Influence of increasing doses of a yeast hydrolyzate obtained from sugarcane processing on *in vitro* rumen fermentation of two different diets and bacterial diversity in batch cultures and Rusitec fermenters

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ABSTRACT

Live yeast cultures and yeast hydrolyzates can be used as rumen fermentation modifiers, but their effects and mode of action are different. Whereas the effects of live yeast cultures on rumen fermentation are well documented, yeast hydrolyzates have received much less attention. The influence of a yeast hydrolyzate from *Saccharomyces cerevisiae*, obtained after ethanol production from sugarcane (YHS), on *in vitro* rumen fermentation was investigated using both batch cultures and Rusitec fermenters inoculated with ruminal fluid from sheep. Batch cultures (300 mg dry matter (DM)) with two mixed diets (AHC, 0.5:0.5 alfalfa hay:concentrate; BSC, 0.15:0.85 barley straw:concentrate) as substrate were supplemented with increasing doses of YHS (0, 3.3, 6.7, 10.0 and 13.3 ml/l) and incubated for 16.5 h at 39 °C. Supplementation of increasing amounts of YHS to AHC-cultures increased ($P < 0.05$) linearly total volatile fatty acid (VFA) production and butyrate molar proportion, and decreased ($P < 0.001$) acetate proportion and acetate:propionate ratio. In contrast, only subtle effects of YHS on $\text{NH}_3\text{-N}$ concentrations and molar proportions of isovalerate and caproate were observed for the BSC diet. Longer-term effects of YHS supplementation on rumen fermentation of AHC diet were investigated using four Rusitec fermenters in a cross-over experimental design with two 14-day incubation periods. Fermenters were given daily 30 g of diet DM, and in each period half of them were supplemented daily with 5 ml of YHS (10.0 ml/l). Supplementing with YHS did not affect ($P > 0.05$) total VFA production, lactate concentrations, DM and aNDFom disappearance or enzymatic activities (amylase, xylanase and carboxymethylcellulase). Compared with the unsupplemented fermenters, YHS treatment increased ($P < 0.001$) $\text{NH}_3\text{-N}$ concentrations and molar proportions of propionate and butyrate at the expense of acetate, and decreased ($P < 0.001$) acetate:propionate ratio. In addition, YHS supplementation tended ($P < 0.07$) to reduce $\text{CH}_4/\text{total VFA}$ ratio and to increase microbial growth in the liquid phase of the fermenters. The automated ribosomal intergenic spacer analysis (ARISA) of samples taken on days 3, 8 and 14 of incubation from solid and liquid content of fermenters revealed that YHS supplementation increased ($P < 0.02$) bacterial

Abbreviations: ADFom, acid detergent fiber expressed exclusive of residual ash; AFOM, apparently fermented OM; aNDFom, neutral detergent fiber with heat-stable amylase and expressed exclusive of residual ash; ARISA, automated ribosomal intergenic spacer analysis; CP, crude protein; BSC, 15:85 barley straw:concentrate; AHC, 50:50 alfalfa hay:concentrate; OM, organic matter; VFA, volatile fatty acids; YH, yeast hydrolyzate; YHS, yeast hydrolyzate obtained from sugarcane processing

diversity in the liquid phase and tended to increase ($P < 0.08$) it in the solid phase. The results indicate that YHS at a dose of 10 ml/l may be a useful dietary additive for ruminants, because it promoted a shift in fermentation toward propionate production, reduced the CH_4 /total VFA ratio and increased microbial growth with a 50:50 alfalfa hay:concentrate diet.

1. Introduction

The identification of substances that modify rumen fermentation to increase its efficiency and decrease the polluting emissions to the environment is a key objective of ruminant nutrition research. Live yeast cultures from *Saccharomyces cerevisiae* have been shown to modify ruminal fermentation parameters and to have beneficial effects on ruminant performance, but such effects can be influenced by both the yeast culture and the diet fed to animals, among other factors (Carro et al., 1992; Chaucheyras-Durand et al., 2012). Most of the studies on yeast as feed additives have been conducted with live yeast cultures, and the potential of yeast hydrolyzates (YH) as ruminal fermentation modifiers has received much less attention, despite that the effects and mode of action of both additives are completely different. Rossi et al. (2004) reported that the peptidic fractions of *S. cerevisiae* stimulated the growth of *Megasphaera elsdenii*, the major lactate utilizer in the rumen, and Meissner et al. (2014) observed that a *S. cerevisiae* YH increased the growth of *Megasphaera elsdenii* and enhanced the *in vitro* ruminal fermentation of a high-fermentable substrate. More recently, Kettunen et al. (2016) and Oeztuerk et al. (2016) have reported the efficacy of a patented *S. cerevisiae* YH product to stimulate the *in vitro* fermentation of different diets in batch cultures and Rusitec fermenters, respectively.

