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FASTING EFFECTS ON *IN VITRO* FERMENTATION PATTERN OF RABBIT CAECAL CONTENTS

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SUMMARY - Caecal contents were collected from six 16h fasted (F) and six non fasted (NF) rabbits, sacrificed between 9 and 10am. They were incubated for 24h/39°C and net productions of volatile fatty acids (VFA) and methane were determined. In a separate preliminary experiment, caecal contents obtained from a F and a NF rabbit were incubated with starch, cellulose or pectins. In F animals, total VFA concentration was lower (P<0.001) and caecal pH significantly higher (P=0.015) than in NF ones: 6.5 *vs* 6.2. Molar proportions of propionate were higher than butyrate (11.3 *vs* 4.0%) in F rabbits. However, the latter became higher after 24h of *in vitro* incubation. Significant (P<0.001) less total VFA (844 *vs* 1776 µmol/flask) but higher (P=0.003) amounts of methane (271 *vs* 131 mmoles/mole VFA) were formed using caecal contents from F rabbits. Calculated hydrogen recoveries were indicative of reductive acetogenesis (RA) in NF but not in F animals (49 *vs* 73%). When one of the substrates was added, effect of fasting was not any more clear for VFA but still for methane and hydrogen recoveries.

RESUME : Influence du jeûne sur le profil fermentaire du contenu caecal du lapin.
Des contenus caecaux de six lapins à jeûn (F) depuis 16h et six autres ayant reçu des aliments ad libitum (NF), sacrifiés entre 9.00h et 10.00h du matin, ont été utilisés pour des incubations in vitro. Ces contenus caecaux ont été incubés pendant 24h à 39°C et la production nette d'acides gras volatils (VFA) et de méthane a été determinée. Dans une autre expérience préliminaire, les contenus caecaux d'un lapin F et d'un autre NF ont été incubés avec 500mg d'amidon, de cellulose ou de pectines, comme substrats. Les contenus caecaux des animaux F ont une concentration totale en VFA plus basse (P<0.001) et un pH caecal significativement plus élevé (P<0.015) que les lapins NF : 6,7 vs 6,2.Le propionate était plus élevé que le butyrate (11.3 vs 4.0%) chez les lapins F, cependant ce dernier devient plus élevé après incubation in vitro. Significativement (P<0.001) moins de VFA (844 vs 1776 µmol/flacon) mais plus de méthane (271 vs 131 mmoles/mole VFA) a été produit chez les lapins F. Les bilans d'hydrogène suggèrent la présence de l'acétogénèse réductrice chez les lapins NF, mais pas chez les F (49 vs 73%). Quand un des substrats était ajouté, l'effet du jeûne était moins clair pour les VFA mais persistait pour le méthane et les bilans d'hydrogène.

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INTRODUCTION

In the rabbit, the caecum is the largest digestive compartment (40% of the whole digestive tract) and it represents a distinct organ for fermentation. It is colonised by an abundant bacterial flora, mainly strictly anaerobic cellulolytic bacteria, that develop strongly around 3 weeks of age and stabilise a few days after weaning (BOULAHROUF *et al.*, 1986; PADILHA *et al.*, 1995). The bacterial flora ferments dietary carbohydrates to volatile fatty acids (VFA), methane (CH_4) and carbon dioxide. Dietary carbohydrates which enter the caecum consist partly of starch not yet totally hydrolysed in the upper gastrointestinal tract and mainly of structural carbohydrates i.e. pectin, hemicellulose and cellulose which can not be digested by endogenous enzymes. Recently MAROUNEK *et al.*, (1995) investigated the caecal fibrolytic activities and reported high pectinolytic activities followed by xylanolytic and cellulolytic ones, a hierarchy corresponding to the one of the cell wall constituent digestibility: pectins>hemicellulose>cellulose (GIDENNE, 1996). As far as microbes are concerned, the VFA are not further used but are largely absorbed prior to defecation, thus representing a major source of absorbed energy for the host animal. Acetate (A) is the major acid followed by butyrate (B) and propionate (P). The latter feature appears to distinguish the rabbit from the other mammalian species.

In contrast to rumen fermentation, the hydrogen sinks in caecal and colon fermentations are characterized by an important contribution of reductive acetogenesis (RA), besides methanogenesis (DEMEYER, 1991). The importance of RA during *in vitro* fermentation of rabbit caecal contents varies with animal age and origin, as evident from calculated hydrogen recoveries (PIATTONI *et al.*, 1996). Before weaning, RA seemed to be the predominant metabolic hydrogen sink present in caecal fermentation, with the exclusion of methanogenesis. The substitution of acetate for methane as hydrogen sink in intestinal fermentations is energetically beneficial for the animal and we have been involved in attempts to induce such substitution in the rumen (DEMEYER *et al.*, 1996; NOLLET *et al.*, 1996; IMMIG *et al.*, 1996). As part of our efforts to understand the factors responsible for the predominant presence of RA over methanogenesis in intestinal fermentations, further experiments were carried out *in vivo* and *in vitro* with rabbit caecal contents, focusing on the effect of a fasting period. At the same time, a preliminary experiment with a F and a NF rabbit was run to study the effect of different added substrates on *in vitro* caecal fermentation.

