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Effect of the ionophore monensin and tannin extracts supplemented to grass silage on populations of ruminal cellulolytics and methanogens *in vitro*

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ABSTRACT

This study examined whether the methane-decreasing effect of monensin (~21%) and different hydrolysable tannins (24%–65%) during *in vitro* fermentation of grass silage was accompanied by changes in abundances of cellulolytics and methanogens. Samples of liquid (LAM) and solid (SAM) associated microbes were obtained from two rumen simulation technique experiments in which grass silage was either tested in combination with monensin (0, 2 or 4 mg d^{-1}) or with different tannin extracts from chestnut, valonea, sumac and grape seed (0 or 1.5 g d^{-1}). Total prokaryotes were quantified by 4′,6-diamidino-2-phenylindol (DAPI) staining of paraformaldehyde-ethanol-fixed cells and relative abundances of ruminal cellulolytic and methanogenic species were assessed by real time quantitative PCR. Results revealed no change in absolute numbers of prokaryotic cells with monensin treatment, neither in LAM nor in SAM. By contrast, supplementation of chestnut and grape seed tannins decreased total prokaryotic counts compared to control. However, relative abundances of total methanogens did not differ between tannin treatments. Thus, the decreased methane production by 65% and 24% observed for chestnut and grape seed tannins, respectively, may have been caused by a lower total number of methanogens, but methane production seemed to be also dependent on changes in the microbial community composition. While the relative abundance of *F. succinogenes* decreased with monensin addition, chestnut and valonea tannins inhibited *R. albus*. Moreover, a decline in relative abundances of *Methanobrevibacter* sp., especially *M. ruminantium*, and *Methanosphaera stadtmanae* was shown with supplementation of monensin or chestnut tannins. Proportions of *Methanomicrobium mobile* were decreased by monensin in LAM while chestnut and valonea had an increasing effect on this methanogenic species. Our results demonstrate a different impact of monensin and tannins on ruminal cellulolytics and gave indication that methane decrease by monensin and chestnut tannins was associated with decreased abundances of *M. ruminantium* and *M. stadtmanae*.

1. Introduction

Monensin is an ionophore used as feed additive for dairy cows and beef cattle to improve the efficiency of feed conversion. Monensin treatment leads to decreased feed intake, higher milk yield [1] or body weight gain [2], improves energy and nitrogen metabolism and has beneficial effects on health [3–6]. The ionophore is thought to act via an antiporter action that causes a cellular energy loss [7] and, rather inhibits ruminal protozoa, fungi and Gram-positive bacteria than Gram-negative bacteria [5,6]. Selection for Gram-negative bacteria is accompanied by increased propionate proportions whereas acetate, butyrate and H_2 mainly produced by Gram-positive bacteria and protozoa decrease. The antimethanogenic effect of monensin found *in vivo* and *in vitro* [8–11] thus, can be explained by a lower supply of ruminal methanogens with H_2 as precursor for methanogenesis [12].

Abbreviations: Rusitec, rumen simulation technique; qPCR, quantitative PCR; NDF, neutral detergent fiber; ADF, acid detergent fiber; TT, total tannins; CT, condensed tannins; HT, hydrolysable tannins; LAM, liquid associated microbes; SAM, solid associated microbes; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindol; PBS, phosphate buffered saline.

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Methane is a climate-relevant gas and its enteric emission via the mouth means a loss of energy estimated to range from 2 to 12% of animal's gross energy intake [13]. Thus, decreasing methane emissions from ruminants is of great interest for nutritionists. However, results from studies on monensin effects are inconsistent [14] ranging from no changes in methane production up to 25% reductions with persistency being also variable from long-to short-term [15]. Different findings seem to be in part a result of different diets used as methane-decreasing effects of monensin were shown to be higher for grass silage than for corn silage and concentrate in batch cultures of mixed rumen microorganisms [16]. Grass silage represents one of the most important forages in ruminant nutrition, but studies on the effect of its supplementation with monensin on methane production are rare. Wischer et al. [17] described changes in fermentation patterns of grass silage with supplementation of monensin and confirmed its methane-lowering effect in a 14 day lasting rumen simulation technique (Rusitec). If these changes were attributed to shifts in the microbial community composition was investigated in the present study as most of our knowledge about monensin's effects on ruminal microorganisms is based on results from pure cultures, while studies on the response of single bacterial [11,18–22] and methanogenic species [23–25] in mixed microbial communities are rare.

Since 2006 the routine use of ionophores as growth promoters has been banned in the European Union to control antibiotic resistance [26]. Thus, during the last decade research on alternative methane mitigation strategies has received a great attention. Tannins are considered to be a promising group of secondary plant compounds for decreasing enteric methane emissions from ruminants by dietary means [27,28]. Chemically, tannins are polyphenols being classified as hydrolysable (polyesters of gallic acid and various individual sugars; HT) and condensed (polymers of flavonoids; CT) tannins, although other tannins combining both basic structures also occur [29]. In the rumen tannins form complexes with many organic compounds and minerals via hydrogen or hydrophobic bonds [29,30]. Mechanisms of their antimicrobial action are poorly understood, but polyphenolics are supposed to react with microbial cell walls and extracellular secreted enzymes [29]. Inhibitory effects on bacteria, methanogens and protozoa were shown for different tannins in a plenty of studies but research mainly focused on CT probably due to the lower risk of toxicity [14]. However, the methane-decreasing effect, in particular that of CT [31], is often accompanied by a decrease in digestion of organic matter, and especially fiber [27]. Instead, HT show less adverse effects on fiber degradation while having a higher methane-decreasing potential [31]. Thus, recently, Wischer et al. [32] tested different HT for their methane-decreasing potential from grass silage *in vitro*. Whether the changes in fermentation patterns observed by these authors were accompanied by changes in the microbial community composition was examined in the present study. In addition to a relative quantification of total methanogens we, in particular, were interested in the relative population size of different cellulolytic as well as methanogenic species as analyzed by the culture-independent method of real-time quantitative (q) PCR.