S. cerevisiae is also the most commonly used microorganism to ferment sugarcane juice and molasses for bioethanol production (Laluce et al., 2016), and the residue generated in this process (yeast cream) has been reported to contain about 60–70% of yeast cells (Pérez et al., 2000; Laluce et al., 2016). The yeast cream is produced in large amounts and has a high pollution potential, but it also contains nutrients and could be used in animal nutrition. The yeast cream can be enzymatically hydrolysed, and the obtained hydrolyzate (YHS) has shown prebiotic effects in broilers (Pérez et al., 2005), but its potential as feed additive in ruminants has not yet been fully analysed. To our best knowledge only Díaz et al. (2009) have tested the effects of YHS and its fractions obtained by centrifugation (supernatant, gross pellet and washed pellet) on *in vitro* fermentation of *Pennisetum purpureum* cv. CT-115, and observed that all of them stimulated the *in vitro* rumen fermentation. However, the influence of this YHS on ruminal fermentation of mixed diets, representative of those used in practical feeding of ruminants, has not yet been tested. Our hypothesis was that the YHS could be effective in modifying rumen fermentation by stimulating the growth of ruminal microbes. The first objective of this study was to test the short-term effects of increasing doses of YHS on *in vitro* ruminal fermentation of two different mixed diets in batch cultures inoculated with ruminal fluid from sheep fed the same diets used as substrates. The second objective was to determine the long-term effects of YHS on *in vitro* ruminal fermentation, microbial growth and bacterial diversity in Rusitec fermenters. Diets were formulated to be representative of those fed to dairy and fattening ruminants, and therefore contained different forages.

Table 1
Ingredient composition and chemical analysis of experimental diets fed to donor sheep and used as substrates for the *in vitro* fermentations in batch cultures.

Ingredient (g/kg of DM)	Diet	
	AHC	BSC
Alfalfa hay	500	–
Barley straw	–	150
Barley	199	425
Corn	96.0	255
Soybean meal	71.0	150
Lupin	60.0	–
Oat	31.5	–
Fullfat soybean	15.0	–
Calcium carbonate	6.9	6.7
Sugarcane molasses	5.0	–
NaCl	3.5	3.7
Dicalcium phosphate	2.1	–
Mineral/vitamin premix ^a	10.0	10.0
Chemical analyses (g/kg of DM)		
Organic matter	935	942
Crude protein	186	150
aNDFom	394	357
ADFom	179	126

^a Declared composition (g/kg mineral/vitamin premix): Vitamin A, 600,000 IU; Vitamin D3, 120,000 IU; Vitamin E, 1 g; Vitamin B1, 33 mg; Niacine, 1.5 g; S, 5 g; IK, 300 mg; SO_4Fe , 1 g; ZnO, 4 g; MnO, 2 g; CoSO_4 , 60 mg; Na_2SeO_3 , 30 mg; Ethoxyquin, 30 mg.

2. Materials and methods

2.1. Animals and diets

Six rumen-cannulated Merino sheep were used as ruminal fluid donors for the *in vitro* incubations and Rusitec fermenters. Three sheep were fed a 50:50 alfalfa hay:concentrate diet (AHC) and the other three received a 15:85 barley straw:concentrate diet (BSC). Diets were representative of those fed to dairy and fattening ruminants, respectively. The diets were fed at a fixed rate of 42 g of dry matter (DM) per kg of body weight^{0.75} distributed in two equal meals at 09:00 and 18:00 h for two weeks before starting the *in vitro* incubations in batch cultures. Ingredient and chemical composition of the diets are shown in Table 1. Samples of the forages and concentrates fed to sheep were ground through a 1 mm screen and mixed in the corresponding proportion to be used as substrates for *in vitro* fermentations. Sheep were cared and handled by trained personnel in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes). The experimental protocols were approved by the León University Institutional Animal Care and Use Committee (Project AIB2010NZ-00190).

2.2. Yeast hydrolyzate (YHS), substrates, and *in vitro* fermentations in batch cultures

The YHS was obtained by enzymatic hydrolysis of the yeast cream resulting from bioethanol production from sugarcane juice and molasses using *S. cerevisiae* at a sugarcane processing plant (Empresa de Producciones Diversificadas José A. Echeverría, Cárdenas, Matanzas, Cuba). The enzymatic hydrolysis was produced by *Bacillus subtilis* E-44 at 45 °C and pH = 6.0, as described in detail by Pérez (2000). The YHS was a brown fluid that contained 0.21 g DM per g fresh matter. One ml of YHS supplied 8.3 mg of nitrogen, 314 µmol of total VFA (158, 52.2, 72.9, 0.34, 1.35, 11.9 and 17.9 µmol of acetate, propionate, butyrate, isobutyrate, isovalerate and caproate, respectively) and 291 µg of lactate. According to Pérez (2000), the YHS is stable up to six months regardless of storage temperature, and it was stored at 4 °C during our study.

Díaz et al. (2011) compared the effects of whole YHS and its fractions obtained by centrifugation (supernatant, gross pellet and washed pellet) on *in vitro* fermentation of *Pennisetum purpureum* cv. CT-115, and concluded that whole YHS was more effective than any isolated fraction; therefore, the whole YHS was used in this study. Four YHS concentrations were selected from the results of Díaz et al. (2011): 0 (control: CON), 100 (YHS1), 200 (YHS2), 300 (YHS3) and 400 µl (YHS4) per culture. The doses were equally increased, and were equivalent to 0, 3.33, 6.7, 10.0 and 13.3 ml/l, respectively. Samples of each diet (300 mg DM) were weighed into 120 ml serum bottles, and the YHS was applied inside the bottles immediately before incubation. In order to achieve the same final volume in all the cultures, CON, YHS1, YHS2 and YHS3 bottles received 400, 300, 200 and 100 µl of distilled water, respectively.

Ruminal contents were obtained immediately before the morning feeding from each sheep, pooled by diet (three sheep per diet), and strained through four layers of cheesecloth into pre-warmed thermal flasks with an O₂-free headspace. The fluid was mixed with the buffer solution of Goering and Van Soest (1970; no trypticase added; pH 7.42) in a proportion 1:4 (vol/vol) at 39 °C under continuous flushing with CO₂. Bottles of each substrate were filled with buffered ruminal fluid (30 ml) from sheep fed the same diet. Bottles were sealed with rubber stoppers and incubated at 39 °C. Samples of ruminal fluid were taken as described below for VFA and NH₃-N analysis.

