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Effects of species-diverse high-alpine forage on *in vitro* ruminal fermentation when used as donor cow's feed or directly incubated

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Alpine forages are assumed to have specific effects on ruminal digestion when fed to cattle. These effects were investigated in an experiment from two perspectives, either by using such forages as a substrate for incubation or as feed for a rumen fluid donor cow. In total, six 24-h in vitro *batch culture runs were performed. Rumen fluid was collected from a non-lactating donor cow after having grazed pastures at ~ 2000 m above sea level for 2, 6 and 10 weeks. These 'alpine runs' were compared with three lowland samplings from before and 2 and 6 weeks after the alpine grazing where a silage–concentrate mix was fed. In each run, nine replicates of four forages each were incubated. These forages differed in type and origin (alpine hay, lowland ryegrass hay, grass–maize silage mix, pure hemicellulose) as well as in the content of nutrients. Concentrations of phenolic compounds in the incubated forages were (g/kg dry matter (DM)): 20 (tannin proportion: 0.47), 8 (0.27), 15 (0.52) and 0 (0), respectively. Crude protein was highest in the silage mix and lowest with hemicellulose, whereas the opposite was the case for fiber. The total phenol contents (g/kg DM) for the high altitude and the lowland diet of the donor cow were 27 (tannins: 0.50 of phenols) and 12 (0.27), respectively. Independent of the origin of the rumen fluid, the incubation with alpine hay decreased (*$P < 0.05$*) bacterial counts, fermentation gas amount, volatile fatty acid (VFA) production as well as ammonia and methane concentrations in fermentation gas (the latter two being not lower when compared with hemicellulose). Alpine grazing of the cow in turn increased (*$P < 0.001$*) bacterial counts and, to a lesser extent, acetate proportion compared with lowland feeding. Further, alpine grazing decreased protozoal count (*$P < 0.05$*) and VFA production (*$P < 0.001$*) to a small extent, whereas methane remained widely unchanged. There were interactions (*$P < 0.05$*) between forage type incubated and feeding period of the donor cow in protozoal counts, acetate : propionate ratio, fermentation gas production and its content of methane,* in vitro *organic matter digestibility and metabolizable energy. Although increased phenolic compounds were the most consistent common property of the applied alpine forages, a clear attribution to certain effects was not possible in this study. As a further result, adaptation (long-term for donor cow, short term for 24 h incubations) appears to influence the expression of alpine forage effects in ruminal fermentation.*

Keywords: rumen, mountain pasture, biodiversity, phenols, methane

Implication

The observation that alpine forages affect *in vitro* ruminal fermentation differently when employed as substrate for incubation and when fed over a longer period to the rumen fluid donor cow is noteworthy. This has implications not only for the evaluation of feeding value and the effect of feeding alpine forage but also for the adaptation of rumen fluid donor animals, as there were several interactions between both dietary interventions. These complex interactions emphasize that alpine forages have specific properties, possibly mediated partly by elevated concentrations of plant secondary compounds, which, however, could still not be proven.

Introduction

Summer grazing at high altitude during the vegetative period is a traditional agricultural practice across the European alps (cf. Leiber *et al.*, 2006). Compared with lowland herbages, alpine pastures have a higher diversity in plant species, especially herbs, and are supposed to be richer in plant secondary compounds (PSC) than intensively managed lowland pastures (Jeangros *et al.*, 1999; Jayanegara *et al.*, 2011a).

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In addition to that, alpine pasture grass and hay are limited in their energy density and therefore may cause a shortage of energy for the rumen microbes (Leiber *et al.*, 2006 and 2009; van Dorland *et al.*, 2006). These two properties were hypothesized to affect those rumen microbes that are involved in ruminal lipid metabolism and might explain the characteristic fatty acid profile of alpine milk (Leiber *et al.*, 2005). Some plant species found in alpine habitats are additionally known for their anti-microbial properties (García-González *et al.*, 2008). Therefore, alpine pasture grass can be expected to modify ruminal fermentation in general, which could also affect methanogenesis. On the level of individual alpine plant species, a limited variation of methanogenesis has been demonstrated *in vitro* (Jayanegara *et al.*, 2011a). However, it may well be that composed swards, which are a mixture of many, possibly interacting, plant species, have other effects than single species. Further, a direct comparison of alpine forages with lowland diets in terms of ruminal fermentation is lacking.

This study tested the hypothesis that forages from high altitude pastures influence ruminal fermentation differently from lowland forages. A second hypothesis was that adaptation to this specific type of forage plays a noticeable role for the expression and the magnitude of this effect. For this purpose, forages from alpine pastures were (i) fed to the rumen fluid donor animal (long-term effect) and (ii) directly incubated in rumen fluid originating either from high altitude or from the lowlands (short-term effect). Comparisons were made with various lowland forages. This approach considers the possibility that adapting donor animals to certain feeds may affect the results of *in vitro* ruminal fermentation (Nagadi *et al.*, 2000; Tejido *et al.*, 2002; Martínez *et al.*, 2010).

