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The effects of feed blocks containing tomato and cucumber by-products on *in vitro* ruminal fermentation, microbiota, and methane production

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ABSTRACT. The aim of this work was to evaluate *in vitro* the effect of replacing 0%, 50%, 75% or 100% of cereal-based concentrate in diets based on lucerne hay with feed blocks containing barley grain or 650 g · kg^{-1} fresh matter of greenhouse waste fruits (tomato, cucumber, or a 1:1 mixture of tomato and cucumber) on ruminal fermentation, methane production, and bacterial and methanogen population sizes. The type of feed-block showed no effect ($p \geq 0.25$). The level of concentrate replacement with blocks did, however, affect ($p \leq 0.042$) the pH, CH_4 concentration, organic matter degradation rate, total gas, CH_4 and total VFA production, acetate/propionate and CH_4/total VFAs ratios, and molar proportions of acetate and butyrate, without changing ($p \leq 0.082$) methanogen and total bacteria abundance. Increasing levels of concentrate replacement with feed blocks modified ruminal fermentation, dry matter and neutral detergent fibre digestibility, and had an antimethanogenic effect.

Introduction

Cereal shortages and increasing prices (FAO, 2010) have stimulated interest in the search for low-cost alternative components of concentrates in ruminant feeding (Vasta et al., 2008). Methane emission from enteric fermentation is of concern worldwide for its contribution to the accumulation of greenhouse gases in the atmosphere and to the loss of energy for the animal (Hook et al., 2010). For these reasons, interest in additives (Mateos et al., 2013) and alternative feeds with potential anti-methanogenic effects for use in animal nutrition (Romero- Huelva et al., 2012) is also increasing.

In the Mediterranean area, greenhouse culture represents 15% of total world horticulture production, with Spain being the main producer (MARM, 2009). This agricultural sector generates abundant fruit wastes, mainly tomato and cucumber, which have to be stored, creating economic and environmental problems. Some by-products from the tomato processing industry, such as tomato pulp and pomace, have previously been evaluated as nutrient sources for ruminants (Ben Salem and Znaidi, 2008), but to our knowledge there is little information on the nutritive value of tomato fruits (Ventura et al., 2009), and data on the use of cucumber fruits in ruminant feeding are scanty. Furthermore, the presence of plant secondary compounds in these by-products could provide them with an added value due to their antimethanogenic effect, although their mechanisms of action are not yet completely understood

(Patra and Saxena, 2010). High moisture in wastes is an inconvenience in their preservation and inclusion in animal feeding, but this limitation may be overcome by using feed block technology.

Our hypothesis is that partial replacement of cereal-based concentrates in ruminal diets with feed blocks containing wastes from greenhouse horticulture could decrease feeding cost and environmental pollution without harmful effects on ruminal fermentation. The objective of the present work was to study the effect of substituting different proportions of a cereal-based concentrate with feed blocks containing 650 g · kg^{-1} fresh matter of fruits of tomato, cucumber, a mixture of both fruits, and barley grain on *in vitro* ruminal fermentation, methane production, and population sizes of bacteria and methanogens.

Material and methods

Diets

A control diet (CO) composed of lucerne hay and a commercial concentrate (35% wheat shorts, 16% barley grain, 12% sunflower meal, 10% maize shorts, 9% soyabean hulls, 9% soyabean meal, 5% maize grain, 2.20% quicklime, 0.45% fatty acid salts, and 0.35% NaCl, UI · kg^{-1} vitamin A 8000 and vitamin D_3 1600) in a 1:1 ratio was used.

The by-products used in this study were tomato and cucumber fruits from greenhouse horticulture. They were collected at the Waste Treatment Factory in Motril (Granada, Spain), homogenized using a commercial blender (model TR330; Danamix, Fordingbridge, UK) and kept at −20°C before use for feed block preparation. Formulated feed-blocks (Table 1) were hand-made following the procedure described in previous works of our group (Molina-Alcaide et al., 2009). Solid ingredients (wheat straw, barley grain and sunflower meal) were mixed with a liquid mixture composed of water, quicklime, salt, urea and fruits of tomato, cucumber, or a mixture (1:1) of both and then heavily packed in an aluminium mold. Compacted blocks were taken out from the mold and air-dried within three to eight days.

