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Original article

In vitro study of the age-dependent caecal fermentation pattern and methanogenesis in young rabbits

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Summary — The caecal fermentation pattern, including methanogenesis, was studied in young rabbits using in vitro batch incubations. Six conventional litters of eight rabbits each were used. At the age of 22, 25, 28, 32, 36, 42 and 56 days, an animal was slaughtered from each litter and its caecal contents were used for in vitro batch incubations at 39 °C/24 h. The incubated samples were analysed for volatile fatty acids (VFA), methane, hydrogen, ammonia nitrogen (NH_3-N) and lactic acid (LA). The net total in vitro VFA production did not differ clearly with age, although a significant decrease was observed on day 36, reflecting the reduced zootechnical performances probably related to an infection with *Clostridium spiroforme* that occurred in the same period. The molar proportions of butyrate and propionate formed a change in the opposite direction with age, starting with a sudden shift from propionate to butyrate at day 25. In vitro NH_3-N production was suggestive of a progressive and significant decrease with age; in vitro LA production was always low. Methane production was almost absent from fermentation until 32 days of age, after which it suddenly shifted from 1.6 to 52.0 µmol/flask/day and increased further with age. A significant litter effect on methanogenesis was observed which suggested the existence of a genetic effect. The hydrogen production was quite low and decreased significantly from day 36 with increasing methanogenesis. The calculated hydrogen recoveries showed a gradual increase from day 32 and were positively correlated ($r = 0.92$) with methane production. In conclusion, it would seem that in young suckling rabbits, reductive acetogenesis is a major characteristic of caecal fermentation, to be replaced gradually and partially by methanogenesis with the increasing intake of solid feed.

rabbit / caecum / in vitro VFA / methane

Résumé — Étude in vitro de l'effet de l'âge sur l'activité fermentaire cæcale et la méthanogenèse chez le lapereau. *L'activité fermentaire cæcale et particulièrement la méthanogenèse ont été étudiées chez les lapereaux à l'aide d'incubations in vitro. Six nichées de huit lapereaux chacune ont été utilisées. À l'âge de 22, 25, 28, 32, 36, 42 et 56 jours un lapereau de chaque nichée a été sacrifié*

et le contenu cæcal échantillonné pour être incubé dans un bain-marie à 39 °C. Après un période d'incubation de 24 heures, la production d'acides gras volatils (AGV), de méthane, d'hydrogène, de lactate (L) et d'azote ammoniacal (NH_3-N) a été déterminée. La production nette des AGV totaux ne varie pas clairement avec l'âge ; on observe une chute significative à 36 jours d'âge reflétant une diminution des performances zootechniques observée entre le 32e et le 36e jour, probablement due à une infection par Clostridium spiroforme*, survenue à cette période. Les proportions molaires du propionate et du butyrate évoluent en sens inverse avec l'âge de telle façon qu'au 25e jour on observe une diminution soudaine du propionate au profit du butyrate. La production in vitro de N-NH_3 diminue progressivement et significativement avec l'âge, alors que celle du lactate reste basse. Il n'y a pas de production de méthane jusqu'au 32e jour. Au-delà de cette période la production de ce gaz est passée brusquement de 1,6 à 52,0 µmoles/flacon/jour et a continué à augmenter par la suite. Quant à la production de méthane, la variabilité entre les nichées suggère un effet génétique sur ce paramètre. La production d'hydrogène est faible et diminue avec l'âge et avec l'augmentation de la méthanogenèse. Le bilan d'hydrogène est positivement corrélé avec l'âge (r = 0,92) et à la production de méthane. En conclusion, il semble que l'acétogenèse réductrice est la principale caractéristique fermentaire dans le cæcum de jeunes lapins. Cette voie serait progressivement et partiellement remplacée par la méthanogenèse lors de l'ingestion des aliments solides.*

lapin / cæcum / AGV in vitro / méthane

INTRODUCTION

The fibre degradating microbial activity in the rabbit takes place mainly in the caecum. The main end products of such microbial fermentation are the volatile fatty acids (VFA) and the gases methane and carbon dioxide. Acetate is the major acid present and the rate of butyrate production exceeds that of propionate (Hoover and Heitmann, 1972). The latter feature appears to distinguish the rabbit from the other mammalian species (Jouany, 1991).

