: olive cake (OC) and tomato pomace (TP).

Spain leads the world production of olive oil. Currently, the most used olive oil extraction system in our country is the two-phase system which generates a by-product called “alperujo”. This “alperujo” can be further processed and other by-products are generated: crude OC (COC), which is generated after drying and removing the olive stones, and exhausted OC (EOC), which is produced after performing a second oil extraction. However, other by-products are generated, such as cyclone OC (CYOC). The objective of the first and second Experiment (Paper 1 and 2) of this Doctoral Thesis was to determine the variability in the chemical composition and *in vitro* ruminal fermentation of OC by-products and to analyse the influence of storage time and further processing (COC, EOC and CYOC) on the nutritive value. Additionally, in the third Experiment (Paper 5), we studied the effect of the particle size on nutritive value of COC samples.

There were marked differences among OC types in their chemical composition. Most notable differences were ether extract (EE) and neutral detergent fibre (NDF) content. Average values for EE in COC, EOC and CYOC samples were 110, 15.9 and 157 g/kg dry matter (DM), respectively and 570, 645 and 441 g/kg DM for NDF. Chemical composition is highly dependent on the processes OC has gone through. For example, as a result of the second oil extraction, EE content decreased and proportionally other fractions, such as NDF, total soluble polyphenols (TSP) and total sugars increased. On the other hand, there were no differences in nitrogen (N) content

among OC types but acid detergent insoluble N (ADIN) content in EOC samples was significantly greater, as a consequence of heat applied during the additional oil extraction. Nevertheless the negative effect of fat in ruminal fermentation reported in the literature (Maia et al., 2007), no negative effect was observed in our studies.

Total sugars content was the most correlated fraction with fermentative parameters, as can be seen in the models obtained in the Experiment 2 (Paper 2). However, NDF content played an important role as well. Exhausted OC had the greatest values for fermentative parameters (potential gas production (PGP): 78.1 ml/g DM; average gas production rate (AGPR): 3.01 ml/h; DM effective degradability (DMED): 245 g/kg DM) followed by CYOC and COC, which correlated with the total sugars content of the samples. Processing of alperujo did not modify the volatile fatty acids (VFA) profile in the in vitro incubations.

In the Experiment 5 (Paper 3), COC samples were compared with other fibrous feeds (results not shown) and overall, we observed that COC samples have lower nutritive value than alfalfa hay, but similar to barley straw. As for the effect of storage time, it was clear that storage time was related with the quality of OC in a way it could be used to determine its AGPR and the amount of fermentable organic matter (FOM). With increasing storage time, nutritive value of OC decreased as a result of the degradation of easily fermentable fractions, such as total sugars. Dry matter effective degradability decreased linearly ($P < 0.001$) by 35.9 and 45.5% as storage time augmented from 1 to 6 months for COC and EOC, respectively. This effect was more remarkable over the first months of storage, so it would be recommended to store the alperujo up to a maximum of 2 months to avoid nutritive losses. Olive cake can be used as a replacement of straw in low production ruminants (Molina-Alcaide and Yáñez-Ruiz, 2008); however, short-stored OC has better nutritive quality than straw and provides other beneficial effects such as fat and polyphenols which can improve the quality of the end product; (meat or milk) (Luciano et al., 2013 and Abbeddou et al., 2011).

A decrease in total soluble polyphenols in OC over the storage period was observed, probably as a result of microbial degradation or enzymes. These changes have been reported by other authors (Alhamad et al., 2017; Alburquerque et al., 2006), and they must be taken in account if the objective of feeding OC is to provide the animal

with the biocompounds present in the olive. Finally, we observed differences in the nutritive value of OC with different particle size. The OC samples differing in particle size showed differences in chemical composition and *in vitro* ruminal fermentation parameters, with samples having more than 3 mm or less than 1 mm showing the greatest fermentability. In samples smaller than 1 mm, the high fermentability could be linked to the lower content of fibre, and in samples greater than 3 mm, highly fermentable fractions amalgam to form bigger particles (Bacha, personal communication). The nutritive value of the analysed OC samples was low, which is in agreement with the literature (Molina-Alcaide and Yáñez-Ruiz, 2008), and the variations observed in the nutritive value was highly dependent on factors such as processing, storage time or particle size. In general, OC could replace straw in animal feeding with a lower cost for the farmer while providing an extra source of fat and biocompounds.

Tomato is one the most common crop worldwide, and the fruits are rich in lycopene, phenols, organic acids, vitamins and other components (Giovanelli and Paradiso, 2002). In the tomato industry, TP is generated as the result of processing tomatoes to produce juices, creams or sauces (Ventura et al., 2009). Tomato pomace is made up of peels, seed and small amounts of pulp. Most agroindustrial by-products are highly variable depending on external factors and TP is not an exception (Lu et al., 2019). The objective of the Experiment 4 (Paper 3) was to determine the variability in nutritive value for ruminants of TP samples obtained at different times from two factories and to analyze its effect on *in vitro* fermentation when it was included in a high-concentrate diet.

Samples of TP were rich in N (average value 25.6 g/kg DM) and EE (82.7 g/kg DM). Tomato pomace is a fibrous byproduct rich in NDF and acid detergent fibre (ADF), and the fibre content observed in our TP samples (average values 572 439 g/kg DM for NDF and ADF, respectively) was greater than that measured for other byproducts in our laboratory, such as sugar beet pulp (487 and 224 g/kg DM for NDF and ADF, respectively) and dried distillers grains with solubles (DDGS) (340 and 88.1 g/kg DM for NDF and ADF, respectively). Although TP samples had a relatively high content of N, a great part is bound to the ADF (ADIN), being this proportion greater than in other by-products (ADIN: 2.05 vs 0.11 and 0.09 g/kg DM for TP, sugar beet pulp and DDGS), and the ruminal degradability and intestinal digestibility of N was low

(510 g/kg and 459 g/kg, respectively). The content of sugars in TP was also considerable (122 g/kg DM), compared with the values reported for sugar beet pulp (48.0 g/kg DM). The high content of easily fermented sugars is probably responsible for the low *Lag* and high AGRP values measured for TP samples (2.45 h and 8.54 ml/h, respectively). Nevertheless, the high lignification of fibre in TP reduces its nutritive value. Values of chemical composition and rumen degradability obtained for TP samples obtained in our study differ from those reported by other authors (Abbeddou et al., 2011; Fondevila et al., 1994), but differences in chemical composition of the original fruits, in cultivation conditions and processing can help to explain the high variability in TP. In our study, no differences were observed in either chemical composition or fermentative parameters among samples from the two processing factories, probably as a result of similar processing in both. However, there were significant differences in EE and lignin content among sampling times, but the composition of other fractions remained unchanged.

Several authors have studied the effect of including tomato by-products in diets for small ruminants on the productive response, as well as on the quality of the meat and milk (Valenti et al., 2018; Romano et al., 2010). Romero-Huelva et al., (2010) also studied the effect of including fresh tomato in dairy goats diets on methane production and reported a reduction of 39% in the methane emissions, but no information on the possible effects of TP on methane production is available. In the Experiment 4, we studied *in vitro* the effect of including TP up to 180 g/kg DM in a diet for fattening lambs. We observed an increase in fermentative parameters, probably as a result of replacing straw for TP, which has a higher nutritive value. On the other hand, we did not detect any antimethanogenic effect as observed with fresh tomato fruits, which could be caused by the deterioration of the biocompounds responsible for the antimethanogenic properties during thermal processing of TP. Therefore, we concluded that TP can be included up to 180 g/kg DM *in vitro* in diets for fattening lambs with no negative effects on ruminal fermentation, although these results should be confirmed in *in vivo* trials. Even though TP has a high content of N, it is not recommended to use it as a protein source because of the low N availability. On the contrary, it could be used as a cheap fibrous feedstuff in farms near to the processing industries, as the high water content precludes its use in remote farms and the drying process or the transport would increase the price of TP substantially.

The ADIN fraction has been used to estimate the N digestibility in forages as well as to determine heat damage. However, in heat treated non-forage feeds, poor relationships have been reported. Despite this, some feeding systems, such as the UK Metabolizable Protein System and the Cornell Net Carbohydrate and Protein System, utilize the ADIN analysis as a measure of N availability. Both systems assume that the ADIN fraction is indigestible, although it has been reported a certain degree of ADIN digestibility. As many by-products contain a substantial proportion of ADIN in Experiment 6 (Paper 4), we proved that ADIN content is overestimated due to the contamination of samples with cetyltrimethylammonium bromide (CTAB) during the ADF analysis previous to ADIN determination. We indicated, as well, that the most reactive fraction and the most susceptible fraction to such contamination are the pectins, and that the observed relationships between ADIN contamination and chemical composition of samples indicate that N-contamination of ADIN increased with increasing structural carbohydrate content of feeds, but it was reduced by ADF lignification. Forages usually have low content of pectins and are highly lignified, whereas non-forage feeds might have greater pectins contents and lower lignification degree. This could be one of the reasons explaining why ADIN analyses have been successfully used in forages to determine N digestibility. The contamination of N due to CTAB might be the responsible for the ADIN digestibility reported in the literature. As we stated earlier, the agroindustrial by-products studied in this Thesis are fibrous and can contain high amounts of ADIN. In this context, analysing ADIN in agroindustrial byproducts could provide useful information about protein degradability and digestibility, and be useful to determine the heat damage of these by-products, as usually they undergo processes where heat is required, such as drying.

