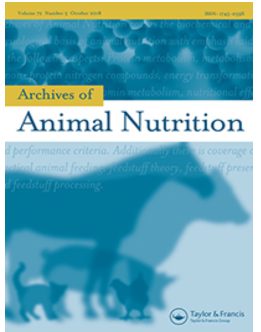

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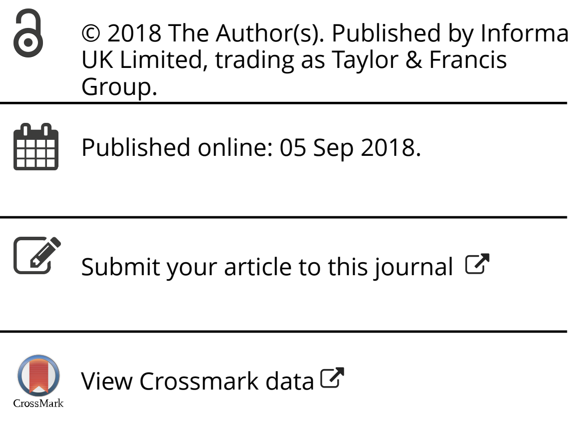

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Effect of dietary nitrogen level and source on mRNA expression of urea transporters in the rumen epithelium of fattening bulls

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ABSTRACT
This paper aims to study the effect of the dietary treatments on mRNA expression of urea transporter B (UT-B) and some aquaporins (AQP) in rumen epithelium of Italian Simmental young bulls. Eighty animals allocated to 16 pens were fed from about 500 to 650 kg body weight with four experimental diets, which resulted from the combination of two crude protein levels (125 and 110 g/kg dry matter, diets M and L, respectively) and two nitrogen sources (soybean meal (SBM) or SBM partly replaced by an isonitrogenous mixture of corn and urea; diets −U and +U, respectively). At slaughtering samples of blood and rumen epithelium were collected from six bulls for each diet. Blood samples were analysed for haematological parameters and quantitative PCR was carried out on the mRNA extracted from the rumen epithelium samples. The bulls fed diets M had lower plasma concentrations of aspartate aminotransferase than those receiving diets L (78.9 vs. 88.3 U/l, $p = 0.04$). Plasma urea was higher ($p = 0.03$) for diets M and lower for diets +U (2.0 vs. 2.5 and 1.73 vs. 2.00 mmol/l, respectively, in M and L diets, $p = 0.04$). The effect of dietary treatments on rumen UT expression were limited to AQP3, which was down regulated ($p = 0.01$) in diets +U. Finally, a high positive correlation ($R^2 = 0.871$) between the expressions of AQP7 and AQP10 was found. In conclusion, the AQP3 appears very responsive to dietary treatments and therefore it is a candidate to be further studied in rumen metabolism experiments. The close relationship between mRNA expression of AQP7 and AQP10 indicates a similar function of these two proteins.

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Aquaporins; bulls; dietary protein; dietary urea; fattening; rumen epithelium; urea transporters

1. Introduction

Ruminants recycle the blood urea into the rumen, where bacterial urease catalyses the hydrolysis of urea into carbon dioxide and ammonia (Jin et al. 2018). The recycling accounts from 40 to 80% of blood urea and is a system of N salvage to increase the nutritional efficiency, particularly in conditions of dietary N shortage (Lapierre and Lobley 2001). Then, rumen bacteria can use ammonia to synthesise amino acids and peptides for their own growth.

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Blood urea crosses the rumen mucosa by simple diffusion, but also facilitative urea transporters (UTs) mediate the movement of urea down a concentration gradient (Stewart et al. 2005; Abdoun et al. 2006). First groups of UT are derived from the UT-A and UT-B genes (Stewart et al. 2005) and UT-B mRNA or protein expressions are involved in the transport across the rumen epithelium (Simmons et al. 2009; Røjen et al. 2011; Lu et al. 2015).

Alternative transport mechanisms for urea are the aquaporins (AQP), a family of membrane-spanning proteins that are mainly responsible for water movement and are expressed in many tissues of mammals. Some AQP (the aquaglyceroporins AQP3, AQP7, AQP9 and AQP10) are permeable to water, glycerol and urea (Zhu et al. 2016; Jin et al. 2018), and Walpole et al. (2015) demonstrated that both AQP and UT-B play significant functional roles in urea transport across rumen epithelium.