In comparison with the rumen, hindgut fermentation is generally more efficient in terms of its potential energy supply for the animal. In fact, reductive acetogenesis replaces, at least partially, methanogenesis as a hydrogen sink in fermentation, as indicated by calculations of the so-called hydrogen recoveries (Demeyer, 1991). Fermentation is an anaerobic oxidation with disposal of electrons to a variety of reduced end products. Such disposal often involves hydrogen gas (H_2) as an intermediate and the latter has to be continuously removed to allow further oxidation. Removal is obtained by H_2 losses in breath and flatus, but also by the activity of H_2 utilising methanogenic, acetogenic and sulphate-reducing bacteria (Demeyer, 1991; Gibson et al, 1993). In the rumen, H_2 disposal is dominated by methanogenesis, as indicated by stoichiometric calculations, based on overall fermentation reactions (Wolin, 1960). Using the amount of end products formed, such stoichiometry allows the calculation of the amounts of "metabolic hydrogen" produced and recovered in reduced end products expressed as percentage of hydrogen recovery. Such calculations test the hypothesis (model) that the amount of metabolic hydrogen liberated in pyruvate and acetate production equals that deposited in the production of methane, propionate and butyrate (Marty and Demeyer, 1973). With rumen contents, such recoveries range between 80 and 90% (Demeyer, 1991), whereas with hindgut contents from different species, including an occasional observation with rabbits, values were much lower, suggesting the presence of other 'hydrogen sinks', as discussed in detail by Demeyer and De Graeve (1991) and Demeyer (1991).

Systematic studies of caecal fermentation in rabbits, including methanogenesis,

are rare. Possible methanogenesis in the rabbit caecum may, however, be affected by the nutritional changes involved in weaning: young rabbits have to change from milk to an exclusively solid and lignocellulose diet within a period of 10–12 days. Such changes affect caecal fermentation pattern. Recently, it has been shown in vivo that a shift from propionate to butyrate occurs very early in age (Padilha et al, 1995; Piattoni et al, 1995). Such age-dependent variation of caecal fermentation pattern has not been studied in detail in young rabbits and data on methanogenesis are lacking.

The objective of the present experiment was to investigate the effect of age on fermentation pattern and methanogenesis in young rabbits. For this purpose, the samples used for the in vivo study of the age-dependent variation of caecal contents composition (Piattoni et al, 1995) were also used for simple batch incubations of the type used for the study of rumen metabolism.

MATERIALS AND METHODS

Animals and diet

Young, conventional rabbits from the experimental strain of the Research Station for Small Stock Husbandry (Maertens, 1992) were used. Initially ten litters born on the same day, from multiparous does were used. The litter size was reduced to eight young rabbits per litter. Only six litters with eight surviving young and with the most homogeneous animal weights, at 21 days of age, were chosen for the experiment. At 28 days of age, the rabbits were separated from the doe (weaning) and litters were kept together in a fattening cage.

Caecotrophy was not prevented. To avoid any change induced by feeding in the intestinal environment, the same standard reproduction diet was fed ad libitum to the does and their young both before and after weaning. The proximate composition of the pellet was in accordance with the recommendations of Lebas (1989) and contained on dry matter (DM) basis: 19.8% crude protein; 16.8% crude fibre; 35.2% NDF; 4.9% crude fat; 22.0% starch and 11.95 MJ metabolizable energy/kg.

