

EFFECTS OF HIGH AMBIENT TEMPERATURE IN RABBITS : METABOLIC CHANGES, CAECAL FERMENTATION AND BACTERIAL FLORA.

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ABSTRACT: Eighteen male New Zealand White rabbits, randomly assigned to three groups, were placed in a climatic chamber at 20 °C. After 10 days of adaptation the temperature was risen every day to 30°C from 08:00 a.m. to 08:00 p.m. The three groups of animals were slaughtered according to the following schedule: six animals at the end of adaptation period (control), six animals 2 days after the beginning of the heat stress, and six animals 14 days after the beginning of the heat stress. At the same schedule blood samples were collected through the ear vein. The exposure to hot environment induced a net decrease of feed consumption in the first two days that was partially recovered in the following days. No relevant changes were observed for plasma

metabolic parameters concentration in the different sampling times, except for an increase of total proteins on day 2, and a tendency to decrease for glucose on day 2 and 14 ($P < 0.08$). Total volatile fatty acids concentration in the caecal content significantly decreased on day 2 and 14 and a parallel trend was observed for acetic, propionic and butyric acid concentration. No relevant changes were observed in the molar proportion of acetic, propionic and butyric acid and pH. Ammonia concentration of the caecal content decreased on day 2. Total anaerobes significantly increased on day 2, clostridia significantly increased on day 2 and 14. No significant changes were recorded for lactobacilli and total coliforms.

RESUME : Effets de la chaleur sur les principaux paramètres sanguins, la fermentation caecale et la flore bactérienne chez le lapin

Dix-huit lapins mâles Neo-Zélandais Blancs, ont été assignés à trois groupes, et placés dans une chambre climatique à 20°C. Après 10 jours d'adaptation, chaque jour la température a été élevée à 30°C de 08:00 heures à 20:00 heures. Les trois groupes d'animaux ont été abattus selon le programme suivant: six animaux à la fin de la période d'adaptation (témoins), six animaux 2 jours après le début de l'exposition thermique, et les six animaux restants 14 jours après le début de l'exposition thermique. Selon la même chronologie des échantillons de sang ont été recueillis à la veine de l'oreille. L'exposition à l'environnement chaud induit une diminution nette de la consommation d'aliment les deux premiers jours ; cette diminution a été partiellement récupérée dans les jours suivants. On n'a observé aucun changement

dans la concentration des paramètres métaboliques du plasma aux différents temps de prélèvement, excepté une augmentation des protéines totales le jour 2, et une tendance à une diminution du glucose les jours 2 et 14 ($P < 0.08$). La concentration en acides gras volatils totaux dans le contenu caecal a diminué de manière significative les jours 2 et 14 et une tendance parallèle a été observée pour la concentration en acides acétique, propionique et butyrique. On n'a observé aucun changement de la proportion molaire en acides acétique, propionique et butyrique et de pH. L'ammoniaque du contenu caecal a diminué le jour 2. Les bactéries anaérobies totales ont augmenté de manière significative le jour 2, les clostridium sont sensiblement accrus les jours 2 et 14. Aucun changement significatif n'a été enregistré pour des lactobacilles et des coliformes totaux.

INTRODUCTION

The effects of heat environmental conditions in rabbits have been extensively discussed in the last two decades mainly as far as regard functional parameters, like growing performances, feed intake (CHIERICATO *et al.*, 1992; PACI *et al.*, 1993) and motorial activity (FINZI *et al.*, 1994). Also metabolic profiles have been deeply investigated but, even in similar experimental conditions, controversial results were obtained (EBERHART, 1980; CHIERICATO 1984; MARAI *et al.*, 1994; EL-MASRY *et al.*, 1994; CHIERICATO *et al.*, 1994; AMICI *et al.*, 1995). Only recently the immune system activity (FRANCI *et al.*, 1996) and oxidative-antioxidative status (AMICI *et al.*, 1995) have been proved to play a role to maintain the homeostasis in heat stressed rabbits. These studies were focused in particular on the long term reaction of the organism to chronic heat stress conditions, including acclimatisation and adaptation (ZULKIFLI and SIEGLE, 1995). In recent studies the short term acute thermal stress was also considered in order to determine its effects on immune

system and to evaluate the functional, metabolic and antioxidative changes at various times (AMICI, unpublished results). On the other hand some studies on feeding regime and composition were performed to evaluate the effects on microbial flora (CANGANELLA *et al.*, 1992), and the relationship with caecal fermentation activity and health status (MORISSE and MAURICE, 1994; PADILHA *et al.*, 1995). The possible effects of the environment and stress on gut microflora was also hypothesised (MORISSE *et al.*, 1985), though only PENNEY *et al.* (1986) observed some variations in a little number of cold acclimatised rabbits.

