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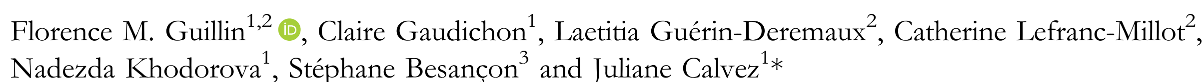
BRIEF REPORT

Caecal digestibility as an approximation of ileal protein digestibility evaluation in rats



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Abstract

The rat model can be used to assess ileal protein digestibility rapidly and in first intention, but no standardised method exists. Our objective was to compare methods to assess protein digestibility, depending on collection site (ileum/caecum) and use of a non-absorbable marker. A meal containing either casein, gluten or pea protein and chromium oxide as non-absorbable marker was given to male Wistar rats and the entire digestive content was collected 6 h later. Total chromium recovery was incomplete and variable, depending on protein source. We observed no significant difference in digestibility between the methods for any of the protein sources tested. Although none of the methods tested is optimal, our results suggest that caecal digestibility can be used as a proxy of ileal digestibility in rats without using a non-absorbable marker. This simple method makes it possible to evaluate protein digestibility of new alternative protein sources for human consumption.

Key words: **Caecum: Chromium oxide: Indigestible marker: Protein digestibility: Rat**

Introduction

Digestibility is a main criteria of protein quality and can be evaluated in human nutrition using animal models such as pigs and rats. Its methodological stakes relies on the measurement of the digestive losses of nitrogen and amino acid. As amino acids are absorbed in the small intestine, it is recommended to evaluate the nitrogen and amino acids losses in the ileum content rather than in the faeces, to avoid bias induced by the activity of the colonic microbiota[1]. The laboratory rat is the simplest model for ileal digestibility assessment *in vivo* and is widely used in practice. However, the digesta cannot be collected continuously by means of a cannula or ileostomy techniques in rats, as it is carried out in pigs. Thus, the digestive content has to be collected at a unique sample time after meal ingestion and complete quantitative collection of ileal digesta containing dietary losses is not possible. To correct for this incomplete collection, a non-absorbable marker is usually used. The marker needs to be totally indigestible and should have a high recovery rate, defined as the ratio between the quantity collected from the total collection and the quantity that was ingested. Some commonly used non-absorbable markers are chromium oxide (Cr_2O_3), titanium dioxide (TiO_2) or acid-insoluble ash. However, there are several issues concerning the use of non-absorbable markers in digestibility studies. The recovery rate may be variable across markers and components of the diet may interact with the non-absorbable marker[2–4]. Therefore, the choice of marker may affect the measurement of ileal digestibility. Another difficulty in the evaluation of ileal digestibility in rats lies in the low amount of digestive content in the

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ileum, which is generally limited to the last 10 cm of the small intestine. Consequently, only a small volume of digestive content is available for analyses.

An alternative technique for ileal digestibility is to use caecal digestibility as an approximation for ileal digestibility[5]. Like in other rodent species, the caecum of the rat is large and accumulates the digesta leaving the small intestine before it enters the colon. It can therefore be assumed that a large part of the losses of dietary nitrogen and amino acids can be collected in the caecum after an appropriate digestion time, which may dispense with the use of an indigestible marker. Indeed, in several studies, most of the dietary nitrogen is found in the caecum 5–6 h after ingestion, which appeared to be an optimal time to collect the digesta[6,7]. This method has been used in previous studies and yielded close digestibility values when compared to studies using ileal sampling. For instance, nitrogen digestibility of whey protein isolate has been determined to be 98·3 ± 0·5 % and 99·0 ± 0·5 % when assessed at caecal and ileal level, respectively[8,9]. This intermediate method avoids the bias related to marker utilization and enables to collect a large amount of digesta for multiple analyses. However, the different methods have not been compared within the same study. The aim of this work was to compare several methods for assessing the true protein ileal digestibility in rats, depending on the collection site (ileum or caecum) and the presence of a non-absorbable marker. The marker chosen in the study was chromium oxide, and three different protein sources were tested: milk casein, wheat gluten and pea protein isolate.