Slaughtering and sampling

Sequential slaughterings were performed between 24 June and 28 July 1994. When the rabbits were 22, 25, 28, 32, 36, 42 and 56 days old, one rabbit was slaughtered from each litter. At the age of 22 days, however, two rabbits were slaughtered in order to collect enough caecal contents for analysis. The rabbits were sacrificed one by one between 0800 and 1200 hours. This range of time was chosen referring to the quite limited fluctuations of the caecal VFA concentration of ad libitum fed rabbits (Bellier and Gidenne, 1992).

Immediately after slaughtering and dissecting, the caecum was isolated by tying off the two extremities with a nylon string, to prevent movement of the digesta. The caecum was weighed, the contents squeezed out into a beaker under CO_2 flushing and carefully mixed with a spatula. Part of it was used to study caecal contents composition (Piattoni et al, 1995) and the rest was used for the in vitro incubations.

Incubations and analyses

The in vitro incubations were run in duplicate unless the sample volume was prohibitively small. The routine technique used over the last 20 years in our laboratory, for the study of rumen and hindgut metabolism (Demeyer, 1991), has been adapted for sample volume (15 vs 50 ml). Caecal contents were incubated within 30 min after slaughtering. Nine grams of caecal contents from each rabbit were divided into three subsamples of equal weight. Each subsample was diluted five-fold under CO_2 flushing with a buffer solution at pH 6.9 (Burroughs et al, 1950). This value is used for rumen and hindgut contents (Demeyer, 1991) and is close to that of preweaned rabbits, but is higher than that of weaned rabbits (Padilha et al, 1995; Piattoni et al, 1995).

One subsample was not incubated (blank) and immediately mixed with 0.3 ml of H_2SO_4 10 N. The other two were transferred into gas-tight incubation flasks. NH_4HCO_3 (8.5 mg/flask) was added to the solution to allow microbial growth. Flasks were filled with CO_2 and incubated

in a shaking waterbath at 39 °C for 24 h. The duration of the incubation period was imposed by practical conditions (overnight) and was analogous to earlier experiments with rumen and hindgut contents (Demeyer, 1991). Incubations were stopped by injection of 0.3 ml of H_2SO_4 10 N. From each flask, 2 ml of the fermentation gas was immediately sampled with a gas-tight syringe and analysed for methane and hydrogen by absorption gas chromatography (Marty and Demeyer, 1973). The remaining liquid sample was centrifuged (10 min at 15 000 *g*), filtered and the filtrate used for VFA analysis by gas-liquid chromatography (Shimadzu; GC 14 A) (Marty and Demeyer, 1973).

The net amount of VFA produced was calculated by substraction of VFA present in the non-incubated samples. Ammonia nitrogen (NH_3-N) and lactic acid (LA) were analysed using the microdiffusion method of Conway (1957).

Hydrogen recoveries (2H rec) were calculated from net amounts of VFA and methane (M) produced as: 2H rec = 100 * (2P + 2B + 4M) / (2A + P + 4B). This stoichiometry recovery expresses the validity of metabolic hydrogen transfer, following the model of fermentation with the net amounts of acetate (A), propionate (P) and butyrate (B) formed as discussed earlier (Marty and Demeyer, 1973; Demeyer, 1991).

Statistical analysis

Analysis of variance using the GLM procedures of SAS/Stat 6.0 (1990) was used to evaluate the effect of age and litter as follows:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \Sigma_{ijk}$$

where Y_{ijk} = depending variable; μ = overall mean; α_i = age effect (i = 1, 7); β_j = litter effect (j = 1, 6) and Σ_{ijk} = random error effect.

Data are presented as least square means and means were compared using the *t*-test. Pearson correlation coefficients were also calculated; significance is declared at $P < 0.05$.