Overall, studies on the expression of rumen UT could be useful for setting feeding strategies to increase the dietary N efficiency in ruminants, especially in conditions of dietary N shortage. In the case of fattening bulls, they receive in the last phase of growth rations low in protein and rich in fermentable substrates, which promote an intensive rumen microbial growth if an adequate supply of degradable N is available. Any progress in knowledge of mechanism of urea influx into the rumen would be useful for an improved prediction of the endogenous availability of rumen degradable N and would support a better calibration of the dietary supply. Therefore, the aim of this paper was to study the impact of the dietary crude protein (CP) level (medium vs. low) and source of N (soybean meal vs. urea) on mRNA expression of UT-B urea transporter and some AQP in rumen epithelium of fattening bulls of the breed Italian Simmental (IS). The paper presents analytical results of biological samples (blood and rumen wall) collected at slaughtering from fattening IS bulls used in a feeding trial described by Spanghero et al. (2017).

2. Materials and methods

2.1. *Animals and treatments*

All experimental procedures met the requirements of the European Community Directive 2010/63/EU on the protection of animals used for scientific purposes and a detailed description of the *in vivo* trial can be found in Spanghero et al. (2017). In brief, 80 IS young bulls were assigned to 16 pens (5 bulls/pen) and entered in the trial when achieving a body weight (BW) of 495 ± 58 kg (around 14 months of age). The entering of bulls in the trial was divided in four steps during four subsequent months (a block of four pens per month), because the bulls were not contemporary. The four pens of each block were randomly assigned to four experimental diets, according to a 2 × 2 experimental design. The diets resulted from a combination of a medium (M) and a low (L) level of CP content (125 and 110 g/kg dry matter [DM], respectively) with two nitrogen (N) sources (SBM alone [−U] or SBM partly replaced by an isonitrogenous mixture of corn and urea [+U]) (Table 1). For CP levels M and L, the diets contained two inclusion rates of SBM (8 and 4% DM, respectively). For replacement of SBM within each protein level (M and L), 4% of SBM was substituted by a mixture of corn and urea (3.5 and 0.5% DM, respectively, treatment +U). The protein evaluation of the experimental diets

Table 1. Ingredients, chemical composition and contents of intestinal digestible protein (PDI) of diets differing in crude protein (CP) level (medium [M] or low [L]) and nitrogen source (soybean meal or urea (adapted from Spanghero et al. 2017).

	Dietary treatments			
	Diets M (~125 g CP/kg DM[#])		Diets L (~110 g CP/kg DM)	
	Without urea (−U)	With urea (+U)	Without urea (−U)	With urea (+U)
Ingredients [g/kg DM]				
Forages and straw	415	415	415	415
Concentrates	435	474	479	517
Urea	0	5	0	5
Soybean meal	87	43	43	0
Supplements	63	63	63	63
Chemical composition [g/kg DM]				
Crude protein	128	127	110	109
Neutral detergent fibre	339	336	337	336
PDI[†]				
- PDIA	46	40	40	34
- PDIN	87	82	74	70
- PDIE	94	87	87	80

[#]DM, dry matter; [†]PDI, sum of by-pass digestible protein (PDIA) and the rumen microbial digestible protein calculated from the fermentable energy or the degradable protein (PDIE or PDIN, respectively) (INRA 2007).

was based on the PDI INRA system ([INRA] Institut National de la research Agronomique 2007) and in all diets the estimated content of available degradable N was lower than the requirements for the bacterial growth (i.e. PDIN less than PDIE, [INRA] Institut National de la research Agronomique 2007). After 4 months from the beginning of the trial, four slaughters sessions were performed once a month, corresponding to the four blocks of pens. Bulls were slaughtered in the same slaughter house at 656 ± 56 kg BW between 08:00 and 09:00 h and samples of blood and rumen tissue were collected immediately after three consecutive slaughtering sessions (eight bulls per session, two animals per diet randomly selected within each pen).