Material and methods

Donor cow treatment and rumen fluid collection

The experiment lasted for 16 weeks in summer 2008. During this period, a rumen-cannulated non-lactating Brown Swiss cow was translocated between two different study sites. The rumen fluid collection periods started at the ETH research station Chamau in central Switzerland at 400 m above sea level (a.s.l.) where the cow had received a mixed diet consisting of maize silage, grass silage and grass hay (6 : 3 : 1) at *ad libitum* access and 1 kg/day of a commercial concentrate for the entire winter and spring. The cow was then moved to the pastures around the buildings of the ETH research station Alp Weissenstein situated at an altitude of 2000 m a.s.l. in the southeast of the Swiss alps. Rumen fluid was collected on the day before transport (reflecting the status after 6 months of adaptation to the lowland diet) and after 2, 6 and 10 weeks of alpine grazing, in order to examine the development of the rumen environment during the entire alpine period (10 to 12 weeks long at this altitude). Subsequently, the cow returned to the lowlands and was fed with the same mixed diet as before. Rumen fluid was collected again 2 and 6 weeks after return. Rumen fluid was always collected at 0900. Other than recommended in the Hohenheim Gas Test protocol (Menke and Steingass, 1988), the cow was not fasted in advance of sampling, because the effect of the feeding on the actual rumen environment was one of the targets of the experiments. During the transport of the rumen fluid to the laboratory, which always lasted for 3.5 h, its temperature was maintained at 38°C. Before use, rumen fluid was filtered through four layers of medicinal gauze (1000 μm pore size, type 17; MedPro Novamed AG, Flawil, Switzerland).

The pastures offered at the alpine site were characterized by two main vegetation types, namely *Crepido aureae–Festucetum rubrae* and *Deschampsio cespitosae–Poetum alpinae.* Always one day before rumen fluid collection, a forage sample representative of the donor cow's diet was collected by mimicking its grazing behavior following the protocol of Berry *et al.* (2000). The assumption was made that the grazing behavior on that respective sampling day was similar to that of the days before. Representative samples from the mixed lowland diet were taken before rumen fluid sampling as well. The nutrient composition of the donor cow's alpine and lowland diets is shown in Table 1. The experiment was approved by the cantonal veterinary office of Zug (permission no. ZG 50/08).

In vitro *incubation experiment*

For *in vitro* incubation, amounts of 200 mg air-dry matter (DM) of four experimental forages were used. In order to be able to establish effects on biohydrogenation as well (results not described here), each forage had been incubated together with 0, 5 and 10 mg of linseed oil as an extra source of unsaturated fatty acids. Although such an oil could possess anti-bacterial effects (Maia *et al.*, 2007), and thus could alter ruminal fermentation, there was actually no oil effect in this study and no significant interaction with the other factors occurred in the variables analyzed. Every treatment combination (forage × oil) was incubated in triplicate. The forages tested were (i) alpine hay produced from a cut of another *Crepido aureae–Festucetum rubrae* pasture; (ii) pure lowland ryegrass hay; (iii) a grass–maize silage mixture (2 : 1) resembling the donor cow's lowland forage; and (iv) purified hemicellulose (xylan; produced by hydrolysis of oat spelt and containing mainly xylose and some arabinose and glucose ($\leqslant$0.1 and $\leqslant$0.15 of total, respectively); Sigma-Aldrich GmbH, Buchs, Switzerland). This set of experimental forages was accompanied by a considerable variation in contents of total and individual phenols (Table 1). Both forages of alpine origin were richer in total extractable phenols (TEP) and tannins compared with the respective lowland feeds, whereas TEP contents in hemicellulose were zero. The alpine forages had a higher fiber content than the lowland feeds as well. In terms of crude protein (CP), the characterization was not as clear because the alpine pasture grass was richer in CP than the lowland feed, whereas the alpine hay was poorer in CP than the other experimental forages. The particularly high CP level of the grass after 10 weeks of grazing at the alpine site probably resulted from regrowth fertilized by the dung of the cow itself.

The *in vitro* method applied was a batch culture technique based on the Hohenheim Gas Test and was operated as described by Soliva and Hess (2007). Briefly, filtered rumen

Table 1 *Composition of nutrients and phenolic compounds of the diets of the donor cow and of the four experimental forages*[†]

	Diet of the donor cow				Experimental forages[§]			
Location Weeks	Alpine 2	Alpine 6	Alpine 10	Lowland[‡] 0, 12, 16	Alpine hay	Ryegrass hay	Silage mix	Hemicellulose
DM (g/kg)	926	926	917	925	916	900	910	928
Organic matter (g/kg DM)	933	938	896	926	930	905	903	874
CP (g/kg DM)	152	143	181	118	101	176	231	19
NDF (g/kg DM)	534	581	467	454	522	530	471	855*
Total fatty acids (g/kg DM)	20.3	18.1	22.7	21.7	13.3	19.0	21.8	0
Total extractable phenols[π] (g/kg DM)	30.6	25.8	23.0	12.5	19.6	8.2	14.9	0
Total tannins[π] (g/kg DM)	17.0	12.4	9.96	3.35	9.20	2.22	7.82	0
Condensed tannins[††] (g/kg DM)	1.91	3.16	0.26	0.26	1.11	0	0.06	0
Hydrolysable tannins (g/kg DM)	15.1	9.26	9.75	3.09	8.09	2.22	7.75	0
Non-tannin phenols[π] (g/kg DM)	13.6	13.4	13.0	9.13	10.4	6.01	7.06	0

DM = dry matter; CP = crude protein; NDF = neutral detergent fiber.
[†]Linseed oil addition not included in nutrient composition of the experimental forages.
[‡]Diet consisted of mixed grass–maize silage, grass hay and concentrate.
[§]Given for forages not supplemented with linseed oil; for the supplemented forages this meant additionally 27 and 55 g total fatty acids/kg DM on average.
*NDF content of the hemicellulose treatment was calculated from organic matter minus crude protein.
[π]Given as gallic acid equivalent.
[††]Given as leucocyanidin equivalent.