RESULTS

Table I shows mean values for the end products of fermentation at different ages of the animals. From the beginning of the experiment (22 days of age), slightly less than 1 mmol of total VFA was formed in fermentation. At 36 days of age only, a significant drop in VFA production was observed, paralleled by a similar drop in growth rate, related to a sanitary problem, probably due to an infection by *Clostridium spiroforme*. The data suggest that the molar proportions of butyrate and propionate formed a change in the opposite direction with age, starting with a sudden shift from propionate to butyrate at 25 days of age. Indeed, although differences between ages did not always reach significance, significant correlations between age and molar proportions of propionate ($r = -0.56$, $P < 0.001$) and of butyrate ($r = 0.70$, $P < 0.001$) could be calculated.

Methanogenesis was almost absent from fermentation, until 32 days of age after which it suddenly shifted from 1.6 to 52.0 μmol/flask/day. A further significant increase with age was observed. Hydrogen production was always low and decreased significantly only after 32 days of age, opposite to the increase in methane production. Hydrogen recovery was very low but a gradual and significant increase between day 32 and day 56 was observed; moreover, it showed a positive correlation ($r = 0.92$) with methane production. In vitro NH_3-N production was suggestive of a progressive and significant decrease with age, reaching a plateau after 42 days at a value of about 196 μmol/flask/day. In vitro LA production was always lower than 3 μmol/flask/day.

Table II shows pooled mean values for the different ages per litter. Significant differences between litters were found for live weight at slaughtering, molar proportions of VFA formed and gas production. Litter 1 was significantly different from all the other litters. Very large differences for methane production were observed (8.2 to

Table I. Age-dependent in vitro caecal fermentation pattern.

	Age (days)							*SEM* [4]	*Statistical significance* [5]
	22 [1]	*25* [2]	*28* [2]	*32* [2]	*36* [2]	*43* [3]	*56* [3]		
Zootechnical performances †									
Live weight at slaughtering (g)	455 ^{a}	609 ^{b}	664 ^{b}	757 ^{c}	859 ^{d}	1 068 ^{e}	1 809 ^{f}	29.9	***
DWG (g/day)	–	48 ^{b}	43 ^{b}	35 ^{ab}	25 ^{a}	39 ^{b}	47 ^{b}	4.8	*
Products formed in vitro									
Total VFA (µmol/flask) ‡	896.7 ^{b}	1 001.3 ^{bc}	931.8 ^{bc}	1 070.8 ^{c}	675.5 ^{a}	856.3 ^{b}	856.1 ^{b}	55.9	***
Acetate (%)	65.6 ^{a}	72.0 ^{b}	72.2 ^{b}	75.9 ^{b}	74.4 ^{b}	75.5 ^{b}	73.6 ^{b}	1.5	***
Propionate (%)	13.6 ^{d}	10.0 ^{bc}	10.4 ^{c}	7.3 ^{a}	8.1 ^{ab}	6.3 ^{a}	6.5 ^{a}	0.8	***
Butyrate (%)	11.3 ^{a}	12.6 ^{a}	11.9 ^{a}	13.2 ^{ab}	12.9 ^{a}	14.9 ^{bc}	16.9 ^{c}	0.6	***
$iC_4 + C_5 + iC_5$ (%)	9.5 ^{c}	5.4 ^{b}	5.5 ^{b}	3.6 ^{ab}	4.7 ^{ab}	3.3 ^{ab}	3.0 ^{a}	0.8	***
Methane (µmol/flask) ‡	0.0 ^{a}	0.1 ^{a}	0.2 ^{a}	1.6 ^{a}	52.0 ^{ab}	86.3 ^{bc}	129.3 ^{c}	20.9	***
Hydrogen (µmol/flask) ‡	6.8 ^{a}	7.2 ^{a}	6.0 ^{a}	6.6 ^{a}	3.9 ^{b}	3.7 ^{b}	2.7 ^{b}	0.6	***
Hydrogen recovery (%)	28 ^{ab}	23 ^{ab}	23 ^{ab}	20 ^{a}	36 ^{bc}	38 ^{bc}	50 ^{c}	5.6	**
NH_3-N (µmol/flask) ‡	477.2 ^{c}	335.8 ^{b}	322.8 ^{b}	298.7 ^{ab}	233.0 ^{ab}	195.0 ^{a}	197.2 ^{a}	39.5	***
LA (µmol/flask) ‡	0.6 ^{ab}	2.1 ^{c}	0.8 ^{ab}	1.0 ^{b}	0.3 ^{ab}	0.1 ^{a}	1.0 ^{ab}	0.3	***