2. Materials and methods

2.1. In vitro incubation and sampling

Samples for microbial analysis were obtained from the *in vitro* experiments described in detail by Wischer et al. [17,32]. In brief, grass silage supplemented with different levels of monensin (experiment 1) or four different tannin extracts (experiment 2) was incubated in a semi-continuous Rusitec system. The inoculum for the *in vitro* experiments was taken from two ruminally fistulated non-lactating Holstein cows before morning feeding. Animals fed grass hay for *ad libitum* intake, a concentrate mix (2 kg d^{-1}) and grazed on pasture during daytime. *In vitro* incubation was performed in fermenters each having a working capacity of 800 mL (at the beginning of incubation filled with 400 mL of strained rumen fluid and 400 mL of buffer solution). Dried (65 °C, 24 h) and ground (1-mm sieve screen) grass silage was weighed into nylon bags (pore size = 100 μm) at an amount of 15 g per bag. In experiment 1 the 15 g of grass silage (organic matter, 865; crude protein, 125; neutral detergent fiber (NDF), 531; and acid detergent fiber (ADF), 363 g kg^{-1} dry matter) per bag remained non-supplemented (control) or were supplemented with 2 or 4 mg monensin (~2.5 and 5 mg l^{-1}; Monensin sodium salt hydrate, Dr. Ehrenstorfer GmbH, Augsburg, Germany) dissolved in 100 μl of ethanol. In experiment 2 grass silage (organic matter, 922; crude protein, 129; neutral detergent fiber (NDF), 444; and acid detergent fiber (ADF), 283 g kg^{-1} dry matter) in bags remained non-supplemented (control) or was supplemented with 1.5 g of one of the four different tannin extracts. The products used were extracts from chestnut (*Castanea sativa*, total tannin (TT) concentration according to the manufacturer ≥ 76%), sumac (*Rhus coriaria*, TT ≥ 62%), valonea (*Quercus valonea*, TT ≥ 67%; all from Baeck GmbH & Co. KG, Norderstedt, Germany) and grape seed (OmniVin 20R; *Vitis vinifera*, polyphenol concentration according to the manufacturer ≥ 95%; S.A. Ajinimoto OmniChem N.V., Louvain-la-Neuve, Belgium). Concentrations of total phenols, non-tannin phenols, and extractable condensed tannins were analyzed as described by Wischer et al. [32]. While grape seed extracts contained 14% CT, chestnut, valonea and sumac extracts contained only HT. Two bags were incubated per fermenter as shown in Fig. S1. At the beginning of incubation one of the two bags contained grass silage with or without any supplement whereas the other one was filled with 60 g of rumen solids. Twenty-four h later the bag with rumen solids was replaced by another feedbag. During the following 12 (experiment 1) or 13 (experiment 2) days feedbags were changed at 24-h intervals, so that each bag was incubated for 48 h. A buffer solution [33] was infused continuously at an average flow rate of 524 mL (experiment 1) or 572 mL (experiment 2) per day and fermenter. In both experiments, incubation of the feed was carried out in quadruplicate per treatment in two consecutive experimental runs of 13 (experiment 1) or 14 (experiment 2) days of duration. At the end of each run microbial cells were obtained from fermenter liquids (liquid associated microbes = LAM) as well as from feed residues (solid associated microbes = SAM) for relative quantification of different microbial species by real-time qPCR and for absolute quantification of total prokaryotic cells by 4′,6-diamidino-2-phenylindol (DAPI) staining. The LAM for real-time qPCR were isolated from 40 mL of fermenter liquids by centrifugation at 27000*g* for 15 min at 4 °C. The resulting cell pellet was stored at −80 °C until DNA extraction. The SAM were detached from particles of feed residues by methylcellulose treatment [34] followed by centrifugation as described for the LAM. For DAPI staining cells were fixed from 1.5 mL of fermenter liquids or 1.5 mL of cell suspension in methylcellulose solution according to Amann et al. [35]. In brief, samples were mixed with 9 mL paraformaldehyde (4% in phosphate buffered saline, PBS, 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM disodium hydrogen phosphate, 1.4 mM potassium dihydrogen phosphate) and 1.5 mL 1 × PBS and incubated for 4 h at 4 °C. Cells from 1.6 mL of this suspension were harvested by centrifugation at 13000*g* for 5 min, washed with 1 × PBS, resuspended in 800 μl of 1 × PBS and 800 μL of absolute ethanol and stored at −20 °C until further analysis.