The experimental diets were formulated by replacing 50%, 75%, or 100% of the concentrate with feed blocks containing fruits of tomato (diets T50, T75 and T100, respectively), cucumber (diets C50, C75 and C100, respectively), a mixture (1:1) of tomato and cucumber (diets TC50, TC75 and TC100, respectively), or barley grain (diets B50, B75 and B100, respectively). Diet ingredients (lucerne hay, concentrate and feed-blocks) were ground (1 mm) before the incubation runs.

Table 1. Ingredients composition of feed blocks, g · kg^{-1} dry matter

Ingredients	Feed blocks[1]			
	T-FB	C-FB	TC-FB	B-FB
Barley grain	–	–	–	83
Tomato	129	–	50	–
Cucumber	–	69	42	–
Wheat straw	598	639	624	631
Quicklime	81	88	87	85
NaCl	49	52	51	51
Sunflower meal	97	104	101	102
Urea	35	36	34	37
Vitamin-mineral mixture[2]	11	12	11	11

[1] B-FB, T-FB, C-FB and TC-FB: barley grain, tomato, cucumber and tomato plus cucumber feed blocks, respectively; [2] contained per kg: g: NaCl 277; ash from the 2-stage dried olive cake 270; $CaH_4(PO_4)_2$ 250; $MgSO_4$ 200; mg: CuO 184; I 25; CoO 8.5; Se 4; ZnO 2.28; IU: vit. A 83,500; vit. D 16,700

Animals

Three adult dry non-pregnant rumen-fistulated Granadina goats (46.9 ± 2.15 kg body weight) were used as inoculum donors for the *in vitro* incubations. The internal diameter of the cannula was 4.5 cm, which allows the collection of rumen content samples from both liquid and solid fractions. Animals were placed in individual boxes and had free access to water. The goats were fed lucerne hay supplied to meet energy maintenance requirements. Animal management and rumen content sampling were carried out by qualified personnel in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 1201/2005 of October 10 on the protection of animals used for experimentation or other scientific purposes) in line with the European Convention for the Protection of Vertebrates used for Experimental and other Scientific Purposes (European Directive 86/609).

Experimental procedure and sampling

The batch culture method was used to study ruminal fermentation, total gas and methane production, as well as the abundance of total bacteria and methanogens promoted by the control and experimental diets (Table 2). Three identical 72 h incubation runs were carried out in three consecutive weeks. Rumen content from each goat was obtained before the morning feeding by using a probe, immediately transported to the laboratory in thermal bottles, mixed and strained through four layers of cheesecloth into a warmed Erlenmeyer flask with an O_2-free headspace. Rumen fluid was always collected from all of the goats for each incubation run.

Table 2. Chemical composition of experimental diets, g · kg^{-1} dry matter

Indices	Diets[1]										
	CO	T50	T75	T100	C50	C75	C100	TC50	TC75	TC100	B50	B75	B100
DM, g · kg^{-1} fresh matter	907	915	920	924	919	925	931	917	923	928	919	925	931
OM	898	872	859	847	869	855	841	870	857	843	872	860	847
Ether extract	20.6	15.1	12.3	9.6	14.8	11.9	9.0	14.9	12.0	9.1	14.8	11.8	8.9
NDF	462	488	502	515	483	494	505	488	501	514	492	507	522
ADF	259	294	311	328	291	308	324	294	311	328	296	315	333
ADL	56.7	62.3	65.1	67.9	62.3	65.1	67.9	62.6	65.6	68.6	63.3	66.6	70.0
Crude protein	151	154	155	157	155	157	159	153	154	155	155	156	158
NSC[2]	264	215	190	165	216	192	168	215	190	165	211	185	159
GE, MJ · kg^{-1} of DM	17.6	17.0	16.7	16.4	16.9	16.5	16.2	17.0	16.6	16.3	16.9	16.5	16.1
Condensed tannins													
free	0.71	0.72	0.72	0.72	0.73	0.75	0.76	0.73	0.73	0.74	0.80	0.84	0.89
fibre-bound	7.28	10.5	12.0	13.6	10.0	11.4	12.7	10.2	11.7	13.2	9.61	10.8	11.9
protein-bound	1.04	1.12	1.16	1.19	1.13	1.17	1.21	1.12	1.16	1.20	1.10	1.13	1.16
total[3]	9.03	12.3	13.9	15.5	11.9	13.3	14.7	12.1	13.6	15.14	11.51	12.77	13.95