The over mentioned results suggest that in rabbits undergoing heat stress, interesting relationships between metabolic parameters, caecal fermentation and microbial population can be found. For these reasons the trial was planned to study the effects of hot environmental conditions roughly resembling summer nictameral variations on feed intake, caecal fermentation and microbial population and the possible relationships with plasma metabolic parameters.

MATERIAL AND METHODS

Eighteen male New Zealand White rabbits (NZW) of 2540 ± 112 g body weight were placed inside a climatic chamber in single cages provided with feed and water *ad libitum* at an environmental temperature of $18.0 \pm 0.5^\circ\text{C}$, and relative humidity of $60 \pm 4\%$. Sixteen hours of day light were provided for the adaptation and experimental period. These environmental conditions correspond to the thermoneutral zone of this species (CASTELLO and ROCHA, 1980). After 10 days of adaptation, the animals were exposed to heat stress conditions resembling the hot summer period. From 08:00 a.m. to 08:00 p.m. the temperature was automatically risen to 30°C and from 08:00 p.m. to 08:00 a.m. the temperature was decreased to 20°C . The environmental conditions were maintained by an automatic conditioning system (Digital thermostat S90TP, and digital humidistat S90HP, Carel, Padova, Italy) and continuously recorded with a thermoidrograph.

During the adaptation and experimental period feed consumption and body weight were individually registered. A commercial balanced feed (185 g/kg DM crude protein, 141 g/kg DM crude fibre, 338 g/kg DM soluble carbohydrates, 11.4 MJ/kg DM of digestible energy) was utilised for the experiment.

The animals were randomly assigned to three groups and slaughtered according to the following schedule: six animals at the end of adaptation period (control), six animals 48 h after the beginning of the heat stress, and six animals 14 days after the beginning of the heat stress. At the same schedule, blood samples were collected (on EDTA- Na_2 , 1 $\mu\text{g}/\text{ml}$) through the ear vein. The plasma obtained by centrifugation (1500g \times 30', at 4°C) was divided in aliquots and frozen at -20°C until analysed. Slaughtering and bleeding started at 07.30 a.m. and lasted less than 20'.

Clinical chemistry parameters were determined by an automatic analyser (Monarch plus, Instrumentation Laboratory, Lexington USA). The following commercial kits from Instrumentation Laboratory were used: 181633-80 for glucose, 181618-80 for cholesterol, 181610-60 for triglycerides, 181621-00 for urea, 181612-80 for total proteins, 181600-10 for AST, 181602-10 for ALT, 181620-80 for albumin.

The caecums were collected from rabbits immediately after sacrifice and disposed in sterile glass containers. Samples of fresh caecal content (at least 2 grams) were then extracted by a disposable spatula inside an anaerobic glove box (Forma Scientific, Mariette, Ohio, USA), resuspended in 5 ml of sodium phosphate buffer (pH 5.5) and stored at -20°C until analysis.

For the enumeration of taxonomic microbiological groups, several media were used. Brain Heart Infusion (Biolife Milano, Italy) medium was used for the isolation of anaerobic micro-organisms. MRS agar (Biolife) was used for the isolation of lactobacilli whereas M17 (Biolife) agar was used for lactic streptococci. Azide Maltose (KF) agar (Biolife) was used for the selective isolation of faecal streptococci, RCM agar (Oxoid S.p.A. Milano, Italy) for the non-selective isolation of clostridia and VRB agar (Biolife) for the selective enumeration of *Escherichia coli*. For the identification of microbial strains, the isolates were cultivated in PTG broth (g/litre): tryptone, 10.0; soy peptone, 5.0; glucose, 15.0; yeast extract, 2.5; K_2HPO_4 , 1.5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5; cysteine-HCl, 0.5; pH, 7.2. Preparations of liquid media and dispersion into Petri dishes were carried out following the common microbiological procedures. All incubations were performed anaerobically in an anaerobic glove box (Forma Scientific) equipped with an internal incubator.