Agroindustrial by-products contain bioactive compounds that might improve the quality of animal products (Romero-Huelva et al., 2012). In the previous experiments we studied *in vitro* the nutritive value of two agroindustrial by-products, OC and TP. The next step would be including such by-products in diets to assess their effect on animal performance and products quality. The objective of the Experiment 5 (Paper 6) was to analyze the effect of including agroindustrial by-products, such as DDGS, dried citrus pulp (DCP) and EOC in a diet for dairy goats on nutrient digestibility, ruminal fermentation, methane production, urinary excretion of purine derivatives (PD) and milk yield and composition. The DDGS replaced soybean meal as a protein source,

whereas the DCP replaced cereals as an energy source and the EOC was included as an extra source of fat. In this Experiment, the objective was not to determine the effect of each single by-product on the parameters studied, but to study the viability of feeding a diet containing a high proportion of agroindustrial by-products, as it could alleviate the environmental impact of agroindustrial and animal production and reduce feeding costs without competing with human food. According to Wilkinson (2011), the potential use of ingredients as human food of the diet based on by-products was 40% lower compared with the control diet. Additionally, the by-product based diet was cheaper (80 €/t). Feeding the diet based on by-products to dairy goats had no negative effect on nutrient utilization, milk yield or ruminal fermentation, and resulted in more milk fat and protein. The milk from the goats fed the by-products diet was richer in unsaturated FA, compared with the milk from goats fed the control diet. A wide list of health benefits have been attributed to those FA, especially to polyunsaturated FA (Parodi, 2009; Banni et al., 2003). For that reason, as an extra positive effect of feeding by-products, the milk from animals receiving such diets could be considered healthier. Some authors have speculated that feeding agroindustrial by-products could reduce methane emissions due to the presence of biocompounds in the by-products (Vasta et al., 2009; Newbold et al., 2005). However, we did not observe any antimethanogenic effect. Nevertheless, the environmental benefit of utilizing agroindustrial by-product is still considerable even though it did not decrease emissions of greenhouse gases. All in one, feeding animals with diets base in by-products could contribute to improve the sustainability of livestock systems (Pascual Vidal, 2015; del Prado et al., 2013).

It is clear, that *in vitro* and *in situ* methods are useful to determine the nutritive value of feedstuffs. Although *in vivo* methods are more accurate, they are much more expensive. As already stated, there are plenty of agroindustrial by-products available today and studying their nutritive value with *in vivo* methods is not feasible, and therefore *in vitro* and *in situ* methods are being used to study their nutritive value. Nevertheless, these methods are still expensive and require considerable amounts of funds and workforce. Furthermore, experimental animals are needed to perform *in vitro* and *in situ* studies. On the other hand, there are tools that could help to predict the nutritive value of feeds which would facilitate their inclusion in diets. This is especially interesting for agroindustrial by-products, especially taking into account the wide range of by-products available. Among these tools, regression analyses have been one of the

most commonly used and they allow to obtain equations similar to those obtained in the Experiment 2 (Paper 2), in which fermentation parameters were estimated from chemical composition, which is easier and cheaper to determine. However, the data used to obtain these equations require fulfilling some statistical assumptions which are not always satisfied, and predictions obtained from these equations are not always accurate.

As technology advances, new tools are available to the user which could provide new methods to predict the nutritive value of feeds. One of these new tools are Neural Networks. Processing the data with neural networks does not require any statistical assumption or condition, which means that information from very heterogeneous sources can be processed. This is a very interesting approach for evaluating agroindustrial by-products, as they are a very heterogeneous group of potential feeds. What is more interesting of Neural Networks is that the more information is provided, the better are the results obtained. As an example, Figure 2 shows a prediction of the AGPR of the four OC samples used to test the accuracy of this procedure (See Materials and methods). When data from TP was included in neural network (Figure 3), the precision increased and was higher ($R^2 = 0.978$; slope close to 1) than that obtained in the Experiment 2 (Paper 2) from regression analyses. In addition, this model could be used to predict fermentation parameters of both OC and TP. These results suggest that neural networks could be a good tool to predict the nutritive value of agroindustrial by-products, as usually the nutritive value is influenced by different chemical fraction, as shown in the Experiments 1 to 4 (Paper 1, 2, 5 and 3) of this Thesis. In the future, these models might be improved by including more data and could provide a universal method to estimate the nutritive value of by-products and other feeds. The possibility of predicting in vivo parameters, such as feed intake or nutrient digestibility should also be explored.

Figure 2. Prediction of AGPR with a neural network including olive cake (OC) data.

Figure 3. Prediction of AGPR with a neural network including olive cake (OC; Green dots) and tomato pomace (TP; Red dot) data.

CONCLUSIONS

1. The nutritive value of olive cake was low, but it was markedly affected by processing, storage time of alperujo before processing, and particle size. Ruminant degradability of cyclone olive cake was as average 1.3 times greater than that of crude and exhausted olive cake obtained at the same storage time. For all types of olive cake, ruminant degradability decreased with advancing storage time, and therefore early processing is recommended. Olive cake samples having more than 3 mm or less than 1 mm showed greater fermentability than those measuring 2 and 3 mm.
2. *In vitro* ruminal fermentation parameters of olive cake can be predicted from the type of olive cake and its content in sugars and total soluble polyphenols. The same ruminal parameters can be predicted from olive cake type and storage time, but the equations had lower R^2 and greater residual standard deviation values than those involving chemical fractions.
3. There was little variability in chemical composition and *in vitro* rumen fermentation of tomato pomace samples obtained from two processing plants at different times over the tomato campaign. Although tomato pomace had relatively high protein content (average 160 g/kg dry matter), both rumen degradability and *in vitro* intestinal digestibility of the undegraded protein were low (average 510 and 459 g/kg, respectively). *In vitro* results indicated that dried TP could be included up to 180 g/kg in a high-concentrate diet without negative effects on rumen fermentation.
4. The use of cetyltrimethylammonium bromide in the analysis of acid detergent insoluble nitrogen (ADIN) resulted in an overestimation of the ADIN fraction, questioning the use of ADIN as a parameter indicative of nitrogen availability. The overestimation was greater for high-fibre samples and was reduced with increasing lignification of the cell wall.
5. A mixture of corn DDGS, DCP and EOC (in proportions of 41, 41 and 18%, respectively) could replace 44% of cereal grains and protein feeds in the concentrate for dairy goats without compromising feed intake, nutrient utilization or ruminal fermentation, and resulted in greater milk fat and protein production, as well as in a more unsaturated and potentially healthier milk FA profile.

Chapter VIII. REFERENCES

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Chapter IX. APPENDIX

IX.1. Influence of particle size of crude olive cake on *in vitro* ruminal fermentation and gas production kinetics

Marcos C. N., Chávez, S., De Blas, C., Molina-Alcaide, E., Ranilla, M. J., and Carro, M. D. (2019). Influence de la taille des particules des grignons d'olive sur la fermentation ruminal et la cinétique de production de gaz. In: Ruiz R., López-Francos A., López Marco L. (eds). Innovation for Sustainability in Sheep and Goats, 2nd Joint Seminar of the Subnetworks on Nutrition and on Production Systems of the FAO-CIHEAM Network for Research and Development in Sheep and Goats. Vitoria-Gasteiz (Spain), 3-5 October 2017. *Options Méditerranéennes: Series A* (Mediterranean Seminars), no. 123. Available at: <http://om.ciheam.org/option.php?IDOM=1034>

Abstract: By-products of olive oil industry can be used in ruminants feeding, but their nutritive value can be affected by the processing method. The aim of this research was to estimate *in vitro* the nutritive value of four samples of crude olive cake (COC) with different particle size obtained during the drying process from the same trommel at different stages of the screening procedure. Particles size was greater than 3 mm (COC3), 2 mm (COC2) and 1 mm (COC1) or smaller than 1 mm (COC0). Alfalfa hay and barley straw were included in the study for comparative purposes. Chemical composition was analysed in all samples. Gas production kinetics was determined in 120-h *in vitro* incubations with sheep rumen fluid as inoculum, and fermentation parameters and *in vitro* dry matter digestibility (IVDMD) were analysed after 24 h of incubation. COC3 and COC0 samples had lower contents of neutral detergent fibre (NDF; 415 and 391 g/kg DM), but greater crude protein (96.6 and 114 g/kg DM) and ether extract (220 and 242 g/kg DM) contents, than COC2 and COC1 (627 and 624; 42.4 and 48.7; 102 and 124 g/kg DM, respectively). IVDMD of COC samples ranged from 39.4 to 58.7%, and was lower than that for alfalfa hay (67.5%) for all samples, but greater than that for barley straw (42.4%) for COC0 and COC3 samples. COC3 and COC0 samples had greater ($P < 0.05$) potential gas production (103 and 66.2 ml/g DM, respectively) and total volatile fatty acids production (608 and 600 μmol , respectively) than COC2 and COC1, but values were lower than those for alfalfa hay (202 ml/g DM and 1023 μmol , respectively) and barley straw (198 ml/g DM and 733 μmol). The results indicate that decreasing particle size to <1 mm increased the fermentation potential of COC, but its nutritive value was still slightly lower than that of barley straw. The similar quality observed for COC3 and COC0 samples was attributed to the high sugar content of COC3 samples.

Keywords: Olive cake – Particle size – Chemical composition – Gas production – Ruminal fermentation.