[1]CO: control diet; T50, T75, T100: diets containing tomato feed blocks; C50, C75, C100: diets containing cucumber feed blocks; TC50, TC75, TC100: diets containing tomato and cucumber feed blocks; B50, B75, B100: diets containing barley grain feed blocks; 50, 75, 100 suffix: increasing levels of greenhouses wastes feed blocks inclusion in experimental diets; [2] non-structural carbohydrates calculated as: NSC: non-structural carbohydrate calculated as OM – (EE + CP + NDF); [3] calculated as free + fibre-bound + protein-bound tannins

The buffer solution of Goering and Van Soest (1970) was previously prepared in an Erlenmeyer flask under a CO_2 stream and kept one hour with an O_2-free headspace after the resazurin colour turnover showed an O_2-free solution. The buffer solution of Goering and Van Soest (1970) was composed of 475 ml distilled water, 237.5 ml macromineral solution (5.7 g Na_2HPO_4 anhydrous, 6.2 g KH_2PO_4 anhydrous, 6.0 g $MgSO_47H_2O$, and 1.0 l distilled water), 1.25 ml micromineral solution (13.2 g $CaCl_2$, $2H_2O$, 10 g $MnCl_2$ $4H_2O$, 1.0 g $CoCl_26H_2O$, 8.0 g $FeCl_36H_2O$ and brought to a volume of 100 ml with distilled water), 237.5 ml buffer (4.0 g NH_4HCO_3, 35 g $NaHCO_3$, and 1.0 l distilled water), 47.5 ml reducing solution (625 mg cysteine solution, 4.0 ml 1N NaOH, 95 ml distilled water, and 625 mg Na_2S9H_2O), and 1.25 resazurin per litre. Strained rumen fluid was mixed with the buffer solution in a 1:4 (v/v) proportion at 39ºC under continuous flushing with CO_2. Buffered ruminal fluid (50 ml) was added to each bottle under CO_2 flushing. Bottles were sealed with butyl rubber stoppers and aluminium caps and placed in a water bath at 39ºC.

Serum bottles of 120 ml (Laboratorios Ovejero S.A., León, Spain) whose weight was previously recorded were used. In each incubation run, triplicate samples (0.5 g dry matter, DM) of control and experimental diets, along with lucerne hay used as a standard, were placed into the bottle and incubated. Blanks were also used, consisting of buffer and strained rumen fluid in a proportion of 1:4 (v/v). Pressure in the bottle headspace and volume of gas produced were measured at 2, 4, 6, 8, 12, 24, 48 and 72 h after inoculation using a Wide Range Pressure Meter (Spec Scientific LTD., Scottsdale, AZ, USA) and a calibrated glass syringe, respectively. After 24 and 72 h of incubation, an aliquot of the gas produced was taken using a 10 ml vacuum tube (Venoject®, Terumo Europe N.V., Leuven, Belgium) for determination of CH_4 concentration. After 24 h, half of the bottles were swirled in ice to stop fermentation and then uncapped to measure pH in the incubation medium with a pH-meter (Metrohm AG, Herisau, Switzerland). The weight of the bottle content was recorded and an aliquot (1 ml) was added to 1 ml of deproteinizing solution (20 g of metaphosphoric acid and 4 g of crotonic acid, as internal standards, per litre of 0.5 M-HCl) for volatile fatty acid (VFA) analysis. The remaining content of bottles was lyophilized for DNA extraction and DM and NDF content analyses. The rest of the bottles were maintained for 72 h after inoculation and fermentation was stopped following a similar procedure to the one previously described for bottles incubated during 24 h.

