IN VIVO AND *IN VITRO* STUDY OF CAECAL FERMENTATION PATTERN AND METHANOGENESIS IN RABBITS

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ABSTRACT

Methane formation and caecal fermentation pattern were studied *in vivo* and *in vitro* in 16 white New Zealand rabbits allocated to four diets formulated with two different sources of fibre (alfalfa hay or sugar beet pulp) and starch (wheat or maize). Animals received the diet for at least two weeks before methane production was measured *in vivo* in a respiratory chamber. Then, animals were slaughtered and caecal contents were sampled for volatile fatty acid (VFA) determination and used as inoculum for *in vitro* incubations performed to determine gas production and methane formation. Total VFA concentration in caecal contents averaged 52.5 mM, with butyrate content higher when alfalfa hay was the main source of fibre compared to sugar beet pulp (9.97 vs. 7.32 mM). Fermentation pattern was also affected by the experimental treatment, acetate being lower (72 vs. 79%) and butyrate higher (20 vs. 14%) when maize was the source of starch in relation to wheat. Fermentation *in vivo* vs. *in vitro* showed some differences (molar proportion of acetate, 75 vs. 72% *in vivo* and *in vitro*, respectively; molar proportion of propionate, 6.9 vs. 9.1% *in vivo* and *in vitro*, respectively), probably due to differences in pH (6.0 vs. 6.7 *in vivo* and *in vitro*, respectively). Only 2 out of 16 rabbits produced a substantial volume of methane *in vivo*, whereas all of them showed some formation in the *in vitro* incubations. No effect of the experimental treatment was observed *in vivo* on methane production due to the high inter-individual variability, whereas *in vitro* an effect of the source of starch was observed, being higher when maize was included in the diet compared to wheat. A similar effect was shown in total gas production, probably due to the stimulation of caecal bacteria in the animals receiving a more resistant starch from maize that cannot be digested in the small intestine and reaches the caecum. H_2 recovery was very low, suggesting the importance of other mechanisms of H_2 disposal than methanogenesis such as reductive acetogenesis, and was again affected by the source of starch in a similar way.

Key words: Rabbit, Caecal fermentation, Methanogenesis, Reductive acetogenesis.

INTRODUCTION

The caecum represents the main fermentation site in the rabbit. Caecal microorganisms convert nutrients leaving the small intestine to volatile fatty acids (VFA), gases (CH_4, CO_2, H_2), ammonia and compounds incorporated into microbial cells. Hydrogen is formed during fermentation, but a high partial pressure of H_2 in some anaerobic ecosystems may reduce the efficiency of fermentation. There are a number of alternative pathways for H_2 disposal, mainly methanogenesis and reductive acetogenesis. Literature data indicate a competition for metabolic H_2 between methanogenic and acetogenic bacteria in the animal digestive tract (De Graeve *et al.*, 1994; Bernalier *et al.*, 1993). Whereas in adult ruminants reductive acetogenesis is relatively unimportant, being methanogenesis the main H_2 sink, in monogastric animals both mechanisms may occur together with other possible pathways for disposal of H_2 (Jensen, 1996). Caecal fermentation has been extensively studied in the rabbit, but methanogenesis has been reported only in *in vitro* studies. Methane production is almost absent from fermentation before weaning (Piattoni *et al.*, 1996). These authors suggest that reductive acetogenesis is a major characteristic of caecal fermentation in young rabbits, being replaced gradually

and partially by methanogenesis as the intake of solid food increases, but reductive acetogenesis still appeared to be important. Factors involved in the partitioning of H_2 between reductive acetogenesis and methanogenesis in the rabbit caecum are not well understood.

The aim of this work was to investigate the effect of different sources of fibre and starch on caecal fermentation pattern in rabbits and to study the importance of methanogenesis in their caecum. For this purpose *in vivo* and *in vitro* experiments were designed to evaluate methane production.

MATERIALS AND METHODS

Animals and experimental design

16 New Zealand White rabbits (70-80 days and 2.27±0.064 kg) were randomly allocated to four different diets based on two sources of fibre (alfalfa hay, AH; and sugar beet pulp, SBP) and two sources of starch (wheat and maize; Table 1).

Table 1: Ingredients and chemical composition of diets

		Alfalfa hay		Sugar beet pulp	
		Maize	Wheat	Maize	Wheat
Ingredients (%)	Sugar beep pulp	19.6	18.5	47. 8	48.5
	Alfalfa hay	50.6	51.6	15.9	16.2
	Maize	18.4	0	14.9	0
	Wheat	0	19.6	0	16.5
	Soybean meal	10.7	9.7	16.1	14.6
	Treated straw	0.5	0	5.1	4.1
	Sunflower oil	0	0.4	0	0
	Mineral and vitamin mix[1]	0.2	0.2	0.2	0.2
Chemical composition (%)	Dry matter	91.0	91.2	90.6	91.0
	Organic matter	89.9	89.9	92.0	92.2
	Crude protein	18.1	18.4	17.0	17.4
	Neutral detergent fibre	33.8	32.4	35.0	34.1
	Acid detergent fibre	17.6	18.1	18.2	18.1
	Lignin	4.6	4.1	2. 8	2.6