IX.1.1. Introduction

Olive oil extraction generates several by-products that can be used in animal feeding, especially the cakes and pomaces obtained in the extraction process, but their characteristics and nutritive value depend markedly on the processing method (Heuzé et al., 2015). The main product is the “alperujo”, which is stored in open ponds until processing, frequently over a period of several months. Alperujo is dried and olive

stones are separated from the pulp by a process of screening. This process consists of placing the alperujo in a trommel (a cylinder of perforated metal, open at both ends and with a slight incline), that rotates classifying the material by their particle size (Savage et al., 2005). The screening for stones removal produces COC with different particle sizes, and may influence the nutritional quality of COC. The objective of this study was to analyse the influence of particle size of COC on their chemical composition, *in vitro* ruminal fermentation and gas production kinetics.

IX.1.2. Materials and methods

Four samples of crude olive cake (COC) obtained from the same trommel at different stages of the screening process were used in this study. Samples had different particle size: bigger than 3 (COC3), 2 (COC2) and 1 mm (COC1), or smaller than 1 mm (COC0). Chemical composition was analysed according to the AOAC (2005). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin analyses were carried out as described by Van Soest et al. (1991).

Substrates were fermented *in vitro* to determine gas production kinetics and ruminal fermentation parameters. One sample of alfalfa hay and barley straw were also incubated for comparative purposes. Samples (200 mg of dry matter (DM)) of each substrate were weighed into 60-mL bottles. Ruminal fluid was obtained from four rumen-cannulated Lacaune sheep (64.7 ± 2.10 kg body weight) fed grass hay and concentrate in 2:1 proportion twice daily. Sheep were managed according to the protocols approved by the Institutional Animal Care and Use Committee of the Technical University of Madrid and had free access to water over the trial. Ruminal contents of each sheep were obtained immediately before the morning feeding, strained through four layers of cheesecloth and mixed with the buffer solution of Goering and Van Soest (1970; no trypticase added) in a proportion 1:4 (vol/vol) at 39 °C under continuous flushing with CO₂. Bottles were prewarmed (39 °C) prior to the addition of 20 ml of buffered rumen fluid, capped and incubated at 39 °C. Two incubation runs were performed. In the first incubation run, gas production was measured at 3, 6, 9, 12, 18, 24, 36, 48, 72, 96 and 120 h using a pressure transducer (Widereager Wide Range Pressure Meter, Sper Scientific LTD, Scottsdale, AZ, USA) and a calibrated syringe, and the gas produced at each measurement time was released. Bottles without sample (blanks) were incubated to correct for endogenous gas production. In the second run,

bottles were incubated for 24 h, gas production was measured as described before and a gas sample (10 ml) was stored in a vacuum tube for CH₄ analysis. Bottles were then uncapped and samples for volatile fatty acid (VFA) and NH₃-N analyses were taken as described by Martínez et al. (2010). Procedures for CH₄, VFA and NH₃-N analyses have been also described by Martínez et al. (2010). The amount of OM apparently fermented (OMAF) in each bottle was estimated from VFA production as described by Demeyer (1991).

In vitro DM digestibility (IVDMD) was determined by weighting 300 mg of substrate in polyester bags (30 µm pore size; Ankom Corp #57, Ankom Technology Corp., Fairport, NY, USA) which were incubated with buffered ruminal fluid in an Ankom Daisy II incubator (Ankom Technology Corp, Fairport, NY, USA) at 39 °C under continuous rotation. After 24 h, bags were washed with cold water, dried at 60 °C for 48 h and weighted to calculate the IVDMD. Three bags were used for each substrate.

Gas production data were fitted with time using the exponential model: $\text{gas} = A (1 - e^{-c(t - \text{Lag})})$, where A is the asymptotic gas production (mL), c is the fractional rate of gas production (h⁻¹), Lag is the initial delay in the onset of gas production (h) and t is the gas measurement time. The parameters A , c and lag were estimated by an iterative least squares procedure using the NLIN procedure of SAS (version 9.2; SAS Inst. Inc., Cary, NC, USA). The average gas production rate (AGPR; mL gas/h) was calculated as $\text{AGPR} = A c / [2 (\ln 2 + c \text{Lag})]$. Data were analysed as a mixed model using the PROC MIXED of SAS. The effect of substrate was considered fixed and that of the inoculum as random. Significance was declared at $P < 0.05$, and comparison of means was performed by the Tukey test.

IX.1.3. Results and discussion

Content of crude protein (CP), NDF, ADF, lignin and ether extract in COC samples ranged from 42.4 to 114, 391 to 627, 273 to 408, 122 to 150, and 102 to 242 g/kg DM, respectively (Table 26). These values are in the range of those reported by others for COC samples from different sources (Molina-Alcaide and Yáñez-Ruiz, 2008; Molina-Alcaide et al., 2003), with the exception of EE content, which was greater in our samples. The high amount of CP bound to the ADF in the COC samples (11 to 43% of total CP) indicated a low N availability, as previously reported for COC (Molina-

Alcaide and Yáñez-Ruiz, 2008). The IVDMD of COC samples ranged from 39.4 to 58.7%, and was lower than that for alfalfa hay (67.5%) for all samples, but greater than that for barley straw (42.4%) for COC0 and COC3 samples.

Table 26. Chemical composition (g/Kg dry matter) and *in vitro* dry matter digestibility (IVDMD) of crude olive cake (COC) samples with different particle size and two forages.

	COC3	COC2	COC1	COC0	Alfalfa hay	Straw
Crude protein (CP)	96.6	42.4	48.7	114	194	55.6
Neutral detergent fibre (NDF)	415	627	624	391	394	697
Acid detergent fibre (ADF)	298	408	418	273	215	388
Lignin	122	148	163	150	45	38.8
Crude protein bound to the ADF	29.4	10.7	12.7	42.5	66.2	19.2
Ether extract	220	102	124	242	47.2	28.5
IVDMD (%)	58.7	39.4	40.9	49.4	67.5	42.4

As shown in Table 27, COC3 had the greatest ($P < 0.05$) asymptotic gas production (A) and COC2 and CO1 had the lowest values. The higher gas production values observed in COC3 would indicate a more extensive fermentation, as cumulative gas production is directly related with the amount of organic matter fermented (Menke et al., 1979). In contrast, there were no differences ($P \geq 0.146$) among COC samples for *c* and *Lag* parameters. COC3 also showed a greater value ($P < 0.05$) of AGPR compared with the other samples. Cumulative gas production over the first 24 h of incubation was greater for COC3 and COC0 than that for barley straw, but COC2 and COC1 showed similar values to straw (Figure 4). Gas production for all COC samples was lower than that for alfalfa hay at all sampling times.

Table 27. Gas production parameters of crude olive cake (COC) samples with different particle size and two forages ($n = 4$)¹.

Substrate ¹	A	c	Lag	AGPR
COC3	103 ^c	0.0558	0.4	3.98 ^b
COC2	29.1 ^a	0.0801	2.08	1.37 ^a
COC1	30.7 ^a	0.0813	1.76	1.54 ^a
COC0	66.2 ^b	0.0531	1.21	2.35 ^a
sem ²	2.14	0.01002	0.23	0.23

P =	<0.001	0.146	0.242	<0.001
Forages				
Alfalfa hay	202	0.0442	1.82	5.73
Barley straw	198	0.0303	15.4	2.57

^{a-c} Means in the same row with different superscript differ ($P < 0.05$)

¹ A: potential gas production; c: fractional rate of gas production; Lag: time until the production of gas begins; AGPR: gas production rate until it has reached half of the A value

² sem: standard error of the mean

Figure 4. *In vitro* cumulative gas production of COC samples with different particle size and two forages.

The results of the 24 h *in vitro* fermentations (Table 28) were in well agreement with those observed for gas production kinetics. Both COC3 and COC0 produced more ($P < 0.05$) total VFA, acetate, propionate and CH_4 than COC2 and COC1, resulting in greater ($P < 0.05$) amount of OMAF. In contrast, there were no differences ($P > 0.05$) among COC in NH_3 concentrations, which may be due to the use of a N-enriched buffer solution. The greater fermentation of COC0 may be explained by its lower stone content, as stones are low fermentable. The high fermentation observed in COC3 may have been due to a high sugar content, as this sample contained small green-coloured balls which were identified as caramelized sugars (Bacha, personal communication). Both VFA and CH_4 production were lower in all COC samples than in the two tested forages, indicating that COC samples were less fermented.

IX.1.4. Conclusions

Olive cake samples differing in particle size showed marked differences in chemical composition and *in vitro* ruminal fermentation parameters, with samples having more than 3 mm or less than 1 mm showing the highest fermentability. Crude olive cake could be used in ruminant diets replacing fibrous feeds with low nutritional value, but due to its high fat content olive cake would also contribute to increase the energy level of the diet.

Table 28. *In vitro* fermentation parameters after 24 h incubation of crude olive cake (COC) samples with different particle size and two forages with buffered ruminal fluid from sheep ($n = 4$)¹.