fluid was mixed with pre-warmed Menke buffer solution (1 : 2). Then, 30 ml of this mixture was added to 200 mg of the air-dry experimental forages which had been weighed into special incubation glass pistons with two outlets. Linseed oil was prepared as an emulsion (50 mg/ml) with 1% (v/v) Tween 80® in aqueous solution following Khiaosa-ard *et al.* (2010) before being added in amounts of either 0.1 or 0.2 ml. In the zero oil treatments, 0.2 ml of the aqueous Tween 80® solution was added. Further, triplicates of incubation units of either blank (rumen fluid buffer mixtures only) and of standard hay and concentrate with known gas production (GP) potential, obtained from the University of Hohenheim, were included in each run to establish baseline values for later correction as outlined in López *et al.* (2010). The pistons were incubated at 39°C for 24 h. Total fermentation gas volume formed in these 24 h was quantified with the help of the calibrated scale printed onto the piston. Subsequently, the residue consisting of rumen fluid, buffer and feed residues (called incubation fluid further on) was removed from the pistons for subsequent analysis. Samples of the fermentation gas remaining in the piston were obtained by a Hamilton syringe through the rubber covering the second outlet.

Laboratory analysis

The incubation fluid samples were analyzed for pH and ammonia concentration immediately upon collection by using a potentiometer (model 173, Metrom, Herisau, Switzerland) equipped with the respective electrodes. Total counts of bacteria and ciliate protozoa (differentiated into holotrichs and entodiniomorphs) present in the incubation fluid samples were determined using 0.02 and 0.1 mm depth Bürker counting chambers (Blau Brand, Wertheim, Germany), respectively. Before counting, the microbial samples were fixed with Hayem solution (mg/ml; $HgCl_2$, 2.5; Na_2SO_4, 25; NaCl, 5.0). Concentrations of volatile fatty acid (VFA) were analyzed by HPLC (System Hitachi Lachrom, Merck, Tokyo, Japan) following the protocol of Ehrlich *et al.* (1981). Methane in fermentation gas was determined using a gas chromatograph (model 5890 Series II, Hewlett Packard, Avondale, PA, USA) equipped with thermal conductivity and flame ionization detectors and a Carboxen-1000 column (15′ × 1/8″ SS, mesh size 60/80; Fluka Chemie AG, Buchs, Switzerland).

Feeds were analyzed following standard protocols (Association of Official Analytical Chemists (AOAC), 1997). DM and total ash contents were determined with an automatic analyzer (TGA-500 Leco Corporation, St. Joseph, MI, USA), ether extract (EE) after petrol ether extraction (required for the calculation of metabolizable energy (ME)), and nitrogen with a C/N analyzer (Type FP-2000, Leco Corporation, St. Joseph, MI, USA). CP content was calculated as 6.25 × N. The ash-corrected content of neutral detergent fiber (NDF) was analyzed as outlined in Van Soest *et al.* (1991) with a Fibertec 1020 system (Tecator, Höganäs, Sweden) using α-amylase (Termamyl 120L, type S; Novo Nodirsk A/S, Bagsværd, Denmark). The total fatty acid content of the feeds was assessed as described in Palmquist and Jenkins (2003). In addition, non-fatty acid compounds were removed using thin layer chromatography (Khiaosa-ard *et al.*, 2009). The determination of TEP (not containing lignin) and tannins was performed as described in detail in Jayanegara *et al.* (2009). For that, the oven-dried (60°C, 24 h) samples were extracted with aqueous acetone water (7 : 3 v/v). Other findings (Muetzel and Becker, 2006; own unpublished tests) suggest that the drying method chosen was probably not systematically affecting extractability and biological activity of tannins in comparison with freeze-dried and freshly frozen

samples. Different from the Folin–Ciocalteu method, polyvinylpolypyrrolidone was used to separate tannin phenols from non-tannin phenols. Butanol-HCl-iron was applied to determine condensed tannins. Hydrolysable tannins were estimated by the difference of total tannins and condensed tannins. This is a simplification and may be slightly biased by applying different standards (leucocyanidin for condensed tannins, gallic acid for the other traits).

In vitro organic matter digestibility (IVOMD) and ME contents were calculated using the adjusted fermentation GP volume and the standard equations given in Menke and Steingass (1988) reading:

$$\begin{aligned}\text{IVOMD (mg/g)} &= 148.8 + 8.893 \times \text{GP (ml)} + 0.448 \\ &\quad \times \text{CP (g/kg DM)} + 0.651 \\ &\quad \times \text{total ash (g/kg DM)};\end{aligned}$$

$$\begin{aligned}\text{ME (MJ/kg)} &= 3.16 + 0.0695 \times \text{GP (ml)} + 0.000730 \\ &\quad \times \text{GP}^2 + 0.00732 \times \text{CP (g/kg DM)} \\ &\quad + 0.02052 \times \text{EE (g/kg DM)}.\end{aligned}$$

Statistical analysis

Data were analyzed using the GLM procedure of SAS (version 9.1, SAS Institute, Cary, NC, USA) considering the factorial experimental arrangement based on rumen fluid origin (lowland *v.* alpine), experimental forage type and their interactions. The linseed oil treatment and its interactions with the other test factors initially included were not further considered in the final statistical model as this resulted in very few significances. Multiple comparisons among main factor means and interaction means were performed using Tukey's method. For the comparisons of the means of the individual measurement periods, as displayed in the figures, a second model (MIXED procedure of SAS; Littell *et al.*, 1998) was used where the repeated measurements character of the data was considered. The Least square means of these periods were compared with Tukey's adjustment for significance within experimental forage type.