MATERIAL AND METHODS

Animals and Diet

Twelve nearly adult rabbits (2.71 ± 0.19kg; 70-77d old) from the experimental strain of the Institute of Small Stock Husbandry (MAERTENS, 1992a) showing a normal weight gain during the fattening period, were randomly chosen and sacrificed. Animals were fed a standard fattening diet, in accordance with recent recommendations (MAERTENS *et al.*, 1992b), with the following composition on DM basis: 18.6% CP, 19.2% ADF, 4.7% Crude Fat, 17.5% Starch and 10.2MJ metabolizable energy/kg. This diet was always fed *ad libitum* since weaning (29d of age). For F animals, feed was removed at 5pm the day before sampling but water was still available *ad libitum*. Animals were kept under normal environmental conditions (12h of light, temperature between 16 and 22°C).

Sampling

Animals were sacrificed between 9 and 10am by cervical dislocation. Thereafter they were dissected and the caecum isolated by tying off the two extremities with a nylon string to prevent movement of digesta. Caecal pH was immediately measured by a glass electrode pHM62 (Radiometer, Copenhagen) *via* a small slit in the caecum. In the first experiment separate caecal samples were collected from each rabbit for *in vivo* and *in vitro* determinations.

In a second, preliminary experiment caecal contents from a F and a NF rabbit were also collected to run separate *in vitro* incubations with 3 different substrates: respectively purified maize starch (Cerestar), purified cellulose (Alphacel) and pectins (Pure Citrus Pectins, UpJohn). Fermentation substrates were added at a level of 500mg/flask.

Incubations and analysis

Fourty grams of caecal contents were fivefold diluted, under CO_2 flushing, with a buffer solution (pH 6.9)(BURROUGHS *et al.*, 1950) containing 705.7mg/l of NH_4HCO_3 to allow microbial growth. Fifty ml of this dilution were immediately transferred into duplicate gastight incubation flasks, then gassed with CO_2 and incubated in a shaking water bath at 39°C. After 24h of incubation, pH was checked before microbial activity was stopped by injection of 1ml of H_2SO_4 10N. The gas phase was analysed by gaschromatography for methane and hydrogen, as described earlier (MARTY and DEMEYER, 1973). A sample, immediately mixed with 1ml of H_2SO_4 10N without being incubated, acted as blank and was used to investigate *in vivo* total VFA concentration and molar proportions.

All the samples were then centrifuged (10 min, 15000 *g*), filtered and the supernatant kept for VFA analysis by gaschromatography (MARTY and DEMEYER 1973). The net amount of VFA produced was obtained after correction for non incubated blanks. Furthermore, when a fermentation substrate was added, net VFA production was obtained after correction for simultaneous incubations without substrate. Hydrogen recoveries were calculated from VFA and methane $[100 \times (2P + 2B + 4CH_4)]/(2A + P + 4B)$ as derived from stoichiometry (MARTY and DEMEYER, 1973; DEMEYER, 1991).

Statistical analysis

Treatment of the data was performed using the program SPSS (1993). Data are presented as mean ± standard deviation and means were compared using a T Test.

Table 1 : Total VFA concentration and molar proportions in caecal contents obtained from fasted (F) or non fasted (NF) rabbits (mean ± SD).

	Total VFA (mmol kg^{-1})	VFA molar proportions			Caecal pH[2]
		% C_2	% C_3	% C_4	
F[1]	18.1±3.2	84.7±4.5	11.3±6.6	4.0±3.2	6.7±0.1
NF[1]	67.7±19.3	76.0±3.3	5.7±1.5	18.3±3.2	6.2±0.2
Stat. Signif.	**<0.001**	**<0.003**	**0.090**	**<0.001**	**0.015**

[1] : n = 6
[2] : caecal pH was determined only on **4** F and **4** NF rabbits.