Substrate	CH ₄ (μmol)	NH ₃ -N (mg/L)	Total	(μmol)				Ac/Pr (mol/mol)	OMAF (mg)
			VFA (μmol)	Ac	Pr	Bt	Minor VFA		
COC3	190 ^b	348	628 ^b	352 ^b	141 ^b	90.2 ^b	45.5 ^b	2.59 ^a	54.5 ^b
COC2	144 ^a	382	406 ^a	226 ^a	73.4 ^a	65.8 ^a	41.0 ^a	3.15 ^b	34.9 ^a
COC1	155 ^a	366	451 ^a	249 ^a	83.5 ^a	73.4 ^{ab}	45.4 ^b	3.07 ^b	38.8 ^a
COC0	187 ^b	349	600 ^b	338 ^b	131 ^b	84.3 ^{ab}	47.3 ^b	2.68 ^a	51.6 ^b
sem ²	6.10	11.2	14.5	7.50	4.85	4.47	3.62	0.052	1.44
P =	<0.001	0.167	<0.001	<0.001	<0.001	0.016	0.007	<0.001	<0.001
Forages									
Alfalfa hay	315	423	1023	625	216	116	55.4	3.03	87.0
Barley straw	250	372	733	463	131	92.2	56.8	3.66	63.0

^{a-c} Means in the same row with different superscript differ ($P < 0.05$)

¹ Minor VFA are calculated as the sum of isobutyrate, isovalerate, valerate and caproate. The amount of organic matter apparently fermented (OMAF) was calculated from VFA production as described by Demeyer (1991).

² sem: standard error of the mean

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IX.2. Effects of agro-industrial by-products supplementation on dairy goats milk characteristics, nutrients utilization, ruminal fermentation and methane production

Marcos C. N., Carro, M. D., Fernández-Yepes, J., Haro, A., Romero-Huelva, M., and Molina-Alcaide, E. (2019). Effects of including a mixture of agroindustrial by-products in the diet of dairy goats on nutrients utilization, ruminal fermentation, methane production and milk yield and composition. *Journal of Dairy Science*. (Under Review: R1 submitted after a “minor revision” decision).

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INTERPRETIVE SUMMARY: Feeding agroindustrial by-products to ruminants can have multiple benefits, such as lowering feeding costs, reducing competition with human food, decreasing environmental impact associated with by-products disposal, and improving the quality of animal products. We evaluated the effect of replacing 44% of conventional feeds with corn distillers grains with solubles (DDGS; 18%), dried citrus pulp (18%) and exhausted olive cake (8%) in a concentrate for dairy goats. Goats fed the by-products tended to produce more milk with more unsaturated fatty acids, and did not show any negative effect in either nutrient utilization or rumen fermentation.

Running head: Agroindustrial by-products in dairy goats feeding

Simple Summary: **Effects of including a mixture of agroindustrial by-products in the diet of dairy goats on nutrients utilization, ruminal fermentation, methane production and milk yield and composition by Navarro et al.** Feeding by-products to ruminants can contribute to sustain ruminant production by decreasing diet cost. Additionally it may reduce both competition with human food and environmental impact, and improve the quality of animal products. The effect of replacing 44% of conventional feeds with DDGS (18%), DCP (18%) and EOC (8%) in a concentrate for dairy goats has been evaluated. Goats fed the diet including by-products tended to produce more milk with a healthier fatty acid profile, and did not show any negative effect in either nutrient utilization or rumen fermentation.

Abstract: The use of agroindustrial by-products, such as dried distillers grains with solubles (DDGS) and dried citrus pulp (DCP), has been widely investigated in dairy cows, but information on their effects in dairy goats is limited. The influence of olive cake (a by-product of olive oil production) feeding to dairy goats has been assessed in some studies, but exhausted olive cake (EOC) has been much less investigated. Twelve Murciano-Granadina goats were used in a cross-over design trial with two periods to assess the effects of including agroindustrial by-products on nutrient digestibility, ruminal fermentation, methane production, urinary excretion of purine derivatives and milk yield and composition. In each period, 6 goats received daily a control diet composed of 1 kg of alfalfa hay and 1 kg of high-cereal concentrate and other 6 goats received a diet (BYP) composed of 1 kg of alfalfa hay and 1 kg of a concentrate including corn DDGS, DCP and EOC in proportions of 180, 180 and 80 g/kg concentrate (as fed basis), respectively. Diet had no effect on total DM intake, but

intake of alfalfa hay, CP and fat was greater ($P \leq 0.042$) for the BYP than for control group. There were no differences between diets in nutrients apparent digestibility, with the exception of fat which was greater ($P = 0.002$) for BYP compared with control diet. Although fecal N tended to be greater ($P = 0.063$) for BYP-diet, there were no differences in N utilization. Compared to control, milk yield tended to be greater ($P = 0.056$) and daily production of milk CP, fat, whey protein and TS, as well as milk gross energy, were greater ($P \leq 0.031$) for BYP diet. Diet BYP changed milk fatty acid (FA) profile, as the concentration of C12:0, C14:0 and C16:0 FA was or tended to be lower ($P \leq 0.080$) and that of polyunsaturated FA was greater ($P = 0.001$) in the milk of BYP-fed goats compared with those fed the control diet. Diet had no effect on ruminal parameters (pH, VFA and $\text{NH}_3\text{-N}$ concentrations) and methane emissions, but urinary excretion of total purine derivatives tended to be lower ($P = 0.070$) in BYP-fed goats than in those fed the control diet. A mixture of corn DDGS (180 g), DCP (180 g) and EOC (80 g) could replace 44% of cereal grains and protein feeds in the concentrate for dairy goats without compromising nutrient utilization, ruminal fermentation or milk yield, and led to a more unsaturated FA profile in milk.

Key words: dairy goats, DDGS, citrus pulp, exhausted olive cake, milk, fatty acids

IX.2.1. Introduction

The use of agroindustrial by-products in animal feeding, particularly in ruminants, is continuously increasing worldwide as it may reduce feeding costs and, other reasons that are equally or even more important to support the growing interest in their use in ruminants feeding. Many by-products are not potentially edible by humans, and therefore they do not compete with human food (Bakshi et al., 2016). Some by-products have high pollution potential, and their inclusion in animal diets can contribute to reduce the environmental problems associated with their accumulation, concurrently contributing to sustainability of livestock production and reducing the carbon footprint of animal products (del Prado et al., 2013). By-products can also contain bioactive compounds that may improve not only animal health and quality of animal products, but also reduce methane emissions and contribute to decrease the environmental impact of livestock production (Romero-Huelva et al., 2012).

Dried distillers grains with solubles (DDGS) are by-products of the ethanol industry. Whereas there is a lot of information on the effects of DDGS in dairy cows

feeding, to our best knowledge only Cais-Sokolińska et al. (2015, 2019) have evaluated their effect in dairy goats focusing on milk fatty acid (FA) profile, basic milk composition and cytological quality (SCC count) and both fermentation process and aroma profile of the resulting kefir, and no information on DDGS effects on the yield of goat's milk is available. Citrus pulp is the main by-product of citrus industry that can be fed fresh, ensiled or dried. Citrus pulp is used as a source of dietary energy, usually as cereal grains replacer. In both dairy cows and sheep, the inclusion of dry citrus pulp (DCP) in the diet up to 30% has been reported to have no negative effect on milk yield, fat content and flavor in some studies (reviewed by Bampidis and Robinson, 2006), but in others inclusion of DCP in diet increased milk yield and fat concentration. In dairy goats, López et al. (2014) observed that totally replacing the dietary corn with DCP had no effect on milk yield but increased milk fat concentration and Ibáñez et al. (2016) observed that dairy goats had greater fat mobilization when DCP completely replaced barley in the diet, although milk yield was not affected.

Olive oil extraction generates a wet solid-waste called “alperujo” that is stored in open-air ponds before being destoned and dried, in a process that generates a by-product called olive cake. Olive cake can be used in animal feeding or be subjected to a chemical extraction (using hexane or other authorized food-grade solvents) to obtain pomace olive oil, generating exhausted olive cake (EOC) as by-product (Marcos et al., 2019). This by-product is a fibrous material with low nutritive value, but rich in polyphenols (Marcos et al., 2019; Molina-Alcaide and Yáñez-Ruiz, 2008) and therefore it might modify ruminal fermentation and improve milk quality. The objective of this study was therefore to assess the effects of replacing conventional ingredients of a concentrate for dairy goats with a mixture of DDGS, DCP and EOC on feed intake and nutrient digestibility, milk yield and composition, urinary excretion of purine derivatives, and methane emissions.

IX.2.2. Materials and methods

Animals used in this trial were cared and handled in accordance with the Spanish guidelines for experimental animal protection, and all experimental procedures were approved by the Ethic Committee for Animal Experimentation of the Spanish Research Council and the Junta de Andalucía (approval number 29/08/2016/145).

IX.2.2.1. Animals and feeding

Twelve Murciano-Granadina dairy goats producing 1837 ± 146.7 g milk/d, with 51.5 ± 1.83 kg of BW, and 12.8 ± 1.17 DIM were divided into 2 homogeneous groups based on BW and milk yield at the beginning of the experiment. Goats were placed in individual boxes with free access to fresh water, and each group was randomly assigned to one of the two experimental diets: a control diet and a diet containing agroindustrial by-products (BYP). The control diet was based on alfalfa hay and a high-cereal concentrate in a 50:50 ratio. The BYP diet was also based on a 50:50 ratio alfalfa hay and concentrate, in which 44% of conventional ingredients (corn, barley, soybean meal, palmiste meal and wheat bran) was replaced with a mixture of 180, 180 and 80 g of corn DDGS, DCP and EOC per kg concentrate, respectively (fresh matter basis). The ingredients of both concentrates, and chemical composition and fatty acid (FA) profile of feeds are shown in Table 29. The diets were formulated to meet the N and energy requirements of Murciano-Granadina goats producing daily 2 kg of milk (Aguilera et al., 1990; Prieto et al., 1990), and were supplied twice a day at 09:00 and 16:00 over the trial.