The pH of caecal samples was determined by a Hanna Instruments pH meter, model 8417. Analysis of fermentation products from the caecal samples was performed with a Carlo Erba gaschromatograph model MEGA (Carlo Erba Milano, Italy) equipped with a HP fused silica capillary column (ID 0.31 mm, length 50 m). Samples were centrifuged for 10 min at 10,000rpm and injection was performed with 0.5 μl of supernatant.

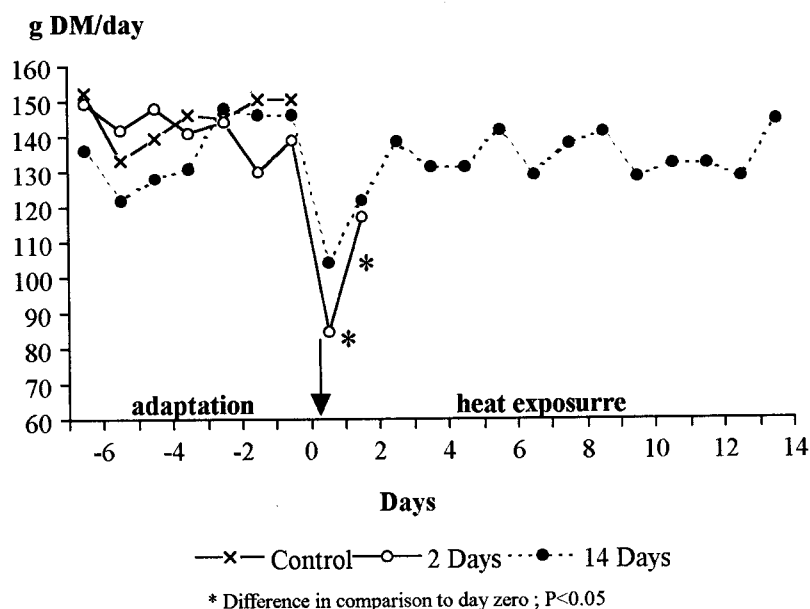
For the taxonomic identification of the most representative isolates the following API kits (Biomerieux, Italy) were used: 20 STREP for streptococci, 20 A for anaerobic bacteria and 20 E for enterobacteria. Analyses were carried out following the manufacturer's guidelines.

Urea and ammonia production in the caecal samples were determined by the spectrophotometric method for Urea/Ammonia (Boehringer Mannheim, Germany). This method is based on the reaction of urease or glutamate dehydrogenase (GIDH) with urea and ammonia, respectively with the resulting UV detection of NH_3 or L-glutamate at 340 nm.

This experiment was performed in conformity with the Italian law on procedures for experimental animals. All procedures to minimise pain and discomfort were adopted during the trial. No signs of disease were registered during the trial and adaptation period.

The data were submitted to analysis of variance according to a randomised blocks design. Comparisons between the means were evaluated with the least significant difference test (SAS, 1993). The effect of group by time was tested by orthogonal contrast. Correlation between the parameters were performed utilising the Pearson test (SAS, 1993).

Figure 1 : Feed intake



RESULTS AND DISCUSSION

Table 1 : Plasma concentration of metabolic parameters in rabbits undergoing heat exposure

Sampling time	Control	2 days	14 days	s.e.
Glucose (mmol/l)	8.47	7.92	7.99	0.189
Cholesterol (mmol/l)	2.00	2.28	1.96	0.257
Triglycerides (mmol/l)	0.83	0.93	0.94	0.106
Urea (mmol/l)	5.40	4.84	5.30	0.430
Total protein (g/l)	56.5 b	57.8 b	63.0 a	1.27
AST/GOT (U/l)	38.0	39.0	42.7	8.85
ALT/GPT (U/l)	47.0	42.2	55.2	6.32
Albumin (g/l)	36.7	37.7	39.1	0.95
Albumin rate (%)	65.2	65.3	62.1	1.54

Different superscript letters on the same row indicate significant differences: a, b $P < 0.05$.