2.2. *Plasma and rumen tissue sampling*

During exsanguinations, blood samples were collected from each bull in EDTA-treated 9-ml tubes (Vacuette; Greiner Bio-One, Kremsmünster, Austria) and for plasma separation they were immediately centrifuged at 2000× *g* for 15 min at room temperature. Plasma was stored at −20°C and then sent to a specialised and certified laboratory (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Padua, Italy) for biochemical analysis, which included total protein, albumin, globulins, urea, glucose, total cholesterol, non-esterified fatty acids, aspartate aminotransferase (AST), gamma glutamyltransferase and phosphorus.

Within 30 min after exsanguination, rumen wall samples were cut from the ventral sac (approximately 100 cm^2) and rinsed with saline solution (NaCl 9 g/l). Approximately 2 cm^2 of rumen epithelium were excised and placed in 2.0-ml RNase-free cryovials (Simport, Beloeil, Canada), immediately frozen in liquid N, and then stored at −80°C until RNA analysis.

2.3. *RNA extraction, reverse transcription and quantitative PCR*

Total RNA was extracted from 40 mg rumen epithelium (powdered with a mortar and a pestle in liquid N) using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, DNase treatment included. Concentration and purity of RNA extracted were assessed using a spectrophotometer NanoDrop 2000c (Thermo Fisher Scientific). Integrity of RNA (presence of intact 18S and 28S rRNA bands) and absence of genomic DNA was verified by agarose gel (1% in TBE buffer) electrophoresis, comparing with molecular weight standards.

To obtain complementary DNA (cDNA), the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) was used. Each 20 µl of reaction contained 4 µl of reverse-transcription reaction mix, 1 µl of iScript reverse transcriptase (Moloney Murine Leukemia Virus-derived RNase H+), a volume of RNA solution to have 50 ng/µl RNA and nuclease-free water to final volume. The mixture was held 5 min at 25°C, 1 h at 42°C, and 5 min at 85°C before being cooled on ice. The cDNA concentration and purity was verified using NanoDrop 2000c.

A qualitative PCR was carried out on a mix of total samples cDNA to validate all primer pairs specificity, for all the genes in exam (urea transporter-B (UT-B), aquaporin3 (AQP3), aquaporin7 (AQP7), aquaporin10 (AQP10)) and reference genes (β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein, large, P0 (RPLP0); Table 2). The PCR was performed using the Bio-Rad CFX96 system (Bio-Rad), on a reaction volume of 20 µl, containing 0.3 µl of each forward and reverse primer (0.3 µmol/l), 10 µl of iQ SYBR Green Supermix (Bio-Rad), 8.4 µl of sterile water, 1 µl of cDNA. Amplification conditions included 3 min at 95°C, 40 PCR cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C, then 1 min at 95°C, followed to a melt curve of 55–95°C with 0.5°C increments every 5 s. Unspecific amplifications and primers-dimers formation were excluded by checking melt curves. Amplicon length was verified by agarose gel (1.5% in TBE buffer) electrophoresis, comparing with MW standards (Table 2).

Table 2. Primer sequences (5′–3′) and quantitative PCR products.

Gene	Primers	Amplicon length [bp]	References	R^2	Efficiency [%]
UT-B[•]	F*: TGGCACTCACCTGGCAAACCC R[#]: TGGCAATCCGACCACAGCCAT	103	Berends et al. (2014)	0.999	100.9
Aquaporin 3	F: CGTGACCCTAGGCATCCTTATT R: AGGTGTACACAGGCAGCTTGATC	118	Berends et al. (2014)	0.995	95.0
Aquaporin 7	F: ATTGTGACTGGCATCCTTG R: TGGTTCTGAAGACTTGTGAG	139	Sauerwein et al. (2013)	0.991	102.4
Aquaporin 10	F: TCCTGGCCGACATGCTATC R: GCCCCAGCCAGCTACGTA	100	Røjen et al. (2011)	0.992	98.3
β-actin	F: CTCTTCCAGCCTTCCTTCCT R: GGGCAGTGATCTCTTTCTGC	177	Duckett et al. (2009)	0.999	96.5
GAPDH[†]	F: TCATCCCTGCTTCTACTGGC R: CCTGCTTCACCACCTTCTTG	176	Bernabucci et al. (2006)	0.997	97.3
RPLP0[‡]	F: CAACCCTGAAGTGCTTGACAT R: AGGCAGATGGATCAGCCA	226	Wang et al. (2009)	0.998	97.2

*F, forward; [#]R, reverse; [•]UT-B, urea transporter B; [†]GAPDH, glyceraldehyde-3-phosphate dehydrogenase; [‡]RPLP0, ribosomal protein large P0.

