MEASUREMENT OF THE BACTERIAL FIBROLYTIC ACTIVITY IN THE CAECUM AND IN THE SOFT FAECES OF THE RABBIT

JEHL N.[1], GIDENNE T.[1], LE ROUX J.F.[2]

[1] Institut National de la Recherche Agronomique, Station de Recherches Cunicoles
BP 27, 31326 CASTANET-TOLOSAN, France
[2] Ralston Purina, 1 pl Charles De Gaulle, 78180 SAINT QUENTIN EN YVELINES, France

Abstract - The present work aimed to develop a new methodology for studying the bacterial fibrolytic enzymatic activity (BFEA) of the rabbit caecal flora, from soft faeces sampling. Twelve New Zealand White rabbits (72 d of age) fed *ad libitum* were used for three trials. After a first soft faeces collection to adapt rabbit to the collar, the measurement of BFEA on the two further samplings of soft faeces (at interval of 6 days), showed a relatively good repeatability (intra-individual variability=11% and 19% respectively for pectinase and cellulase). In a second trial, seven rabbits were fed successively a control diet (31.1% NDF) and a low fibre diet (19.4% NDF). No significant difference between diets, was found either for cellulasic or for pectinasic activity in soft faeces. Pectinasic activity was about ten folds higher than cellulasic one. In a third trial, BFEA in the caecum and in the soft faeces were compared, and no significant difference was observed. This suggests that soft faeces could be used in place of caecal content for BFEA measurement. This new approach could then allow repetitive BFEA measurements on the same animal, as soon as the rabbit produce soft faeces.

INTRODUCTION

Caecal microbial activity (CMA) could be addressed through several approaches. A quantitative one is given by microbiological enumeration. However this method is time consuming and of relatively low precision. Furthermore, qualitative fractionation of bacterial strains remains very difficult. It is also possible to determine the daily biomass production using an internal microbial marker, such as D-aminopimelic acid. Nevertheless, this method does not give any qualitative indication about the degrading activity of the bacteria for a specific substrate. On the other hand, the measurement of the caecal concentrations of the fermentation end-products gives more qualitative information than quantitative one (BELLIER and GIDENNE, 1996). Recently, the CMA has been evaluated quantitatively and qualitatively by determining the activity of various enzymes specific of the caecal flora (MAROUNEK, 1995), such as fibrolytic activity (cellulolytic, hemicellulolytic and pectinasic). However, the important variability of enzyme activity between animals reported by Jehl et al. (1995) suggests to use the animal as its own control, but it is not possible to sample caecal digesta on cannulated rabbits before 42 days of age (GIDENNE et BELLIER, 1992). The use of soft faeces to analyse the bacterial fibrolytic activity (BFEA) could thus overcome the problem of repetitive sampling on the same animal. Consequently, the present study aimed to control the following points to validate such a method: the repeatability of the soft faeces enzymatic activity measurement, the comparison of enzymatic activity in the caecum and the soft faeces. In addition, the effect of the dietary fibre level will be evaluated.

MATERIAL AND METHODS

Animals and feeding

Twelve New Zealand white rabbits (72 days of age, average wheight=1887g) were housed in individual metabolism cages, under a 12:12 light dark/schedule (8:30-20:30h). Experimental diets and water were given *ad libitum*. Two diets were formulated (table 1) to obtain a decrease of the dietary fibre level (NDF), by substituting starch to fibre sources (mainly beet pulp), without change in the proportions of the different fibre fractions. The ADF content of the low fibre diet was under the current recommendations.

(1) :phone (33) 61285103 ; Fax: (33) 61285319; E.mail: gidenne@toulouse.inra.fr

Experimental design

A first trial was designed to measure the repeatability of the BFEA measurement between 3 successive sampling of soft faeces, at 85, 89 and 95 days of age. Soft faeces were collected on 5 rabbits fed the control diet after a 2 weeks period of adaptation to the diet A second trial was designed to compare BFEA in caecal digesta and soft faeces. After the last collection of soft faeces (Trial I), animals were sacrificed and caecal digesta were sampled to compare the BFEA with soft faeces of the same animals. A third trial was designed to evaluate the effect of the dietary fibre level on the BFEA, using a group of 7 rabbits fed successively a control and then a low fibre diet, during two periods of three weeks (72 to 109 d of age ; adaptation period of 2 weeks, and 1 week for two samplings of soft faeces ; table 4).