[1]Composition of vitamin-mineral mix: 200 mg/kg Co ($CoSO_4$ 7 H_2O), 3 g/kg Cu ($CuSO_4$ 5 H_2O), 20 g/kg Fe ($FeSO_4$ 5 H_2O), 8 g/kg Mn (MnO_2), 30 g/kg Zn (ZnO), 30 mg/kg Se (Na_2SeO_3), 500 mg/kg I (KI), 4500000 IU/kg vit A, 550000 IU/kg vit D_3, 1100 mg/kg vit E, 250 mg/kg vit B_1, 1500 mg/kg vit B_2, 100 mg/kg vit B_6, 6000 mg/kg vit B_{12}, 500 mg/kg vit K, 5000 mg/kg D-pantothenate, 12.5 g/kg niacin, 100 g/kg choline chloride

Food was restricted to 100 g/d and animals had free access to drinking water. Each animal received the experimental diet for at least two weeks before they were individually confined for a short period of time (<8 h) and during three consecutive times into an hermetically sealed respiratory chamber for measuring methane production from the change in CH_4 concentration within the fixed volume of the system (Lachica *et al.*, 1995). Then, animals were slaughtered and the caecum excised and dissected. The pH of caecal content was measured and concentration of VFA determined. The remaining caecal contents (approximately 90 g) were diluted in 900 ml of incubation medium (Theodorou *et al.*, 1994) under anaerobic conditions, and this solution was used as inoculum for an *in vitro* incubation trial. Fermentation trial was conducted in triplicates using 120 ml bottles. Sterilized caecal contents from adult female rabbits, receiving one of the four experimental diets, were used as substrate (approximately 800 mg per bottle). Bottles were filled with the inoculum (80 ml, except 90 ml for those to measure methane formation) and incubated at 39ºC in anaerobic conditions, and bottles without substrate were also included as blanks. The microbial fermentation pattern was studied by measuring the gas produced during the *in vitro* incubation of substrate (Theodorou *et al.*, 1994). Gas from additional bottles was sampled for measuring methane concentration. The pressure of gas produced in each bottle was recorded on a HD8804 manometer with a TP804 pressure gauge (DELTA OHM, Italy) after 2, 4 and 6 h of incubation. Readings were converted into volume (ml) by using the following pre-established linear regression between pressure recorded (mbar) in the same type of bottles and known air volumes:

volume = (pressure – 0.981) / 30.375 (r = 0.996; n = 64)

Inoculum was sampled (4 ml) initially for determination of VFA concentration. After 6 h of incubation, total gas production was measured and a gas sample (about 15 ml) was removed from each bottle and stored in a Haemoguard Vacutainer (Terumo Europe N.V., Leuven, Belgium) before analysis for CH_4 concentration. Bottles were uncapped, the pH was measured immediately with a pH meter, and bottles content was sampled (4 ml) for determination of VFA concentration.

Chemical Analyses

VFA concentration was analysed by GLC following the procedure described by Jouany (1982). Net production of VFA was calculated by subtraction of VFA present in the bottles incubated without substrate. Methane concentration was analyzed with a Shimadzu GC 14B (Shimadzu Corporation, Kyoto, Japan) equipped with flame ionization detector and a column packed with Carboxen 1000 (Supelco, Madrid, Spain) as described by Carro and Ranilla (2003).

Statistical Analysis and calculations

Data were analysed by ANOVA as a 2 x 2 factorial design, with the source of fibre and starch as main factors. Interaction of main effects was not significant in most cases, and where necessary it is indicated. H_2 recoveries ($2H_{rec}$) were calculated according to Demeyer (1991) as: $2H_{rec}$=100 x (2P+2B+4M)/(2A+P+4B), where A, P, B and M represent net molar production values of acetate, propionate, butyrate and methane, respectively.

RESULTS AND DISCUSSION

In caecal contents the VFA concentration averaged 52.5±3.5 mM, and AH induced a higher butyrate concentration compared to SBP, the differences being more pronounced with maize as source of starch (interaction fibre x starch; P=0.05). The source of starch affected acetate concentration only when AH was the main source of fibre, being lower with maize (interaction fibre x starch; P=0.04), and similar results were obtained with propionate (P=0.03) and total VFA (P=0.03). Fermentation pattern of VFA, either in the caecal contents or *in vitro* cultures, was affected by diet, acetate proportion being higher and butyrate lower with wheat than with maize. A higher amount of resistant starch from maize reaching the caecum may lead to butyrogenic fermentation (Bird *et al.*, 2007). Molar acetate and propionate proportions were higher and lower, respectively (75.5 and 6.9 vs. 72.6 and 9.1% acetate and propionate, respectively) *in vivo* than *in vitro*, probably due to pH differences (6.0 vs. 6.7 *in vivo* and *in vitro*, respectively).