After 16.5 h of incubation (corresponding to a passage rate from the rumen of 0.06 per h), gas production was measured using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a plastic syringe, and a gas sample (10 ml) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for analysis of CH₄. Bottles were then uncapped, pH was measured immediately (Crison Basic 20 pH-meter, Crisson Instruments, Barcelona, Spain) and bottles were placed in iced water to slow down fermentation. One ml of content was added to 0.625 ml of deproteinising solution (20 g metaphosphoric acid and 4 g crotonic acid per L) for VFA determination, 1 ml was added to 1 ml 0.5 M HCl for NH₃-N analysis, and 1 ml was immediately frozen for lactate analysis.

The experiment was repeated in four non-consecutive days to get four replicates per experimental treatment. Each incubation day, two bottles were incubated for each diet (AHC and BSC) and experimental treatment (CON, YHS1, YHS2, YHS3 and YHS4). A total of 20 bottles with substrate (two per diet and treatment) and 4 bottles without substrate (blanks; two per inoculum) were incubated. Blanks were used to correct the gas production values for gas release from endogenous substrates. Values measured in the two bottles incubated for each diet and experimental treatment were averaged before statistical analysis, and therefore there were four values per diet and experimental treatment.

2.3. Rusitec trial

2.3.1. Experimental design and sampling

Two identical 14-d incubation runs were carried out using four Rusitec fermenters (500 ml effective volume) in a cross-over design. The general incubation procedure was as described by Martínez et al. (2009), and in each run two fermenters received one of the experimental treatments: control (CON) and supplemented daily with 5 ml of YHS (YHS). The YHS dose was selected from the results of the batch cultures trial and the supply of YHS started on day 2 of incubation. On the first day of each incubation run, each fermenter was inoculated with 250 ml of strained rumen fluid, 200 ml of artificial saliva, and 80 g of solid rumen content supplied into a nylon bag. Ruminal contents (liquid and solid) from three sheep fed a 50:50 alfalfa hay:concentrate diet were collected immediately before the morning feeding, mixed, strained through two layers of cheesecloth, and transferred to the corresponding

fermenters within 30 min of collection. The liquid flow through fermenters was maintained by continuous infusion of artificial saliva (McDougall, 1948) at a rate of 650 ml/d (dilution rate of 5.42%/h). Each fermenter received daily 30 g of DM of the AHC diet (15 g of alfalfa hay and 15 g of concentrate). Alfalfa hay was chopped (about 0.5-cm pieces) and concentrate was ground through a 3-mm sieve. Each day at 10:00 h, forage and concentrate were fed into separate nylon bags (100 µm pore; 8 × 15 cm), which were incubated for 48 and 24 h, respectively. Three bags (two with forage and one with concentrate) were consistently kept in each fermenter. Dilution rate and solids retention time were chosen to resemble values previously observed *in vivo* in sheep (Ranilla et al., 1997, 1998). Liquid effluent was collected daily in flasks containing 20 ml of H₂SO₄ (20%; vol/vol) to maintain pH below 2. On day 13 a water solution saturated with HgCl₂ (5 ml) was added (replacing the H₂SO₄ solution that could cause bacterial lysis) to the effluent containers, which were held in an iced water bath to impede microbial growth.

After 8 d of adaptation, on days 9, 10, 11 and 12, samples for gas and for VFA, NH₃-N and lactate determination in the effluents were collected, and diet apparent disappearance was measured following the procedures described by Martínez et al. (2009). In addition, 3 ml of fermenters' fluid were frozen (−80 °C) for determination of amylase, xylanase and carboxymethylcellulase activities. On days 3, 8 and 14 of incubation, 3 ml of fluid from each fermenter were taken into a sterile container and immediately frozen at −80 °C for DNA extraction. In addition, the two nylon bags (one with forage and one with concentrate) collected from each fermenter were washed twice with 40 ml of fermenter liquid and the washing fluid was returned to the fermenter. Bags were then opened, their contents were transferred to a sterile container, thoroughly mixed and, about 5 g were frozen at −80 °C for DNA extraction.

On day 10, dose of 5.6 mg of ¹⁵N (¹⁵NH₄Cl) was added to each fermenter to label instantaneously the NH₃-N pool. Then, a solution of ¹⁵NH₄Cl was added daily to the artificial saliva at a rate of 4.0 mg of ¹⁵N per g of dietary N for measuring microbial growth. On days 13 and 14, both effluent and the solid digesta were sampled to determine microbial protein synthesis as described by Carro and Miller (1999). Briefly, about 500 ml of effluent were used for liquid-associated bacteria isolation as described by Martínez et al. (2009) and the rest of the effluent was freeze-dried for DM determination and ¹⁵N enrichment analysis. The contents of nylon bags were thoroughly mixed and used for DM determination, ¹⁵N enrichment analysis and isolation of solid-digesta associated bacteria as detailed by Martínez et al. (2009).