Assessment of microbial population size

The abundance of total bacteria and methanogenic archaea in samples of bottle contents was determined by quantitative real time PCR (qPCR). Extraction and purification of DNA were performed from approximately 50 mg freeze-dried samples using the QIAamp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer's instructions with a modification: a higher temperature (95ºC) was used for lysis incubation. Extracted DNA yield and purity were assessed by using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA samples were used as templates to quantify the copy numbers of 16SrRNA (for bacteria) and of methyl coenzyme M reductase A (mcrA) gene for methanogens. For each microbial group, three different aliquots of DNA were analysed using qPCR with the following primer pairs: forward: 5′-GTGSTGCAYGGYTGTCGTCA-3′ and reverse: 5′-ACGTCRTCCMCACCTTCCTC-3′ for total bacteria (Maeda et al., 2003), and forward: 5′-TTCGGTGGATCDCARAGRGC-3′ and reverse: 5′-GBARGTCGWAWCCGTAGAATCC-3′for methanogens (Denman et al., 2007). A negative control (sterile distilled water) was also loaded on each plate run to screen for possible contamination and dimer formation and to set the background fluorescence for plate normalization. The qPCR was performed using an iQ5multicolor Real-Time PCR Detection System (BioRad, Laboratories Inc., Hercules, CA, USA). One µl of DNA extract was added to amplification reactions (25 µl) containing 0.2 µl of each primer (10 µM) and 12.5 µl of iQSYBR Green Supermix (Biorad Laboratories Inc.). Cycling conditions were 95ºC for 5 min, 40 cycles at 95ºC for 15 s, 60ºC for 30 s and 72ºC for 55 s, and a final extension at 72ºC for 1 min. The threshold cycle (i.e. the amplification cycle in which product formation exceeds background fluorescence) of each sample was determined during the exponential phase of amplification. All post run data analyses were performed using Bio Rad CFX Manager Software (version 1.6.541.1028).

The external standards used for the qPCR amplifications had been validated previously for rumen bacteria (Maeda et al., 2003) and methanogenic archaea (Denman et al., 2007).

Chemical analysis

Ground (1 mm) samples of ingredients and diets were analysed for dry matter (DM), organic matter (OM) and ether extract (EE) according to AOAC (2005) methods. After N determination using a LecoTruSpecCN® (St. Joseph, MI, USA), crude protein (CP) was calculated by multiplying by 6.25. NDF and ADF were analysed according to Van Soest et al. (1991) using an ANKOM Model 220 Fiber Analyzer (Macedon, NY, USA) with α-amylase for NDF analysis in concentrate samples and both NDF and ADF contents referred to ash-free weight. The ADL was determined by solubilization of cellulose with 72% sulphuric acid. The energy content was determined using an oxygen bomb calorimeter (PARR 1356, Biometer®). Free, protein- and fibre-bound condensed tannins were sequentially extracted and quantified following the procedure described by Perez-Maldonado and Norton (1996). Condensed tannins from quebracho powder (Roy Wilson Dickson Ltd., Mold, UK) were used as a standard.

The CH_4 concentration was determined by gas chromatography (GC) using an HP 5890 Hewlett Packard Series II gas chromatograph (Waldbronn, Germany) equipped with a flame ionization detector (FID) and an HP-INNOWAX cross linked polyethylene glycol column (25 m × 0.2 mm × 0.2 µm). The carrier gas was He and peaks were identified by comparison with a standard of known composition. A sample of 0.5 ml of gas was injected using a 1 ml Sample-Lock® syringe (Hamilton, Nevada, USA). Total and individual VFA were analysed by gas chromatography following the methodology described by Isac et al. (1994).

Calculations and statistical analysis

The gas produced in batch cultures was adjusted to the expression:

$$y = A\,[1 - e - c \cdot t]$$

where: y – the cumulative gas production (ml), A – the asymptote (total gas; ml), c – the organic matter degradationrate, t – the incubation time (h).

The amount of VFA in the batch culture bottle contents after 24 h of incubation both for control and experimental diets was corrected for the amount of VFA in the rumen liquid used as inoculum. The DM truly digested for control and experimental diets was calculated according to Van Soest et al. (1966) as:

$$\text{True DM digestibility} = (\text{DM input} - \text{NDF output})/\text{DM input}$$

NDF output being that analysed in the residue after 24 h of incubation.

The total amount of CH_4 produced was calculated from total gas produced and its CH_4 concentration.

