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Effect of nitrogen sources on *in vitro* fermentation profiles and microbial yield using equine caecal contents

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ABSTRACT

The effect of different nitrogen and carbohydrate sources on *in vitro* fermentation profile and microbial yield of equine caecal contents was assessed. For this purpose, caecal contents were collected from 3 geldings, fed at maintenance level twice a day, and diluted with a buffer mineral solution (1:10, v/v). Caecal inoculum was then used in incubations using the gas production technique in one of the following treatments: mixture of rapidly fermentable carbohydrates (glucose, xylose and starch); 25 mg of N in the form of casein; 25 mg of N in the form of urea; mixture of rapidly fermentable carbohydrates plus 25 mg of N in the form of casein; mixture of rapidly fermentable carbohydrates plus 25 mg of N in the form of urea; no substrate. Total volatile fatty acids (VFA), ammonia nitrogen and gas production were measured after a 24-h incubation. Microbial biomass was estimated using adenine and guanine bases as internal markers, and microbial growth efficiency (Y_{ATP}) and gas efficiency (E_{gas}) were estimated. Results showed a higher fermentative activity when nitrogen together with soluble carbohydrates was provided in contrast to treatments that were energy or nitrogen limited. When nitrogen and carbohydrates are provided, besides an efficient growth of the microbial population, there is a marked increase in total VFA and gas production. Although the caecal microbial population will respond to casein addition with an increase in VFA and gas production, microbial growth efficiency will be lower compared to the addition of urea as nitrogen source, indicating that the microbial population would mainly use non protein nitrogen for growth.

Abbreviations: A:P ratio, acetate:propionate ratio; ATP(A), ATP estimation by acrylate pathway; ATP(S), ATP estimation by succinate pathway; BW, body weight; DM, dry matter; E_{gas}, microbial growth efficiency related to gas production; Pp, proportion; SEM, standard error of the mean; VFA, volatile fatty acids; Y_{ATP}, microbial growth efficiency; Y_{ATP}(A), microbial growth efficiency estimated by the acrylate pathway; Y_{ATP}(S), microbial growth efficiency estimated by the succinate pathway.

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1. Introduction

The equine caecum and colon are the major sites for fermentation were a vast microbial population maintains, under normal feeding conditions, a balance with its host, keeping the integrity of the ecosystem (Julliand, 1998). Factors that distress the digestive microbial ecosystem are as numerous and complex as the microorganisms that inhabit it (Hespell and Bryant, 1979). Despite the fact that the understanding of equine hindgut microbial population is essential, knowledge concerning the metabolism of the caecum–colon microbial population and its requirements is scarce.

Microbial growth and consequent fiber degradation in the hindgut environment relies on the energy and nitrogen availability. A sizable proportion of the available protein and carbohydrate is digested and absorbed prior to reaching the caecum–colon environment. This means that feed cytoplasmic protein (nitrogen) and soluble sugars reach the hindgut in low amounts, whereas the majority of cell wall carbohydrates and linked nitrogen will reach the hindgut, leading to a probable protein limiting environment (Santos et al., 2011). In the rumen, when microbial growth is limited by nutrients other than energy (*e.g.* nitrogen), a situation of energetic uncoupling can occur (Russell and Cook, 1995; Russell, 2007). In a situation of energetic uncoupling, there is a higher heat production, an increase in volatile fatty acids (VFA) production and a low cell yield (Russell, 2007).

It is assumed that the microbial population of the equine hindgut will respond rumen like in terms of its metabolism. In previous studies conducted *in vitro* using the gas production technique, we observed different microbial yields and fermentation profiles when caecal contents were provided with energy and increasing amounts of either casein or urea as nitrogen sources (Santos et al., 2012). In this previous study, we observed a positive response in terms of VFA and microbial yield to urea addition, which we intended to verify in the present work. Other nutrient limiting situations may also exist but have, so far, not been explored.