Results

The pH of the fluid after 24 h of incubation ranged between 6.7 (hemicellulose) and ~7 (other forages) ($P<0.05$, Table 2). Although ruminal pH was lower ($P<0.05$) in the alpine period, the difference was small in magnitude. Ammonia concentration in incubation fluid was lowest after fermenting pure hemicellulose. With alpine hay, ammonia was lower compared with the two other forages as well ($P<0.05$). During alpine grazing, ammonia concentration was lower ($P<0.01$) than during the lowland period, but again the difference was small. Despite the presence of a time effect ($P<0.001$, as determined by repeated measurement analysis; Figure 1), there was also no pronounced development with time in this trait. Total bacterial count was higher ($P<0.001$) with alpine grazing than with lowland feeding (~+36% on average across all groups; Table 2). Total bacterial count were low with alpine hay and the grass–maize silage mix and highest with ryegrass hay ($P<0.05$). Bacterial counts clearly increased

Table 2 *Effects of experimental forages on fermentation traits when incubated with rumen fluid collected in the lowlands and at the high altitude site (sub-cells,* n = *9)*

	Origin of rumen fluid	Experimental forage					s.e.	*P*-level		
		Alpine hay	Ryegrass hay	Silage mix	Hemicellulose	Mean		O	F	O × F
pH	Mean	6.96^{A}	6.94^{AB}	6.91^{B}	6.75^{C}		0.018	**	***	ns
	Alpine	6.94^{abc}	6.91^{bc}	6.89^{c}	6.76^{d}	6.88^{Z}				
	Lowland	7.00^{a}	6.98^{ab}	6.93^{abc}	6.74^{d}	6.91^{Y}				
Ammonia (mmol/l)	Mean	10.6^{B}	12.0^{A}	12.2^{A}	6.0^{C}		0.191	**	***	ns
	Alpine	10.5^{b}	11.8^{a}	11.8^{a}	5.8^{c}	10.0^{Z}				
	Lowland	10.7^{b}	11.2^{a}	12.2^{a}	6.2^{c}	10.4^{Y}				
Total bacteria ($\times 10^8$/ml)	Mean	26.2^{C}	33.4^{A}	26.8^{BC}	30.7^{AB}		1.59	***	***	ns
	Alpine	31.3^{abc}	37.0^{a}	31.5^{abc}	35.0^{ab}	33.7^{Y}				
	Lowland	21.0^{d}	29.8^{bc}	22.1^{d}	26.5^{cd}	24.8^{Z}				
Total protozoa ($\times 10^4$/ml)	Mean	5.04^{B}	4.28^{BC}	7.70^{A}	3.40^{C}		0.417	*	***	***
	Alpine	4.36^{cd}	3.63^{de}	8.68^{a}	2.41^{e}	4.77^{Z}				
	Lowland	5.72^{bc}	4.93^{bcd}	6.72^{b}	4.39^{cd}	5.44^{Y}				
Holotrichs (% of total protozoa)	Mean	19.0^{A}	16.8^{A}	6.81^{B}	7.02^{B}		1.820	**	***	***
	Alpine	25.4^{a}	21.8^{a}	5.21^{b}	5.01^{b}	14.4^{Y}				
	Lowland	11.8^{b}	11.8^{b}	8.40^{b}	9.04^{b}	10.3^{Z}				
Entodiniomorphs	Mean	81.4^{B}	83.2^{B}	93.2^{A}	93.0^{A}		1.82	**	***	***
(% of total protozoa)	Alpine	74.6^{b}	78.2^{b}	94.8^{a}	95.0^{a}	85.7^{Z}				
	Lowland	88.2^{a}	88.2^{a}	91.6^{a}	91.0^{a}	89.7^{Y}				

O = origin; F = forage.

Means of periods and of incubated forages, respectively, sharing no common capital letters are significantly different at $P<0.05$.

Period × forage interaction means sharing no common small letters are significantly different at $P<0.05$. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ns, not significant.