Data were analysed by an unvaried model using the GLM procedure of SPSS (IBM SPSS Statistics v.19, IBM Corp., Somers, NY). The linear model.

Table 3. Effect of the type of feed block and the level of concentrate replacement on the ruminal fermentation parameters and microbial abundances in batch cultures after 24 h of incubation, log gene copies · g^{-1} fresh matter

Indices	Diet[1]													SEM	*P*-value		
	CO	T50	T75	T100	C50	C75	C100	TC50	TC75	TC100	B50	B75	B100		Block type (BT)	Substitution level (SL)	BT × SL
pH	6.61^{a}	6.66^{b}	6.67^{c}	6.71^{d}	6.66^{b}	6.68^{c}	6.72^{d}	6.66^{b}	6.69^{c}	6.71^{d}	6.65^{b}	6.68^{c}	6.70^{d}	0.002	0.74	<0.001	0.75
Gas, ml · g^{-1} incubated DM	214^{d}	189^{c}	179^{b}	161^{a}	186^{c}	174^{b}	160^{a}	187^{c}	175^{b}	165^{a}	191^{c}	176^{b}	164^{a}	0.53	0.27	<0.001	0.81
CH_4, ml· ml^{-1} total gas prod	0.20^{b}	0.20^{b}	0.20^{ab}	0.18^{a}	0.20^{b}	0.19^{ab}	0.19^{a}	0.20^{b}	0.19^{ab}	0.19^{a}	0.20^{b}	0.19^{ab}	0.19^{a}	0.001	0.99	<0.001	0.93
CH_4, ml · g^{-1} incubated DM	42.2^{d}	37.5^{c}	35.0^{b}	29.4^{a}	37.4^{c}	32.7^{b}	29.8^{a}	37.0^{c}	33.2^{b}	31.1^{a}	38.0^{c}	33.1^{b}	30.6^{a}	0.25	0.89	<0.001	0.85
CH_4/VFA, ml · $mmol^{-1}$	7.83^{c}	7.51^{bc}	7.04^{ab}	6.27^{a}	7.59^{bc}	6.62^{ab}	6.21^{a}	7.54^{bc}	6.45^{ab}	6.49^{a}	7.78^{bc}	6.86^{ab}	6.65^{a}	0.12	0.91	<0.001	0.99
A, ml	120^{c}	109^{b}	105^{a}	98^{a}	108^{b}	104^{a}	99^{a}	109^{b}	104^{a}	100^{a}	112^{b}	106^{a}	103^{a}	0.63	0.51	<0.001	0.99
c, h^{-1}	0.092^{c}	0.086^{b}	0.081^{ab}	0.073^{a}	0.082^{b}	0.078^{ab}	0.070^{a}	0.082^{b}	0.078^{ab}	0.072^{a}	0.081^{b}	0.074^{ab}	0.067^{a}	0.001	0.58	<0.001	0.99
Total VFA, mmol	2.71^{b}	2.53^{ab}	2.52^{ab}	2.36^{a}	2.49^{ab}	2.52^{ab}	2.42^{a}	2.47^{ab}	2.61^{ab}	2.40^{a}	2.47^{ab}	2.43^{ab}	2.31^{a}	0.041	0.93	0.042	0.99
VFA, mmol ·100 mol^{-1}																	
acetate	69.7^{a}	70.0^{a}	71.0^{b}	71.7^{b}	70.5^{a}	72.0^{b}	72.3^{b}	70.3^{a}	72.0^{b}	71.3^{b}	69.8^{a}	72.2^{b}	72.1^{b}	0.17	0.74	<0.001	0.97
propionate	16.6	16.4	16.5	16.6	16.2	16.4	16.5	16.4	16.5	16.6	16.5	16.3	16.3	0.057	0.80	0.33	0.98
iso-butyrate	0.81	0.88	0.81	0.74	0.95	0.76	0.77	0.52	0.75	1.01	0.82	0.76	0.89	0.024	0.83	0.65	0.14
butyrate	10.7^{c}	9.95^{bc}	9.42^{ab}	8.79^{a}	9.72^{bc}	9.06^{ab}	8.27^{a}	10.0^{bc}	9.02^{ab}	8.59^{a}	10.3^{bc}	8.91^{ab}	8.70^{a}	0.16	0.93	<0.001	0.99
iso-valerate	1.40	1.52	1.28	1.28	1.56	1.10	1.34	1.40	1.00	1.42	1.34	1.12	1.16	0.063	0.92	0.31	0.99
valerate	0.74	1.19	0.98	0.95	1.07	0.68	0.77	1.10	0.65	1.07	1.24	0.74	0.92	0.086	0.94	0.27	0.99
Acetate/propionate	4.19^{a}	4.26^{ab}	4.29^{b}	4.32^{b}	4.35^{ab}	4.39^{b}	4.40^{b}	4.30^{ab}	4.35^{b}	4.31^{b}	4.23^{ab}	4.44^{b}	4.44^{b}	0.022	0.69	0.023	0.97
Apparent DM digestibility, g · g^{-1}	0.45^{c}	0.37^{b}	0.33^{a}	0.32^{a}	0.38^{b}	0.29^{a}	0.28^{a}	0.41^{b}	0.31^{a}	0.28^{a}	0.38^{b}	0.35^{a}	0.28^{a}	0.006	0.67	<0.001	0.72
True DM digestibility [2], g · g^{-1}	0.75^{d}	0.71^{c}	0.68^{b}	0.66^{a}	0.72^{c}	0.71^{b}	0.65^{a}	0.70^{c}	0.70^{b}	0.66^{a}	0.73^{c}	0.70^{b}	0.64^{a}	0.002	0.25	<0.001	0.83
NDF digestibility, g · g^{-1}	0.45^{d}	0.40^{c}	0.36^{b}	0.34^{a}	0.42^{c}	0.41^{b}	0.31^{a}	0.38^{c}	0.39^{b}	0.34^{a}	0.46^{c}	0.40^{b}	0.30^{a}	0.003	0.29	<0.001	0.87
Total bacteria	9.45	9.55	9.60	9.52	9.49	9.48	9.53	9.61	9.52	9.60	9.58	10.0	9.41	0.027	0.43	0.08	0.13
Methanogens	8.94	8.95	9.05	8.43	9.00	8.84	8.39	8.96	8.15	8.42	9.07	8.34	8.17	0.13	0.89	0.27	0.99