Method

Experimental procedure

This study was conducted in compliance with the EU directive 2010/63/EU for animal experiments and approved by the Ethics Committee in Animal Experiment of INRAE Jouy-en-Josas (Comethea, registration number: 17-20) and the French Ministry of Higher Education and Research (APAFIS #11921-2017091818236657). Thirty-six male Wistar rats weighing 250 g were obtained from Envigo laboratories. They were housed under controlled conditions (room temperature 22 ± 2°C, reversed light–dark cycle), in individual cages with wire bottoms to prevent coprophagia.

All the experiments started after a 6-d adaptation period during which the animals were fed a standard chow diet. The rats were then randomly split into experimental groups according to diets: casein, gluten, pea or protein-free diet (n = 9/group). All diets were AIN-modified standard diets, isocaloric and isonitrogenous (Table 1). The rats had free access to water throughout the duration of the experiment. Due to differences in the experimental diets, experimenters were not blind.

Rats were trained to eat a 4-g calibrated meal in a short time at the beginning of the dark period, as described previously[7]. After a 10-d habituation period to the diets, the rats were given a calibrated meal of 4 g (dry weight) of their respective diets containing 16 mg of Cr_2O_3 (10·9 mg of chromium) and were euthanized 6 h later, by cardiac puncture under gaseous anaesthesia and decapitation. For each rat, gastro-intestinal segments were identified as stomach, proximal intestine, ileum (last 10 cm of the small intestine), caecum and colon. The luminal content of each segment was individually collected entirely, weighed, stored at −20°C and freeze-dried. The faeces lost from the test meal were identified by the green colour of chromium oxide and picked up from 3 h after ingestion to euthanasia.

Table 1. Composition of the four experimental diets

	Casein diet	Gluten diet	Pea diet	Protein-free diet
Weight content (g/kg DM)				
Micellar casein isolate[1],*	105	0	0	0
Hydrolysed wheat gluten[2],*	0	105	0	0
Pea protein isolate[3],*	0	0	105	0
Starch	564	564	564	658
Sucrose	93	93	93	109
Soybean oil	38	38	38	45
Mineral mix[4]	35	35	35	35
Vitamin mix[4]	10	10	10	10
Cellulose	50	50	50	50
Choline	2·3	2·3	2·3	2·3
Energy content (%)				
Protein	14	14	14	0
Carbohydrate	75	75	75	88
Fat	11	11	11	12
Energy (kJ/g)	15·5	15·5	15·5	15·5

DM, dry matter.
* The amount of test protein was calculated using the nitrogen-to-protein conversion factor of 6·25 (total N content × 6·25).
[1] PRODIET® 85B from Ingredia (Arras, France).
[2] NUTRALYS® W.
[3] NUTRALYS® S85F from Roquette (Lestrem, France).
[4] Formulated from AIN-93M.

Chemical analysis

Nitrogen content of dried diets and digesta was measured with an elementary-analyzer based on the Dumas method[10] (Vario Micro Cube, Elementar, Lyon, France). Endogenous losses of nitrogen were estimated from rats fed the protein-free diet. Chromium content was assessed in diets and digesta on an atomic absorption spectrophotometer[11] (contrAA® 800, Analytik Jena). The samples were turned to ashes at 550°C and solubilised in nitric acid prior to reading at 357 nm in flame mode.

Calculation

Three methods were tested: true ileal digestibility calculated from non-absorbable marker recovery (Cr-ileum), true caecal digestibility calculated from non-absorbable marker recovery (Cr-caecum) and true caecal digestibility calculated with the assumption that we quantitatively collected all the dietary losses and that a large majority remained in the caecum 6 h after meal ingestion (no marker-caecum).