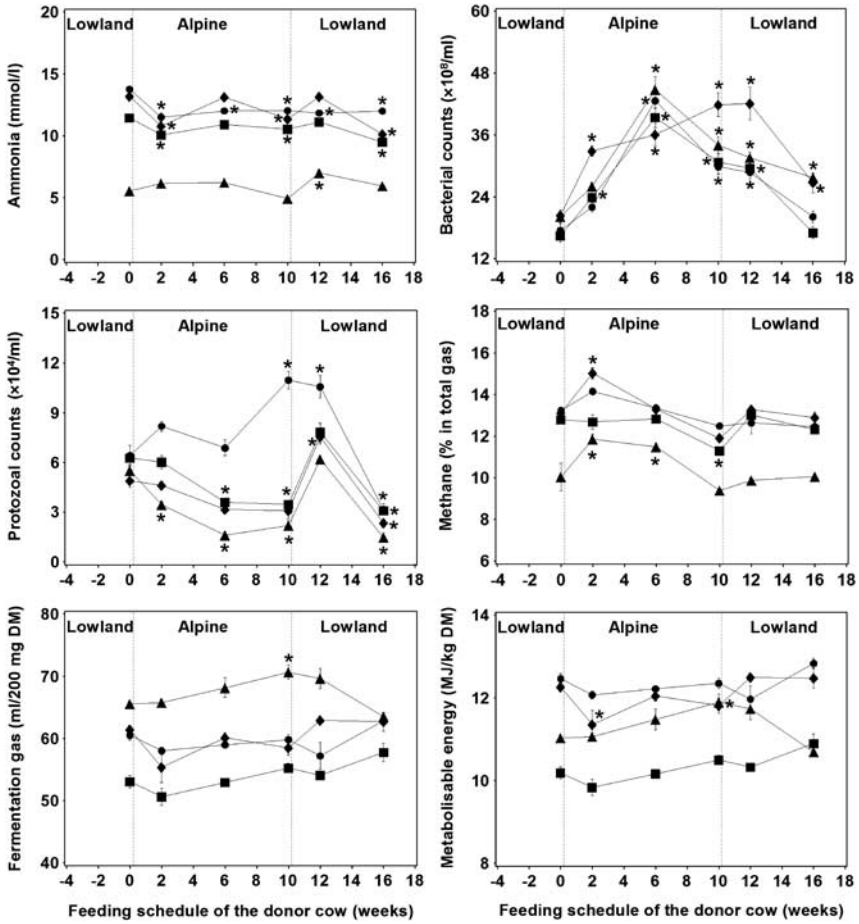

Figure 1 Changes in the effect of forage type (■, alpine hay; ◆, ryegrass hay; ●, grass-maize silage; ▲, hemicellulose) on selected ruminal fermentation traits when incubated for 24 h *in vitro* with rumen fluid collected from the lowlands and at high altitude ($n = 9$ per data point). Overall, effects of experimental forage and period as well as their interactions were significant at $P < 0.001$ for all traits. Asterisks denote means within experimental forage type, which differed ($P < 0.05$) from the first assessment made in the lowlands.

after 2 and 6 weeks of alpine grazing with all forages incubated decreased slightly after 10 weeks and very clearly 6 weeks after the end of the alpine sojourn (Figure 1). The total protozoal count was found to be lower in rumen fluid collected at high altitude when alpine hay, ryegrass hay and hemicellulose were incubated (−31% on average), whereas the opposite was observed with the grass–maize silage mix (+29%, $P < 0.05$; interaction, $P < 0.001$; Table 2). In more detail, protozoal count was immediately increased with hemicellulose when switching to the alpine period (2 weeks), whereas with alpine hay and the grass–maize silage effects only occurred after 6 and 10 weeks of grazing had passed (Figure 1). Two weeks after returning to the lowlands, protozoal counts increased across all forages incubated before declining again to levels lower than initially ($P < 0.05$). Incubation with rumen fluid of alpine origin and incubation of alpine hay and ryegrass hay increased ($P < 0.05$) the holotrich protozoa proportion of total protozoa (Table 2).

Total fermentation GP across all forages incubated was lower ($P < 0.05$) when using rumen fluid from the alpine site compared with that from the lowlands, but the difference was minor in extent. When considering the time-dependent development, no clear shifts of gas formation occurred during the entire experiment (Figure 1). Among the forages, hemicellulose resulted in the highest GP, the alpine hay in the lowest value and the other two forages were found to be intermediate ($P < 0.05$; Table 3). There was an interaction ($P < 0.001$) of rumen fluid origin and forage type because the use of rumen fluid of alpine origin caused a lower GP with all incubated forages except with hemicellulose, where the GP increased. There was a minor increase ($P < 0.05$) in methane proportion of total gas (+1%) when using rumen fluid of alpine origin, but absolute methane production was similar to that found with rumen fluid from the lowlands (Table 3). Methane formation was lower ($P < 0.05$) in amount and proportion with hemicellulose and alpine hay than with the other two forages. There was a sharp increase in methane proportion of total gas after moving the donor cow to the alpine site with three of the forages incubated ($P < 0.05$ relative to initial), but not with the alpine hay (Figure 1). Afterwards the methane proportion gradually declined to the level found initially, and this development persisted until the end of the experiment. With the exception that IVOMD of hemicellulose was lower than that of the silage mix, IVOMD and ME contents varied basically as did total fermentation gas (Table 3, Figure 1).

Table 3 *Effects of experimental forages on gas and methane production and calculated energy content when incubated with rumen fluid collected in the lowlands and at the high altitude site (sub-cells,* n = *9)*

	Origin of rumen fluid	Experimental forage					s.e.	*P*-level		
		Alpine hay	Ryegrass hay	Silage mix	Hemicellulose	Mean		O	F	O × F
Fermentation gas (ml/200 mg DM)	Mean	52.5^{C}	58.5^{B}	58.0^{B}	65.5^{A}		0.74	**	***	***
	Alpine	51.5^{e}	56.4^{cd}	57.4^{c}	66.4^{a}	57.9^{Z}				
	Lowland	53.5^{de}	60.7^{b}	58.6^{bc}	64.5^{a}	59.3^{Y}				
Methane (ml/200 mg DM)	Mean	6.72^{B}	7.95^{A}	7.76^{A}	7.01^{B}		0.122	ns	***	***
	Alpine	6.48^{d}	7.74^{ab}	7.85^{ab}	7.41^{bc}	7.37				
	Lowland	6.98^{cd}	8.15^{a}	7.68^{ab}	6.61^{d}	7.36				
Methane (% offermentation gas)	Mean	12.5^{B}	13.2^{A}	13.0^{A}	10.4^{C}		0.19	*	***	**
	Alpine	12.3^{b}	13.4^{a}	13.3^{a}	10.9^{c}	12.5^{Y}				
	Lowland	12.7^{ab}	13.1^{ab}	12.8^{ab}	10.0^{d}	12.1^{Z}				
In vitro organic matter digestibility (mg/g DM)	Mean	723^{C}	832^{B}	855^{A}	838^{B}		6.6	**	***	***
	Alpine	714^{d}	812^{c}	849^{ab}	846^{ab}	806^{Z}				
	Lowland	733^{d}	851^{ab}	860^{a}	829^{bc}	818^{Y}				
Metabolizable energy (MJ/kg DM)	Mean	10.3^{C}	12.1^{A}	12.3^{A}	11.3^{B}		0.11	**	***	***
	Alpine	10.2^{e}	11.7^{bc}	12.2^{ab}	11.5^{cd}	11.4^{Z}				
	Lowland	10.5^{e}	12.4^{a}	12.4^{a}	11.2^{d}	11.6^{Y}				