[1] CO – control diet; T50, T75, T100: diets containing tomato feed blocks; C50, C75, C100: diets containing cucumber feed blocks; TC50, TC75, TC100: diets containing tomato and cucumber feed blocks; B50, B75, B100: diets containing barley grain feed blocks; 50, 75, 100 suffix: increasing levels of green-houses wastes feed blocks inclusion in experimental diets; [2] calculated as proposed by van Soest et al. (1966): true digested DM = (DM input – NDF output)/DM input, NDF output being that analyzed in the residue after 24 h incubation

used for each dependent variable accounted for the effects of feed block type (BT), substitution level of concentrate, with feed blocks in the corresponding diet (SL), and BT × SL interaction. Differences between individual experimental units (bottles) and between incubation runs were considered random effects. Effects were considered significant at $p \leq 0.05$ and a trend when the *P*-value was between 0.05 and 0.1. Differences among means were tested using the Tukey comparison test.

Results

The type of block did not affect (Table 3) pH values ($p = 0.74$), gas or methane production ($p \geq 0.27$), VFA production ($p = 0.93$) or molar proportions ($p \geq 0.69$), digestibility ($p \geq 0.69$), or abundance of total bacteria ($p = 0.43$) and methanogens ($p = 0.89$). In contrast, the level of concentrate replacement with feed blocks promoted differences in most of the studied parameters. The pH values were higher ($P < 0.001$) for diets containing blocks in comparison with the control one. The gas produced ($ml \cdot g^{-1}$ incubated DM) decreased ($P < 0.001$) with the level of any type of feed block in the diet. The methane concentration decreased ($P \geq 0.001$) with the highest levels of feed blocks in the diet. The effect of increasing the percentage of feed block in the diet was observed ($P < 0.001$) on methane production independently of both the type of block and value expression. The response of the potential gas production (*A*) and the organic matter degradation rate (*c*) to the dietary treatment was the same ($P < 0.001$). Total VFA production decreased ($p = 0.042$) with concentrate replacement, especially for the highest level of block included in the diet. The acetate/propionate ratio generally increased ($p \leq 0.023$) with feed block inclusion in diet. The molar proportions of propionate, isobutyrate, isovalerate and valerate were not affected ($p \geq 0.27$) by increasing the levels of blocks in the diet. In contrast, the molar proportions of acetate and butyrate increased ($P < 0.001$) and decreased ($P < 0.001$), respectively, in relation to the level of replacement with feed blocks. Apparent and true DM digestibility and NDF digestibility decreased ($P < 0.001$) with increasing levels of feed blocks in the diet. The abundance of total bacteria and methanogens was not affected ($p \geq 0.08$) by changes in the level of feed blocks in the diet. No block type × replacement level interaction ($p \geq 0.13$) was found for any of the analysed or calculated parameters.