RESULTS AND DISCUSSION

In F rabbits caecum weight was lower, although not significantly (P=0.2) than in NF ones (128.8±19.1g and 150.9±34.7g, respectively). Table 1 shows that *in vivo* total VFA concentration and molar proportions were significantly influenced by the fasting period prior to the collection of the caecal contents. In F animals, total VFA concentration was almost 4 times lower (P<0.001) than in NF ones, obviously reflecting the low concentration of fermentable substrate. As a consequence caecal pH is higher (P=0.015). As regards the molar proportions, a significant and sharp decrease in butyrate (P<0.001) paralleled by an increase in acetate (P=0.003) is observed in F animals. Because of the high variability in F group, no significant changes were observed for propionate, although NF rabbits showed on average a decrease of 50%. In caecal contents of F rabbits butyrate was surprisingly lower than propionate. It is worthwhile to mention that in two fasted rabbits butyrate was even completely absent. A similar, but less pronounced, phenomenon was shown by GIDENNE and BELLIER (1992) when caecal samples from adult cannulated rabbits were collected *in vivo* after 13.5h of fasting and by MAERTENS and PEETERS (1988) with restricted fed rabbits. The latter authors observed a decrease in total VFA concentration and an inversed ratio of propionic and butyric acid molar proportions during the first week after weaning, when rabbits received only 65% of their *ad libitum* feed intake. Differences in caecal traits between F and NF rabbits are also in line with the diurnal rhythm in feed intake and in caecal fermentation pattern (GIDENNE *et al.*, 1986). VFA circadian pattern is characterised, in adult rabbits, by a decrease in total VFA concentration after 12am, following the lowest feed intake between 8 and 10am (BELLIER *et al.*, 1995). A further

Table 2 : Net fermentation products[3] of caecal contents obtained from fasted (F) or non fasted (NF) rabbits (mean ± SD).

	Total VFA	mmoles/mole VFA[1]				2H rec.	pH [2]
	(µmoles/flask)	C_2	C_3	C_4	CH_4	(%)	after incubation
F[1]	844±92	750±24	101±27	149±37	271±56	73±8	6.1±0.1
NF[1]	1776±320	653±114	72±8	275±120	131±53	49±7	5.5±0.1
Stat. Signif.	**<0.001**	**0.05**	**0.09**	**0.02**	**0.003**	**0.001**	**0.02**

[1] [2] : see Table 1 ; [3] = **10g of caecal contents in 40ml of buffer solution.**

Table 3 - Effect of substrate on *in vitro* caecal fermentation pattern using caecal contents from a fasted (F) and a non fasted (NF) rabbit: preliminary results.

		Total VFA	mmoles/mole VFA				2H rec.
		(mmoles/flask)	C_2	C_3	C_4	CH_4	(%)
Animal F	No *Substrate*	762	811	84	105	357	85
	Starch	2204	600	50	350	460	99
	Cellulose	1596	770	110	120	269	72
	Pectins	3492	840	40	120	34	21
Animal NF	No *Substrate*	1861	770	74	156	153	48
	Starch	2835	660	30	310	144	49
	Cellulose	1529	770	50	180	188	54
	Pectins	3320	880	10	110	7	12

explanation could be found in the velocity of disappearance of VFA from intestinal loops, with butyrate as the best respiratory fuel for the caecal wall and colon as well (MARTY and VERNAY, 1984). Hence, when caecal contents were incubated for 24h in a closed *in vitro* system, butyrate is higher than propionate (Table 2) even for F rabbits thus restoring the typical fermentation pattern of the adult rabbit caecum. In incubations without substrate, the effect of fasting is still reflected in a lower total VFA production ($P<0.001$). Once again, caecal pH is significantly higher ($P=0.02$). However, F animals yield caecal contents that significantly produce more methane ($P=0.003$) and acetate ($P=0.05$) and less butyrate ($P=0.02$). Their fermentation end products fit the model of rumen fermentation, as suggested by the high metabolic hydrogen recoveries (DEMEYER, 1991). On the contrary, NF animals yield much lower ($P=0.001$) hydrogen recoveries, indicative of an important contribution of RA (DEMEYER, 1991). Although animal differences may be involved, the *in vivo* and *in vitro* results strongly suggest that withdrawal of substrate by fasting inhibits the bacteria involved in RA. The data also suggest that butyrate is formed from the acetate produced by RA. Similarly, a substantial synthesis of butyric from acetic acid was observed by PARKER (1976).

Preliminary results from Table 3 indicate that fasting and/or animal origin determines to a large extent substrate fermentation pattern, as obvious from methane production and the calculated hydrogen recoveries. However, when a substrate was added, the effect of fasting was not pronounced any more, as total VFA production and individual molar proportions showed quite similar trends. Hence, the hierarchy in VFA production: pectins>starch>cellulose was the same for F and NF rabbits. Moreover, the characteristics of the substrate added seem to affect the fermentation pattern. Low methylated pectins, for example, represent a special case because of methoxyl groups which shift the VFA fermentation pattern towards more acetate, less butyrate and a decrease in methane production, in accordance with DENIS *et al.* (1990). The addition of starch, resulted in high butyrate and low acetate as also observed by GOODLAD and MATHERS (1988) with rat caecal contents. Cellulose proved to be a poor fermentation substrate with little VFA production because of its low digestibility, as recently reported (GIDENNE and PEREZ, 1996, cited by GIDENNE, 1996).

In conclusion, the physiological differences in caecal fermentation pattern and stoichiometry evidenced by fasting and substrate addition need to be studied in details on a larger number of animals. However, the results with the F rabbits suggest that standardisation of the feeding pattern and collection time is necessary to obtain reliable results. Finally, the rabbit may be a good model to study the fundamental factors determining the relative importance of methanogenesis and RA in hindgut fermentation.

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