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In vitro methane formation and carbohydrate fermentation by rumen microbes as influenced by selected rumen ciliate species

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Abstract

Ciliate protozoa contribute to ruminal digestion and emission of the greenhouse gas methane. Individual species of ciliates co-cultured with mixed prokaryote populations were hypothesized to utilize carbohydrate types differently. In an *in vitro* batch culture experiment, 0.6 g of pure cellulose or xylan was incubated for 24 h in 40-mL cultures of *Entodinium caudatum*, *Epidinium ecaudatum*, and *Eudiplodinium maggii* with accompanying prokaryotes. Irrespective of ciliate species, gas formation (mL) and short-chain fatty acids (SCFA) concentrations (mmol L^{-1}) were higher with xylan (71; 156) than with cellulose (52; 105). Methane did not differ (7.9% of total gas). The SCFA profiles resulting from fermentation of the carbohydrates were similar before and after removing the ciliates from the mixed microbial population. However, absolute methane production (mL $24\ h^{-1}$) was lower by 50% on average after removing *E. caudatum* and *E. maggii.* Methanogen copies were less without *E. maggii*, but not without *E. ecaudatum*. Within 3 weeks part of this difference was compensated. Butyrate proportion was higher in cultures with *E. maggii* and *E. ecaudatum* than with *E. caudatum* and only when fermenting xylan. In conclusion, the three ciliate species partly differed in their response to carbohydrate type and in supporting methane formation.

Keywords: Cellulose; *Entodinium*; *Epidinium*; *Eudiplodinium*; Fiber; Hemicellulose

Introduction

Ciliate protozoa are an integral part of the rumen microbial ecosystem. Apart from degrading carbohydrates like other ruminal microbes, they specifically influence growth and activity of rumen bacteria and methanogenic Archaea. A general influence of the protozoa in this respect has been mainly explored by comparing the fermentation capacity of ruminants with intact or defaunated, *i.e.*, protozoa-free, rumen microbial populations (Eugène et al. 2004; Morgavi et al. 2008; Belanche et al. 2011a). However, defaunation-induced changes like those found for instance in ruminal short-chain fatty acid (SCFA) profile (Williams and Coleman 1992; Eugène et al. 2004; Belanche et al. 2011a) and methanogenesis (Soliva et al. 2003) were not consistent between studies. Likewise, digestibility of organic matter (OM) and fiber is not always (Kasuya et al. 2007; Zeitz et al. 2012), but often (Eugène et al. 2004; Belanche et al. 2011a) higher in faunated animals. In the latter case, hemicellulose degradation seems to be stronger inhibited by defaunation than cellulose digestion (Chaudhary et al. 1995). Further, the rumen microbes in defaunated animals may focus on easily degradable cell wall tissue and discriminate against poorly degradable cell walls whereas discrimination is less in faunated animals (Kasuya et al. 2007).

As the composition of the protozoal fauna is known to differ between diets (Hristov et al. 2001; Tymensen et al. 2012), inconsistent effects of defaunation between studies

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might result from differences in the fermentative activity of individual ciliate species. There are indications from studies performed *in vitro* (Jouany and Toillon 1997; Ranilla et al. 2007) and *in vivo* (Jouany et al. 1981) that individual ciliate species affect methane (CH_4) formation differently. However, this is not supported by the findings of Belanche et al. (2011b). Besides, the different rumen ciliate species have different constitution of genes necessary for (fiber) degradation (Bera-Maillet et al. 2005; Ricard et al. 2006) and differ in their capacity to degrade structural carbohydrates (Michałowski et al. 2001, 2003). The SCFA fermentation pattern and CH_4 formation are known to be influenced by different pure carbohydrates (Weimer 2011; Poulsen et al. 2012). However, data on the contribution of individual ciliate species to total microbial fermentation of pure carbohydrates is lacking.

In the present study, the hypotheses tested were (i) that the ruminal prokaryote population (mainly consisting of bacteria and methanogens) differs in fermentation pattern and CH_4 forming potential from different structural carbohydrates in the presence of different ruminal ciliate species, and (ii) that the ciliate species influence the ecosystem in a way that even after their removal the remaining prokaryotes continue to differ in their activity. To verify both hypotheses, three different ciliate species were isolated and cultivated *in vitro* in mixed ciliate-prokaryotic cultures. The cultures were incubated with cellulose or xylan either in the presence or absence of the respective ciliate species, and the end products of carbohydrate fermentation, protein deamination as well as CH_4 formation, and the microbial population densities, were quantified.