[1] $n = 12$ (two young rabbits from each litter); [2] $n = 6$ (one young rabbit from each litter); [3] $n = 5$ (one young rabbit died); [4] SEM: standard error of the mean; [5] * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; means with different letters on the same row are significantly different ($P < 0.05$); † From Piattoni et al (1995); ‡ net production after 24 h of incubation; VFA = volatile fatty acids.

Table II. In vitro caecal fermentation pattern for different litters [1].

	Litters						*SEM* [4]	*Statistical significance* [5]
	1 [2]	*2* [2]	*3* [2]	*4* [2]	*5* [2]	*6* [3]		
Live weight at slaughtering (g) †	821 [a]	862 [ab]	1008 [c]	860 [ab]	933 [bc]	850 [ab]	27.8	***
Products formed in vitro								
Total VFA (μmol/flask) ‡	950.6	943.6	965.1	867.3	859.9	803.7	51.9	NS
Acetate (%)	67.4 [a]	75.1 [b]	74.3 [b]	73.4 [b]	72.4 [b]	73.9 [b]	1.5	**
Propionate (%)	12.1 [b]	7.6 [a]	8.2 [a]	8.5 [a]	9.4 [a]	7.5 [a]	0.7	**
Butyrate (%)	14.5 [b]	13.1 [ab]	13.4 [ab]	11.7 [a]	14.6 [b]	12.9 [ab]	0.6	*
$iC_4 + C_5 + iC_5$ (%)	6.1 [bc]	4.2 [ab]	4.1 [ab]	6.4 [c]	3.6 [a]	5.7 [abc]	0.7	*
Methane (μmol/flask) ‡	8.8 [a]	52.6 [ab]	83.4 [b]	8.2 [a]	22.2 [a]	55.9 [ab]	19.4	*
Hydrogen (μmol/flask) ‡	6.3 [b]	5.2 [ab]	4.6 [a]	6.3 [b]	5.3 [ab]	3.7 [a]	0.5	*
Hydrogen recovery (%)	28	30	39	22	28	38	5.2	NS
NH_3-N (μmol/flask) ‡	323.1	263.7	276.3	352.7	243.0	306.6	36.7	NS
LA (μmol/flask) ‡	1.2	0.7	0.9	0.9	1.0	0.2	0.3	NS

[1] The values are pooled means for the different ages; [2] *n* = eight rabbits per litter; [3] *n* = six rabbits (two rabbits died in this litter); [4,5,†,‡] see table I.

83.4 µmol/flask) despite a large within-litter variability.

DISCUSSION

It is clear that our experiment was not aimed at the prediction of VFA production in vivo. Its aim was to investigate eventual changes of fermentation pattern with advancing age of the rabbit, particularly in relation to the period immediately after weaning. Indeed, this period is associated with increased mortality due to diarrhoea (Peeters, 1986), clearly related to a defective bacterial fermentation in the caecum. Earlier work from this laboratory has clearly shown that the in vitro batch incubation technique used here, reflects the pattern but not the extent of fermentation with both rumen and caecal contents (Demeyer, 1991). A further argument in favour of such a technique is the impossibility to cannulate rabbits before the age of 5 weeks (Gidenne and Bellier, 1992; Bellier and Gidenne, 1992), excluding in vivo experiments with very young rabbits. It should be realized that the results obtained can only be used in terms of characterization of fermentation pattern; no extrapolations relating to amounts of end products produced and/or absorbed in vivo can be made.