DM = dry matter; O = origin; F = forage.
Means of periods and of incubated forages, respectively, sharing no common capital letters are significantly different at $P < 0.05$.
Period × forage interaction means sharing no common small letters are significantly different at $P < 0.05$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

Alpine grazing of the donor cow led to clearly reduced ($P < 0.001$) total VFA concentrations in the incubation fluid (−8%) compared with lowland feeding (Table 4). Because in the incubations equal amounts of rumen fluid and forages had been used, this is equivalent to a corresponding reduction in actual VFA production. Incubating the hemicellulose enhanced VFA concentration compared with the other forages, especially when using rumen fluid originating from the high altitude site. There were large fluctuations in VFA concentration during the experimental periods across all forages incubated (Figure 2). Lower values were found at the first and the last alpine measurement and at the last lowland measurement date in comparison with the initial value ($P < 0.05$; especially pronounced with the two hay types). Alpine grazing increased ($P < 0.05$) proportions of acetate and iso-butyrate at cost of butyrate, valerate and iso-valerate (Table 4). The increase in acetate was accompanied by a higher ($P < 0.05$) acetate : propionate ratio. Incubating different forages altered ($P < 0.05$) VFA profile as well. Especially the hemicellulose treatment resulted in a different profile with largely elevated propionate proportions and lower proportions of all other VFA ($P < 0.05$). Compared with that, the other three forages appeared to have similar effects, except that the incubation of the alpine hay resulted in lower ($P < 0.05$) proportions of the iso-acids. A more detailed view on the fluctuations between the individual measurement periods (Figure 2) did not reveal additional systematic changes in acetate, propionate and the acetate : propionate ratio. Interactions ($P < 0.01$) between origin of rumen fluid and forage type were only found with propionate proportion and, consequently, in the acetate : propionate ratio, but these interactions were of minor quantitative importance.

Discussion

In this study, alpine forages were tested in their effect either as a feed for the donor cow or as a substrate for *in vitro* incubations. Both alpine forage sources were characterized by an elevated content of PSC, as indicated here by TEP, as had been expected from the literature (Jeangros *et al.*, 1999; Fraisse *et al.*, 2007). Purified PSC or plant extracts and plants rich in PSC have been occasionally demonstrated to alter ruminal fermentation processes *in vitro* in the short term (Makkar *et al.*, 1995; Patra *et al.*, 2006; Soliva *et al.*, 2008; Jayanegara *et al.*, 2011b). Therefore, in this study clear TEP-related effects of the alpine forages on ruminal fermentation were expected.

The experimental set-up allowed testing the effects of forage of alpine origin from two different perspectives including short-term and longer-term responses. When comparing these alpine forage effects, it has to be kept in mind that the respective controls had been different. The lowland diet resembled best the silage mix among the forages incubated, whereas the monoculture lowland hay was an ideal control for the identification of a direct hay origin effect. Hemicellulose was employed as a feed free of PSC. One clear limitation of this study was that only one donor animal could be employed. Since variation between animals exists (Franzolin and Dehority, 1996), which could not be ruled out in the current experimental setting, the results have a preliminary character. However, especially the apparent contrast found between the long-term and the short-term effects is an important information to be further investigated in more detail.

Effects of alpine forage on rumen microbes

A lower bacterial count was expected from the possible antimicrobial effect of phenolic compounds and other PSC

Table 4 *Effects of experimental forages on VFA concentration and profile when incubated with rumen fluid collected in the lowlands and at the high altitude site (sub-cells,* n = *9)*

	Origin of rumen fluid	Experimental forage					s.e.	*P*-level		
		Alpine hay	Ryegrass hay	Silage mix	Hemicellulose	Mean		O	F	O × F
Total VFA (mmol/l)	Mean	82.3^{B}	86.0^{AB}	84.8^{B}	93.2^{A}		2.58	***	**	ns
	Alpine	77.2^{c}	80.7^{bc}	82.1^{bc}	90.5^{ab}	82.6^{Z}				
	Lowland	87.3^{abc}	91.3^{ab}	87.4^{abc}	94.1^{a}	90.0^{Y}				
Acetate (%)	Mean	71.0^{A}	70.5^{A}	70.5^{A}	66.3^{B}		0.39	***	***	ns
	Alpine	72.2^{a}	71.7^{a}	71.8^{a}	66.7^{c}	70.6^{Y}				
	Lowland	69.9^{b}	69.4^{b}	69.3^{b}	66.0^{c}	68.6^{Z}				
Propionate (%)	Mean	18.4^{BC}	19.1^{B}	17.9^{C}	24.7^{A}		0.40	ns	***	**
	Alpine	18.1^{bc}	18.8^{bc}	17.2^{c}	25.3^{a}	19.9				
	Lowland	18.6^{bc}	19.4^{b}	18.7^{bc}	24.1^{a}	20.2				
Butyrate (%)	Mean	8.44^{A}	7.64^{B}	8.85^{A}	7.00^{C}		0.216	***	***	ns
	Alpine	7.56^{de}	6.75^{ef}	8.22^{cd}	6.12^{f}	7.16^{Z}				
	Lowland	9.32^{ab}	8.53^{bc}	9.49^{a}	7.89^{cd}	8.81^{Y}				
Iso-butyrate (%)	Mean	0.80^{B}	1.07^{AB}	1.10^{A}	1.11^{A}		0.156	***	*	ns
	Alpine	0.97^{abc}	1.43^{a}	1.37^{a}	1.21^{ab}	1.24^{Y}				
	Lowland	0.63^{c}	0.72^{c}	0.83^{bc}	1.00^{abc}	0.80^{Z}				
Valerate (%)	Mean	0.64^{A}	0.73^{A}	0.72^{A}	0.46^{B}		0.036	***	***	ns
	Alpine	0.49^{b}	0.51^{b}	0.58^{b}	0.33^{c}	0.48^{Z}				
	Lowland	0.80^{a}	0.94^{a}	0.86^{a}	0.60^{b}	0.80^{Y}				
Iso-valerate (%)	Mean	0.71^{B}	0.93^{A}	0.85^{A}	0.39^{C}		0.040	*	***	ns
	Alpine	0.68^{c}	0.88^{ab}	0.87^{ab}	0.31^{d}	0.68^{Z}				
	Lowland	0.74^{bc}	0.98^{a}	0.83^{abc}	0.46^{d}	0.76^{Y}				
Acetate : propionate	Mean	3.91^{AB}	3.74^{B}	3.97^{A}	2.72^{C}		0.081	***	***	**
	Alpine	4.05^{ab}	3.89^{abc}	4.19^{a}	2.67^{d}	3.70^{Y}				
	Lowland	3.77^{bc}	3.60^{c}	3.76^{bc}	2.77^{d}	3.47^{Z}				