Discussion

There is no information in the literature concerning gas production from diets containing tomato or cucumber fruits. The volume of gas produced after 24 h of incubation ranged from 163 for diets containing 100% of feed block (FB) to 214 $ml \cdot g^{-1}$ DM for diets without FB. These values are similar to those found for grass silage, oat-whole crop silage and maize stover leaves (200, 214 and 201 $ml \cdot g^{-1}$ DM, respectively) after 24 h of incubation (Blümmel et al., 1999; Gierus et al., 2008). The volume of gas produced from the fermented diet components is closely related to the digestibility and energy value for ruminants. In the present work, lower gas production and slower *c* values were observed as concentrate decreased in the experimental diets, resulting in decreased DM and NDF digestibility. The *c* value decrease was more pronounced for FB containing barley than for those containing by-products. For diets containing FB, the values were higher (0.067 to 0.086) than those reported for shrubs (Gasmi-Boubaker et al., 2006), but differences among results from *in vitro* studies could be due to the amount and characteristics of the substrate incubated, as well as the animal species used as donors of inoculum. The antimethanogenic effect observed for diets containing FB, which increased with the FB levels in the diet, was in agreement with Johnson and Johnson (1995) who observed *in vivo* a reduction in methane production with increased substitution of concentrate in diet. The results obtained in the present work are in line with those obtained when barley grain was replaced with citrus pulp, a by-product with low starch content (Ben-Ghedalia et al., 1989).The increased antimethanogenic effect observed as the amount of tomato and cucumber by-products increased in the diet could be due, to some extent, to tannins present in the studied by-products. Secondary plant compounds have been shown to modify rumen fermentation, inhibiting enteric methanogenesis, although their mechanisms of action still are not well understood (Patra and Saxena, 2010). Condensed tannins are thought to directly inhibit methanogens, as well as indirectly limit methanogenesis through a reduction in hydrogen availability, which could be the case in the present work. The antimethanogenic effect of diets containing FB, similar in composition to those used in the present study, has also been observed *in vivo* in both lactating (Romero-Huelva et al., 2012) and nonproductive (Romero-Huelva and Molina-Alcaide, 2013) goats.

The VFA produced by diet fermentation was not reduced by the substitution of 50% or 75% of the concentrate with FB. The higher content of structural carbohydrates in diets containing FB may explain the higher proportion of acetate for those diets in comparison with the control. The decreased molar proportion of butyrate observed when ≥75% of concentrate was replaced with FB may point to a lower content of non-structural carbohydrates in those FB-containing diets. The increased acetate and reduced butyrate molar proportions as results of the substitution of concentrate with FB-containing diets, together with the lack of effect on the molar proportion of propionate, support the idea that FB directly inhibit methanogenesis. A reduction in methane production per unit of released VFA in the rumen without increased propionate molar proportion, as observed in the present work, could be possible when metabolic pathways like homoacetogenesis act as hydrogen sinks alternative to propionate. There is scarce information on the effect of replacing concentrate with FB-containing by-products on ruminal VFA. The replacement of 50% of concentrate in lucerne hay-based diets with FB containing olive cake decreased the propionate molar proportion in continuous-culture fermenters, but no effect on total or individual VFA was observed in non-productive goats (Molina-Alcaide et al., 2009). Also in non-productive goats (Romero-Huelva and Molina-Alcaide, 2013), the replacement of 50% of the concentrate with FB containing tomato and cucumber by-products increased the ruminal concentration of total VFA and molar proportion of propionate and reduced that of butyrate. In lactating goats, however, the replacement of 25% of concentrate with similar FB decreased total VFA, acetate and isoacid concentrations in the rumen (Romero-Huelva et al., 2012).