Table 29. Ingredient and chemical composition of experimental concentrates and potential use as human food of concentrate ingredients.

Item	Concentrate	
	Control	BYP
Ingredients, g/kg as fed		
Corn	330	268
Barley	200	-
Wheat	100	100
Soybean meal	122	102
Palm meal	88	-
Colza meal	25	25
Weat bran	100	30
Corn DDGS	-	180
Dry citrus pulp	-	180
Exhausted olive cake	-	80
Calcium soap	12.0	12.0
CaCO ₃	10.0	10.0
NaHCO ₃	8.0	8.0
NaCl	3.0	3.0
Vitamin-mineral mixture ²	2.0	2.0
Chemical composition, g/kg as fed basis		
DM	894	883
OM	843	824
CP	160	174
NDF	205	208
ADF	75.9	85.7
Lignin	17.4	23.4
Ether extract	28.2	47.0
Total soluble polyphenols	1.95	6.05
Fatty acids, g/100 g total fatty acids		
C12:0	7.01	0.282
C14:0	2.78	0.371
C14:1 <i>cis</i> -9	ND ¹	0.001
C16:0	21.6	20.6
C16:1 <i>cis</i> -9	0.169	0.001
C18:0	2.78	2.76
C18:1 <i>trans</i> -9	0.077	0.004
C18:1 <i>trans</i> -11	0.080	0.002
C18:1 <i>cis</i> -9	26.6	34.5
C18:1 <i>cis</i> -11	0.828	0.875
C18:2 <i>trans</i> -9, <i>trans</i> -12	0.004	0.009
C18:2 <i>cis</i> -9, <i>cis</i> -12	34.1	36.7
C20:0	0.343	0.427
Potential use of ingredients as human food (%) ³	64.4	38.7

¹ ND: not detected.

² Contained (per kg): 40 mg Se; 250 mg I; 80 mg Co; 3,000 mg Cu; 6,000 mg Fe; 23.4 g Zn; 29 g Mn; 60 g S; 60 g Mg; 2,000,000 IU vitamin A; 400,000 IU vitamin D3; 2 g vitamin E; 10 g nicotinic acid; 20.3 g choline (Nacoop S.A., Madrid, Spain).

³ Calculated according to Wilkinson (2011)

IX.2.2.2. Experimental procedure and measurements

The experiment was conducted as a cross-over design with 2 periods of 37 days each, comprising 21 d of adaptation to diets, and 16 d for measurements and sampling. Over the trial, animals were milked once a day in the morning in a 1 x 10 stall milking parlour (DeLaval, Madrid, Spain), and were weighted at the beginning and end of each experimental period. On d 22 of each experimental period, animals were moved to individual metabolism crates and after 3-d of adaptation, total feces and urine voided by each animal were quantitatively collected for 5 d. Urine was collected into buckets containing 10% HCl (vol / vol) to keep the pH below 3 and avoid N losses. Aliquots (10%) of urine were taken daily for density measurement, and immediately frozen until analyses of N content, energy and purine derivative (PD) and creatinine concentrations. Samples (20%) of total fecal output were collected each day for digestibility determination and stored at -20°C until chemical analyses. Samples of feces and urine were pooled for each goat to form composite samples.

On d 30 goats were moved to floor pens, and on d 31 approximately 20 mL of ruminal fluid was collected from each animal before feeding using a stomach tube attached to a vacuum pump. The fluid was strained through 4 layers of cheesecloth, the pH was immediately measured, and 3 ml of ruminal fluid were mixed with 3 ml 0.5 M HCl and stored frozen until $\text{NH}_3\text{-N}$ and VFA analyses. From d 32 to 37, 4 goats from each dietary treatment were sequentially placed in 4 individual open circuit respiration chambers (1 goat per chamber; 2 d of measurement) to measure individual methane emissions as described by Romero-Huelva and Molina-Alcaide (2013). Short methane measurement interruptions (10 min) occurred daily at 09:00 h for diet supply, milking, and chamber floor cleaning, but they had little effect on daily methane emissions as the corresponding exhaust duct was not sampled during this period. Fluxes were determined twice per day and averaged to calculate the 24-h emissions.

Individual feed and water intake were measured over the trial, but only values measured during the sampling period were used for the statistical analysis. During the sampling period, alfalfa hay and concentrate refusals from each animal were weighted daily, pooled, and stored at -20°C until analyses. Each day of the sampling period, milk yield was recorded using a 0.1 g precision balance, milk density was measured, and aliquots were stored at -30°C without preservatives for chemical analyses.

IX.2.2.3. Chemical analyses

Dry matter (method 934.01), ash (method 942.05) and ether extract (method 920.39) content in samples of diet ingredients and refusals and feces were analyzed according to AOAC (2005). Total soluble polyphenols in feeds were analyzed by the Folin-Ciocalteu assay as described by Singleton et al. (1965). The N content of diet ingredients, refusals, and of feces and urine was determined according to the Dumas method using a TruSpec CN equipment (Leco Corp. St. Joseph, MI, USA). The analyses of NDF in diet ingredients and refusals were carried out according to Van Soest et al. (1991) using an Ankom 220 Fiber Analyzer unit (Ankom Technology Corp., Macedon, NY, USA), whereas ADF and acid detergent lignin were determined as described by Robertson and Van Soest (1981), and all results were expressed exclusive of residual ash. Energy content of diet ingredients, refusals, and of feces and urine was determined by using an adiabatic calorimeter (model 1356, Parr Instruments Co., Moline, IL).

The analyses of VFA and $\text{NH}_3\text{-N}$ concentrations in ruminal fluid were performed by gas chromatography and a colorimetric method, respectively, as described by Romero-Huelva and Molina-Alcaide (2013). Total solids in milk were determined by lyophilization of milk samples, and total N content was analyzed by the Dumas method. Non protein-N was analyzed in samples of milk filtrates after precipitation with 12% (wt/vol) trichloroacetic acid solution. Non casein-N was determined in milk filtrates after precipitation with 10% (wt/vol) acetic acid at a pH of 4.1 (Recio et al., 1997). Values of N in milk were converted to the corresponding protein by multiplying by 6.38. The milk fat content was measured following the Gerber method (Pearson, 1976).

Extraction of total FA in samples of alfalfa hay and concentrates was performed following the procedure of Folch et al. (1957), and the FA were methylated according to Kramer and Zhou (2001), with slight modifications, as a double methylation was carried out, using first NaOH/methanol, at 50 °C for 15 min, followed by HCl/methanol at 50 °C for 1 h to obtain the FA methyl esters (FAME). For milk FA composition analysis, FA were extracted and transesterified to FAME as described by Abecia et al. (2012). The FAME were separated and quantified using a gas chromatograph (Model Focus GC, Thermo Scientific, Milan, Italy) equipped with a flame-ionization detector and a 100-m fused silica capillary column (0.25 mm i.d., 0.2- μm film thickness; SP-Supelco,

Bellefonte, PA) and helium as the carrier gas. Total FAME profile in a 1- μ L sample volume at a split ratio of 1:50 was determined using a temperature gradient program (Shingfield et al., 2003). Nonadecanoic acid (C19:0; Sigma-Aldrich-N5252) was used as an internal standard, and FAME peaks were identified by comparing their retention times with those of standards (47885 U and O5632: Sigma Aldrich, Madrid, Spain; 90-1093, Larodan Fine Chemicals, Stockholm, Sweden). Results are expressed as percentage of total FAME identified.

Methane concentrations were measured continuously in the air stream in each duct using a gas analyzer ADC MGA3000 (Spurling Works, Herts, UK) as detailed by Romero-Huelva et al. (2017). The concentration of creatinine and purine derivatives (PD; allantoin, uric acid, xanthine and hypoxanthine) in urine were determined by HPLC following the method described by Balcells et al. (1992) and the equipment detailed by Romero-Huelva et al. (2017).

IX.2.2.4. Calculations and statistical analyses

Nitrogen and energy balances were calculated as the difference between the amount of N and energy ingested and that lost in urine, feces and milk, and the energy lost as CH₄ (0.890 MJ / mol of CH₄; Newbold et al., 2007). Milk protein-N content was calculated as the difference between total and non-protein N in milk, whereas the casein-N concentration in milk was calculated as the difference between total N and non casein-N. The amount of whey-N in milk was calculated as the difference between milk protein and casein-N. Milk lactose was calculated as the difference between the amount of total solids and that of protein, fat, and total ash in milk.

Data were analyzed as a mixed model using the PROC MIXED of SAS (SAS Institute Inc., Cary, NC), in which the effects of diet, period and their interaction were considered fixed and that of goat was considered random. Effects were declared significant at $P < 0.05$, and P -values between 0.05 and 0.10 were considered as a trend.

