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In vitro assessment of the factors that determine the activity of the rumen microbiota for further applications as inoculum

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Running title: Factors affecting rumen microbial activity *in vitro*

Abstract

BACKGROUND: Rumen microbiota has been used as inoculum for *in vitro* studies and as probiotics to improve productivity in young animals. However great variability across studies has been noted depending on the inoculum considered. This paper aims to assess the relevance of different factors (microbial fraction, collection time, donor animal diet, fermentation substrate and inoculum preservation method) to maximize the rumen inoculum activity to set the standards for further *in vitro* and *in vivo* applications.

RESULTS: Rumen inoculum sampled at 3h after feeding led to greater microbial growth and activity (+12% VFA, +17% ammonia) than before feeding. Similar results were noted when rumen liquid or rumen content were used as inocula. Rumen inoculum adapted to concentrate diets increased microbial activity (+19% VFA) independently of the substrate used *in vitro*. Freezing-thawing the inoculum, in comparison to fresh inoculum, decreased microbial activity (-14% VFA, -96% ammonia), anaerobic fungi and protozoa, being holotrichs protozoa particularly vulnerable. Inoculum lyophilisation had a stronger negative effect on the microbial activity (-51% VFA) and delayed the re-activation of the microbes leading to lower levels of methanogens, anaerobic fungi and nearly to the complete wipe out of rumen protozoa.

CONCLUSIONS: Fresh rumen fluid sampled at 3h after feeding from donor animals fed concentrate diets should be chosen when the aim is to provide the most diverse and active rumen microbial inoculum.

Keywords: *in vitro*, microbial activity, preservation method, probiotics, rumen inoculum

INTRODUCTION

There is a raising pressure to develop safe and effective rumen modulators in ruminant production as concerns over the use of antibiotics in livestock production and the need for increasing productivity continues to grow (1). Among the range of options, probiotics offer a source of microorganisms to provide a beneficial microbial balance in the gastrointestinal tract (2). Although the rumen is home to a highly diverse microbiota comprising bacteria, protozoa, anaerobic fungi, methanogens and viruses (3), only a few autochthonous members of this complex community and few allochthonous species (mainly *Saccharomyces cerevisae, Lactobacillus spp* and *Enterococcus spp*) have been explored for their potential as probiotics (2). The results of using probiotics on rumen function are highly variable and dependent on various factors such as the type of probiotic, dose, preservation method, diet and host animal, however the best results have generally been achieved by generating a multi-factorial response through the administration of a combination of various microbial species to maximize their activity (2, 4). Thus, it may be hypothesized that probiotics collected from the rumen may establish more easily into the microbial community than foreign species. Inoculation of rumen fluid from healthy animals to sick animals has often been conducted by producers and veterinarians to re-stablish the rumen balance (specially in animals that have been off feed) (5) and to prevent sickness associated to grain overfeeding (6), however there is not such commercial product based on this concept. Several studies have used microbial factions harvested from the rumen content as probiotics to improve the health and growth of young ruminants before weaning (7, 8). An early study showed that a single inoculation with fresh rumen fluid to growing lambs improved N balance (9) although results varied depending on the donor animal diet (10). Moreover, different types and preservation methods of the rumen inocula have been explored. For example placing cuds from adult animals into the mouth of young calves facilitated the establishment of rumen protozoa (11) while the administration of lyophilized (12) or dry stabilized rumen extract (13) showed positive effects on the average weight gain and feed digestibility in young animals. On the contrary, two studies (8, 14) noted no beneficial effects of inoculating lyophilized rumen microbiota to growing lambs. Thus, the

great variability observed across these and other studies suggests that more effort is needed to identify and describe the relevance of different factors which define the inoculum microbial activity under standardized laboratory conditions prior conducting further *in vivo* studies.

Rumen microbiota has extensively been used as inoculum for *in vitro* fermentation techniques to evaluate the nutritional value of ruminant feeds and the effectiveness of feed additives (15). As a result, several recommendations have been provided in relation to donor animal, microbial adaptation to the diet, type of rumen inoculum, sampling time or preservation method (16, 17). Although there are not standard protocols, fresh rumen liquid sampled before feeding from donor animals adapted to the same (or similar) diets than used as fermentation substrate represent the inoculum most commonly used when the aim is to minimize the inter-animal variation and allow comparisons among *in vitro* studies (16, 17). However, to date no studies have assessed how these technical aspects should be addressed when the aim is to achieve the rumen microbiota with the highest possible activity. Therefore, this paper aims to expand knowledge on the factors which maximize and maintain the rumen microbial activity in terms of anaerobic fermentation and abundance of the main microbial groups, once collected from the animal, in order to describe the most suitable inoculum to be directly inoculated *in vivo* as probiotics or used for *in vitro* studies.

MATERIALS AND METHODS

A multi-factorial approach was chosen to assess the impact of different factors on maintaining the rumen microbial activity; first, an *in vivo* monitoring of the diurnal changes in the rumen pH was performed to identify the most suitable sampling time depending on the donor´s diet. Then, a series of three consecutive *in vitro* experiments were performed to determine the effect of the type of incubation buffer (Exp. 1), sampling time, rumen fraction (Exp. 2), inoculum adaptation to the diet and preservation method (Exp. 3) on the microbial activity. The findings from each trial were implemented on the subsequent experimental design. All incubations were performed using two different diets as substrates to elucidate whether these findings could be applied across various production systems. Animal procedures were carried out according to the Spanish guidelines (RD

153/2013) and protocols were approved by the Ethical Committee for Animal Research (EEZ-CSIC) regional government (09/03/2017). Eight Murciano-Granadina female goats fitted with permanent rumen fistula were randomly distributed into two groups fed either a forage diet (DF, 50:50 proportions of alfalfa hay and oat hay) or a concentrate diet (DC, ratio 70:30 concentrate to forage ratio). Chemical composition (in g kg^{-1} DM) of the concentrate was 949 OM, 36.1 N, 319 NDF, 87.2 ADF, 34 ADL and 48.3 EE, while for the forage was 898 OM, 31 N, 576 NDF, 335 ADF, 68 ADL and 19 EE. Animals were kept in individual pens and diet was offered at 1.2 times maintenance level divided into two equal meals at 08:00h and 16:00h (18).