Table 2 : Caecal concentration of fermentative products and pH in rabbits undergoing heat exposure

Sampling time	Control	48 h	14 d	s.e.
Total VFA(1) (mmol/kg)	81.2 A	6.8 B	31.6 B	8.5
Acetic ac. (mmol/kg)	59.4 A	4.7 B	22.2 B	7.0
Propionic ac. (mmol/kg)	8.8 A	0.7 B	3.4 B	0.9
Butyric ac. (mmol/kg)	13.0 A	1.4 B	6.0 AB	1.8
Acetic ac. (% total VFA)	72.3	68.8	69.5	3.0
Propionic ac. (% total VFA)	11.9	12.3	10.9	1.9
Butyric ac. (% total VFA)	15.8	18.9	19.6	2.8
Ammonia (mmol/kg)	11.0 Aa	3.3 Bc	6.1 Ab	1.8
pH	6.82	6.85	6.62	0.15

Different superscript letters on the same row indicate significant differences A, B: $P < 0.01$; a, b: $P < 0.05$.
(1) Acetic + propionic + butyric.

As reported in Figure 1, the exposure to hot environment induced a net decrease of feed consumption in the first two day that was mostly recovered in the following days, and then remained stable. No relevant changes were observed for the concentration of plasma metabolic parameters in the different sampling days (Table 1), except for total proteins that raised after 14 days of exposure to hot environment ($P < 0.05$) and glucose that showed a tendency to decrease at 2 and 14 days ($P < 0.08$). If we also consider the decrease of triglycerides, not significant but at probability level of 12%, it can be supposed that these could reflect the consequences of feed intake reduction.

The exposure to heat stress induced a dramatic decrease of volatile fatty acid (VFA) concentrations in the caecal content at 2 and 14 days ($P < 0.01$) and a similar trend was observed for acetic, propionic and butyric acid concentration (Table 2). The intense decrease of mains VFA, as a consequence of heat exposure, did not affect the molar proportion of acetic, propionic and butyric acid that remained unchanged during the experiment.

Table 3 : Microbial flora count in caecal content of rabbits undergoing heat exposure

Sampling time		Control	2 days	14 days	RMSE
Total anaerobes	(CFU/g)	1.3 × 10 ⁴ B	7.3 × 10 ⁵ A	1.5 × 10 ⁵ B	3.0 × 10 ⁵
Clostridia spp.	(CFU/g)	7.0 × 10 ³ b	4.0 × 10 ⁴ a	4.1 × 10 ⁴ a	2.1 × 10 ⁴
Total coliforms	(CFU/g)	1.7 × 10 ⁴	1.4 × 10 ⁶	1.8 × 10 ⁴	1.6 × 10 ⁶
Lactobacilli	(CFU/g)	2.3 × 10 ⁴	1.1 × 10 ⁶	1.3 × 10 ⁵	1.3 × 10 ⁶

Different superscript letters on the same row indicate significant differences A, B: P<0.01; a, b: P<0.05.

Ammonia concentration of the caecal content decreased after 2 days (P<0.01) and partially recovered at 14 days. No significant changes were observed for pH values. Concerning the microbial population (Table 3), total anaerobes count resulted significantly higher values (P<0.01) after 2 days in comparison to control time and 14 days. Clostridia were significantly higher at 2 and 14 days (P<0.05). No significant changes were recorded for lactobacilli and total coliforms although an increase of microbial count was recorded at 2 days. Faecal streptococci always resulted below 10² cfu/g and no significant changes were observed throughout the experiment.

The decrease of the caecal material as a consequence of soft faeces production was reported by GIDENNE and LEBAS (1984). The hour for sampling caecal content should be carefully considered in order to avoid misinterpretation of results. BELLIER *et al.* (1995) observed circadian variations of the caecal traits of adult rabbits mainly concerning pH and NH₃ nitrogen. GIDENNE (1986) reported no significant variations concerning NH₃. Biochemical changes of caecal content in the time preceding soft faeces excretion was observed by DEMAUX *et al.* (1980).

According to our results this trait, namely microbial flora (Table 3), was affected by exposure of the animals to heat environment. Particularly total anaerobes significantly increased (P<0.01) after two days of heat exposure, and then decreased suggesting an adaptation response. This trend could be related to the inverse trend of caecal ammonia that is widely utilised for protein microbial synthesis as also suggested by EMALDI *et al.* (1979) and CROCIANI *et al.* (1984). In accordance with such explanation the decrease of blood urea (about 10%), though not significant, should be noticed. In fact, it is well known that a large amount of metabolic urea (38%) moves in caecum where it represent a very important source of nitrogen for the caecal microflora synthesis (FORSYTE and PARKER, 1985). It must be also underlined that total anaerobes counts are lower than that observed by other researchers (PENNEY *et al.*, 1986; TEDESCO *et al.*, 1994; PADILHA *et al.*, 1995), but this could be related to the level of soluble carbohydrates in the feed since it was reported

that increasing cellular carbohydrates induce a decrease of non sporulated anaerobial flora (MORISSE *et al.*, 1985).