Material and Methods

Initial cultivation period

The selected ruminal ciliate species included *Entodinium caudatum*, which feeds mainly on starch, as well as *Epidinium ecaudatum* and *Eudiplodinium maggii*, which are amongst the ciliates with the highest fibrolytic activity (Williams and Coleman 1992). They were isolated from a sheep rumen by collecting 10–20 individuals per species, which were identified on the basis of morphological features (Zeitz et al. 2011). All cells isolated per species were transferred into 50-mL Erlenmeyer flasks sealed with a natural rubber stopper. The flasks contained 40 mL of Caudatum-type medium which is free of ruminal fluid (Coleman et al. 1972). Following the protocol of Michałowski (1995), ciliate cultures were incubated at 39 °C under exclusive CO_2 atmosphere for 2 weeks. Afterwards, cultures were propagated in medium M (Dehority 1998) for approximately 2 months before the experiment started. Medium M had well supported growth and viability of all selected species in a previous study (Zeitz et al. 2011). The media were prepared aerobically every second week, and stored at 4 °C. Ciliate protozoa were cultivated in the presence of prokaryotes in the present study. The prokaryotes had been obtained in the course of isolating the ciliates, *i.e.*, they originated either from the sheep's original rumen fluid or represented the prokaryotes which had been associated extra- or endocellularly with the picked ciliate cells. The ciliate cultures received 15 mg day^{-1} of a powdered feed composed of ryegrass hay, wheat gluten, barley flour, and crystalline cellulose (Sigmacell type 20, S3504; Sigma–Aldrich, Buchs, Switzerland) in a ratio of 0.6: 0.16: 0.12: 0.12 as described in Zeitz et al. (2011). After feeding, the culture flasks were gassed with CO_2 for 3 min at 39 °C. Cultures were transferred into fresh medium every 4 days (Zeitz et al. 2011).

Experimental design and protocol

The same source of cellulose as used during cultivation was tested as a pure carbohydrate. Both crystalline and amorphous cellulose are occurring in forages (Weimer 1992). The other carbohydrate type used was xylan (a form of highly complex polysaccharides; produced from oat spelts; product number 38500 (discontinued product; Serva, Heidelberg, Germany), serving as a model for hemicellulose. In order to standardize the carbohydrate particle size as far as possible, xylan was ground for 40 s with a ball mill (Retsch MM200, Schieritz & Hauenstein, Arlersheim, Switzerland) at 30 m s^{-1}. Additionally, xylan was sieved to exclude all particles >63 μm.

Incubations were carried out at 39 °C for 24 h in 120-mL serum bottles sealed with butyl rubber stoppers (Sigma–Aldrich, Buchs, Switzerland). Ciliate cultures were used after being allowed to grow for 4 days in fresh medium. Besides these ciliate cultures (*i.e.*, ciliates plus prokaryotes), prokaryotic cultures free of ciliates were tested. These cultures were prepared by removing the ciliates just before the incubation started through using the supernatant after centrifuging the ciliate cultures for 3 min at 2147 × *g* at ambient temperature (Kisidayova et al. 2000). From each of the ciliate-prokaryotic co-cultures ($n = 7$) and the ciliate-free cultures ($n = 4$ only, due to technical problems) 20 mL were then transferred into incubation bottles. These were filled with 20 mL of pre-warmed medium M, 0.6 g of one of the pure carbohydrates and 0.18 g of wheat gluten (G5004-500G; Sigma–Aldrich, Buchs, Switzerland) as protein source. Cultures were then gassed for 3 min with CO_2 to remove residual oxygen. Additionally, two bottles without feed served as blanks for each ciliate-prokaryotic and for each ciliate-free prokaryotic culture. The amount of gas and CH_4 produced in these blanks was subtracted from that produced by the carbohydrate-treated cultures in order to calculate net gas and CH_4 formation.

Gas production was measured after 4, 8, 20, and 24 h of incubation by using 5-mL, 10-mL and 20-mL glass syringes (Eterna Matic; Sanitex, Bassecourt, Switzerland) with disposable needles (Erosa; 23 gauge; Rose GmbH, Trier, Germany) that were plunged through the rubber stopper. The amounts of gas produced were then read from the calibrated scale of the syringe. In a preliminary test, a very high