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Influence of rumen contents' processing method on microbial populations in the fluid and subsequent *in vitro* fermentation of substrates of variable composition

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ABSTRACT

The *in vitro* batch culture technique is being increasingly used to study rumen fermentation, but the results are affected by several factors, being the source of the inoculum one of the most relevant. This work was conducted to assess the effects of different processing methods of ruminal contents on microbial populations in the obtained fluid, and its influence on fermentation parameters when the fluid was used as inoculum for *in vitro* incubations. Rumen contents were obtained from four rumen-fistulated sheep fed a 2:1 alfalfa hay:concentrate diet and subjected to the following treatments: SQ: squeezed through four layers of cheesecloth; FL: SQ treatment and further filtration through a 100-μm nylon cloth; STO: blended for three min at 230 rev min^{-1} in a Stomacher® and further filtrations as in SQ. Microbial populations' abundance and bacterial diversity in the ruminal fluids were analysed by quantitative PCR (qPCR) and automated ribosomal intergenic spacer analysis (ARISA), respectively. Three forages (alfalfa hay, grass hay and barley straw) were incubated *in vitro*, either alone or mixed with concentrate (1:1), using each of the ruminal fluids as inoculum. There were no differences between SQ and FL methods in any of the microbial populations analysed, but STO increased the relative abundance of *Fibrobacter succinogenes* and *Ruminococcus albus* ($P < 0.05$) and decreased ($P < 0.05$) the concentration of protozoal DNA compared with SQ. Bacterial diversity was not affected ($P > 0.05$) by the processing methods. There were no interactions ($P > 0.05$) between the processing method and the characteristics of the substrates for any of the fermentation parameters analysed. Compared with SQ, the STO method resulted in greater ($P < 0.05$) methane production and ammonia-N concentrations in 8 h incubations. After 24 h of incubation, the use of STO inoculum increased ($P < 0.05$) methane production and dry matter degradability compared with SQ, with no differences in the rest of the parameters.

Abbreviations: ARISA, automated ribosomal intergenic spacer analysis; CCA, canonical correspondence analysis; FC, forage:concentrate; FL, squeezed through four layers of cheesecloth and further filtration through a 100-μm nylon cloth; OTUs, operational taxonomic units; qPCR, quantitative PCR; SQ, squeezed through four layers of cheesecloth; ST, blended for three min at 230 rev min^{-1} in a Stomacher® before being squeezed through four layers of cheesecloth; TDMD, true dry matter digestibility.

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No differences between SQ and FL methods were detected in any parameter. The results show that stomaching the rumen contents prior to inoculation of *in vitro* cultures modified some microbial populations, but had only subtle effects on fermentation parameters.

1. Introduction

The *in vitro* batch culture technique is being increasingly used to study ruminal fermentation by incubating substrates in buffered rumen fluid and measuring the main fermentation parameters, such as the concentration of ammonia-N and the production of volatile fatty acid (VFA) and methane. However, *in vitro* fermentation characteristics are influenced by many factors, such as the source, activity and preservation method of the inoculum, characteristics of the incubated substrate, the incubation medium, and the equipment used, among many others (Rymer et al., 2005; Mould et al., 2005; Yáñez-Ruiz et al., 2016). The diet of the donor animals and the sampling time of rumen contents have been identified as main factors affecting the fermentation characteristics in the *in vitro* batch culture technique (Mould et al., 2005; Martinez et al., 2010; Mateos et al., 2015), but another relevant factor is the processing method, as microbial populations differ between the solid and liquid phases of rumen contents and different substrates are more or less sensitive to fermentation by more or less strictly anaerobic bacterial species.

Some studies have analysed the effects of using different combinations of solid and liquid rumen contents as inoculum for *in vitro* incubations, but most of this work has focused on gas and VFA production and has produced contrasting results. Lee et al. (2004) observed that using solid-associated bacteria as inoculum for *in vitro* incubations of different substrates increased the amount of gas and VFA produced after 72 h of fermentation compared with the use of an inoculum of only liquid-associated bacteria. In contrast, Rymer et al. (1999) and Bueno et al. (2005) found no effects of the inclusion of solid-associated bacteria in the inoculum. The research on the influence of the processing method of rumen contents on microbial populations in the obtained fluid is limited to bacterial and protozoal populations (Senshu et al., 1980; Mackie et al., 1983; Fliegerova et al., 2014), and there is no information on other microbial populations which play an important role in the fermentative process, such as fungi and methanogenic archaea. Moreover, to our knowledge the influence of the processing method on both the microbial populations in the fluid and subsequent *in vitro* fermentation characteristics has not yet been addressed.

The aim of this study was therefore to evaluate the effect of three different processing methods of rumen contents on the microbial populations (total and cellulolytic bacteria, protozoa, fungi and methanogenic archaea) and bacterial diversity in the fluid obtained, and to assess their influence on the *in vitro* fermentation characteristics when the fluid was used to inoculate batch cultures. Six substrates of variable composition were used as it is likely that some processing methods will have a greater influence than others on the *in vitro* fermentation of a substrate. Our hypothesis was that the fermentation of fibrous substrates would be more affected by the inclusion of solid-associated bacteria in the inoculum than that of starchy substrates. Moreover, the study examined the effects of the processing method at two incubation times (8 and 24 h) because it was hypothesized that any changes in fermentation due to differences in the microbial populations in the ruminal fluids would become less marked as incubation time increased. Finally, the variability among individual donor animals in microbial populations in the obtained rumen fluids was also assessed.

2. Materials and methods

2.1. Donor animals and feeding

Four adult rumen-fistulated sheep (64.5 ± 2.10 kg body weight) were used as donors of ruminal contents. Animals were housed in individual pens with free access to water and a mineral-vitamin mixture. Sheep were fed a 2:1 mixed diet of lucerne hay and a commercial concentrate at energy maintenance level (ARC, 1984) distributed in two equal meals. The diet contained 913, 168, 426 and 269 g of organic matter, crude protein, neutral detergent fibre (aNDF) and acid detergent fibre (ADF) per kg of dry matter (DM), respectively. Sheep management and rumen content withdrawal were carried out 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) in line with the European regulations. All the experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of León.

2.2. Processing methods of ruminal digesta

The experimental treatments and *in vitro* incubations were conducted on four different days, and the rumen digesta of one sheep was used in each of them. Samples (mix of liquid and solid) from the dorsal, central and ventral regions of the rumen were collected from one sheep immediately before the morning feeding with a pair of tongs inserted through the rumen cannula to form one composited sample (about 600 g). Rumen contents were placed into thermos flasks pre-heated at 39 °C by filling with hot water and transferred to the laboratory within 15 min. Rumen digesta was divided into three