IX.2.3. Results and discussion

IX.2.3.1. By-products and concentrates composition

Corn DDGS, DCP and EOC contained (as fed basis) 900, 897 and 823 g DM; 847, 840 and 741 g of OM; 264, 48.6 and 74.8 g of CP; 371, 179 and 464 g of NDF, and 98.1, 32.8 and 29.1 g of EE per kg, respectively. Alfalfa hay contained (as fed basis)

935, 807, 159, 447, 321, 74.7 and 12.8 g of DM, OM, CP, NDF, ADF, lignin and EE per kg, respectively. The composition of by-products was in the range of previously published values for corn DDGS (NRC, 2001), DCP (NRC, 2001), and EOC (Marcos et al., 2019; Molina-Alcaide and Yañez-Ruiz, 2008). Both control and BYP concentrates were formulated to have similar CP and NDF contents, although BYP concentrate resulted in slightly greater CP content compared to control one. In addition, EE content was greater in BYP concentrate due to the high EE concentration in DDGS. The greater content in total soluble polyphenols of BYP compared with the control concentrate was attributed to the inclusion of DCP and EOC, which have greater polyphenols content (17.1 and 24.2 g/kg in our study) than other conventional feeds used in ruminant diets (Marcos et al., 2019).

Compared with control, BYP concentrate had greater content of oleic (C18:1 cis-9) and linoleic (C18:2 cis-9, cis-12) acids and lower content of lauric (C12:0), myristic (C14:0) and palmitic (C16:0), acids, which is in agreement with previous results indicating that the inclusion of olive cake in the diet usually increases the amount of oleic acid (Luciano et al., 2013; Molina-Alcaide et al., 2010). Furthermore, about 80% of FA in DDGS are unsaturated (Pecka-Kielb et al., 2017), and the FA in citrus pulp are mainly oleic, linoleic, linolenic (C18:3), palmitic and stearic acids (C18:0) (Bampidis and Robinson, 2006), which might have contributed to the differences observed in FA profile of the two concentrates. The most abundant FA in alfalfa hay were palmitic, linoleic and linolenic (24.7, 21.9 and 34.6% of total FA, respectively), which is in agreement with previous results (Romero-Huelva et al., 2017; Molina-Alcaide et al., 2010).

The proportion of each by-product in the diet was selected from *in vitro* studies by our group (unpublished results) and from previously *in vivo* published studies (Ibáñez et al., 2016; Molina-Alcaide et al., 2010; Molina-Alcaide and Yañez-Ruiz, 2008; Bampidis and Robinson, 2006). According to Wilkinson (2011), the estimated human edible proportion of ingredients in the concentrate was 25.7 percentage units lower in BYP than in control concentrate (64.4 vs. 38.7%, respectively; Table 29), reflecting a lower use of human-edible foods for BYP diet.

IX.2.3.2. Intake, apparent digestibility of nutrients, and energy and nitrogen balance

As shown in Table 30, there were no differences between groups in total DM, concentrate DM and water intake ($P \geq 0.17$), but BYP-fed goats consumed more DM alfalfa hay ($P = 0.04$) than those fed the control diet. An increase in alfalfa hay was also observed by Romero-Huelva et al. (2017) when dairy goats were fed a concentrate including by-products (tomato fruits, citrus pulp, brewer's grain and brewer's yeast) compared with those fed a high-cereal concentrate. Although in our study both concentrates had the same NDF concentration (Table 29), the NDF of DDGS and DCP is rapidly degraded in the rumen and might have a lower effectiveness in stimulating rumination, leading to a greater alfalfa hay intake to supply physically effective fiber. Intake of CP and fat in goats fed the BYP diet was greater ($P = 0.003$ and < 0.001 , respectively) than that in control-fed goats, which is consistent with the greater CP and fat content of BYP diet.

Table 30. Average values of feed and water intake and apparent digestibility of nutrients in dairy goats fed the experimental diets.

Item	Diet ¹		SEM	P-value
	Control	BYP		
Intake, g/d				
Total DM	1591	1637	21.3	0.17
Alfalfa hay	710	770	17.6	0.042
Concentrate	880	883	9.3	0.83
OM	1482	1508	19.0	0.37
CP	285	311	4.6	0.003
EE	38.5	58.4	0.50	<0.001
NDF	553	577	9.8	0.12
ADF	330	353	7.5	0.07
Water, L/d	4.99	5.18	0.29	0.65
Apparent digestibility, g/g				
MO	0.757	0.746	0.0067	0.27
CP	0.753	0.746	0.0066	0.48
EE	0.795	0.832	0.0059	0.002
NDF	0.553	0.572	0.0109	0.26
ADF	0.522	0.555	0.0145	0.15

¹ Control: 40:60 alfalfa hay:high-cereal concentrate; BYP: 40:60 alfalfa hay:concentrate containing by-products

There were no differences ($P \geq 0.15$) between diets in the nutrients apparent digestibility, with the exception of fat digestibility. The greater ($P = 0.002$) fat digestibility in BYP-fed compared with control-fed goats could be due to differences in the FA profile of the diets, as DDGS fat has been reported to be highly digestible (Kurokawa et al., 2012).

As there were no differences between diets in CP digestibility, the greater N fecal excretion in BYP-fed goats was expected due to their greater N intake (Table 31). There were no differences between diets in urine N excretion, but milk total N, milk protein N, and N retained in the body (calculated as the difference between N intake and N excreted in feces and urine) tended to be greater ($P \leq 0.07$) in goats fed the BYP diet compared with the control one. In contrast, total N retained (calculated as the difference between N intake and N excreted in feces, urine and milk) was not affected ($P = 0.12$) by the diet. Values for fecal N, urinary N, N retained in the body, and total retained N in the present study were greater than those reported by Romero-Huelva et al. (2017) for Murciano-Granadina goats fed a control diet or a diet in which the concentrate included by-products (averaged values 0.477, 0.765, 1.16 and 0.706 g /kg BW^{0.75}, respectively). In another study by the same group (Romero-Huelva et al., 2012), values for fecal N and urinary N measured in Murciano-Granadina goats fed diets containing different by-products (averaged values 0.733 and 1.39 g /kg BW^{0.75}, respectively) were similar to those observed in our study, but the amount of N retained in the body and total N retained were greater (1.0 and 0.551 g /kg BW^{0.75}, respectively). The lower values observed in our study would indicate an inefficient use of dietary N, possibly caused by an excess of RDP, as indicated by the high excretion of N in urine that was 52.8 and 46.1% of total N intake for control and BYP diets, respectively. The CP content used in the present study was chosen to be representative of those used in the practice in the feeding of high-producing dairy goats, although lower CP levels were used in the previously cited studies. In addition, the different lactation stage of the animals in the different trials (middle lactation in the study of Romero-Huelva et al. (2017, 2012) and early lactation in the present one) could help to explain the observed differences.

The lack of differences ($P \geq 0.48$) between diets in any parameter of N utilization indicates that the inclusion of DDGS, DCP and EOC in the concentrate did not affect negatively the efficiency of N utilization. In addition, the BYP diet tended ($P = 0.10$) to increase the N retained body/digestible N ratio, which is in agreement with

the numerically lower weight loss observed in BYP-fed goats (Table 31).

There were no differences ($P \geq 0.11$) between diets in gross energy intake, fecal and urinary energy losses, and the intake of digestible and ME energy (Table 31). In contrast, milk energy, either expressed as MJ/kg of $BW^{0.75}$ or as a proportion of both digestible and ME energy intake, was greater ($P \leq 0.04$) in BYP-fed goats compared with those fed the control diet, suggesting an improvement of the efficiency of energy utilisation in BYP-fed goats.

IX.2.3.3. Milk yield and composition

Milk yield tended to be greater ($P = 0.06$) and the daily production of milk fat, protein, whey protein and TS, as well as milk gross energy, were greater ($P \leq 0.03$) for goats fed the BYP diet compared with those fed the control diet. In agreement with our results, the inclusion of 600 g of DCP per kg in the diet of dairy goats has been reported to have no negative effect on milk yield and to increase the production of milk fat (Ibáñez et al., 2016; López et al., 2014). Milk yield response to DDGS inclusion in the diet has also been positive in dairy cows (Hollmann et al., 2011), and an increase in milk protein and lactose yield has been reported in several studies (Janicek et al., 2008; Anderson et al., 2006; Kleinschmit et al., 2006). In contrast, no effect on milk yield was observed in sheep fed 20% of DDGS (Pecka-Kielb et al., 2017), and even a reduction in milk protein, fat and TS has been observed by Radunz et al. (2011). The results on the influence of olive cake feeding on milk yield and composition are also controversial. Chiofalo et al. (2004) reported an increase in milk yield when including 200 g/kg of olive cake in the diet of dairy ewes, but Vargas-Bello-Pérez et al. (2013) observed no effect on milk yield by including up to 250 g/kg of olive cake in dairy ewes, and Molina-Alcaide et al. (2010) found a decrease in milk yield in goats which received a diet with 120 g/kg of olive cake. The variable composition of olive cake, especially in their sugars and fat content (Marcos et al., 2019), can explain these contrasting results.

Table 31. Average BW and BW change over the trial and values of N and energy utilization in dairy goats fed the experimental diets.