Methane formation *in vivo* was small except for two rabbits that produced substantial volumes. The individual variability was very high (CV=250%) in the observed values of methane production, what explains the lack of significant differences (Table 2). The total volume of gas produced *in vitro* increased with time (Table 2) and was not affected by the fibre source. Between the sources of starch, maize induced a higher gas production than wheat at any time (P<0.05 at 2 and 6 h, and P<0.10 at 4 h). On average, methane represented 0.8% of the total gas, and its production was low, but less variable than *in vivo* (Table 2). No treatment differences were observed in CH_4 concentration, but those animals given maize diets tended to a higher methane concentration (P<0.10) than those given the wheat diets. When expressed on substrate weight basis, CH_4 production was higher in animals given maize than wheat diets (P<0.01).

Whereas methane production is one of the main pathways to dispose of reducing equivalents generated during ruminal fermentation, methanogenic organisms are less abundant in the large intestine (Morvan *et al.*, 1996). The small values of 2H recovery observed suggest a greater importance of other pathways of H_2 disposal than methanogenesis, such as reductive acetogenesis.

Table 2: Volatile fatty acids (VFA) concentration in caecal contents, *in vitro* gas production over time, *in vivo* or *in vitro* methane production and 2H recovery

		Fibre		Starch			Probability	
		AH	SBP	Maize	Wheat	SEM	Fibre	Starch
Volatile fatty acids:	Acetic (mM)	36.7	42.9	35.8	43.8	4.76	NS	NS
	Propionic (mM)	3.70	3.44	3.43	3.71	0.49	NS	NS
	Butyric (mM)	9.97	7.32	9.61	7.69	1.09	*	NS
	Total (mM)	57.1	47.9	49.5	55.51	5.84	NS	NS
	Acetic (%)	75.7	75.3	72.1	78.8	1.69	NS	**
	Propionic (%)	7.32	6.51	7.14	6.68	0.77	NS	NS
	Butyric (%)	17.4	16.0	19.5	13.9	1.12	NS	***
Total gas production	2 h	7.98	7.88	8.73	7.34	0.647	NS	*
(ml/g DM):	4 h	16.20	16.79	17.57	15.32	0.803	NS	T
	6 h	22.47	23.54	24.77	21.40	1.358	NS	*
Methane production: *In vivo*	$ml/BW^{0.75}/d$	2.74	1.20	3.41	0.52	2.458	NS	NS
In vitro	µl/ml gas	8.18	8.07	8.69	7.56	0.624	NS	T
	µmol/g substrate	8.29	8.64	9.85	7.22	0.853	NS	**
	2H recovery (%)	24.39	23.57	25.75	22.22	0.989	NS	**

NS, not significant; T, P<0.10; *, P<0.05; **, P<0.01; ***, P<0.001

Our values are lower than those reported by Piattoni *et al*. (1996) in 56-day-old rabbits (50%), but similar to those observed by Marounek *et al*. (1999) (26-29.6% in rabbits 11 weeks old). Again, 2H recovery was higher when maize was used as source of starch, due to a higher methane production. Previous *in vitro* studies (Piattoni *et al*., 1996) have suggested that reductive acetogenesis may be the main H_2 sink in the caecum of young rabbits, but it can be replaced gradually and partially by methanogenesis after weaning with intake of solid food. Methanogenesis seems to be almost absent in animals before weaning, although it increases afterwards (Marounek *et al*., 1999). Unlike our *in vitro* study, where all animals showed some CH_4 production, *in vivo* only 2 out of 16 rabbits showed a considerable methane production in the respiratory chamber. Our results suggest that methanogenic bacteria exist in the rabbit caecum, but only some rabbits exhibit a remarkable CH_4 production, in agreement with Piattoni *et al*. (1995) who suggested the existence of a genetic effect. Besides, the individual variability has to be considered *in vivo*.

A competition among three main H_2-consuming organisms, methanogenic Archaea, acetogenic bacteria and sulphate reducing bacteria has been described in the large intestine. The latter have a higher substrate affinity for H_2 than methanogenic Archaea (Gibson *et al*., 1990), but their growth depends on sulphate availability. An alternative route for H_2 disposal is reductive acetogenesis. The relative substrate affinities of methanogens for H_2 ought to favour methanogenesis in a competitive environment (Macfarlane and Gibson, 1997). Therefore, methanogenesis should usually dominate H_2-dependent acetate production in anaerobic ecosystems, but the major presence of acetogenesis in rabbits might lie in the higher acid sensitivity of sulphate reducing bacteria and methanogens (Gibson *et al*., 1990). This may also explain the greater methanogenic activity *in vitro* vs *in vivo* due to the higher pH *in vitro* (6.7 vs. 6.0). Furthermore, acetogens can grow better in a low-substrate environment due to their ability to grow on substrates other than H_2/CO_2, such as monosaccharides (Drake, 1994), and are more resistant to bile salts (Jezierny *et al*., 2007). These capacities would allow them to be more competitive than methanogens in a digestive tract with a fast passage rate (Morvan *et al*., 1996) and might explain the lower presence of methanogens in the caecum and colon of rabbits than in other fermentation compartments.

CONCLUSIONS

In conclusion, this study seems to confirm that, in detriment of methanogenesis, acetogenesis might be one of the major H_2 sinks in rabbit caecal environment unlike other fermentation compartments, although methanogenic Archaea would exist and methane formation becomes remarkable in favourable conditions.

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