Recovery of chromium (Cr) was calculated as follows:

$$\text{Cr recovery in segment (\%)} = \frac{\text{Cr}_{\text{segment}}}{\text{Cr}_{\text{ingested}}}$$

Distribution of ingested meal throughout the digestive tract was determined based on chromium recovery distribution. True caecal and ileal nitrogen (N) digestibilities in the marker techniques (Cr-caecum, Cr-ileum) were calculated as follows:

$$\text{True N digestibility segment (ileum or cecum) (\%)} = \frac{\left(\text{Cr recovery in segment} \times \frac{N_{\text{ingested}}}{100}\right) - N_{\text{dietary}}\ \text{segment}}{\text{Cr recovery in segment} \times \frac{N_{\text{ingested}}}{100}}$$

True caecal nitrogen digestibility in the no marker technique was calculated as follows:

$$\text{True cecal N digestibility (\%)} = \frac{N_{\text{ingested}} - (N_{\text{ileum dietary}} + N_{\text{caecum dietary}})}{N_{\text{ingested}}} \times 100$$

N_{dietary} in ileum and caecum corresponded to nitrogen from the meal and was defined as:

$$N_{\text{dietary}} = N_{\text{total}} - N_{\text{endogenous}}$$

$N_{\text{endogenous}}$ were the endogenous losses of nitrogen in ileum and caecum, measured through the digestive losses in the protein-free group. In the no marker technique, N_{ingested} excluded the residual amount of dietary nitrogen recovered in the stomach.

Statistical analysis

The power calculation performed to determine the sample size required was detailed in our previous publication[12] on the main outcome (nitrogen digestibility). The values are expressed as means ± standard deviation (SD). The data were analysed using Prism 6.04 (Graph Pad Software Inc.). Normality of data was tested with Quantile *v.* Quantile Plots and Shapiro–Wilk tests[13]. The influence of the protein on the quantity of digesta, chromium recovery and total nitrogen recovery in each segment was evaluated using a one-way ANOVA or Kruskal–Wallis test (depending on the normality of data) with the protein as a factor[14]. Dietary nitrogen recovery in each segment between casein and gluten was evaluated using unpaired *T*-test or Mann–Whitney test. Since nitrogen digestibility was normally distributed, the influence of the method of estimation of true nitrogen digestibility was tested using a mixed model with method and protein as fixed effects and animal as random effect[15]. When an overall significant difference was observed ($P < 0{\cdot}05$), a side-by-side comparison was made between methods and within each protein using Bonferroni or Dunn's correction.

Results

The quantity of dried digesta collected in the different segments of the gastro-intestinal tract is presented in Table 2. In the ileum, between 43 ± 25 mg (dry matter) of digesta for the pea group and 66 ± 33 mg for the gluten group was collected 6 h after meal ingestion, with a mean of 55 ± 9 mg for all rats. In the caecum, the larger amount of digesta was collected, varying from 414 ± 88 mg for the protein-free group to 510 ± 143 for the casein group and with a mean of 454 ± 41 mg for all rats. No effect of the protein source ingested was observed on the quantity of digesta recovered in the different parts of the digestive tract.

Chromium recovery is presented in Table 2. Total chromium recovery in the entire digestive tract was incomplete and variable depending on the diet ($P < 0{\cdot}0001$), reaching 81·2 ± 10·7% for the casein group, 68·1 ± 15·4% for the gluten group and 75·3 ± 12·0% for the protein-free group. Total chromium recovery was only 25·0 ± 12·3% in the pea protein group, much lower than in the other groups ($P < 0{\cdot}0001$). Consequently, the pea protein group was removed from the study for digestive losses evaluation. The majority of ingested chromium was found in the caecum of the rats. This result was in accordance with the high amount of digesta collected in this segment, representing in average 42% of the total digestive content after 6 h of digestion. When expressed as the percentage of total marker recovery along the digestive tract (excluding stomach), with total chromium recovery artificially increased to 100%, 80% of the meal was located in the ileum and caecum 6 h after ingestion for casein and gluten groups, and 65% for the protein-free group.

The total nitrogen recovered from the digestive tract of the rats came in majority from the proximal intestine, with a high contribution of endogenous nitrogen, according to the values obtained in the protein-free group (Table 2). No difference between protein source was observed on total nitrogen recovery in the different segments of the digestive tract, except in the caecum where significantly higher nitrogen was found after casein intake than protein-free meal ($P = 0{\cdot}0014$). Dietary nitrogen stands for the digestive losses of nitrogen from the test proteins (Table 2). The amount of dietary nitrogen was obtained by removing the nitrogen content found in the digesta of protein-free rats from the nitrogen content in the other groups, for each segment. Due to the low chromium recovery in the pea group, only casein and gluten were compared. The proximal intestine and the caecum concentrated the majority of the dietary nitrogen, with a strong inter-individual variability. The quantity found in ileum was low explained by the small amount of digestive content in this segment (only 4% in average). No difference was observed on dietary nitrogen recovery in the different parts of the digestive tract after casein and gluten intake, except for lower dietary nitrogen in the stomach after gluten ingestion ($P = 0{\cdot}0060$).

Four rats had to be removed from the calculation of ileal digestibility for the Cr-ileum method in the casein group and three rats in the gluten group because digestive content in ileum was lacking for chromium analysis, resulting in n = 5 and n = 6 in casein and gluten groups, respectively. True nitrogen digestibility ranged from 93 to 96% for gluten and from 89 to 94% for casein, depending on the method (Fig. 1). No effect of protein or method was observed on nitrogen digestibility and the variability was high, especially in the casein

Table 2. Quantity of digesta, chromium recovery and total and dietary nitrogen content in each gastro-intestinal segment of the rats, 6 h after meal ingestion

	Protein-free	Casein	Gluten	Pea	*P*-value
Quantity of digesta (mg DM)					
Stomach	142 ± 57	188 ± 124	143 ± 58	128 ± 114	*n.s.*[1]
Proximal intestine	270 ± 148	334 ± 205	307 ± 115	314 ± 221	*n.s.*[1]
Ileum	57 ± 24	54 ± 16	66 ± 33	43 ± 24	*n.s.*
Caecum	414 ± 88	510 ± 143	433 ± 118	459 ± 133	*n.s.*
Colon + faeces	275 ± 167	235 ± 146	246 ± 219	177 ± 88	*n.s.*[1]
Chromium recovery (%)[2]					
Stomach	3·4 ± 7·4[ab]	4·5 ± 8·7[a]	1·5 ± 0·9[ab]	0·4 ± 0·3[b]	*0·0081*[1]
Proximal intestine	2·3 ± 2·9	2·0 ± 2·6	2·4 ± 3·7	0·4 ± 0·9	*n.s.*[1]
Ileum	3·2 ± 2·7[a]	5·5 ± 2·2[ab]	9·5 ± 3·9[a]	2·3 ± 1·6[b]	*0·0030*
Caecum	43·5 ± 18·4[a]	56·7 ± 16·2[a]	50·0 ± 13·4[a]	19·5 ± 12·2[b]	*<0·0001*
Colon + faeces	23·7 ± 21·7[a]	13·2 ± 11·4[ab]	8·0 ± 8·8[ab]	3·9 ± 2·2[b]	*0·0066*
Total	75·3 ± 12·0[a]	81·2 ± 10·7[a]	68·1 ± 15·4[a]	25·0 ± 12·3[b]	*<0·0001*
Total nitrogen (mg DM)[3]					
Stomach	3·4 ± 2·3	4·9 ± 2·6	2·9 ± 0·7	4·0 ± 2·9	*n.s.*[1]
Proximal intestine	13·8 ± 11·8	26·3 ± 20·3	17·3 ± 11·0	20·4 ± 13·5	*n.s.*[1]
Ileum	1·2 ± 0·6	1·8 ± 0·8	1·5 ± 0·9	1·1 ± 0·6	*n.s.*[1]
Caecum	7·1 ± 1·5[a]	12·9 ± 3·5[b]	10·3 ± 2·6[ab]	10·6 ± 3·6[ab]	*0·0027*
Colon + faeces	3·2 ± 1·6	5·0 ± 2·8	4·7 ± 4·0	3·1 ± 1·3	*n.s.*[1]
Dietary nitrogen (mg DM)[4]					
Stomach	–	1·7 ± 2·4[a]	0·1 ± 0·3[b]	–	*0·0060*[1]
Proximal intestine	–	14·8 ± 18·1	5·9 ± 8·9	–	*n.s.*[1]
Ileum	–	0·7 ± 0·8	0·5 ± 0·8	–	*n.s.*[1]
Caecum	–	5·8 ± 3·5	3·3 ± 2·4	–	*n.s.*
Colon + faeces	–	2·3 ± 2·1	1·7 ± 3·8	–	*n.s.*[1]

DM, dry matter; n.s., non-significant.
[1] *P*-value of the non-parametric tests performed for not-normally distributed data (Kruskal–Wallis test for quantity of digesta, chromium recovery and total nitrogen and Mann–Whitney test for dietary nitrogen), otherwise parametric tests were performed (one-way ANOVA and unpaired *T*-test).
[2] Chromium recovery was determined by atomic absorption spectrophotometry.
[3] Total nitrogen content was assessed by elementary analysis of dried digesta based on the Dumas method.
[4] Data from the protein-free group are the endogenous losses of nitrogen and are used to calculate dietary nitrogen. Pea data were removed for calculation of dietary nitrogen due to abnormally low total chromium recovery.
Values are means ± standard deviation (SD), *n* 9/group. Within each segment, values with different letters are statistically different.

group. The choice of gastro-intestinal segment for the assessment of digestive losses (ileum or caecum) had no effect on digestibility results, as shown by side-by-side comparison between Cr-ileum and Cr-caecum methods ($P = 0{\cdot}17$ and 1·0 in casein and gluten groups, respectively). The use of the non-absorbable marker in the calculations had no effect on true digestibility, as revealed by the comparison between Cr-caecum and no marker-caecum methods ($P = 0{\cdot}54$ and 0·59 in casein and gluten groups, respectively).

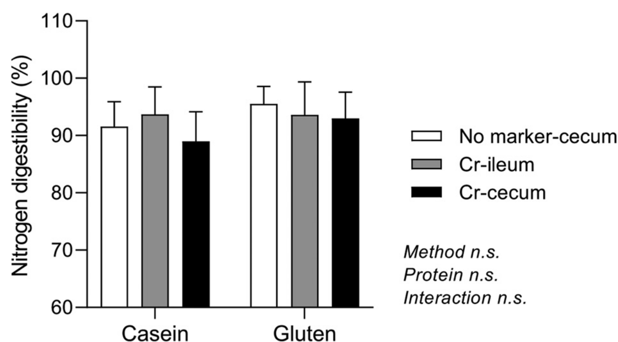

Fig. 1. True nitrogen digestibility (%) of proteins calculated from the three methods, using different gastro-intestinal segments for nitrogen losses assessment (ileum or caecum) and with or without the use of chromium oxide marker. Values are means ± standard deviation (SD). Rats were removed from the calculation of ileal digestibility due to limited digesta quantity: n = 5 in casein group and n = 6 in gluten group for Cr-ileum method; n = 9 per group for Cr-caecum and no marker-caecum methods. The influence of the method of estimation of true nitrogen digestibility was tested using a mixed model with method and protein as fixed effects and animal as random effect. Cr-ileum, ileal digestibility calculated from marker technique; Cr-caecum, caecal digestibility calculated from marker technique; No marker-caecum, caecal digestibility calculated without the use of Cr. n.s., non-significant.

Discussion

A main objective of the study was to evaluate the effectiveness of the use of a non-absorbable marker for ileal digestibility assessment. In the present study, the recovery rate of chromium was incomplete, considerably below 100% in some cases, which was also described in previous studies[16,17]. In the present study, while we collected the entire gastro-intestinal content, chromium recovery values were highly variable depending on the diet, ranging from 25% for rats fed pea protein to 81% for rats fed casein. The food matrix appeared to have a strong influence on chromium determination, which was a significant weakness for a marker. Vicente *et al.* also highlighted the interaction between sample types and marker[18]. Chromium is susceptible to interact with other cations and interference with iron in atomic absorption spectrophotometry (AAS) trials has been described previously[19]. Gunčaga *et al.* showed that the presence of traces of iron in faeces samples may decrease the readings by 10–20% during AAS[20]. The composition of pea protein isolate and especially its iron content (0·2 g/kg) could have interfered with chromium analysis on digesta samples, leading to a low recovery rate of the