The main objective of this study was to evaluate the effects of protein (casein), non-protein (urea) N, and soluble carbohydrates availability on equine caecal fluid fermentation parameters and microbial growth efficiency using the gas production technique.

2. Materials and methods

2.1. Gas production incubations

Three horses (350 ± 10 kg body weight, BW) fitted with permanent caecal cannulas were used as caecal fluid donors. All horses were fitted with a permanent caecal cannula close to the ileo-caecal junction. The barrel of the cannula was about 150 mm long with a diameter of 40 mm (Rosenfeld and Astbø, 2009). Animals were fed with a diet that consisted of 1.5 kg of a commercial concentrate feed that contained on a DM basis: 185 g/kg CP, 304 g/kg NDFom and 183 g/kg ADFom offered in two meals (9:00 h and 16:00 h) and *ad libitum* meadow hay (75 g/kg CP, 746 g/kg NDFom and 526 g/kg ADFom, in DM basis). The diet level was based on the criteria previously defined by INRA for a resting horse, housed in a box (INRA, 1990). Animals had free access to water and to mineral–vitamin blocks, and were allowed to walk 20 min per day. Caecal fluid was pooled from all the animals and withdrawn 2 h after the morning meal into pre-warmed insulated flasks, previously filled with CO_2, strained through eight layers of cheesecloth and kept at 39 °C under a constant flux of CO_2. Incubations were performed using the methods described by Cone et al. (1996) for rumen fluid, with minor modifications. The buffer solution was N-free and contained 10.03 g/l $NaHCO_3$, 1.43 g/l Na_2HPO_4, 1.55 g/l KH_2PO_4, 0.15 g/l $MgSO_4$–$7H_2O$, 0.52 g/l Na_2S, 0.017 g/l $CaCl_2{\cdot}2H_2O$, 0.015 g/l $MnCl_2$–$4H_2O$, 0.002 g/l $CoCl_3$–$6H_2O$, 0.012 g/l $FeCl_3$–$6H_2O$ and 0.125 mg/l resazurin. To avoid a high input of N, caecal fluid was diluted 1:10 (v/v) with the buffer/mineral solution.

Incubations were performed using 60 ml of the buffered caecal fluid in 250 ml fermentation bottles (Schott, Mainz, Germany), containing one of the following substrates: E – 360 mg of rapidly fermentable carbohydrates (120 mg of glucose – Fluca®, 99% purity, 120 mg of xylose – Sigma®, 99% purity and 120 mg of soluble starch – Merck®); C – 25 mg of N in the form of casein (Sigma®, 90% purity); U – 25 mg of N in the form of urea (Acros organics®, 99.5% purity); EC – 360 mg of rapidly fermentable carbohydrates (120 mg of glucose, 120 mg of xylose and 120 mg of starch) plus 25 mg of N in the form of casein; EU – 360 mg of rapidly fermentable carbohydrates (120 mg of glucose, 120 mg of xylose and 120 mg of starch) plus 25 mg of N in the form of urea; B – control (no substrate).

All laboratory handlings were conducted under continuous flushing with CO_2. All samples were incubated in duplicate with 3 repetitions in time (independent runs). Gas production was recorded for 24 h, using a fully automated system (Cone et al., 1996).

2.2. Chemical analysis

Determination of pH, VFA, ammonia nitrogen (NH_3-N) and quantification of purine bases were performed after the 24 h fermentation. The pH value of the inoculum and fermentation medium was measured with a digital pH meter (pH 530, Wissenschaftlich-Technische Werkstätten Weilheim, Germany).

Fermentation fluids from the gas production experiment were sequentially centrifuged at 500 × *g* for 5 min at 4 °C, and the supernatants were centrifuged at 20,000 × *g* for 20 min, at the same temperature. The final supernatants were frozen (−20 °C) for subsequent VFA and NH_3-N analysis. The residues (bacterial pellets) obtained were freeze dried within a period