2.3.2. DNA extraction and automated ribosomal intergenic spacer analysis (ARISA)

DNA was isolated in triplicate from pellets of centrifuged liquid samples (1 ml, 20,000 × g, 5 min, 4 °C) and lyophilized solid digesta samples (200 mg DM) after mechanical disruption of microorganisms with a MiniBead-beater (3 min; Biospec Products, Bartlesville, OK, USA). The DNA was extracted following the procedure described by Yu and Morrison (2004) with the exception that an additional step involving the treatment of samples with cetyltrimethylammonium bromide (CTAB) was included to remove PCR inhibitors (Saro et al., 2012), and the QIAamp DNA Stool Mini Kit columns (QIAGEN, Valencia, CA, USA) were used to purify the DNA. Absorbance ratios (A₂₆₀:A₂₈₀) of eluted DNA were measured in a Nanodrop ND-1000 (Nano-Drop Technologies, Wilmington, DE) to assess the purity of DNA.

For ARISA analyses, the internal transcribed spacer of DNA was amplified using universal bacterial primers 16S-1392F and 23S-125R as described by Saro et al. (2012). Thermocycling and ARISA technique were conducted in a 2720 Thermal Cycler (Applied Biosystem, Foster City, CA, USA) and a MegaBACE 500 (Amersham Biosciences, Little Chalfont, Bucks, United Kingdom), respectively. Peaks were identified by comparison with an internal size standard using the GeneMarker Software v1.80 (SoftGenetics, State College, PA, USA) and the presence/absence of the different peaks was considered to compare the electropherograms profiles by using a similarity matrix. The Shannon's diversity index was calculated to evaluate the diversity of bacterial communities, and dendrograms were constructed using the Pearson coefficient and the unweighted pair-group method using arithmetic averages (UPGMA) options in the MVSP v3.12d software (Kovach Computing Service, Anglesey, Wales, UK).

2.4. Analytical procedures

Dry matter (ID 934.01), ash (ID 942.05), N (ID 984.13) and ADFom (ID 973.18) were determined according to the Association of Official Analytical Chemists (1999). Analyses of aNDFom were carried out according to Van Soest et al. (1991) using an ANKOM²²⁰ Fibre Analyzer unit (ANKOM Technology Corporation, Fairport, NY, USA). Sodium sulphite and heat-stable amylase were used in analysis of aNDFom. Both aNDFom and ADFom were expressed exclusive of residual ash.

Concentrations of VFA, NH₃-N and total lactate were determined as described by Garcia-Martinez et al. (2005), and CH₄ was analysed by gas chromatography following the procedure described by Martínez et al. (2010). Preparation and analysis of samples for ¹⁵N followed the procedures described by Carro and Miller (1999). Amylase, xylanase and carboxymethylcellulase activities in fermenters' content were determined as described by Giraldo et al. (2007) using soluble starch, oat spelt xylan and carboxymethylcellulose as substrates, respectively.

2.5. Calculations and statistical analyses

The amounts of VFA produced in each bottle were calculated by subtracting the amount present initially in the incubation medium from that determined at the end of the incubation period. The amount of organic matter apparently fermented (AFOM) in each culture was estimated from VFA production using the equation proposed by Demeyer (1991).

Effects of YHS supplementation and incubated diet in the experiment with batch cultures were assessed by ANOVA as a mixed model using the PROC MIXED of SAS (version 9.4; SAS Inst. Inc., Cary, NC, USA). Five concentrations of YHS (0, 3.33, 6.7, 10.0 and

13.3 ml/l), diet (AHC and BSC) and the YHS $\times$ diet interaction were included in the model as a fixed effects, whereas incubation day was considered as a random effect. Orthogonal polynomial contrasts were used to test for linear and quadratic effects of YHS. Significance was declared at $P < 0.05$, whereas $P < 0.10$ values were considered to be a trend. When a significant effect of YHS dose was detected, treatment means were compared using the Tukey's test ($P < 0.05$). Because each diet was only incubated with ruminal fluid from sheep fed the same diet used as substrate in the batch cultures, the effects of the two factors (inoculum and diet used as substrate) are confounded.

Fermentation and bacterial diversity data from Rusitec fermenters were analysed as a mixed model with repeated measures using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA). The statistical model used included the YHS treatment, incubation run, time and treatment $\times$ time as fixed effects, and fermenter as a random effect. Data on microbial growth were analysed independently for each digesta phase as a mixed model which YHS treatment, incubation run, and YHS treatment $\times$ incubation run as fixed effects, and fermenter as a random effect. Effects were declared significant at $P < 0.05$, and $P < 0.10$ was considered a trend.

3. Results

The two types of inoculum used in this experiment differed in fermentation characteristics (data not shown). The fluid from AHC-fed sheep had greater ($P < 0.05$) pH (6.68 vs. 6.13), $\text{NH}_3\text{-N}$ concentrations (132 vs. 115 mg/l), acetate proportions (64.9 vs. 57.8 mol/100 mol) and acetate:propionate ratio (3.71 vs. 2.70), but lower ($P < 0.05$) propionate proportion (17.5 vs. 21.4 mol/100 mol), compared with the BSC-inoculum.

3.1. Batch cultures fermentations

Effects of supplementing batch cultures with YHS on rumen fermentation parameters are shown in Tables 2 and 3. Yeast hydrolyzate $\times$ diet interactions ($P < 0.001\text{--}0.007$) were detected for molar proportions of acetate, propionate and caproate and acetate:propionate ratio, and a trend ($P = 0.063$) to interaction was observed for final pH. Increasing levels of YHS linearly augmented total VFA production ($P = 0.031$), butyrate ($P = 0.007$) and valerate ($P = 0.023$) proportions, and $\text{NH}_3\text{-N}$ concentrations ($P = 0.040$) but decreased ($P < 0.001$) acetate proportions and acetate:propionate ratio. Effects of YHS on total VFA production, proportions of acetate, butyrate, isovalerate, valerate and acetate:propionate were only observed for the AHC diet, whereas those on $\text{NH}_3\text{-N}$ concentrations and isovalerate proportions were only detected for the BSC diet. Neither linear nor quadratic effects ($P = 0.127\text{--}0.855$) of YHS supplementation were observed for final pH, total lactate concentrations, or production of gas and CH_4 for any diet. The amount of AFOM increased ($P = 0.029$) linearly with increasing doses of YHS for AHC diet, but no effect was observed for the BSC diet. The incubated diet affected ($P < 0.001\text{--}0.026$) all parameters measured, with the exception of total lactate concentrations ($P = 0.740$).