Digestibility of DM and NDF decreased when concentrate was replaced with FB, which may be related to the antimethanogenic effect of FB-containing diets. According to our knowledge, little or no information on the nutritive value of the studied by-products exists. Ventura et al. (2009) reported a low nutritive value of tomato, which may explain, to some extent, the observed reduction in gas and VFA production and nutrient digestibility for diets containing this by-product in comparison with the control. Our results agree with those obtained in lambs fed diets including tomato and olive cake-based FB (Ben Salem and Znaidi, 2008) but differ from those obtained in lactating goats with FB containing tomato and cucumber by-products (Romero-Huelva et al., 2012). In non-productive goats DM digestibility decreased with FB containing tomato and cucumber by-products, but NDF digestibility was not affected (Romero-Huelva and Molina-Alcaide, 2013). Results from different experiments differ depending on factors such as FB composition, level of concentrate replacement and animal species used as experimental animals. A reduction in the abundance or activity of fungi with FB-diets could also contribute to the decreased digestibility of fibre, as fungi are relevant in fibre degradation and lignin solubilization (Mountfort, 1987).

Although microbiota in *in vitro* systems is a key point to support their ability for simulating rumen fermentation, available information on this topic is very scarce. Soto et al. (2012) stated that manipulation of rumen content to prepare inoculum for *in vitro* systems reduced the abundance of different microbial groups due to filtration and exposure to oxygen, which remove solid-associated and strict anaerobic microorganisms. Batch cultures are able to maintain bacterial and protozoal densities during 24 h of incubation (Soto et al., 2013), however, while fibrolytic bacteria and fungi densities increased after 24 h of incubation, maybe to compensate the discarding of most of the solid-attached microorganisms taking place during inoculum preparation (Soto et al., 2012). Our results concerning decreased NDF digestibility with increasing levels of FB in the diet could indicate decreased abundance or activity of fibrolytic microorganisms. This is not consistent, however, with the increase in the molar proportion of acetate observed with the higher levels of FB in diet, which would indicate increased activity of acetogenic microorganisms. Shifts in the diversity of microbial groups and not in their abundance could be the underlying cause of the observed differences associated with diet composition affecting the proportions of VFA, acetate and butyrate, digestibility and methane production, with the less abundant microorganisms being the most affected by changes in the diet. The pH could be responsible for shifts in ruminal microbial diversity, but in the present work pH values were within physiological limits for all of the studied diets. In agreement with our results, many studies have indicated that methane production in the rumen does not depend on the abundance of methanogens, but on their diversity (Morgavi et al., 2010). The mechanism of action of FB-containing diets, which reduce methane production, does not seem to involve propionate as a sink for H_2, but a different metabolic pathway such as homoacetogenesis, as previously mentioned. The activity of specific methanogens may have been

inhibited with FB-containing diets as well. In general, it is difficult to extrapolate results from *in vitro* to *in vivo* conditions, but observations in the present work are in line with those observed in dairy goats (Romero-Huelva et al., 2012). In contrast, our results do not agree with observations on non-productive goats (Romero-Huelva and Molina-Alcaide, 2013), although both lactating and non-productive goats were fed diets similar to those studied in the present work. Our results are not in line with those obtained in experiments performed *in vivo* by Johnson and Johnson (1995), in which the type of carbohydrate influenced pH and subsequently altered the ruminal microbiota.

Conclusions

The use of feed blocks containing tomato and cucumber by-products as concentrate replacers in ruminant feeding is an interesting low-cost strategy for its potential to reduce methane emissions that can be mediated by direct inhibition of some methanogen activity or a metabolic pathway different from the one using propionate as an acceptor of H_2 alternative to methane. Additionally, volatile fatty acids production is unaffected unless the replacement level of concentrate per block exceeds 75%. The detrimental effect of block-containing diets on the digestibility of dry matter and fibre may limit interest in this dietary strategy, however.

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