Item	Diet ¹		SEM	P-value
	Control	BYP		
BW, kg	44.8	45.8	0.60	0.29
BW change, kg	- 6.78	- 5.86	0.453	0.18
BW ^{0,75}	17.3	17.6	0.18	0.24
g/kg of BW ^{0,75}				
N intake	2.65	2.84	0.051	0.031
Fecal N	0.655	0.724	0.0228	0.06
Urine N	1.40	1.31	0.056	0.27
Milk total N	0.569	0.615	0.0145	0.06
Milk protein N	0.494	0.535	0.0129	0.06
Digestible N ²	2.00	2.12	0.049	0.10
N retained body ³	0.594	0.829	0.0811	0.071
Total N retained ⁴	0.025	0.218	0.0788	0.12
N utilization, g/g				
Digestible N/intake N	0.753	0.746	0.0066	0.48
N retained body/digestible N	0.284	0.377	0.0360	0.10
Milk protein N/digestible N	0.252	0.256	0.0076	0.74
Milk protein N/milk total N	0.868	0.869	0.0035	0.82
Milk protein N/intake N	0.189	0.190	0.0047	0.81
MJ/kg of BW ^{0,75}				
Gross energy intake	1.73	1.73	0.027	0.98
Fecal energy	0.432	0.461	0.0130	0.15
Urine energy	0.073	0.082	0.0036	0.11
Milk energy	0.393	0.428	0.0102	0.042
Digestible energy intake ⁵	1.30	1.27	0.024	0.51
ME intake ⁶	1.14	1.12	0.024	0.45
Energy utilization, MJ/MJ				
Digestible energy/energy intake	0.751	0.735	0.0064	0.11
Milk energy/digestible energy	0.307	0.338	0.0085	0.031
Milk energy/ME	0.349	0.388	0.0104	0.030
ME/energy intake	0.661	0.642	0.0070	0.09
ME/digestible energy	0.881	0.874	0.0035	0.24

¹ Control: 40:60 alfalfa hay:high-cereal concentrate; BYP: 40:60 alfalfa hay:concentrate containing by-products

² Digestible N = N intake – fecal N.

³ N retained body = N intake – fecal N – urine N.

⁴ Total N retained = N intake – fecal N – urine N– milk total N.

⁵ Digestible energy = energy intake – fecal energy.

⁶ ME = digestible energy – urine energy – methane energy; A value of 0.890 MJ/mol of methane (Newbold et al., 2007) was used for calculations.

Table 32. Average values of milk yield and composition and feed efficiency in dairy goats fed the experimental diets.

Item	Diet ¹		SEM	P-value
	Control	BYP		
Milk yield, g/d				
Milk	1967	2123	49.5	0.06
Fat	91.0	102	2.8	0.025
Protein	62.6	68.9	1.71	0.031
Casein	39.2	42.8	1.42	0.11
Whey protein	22.9	25.5	0.51	0.007
Lactose	102	109	3.4	0.17
TS	269	298	7.6	0.028
Gross energy, MJ/d	6.77	7.50	0.202	0.033
Milk composition, g/kg				
Fat	45.8	47.8	1.00	0.20
Protein	31.8	32.9	0.26	0.026
Casein	20.0	20.5	0.31	0.38
Whey protein	11.6	12.0	0.26	0.22
Lactose	52.1	52.9	0.67	0.44
TS	137	141	1.4	0.10
Gross energy, MJ/kg	3.44	3.53	0.054	0.25
Feed efficiency ²	23.1	24.8	0.62	0.08

¹ Control: 40:60 alfalfa hay:high-cereal concentrate; BYP: 40:60 alfalfa hay:concentrate containing by-products

² Efficiency of feed utilization; feed efficiency = (Milk energy/energy intake) × 100.

The gross energy in milk observed for BYP-fed goats (Table 32) is consistent with the greater proportion of digestible ME used for milk production (Table 31), which would indicate a greater energy efficiency for milk production. In fact, feed efficiency tended ($P = 0.08$) to be higher in goats fed the BYP diet. Overall, there was no difference in milk composition between the two diets, excepting the greater ($P = 0.026$) protein content obtained with diet BYP compared with control diet (Table 32), which is consistent with the trend ($P = 0.10$; Table 31) to greater digestible N in these goats.

The FA profile of goat milk is affected by the FA and chemical composition of the diet (Vasta et al., 2008). Total saturated FA (SFA) in milk from goats fed BYP diet were significantly lower ($P = 0.035$) compared with control diet (Table 33), but there were differences in the response to diet among the individual SFA. Compared with milk

from control goats, concentrations of C14:0 were lower ($P = 0.008$) and those of C12:0 and C16:0 tended to be lower ($P = 0.08$ and 0.05 , respectively) in the milk from BYP-fed goats, whereas goats fed BYP had greater ($P = 0.002$) concentrations of stearic acid. These results indicate a potentially healthier profile of the milk from BYP-fed goats, as C12:0, C14:0 and C16:0 are atherogenic and have been related to an increase in total and low density lipoprotein cholesterol concentrations (Williams, 2000), whereas C18:0 is considered to have no effect on cholesterol values. There was no difference ($P \geq 0.63$) between diets in any branched-chain FA (C15:0 iso, C17:0 iso and C18:0 anteiso). In agreement with our results, Cais-Sokolińska et al. (2019) observed an increase in stearic acid concentration in the milk of dairy goats when DGGS were included in the diet at a rate of 120 g/kg, but no information on shorter chain FA was reported in their study. Ibáñez et al. (2016) reported no effect of including 600 g of DCP per kg of diet in the concentration of C4 to C15 FA in goat milk.

Olive cake is characterized by a high monounsaturated FA (MUFA) content, especially of oleic acid (Chiofalo et al., 2004), and its inclusion in the diet of dairy ewes has been shown to reduce SFA and increase MUFA content in milk (Vargas-Bello-Pérez et al., 2013; Chiofalo et al., 2004). In our study, milk from BYP-fed goats tended ($P = 0.08$) to have greater total MUFA content, but there were differences in the individual FA. Concentrations of 9-hexadecanoic acid (C16:1 trans-9) and elaidic acid (C18:1 trans-9) were greater ($P = 0.004$ and 0.036 , respectively) and those of oleic (C18:1 cis-9) and 11-octadecenoic acids (C18:1 cis-11) tended to be greater ($P \leq 0.086$) in the milk from goats fed BYP diet compared with control diet, with no differences in the other MUFA analyzed. The intake of trans FA is considered a risk factor for coronary heart disease and diabetes in consumers (Salmeron et al., 2001), but oleic acid is considered a beneficial FA for human health (Dewhurst et al., 2006). The content of vaccenic acid (C18:1 trans-11) was similar for both diets, and values were greater than those reported for goat milk in previous studies (0.95 g/100 g of total FA identified; Romero-Huelva et al., 2017; Ibáñez et al., 2016; Molina-Alcaide et al., 2010). As discussed by Ibáñez et al. (2016), greater vaccenic acid contents seem to be positively correlated with negative energy balances in goats and cows.

Table 33. Average values of fatty acids (FA) composition (g/100 g of identified FA) in milk fat from dairy goats fed the experimental diets.

Item	Diet ¹		SEM	P-value
	Control	BYP		
C4:0	2.44	2.65	0.042	0.07
C6:0	2.97	3.09	0.058	0.36
C8:0	3.53	3.60	0.080	0.70
C10:0	11.5	10.9	0.24	0.32
C12:0	4.76	3.98	0.167	0.08
C14:0	9.95	8.34	0.164	0.008
C15:0 <i>iso</i>	0.131	0.127	0.0059	0.78
C16:0	28.7	26.8	0.36	0.05
C17:0 <i>iso</i>	0.374	0.359	0.0143	0.64
C18:0	7.76	9.05	0.084	0.002
C18:0 <i>anteiso</i>	0.363	0.382	0.0185	0.63
C20:0	0.186	0.152	0.0260	0.56
Total saturated FA	74.7	71.5	0.51	0.035
C14:1 <i>cis</i> -9	0.123	0.117	0.0086	0.76
C16:1 <i>cis</i> -9	0.691	0.728	0.0098	0.13
C16:1 <i>trans</i> -9	0.092	0.165	0.0062	0.004
C18:1 <i>cis</i> -9 ²	18.3	20.4	0.46	0.09
C18:1 <i>trans</i> -9	0.322	0.369	0.0076	0.036
C18:1 <i>cis</i> -11	0.438	0.509	0.0158	0.09
C18:1 <i>trans</i> -11	1.333	1.311	0.1096	0.93
Total monounsaturated FA	21.3	23.6	0.48	0.08
C18:2 <i>cis</i> -9, <i>cis</i> -12	2.42	2.96	0.038	0.002
C18:2 <i>cis</i> -9, <i>trans</i> -11	0.540	0.938	0.0260	0.002
C18:3 <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12	0.046	0.039	0.0021	0.17
C18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	0.326	0.318	0.0070	0.62
C20:5 <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17	0.083	0.087	0.0055	0.76
C22:6 <i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19	0.011	0.013	0.0007	0.24
Total polyunsaturated FA	3.90	4.85	0.052	0.001
According to origin ³				
< 16 carbon	36.4	33.9	0.70	0.139
16 carbon	29.7	27.8	0.35	0.054
> 16 carbon	33.8	38.3	0.56	0.016
$\Sigma n-3/\Sigma n-6$	0.148	0.111	0.0029	0.003

¹ Control: 40:60 alfalfa hay:high-cereal concentrate; BYP: 40:60 alfalfa hay:concentrate containing by-products

² Contains C18:1 *trans*-12 and C18:1 *cis*-10 as minor components.

³ <16-carbon FA represent de novo synthesized FA, >16-carbon FA represent preformed FA taken up from circulation, and 16-carbon FA are derived from both sources.

