Use of real-time polymerase chain reaction assay for monitoring *in vitro* ruminal cellulolytic bacteria population as affected by non-structural carbohydrates

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Introduction Cellulose is the most abundant polymer in nature, but mammals do not synthesize enzymes to digest cellulose. Mosoni *et al* (2007) demonstrated that the dynamics of cellulolytic bacteria were in good correlation with the response to diet shift, particularly the changes of concentrate. The aim of the present study was to determine the effect of adding of non-structural carbohydrates (NSC) including sucrose, starch and equal mixture of them to an *in vitro* medium containing cellulose (Cell) as sole nutrient on both ruminal total anaerobic bacteria population and two major cellulolytic bacterial species (*Fibrobacter succinogenes* and *Ruminococcus Albus*) using SYBR Green real-time polymerase chain reaction (PCR) assay.

Material and methods Experimental treatments consisted of non-supplemented Cell (150 mg) and Cell plus NSC (70 mg) as sucrose (CellSu) or starch (CellSt) or a 1:1 mixture of sucrose and starch (CellSuSt). Treatments were incubated in a 40% rumen fluid medium prepared as described by Arroquy *et al*. (2005). Forty five ml of medium were distributed into a 100 ml bottle. Then, each bottle was inoculated with 5 ml of strained rumen fluid, taken from 3 sheep before the morning feeding, and finely bubbled with CO_2. The bottles (three bottles per each treatment) were incubated under anaerobic conditions for 48 h at 39 ºC. Then, each bottle contents were filtered through a 22 µm filter, and liquid phase was used for DNA extraction. DNA was extracted from the samples using the QIAamp® DNA stool mini kit (Qiagen Ltd, Crawley, West Sussex, UK) following the manufacturer's instructions. The designed 16s rRNA gene-targeted primer sets used for the real-time PCR are described in Table 1. Cellulolytic bacterial rDNA concentrations were measured by real time PCR relative to total bacteria amplification (ΔΔCt). PCR conditions for all species were as follows: 15 s at 95°C for denaturing, 15 s at 61°C for annealing and 30 s at 72°C for extension (40 cycles), except for 5 min denaturation in the first cycle. Data are expressed relative to quantification of the total bacterial population. Data were analyzed as a complete randomized design using GLM procedure of SAS (2003). Model was: Y = Mean + Treatment + residual.

Table 1 PCR primers for real-time PCR assay

Target species	Forward primer	Reverse primer	Size (bp)
Total bacteria	5′-GTGSTGCAYGGYTGTCGTCA-3′	5′-ACGTCRTCCMCACCTTCCTC-3′	120
Fibrobacter succinogenes	5′-GTTCGGAATTACTGGGCGTAAA-3′	5′-CGCCTGCCCCTGAACTATC-3′	175
Ruminococcus Albus	5′-CCCTAAAAGCAGTCTTAGTTCG-3′	5′-CCTCCTTGCGGTTAGAACA-3′	122

Results Table 2 shows the population of the *in vitro* target cellulolytic and total bacteria in the medium containing cellulose while their responses to supplementing NSC enumerated by the real-time PCR assays. The results of the present study showed that adding different types of NSC did not have any significant effect on F. succinogenes and R. Albus as representatives of cellulolytic bacteria. However, it tended (P=0.09) to increase the total bacteria population.

Table 2 *In vitro* DNA concentration of total bacteria and the population of the cellulolytic bacteria relative to total bacteria in the medium containing cellulose and different types of non-structural carbohydrates

Bacteria	Treatments				s.e.m	P
	Cell	CellSu	CellSt	CellSuSt		
Total bacteria (ng/µl)	0.0200	0.0226	0.0297	0.0557	0.0094	0.09
Fibrobacter succinogenes	0.3992	0.5691	0.5612	0.5152	0.0940	0.58
Ruminococcus Albus	0.0191	0.0218	0.0216	0.0118	0.0099	0.87

Conclusions Generally, treatments used in the present study had no significant effect on ruminal cellulolytic bacteria population relative to the total bacteria. Similarly, the use of competitive PCR to quantify cellulolytic bacterial species did not clearly demonstrate that high energy diets could result in a decrease in the cellulolytic bacterial populations (Koike and Kobayashi 2001). Martin *et al.* (2002) argued that this type of diet affected the fibrolytic enzyme activities more than the number of cellulolytic bacteria. However, there is a need to evaluate the effect of type of NSC on these bacteria under *in vivo* condition.

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https://doi.org/10.1017/S2040470010003924 Published online by Cambridge University Press