Experiment 1: In vivo rumen pH monitoring and in vitro buffering capacity

This experiment aimed to identify the rumen microbiota with the greatest activity to be directly used as inoculum and the most appropriated *in vitro* conditions to evaluate its activity once removed from the rumen. Rumen pH was monitored *in vivo* to determine the time after feeding with the greatest fermentation peak (lowest pH) depending on the diet (DC vs DF) consumed by the donor animal. Three incubation solutions with different buffering capacity were used to identify whether the incubation pH could influence the results depending on the substrate considered (interaction Diet × Buffer). After 2 weeks of adaptation to the diets, rumen digesta samples were collected through the cannula at 0, 2, 4 and 6 hours after the morning feeding to determine the diurnal changes in rumen pH (Figure 1). Based on previous recommendations (19) three combinations of salts consisting on decreasing levels of $NaHCO_3$ (35, 10.3 and 3.15 g L^{-1}) and increasing levels of NH_4HCO_3 (4.0, 1.07 and 0.25 g $L1^{-1}$) were used for adjusting the *in vitro* conditions to targeted high (6.80), medium (6.25) and low pH (5.75).

The batch culture incubation (20) used a total of 48 Wheaton bottles in a single run according to the following 3×2×4×2 factorial design: 3 buffer solutions (high vs medium vs low pH) × 2 diets as incubation substrate (DC vs DF) × 4 animal inocula (n=4) × 2 analytical replicates. Rumen contents were individually collected from donor goats at 3 hours after the morning feeding, time at which the greatest differences in rumen pH among diets were generated (based on the preliminary study),

filtered through a double layer of muslin and bubbled with CO_2. This inoculum was immediately diluted with preheated incubation solutions (in a 1:3 ratio) and anaerobically dispensed to 120-mL Wheaton bottles containing 500 mg DM of either DC or DF and 50 mL of total volume. Experimental diets were ground using a hammer mill with $1mm^2$ pore size and the incubation of each substrate was performed using rumen inoculum from animals adapted to the equivalent diet. Bottles were sealed and kept static in an incubator at 39ºC receiving a gentle mix before each measurement. *In vitro* incubations lasted for 96h and microbial activity was determined after 24h incubation; a sample representing 7% of the incubation fluid was taken by aspiration using a 14G needle, the pH was immediately recorded and the sample was divided in two subsamples: the first sample (1.6 mL) was diluted with 0.4 mL of an acid solution (0.5N HCl, 200 g L^{-1} metaphosphoric acid containing 0.8g L^{-1} of crotonic acid as internal standard) and stored at -20ºC until volatile fatty acid determination (VFA). The second sample (0.8mL) was diluted with 0.2 mL of trichloroacetate solution (25 g L^{-1}) for ammonia analysis. In order to determine the fermentation kinetics, gas pressure in the headspace of the bottles was measured and released at 2, 4, 7, 10 and 24, 48, 72 and 96h using a Wide Range Pressure Meter (Sper Scientific LTD, Scottsdale, AZ, USA).

Experiment 2: Effect of microbial fraction, collection time and fermentation substrate

The incubation buffer that promoted the highest pH (target 6.80) also promoted the highest microbial fermentation (see below), thus it was chosen for further experiments to prevent a possible limitation of the microbial activity induced by the buffer. Similarly, rumen sampling times (0 and 3h after feeding) were chosen because they led to the greatest *in vivo* differences in the rumen pH among diets. A total of 72 Wheaton bottles were used according to a 2×2×2×4×2 factorial design distributed into two consecutive incubation runs as follows: 2 inoculum fractions (rumen liquid vs rumen liquid plus solids) × 2 collection times (0h vs 3h after feeding) × 2 diets as substrate (DC vs DF) × 4 animal inocula (n=4) × 2 analytical replicates plus 8 bottles used as positive controls. Two consecutive incubation runs with 3h time difference between them were conducted; thus positive control bottles containing identical frozen-thawed inocula were added into each incubation run to

detect abnormalities between the runs. These positive control bottles were composed by rumen inocula from each donor animal (containing dimethyl-sulfoxide, DMSO, as cryopreservant), incubation buffer and the same substrate than that consumed by the donor animal. Before conducting experiment 2, rumen content (200g FM) was collected from each donor goats, filtrated through double cheesecloth and manually squeezed to separate rumen liquid and solids in order to: i) determine the proportions of liquid and solids in the rumen contents (which resulted to be 250 and 750 g kg^{-1} of fresh rumen content, respectively) and ii) to obtain lyophilized and autoclaved rumen solids for subsequent experiments. The starting day of each incubation run rumen contents were fractionated into liquid and solids: the liquid fraction (LIQ) was mixed with the incubation buffer placed into incubation bottles containing the experimental diets. The rumen content fraction was artificially reconstituted by mixing rumen liquid and solids (LS) in the proportions described above. Moreover, each LIQ incubation bottle was supplemented with autoclaved rumen solid (113 mg DM) obtained from the same donor animal to compensate the extra fermentable supply provided by the solids in the LS fraction. Each type of inoculum was incubated with its own related substrate. *In vitro* incubations and sampling procedures were the same than described in Experiment 1 in terms of pH, VFA, ammonia and gas production but three additional samples were taken after 24h of incubation: a gas sample was collected from the headspace (5mL into a vacuum tube) after gas pressure excess was released for further methane determination, moreover one incubation sample (0.2mL) was frozen in liquid N for microbial quantification and other sample (0.8mL) was diluted with 0.8mL of formaldehyde solution (80 mL L^{-1}) for protozoal counting.