Finally, total polyunsaturated FA (PUFA) content in milk from BYP-fed goats was 24.4% greater ($P = 0.001$) compared with control diet, and more specifically concentrations of linoleic acid (C18:2 cis-9, cis-12) and rumenic (CLA; C18:2 cis-9, trans-11) acids were 22.3 and 73.7%, respectively, greater in milk from goats fed BYP diet compared with that from goats fed control diet. No differences ($P \geq 0.167$) between diets were observed in the rest of PUFA identified. The PUFA are considered healthy for humans, and a wide list of health benefits have been attributed to those FA, but especially to CLA (Parodi, 2009; Banni et al., 2003), as its intake has been negatively related to the risk of colorectal cancer (Larsson et al., 2005), being its anti-mutagenic properties attributed mainly to rumenic acid (Lock et al., 2004). The increase in PUFA concentrations observed in milk from BYP-fed goats is in agreement with previous studies reporting increases in PUFA, and especially rumenic and linoleic acids, when DDGS (Cais-Sokolińska et al., 2019, 2015) and DCP (Ibáñez et al., 2016) replaced high-starch sources in the diet of dairy goats. Differences in milk FA profile can be due to differences in FA intake, but also to other factors as the intake of energy (Leiber et al., 2005) or plant secondary compounds that can influence the biohydrogenation of unsaturated FA in the rumen (Vasta et al., 2008). Altogether, these results indicate that BYP feeding changed milk fat quality.

No differences between diets were observed in milk content of $<16C$ FA, but $C16$ FA tended ($P = 0.05$) to be greater in milk from control-fed goats. The concentration of $>16C$ FA was 14.8% greater ($P = 0.016$) in the milk from goats fed BYP diet compared with control diet, which would indicate that a greater proportion of FA were taken up directly by the mammary gland, and therefore a greater amount of PUFA reaching the small intestine without being biohydrogenated in the rumen. Finally, feeding BYP decreased ($P = 0.003$) by 25% the $\Sigma n3$ -to- $\Sigma n6$ ratio in milk compared with control diet. In agreement with our results Cais-Sokolińska et al. (2019) observed a reduction in the $\Sigma n3$ -to- $\Sigma n6$ ratio (from 0.171 to 0.091) in the milk of dairy goats by including 120 g of DDGS per kg of diet, Increasing this ratio in the diet has been considered favorable for human health (Simopoulos, 2002), although some limitations when considering the ratio between these two families of PUFA have also been risen (Marventano et al., 2015).

IX.2.3.4. Ruminal fermentation, methane emissions, and purine derivatives excretion

There was no effect of diet ($P \geq 0.33$) on pH, total VFA and NH_3 concentrations and VFA profile (Table 34). Low VFA concentrations and greater pH values are often obtained when rumen content is collected through stomach tubing instead of a rumen cannula (Ramos-Morales et al., 2014). The values observed in our study were similar to those obtained in other studies in Murciano-Granadina goats receiving similar diets and using the same sampling procedure (Romero-Huelva et al., 2017; 2012). Protein of DDGS has been reported to have a lower degradability than other protein sources commonly used in ruminants feeding (Beenchar et al., 2013; NRC, 2001), but $\text{NH}_3\text{-N}$ concentrations in the rumen were adequate for microbial growth with both diets (Firkins et al., 2007).

As shown in Table 34, there were no differences ($P \geq 0.655$) between diets in methane emissions, indicating the lack of antimethanogenic compounds in the BYP diet. In agreement with our results, López et al. (2014) and Ibáñez et al. (2016) reported no effect of DCP on methane production by dairy goats. In contrast, Benchaar et al. (2013) observed a reduction in methane emissions when DDGS were fed to dairy cows, and Pecka-Kielb et al. (2017) observed the same effect in sheep. Benchaar et al. (2013) attributed this antimethanogenic effect to a decrease in the fibre degradation in the rumen caused by the high EE content of DDGS, but in our study the EE content of BYP concentrate (47.0 g/kg; Table 29) was below the fat level recommended in the diet of ruminants to avoid reductions in fiber degradability (NRC, 2001). The lack of differences between diets in both NDF digestibility (Table 30) and VFA profile (Table 34) supports the absence of any antimethanogenic compound in BYP diet. In contrast, several studies (Romero-Huelva et al., 2017, 2012) have reported a decrease in methane emissions when other by-products, such as tomato wastes, were included in the diet.

Table 34. Average values of ruminal fermentation parameters, methane emissions, daily urinary excretion of creatinine and purine derivatives and estimated microbial N flow in dairy goats fed the experimental diets.

Item	Diet ¹		SEM	P-value
	Control	BYP		
Ruminal fermentation parameters				
pH	6.94	6.93	0.063	0.90
Total VFA, mM	70.1	74.1	13.67	0.84
Molar proportions, mmol/100 mol				
Acetate	65.6	66.6	1.34	0.62
Propionate	11.2	12.9	1.21	0.35
Butyrate	14.6	12.5	1.45	0.33
Minor VFA ²	8.54	7.97	1.184	0.74
Acetate/Propionate	6.07	5.52	0.644	0.56
NH ₃ , mg/100 ml	12.6	11.0	1.47	0.45
Methane emissions				
CH ₄ , L/d	36.7	34.4	3.52	0.66
CH ₄ , L/kg of DMI	21.7	20.7	1.89	0.71
CH ₄ , L/kg of BW ^{0.75}	2.21	2.06	0.220	0.66
Urinary excretion, mmol/d	5.69	3.50	0.400	0.003
Allantoin	9.04	7.19	0.576	0.05
Hypoxanthine	0.298	0.367	0.0697	0.50
Xanthine	0.112	0.117	0.0124	0.80
Uric acid	2.65	2.23	0.175	0.11
Total purine derivatives ³	12.1	9.90	0.770	0.07

¹ Control: 40:60 alfalfa hay:high-cereal concentrate; BYP: 40:60 alfalfa hay:concentrate containing by-products

² Calculated as the sum of isobutyrate, isovalerate and valerate

³ Calculated as the sum of allantoin, hypoxanthine, xanthine and uric acid

The greater ($P = 0.003$) values of urinary excretion of creatinine observed in the control goats than in those BYP-fed are in agreement with the numerically greater body weight loss of these goats (Table 31), as creatinine is a metabolic product of creatine and phosphocreatine, both being found almost exclusively in muscle (Van Niekerk et al., 1963). Feeding BYP diet decreased the urinary excretion of allantoin ($P = 0.046$)

and tended to decrease ($P = 0.07$) the excretion of total purine derivatives, which would indicate a lower microbial protein synthesis in BYP-fed goats, as urinary excretion of purine derivatives is used as an index of ruminal microbial protein synthesis (Balcells et al., 1991). The low ruminal degradability of DDGS protein might have caused a deficit of RDP in BYP concentrate, but $\text{NH}_3\text{-N}$ and minor VFA concentrations measured in the rumen do not support this hypothesis, as both values were adequate for optimal microbial growth (Zhang et al., 2013; Firkins et al., 2007). For both diets, ratios of creatinine/allantoin and creatinine/total purine derivatives were in the range of values previously reported for goats (Romero-Huelva et al., 2017).

IX.2.4. Conclusions

A mixture of corn DDGS, DCP and EOC could replace 44% of cereal grains and protein feeds in the concentrate for dairy goats without compromising nutrient utilization or ruminal fermentation, and resulted in greater milk fat and protein production as well as a more unsaturated milk FA profile. The use of these by-products did not affect methane emissions, but can reduce the negative environmental impact associated with their disposal. Our results indicate that the CP content in the diets used in our study was in excess of goats requirements.

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IX.3. Python Script

```
import numpy as np
import sklearn
from sklearn.model_selection import train_test_split
lista_variables = []
lista_d = []
fichero = open("C:/Users/usuario/Desktop/REDES/hoc.csv")
for linea in fichero:
    lista_sub=[]
    campos = linea.split(';')
    lista_d.append(campos[12])
    lista_sub=(campos[0],campos[1],campos[2],campos[3],campos[4],campos[5],
campos[6], campos[7],campos[8],campos[9],campos[10],campos[11])
    lista_variables.append(lista_sub)

x = np.array(lista_variables).astype(np.float)
y = np.array(lista_d).astype(np.float)
x_train, x_test, y_train, y_test = sklearn.model_selection.train_test_split(x, y,
test_size = 0.33)
(train_data, train_targets), (test_data, test_targets)= (x_train, y_train), (x_test,
y_test)

mean = train_data.mean(axis=0)
train_data -= mean
std = train_data.std(axis=0)
train_data /= std
test_data -= mean
test_data /= std

from keras import models
from keras import layers
def build_model():
    model = models.Sequential()
    model.add(layers.Dense(4, activation='relu',
input_shape=(train_data.shape[1],)))
    model.add(layers.Dense(4, activation='relu'))
    model.add(layers.Dense(1))
    model.compile(optimizer='rmsprop', loss='mse', metrics=['mae'])
    return model

model = build_model()
model.fit(train_data, train_targets,
epochs=80, batch_size=16, verbose=0)
test_mse_score, test_mae_score = model.evaluate(test_data, test_targets)
test_mae_score
b = []
```

```
fichero = open("C:/Users/usuario/Desktop/REDES/comph.csv")
for linea in fichero:
    lista_sub=[]
    campos = linea.split(';')
    lista_sub=(campos[0],campos[1],campos[2],campos[3],campos[4],campos[5],
campos[6], campos[7],campos[8],campos[9],campos[10],campos[11])
    b.append(lista_sub)

x = np.array(b).astype(np.float)
y = model.predict(x)
print(y)
```