Table 2
Effects of increasing doses of a yeast hydrolyzate (YHS) on volatile fatty acids (VFA) production after 16-h *in vitro* fermentation of two diets by mixed rumen micro-organisms.¹

Item	Diet	Treatment					SEM	P value			Diet	YHS $\times$ Diet
		CON	YHS1	YHS2	YHS3	YHS4		L ²	Q ²			
Total VFA (μmol)	AHC	2090 ^a	2075 ^a	2086 ^{ab}	2133 ^{ab}	2162 ^b	24.7	0.031	0.267		< 0.001	0.407
	BSC	2239	2203	2259	2230	2250						
Molar proportion (mol/100 mol) of:												
Acetate	AHC	65.8 ^b	64.8 ^b	63.3 ^a	63.4 ^a	63.2 ^a	0.37	< 0.001	0.147		< 0.001	0.007
	BSC	51.8	51.9	52.0	51.5	51.7						
Propionate	AHC	19.1	19.8	20.2	20.7	20.8	0.31	0.078	0.222		< 0.001	0.002
	BSC	31.5	30.4	30.0	30.5	30.6						
Butyrate	AHC	10.3 ^a	10.5 ^a	11.4 ^b	11.4 ^b	11.2 ^b	0.23	0.007	0.006		< 0.001	0.161
	BSC	14.3	14.9	14.9	14.8	14.6						
Isobutyrate	AHC	1.19	1.65	1.58	1.16	1.20	0.20	0.804	0.109		0.026	0.691
	BSC	0.28	0.44	0.51	0.45	0.42						
Isovalerate	AHC	1.33	1.36	1.40	1.34	1.36	0.03	0.075	0.024		< 0.001	0.429
	BSC	0.62 ^a	0.73 ^b	0.77 ^b	0.76 ^b	0.73 ^b						
Valerate	AHC	1.61 ^a	1.66 ^{ab}	1.74 ^b	1.61 ^a	1.88 ^b	0.07	0.023	0.682		< 0.001	0.390
	BSC	1.35	1.39	1.48	1.51	1.45						
Caproate	AHC	0.66 ^c	0.27 ^a	0.41 ^b	0.44 ^b	0.46 ^b	0.03	< 0.001	< 0.001		< 0.001	< 0.001
	BSC	0.23 ^a	0.30 ^{ab}	0.37 ^b	0.49 ^c	0.50 ^c						
Acetate:propionate (mol/mol)	AHC	3.48 ^c	3.30 ^b	3.19 ^a	3.13 ^a	3.10 ^a	0.04	< 0.001	0.378		< 0.001	< 0.001
	BSC	1.66	1.71	1.73	1.69	1.69						

^{a,b,c} For each diet and variable, values not sharing a common superscript differ ($P < 0.05$).

¹ 0, 100, 200, 300 and 400 μl of YHS per batch culture (30 ml) were added for CON, YHS1, YHS2, YHS3 and YHS4, respectively ($n = 4$). AHC and BSC diets were composed of 50:50 alfalfa hay:concentrate and 15:85 barley straw:concentrate, respectively.

² L: linear effect of YHS dose; Q: quadratic effect of YHS dose.

Table 3

Effects of increasing doses of a yeast hydrolyzate (YHS) on final pH, concentrations of $\text{NH}_3\text{-N}$ and total lactate, production of gas and CH_4 , CH_4 /volatile fatty acid (VFA) ratio and apparently fermented organic matter (AFOM) after 16-h *in vitro* fermentation of two diets by mixed rumen micro-organisms.¹

Item	Diet	Treatment					SEM	P value			
		CON	YHS1	YHS2	YHS3	YHS4		L ²	Q ²	Diet	YHS × Diet
pH	AHC	6.63	6.62	6.61	6.59	6.58	0.01	0.127	0.370	< 0.001	0.063
	BSC	6.50	6.48	6.48	6.50	6.51					
$\text{NH}_3\text{-N}$ (mg/l)	AHC	224	221	213	212	238	12.1	0.040	0.843	< 0.001	0.145
	BSC	99.7 ^a	139 ^b	142 ^b	147 ^b	146 ^b					
Total lactate (mg/l)	AHC	10.5	9.71	8.55	10.1	9.26	0.64	0.542	0.217	0.740	0.808
	BSC	9.22	9.21	8.47	9.12	9.36					
Gas (μmol)	AHC	3246	3243	3271	3312	3301	29.9	0.202	0.641	< 0.001	0.561
	BSC	3431	3459	3456	3441	3438					
CH_4 (μmol)	AHC	565	550	551	535	551	15.6	0.505	0.816	0.170	0.907
	BSC	460	463	475	455	461					
CH_4 /VFA (mol/mol)	AHC	0.270	0.266	0.265	0.251	0.255	0.0079	0.170	0.828	0.002	0.825
	BSC	0.205	0.210	0.211	0.204	0.206					
AFOM (mg) ³	AHC	179 ^a	178 ^a	180 ^b	185 ^b	186 ^b	2.34	0.029	0.459	< 0.001	0.254
	BSC	203	200	205	202	203					

^{a,b} For each diet and variable, values not sharing a common superscript differ ($P < 0.05$).