VFA = volatile fatty acid; O = origin; F = forage.
Means of periods and of incubated forages, respectively, sharing no common capital letters are significantly different at $P < 0.05$.
Period × forage interaction means sharing no common small letters are significantly different at $P < 0.05$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.

(Kamra *et al.*, 2006). Indeed, the incubation of the alpine hay reduced bacterial counts, but the rumen fluid collected from the alpine site had higher bacterial counts, indicating that the adaptation of the donor animal to a certain feed has a different influence on the result than the short-term incubation of a similar forage. Furthermore, bacterial counts peaked after 6 weeks of alpine grazing, which was when the forage had the highest content of condensed tannins. The increase in bacterial count was, however, found earlier with condensed tannin supplementation *in vitro* (Khiaosa-ard *et al.*, 2009) and with herbal preparations studied *in vivo* (Bhatt *et al.*, 2009). Therefore, no general anti-bacterial effect of the alpine pasture plants can be assumed, which is also in agreement with the results of Jayanegara *et al.* (2011a).

Also in the short-term perspective, based on the differences between the incubated forage types, no general influence of the TEP present in the alpine hay can be deducted. In fact, there are various factors influencing bacterial counts, and some of them could even have been counteractive in this study. For instance, a lower bacterial count is likely to have resulted from the lower content of fermentable organic matter of the alpine hay compared with the other forages (Grubb and Dehority, 1975). Besides, the alpine hay had a lower CP content than the lowland forages incubated. By contrast, a higher average CP concentration of the alpine meadows compared with the lowland diet ingested by the cow may have contributed to an improved microbial growth during the alpine grazing periods of the donor cow. In addition, bacterial counts might have been higher in the rumen fluid of alpine origin as a compensation for the concomitantly decreased protozoa, which are predators of bacteria (Hess *et al.*, 2003). It is obvious that rumen bacteria got adapted to the TEP within the 70-day period of alpine grazing. Resistant rumen bacteria can overcome the inhibitory effects of PSC (see reviews by Smith *et al.*, 2005; Makkar *et al.*, 2007). This was shown for instance in continuous culture where rumen microbes got adapted to PSC from plant extracts already after 6 days (Cardozo *et al.*, 2004). Finally, a clearly reduced water intake with alpine forage, as observed by Leiber *et al.* (2009) when comparing lowland and alpine hay, might explain why bacterial concentrations in rumen fluid were higher in the animal (little dilution) compared with alpine forage incubated *in vitro*.

Alpine forage, tested in either way, resulted in lower protozoal counts compared with the corresponding silage-based lowland diets (although not when compared with the lowland hay). Although the effects of PSC on protozoal populations reported are quite variable (Benchaar *et al.*, 2008; Vasta *et al.*, 2010), and type and dose of PSC are important, several PSC are known to act specifically against

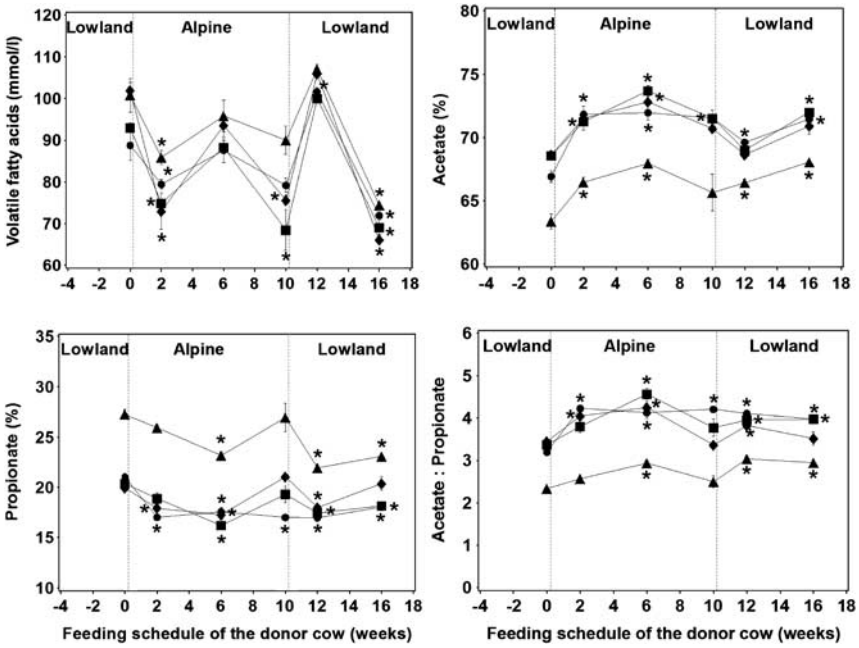

Figure 2 Changes in the effect of forage type (■, alpine hay; ◆, ryegrass hay; ●, grass-maize silage; ▲, hemicellulose) on traits concerning volatile fatty acids when incubated for 24 h *in vitro* with rumen fluid collected from the lowlands and at high altitude ($n = 9$ per data point). Overall, effects of experimental forage and period as well as their interactions were significant at $P < 0.001$ for all traits. Asterisks denote means within experimental forage type, which differed ($P < 0.05$) from the first assessment made in the lowlands.