Experiment 3: Effect of donor animal diet, fermentation substrate and inoculum preservation method

Based on the results from Experiment 2 (see below), liquid inoculum was chosen for further experiments because is easier to obtain, homogenize and handle in the lab than solid inoculum. Similarly, inoculum collected at 3h after feeding instead of before feeding was chosen for further experiments given its greater activity independently of the diet considered (see below). A total of

104 bottles were used according to a 2×3×2×4×2 factorial design distributed into two incubation runs as follows: 2 inocula adapted to either forage (AF) or concentrate (AC) × 3 inocula preservation methods (fresh (FRE) vs defrosted (DEF) vs lyophilized (LIO)) × 2 diets used as substrate (DF vs DC) × 4 animal inocula (*n*=4) × 2 analytical replicates plus 8 bottles used as positive controls. Incubation runs were conducted in two consecutive weeks and positive control bottles were used as described before. The day of the first incubation, rumen content (500mL) was extracted from each goat, filtered and divided into two samples: one was immediate incubated as fresh inocula (FRE), while the other sample was distributed into falcon tubes containing DMSO at 5% as cryopreservant (35mL total volume). These tubes were gently mixed and placed in a cooling water bath contain ice for 30 minutes (equilibrium time), prior being frozen at -80ºC for 7 days. Half of these tubes remained frozen and the rest were lyophilised to determine the DM content. The day of the second incubation run frozen inocula were defrosted (DEF) in a water bath at 39ºC for 5 min prior mixing with the incubation buffer under CO_2 bubbling. An equivalent amount of lyophilized rumen fluid (LIO) to 16.67mL of fresh rumen fluid was dispensed into the incubation bottles along with 16.67mL of distilled water and incubation buffer (33.3mL) to make 50mL of total volume. Cryopreservant was also spiked into FRE bottles to prevent potential bias across treatments. Incubation procedures were performed as described in experiment 2.

Sample analyses

Feed composition was measured as previously described (21). Methane concentration in the headspace samples was determined by gas chromatography (Waldbronn, Germany). Concentrations of individual VFA were determined by a GC system coupled with a Flame Ionization Detector (Auto-system Perkin-Elmer Cor., Connecticut, USA), while ammonia concentration was measured using a colorimetric method (22). Protozoal concentration and classification were determined by optical microscopy (23). DNA was extracted from frozen samples using a commercial kit (FavorPrep Stool DNA Isolation Mini Kit, Taiwan) and concentrations of total bacteria, methanogenic archaea, protozoa and anaerobic fungi were determined by quantitative PCR as previously described (24).

Calculations and statistical analyses

Organic matter fermentation of total hexoses (FOM) as well as metabolic hydrogen production and incorporation into fermentation products were stoichiometrically calculated (25). For gas production (GP), pressure measurements were adjusted for the amount of headspace available and converted to volume units using the ideal gas law. Then, cumulative GP data were fitted to the predictive equation described by France *et al.,* (26): $Y=A(1-e^{-ct})$ where *Y* (mL) is the cumulative GP at time *t* (h), *A* is the asymptotic or potential GP (mL) and *c* is the GP rate (µL h^{-1}). Data from analytical replicates were averaged and data from qPCR and protozoal counting were log transformed prior statistical analyses. Data from *in vivo* rumen pH monitoring were analysed by repeated measurements, while data from *in vitro* experiments were analysed by ANOVA as follows:

Experiment 1: $Y_{ijk} = \mu + D_i + B_j + G_k + e_{ijk}$

Experiment 2: $Y_{ilmk} = \mu + D_i + F_l + T_m + G_k + e_{ilmk}$

Experiment 3: $Y_{inok} = \mu + D_i + A_n + M_o + G_k + e_{inok}$

where *Y* is the dependent, continuous variable, μ is the overall population of the mean, D_i is the fix effect of the diet used as substrate (*i*= DC, DF), B_j is the fix effect of the incubation buffer (*j* = low, medium, high pH), F_l is the fix effect of the microbial fraction (*l* = LIQ, LS), T_m is the fix effect of the inoculum collection time (*m* = 0h, 3h after feeding), A_n is the fix effect of the inoculum adaptation to the diet (*n* = AF, AC), M_o is the fix effect of the inoculum preservation method (*o* = FRE, DEF, LIO), G_k is the random effect of the goat used as donor (*k* = 1, 2, 3, 4) and *e* is the residual error. When significant effects were detected means were compared by Fisher´s protected LSD test using the SPSS software (IBM Corp., Version 21.0, New York, USA). Significant effects were declared at $P < 0.05$ and tendencies to differences at $P < 0.10$.

RESULTS

Experiment 1: In vivo rumen pH monitoring and in vitro buffering capacity

In vivo monitoring of the rumen pH showed a significant effect of the diet ($P = 0.003$) and sampling time ($P = 0.011$). Rumen pH for DC was lower than observed for DF diets with the greatest

differences among diets observed between 2 and 4h after feeding (Figure 1). Experiment 1 showed that incubation of DC diet with rumen inoculum from animals adapted to such diet resulted in greater fermentation than those adapted to DF in terms of ammonia and total VFA concentrations, FOM, H production, asymptotic GP, and higher molar proportions of propionate and butyrate (Table 1). On the contrary, DF led to higher rumen pH and acetate molar proportion than DC diet. As expected, incubation pH was affected by the buffer used. The buffer which generated the highest pH led to the greatest *in vitro* fermentation in terms of ammonia, total VFA concentrations, FOM, H production and asymptotic GP without affecting the VFA profile.

(Figure 1 and Table 1 here)

Experiment 2: Effect of microbial fraction, collection time and fermentation substrate

No differences in the fermentation parameters were noted between the positive controls used in both incubation runs. Significant interactions were observed between the effect of the diet used as substrate and the sampling time. Thus, in Table 2 the fix effect of the rumen fraction and the interaction between diet and sampling time are depicted. The rumen fraction used as inoculum had a minor impact on rumen fermentation: LIQ fraction in comparison to LS tended to increase propionate molar proportion and to decrease the levels of anaerobic fungi and methane production per unit of FOM. On the contrary, the effects of diet and sampling time were more evident: the use of DC as substrate, in comparison to DF, promoted a greater fermentation extent in terms of higher levels of VFA and FOM, as well as higher asymptotic GP, GP rate, H production, H capture and methane emissions (mmol d^{-1}). This DC diet promoted a lower concentration of anaerobic fungi than DF diets but increased the protozoa levels measured either by qPCR or optical counting without affecting the relative proportions of the main protozoal groups. Regarding to the rumen sampling time, inocula taken 3h after feeding, in comparison to 0h, promoted a greater fermentation activity in terms of ammonia, total VFA, acetate molar proportion, FOM, asymptotic GP, GP rate, methane and H productions. Inocula sampled at 3h after feeding, in comparison to 0h, tended to increase the levels of bacteria and methanogens but lowered the levels of anaerobic fungi and protozoa

promoting favouring the growth of *Diplodiniinae* in detriment to *Entodiniinae*. The differences between DC and DF diets in terms of *in vitro* pH, propionate and *Isotricha* proportion were more obvious when the inocula was taken at 3h after feeding than before feeding, while the opposite was true for the molar proportion of isobutyrate, isovalerate, total protozoal counts (interaction D×T).