Table 4 : Zootechnical performances for rabbits fed successively a control or a low-fibre diet (trial III).

Diet	**Control**	**Low fibre**		
Period	72-91j	91-109j		
Number of rabbits	n=7	n=7	*T*[3]	*Stat signif.*
Live Weight (g)	1951	2553		
Daily feed intake (g/d)	130	110		
Feed intake (g/kg LW)	66.7 ± 5.6	43.4 ± 3.1	*3.6*	*0.011*
Feed conversion	4.2 ± 0.8	3.9 ± 0.5	*0.6*	*0.57*

[3] : see table 3.

Soft faeces and caecal content collection

A light plastic collar (50g, GIDENNE and LEBAS, 1987) was placed (from 17:00 till 12:00h) on each animal to prevent caecotrophy and thus allowed to collect soft faeces. Soft faeces were collected on the morning as soon as possible after their excretion and were put as soon as possible in a pre-cooled (4°C) anaerobic buffer (N-morpholino) ethane-sulphonic acid (MES) + dithiothreitol (DTT), pH 6.5). We also assessed the effect of the time delay between the caecotrophe sampling and its treatment (mixing in the buffer at 4°C) on the BFEA. On 3 rabbits having a sufficient amount of soft faeces, an aliquote was treated about four hours after its excretion and compared with treatment of freshly excreted soft faeces.
A recovering period (at least 3 d) was respected between two consecutive soft faeces collections to prevent a change in the microbial and biochemical conditions of the hindgut. Caecal contents were obtained on 5 animals after slaughtering by cervical dislocation, at the end of the caecotrophy period (about 11:00 h).
During the experimental period feed intake and live-weight were recorded once a week. The feed intake was also recorded for the day of the soft faeces sampling, in order to control the impact of wearing a collar.

Enzyme extraction from soft faeces and caecal content

The digesta solution (caecotrophes+anaerobic buffer 1g / 2.5 ml) was frozen and thawed in order to weaken microbial cells and to improve its rupture. Enzymes were extracted by sonication of the digesta solution (four 30s. sonication periods with 30s. intervals at 4°C) to released cellular membranes-associated and intracellular enzymes. Unbroken cell material and dietary particles were removed by centrifugation (20 000 x g 15' at 4°C). The supernatant fraction was duplicate and store at -80°C before enzyme assay. All phases of the enzyme extraction procedure were carried out at 4°C to minimise the effects of proteases released by cell lysis on fibrolytic enzymes and under anaerobic conditions.

Table 1 : Composition of the experimental diets

Diets	**Control**	**Low fibre**
Ingredients (g.kg^{-1} air dry basis)		
Beet pulp	31.0	13.0
Dehydrated Alfalfa	9.5	4.0
Straw	9.5	4.0
Soya bean meal	17.2	18.0
Wheat	30.0	57.6
Vitamins and minerals	2.8	3.4
Chemical analysis (g.kg-1 DM)		
Dry matter	90.4	90.3
Starch	19.6	37.4
Crude protein (N x 6.25)	16.2	18.5
N.D.F.	31.1	19.4
A.D.F.	16.5	9.2
Lignin (A.D.L.)	2.4	1.8
Pectins [1]	13.6	7.6