The trend of clostridia observed during the trial can lead to several and controversial considerations. As reported by MORISSE and CHEEKE (1986) rabbits showing digestive troubles (diarrhoea) also had high pH values of the caecal

content (above 7.0). These conditions are unfavourable for the caecal bacterial flora (non sporulated anaerobes) and allow the development of bacteria, as *E. coli* and clostridia, able to produce toxins that are lethal for rabbits. MORISSE *et al.* (1985) observing no signs of disease in rabbits with an abnormal growth of caecal clostridia and *E. coli* suggested that the toxins production is inhibited by low pH values (5.8) and/or that the caecum was colonised by non pathogenic strains. In the same paper the authors also suggested the relationships between caecal microbial flora, AGV and NH₃ concentration, pH, and feed composition. Our results are in agreement with these hypothesis since no signs of digestive troubles related to clostridia were observed in the investigated animals, but according to the different AGV concentration differences in pH values should be expected.

Moreover, it was also observed a negative correlation between pH and butyrate proportion (r = -0.71, P<0.005) and a positive correlation between pH and acetate proportion (r = 0.55, P<0.042). In the latter case, the increase of acetic acid might be due to a better development of acetogenic clostridia at higher pH values but we have no evidence of that.

As far as regard the population of lactobacilli in the caecum, no significant differences were observed during the trial, nevertheless an increase was detected after the heat stress. The presence of lactobacilli in the rabbit caecum is controversial; TEDESCO *et al.* (1994) reported that these bacteria is part of the rabbit microflora at a power of 10⁵, Penney *et al.* (1986) observed that even the rabbits supplemented with *L. acidophilus* failed to show lactobacilli in the caecum also when fed with hay, apple, apple-twigs or pellets. Unfortunately the lack of recent data concerning lactobacilli in the caecum of rabbit does not allow to formulate more reliable hypothesis on so contrasting results.

Different explanations are necessary to discuss the observed increase of total coliforms in the caecum. NETTELBLADT *et al.* (1997) observed an increased number of coliforms in the caecal content of starving rats. In addition although there are no specific reports

on the effects of restricted feeding or fasting in rabbits, the role of volatile fatty acids (VFA), in particular the decreased amount of butyrate, can be underlined since this substance is reported to be a limiting factor for *E. coli* proliferation in rabbits (MORISSE *et al.*, 1985). Anyway, it should be underlined that the rate of the main VFA remained unchanged and, as usually reported, it was characterised by a higher proportion of butyrate in comparison to propionate (VERNAY and RAYNAUD, 1975). The marked decrease of VFA concentration in caecal content can be the consequence of the intense absorption of VFA from the caecum as suggested by PROHASZKA and SZEMEREDI (1984) in starving rabbits. The same authors also suggest that VFA concentration is affected by a bioregulatory mechanism able to optimise the antibacterial activity of these substances. At the light of these results it is also possible to explain the unchanged pH of the caecal content, and the increased absorption of VFA can be related to the scarce variations of blood metabolic parameters, e.g. the glucose concentration as previously suggested (CHIERICATO *et al.*, 1994; AMICI *et al.*, 1995). Relevant changes of blood metabolic parameters can be only observed as a consequence of acute thermal stress, as short as one hour, mainly concerning glucose, triglycerides and urea (AMICI, unpublished results).

A comprehensive interpretation of the present results enforce the opinion that reduced feed intake play an important role as intermediate stress factor in heat stressed animals as previously suggested for immune system activity and/or antioxidative status (AMICI *et al.*, 1995; FRANCI *et al.*, 1996). In these previous studies it was also reported that the most sensitive indicators of stress can be identified in some parameters of immune and oxidative-antioxidative systems. The alterations of caecal microflora, though no signs of disease were registered in the present trial, can represent a risk for the homeostasis of rabbit intestine and the use of additive substances with probiotic activity could be usefully utilised to avoid the development of pathogenic bacterial strains.

Acknowledgements : Research supported by National Research Council of Italy, Special Project RAISA, Sub-project no. 3.

Received : May 29th, 1998

Accepted : October 13th, 1998

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