The quantitative PCR was performed for each gene in exam and in triplicate for each sample, using the same instrument, reagents, volumes and amplification cycles of qualitative PCR. Relative gene expression was calculated according to the Livak method (Livak and Schmittgen 2001). Within the reference genes considered, ACTB was used for normalisation of RT-qPCR data because it showed the highest gene stability measure. Data were presented as fold-change ratio having the group with low protein level and without urea (group L, –U) as reference. Primers efficiency was calculated and verified using the standard curve obtained by amplification of serial dilution of the pooled cDNA (Pfaffl 2001) (Table 2).

2.4. *Statistical analysis*

Data were statistically analysed as a factorial 2 × 2 completely randomised block (3 blocks) design ([R Core Team] 2017):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{ik} + \varepsilon_{ijk}$$

where

μ is the overall mean, α_i is the random effect (block) of the group of pens (i = 1,3); β_j is the fixed effect of the dietary CP concentration (j = 1,2); γ_k is the fixed effect of the N source (k = 1,2) and ε_{ijk} is the random error.

The simple correlations between gene expression of all UT (as ΔC_t, calculated according to the Livak method, Livak and Schmittgen 2001) were studied. One outlier belonging to group M–U was detected by Grubbs' test and therefore excluded from analysis.

3. Results

Diets differed in terms of CP concentration (125 and 110 g/kg DM) and N source (SBM or SBM partly replaced by urea, Table 1). Results from the parent study (Spanghero et al. 2017) showed no difference between experimental groups regarding DM intakes (range 9.28–9.32 kg/d), while growth rates tended to be more favourable for diets M than for L (1.37 vs. 1.28 kg/d, $p < 0.10$).

Haematological variables of samples collected at slaughterhouse are shown in Table 3. The bulls fed diets M had lower plasma concentrations of AST than those receiving diets L (78.9 vs. 88.3 U/L, $p = 0.04$). Plasma urea (PU) was higher ($p = 0.03$) for M diets and lower for +U diets ($p = 0.04$). The other measured parameters were not significantly affected by dietary treatments.

The effect of dietary treatments on rumen UT expression (Table 4) were limited to AQP3, which was down regulated in +U diets ($p = 0.01$). Finally, a high positive correlation ($R^2 = 0.871$) between the expressions of AQP7 and AQP10 was found (Figure 1).

4. Discussion

Bulls fed diets high in protein tended to increase the growth rates and had a 20% increment in the PU levels. The positive relationship between the dietary protein and

Table 3. Effect of diets differing in crude protein (CP) level (medium [M] vs. low [L]), and nitrogen source (urea vs. soybean meal) on haematological variables of Italian Simmental young bulls. Data are expressed as mean ± standard error.

	Dietary treatments							
	Diets M (~125 g CP/kg DM*)		Diets L (~110 g CP/kg DM)		*p*-Value			
	Without urea (−U)	With urea (+U)	Without urea (−U)	With urea (+U)	Block	P[♦]	U[◊]	P × U
Total protein [g/l]	76.4 ± 0.81	74.7 ± 1.23	72.8 ± 3.49	76.3 ± 2.23	0.96	0.57	0.61	0.13
Albumin [g/l]	35.6 ± 0.98	36.0 ± 0.63	34.0 ± 0.45	35.5 ± 0.85	0.58	0.20	0.20	0.52
Globulins [g/l]	40.8 ± 0.97	38.7 ± 1.59	38.8 ± 1.74	40.8 ± 1.85	0.75	0.99	0.93	0.22
Glucose [mmol/l]	5.6 ± 0.20	6.8 ± 0.98	5.6 ± 0.26	5.5 ± 0.30	0.22	0.21	0.38	0.25
Cholesterol [mmol/l]	1.63 ± 0.193	1.76 ± 0.131	1.68 ± 0.197	1.94 ± 0.104	0.04	0.52	0.23	0.54
NEFA[†] [meq/l]	0.14 ± 0.048	0.10 ± 0.026	0.12 ± 0.016	0.10 ± 0.023	0.02	0.47	0.14	0.47
AST[‡] [U/l]	76.0 ± 4.83	81.8 ± 3.90	92.3 ± 3.77	84.3 ± 5.46	0.06	0.04	0.69	0.14
GGT[#] [U/l]	20.6 ± 2.71	18.7 ± 1.15	22.3 ± 3.74	19.5 ± 1.71	0.54	0.59	0.39	0.83
Phosphorus [mmol/l]	2.47 ± 0.136	2.63 ± 0.179	2.71 ± 0.212	2.74 ± 0.099	0.14	0.34	0.64	0.76
Urea [mmol/l]	2.5 ± 0.27	2.0 ± 0.27	2.0 ± 0.22	1.73 ± 0.25	<0.01	0.03	0.04	0.34