protozoa. This includes tannins among the phenolic compounds and especially saponins (Śliwiński *et al.*, 2002; Hess *et al.*, 2003; Carulla *et al.*, 2005). Another explanation for low protozoal counts is the low content of the alpine forages of easily digestible non-fiber carbohydrates, the nutrients preferred by the protozoa (Dijkstra, 1994). This is particularly obvious from the strong anti-protozoal effect of the PSC-free hemicellulose. The holotrich protozoa seemed to be less susceptible to alpine forage than the entodiniomorphs. The same was reported by Patra *et al.* (2006) when investigating the responses of protozoa populations to extracts of PSC-containing plants.

Effects of alpine forage on the production of ammonia and methane

The ammonia concentration in the incubation fluid was clearly lower with the alpine hay than with the lowland hay and the silage mix, whereas the corresponding decline with alpine forage found in the rumen fluid of the donor cow was only small. This coincides with the differences in CP contents in relation to the respective controls and is further illustrated by the exceptionally low ammonia concentration found with the low-CP feed hemicellulose. Tannins strongly bind to protein at pH 6.0 to 7.0 (Cortés *et al.*, 2009) and thus inhibit ruminal microbial protein degradation and ammonia formation (Śliwiński *et al.*, 2002). Since dietary tannin concentrations clearly varied among forages in this study, an effect could have been possible. However, the large variation in CP content between the forages has such a high relevance for the ruminal ammonia production that distinguishing of tannin effects from those of CP seems to be impossible, the more because the concentration of tannins in the alpine forages was rather low compared with the dietary levels investigated in other studies (Carulla *et al.*, 2005; Khiaosa-ard *et al.*, 2009).

The short-term incubation of alpine hay led to a lower methane production than that from lowland hay and the mixed silages. On the basis of the studies of Jayanegara *et al.* (2011a and 2011b), it can be assumed that total phenolic compounds at a concentration of ~20 g/kg in the alpine hay contributed at least partly to the decrease in methane production. Different from that, in the long-term perspective, there was no systematic effect of alpine grazing on methane formation and its proportion of total gas *in vitro*. The fluctuation (increase and then gradual decline) in methane formation among the alpine sub-periods indicates that changes in microbial population or their activity in the native rumen fluid or both took place. The fluctuations did not coincide with those found in numbers of protozoa, microbes where a substantial proportion of methanogens are closely associated with (Hess *et al.*, 2003). As discussed earlier, short-term anti-methanogenic effects may have been overcome by gradual microbial adaptation. The lowest methane proportion found when incubating hemicellulose is likely to be explained by the concomitant increase in total GP, which was more than proportionate compared with methane.

Effects of alpine forage on VFA and its energy content

The fluctuations found on the alpine site in acetate proportion coincided with those found for methane production, which was expected as both are related to fiber degradation. This also suggests that the hydrogen supply for the methanogens (Martínez *et al.*, 2010) was initially elevated with

alpine grazing. The most peculiar VFA profile (acetate and propionate proportion) was found with the pure fiber coming from the hemicellulose treatment while the alpine and the lowland forages, containing quite similar NDF contents, showed no major differences. Similar to the low ammonia concentration, the clear decrease in the iso-fatty acids found with the alpine hay compared with the lowland hay incubated can be attributed to the low-CP concentrations of the alpine hay. The significant shift in the acetate : propionate ratio between the lowland and the alpine periods, that is, the long-term effect, has to be attributed to the lower fiber and higher soluble carbohydrate contents (data for soluble carbohydrates not shown) of the lowland compared with the alpine diet.

Total VFA concentration (and therefore production) as well as total fermentation GP were lower with the alpine forage compared with the respective lowland controls. This means that forage quality, in terms of digestibility (here estimated by the IVOMD) and ME concentration, was lower although not very much. Similar observations on alpine forage quality have been made in other studies (e.g. Leiber *et al.*, 2006). In the present investigation, there was a minor increase with time on the alpine pasture, and also some interactions with forage type occurred. The differences in IVOMD and ME between alpine forage and the corresponding lowland feeds were again rather small when compared with the difference to the well digestible pure hemicellulose.

Conclusion

This *in vitro* study showed that employing alpine forages influences ruminal fermentation compared with lowland feeding systems. This adds to earlier findings on the specific properties and effects of such forages on intake, digestion, performance and milk quality, some of which are favorable, others unfavorable. Overall, although the TEP concentrations were high in both alpine forages compared with the other feeds, it was not possible to clearly distinguish the effects of these compounds from other dietary properties on ruminal fermentation parameters. However, in comparison with the different controls used, it appears most likely that the effects were the result of a combination of a rather limited digestibility and energy content but also of constituents of the alpine forage, which are present in minor proportions. The frequent occurrence of significant interactions between test forage and rumen fluid origin, although not being of large magnitude, may be a result of the different degree of microbial adaptation to alpine forage. Being aware about the fact, that only one donor cow could be used, the results, however, underline the importance of adapting the cow donating rumen fluid for short-term *in vitro* experiments to forage with specific properties (Nagadi *et al.*, 2000; Tejido *et al.*, 2002; Martínez *et al.*, 2010).

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