The validity of the technique used is illustrated by the relation of the pattern of VFA production (table I) to that found in vivo. The age-dependent shift from propionate to butyrate proportions reflects a similar change observed for VFA proportions in vivo with the same animals (Piattoni et al, 1995) or even with SPF rabbits (Padilha et al, 1995). This finding is complementary to a recent observation made by Bellier et al (1995), who observed lower butyrate proportions in the caecum of 6-week-old rabbits compared to adult animals. The authors related this change to the establishment of caecotrophy and dry feed intake, whereas our results suggest the effect of age. A further indication of the validity of the in vitro technique for the study of factors affecting in vivo fermentation, is the sudden drop in total VFA production, associated with a drop in daily weight gain (table I). The factors underlying both results are probably related to an infection with *Clostridum spiroforme*, as two rabbits died from enterotoxemia. Recently, it has been shown that experimental infection with *C spiroforme* tends to decrease caecal VFA concentration (Peeters et al, 1995).

The in vitro NH_3-N production is suggestive of a progressive and significant decrease with age. As NH_3-N is mainly derived from protein metabolism, through amino acid deamination, it is tempting to speculate that this decrease was related to a lower protein substrate in caecal contents with age.

The most interesting and clear observation from the experiments described here, however, concerns the age-dependent change in methanogenesis. Up to the age of 36 days, almost no methane could be detected, although considerable amounts of VFA were formed, in similar proportions to those formed at later ages. At day 36, the methane production suddenly shifted from 1.6 to 52 µmol/flask/day. The late production of methane could be ascribed to the slow and low level of colonization of the cellulolytic microflora in the caecum as suggested by the results of Padilha et al (1995).

Methanogenesis, however, did not occur in all the litters at the same age. Indeed, the statistical analysis showed a large variability between litters in methane production, contrasting with a relative constancy for VFA production. This was further underlined by the coefficients of variation showing very high values for methane production (190%) in contrast to VFA production (10%). It was even more striking that significant litter effects were found for the molar ratio of individual VFA, as well as for the methane and the hydrogen production. Anyway, the high

variability and the significant litter effect for both the gas production and VFA pattern suggests the existence of a genetic effect on hindgut methanogenesis and fermentation pattern, as has been observed for humans (Gibson et al, 1993) and other animals (Hackstein et al, 1996). Detailed zootechnical data (eg, daily ratio milk – solid feed) were not determined in our experiment and differences between litters may not be totally explained by genetic effects.

Although the production of both methane and hydrogen varied in opposite directions, in line with the role of hydrogen gas as precursor for methane in rumen, only very small amounts of hydrogen gas were produced, suggesting the presence of a hydrogen sink other than methanogenesis. This suggestion is also evident from the calculated metabolic hydrogen recoveries, yielding values of 20–50%. We have considered such low values as indicative of the presence of reductive acetogenesis (Henderson and Demeyer, 1989). Based on similar experiments and stoichiometric considerations, reductive acetogenesis has been identified as a substantial source of acetate in the termite gut and in the hindgut of a number of animals (see reviews Demeyer and De Graeve, 1991 and Durand and Bernalier, 1995).

Although it is clear that more definite evidence should be sought, using, for example, experiments on incorporation of labelled carbon dioxide and identification of the bacterial species involved, our results suggest that in the young suckling rabbit, reductive acetogenesis is a major characteristic of caecal fermentation, to be replaced gradually and partially by methanogenesis with the intake of solid feed after weaning. The factors determining such change are unknown but are most probably related to the radical change of substrate for the bacterial population. Similar evidence exists for the initial presence of reductive acetogenesis in the rumen of newborn lambs, replaced by methanogenesis very early in development (Doré et al, 1992). The importance of such change in sheep and rabbits is not clear but may be related to the establishment of a hindgut flora, more resistant to invasion of foreign organisms. In terms of efficiency of feed utilisation, however, reductive acetogenesis seems to be preferable over methanogenesis.

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