*DM, dry matter; [♦]P, dietary CP level; [◊]U, urea supplementation [†]NEFA, non-esterified fatty acids; [‡]AST, aspartate aminotransferase; [#]GGT, gamma-glutamyltransferase.

Table 4. Effect of diets differing in crude protein (CP) level (medium [M] vs. low [L]), and nitrogen source (urea vs. soybean meal) on urea transporter B (UT-B), aquaporin 3 (AQP3), aquaporin 7 (AQP7), and aquaporin 10 (AQP10) gene expression. Data are expressed as fold change ratio ± standard error in relative mRNA expression, groups were compared to low protein level and without urea group (L, −U).

	Dietary treatments							
	Diets M (~125 g CP/kg DM[#])		Diets L (~110 g CP/kg DM)		*p*-Value			
	Without urea (−U)	With urea (+U)	Without urea (−U)	With urea (+U)	Block	P[♦]	U[◊]	P × U
UT-B	1.00 ± 0.375	0.87 ± 0.273	1.00 ± 0.401	1.02 ± 0.549	0.03	0.46	0.60	0.51
AQP3	0.94 ± 0.336	0.84 ± 0.267	1.00 ± 0.454	0.70 ± 0.426	<0.01	0.53	0.01	0.11
AQP7	0.49 ± 0.241	0.79 ± 0.364	1.00 ± 0.454	0.91 ± 0.493	0.20	0.51	0.83	0.69
AQP10	0.29 ± 0.174	0.73 ± 0.456	1.00 ± 0.721	0.91 ± 0.565	0.16	0.40	0.67	0.58

[#]DM, dry matter; [♦]P, dietary CP level; [◊]U, urea supplementation.

the level of PU is well documented (Archibeque et al. 2002; Marini and Van Amburgh 2003; Schiavon et al. 2012), while it is apparently surprising that +U diets lowered the PU concentrations with respect to the −U diets (minus 10–25%). Indeed, the criteria of preparation of diets, regardless the CP level, was to create an excess of rumen fermentable energy to allow that the rumen ammonia originating from fermentation should have been used for bacterial growth, with probable scarce ammonia absorption through the rumen wall. It can be speculated that a greater availability of essential amino acids in the intestinal absorbed protein coming from microbial protein could have reduced the catabolic degradation to urea and lowered the PU levels. However, such favourable rumen conditions were probably not sufficient to induce difference in the growth performance of bulls fed urea. The high protein level of diets, in addition to increase the PU levels also significantly reduced the blood AST, as found recently in a trial with