(Table 2 here)

Experiment 3: Effect of donor animal diet, fermentation substrate and inoculum preservation method

No differences in the fermentation parameters were detected between the positive controls across runs. Only two parameters showed significant interactions between the inoculum adaptation to the diet and the preservation method, thus in Table 3 results were presented for the fix effect of the diet used as substrate and the interaction between the inoculum adaptation and the preservation method. As mentioned before, DC diet promoted a greater microbial activity in terms of total VFA, FOM, GP rate, H production and H incorporation than DF diet. The microbial adaptation to the diet had a strong effect on the microbial activity *in vitro*: AC inocula, in comparison to AF, promoted higher levels of total VFA, FOM, asymptotic GP, GP rate, H production, H incorporation, propionate and butyrate molar proportions. On the contrary, AF inocula led to higher acetate molar proportions than AC inocula. Microbial adaptation to the diet and diet used as substrate did not show significant interactions. Anaerobic fungi reached their highest concentration when the AF inoculum was incubated with DF diets *in vitro*, while subfamily *Diplodiniinae* decreased their proportion when AF inoculum was incubated with DC diet (D×A).

Inocula preservation method was by far the factor with the greatest impact on the microbial activity reaching the highest values for FRE inoculum. Microbial activity substantially decreased when using DEF inocula while the LIO inoculum showed the lowest fermentative activity *in vitro*. This general pattern FRE > DEF > LIO was observed in terms of ammonia, total VFA, FOM, asymptotic GP, methane production H production, H incorporation and *in vitro* concentration of methanogens, anaerobic fungi and protozoa. Holotrich protozoa were the most negatively affected by the preservation

method, while the subfamily *Entodiniinae* was the most resilient. The effect of the preservation method was similar across substrates but was highly affected by the donor animal diet (A×M): the negative impact of the preservation was more evident when the inoculum was adapted to forage than to concentrate diets. Thus, the lowest *in vitro* microbial activity (total VFA, GP, methane, methanogens and protozoa) were observed when AF inocula were lyophilized.

(Table 3 here)

DISCUSSION

Experiment 1: In vivo rumen pH monitoring and in vitro buffering capacity

Rumen pH affects the rate and extent of microbial fermentation of feeds as a result of the balance between production and absorption of fermentation products and the saliva buffering capacity (27). The *in vivo* study of the postprandial pH fluctuations showed the greatest differences among diets occurred between 2 and 4h after feeding (fermentation peak), thus an intermediate time (3h after feeding) was considered as the most appropriate time for rumen sampling.

Different *in vitro* approaches based on batch cultures have been developed to simulate the pattern of *in vivo* microbial fermentation in the rumen (20, 28, 29). These systems have been designed for maintaining the incubation pH relatively high and constant (6.7 to 6.8) by including bicarbonate buffer with a minor proportion of phosphate buffer in the medium. However, most rumen microbes are only metabolically active under a specific pH range (3) which may limit the *in vitro* fermentation if incubation buffering capacity is exceeded. To address this problem, three different bicarbonate concentrations in the incubation buffer were used (19) to cover the pH range observed *in vivo*. Our results showed that lowering concentrations of bicarbonate promoted a progressive and concomitant decline in the pH and in the microbial activity. Consequently, a decrease in the VFA concentration (-9%) and *in vitro* GP (-19%) was noted when pH dropped from 6.5 to 5.8, and similar further reductions in VFA (-9%) and GP (-21%) were noted when pH declined from 5.8 to 5.4. These observations indicated that incubation pH affected the extent of the microbial fermentation across diets but kept similar VFA profile. More research is needed to reveal if this situation was also

associated to minor changes in the microbial community (3), but indicated that maximizing the incubation buffering capacity is recommended to prevent a buffer-related limitation of the microbial activity in further experiments.

Experiment 2: Effect of microbial fraction, collection time and fermentation substrate

Several studies have demonstrated substantial differences between the rumen microbiota associated to either the liquid or the solid phase in relation to their chemical and microbiological composition (30-32). Some authors suggested that liquid-associated bacteria have higher activity as a result of a greater access to soluble and easily digestible substrates (33). Nevertheless, a recent study demonstrated that the rumen bacterial community associated to the feed particles had higher levels of fibrolytic microbes (i.e. *Firmicutes*), bacterial diversity and established more complex microbial networks than liquid-associated bacteria (34). Theoretically, using rumen content (liquids plus solids) as inoculum for *in vitro* incubations should increase the microbial fermentation as a result of a combination of greater supply of fermentable material and solid-associated microbes. To discern between these two confounding factors, the LIQ inoculum was supplemented with autoclaved rumen solids in order to provide the proportional part of fermentable material but without viable solid-associated microbes. Results showed that LS inoculum led to higher *in vitro* concentrations of anaerobic fungi (+0.29 log units) and methane production per unit of FOM (+17%), possibly as a result of the anaerobic fungi chemotaxis toward solid material (35) and more active degradation of structural carbohydrates (36). However, it seems that the absence of viable solid-associated microbes in the LIQ inoculum was compensated by a greater abundance of liquid-associated bacteria resulting on similar microbial numbers and fermentation pattern than LS inoculum when incubated with an identical substrate. These findings are in line with a recent study (37) which revealed that rumen bacterial distribution in the rumen of Holstein cows determined by using Next Generation Sequencing was mainly affected by diet and individual cows rather than by the rumen fractions.