¹ 0, 100, 200, 300 and 400 μl of YHS per batch culture (30 ml) were added for CON, YHS1, YHS2, YHS3 and YHS4, respectively ($n = 4$). AHC and BSC diets were composed of 50:50 alfalfa hay:concentrate and 15:85 barley straw:concentrate, respectively.

² L: linear effect of YHS dose; Q: quadratic effect of YHS dose.

³ Apparently fermented organic matter estimated from volatile fatty acids production according to Demeyer (1991).

Table 4

Effects of a yeast hydrolyzate (YHS) supplementation on fermentation parameters, diet digestibility, enzymatic activity and microbial protein synthesis in *Rusitec* fermenters fed a 50:50 forage:concentrate diet ($n = 4$).

Item	Treatment		SEM	P =
	CON	YHS		
pH	6.50	6.52	0.051	0.817
NH_3 (mg/l)	339	405	12.3	< 0.001
Total VFA (mmol/d)	105	106	3.3	0.801
Molar proportions (mol/100 mol):				
Acetate	54.5	51.1	0.48	< 0.001
Propionate	14.9	17.7	0.28	< 0.001
Butyrate	17.2	18.6	0.30	< 0.001
Isobutyrate	1.22	1.35	0.041	0.002
Isovalerate	4.30	3.56	0.188	< 0.001
Valerate	4.85	5.31	0.154	0.003
Caproate	3.06	2.35	0.138	< 0.001
Acetate:propionate (mol/mol)	3.67	2.89	0.085	< 0.001
CH_4 (mmol/d)	29.5	28.3	0.75	0.059
CH_4 /Total VFA (mol/mol)	0.281	0.267	0.0090	0.064
Lactate (g/L)	6.25	6.69	0.907	0.559
Enzymatic activity ^a				
Amylase	0.269	0.270	0.0330	0.973
Xylanase	0.627	0.641	0.0361	0.616
Carboxymethylcellulase	0.078	0.083	0.0092	0.536
Diet disappearance (g/g)				
Dry matter	0.629	0.641	0.0096	0.158
aNDFom	0.468	0.474	0.0139	0.636
ADFom	0.282	0.293	0.0211	0.556
Microbial growth (mg N/d)				
Solid phase	128	129	7.1	0.899
Liquid phase	110	129	3.6	0.065
Total	237	258	6.8	0.165
Efficiency of microbial growth (mg N/g organic matter apparently fermented)	24.2	23.6	0.61	0.589

^a Amylase and carboxymethylcellulase activities are expressed as nanomoles of glucose released from soluble starch or carboxymethylcellulose by 1 ml of liquid fermenters' content in 1 min at 39 °C and pH 6.5. Xylanase activity is expressed as nanomoles of xylose liberated from oat spelt xylan by 1 ml of liquid fermenters' content in 1 min at 39 °C and pH 6.5.

3.2. Fermentation in the Rusitec system

Effects of YHS on fermentation parameters and bacterial diversity in Rusitec fermenters are shown in Tables 4 and 5, respectively. The addition of YHS did not affect ($P \geq 0.536$) pH before feeding, total VFA production, enzymatic activities or diet disappearance, but modified VFA profile. Compared with the control, the addition of YHS increased ($P \leq 0.003$) the proportion of propionate, butyrate, isobutyrate and valerate, and decreased ($P < 0.001$) acetate, isovalerate and caproate proportions, and tended to decrease ($P = 0.059$) CH_4 production. As a consequence of these changes, YHS-supplemented fermenters had lower ($P < 0.001$) acetate:propionate ratios and tended to lower ($P = 0.064$) CH_4 /total VFA ratios. Supplementation with YHS did not affect ($P = 0.899$) microbial growth in the solid phase of fermenters, but tended to increase ($P = 0.065$) microbial growth in the liquid digesta. Efficiency of microbial growth was not affected by YHS supplementation.

As shown in Table 5, both the number of peaks and Shannon index values in the ARISA electropherograms of samples from the liquid digesta were greater ($P = 0.002$ and 0.019 , respectively) in the YHS-supplemented fermenters compared with those un-supplemented. A similar trend ($P \leq 0.077$) was observed in the solid digesta. Bacterial diversity in the liquid digesta increased ($P < 0.001$) with incubation time for both treatments (control and YHS), and that in the solid digesta also tended ($P \leq 0.061$) to increase as time progressed. In addition, the similarity index between the solid and liquid digesta was greater ($P = 0.036$) in YHS-supplemented fermenters than in control ones.

The dendrograms in Fig. 1 clearly show changes in the structure of bacterial populations over the incubation period in our study. It is worth to notice that all samples from day 3 clustered together, for both liquid and solid digesta, although no further subclustering according to treatment or incubation run was observed. In contrast, no clear pattern of clustering according to YHS treatment, sampling day or incubation run was observed for samples taken on days 8 and 14. In general, fermenters 1 and 2 were more similar to each other than to the rest of fermenters, and the same was observed for fermenters 3 and 4.

4. Discussion

The study with batch cultures was designed to investigate the effects of increasing doses of YHS on *in vitro* ruminal fermentation as influenced by the type of diet fed to donor sheep and incubated as substrate. The type of inocula has been identified as one of the most important factors influencing *in vitro* fermentation, and previous studies have shown the influence of the inoculum on the main fermentative parameters and the bacterial populations developed in batch cultures (Mateos et al., 2015; Vanegas et al., 2017). Therefore, ruminal fluid from sheep fed the same diet as that incubated in the batch cultures were used to inoculate the cultures with a microbiota representative of that existing in the rumen of sheep fed these diets. The observed values for the fermentation parameters in the two inocula were in accordance with those previously reported in sheep fed similar diets (Carro et al., 2000; Ramos et al., 2009), and seem to indicate the existence of different microbial communities in each of them. Overall, YHS supplementation had only slight effects on the fermentation of BSC diet. In contrast, YHS stimulated the fermentation of AHC diet, as indicated by the increased amount of both total VFA and AFOM. The VFA represent the main supply of metabolizable energy for ruminants, and therefore an increase in their production would be nutritionally favorable for the host animal.

In addition, YHS supplementation of AHC-cultures shifted the rumen VFA profile to less acetate and more propionate (only a trend was observed for propionate), indicating that YHS resulted in more efficient rumen fermentation. Differences in the response to a different YH supplementation to batch cultures containing substrates varying in grass silage to concentrate ratio (0.75:0.25–0.25:0.75) have also been reported by Kettunen et al. (2016). These authors observed a maximum increase of microbial cell density (20%) with the substrate containing 0.75 grass silage, whereas lower increases (9–12%) were detected for the substrates containing lower silage proportions. Similarly, Ospi et al. (2012) reported that the effects of inactivated cells of *S. cerevisiae* on *in vitro*

Table 5

Effects of a yeast hydrolyzate (YHS) supplementation on the evolution through the incubation period of number of peaks and Shannon index values detected in the automated ribosomal intergenic spacer analysis (ARISA) electropherograms in solid and liquid contents of Rusitec fermenters, and similarity index of ARISA profiles between solid and liquid contents.

Sample	Item	Treatment	Incubation day			SEM	P-value		
			3	8	14		Treatment	Time	Treatment × time
Solid content	Number of peaks	CON	38.0	40.0	41.8	1.81	0.056	0.057	0.213
		YHS	40.0	46.5	41.5				
	Shannon index	CON	3.60	3.69	3.73	0.048	0.077	0.061	0.270
		YHS	3.68	3.84	3.72				
Liquid content	Number of peaks	CON	36.0 ^a	44.3 ^b	43.0 ^b	0.98	0.002	< 0.001	0.399
		YHS	37.8 ^a	47.5 ^b	46.8 ^b				
	Shannon index	CON	3.58 ^a	3.79 ^b	3.74 ^b	0.023	0.019	< 0.001	0.493
		YHS	3.63 ^a	3.86 ^b	3.84 ^b				
Solid-Liquid	Similarity index, %	CON	64.9 ^a	78.3 ^c	73.4 ^b	1.893	0.036	< 0.001	0.181
		YHS	71.5 ^a	77.6 ^b	79.2 ^b				

^{a,b} Within each row, means without a common superscript differ ($P < 0.05$). Superscripts are only shown when a significant ($P < 0.05$) effect of time was detected.

Fig. 1. Dendrograms of ARISA profiles of bacterial communities in solid (A) and liquid (B) phase from Rusitec fermenters on days 3, 8 and 14 of incubation and either supplemented with a yeast hydrolyzate (YHS) or not (CON). Numbers 1–4 correspond to individual fermenters (FER). Two identical incubation runs (1, 2) were performed.

ruminal fermentation of substrates with different forage to concentrate ratio were more noticeable with a high-fiber substrate and only subtle effects were detected with a high-concentrate substrate. These results agree with the higher effects of YHS observed in our study for the AHC compared with the BSC diet. Based on these results we decided to investigate the long-term effects of YHS on *in vitro* fermentation of AHC diet using Rusitec fermenters.

Our results from batch cultures agree with previous studies (Gómez et al., 2005; Martínez et al., 2010) showing that CH₄ production *in vitro* was affected by forage:concentrate ratio in the incubated diet, although YHS did not affect CH₄ production and there were no YHS x diet interactions. Production of CH₄ was lower in BSC-cultures than in those containing AHC diet, but it has to be noticed that batch cultures are heavily buffered, and the pH decrease usually observed in BSC-fed animals diets could not be simulated; therefore, the influence of YHS on rumen pH could have not been properly tested. Although *in vitro* studies have some limitations, they constitute a useful tool to test a high number of experimental treatments before performing *in vivo* trials (Mateos et al., 2015). The higher NH₃-N concentrations in the AHC-cultures are in accordance with the higher crude protein content of AHC diet compared with BSC-diet (Table 1).

Because no differences among the three highest doses used in the batch cultures (6.7, 10.0 and 13.3 ml/l) were detected in most of the measured parameters, a daily dose of 10.0 ml/l of YHS was chosen for the Rusitec trial (5 ml per day for an effective fermenters' volume of 500 ml). As observed in the batch cultures, YHS supplementation did not influence fermenters' pH. However, the increase in total VFA production observed in the batch cultures supplemented with YHS was not observed in the fermenters. Although YHS was used at a concentration of 10.0 ml/l in both YHS3-cultures and Rusitec fermenters, differences in solid/liquid ratio between the two *in vitro* systems originated marked differences in the YHS dose expressed as ml/g diet DM (1.00 and 0.17 ml/g diet DM for YHS3-