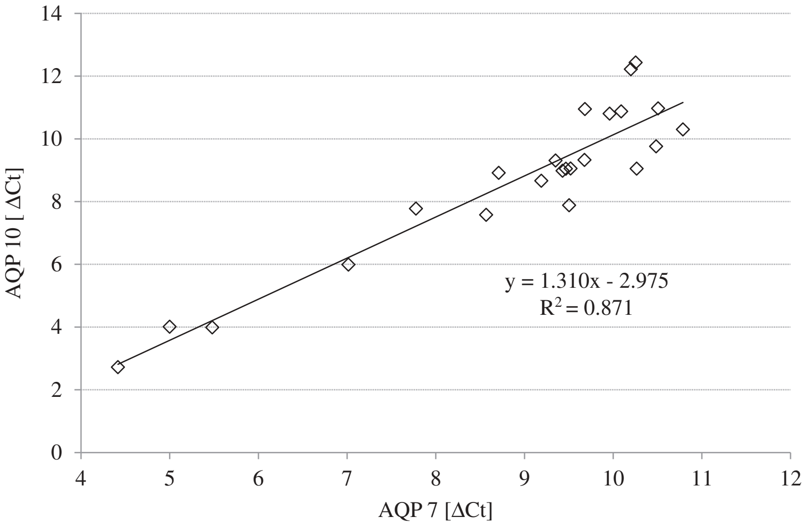

Figure 1. Relationship between aquaporin 7 (AQP7) and aquaporin 10 (AQP10) gene expression, in terms of ΔC_t calculated according to the Livak method (Livak and Schmittgen 2001).

dairy cows (Amanlou et al. 2017): as this enzyme is involved in urea formation process, its blood levels could be controlled by the PU levels via a negative feedback.

The effect of dietary treatments on the UT gene expression were limited to AQP3, which was down regulated in diets containing urea, while the dietary protein concentration did not determine any effect on expression of this channel. The functional role of AQP3 in the rumen epithelium transfer of urea was firstly demonstrated by the recent work of Walpole et al. (2015) in an experiment with calves, which provided the evidence that a portion of urea flux occurs via facilitated diffusion through AQP. A further indication of the role of AQP3 was given by Berends et al. (2014) who stimulated rumen urea influx in calves by feeding incremental amounts of solid feeds, in addition to a basal liquid milk replacer, and observed an increased mRNA expression of AQP3, but not that of AQP7, and UT-B.

Røjen et al. (2011) used lactating cows fed two diets differing in the CP content (17.1 vs. 12.7% DM) and animals fed the higher protein diets showed an up regulation of genes for AQP3, AQP7 and AQP10, but not for UT-B. In our study, the CP difference of diets (11.0 and 12.5% DM) was much lower and, probably, this had a limited impact on urea influx. In a recent meta-analysis Batista et al. (2017) studied the relationship between dietary CP levels and the rumen urea N entry rate in a data set of trials involving several cattle categories. According to such a model, the different CP intakes in the trial of Røjen et al. (2011) caused a variation of approx. 67% in the urea entry rate in the rumen, while in our work the difference accounts to only 23% and this could help to explain the lack of expression of UT-B with respect to the protein level of diets.

Regarding the down regulation of the AQP3 channel in +U diets it must be considered that a high rumen ammonia level is negatively correlated with urea influx in the rumen (Kennedy and Milligan 1980; Doranalli et al. 2011). The diets containing

urea had a significant post feeding increment of rumen ammonia concentration *in vitro* (Spanghero et al. 2017) and this probably attenuated the rumen influx of urea by inactivation of the AQP3 channel.

Finally, in our study, the mRNA expression of AQP7 and 10 has not shown differences between diets, but we have highlighted a high positive correlation (R^2 = 0.871) between the expressions of these two channels. This fact put in evidence a close relation in the function between these two proteins. No literature data are available to support this finding, but Del Bianco et al. (2018) recently investigated the gene involved in rumen urea transport in bulls having different residual feed intake (RFI): despite no difference were found between high, medium and low RFI groups, the AQP7 and AQP10 showed the same numerical trend in the three experimental groups of bulls, indirectly supporting our relationship. Studies on AQP expression in mammals suggest similarities between AQP7 and AQP10. Indeed, these two proteins are preferentially expressed in the apical site of small intestine enterocytes, while AQP3 is more characteristic of the baso-lateral site of the stomach epithelial cells, suggesting distinct functions between AQP7/10 and AQP3 (Laforenza 2012; Zhu et al. 2016).

5. Conclusions

In conclusion, in the present study the expression of only one AQP was changed by dietary treatments. It is possible that the other UT could be less sensible and/or not sufficiently stimulated by the modest experimental differences between our diets. On the contrary the AQP3 appears very responsive to dietary treatments and therefore it is candidate to be further studied in rumen metabolism experiments, even in conditions of small inter-diets variation. Finally, the close relationship between mRNA expression of AQP7 and AQP10 indicates a close relation in the function between these two proteins.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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