A diurnal variation in the availability of digestible nutrients in the rumen has been linked to the proliferation of rumen microbes, the accumulation of fermentation products and variations in the

rumen pH (38). In a study in which dairy cows were fed diets varying in protein and starch levels, it was noted than rumen samples taken at 2.5h after feeding, in contrast to those taken before feeding, had increased levels of fermentation products but lower microbial concentrations per unit of dry matter as a result of their dilution with feed (36). In our study, inocula taken 3h after feeding also lowered the incubation pH and increased the microbial fermentation in terms of VFA (+12%), ammonia-N (+17%), asymptotic GP (+24%) and GP rate (+11%) in comparison to inocula taken before feeding. This fermentative situation was associated to higher levels of bacteria (+0.25 logs) and methanogens (+0.23 logs) in detriment of slow growing microbes such as protozoa (-0.53 logs) and anaerobic fungi (-0.27 logs). Published literature shows important discrepancies in relation to the ideal rumen sampling time aiming to minimize the diurnal variation in the microbial activity (39), possibly because this microbial activity is determined as much by the time of rumen sampling relative to feeding as by feeding pattern, eating behaviour and other factors. In order to minimize the inocula variability across different donor animals, rumen sampling before feeding is generally recommended for *in vitro* feed evaluation studies because the microbial activity and diversity of the inoculum are at their lowest (16). However, this sampling strategy may not capture the "true potential" that a nutritional strategy (e.g. rumen microbiota as probiotics) may have if a more diverse and active microbiome is sampled after feeding as was proven in the present study.

Experiment 3: Effect of donor animal diet, fermentation substrate and inoculum preservation method

Results indicated that the diet consumed by the donor animal highly determined the microbial activity in the inoculum, but did not affect neither methane emissions nor the abundance of the main microbial groups, including protozoa. Thus, the importance of using diet-adapted rumen inoculum to the same substrate used *in vitro* is still debatable (A×D interaction) (16). A study noted a higher fermentation rate of native potato starch *in vitro* when the rumen inocula were obtained from donor animals adapted to a diet containing potato in contrast to non-diet-adapted rumen inocula (40). Thus, feeding donor animals a similar diet to the substrate used *in vitro* may be advantageous to

prevent carry over effects in feed evaluation studies (16). Nevertheless, our study revealed a lack of interaction between the inoculum adaptation and the diet used *in vitro* suggesting that the effect of the adaptation was substrate-independent. Several studies have noted that *in vitro* VFA production, bacterial concentration and degradability parameters of various feeds increased by raising the proportion of concentrate in the diet consumed by the donor ruminant (41, 42). As a result, it has been hypothesised that using an inoculum with high microbial activity can result in a significant proportion of degraded carbohydrates been transformed into fermentation products and CO_2 gas rather than incorporated into new microbial matter (43). This hypothesis was supported by our findings because AC inocula, in contrast to AF inocula, led to a greater yield of fermentation products, in terms of VFA (+19%), asymptotic GP (+16), GP rate (+26%) as well as a higher propionate production (+16%), but without differences in the microbial numbers after 24h of incubation. Two studies have shown similar positive effects on productivity when young animals were inoculated with fresh rumen fluids regardless of the diet consumed by the donor animal (7, 44). However, this factor had long lasting effects on the structure of the rumen microbial community of the inoculated animals (10).

More controversy appears regarding the ideal preservation method for maintaining the microbial activity: Cone *et al.* (45), after storing rumen inocula either at 39ºC or -20ºC for different time periods, reported similar *in vitro* GP values for rumen inocula stored for up to 4h, but decreased for longer storage times and freezing procedures. In our experiment the greatest values of microbial fermentation and activity also were detected when FRE inoculum was used *in vitro*, possibly as a result of the highest concentrations of the main microbial groups (i.e. methanogens, anaerobic fungi and protozoa). The use of DEF inoculum led to moderate decreases in the *in vitro* microbial activity in terms of VFA (-14%), asymptotic GP (-13%) and H production (-19%). Moreover, a substantial decrease in the ammonia-N levels (-96%) resulted in values below 50 mg L^{-1}, which is considered the minimum concentration required for optimal microbial growth *in vitro* (46), and could partially explain the lower microbial numbers observed in DEF inocula. Our findings agree with previous

studies which noted that freezing the rumen inocula (without cryopreservant) implied a delay in the initiation of the fermentation (47) and negatively affected the *in vitro* fermentation of fibrous feedstuff (-13%), but not that of starch as substrate (17, 48). Eukaryotic cells and gram-negative bacteria involved in fibre degradation (i.e. *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* or *Butyrivibrio fibrisolvens*) are particularly sensitive to mechanical damage caused by ice crystals and disruption of cell membranes, however cryopreservants can partially overcome this problem (49). Glycerol is one of the most common cryopreservants used in microbiology, however it can be rapidly used as an energy source by rumen bacteria once the sample has been thawed, originating an increase in the VFA production and propionate molar proportion. Such indirect artefact may bias the results (47). To avoid this inconvenient DMSO was used, despite its toxicity, to freeze the inoculum because it has been shown that promotes similar *in vitro* digestibility than fresh inocula (50), and allows higher protozoal recovery rate (90% after six months storage) than glycerol or ethylene glycol (51). Since the toxicity of DMSO to cells increases with temperature, a sample equilibration step at 4ºC was used to maximize its effectiveness (52). Despite these considerations, our freezing-thawing procedure had an important negative impact on the rumen eukaryotic cells such as anaerobic fungi (-0.14 logs) and protozoa cells (-0.70 logs), being holotrich protozoa particularly vulnerable to freezing (-86%). These results on protozoal impact should be carefully interpreted due to the intrinsic methodological limitations to study rumen protozoa as a result of their slow growth *in vitro* and their variable number of 18S rDNA copies per cell across species (53).

The use of DEF inocula favoured the proliferation of total bacteria (+24 logs) which may explain the observed increase in the propionate percentage (+25%), possibly because rumen amylases are more resistant to freezing than cellulases or xylanases (54). The release of bacteria attached to the feed particles has been considered to justify the higher values of viable colony counts in rumen fluid stored at 0ºC for 8h compared to fresh rumen fluid (55). However, the higher bacterial concentration observed when DEF (+0.24 logs) and LIO inocula (+0.08 logs) were incubated *in vitro*, in comparison to FRE inocula, was most likely due to: i) a higher bacterial recovery rate after the preservation in

comparison to other microbial groups (49) and ii) a greater bacterial growth during the *in vitro* incubation due to a lower competition with other microbial groups such as rumen protozoa (53, 56). A study which compared different preservation methods of rumen inoculum (47) concluded that cryopreservants are not required if the freezing procedure is carried out quickly using liquid N and a high container surface to sample volume ratio, resulting on similar *in vitro* gas production, VFA and bacterial diversity in fresh and defrosted inocula. Unfortunately, this later study did not assess the impact of the preservation method on the eukaryotic cells which are considered the most vulnerable rumen microbes (52).

Rumen fluid lyophilisation has been explored as an alternative to standardize *in vitro* studies by decreasing the variation inherent to the technique and to eliminate the need of constant access to donor animals. Results on the viability of rumen microbes after lyophilisation are highly variable across studies depending on the freezing method, use of cryopreservant, storage conditions and microbial group considered (57-59). Our study showed that lyophilisation had a strong negative impact on microbial activity in terms of VFA (-51%), ammonia-N (-96%), H production (-51%) and asymptotic GP (-19%) in comparison to FRE inocula. Moreover, the lower GP rate (-69%) indicated a substantial delay in the re-activation of the rumen microbiome which led to lower values of methanogens (-1.71 logs), anaerobic fungi (-0.18 logs) and nearly the complete wipe out of rumen protozoa (-2.66 logs). Subfamily *Entodiniiane* was the protozoa group which better dealt with the lyophilisation process, possibly as a result of their small size (55). These findings justify the less positive effects of inoculating lyophilized rumen fluid (14), in comparison to fresh inoculum (9), to growing lambs in terms of N retention and growth. Our study showed a substantial interaction between the rumen microbial adaptation to the diet and the preservation method considered: in general FRE and LIO procedures had less damaging effects if rumen microbes were adapted to concentrate than to forage diets. This interaction may rely on the different community structure and lower bacterial and protozoal diversity generally reported in the rumen of animals fed high concentrate diets (36, 60). Further microbial adaptation processes based on the acceleration of the

metabolic rate, energy spilling reactions and accumulation of starch particles have been described in presence of high levels of available energy (61). These adaptation strategies could help rumen microbes to better prepare their metabolism for further damaging processes, being the protective effect on the microbes proportional to the severity of the preservation method.

CONCLUSIONS

This study revealed that fresh rumen fluid sampled at 3h after feeding from donor animals fed a high concentrate diet led to the greatest microbial numbers and *in vitro* fermentation rates. Thus, this inoculum is suggested to be the choice if the aim is to provide the most active rumen microbiota. Alternatively, defrosted rumen inoculum could be used, but taking in consideration that a moderate decrease in the microbial activity but substantial in terms of rumen protozoa and anaerobic fungi may occur. On the contrary, lyophilized rumen inoculum promoted a substantial delay in the microbial reactivation process and a low microbial activity which could compromise its effectiveness as inoculum. This study also noted that the adaptation of the donor animal to the diet used as fermentation substrate provided no advantages in comparison to non-adapted inocula. Although, similar experiments are still needed to proof these results in different ruminant species and under *in vivo* conditions, these findings should be considered in the design of further studies.

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Table 1. Effect of the diet used as substrate and incubation buffer on the rumen microbial activity *in vitro* (Experiment 1)

Diet	DC			DF				P-values		
Buffer	Low pH	Medium pH	High pH	Low pH	Medium pH	High pH	SED[b]	D	B	DxB
FERMENTATION										
pH	5.19	5.51	6.32	5.68	6.08	6.64	0.131	**	***	ns
Ammonia-N (mg L^{-1})	435	322	661	211	192	276	118.9	*	**	ns
Total VFA (mmol L^{-1})	80.5	91.3	103.1	66.4	70.5	75.3	9.890	†	***	ns
Acetate (mmol mol^{-1})	544	538	538	699	702	699	22.90	***	ns	ns
Propionate (mmol mol^{-1})	230	230	230	160	161	169	20.10	**	ns	ns
Butyrate (mmol mol^{-1})	161	162	154	106	102	95.9	11.50	***	ns	ns
Isobutyrate (mmol mol^{-1})	12.8	14.1	17.1	10.3	10.4	10.8	2.556	†	ns	ns
Valerate (mmol mol^{-1})	32.1	34.3	32.6	11.2	11.0	11.3	4.770	***	ns	ns
Isovalerate (mmol mol^{-1})	20.1	22.8	28.1	13.2	13.6	14.2	4.360	*	ns	ns
FOM[a] (mg)	422	476	529	327	346	367	51.30	*	**	ns
GAS PRODUCTION										
Asymptotic GP (mL)	82.7	106	130	50.2	62.8	77.8	12.55	**	***	ns
GP rate (µL h^{-1})	147	139	139	132	114	94.9	18.38	ns	*	ns
H production[a] (mmol)	8.32	9.41	10.44	6.69	7.08	7.48	1.057	*	*	ns

[a]Data stoichiometrically calculated based on the VFA production (25)

[b]Standard error of the difference among means (*n*=4) for the interaction among diets (D: DC concentrate vs DF forage diet) and buffers (B: Low vs Medium vs High pH).

Within a row means without a common superscript differ (P<0.05). *** *P*<0.001, ** *P*<0.01, * *P*<0.05, † *P*<0.10, ns: not significant.

Table 2. Effect of the microbial fraction used as inoculum, collection time and diet used as substrate on the rumen microbial activity *in vitro* (Experiment 2).

Treatments	Fraction		Time post-feeding &diet					P-values						
			0h		3h									
	LIQ	LS	DC	DF	DC	DF	SED[b]	D	F	T	D×F	D×T	F×T	D×F×T
FERMENTATION														
pH	6.38	6.25	6.38[b]	6.75[a]	5.63[c]	6.51[ab]	0.204	**	ns	***	ns	**	*	ns
Ammonia-N (mg L^{-1})	237	296	296	195	319	256	57.70	ns	ns	†	ns	ns	**	ns
Total VFA (mmol L^{-1})	95.9	93.5	98.2	80.8	109	90.8	9.040	**	ns	*	ns	ns	ns	ns
Acetate (mmol mol^{-1})	642	656	578	665	636	719	20.10	**	ns	***	ns	ns	ns	ns
Propionate (mmol mol^{-1})	191	181	197[a]	187[a]	197[a]	165[b]	16.03	ns	†	†	ns	*	†	ns
Butyrate (mmol mol^{-1})	123	123	151	101	142	98.1	11.91	**	ns	ns	ns	ns	ns	ns
Isobutyrate (mmol mol^{-1})	11.5	9.94	18.9[a]	14.2[b]	4.81[c]	4.88[c]	1.569	ns	***	***	ns	***	***	ns
Valerate (mmol mol^{-1})	13.1	13.0	22.3	13.4	10.8	5.75	2.079	**	ns	***	ns	†	ns	ns
Isovalerate (mmol mol^{-1})	18.7	16.9	33.5[a]	20.2[b]	9.56[c]	7.94[c]	3.407	*	ns	***	ns	***	*	ns
FOM[a] (mg)	479	467	496	393	558	446	45.20	**	ns	*	ns	ns	ns	ns
GAS PRODUCTION														
Asymptotic GP (mL)	198	207	206	156	252	197	22.52	*	ns	***	ns	ns	ns	ns
GP rate (µL h^{-1})	58.2	63.2	63.8[b]	51.3[c]	76.0[a]	51.5[c]	6.780	***	ns	†	ns	ns	*	ns
Methane (mmol L^{-1})	5.77	6.04	5.83	5.29	6.51	5.99	1.166	†	ns	**	ns	ns	ns	ns
Methane (mmol d^{-1})	0.86	0.95	0.78	0.65	1.18	1.01	0.198	*	ns	***	ns	ns	ns	ns
Methane (mmol g FOM^{-1})	1.72	2.01	1.56	1.69	2.19	2.30	0.340	†	*	***	ns	ns	ns	ns
H produced[a] (mmol)	9.65	9.47	9.94	7.92	11.25	9.13	0.902	**	ns	*	ns	ns	ns	ns
H incorporated[a] (mmol)	4.17	4.07	4.66	3.20	5.12	3.50	0.512	***	ns	ns	ns	ns	ns	ns
H incorporation rate (mmol mol^{-1})	424	425	465	405	458	386	20.65	***	ns	ns	ns	ns	ns	ns
MICROBES (log copy g DM^{-1})														
Bacteria	11.9	11.9	11.8	11.7	12.2	11.9	0.304	ns	ns	†	†	ns	ns	ns
Methanogens	8.78	8.59	8.55	8.59	8.86	8.74	0.241	ns	ns	†	ns	ns	ns	ns
Anaerobic fungi	6.79	7.08	6.84	7.57	6.45	6.90	0.204	**	**	***	†	ns	ns	ns
Protozoa	9.70	9.69	10.2	9.47	9.69	9.43	0.280	*	ns	†	ns	ns	ns	ns
PROTOZOAL COUNTS														
Total (log cells mL^{-1})	4.73	4.72	5.37[a]	4.69[b]	4.43[c]	4.43[c]	0.181	†	ns	***	ns	***	ns	ns
Subf. Entodiniinae (%)	87.3	86.6	92.6	88.6	80.3	86.3	10.79	ns	ns	*	ns	†	ns	ns
Subf. Diplodiniinae (%)	4.28	5.50	1.93	5.28	6.13	6.22	3.351	ns	ns	*	†	ns	ns	ns
Ophryoscolex (%)	2.45	2.86	0.88	0.03	9.65	0.07	6.600	ns	ns	†	ns	ns	ns	ns
Isotricha (%)	0.80	0.52	0.82[a]	0.70[a]	0.03[b]	1.09[a]	0.518	ns	ns	ns	ns	*	ns	ns
Dasytricha (%)	5.21	4.50	3.80	5.41	3.88	6.32	4.369	ns	ns	ns	ns	ns	ns	ns

[a]Data stoichiometrically calculated based on the VFA production (25)

[b]Standard error of the difference among means (n=4) for the interaction among diets (D: DC concentrate, DF forage), microbial fraction (F: LIQ liquid vs LS liquid + solid) and sampling time (T: 0h vs 3h after feeding). Within a row means without a common superscript differ (P<0.05). *** *P*<0.001, ** *P*<0.01, * *P*<0.05, † *P*<0.10, ns: not significant.

Table 3. Effect of the donor animal diet, diet used as substrate and preservation method on the rumen microbial activity *in vitro* (Experiment 3)

Treatments	Diet		Inoculum adaptation & preservation							P-values						
			FRE		DEF		LIO									
	DC	DF	AC	AF	AC	AF	AC	AF	SED[b]	D	A	M	D×A	D×M	A×M	D×A×M
FERMENTATION																
pH	6.34	6.54	6.00	6.56	6.27	6.71	6.37	6.75	0.171	***	*	***	ns	ns	†	ns
Ammonia-N (mg L^{-1})	125	129	348	359	12.8	12.4	13.4	15.9	59.60	ns	ns	***	ns	ns	†	ns
Total VFA (mmol L^{-1})	109	103	149^{a}	122^{c}	131^{b}	102^{d}	88.2^{e}	45.2^{f}	14.99	*	†	***	ns	ns	*	ns
Acetate (mmol mol^{-1})	607	627	568^{c}	660^{b}	537^{cd}	657^{b}	527^{d}	754^{a}	26.49	†	***	**	ns	ns	***	ns
Propionate (mmol mol^{-1})	216	206	199^{b}	186^{b}	264^{a}	217^{b}	265^{a}	137^{c}	19.85	ns	**	**	ns	ns	***	ns
Butyrate (mmol mol^{-1})	123	114	161	107	130	81.0	152	79.4	27.83	ns	**	*	ns	ns	ns	ns
Isobutyrate (mmol mol^{-1})	9.71	9.90	13.6^{a}	11.3^{b}	11.8^{b}	9.44^{c}	5.38^{e}	7.38^{f}	1.550	ns	ns	***	ns	†	***	ns
Valerate (mmol mol^{-1})	27.3	25.4	31.9^{b}	18.3^{c}	33.8^{b}	18.6^{c}	42.9^{a}	12.6^{c}	7.960	ns	*	ns	ns	ns	**	ns
Isovalerate (mmol mol^{-1})	16.3	17.5	26.1^{a}	17.3^{b}	22.9^{a}	16.9^{b}	8.00^{c}	10.1^{c}	4.640	ns	ns	***	ns	ns	**	ns
FOM[a] (mg)	554	517	772^{a}	603^{c}	662^{b}	491^{d}	467^{d}	218^{e}	71.49	**	*	***	ns	ns	*	ns
GAS PRODUCTION																
Asymptotic GP (mL)	178	173	235^{a}	156^{c}	198^{b}	143^{c}	179^{b}	140^{c}	26.01	ns	*	***	ns	**	*	**
GP rate (µL h^{-1})	83.1	71.1	117^{a}	78.4^{b}	125^{a}	81.1^{d}	37.3^{c}	23.3^{c}	15.32	*	*	***	ns	ns	*	ns
Methane (mmol L^{-1})	4.04	4.18	6.24	5.75	5.18	5.34	0.83	1.32	1.016	ns	ns	***	ns	ns	ns	ns
Methane (mmol d^{-1})	0.64	0.58	1.26^{a}	0.71^{bc}	0.91^{b}	0.60^{c}	0.10^{b}	0.06^{d}	0.226	ns	ns	***	ns	ns	*	ns
Methane (mmol g FOM^{-1})	1.01	0.94	1.59	1.17	1.35	1.23	0.19	0.30	0.284	ns	ns	***	ns	ns	ns	ns
H produced[a] (mmol)	10.9	10.3	15.4^{a}	12.2^{b}	12.7^{b}	9.72^{c}	8.95^{c}	4.51^{d}	1.347	**	*	***	ns	ns	*	ns
H incorporated[a] (mmol)	5.14	4.55	7.54	4.74	6.98	4.01	4.65	1.15	0.857	**	**	***	ns	ns	ns	ns
H incorporation rate (mmol mol^{-1})	444	420	489^{b}	389^{c}	543^{a}	412^{c}	501^{b}	256^{d}	33.90	*	***	***	ns	ns	***	ns
MICROBES (log copy g DM^{-1})																
Bacteria	12.8	12.8	12.9	12.5	13.1	12.8	12.9	12.6	1.309	ns	ns	**	ns	ns	ns	ns
Methanogens	8.00	7.96	8.61^{ab}	8.46^{b}	8.51^{ab}	8.66^{a}	7.15^{c}	6.49^{d}	0.189	ns	ns	***	ns	ns	***	ns
Anaerobic fungi	7.54	7.57	7.56^{b}	7.76^{a}	7.54^{b}	7.50^{bc}	7.46^{c}	7.49^{bc}	0.083	ns	ns	***	*	ns	**	**
Protozoa	8.52	8.42	9.40	9.50	7.82	8.69	7.58	7.81	0.573	ns	ns	***	ns	ns	†	ns
PROTOZOAL COUNTS																
Total (log cells mL^{-1})	3.62	3.75	4.71^{a}	4.90^{a}	4.05^{b}	4.17^{b}	2.68^{c}	1.61^{d}	0.615	ns	ns	***	ns	*	***	*
Subf. Entodiniinae (%)	91.9	89.4	84.5	88.0	91.5	94.5	90.4	96.9	7.050	†	ns	**	†	ns	ns	ns
Subf. Diplodiniinae (%)	4.37	6.02	4.52^{bc}	5.84^{ab}	5.38^{ab}	5.14^{b}	7.97^{a}	1.25^{c}	4.505	**	ns	ns	*	ns	***	ns
Ophryoscolex (%)	0.70	0.53	1.58	0.21	1.13	0.13	0.45	0	0.752	ns	†	ns	ns	ns	ns	ns
Isotricha (%)	0.18	0.71	0.45	1.31	0.25	0.09	0.52	0	0.726	*	ns	†	ns	ns	†	ns
Dasytricha (%)	2.83	3.38	8.92	4.65	1.76	0.10	0.63	1.88	3.862	ns	ns	**	ns	ns	ns	ns

[a]Data stoichiometrically calculated based on the VFA production (25)

[b]Standard error of the difference among means (*n*=4) for the interaction among diets (D: DC concentrated vs DF forage), inoculum adaptation (A: AC concentrate vs AF forage) and preservation method (M: FRE fresh vs DEF defrosted vs LIO lyophilized). Within a row means without a common superscript differ (P<0.05). *** *P*<0.001, ** *P*<0.01, * *P*<0.05, † *P*<0.10, ns: not significant.

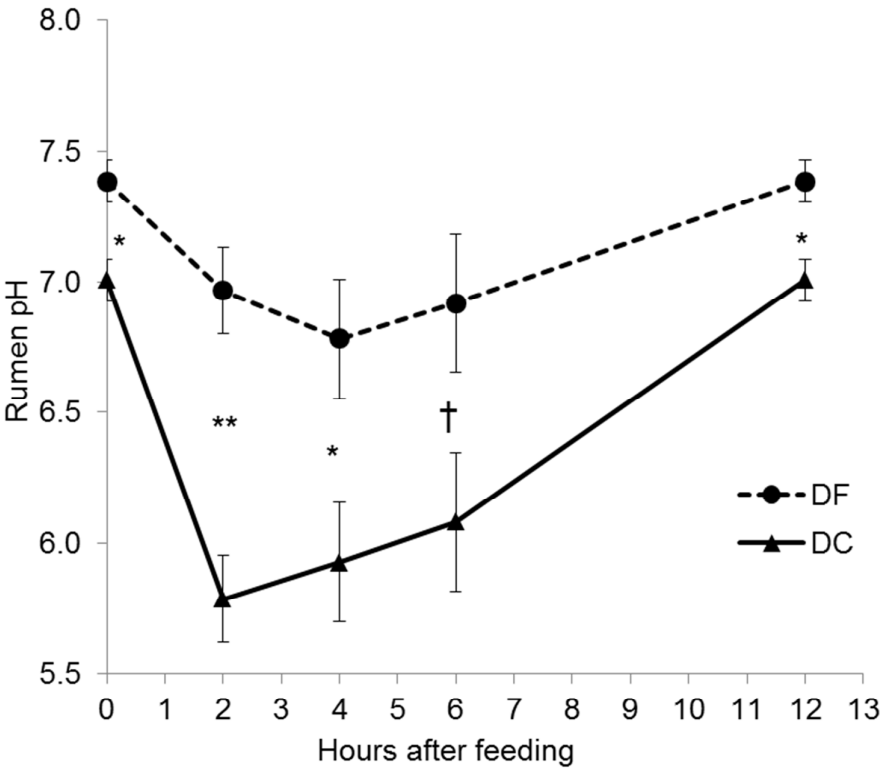

Figure 1. Dynamics of the rumen pH after feeding in goats fed forage (DF) vs concentrate (DC) based diets. ** P<0.01, * P<0.05, † P<0.10.