Evaluation of physiological parameters of the plasma oxidative status in rabbits
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ABSTRACT
The aim of our work was to analyse oxidative parameters in rabbits, under nutritional physiological conditions of feeding and rearing. We evaluated zootechnical parameters and the nutritional status of rabbits, the latter by analysing serum triglycerides and albumin levels. All these parameters were found to be in the physiological range at the beginning and end of the test. For assessing the oxidative status of the animals, we measured the level of serum reactive oxygen metabolites (ROMs) and the level of serum antioxidants (d-ROMs and anti-ROMs tests, respectively). The levels of d-ROMs (CARR U) showed values of 299.9 ± 6.1 at the beginning and of 296.8 ± 6.4 at the end of the study. The levels of anti-ROMs (fast antioxidants) showed values of 85.2 ± 2.1 µEq/L in rabbits 70 days old and of 85.4 ± 2.7 µEq/L after 60 days. Statistical analysis of the values, for all considered parameters, showed that the mean of the differences between the beginning and the end of the experimental period was not significantly different from 0. Further studies are needed to assess the variations of these parameters related to oxidative stress in other physiological states of rabbits (pregnancy or weaning) or in rabbits affected by diseases.

1. Introduction
In recent years, considerable attention has been given to the preservation of livestock welfare in order to ensure the optimal growth conditions of the animals and to preserve them from multifactorial diseases, which can have a heavy impact on zootechnical productivity (Cerioli et al. 2006, 2008; Ludwig et al. 2006, 2007, 2008; Luzi et al. 2007). The intensive breeding of rabbits in recent decades has resulted in many problems related to the appearance of certain enteric and metabolic infections that have caused high mortality in the animals, thus weighing heavily on the productivity of farms (Luzi et al. 2007). These pathologies have multifactorial etiologies and occur at specific delicate stages of rabbit production due to predisposing factors, such as imbalances in the diet, hygiene deficiencies, environmental and climatic factors, overcrowding, and stress (Costantini & Castellini 1990; Broom & Johnson 1993). In addition to the zootechnical parameters, evaluating the oxidative plasmatic status in farm animals is important for monitoring animal welfare (Vassalle 2009). This assessment is carried out in order to verify whether the animal can maintain a homeostatic condition in spite of stressful environmental stimuli directed at elevated meat productivity (Chirase et al. 2004). According to Brambilla et al. (2003), from a prognostic point of view in the state of animal welfare, two different stressful situations, namely physiological or pathological, can be considered. In the case of physiological stress, animals are able to develop an adaptive response that is expressed by activating endogenous antioxidant mechanisms, which can compensate for the imbalance of oxidative status. Conversely, under conditions of pathological stress, the adaptive response of the organism is inadequate and leads to excessive production of free radicals, which results in oxidative stress (Brambilla et al. 2003; Khadija et al. 2009). In fact, the excessive generation and/or inadequate removal of free radicals results in destructive and irreversible cell damage (Lopaczynski & Zeisel 2001). Oxidative stress can be measured directly, by detecting free radical production, or indirectly, by detecting antioxidant defenses of the organism. Cellular antioxidant defenses consist of a complex interacting network, and more than 40 molecules are involved in the oxido-reduction metabolism (Montuschi et al. 2004; Shishehbor & Hazen 2004; Tsimikas 2008). Evaluation of the oxidative plasmatic status (the level of serum reactive oxygen metabolites – ROMs – and the level of serum antioxidants) may have several implications of veterinary interest. For instance, negative situations within the farm could be identified, thus suggesting the most appropriate interventions to create optimal conditions for the animals. In fact, in farm animals, oxidative stress is involved in a number of pathological conditions, including those associated with animal production, reproduction, and welfare (Lykkesfeldt & Svendsen 2007; Pastorelli et al. 2010). Evaluating plasma oxidative levels in animals would therefore be very useful in considering the health status of animals (Brambilla et al. 2002; Pasquini et al. 2008). Unfortunately, oxidative parameters in healthy rabbits, under standard conditions of nutritional and zootechnical breeding, are not well defined in the literature. To fill this gap of knowledge, the aim of our work was to evaluate the plasma oxidative status (ROMs and serum antioxidants) in healthy rabbits fed balanced diets for growth, and reared...
under standard environmental conditions (such as the size of cages and climatic conditions). For the purpose of this study, we also evaluated the stability of nutritional status of rabbits under physiological conditions; we therefore evaluated serum nutritional parameters, namely lipid levels (triglycerides) and serum protein levels (albumin) at the beginning and end of the trial. Furthermore, we evaluated the zootechnical parameters of rabbits.

2. Materials and methods

2.1. Animals, diets, and experimental design

A total of 30 growing crossbred New Zealand female rabbits aged 70 days and weighing, on average, 2318 ± 40 g, were individually housed in standard conditions at a temperature of 22 ± 2°C, a relative humidity of 70% ± 5, and at an estimated temperature–humidity index (THI, Marai et al. 2002) of about 26, in wire cages of dimensions 70 × 50 cm, at a height of 90 cm from the concrete floor. For the study, we carefully selected 30 rabbits in good health from hundreds of farmed rabbits examined. The animals were fed with a standard pellet diet, which was balanced and formulated for covering all the needs of the rabbits, including vitamins and minerals, according to the requirements of the NRC (1977). This diet was similar to the diets usually used for rabbits on farms, and was suitable for the physiological conditions of the rabbits (NRC 1977) (Table 1). The diet was stored in darkness to avoid auto-oxidation of the lipid sources. The experimental period lasted for 60 days. Food and water were available ad libitum to the animals. At the beginning and end of the trial, we performed blood sampling of the rabbits for nutritional evaluation: serum lipid levels (triglycerides) and serum protein levels (albumin) were measured, for evaluation of serum oxidative parameters. During the test, we also evaluated the zootechnical performances of the rabbits, namely initial weight, final weight, food consumption, and feed conversion, and we also performed general clinical external evaluations, that is, state of the fur, eyes, ears, mouth, teeth, and legs, and the appearance of faeces and urine. Furthermore, we observed the behaviour of the animals. At the end of the experimental period, all the rabbits were weighed and were slaughtered according to Italian regulations (Italian Legislative Decree No. 333 of 1/9/1998: implementation of Directive 93/119/EC).

2.2. Analytical determinations of diet

The approximate composition of the diet was determined following AOAC procedures (1990). Three representative samples of the diet were taken at different times during the trial period and were used (mixed together) for the analyses. The diet was analysed to determine dry matter, organic matter, crude protein, crude fibre, ether extract (EE) (using the Soxlet method), ash (by ignition at 550°C), and nitrogen-free extract. The diet was also analysed to determine the neutral detergent fibre without sodium sulphite or α-amylase, and acid detergent fibre (ADF), as described by Van Soest et al. (1991), expressed as exclusive of residual ash, acid detergent lignin, determined by solubilization of cellulose with sulphuric acid, as described by Robertson and Van Soest (1981), and gross energy (GE), by means of an adiabatic bomb calorimeter (IKA C7000, Staufen, Germany).

2.3. Biochemical analyses of rabbit serum

Blood samples were collected in non-heparinized tubes at the beginning and at the end of the experimental period. Blood samples were taken in the morning from the ear vessels of the rabbits. The blood was allowed to clot and was then centrifuged at 3000 rpm for 15 min at room temperature. The serum was then separated and stored at −70°C until required for analysis. The assessment of albumin and triglyceride levels was carried out in the laboratories of the Department of Veterinary Sciences at the University of Turin, