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A NEW METHOD TO MEASURE THE REDOX POTENTIAL (Eh) IN RABBIT CAECUM: RELATIONSHIP WITH pH AND FERMENTATION PATTERN

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Abstract: This study aimed to assess the anaerobic status of the caecal biotope in the rabbit through the measurement of its redox potential (Eh). Since the caecal content has a high viscosity, the duration of the Eh measurement is high (10 to 20 min) and two methods were compared in 10 week- old rabbits: *in vivo vs. post-mortem*. In addition, Eh, pH and temperature of the caecal digesta were analysed according to caecotrophy and three periods in the day (soft faeces production: 08:00-10:00 h and 12:00-14:00 h; hard faeces production: 17:00-19:00 h) were compared, using 34 rabbits aged 65 d and weighing 2.3 kg. Caecal Eh decreased 2 min after measurement began, and then stabilised from 20 min onwards (from −152 to 221 mV, $P<0.001$), in contrast to caecal pH which remained constant over time. Mean values for Eh (at 20 min) and pH were - 219 mV and 6.2 respectively, and did not change according to method or collection period. Only the caecal temperature was 2°C higher ($P<0.001$) for the *in vivo* (39°C) than for the *post-mortem* (37°C) method. Average caecal dry matter and total volatile fatty acid were on average 22 % and 106 mmol/L, and were affected neither by the method nor by the collection period. Caecal Eh was negatively correlated to caecal pH ($R^2=0.22$; P=0.006, n=34), but not to other biotope traits. The Eh measurement in rabbit caecal content could be performed with a minimum recommended duration of 20 min, under anaesthesia or *post-mortem*. We confirmed that the rabbit caecal ecosystem is highly anaerobic.

Key Words: redox potential, fermentation pattern, pH, caecum, rabbit, methodology.

INTRODUCTION

In herbivorous animals, some parts of the gastro-intestinal tract where the fermentation process occurs (rumen, caecum, and colon) play a key role in the digestive process. Nutritional studies conducted in these animals have resulted in the characterisation of a group of parameters i.e. pH, ammonia, and volatile fatty acids (VFA), which define the normal state of such anaerobic biotopes and could give a diagnostic value when a metabolic disorder occurs. Redox potential (Eh) is another intrinsic physico-chemical parameter of the biotope which has been measured essentially to characterise rumen area. For instance, Marounek and Wallace (1984) report that the redox potential influences the microbiota composition and metabolic activity of the microbial ecosystem. In normal conditions, the ruminal medium is anaerobic with a markedly negative Eh (−150 to −220 mV) that reflects the absence of oxygen and a strongly reducing power.

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The caecum represents the main area of microbial activity for the rabbit. It is important that the reductive characteristics of the caecum environment are investigated to gain a better understanding of the microbial activity and dynamics of fermentation. Therefore, we assumed that caecal microbiota structure and/or activity would be affected by the redox potential, and thus it may be a good candidate as a predictive indicator of the digestive health of young rabbits. To our knowledge, no measurements of the Eh have been done on the caecal content. Thus, this work aims to measure the Eh and relationship with pH and fermentation pattern in adult rabbit caecal content, at three periods over 24 h to assess the potential impact of feeding behaviour and caecotrophy, as found for caecal fermentative activity in the growing rabbit (Bellier *et al*., 1995).

According to preliminary observations (Kimsé, 2009), Eh value is obtained within 1 min in redox standard solution and in aqueous media (such rumen fluid). But in more viscous digesta, such the caecal content (20-23% dry matter), Eh value decreased rapidly during the first 15 min, then became stable. Therefore, this study aimed to evaluate the optimal time delay to reach a stable value. In addition, since the measurement duration is relatively long (>15 min), we aimed to verify if it is necessary to keep the animal anaesthetised or not, according to parameters such as body temperature and fermentation pattern. For this reason, Eh measurements were performed according to two methods: under anaesthesia and *post-mortem* (after euthanasia).

MATERIALS AND METHODS

Animals and diet

This experiment was conducted using 34 New Zealand White×Californian rabbits. Rabbits were 65 d of age, and they were kept in individual metabolism cages (55×40 cm) and submitted to a 12-h light (07:00 to 19:00 h) and 12-h dark schedule. The temperature (18 ± 2°C) was automatically regulated in the breeding room. They were given ad libitum access to water and to an experimental pelleted diet (diameter size 5 mm) convenient for growing and adult rabbits (Table 1). Animals were handled in compliance with the regime for care of animals in experimentation, in accordance with French national legislation (decree 2001-486, 06/06/2001).

Eh, temperature and pH measurement in the caecum and sample preparation

Redox potential, temperature and pH measurements were carried out according to a 2×3 factorial experimental design. Two groups of 17 rabbits were assigned to the two methods, *in vivo* and *post-mortem*. Within each method, three collection periods (08:00 to 10:00 h, 12:00 to 14:00 h and 17:00 to 19:00 h) were assessed by measuring caecal criteria on 6 animals (on samples obtained after the animals were put to sleep), except for the last period where 5 rabbits were used. The periods 08:00 to 10:00 h and 12:00 to 14:00 h corresponded to the soft faeces production and ingestion by the rabbit (caecotrophy), whilst hard faeces were excreted during 17:00 to 19:00 h period and after.

Relative to the *in vivo* method, 17 rabbits were anaesthetised, using an intramuscular injection of xylazine (Rompun®, Bayer, Leverkusen, Germany, 0.5 mL/kg of BW) and 10 min after an intramuscular injection of ketamine (Imalgène®, Rhône Merieux, France, 0.4 mL/kg BW). Anaesthesia was maintained during Eh, temperature and pH measurements. With respect to the *post-mortem* method, the 17 animals were first anaesthetised with xylazine injection, and 10 min after they were sacrificed with T61® (0.5 mL/kg of BW, endo-cardiac injection), before Eh measurements.

The caecum was reached through a small midline incision (5 to 8 cm) on the abdomen then a second small midline incision (2 to 2.5 cm) was made on the caecum to insert Eh and pH electrodes. The Eh, pH and

Table 1: Ingredients and chemical composition of the experimental diet.

Ingredients (%)	
Wheat	7.50
Beet pulp	15.8
Soya-bean hulls	10.9
Wheat bran	35.2
Sunflower meal 35	24.0
Soy molasses	3.57
L-Lysine	0.12
Salt	0.50
$CaCO_3$	0.97
Vitamin/mineral mix[1]	1.50
Nutrient analysis (g/kg dry matter)	
Crude ash	78
Crude Protein (N x 6.25)	160
Crude fat	23
Crude fibre	167
Neutral detergent fibre	402
Acid detergent fibre	200
Acid detergent lignin	46
Digestible energy (kcal/kg)[2]	2250

[1]Values calculated from Maertens *et al*. (2002).
[2]include a coccidiostatic (salinomycin)

T measurements were made with two electrodes connected to a digital pH meter (Metrohm® model 713 CH-9101, Herisau, Switzerland). A glass pH and temperature electrode "Unitrode" (Pt1000/B/2/3MKCl; Metrohm®) and an Eh platinum electrode "Combined" (Pt-ring electrode; Pt/−2 to 80°C; Metrohm®) were used in both methods. Before caecum incision, the pH electrode was calibrated and the Eh electrode was tested by using a redox standard solution (Metrohm® Ltd. CH-9101, Herisau, Switzerland).The electrodes were inserted in the caecal content between 5th and 7th caecal spire. During measurements, animals were covered with a towel to minimise contact with atmospheric air and to avoid loss of body temperature. It is acknowledged that the time delay for obtaining a stable Eh measurement is longer in a medium having a relatively high viscosity, such as in the caecum (20-23% dry matter), compared to a ruminal content (5-8%). Consequently, Eh (and pH) were recorded first 2 min after caecum incision and then 7 measurements were performed each 5 min for 35 min, to estimate the optimal delay period to reach the stabilisation of the Eh value.

To avoid any oxygen flow inside the caecal content during Eh measurements, digesta were sampled immediately after the last measurement of Eh for chemical analyses. Samples for VFA assay were placed in polyvinyl chloride tubes containing 2 g caecal digesta per 2 mL $HgCl_2$ (2%, w/v), and stored immediately at −18 °C. After defrosting and centrifugation, concentrations of VFA were determined on supernatant samples using gas chromatography (5890A, Hewlett Packard, Avondale, PA).

Eh calculation

By definition, the Eh is the difference of potential between a platinum electrode and a standard hydrogen electrode (The International Hydrogen Zero). Given that in the measurements actually made the reference electrode was not an hydrogen electrode, all records of the potential difference must be corrected using the formula: $Eh=E_0 + C$; where E_0 is the potential of the platinum electrode, and C is the potential of the reference electrode relative to the standard hydrogen electrode for a given temperature (Kjaergaard, 1977; Nordstrom, 1977; Sauer and Teather, 1987).

Chemical analyses

The following chemical analyses were carried out on feed. Dry matter was determined on triplicate samples by heating at 103°C for 24 h, organic matter after ashing at 550°C for 5 h, nitrogen by Dumas procedure (auto-analyseur LECO, mod. FP428), and neutral detergent fibre, acid detergent fibre and acid detergent lignin, according to the sequential procedures of Van Soest *et al*. (1991), with an amylolytic pre-treatment using thermostable amylase for feed samples (AFNOR, 1997; EGRAN, 2001). The dry matter level of the caecal digesta was determined on duplicate samples by heating for 24 h at 103°C. Separation of VFA was performed by gas liquid chromatography (Hewlett Packard 5890 Series II) using the technique adapted from Playne (1985).

Statistical analysis

Caecal VFA and dry matter were analysed according to a 2×3 factorial model (GLM procedure, SAS online user guide), as follows: $Y_{ijk} = \mu + M_i + P_j + DM_k + (M\times P)_{ij} + (M\times DM)_{ik} + e_{ijk}$. , where Y is the random variable, μ is the overall mean, M is the effect of method (*in vivo*; *post-mortem*), P is the effect of period (08:00 to 10:00 h; 12:00 to 14:00 h; 17:00 to 19:00 h), DM_k is the duration of measurement (5, 10, 15, 20, 25, 30 ans 35 min), (M×P) is the interaction of method and period collection, (M×DM) is the effect of the interaction of method and duration of measurement and e_{ijk} is the residual error. Eh, pH and caecal temperature were statistically analysed according to a split-splot model, including the effect of the animal (nested in the interaction between method and period); the main effects were tested on the mean square error (MSE) of the interaction between method and period, and accordingly the means were compared using the same MSE (Pdiff test, SAS). Relationship between Eh and pH was submitted to a regression analysis (REG procedure, SAS), using the values obtained after the time delay (at 20 min) for Eh and pH.

RESULTS AND DISCUSSION

The live-weight of animals was of 2.3 ± 0.2 kg (mean ± sd, n=34), while their feed intake during the week previous to the measurement period averaged 145 ± 21 g/d.

Methodology for measuring the redox potential (Eh) in caecal content.

With both methods, caecum Eh dropped rapidly from –152 to –211 mV for 15th min after insertion of the electrodes (Figure 1), and the Eh value recorded at 2 min (−152 mV) was significantly higher than those recorded from 20 to 35 min (- 219 mV on average). Then, Eh slowly decreased from –216 to –221 mV, and we recommended a time delay of 20 minutes for Eh measurement, whatever the method of measurement.

This decrease of Eh during the first 10 min of the measurement may be attributed to the entrance of oxygen into the caecum when electrodes were inserted, and that would be quickly consumed by facultative anaerobic bacteria, thus maintaining anaerobic conditions (Broberg, 1957b). For instance, Marden *et al*. (2005) noticed an Eh increase in the rumen (−220 to −200 mV) after the meal, probably due to an oxygen supply associated to feed intake, mastication, and water intake. However, care has been taken here to

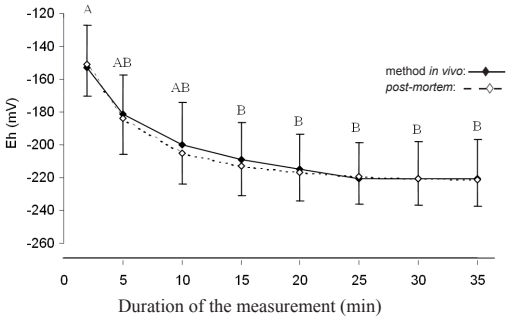

Figure 1: Kinetics of redox potential (Eh) in the caecal content, according to the method of measurement in the rabbit (data obtained on 10 week-old rabbits). Effect of method on Eh of the caecal content: P=0.96.
[A,B] Means (between duration and among methods) having a common letter are not different at P<0.05.

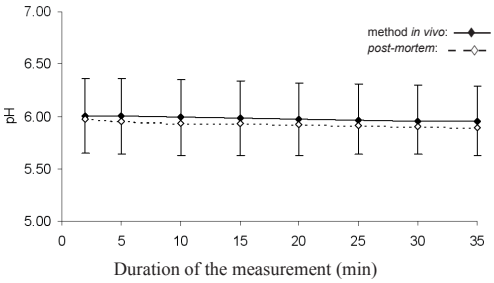

Figure 2: Kinetics of caecal pH in rabbit (data obtained on 10 week-old rabbits) according to the method of Eh measurement. Effect of duration on pH of the caecal content: P=0.99; Effect of method: P=0.66.

reduce oxygen entry during electrodes insertion. Even so, we assumed that the decrease of Eh originated more probably from the time delay for stabilisation of the Eh electrodes in a viscous media such the caecal digesta (20-23% of dry matter). For instance, Eh is carried using 2 half-cells connected by a salt bridge that allows exchange of electrons. Because caecal digesta are pasty, the time delay to establish the bridge and a correct exchange of electrons could be longer than in a non-viscous water solution, and here reached 20 min. Thus, the average value of −219 mV seemed relevant and representative of the high anaerobic environment in the caecal biotope.

No significant interaction on caecal Eh, pH and temperature were found between methods and periods or duration of the measurements (Table 2). Redox potential and pH were affected neither by the method nor by the period of measurement. Therefore, this last result indicated that the two physico-chemical parameters seemed not greatly affected by the partial emptying (about −20%) of the caecum during caecotrophy (Leng and Hörnicke, 1976; Gidenne and Lebas, 1984). Mean Eh values were −206 and −204 mV for *in vivo* and *post-mortem* methods respectively. In normal conditions, the caecal biotope is anaerobic and the markedly negative values of Eh reflect the absence of oxygen and strong reducing conditions. Values reported in our study were in accordance with those found in the hindgut of other herbivores: −200 and −220 mV respectively in the caecum and the colon of goat (Marounek *et al*., 1987). The observed Eh values were significantly higher in more aerobic biotopes, such the abomasum (+100 mV) and duodenum (+10 mV) of goat (Marounek *et al*., 1987). Eh also vary in the rumen according to species, with Eh ranging from −174 to −217 mV in dairy cows (Marden *et al*., 2005), from −103 to −260 mV in sheep (Broberg, 1957a; Barry *et al*., 1977; Mathieu *et al*., 1996) and from −128 to −190 mV in goats (Marounek *et al*., 1982; Andrade *et al*., 2002).. The Eh differences reported among the different segments of the gut are related to the anaerobic status of the microbiota, thus suggesting large variations in microbial composition. So, the metabolic activity of anaerobic bacteria depends largely on enzymes which operate normally when the medium is reduced (Hewitt, 1950; Sasaki *et al*., 2001). Accordingly, aerobes have a normal metabolism when the Eh of their environment ranged from +400 to −200 mV, while anaerobes require an Eh between +50 to −400 mV (Baldwin and Emery, 1960).

Relationship between Eh and caecal biotope traits.

The pH of the caecal digesta did not differ significantly according to the two methods, although pH seemed slightly lower in *post-mortem* compared to in-vivo method (5.9 *vs*. 6.0, respectively; Figure 2).

Table 2: Physico-chemical parameters in the caecum (data obtained on 10 week-old rabbits) according to Eh measurement method and collection period.

	Method		Period				*P*-value			
	Post-mortem	*In vivo*	08:00-10:00 h	12:00-14:00 h	17:00-19:00 h	RMSE[1]	Method	Period	DM[2]	Method×Period
n[3]	17	17	12	12	10					
Eh (mV)[4]	−204	−206	−196	−205	−208	10	0.96	0.31	<0.001	0.74
pH	5.9	6.0	5.8	6.0	6.1	0.0	0.66	0.22	0.99	0.62
T (°C)[5]	37.2	38.9	38.2	37.8	38.2	0.3	<0.001	0.43	0.99	0.38

[1] RMSE: root mean square error, [2] DM: duration of measurement, [3] n: number of rabbits, [4] Eh: redox potential, [5]T: temperature. Interactions DM×Method, Period×DM and Period×Method×DM: *P*-value=1.

The pH was here in agreement with previous studies on the fattened rabbit (Bellier *et al*., 1995; Gidenne *et al*., 2002; Garcia *et al*., 2002). The caecal pH remained constant throughout the measurement duration (35 min). Kohn and Dunlap (1998) had demonstrated the strong relationship between the pH in rumen and partial pressure of CO_2. Variations in the amount of CO_2 dissolved in an aqueous medium would automatically change the amount of H^+ ions. Since the caecal content is pasty the dissolution of CO_2 would not be favoured, and this contributes to low fluctuations in pH.

Conversely, temperature of the caecal content was 2°C higher ($P<0.001$) for *in vivo* compared to *post-mortem* method, whatever the period or the duration of the measurement (Table 2). Even so, the caecal temperature did not vary with duration of the measurement (Figure 3). Whatever the method used, the constant temperature and pH values during the Eh measurements would suggest that microbial activity would probably not be greatly modified.

The Eh was negatively correlated only to the caecal pH: Eh (mV)= −33.0–29.2 pH (Figure 4; R^2= −0.22; P =0.006; n=34). This result was in agreement with those of Baldwin and Emery (1960), Marounek *et al*. (1987) and Giger-Reverdin *et al*. (2006) who found a negative linear correlation between Eh and pH, but with a higher correlation coefficient (0.64 to 0.71) and a higher slope (−60 to −70). The relationship between these two parameters reflected that many biochemical reactions in the caecum ecosystem depended on redox couples with exchanges of protons.

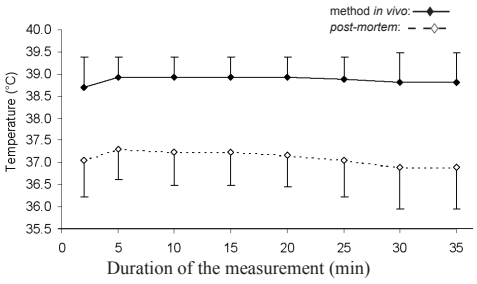

Figure 3: Kinetics of the caecal content temperature, according to the method of Eh measurement in the rabbit (data obtained on 10 week-old rabbits). Effect of duration: P=0.99. Effect of method: P<0.001.

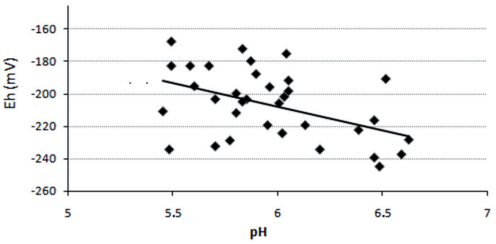

Figure 4: Relationship between Eh and pH in the caecal content of the rabbit (data obtained on 10 week-old rabbits, Eh values are those measured at stabilisation time (20 min)).
Eh (mV)= −33.0(±58.7) – 29.2(±9.8) pH,
R^2 =0.22 (P=0.006, n=34).

Table 3: Fermentation pattern in the caecum (data obtained on 10 week-old rabbits) according to Eh measurement method and collection period.

	Method		Period				*P*-value		
	In vivo	*Post-mortem*	08:00-10:00 h	12:00-14:00 h	17:00-19:00 h	RMSE[1]	Method	Period	Method×Period
Dry Matter (%)	22.2	22.1	22.2	22.4	21.7	1.8	0.83	0.72	0.96
Total VFA (mmol/L)[2]	111.1	108.7	112.7	110.3	105.7	18.7	0.75	0.64	0.38
Acetate (%)	77.1	77.1	76.0	77.5	78.1	4.4	0.95	0.54	0.93
Propionate (%)	5.0	5.2	4.8	5.4	5.1	1.0	0.73	0.49	0.64
Butyrate (%)	17.1	16.9	18.4	16.3	16.1	4.4	0.98	0.41	0.86
Butyrate/Propionate	3.5	3.4	4.0	3.2	3.3	1.2	0.93	0.26	0.48

[1]RMSE: root mean square error, [2] VFA: volatile faty acids.

Neither dry matter nor total VFA concentration nor VFA proportions in the caecal digesta were influenced by the method and the collection period (Table 3). The total VFA concentration averaged 110 mmol/L, that is in agreement with the literature for fattened or adult rabbits. (Bellier *et al*., 1995; Carabaño *et al*., 2006, Falcao-E-Cunha *et al*., 2006). The caecal fermentation pattern also ranged within classical values observed in the adult rabbit: acetate (77%), butyrate (17%) and propionate (5%). Accordingly, the butyrate to propionate ratio had a mean value of three (Gidenne, 1986; Garcia *et al*., 2000), whereas in most herbivorous species it remains under 1. Adjiri *et al*. (1992) showed that this particular VFA pattern originated from the caecal microbiota and not from the substrate fermented. The dominance of Flexibacter-Cytophaga-Bacteroides group, known to include several butyrate producing strains, was shown by Bennegadi *et al*. (2003), and could explain the high butyrate proportions in the caecum.

The results of our study indicated that the caecal pH and the level and the profile of the caecal VFA were not affected by the period of measurement in the fattened rabbit, as previously observed in adult animals by Bellier *et al*. (1995) for VFA level. This may suggest that the caecal microbiota could maintain a constant environment throughout the day. The regular rhythm of feed intake of the rabbit, including soft faeces intake (Gidenne and Lebas, 2006) would be a favourable factor for so few changes in the daily microbial activity (at least for adult animals). Moreover, Bellier *et al*. (1995) mentioned nycthemeral changes in caecal pH and VFA for the growing rabbit. Additionally, previous studies reported higher caecal pH (Fraga *et al*., 1984) and lower VFA concentrations (Gidenne, 1986) during the caecotrophy period (in the morning) when the feed intake is low.

CONCLUSION

The Eh measurement in rabbit caecal content could be equally performed under anaesthesia or on a dead animal. The minimum time delay required for measurements was 20 min. Caecal Eh, pH or fermentation pattern were not affected by the period of sampling (according to caecotrophy), and Eh was negatively correlated with pH.

In the future, caecal Eh might be associated with other physical and chemical parameters of the caecal media to improve our understanding of the functioning of the rabbit caecal ecosystem, particularly in case of dysbiosis leading to digestive troubles in the young animal.

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