cultures and fermenters, respectively). As a consequence, the amount of DM from YHS supplied to the cultures accounted for 21.0% of the DM incubated in the YHS3-cultures (300 mg DM diet), but only for 3.5% in the fermenters (30 g DM diet). The increase in VFA production observed in the YHS3-cultures might have been due to the fermentation of the DM added with YHS more than to a stimulation of microbial activity. As discussed by [Ospi et al. \(2012\)](#), yeast do not induce prompt changes in the ruminal microbial population, and thus short-term shifts in fermentation pattern cannot be expected in response to yeast products. However, the observed response could have been mediated by a prebiotic effect of yeast cells, as the YHS used in this study has been reported to contain glucan and mannan oligosaccharide that can be easily fermented by ruminal microbes ([Pérez et al., 2016](#)). The increased $\text{NH}_3\text{-N}$ concentrations observed in both *in vitro* systems might also have been due to the microbial degradation of yeast cells because of their high protein content ([Oeztuerk, 2009](#)). In fact, daily $\text{NH}_3\text{-N}$ outflow was 220 and 263 mg for control and YHS-fermenters, respectively, and the difference between treatments (43 mg $\text{NH}_3\text{-N}$) is consistent with the 41.5 mg of N supplied daily by YHS (1 ml of YHS contained 8.3 mg of N). This hypothesis is also supported by the greater isovalerate and valerate proportions in YHS-fermenters compared with control ones, as both VFA are produced mainly from degradation of amino acids by rumen microorganisms. [Oeztuerk \(2009\)](#) observed that the increase in $\text{NH}_3\text{-N}$ concentration in Rusitec fermenters produced by a live yeast culture from *S. cerevisiae* was higher than that caused by the same cultures autoclaved, and concluded that the difference between both yeast preparations might have been associated with a stimulation of proteolytic activity of rumen bacteria by live yeast culture. The increased amounts of $\text{NH}_3\text{-N}$ concentrations in our study seem to be due to the N supply by YHS, and suggest that this YHS would be especially useful to improve ruminal fermentation of nitrogen-deficient diets.

In addition to N, dead yeasts can provide growth factors, such as amino acids, peptides and other compounds essential for some ruminal microbes ([Pacheco et al., 1997](#); [Pérez et al., 2016](#)), whose growth can be stimulated. This idea is consistent with the trend to increased microbial growth observed in the liquid phase of the YHS-fermenters compared with control ones. A stimulation of the growth of ruminal bacteria by live ([Miller-Webster et al., 2002](#)) and dead ([Kettunen et al., 2016](#)) yeast cultures has been shown in some studies, but others have reported no effect ([Carro et al., 1992](#)). Moreover, YHS supplementation promoted a greater bacterial diversity in both digesta phases of YHS-fermenters (Table 5). This could indicate that YHS promoted the growth of bacteria that could not be detected in the control-fermenters due to their low abundance. The ARISA is a qualitative technique and does not allow identification of bacterial species, but it is assumed that each peak in the electropherograms reflects the predominant bacterial species or populations present in the samples. However, no clear grouping of samples according YHS treatment was observed in the den-drograms of ARISA profiles, suggesting a high complexity of Rusitec bacteria populations but only subtle differences in their structure between YHS and control fermenters.

In Rusitec fermenters, an adaptation period is required to reach steady-state conditions, and this is reflected both in the fermentation parameters and the structure of bacterial populations that establish in the system. In our experiment, all samples from day 3 clustered together, for both liquid and solid digesta, with no clear pattern of clustering according to sampling day for samples taken on days 8 and 14, indicating the changes in bacterial populations over time in the first days of incubation and the stability reached after adaptation period. Bacterial diversity in the liquid digesta increased from day 3 to days 8 and 14, and a similar trend was observed in the solid digesta for both treatments (control and YHS), suggesting that predominant bacteria in Rusitec fermenters differed from those in the inoculum and that populations developed in the Rusitec are as complex as those in the rumen.

Differences in bacterial populations and/or in their activity in response to YHS supplementation could explain the change in VFA profile observed in the fermenters. Supplementation with YHS shifted VFA profile from acetate to propionate, reducing the acetate:propionate ratio. Similar results have been recently reported by supplementing different YH to batch cultures ([Kettunen et al., 2016](#)) and Rusitec fermenters ([Oeztuerk et al., 2016](#)). The trend to reduced CH_4 production observed in YHS-fermenters in our study is consistent with the increased propionate proportions, as production of propionate serves as a competitive pathway for hydrogen. As a result, CH_4 /total VFA tended to be lower in YHS-fermenters, which would indicate a more efficient fermentation.

Finally, the lack of effect of YHS on lactate concentrations in both *in vitro* experiments is consistent with the idea that dead yeast cells had no effect on lactate production ([Miller-Webster et al., 2002](#); [Chaucheyras-Durand et al., 2012](#)). In contrast, live yeasts have been reported to stimulate the growth of rumen lactate-utilizers (*M. elsdenii* and *S. ruminantium*) by supplying metabolites (i.e. peptides, vitamins, organic acids, ...) that are essential for these bacteria and to reduce lactate concentrations by outcompeting *S. bovis* for the utilization of sugars ([Chaucheyras-Durand et al., 2012](#)).

5. Conclusions

The effectiveness of the yeast hydrolysate used in this study to modify rumen fermentation was diet-dependent, being more effective with a medium-concentrate than with a high-concentrate diet in short-term *in vitro* incubations. In long-term *in vitro* experiments, the yeast hydrolysate stimulated the rumen fermentation of a 50:50 alfalfa hay:concentrate diet and increased microbial growth. If results are confirmed *in vivo*, the yeast hydrolysate could be used as a low-cost feed additive.

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