[1] : calculated values from tables

Biochemical analysis and enzymatic assay procedure

Measurements of Van-Soest fibre fractions (NDF, ADF, ADL) were made according to VAN SOEST *et al.* (1991) using an amylolytic pre-treatment with a thermostable amylase. Nitrogen was determined by DUMAS combustion method using Leco apparatus model FP-428 (Leco Corp., St Joseph, MI, USA), and converted to crude protein using the factor 6·25. Starch was determined enzymically after gelatinisation (autoclaving) by using the hexokinase (EC 2.7.1.1) G6PDH (NAD) (EC 1.1.1.49) system (Böehringer Mannheim).
Pectinolytic and cellulolytic activities were assayed respectively on citrus and carboxymetylcellulase (CMC) substrate by measuring the amount of reducing sugars released after incubation of 0.1ml of enzyme preparation and 1ml of substrate (CMC or pectin 2mg/ml) for 60' at 39°C. The reaction was stopped by heating 5' at 100°C. Reducing sugars were quantified by the p-Hydroxybenzoic acid hydrazide method (LEVER, 1977). Enzymatic activity was expressed as nmol of reducing sugars released per h. and per g. of digesta (DM basis).

Statistical analysis

Repeatability of the measurement on different days was determined according to the GLM model of SAS (1988) and using a 2 factorial crossed design (rabbit x collection day). Effect of the diet (trial III) was also determined according to two factorial crossed design (rabbit x diet). The comparison between caecal digesta and soft faeces (trial II) was made according to a student t test for the intra-individual differences of enzyme activity in the caecum and in the soft faeces for the same animal (paired t test).

RESULTS

On average, activity of pectinase was about ten folds higher than CMCase (table 2). A significantly lower (-20%) pectinase activity was observed for the first collection compared to the two further collections (trial I), whereas no significant difference was observed for CMCase. During the first soft faeces sampling, the feed intake also showed a significant fall (animals weared the collar for the first time) compared to the two further collections (meanly 62 and 105 g respectively). The intra individual variation of soft faeces between the second and the third collection expressed as variation coefficient was about 11% for pectinase and 19% for CMCase.

Table 2 : Effect of the collection period on soft faeces bacterial fibrolytic activity (trial I).

Enzyme activity	Collection 1 (n=5)[2]	Collection 2 (n=4)	Collection 3 (n=5)	*SEM*[1]	*Stat signifi.*
(nmole of reducing sugars released/g DM/hour)					
Pectinase	176.8[a]	214.8[b]	234.0[b]	*8.9*	*0.007*
CMCase	20.3	18.9	19.2	*2.1*	*0.69*

(a,b): Mean values with common superscripts were not significantly different (P<0.05). ; [1] : residual standard error of the mean (two factorial crossed design). ; [2] : number of animals.

The BFEA of freshly excreted caecotrophes was higher than that of dry ones both for pectinolytic and cellulasic activity (+30%), suggesting to sample and to treat soft faeces as soon as possible.
No significant difference was observed between soft faeces and caecum (Trial II), either for CMCase or for pectinolytic activity (table 3). Though, fibrolytic activities tend to be slightly higher in soft faeces . A lower dietary fibre level reduced the feed intake (table 4, Trial III), but it did not affect significantly , the BFEA either for pectinase or for CMCase (table 5).

DISCUSSION

It is well acknowledged that the chemical composition of the soft faeces is close to that of caecal contents (PROTO, 1965; CARABAÑO *et al.*, 1988). Our results suggest also that BFEA in soft faeces was similar to that found in the caecum. Reliable results were obtained by EMALDI *et al.* (1978) who found a similar cellulolytic flora in caecotrophes and in the caecum.

Table 3 : Comparative enzymatic activity in the caecum and in the soft faeces (trial II)

Enzyme Activity	Soft faeces[1] (n=5)[2]	Caecum[1] (n=5)	T[3]	Stat.sign.
(nmole of reducing sugars released/g DM/hour)				
Pectinase	234 ± 43.5	206.9 ± 56.7	2.3	0.083
CMCase	19.2 ± 7.2	14.4 ± 9.5	2.3	0.084

[1] : inter-individual mean values ± standard deviation; [2] : number of animals.
[3] : Student t value testing the null hypothesis of intra-individual differences (paired t test).

Table 5 : Effect of the dietary fibre level on bacterial fibrolytic activity in the soft faeces (trial III).