marker. As a comparison, the iron content of the hydrolysed wheat gluten we used was much lower (< 0·01 g/kg). Consequently, in our conditions, the marker method do not seem optimal in the determination of ileal digestibility. Testing other non-absorbable markers would be necessary for further discussion about the effectiveness of the marker technique in general. For the present study, chromium oxide was chosen because of its current use as a non-absorbable marker for digestibility studies in animal models and the applicability of chromium analysis in our laboratory. Titanium dioxide and acid-insoluble ash may display higher recovery rates[16]. In a study carried out by Jagger *et al.* in pigs using several non-absorbable markers, chromium oxide recovery was 75% when titanium oxide recovery reached 98%[2]. In our study, the complete collection of digesta along the entire digestive tract enabled to calculate the total recovery rate of the marker. But in rat studies, the recovery value of the marker is generally not assessed because the collection of digestive content is limited to the ileum.

The comparison between the methods revealed that neither the selected segment nor the use of an indigestible marker had an effect on true digestibility results. Inter-individual variability was high in the two marker methods, explained by the variable recovery rate of chromium and the reduced number of rats and leading to a high SD for protein digestibility results. The SD was lower in no marker-caecum technique. The results we obtained suggest that true digestibility could be assessed in caecum instead of ileum and without using a marker. However, none of the method is optimal and they all lead to some uncertainties. The limitations of the marker method in ileal digestibility measurements are related to the low and variable recovery rate of the marker. Marker technique is also negatively impacted by the large amount of sample needed for chromium analysis compared with the limited amount of digesta available in the ileum (around 50 mg in our study). For other markers, the quantity of sample needed for analyses are also important for accurate measurements. A solution is to pool ileal content of several rats, but increasing the total number of rats would be necessary which does not respect the 3R principle of animal experimentation. Caecal digestibility method allows the collection of larger amount of digesta, but also induces its own bias. It neglects the dietary losses of nitrogen in proximal intestine and colon, which may overestimate digestibility. The fate of the majority of dietary nitrogen in the proximal intestine is to be absorbed, especially for highly digestible protein, and the dietary nitrogen in the colon is low which should minimise the overestimation of digestibility. In the caecal method, the entire content of the digestive tract has to be collected and analysed to make sure dietary nitrogen losses are mainly in the caecum. Furthermore, caecum microbial activity may be reduced in comparison to colon but yet existing, which could impact the results especially for amino acid digestibility assessment. Nevertheless, evaluating protein digestibility at the caecum level enables to avoid the bias induced by the variation in recovery rate of marker. It also decreases the number of analytical methods. The quantity of digesta provided by caecum 6 h after ingestion (around 450 mg in this study, nine times higher than in ileum) is a significant advantage especially when proteins are labelled, as isotopic analysis for amino acid digestibility assessment requires large amounts of samples (100 mg for isotopic enrichment analysis in individual amino acid by GC-C-IRMS, 10 mg for amino acid content analysis by UHPLC).

In conclusion, the results of the present study suggested that true caecal digestibility could approximate true ileal digestibility and dispense with the use of a non-absorbable marker, provided that the amount of nitrogen in each segment of the digestive tract is known. It would bring notable benefits, such as increasing the amount of digesta available for analysis and avoiding bias related to marker recovery. This simple method is thus a useful tool to determine ileal nitrogen and amino acid digestibility of a large variety of new, alternative and sustainable protein ingredients, in the context of diversification of the protein sources for human consumption.

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The authors' responsibilities were as follows: C. G., J. C., L. G. D. and C. L. M. contributed to the conception and design of the study; F. G., J. C., S. B. and N. K. conducted the research; F. G. and J. C. analysed the data; F. G. wrote the original draft of the paper; J. C., C. G., L. G. D., and C. L. M. reviewed and edited the manuscript; C. G. administered the project; and all authors read and approved the final manuscript.

Data described in the manuscript will be made available upon reasonable request, pending application and approval.

C. G., N. K., S. B. and J. C. declare that they have no conflict of interest. F. G., L. G. D. and C. L. M. are employed by Roquette.

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