Diets Enzyme activity	Control (n=6)[2]	Low fibre (n=7)	SEM[1]	Stat Signifi.
nmole of reducing sugars released/g DM/hour				
Pectinase	168.2	148.7	11.0	0.80
CMCase	18.8	11.3	2.8	0.20

[1], [2] : see table 2.

BFEA values showed a relatively good repeatibility in the soft faeces, after rabbits have been adapted to the collar. During the first soft faeces sampling, the lower BFEA was associated to a lower feed intake. This corresponds to a reduced flow of nutrients entering the caecum, and in turn could lead to a lower microbial activity. A sharp decrease of the caecal fermentative activity was also observed for restricted rabbit (PEETERS and MAERTENS, 1988) or before feeding (GIDENNE and BELLIER, 1992). This suggests the necessity to respect an adaptation period to the collar, before to sample soft faeces.

Pectinasic activity was tenfold higher compared to cellulolytic one. It may be due to the high level of digestible fibre in the experimental diet. But, our values were close to those obtained by MAROUNEK *et al.* (1995) in the caecum of adult rabbits fed a commercial diet. Compared to cellulase, a higher xylanasic activity was also reported by JEHL *et al.* (1995), but remained lower than pectinolytic one (MAROUNEK *et al.*, 1995). These results are also supported by microbiological enumeration reporting higher counts of pectinolytic and hemicellulolytic bacteria compare to cellulolytic ones (BOULAHROUF *et al.*, 1991). The cellulose hydrolysis is a relatively slow process (over 24h), and the rate of passage in the rabbit caeco-colic compartments is relatively short (6-10h). So, we could hypothesise that cellulolytic bacteria have not enough time in the rabbit caecum to colonise and degrade extensively the cellulose , thus explaining the relatively low cellulasic activity.

No significant effect of the dietary fibre level was here noticed for animals receiving successively the control and the low fibre diet. However, GIDENNE *et al.* (1991) observed that rabbits, fed successively a high fibre and a low fibre diet, keep their initial fibre degradation efficiency (NDF digestibility, VFA level) 3 weeks after switching the diet. These animals also showed a transit time faster than those fed the low fibre diet throughout the experiment. Thus, it could be considered that the dietary fibre content, even in our low fibre diet was not a limiting factor able to affect, in two weeks, the BFEA.

In conclusion soft faeces can be used to evaluate the enzymatic activity of the caecal flora. This method allow repetitive measurement on the same animal (then avoiding inter-individual variations), and would thus overcome the problem of the in vivo evaluation of the caecal flora activity in the weanling rabbits.

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Mesure de l'activité fibrolytique bactérienne, dans le caecum et les caecotrophes du lapin - Cette étude a pour objet de tester chez le lapin, une nouvelle méthode d'estimation de l'activité fibrolytique de la microflore caecale (AFMC) à partir d'échantillons de caecotrophes. Trois essais ont été réalisés avec douze lapins Néo-Zélandais Blanc (72 jours d'âge) nourris *ad libitum*. Après une première collecte de caecotrophes destinée à adapter le lapin au collier, la variabilité intra-individuelle de la mesure d'AFMC sur les deux collectes suivantes (6 jours d'intervalle) est de 11 et 19% respectivement pour les activités pectinasiques et cellulasiques. L'activité pectinasique est environ dix fois supérieure à l'activité cellulasique. Une seconde expérimentation a porté sur sept lapins recevant successivement un aliment témoin (31% NDF) et un aliment pauvre en fibres (19.4% NDF). Aucune différence significative d'activité pectinasique et cellulasique des caecotrophes n'a été observée. L'AFMC mesurée sur un échantillon de contenu caecal ne diffère pas significativement de celle des caecotrophes (troisième essai). L'activité fibrolytique de la microflore caecale pourrait donc être estimée par l'intermédiaire des caecotrophes. Cette nouvelle approche devrait permettre des mesures répétées d'AFMC chez un même animal, dès qu'il pratique la caecotrophie.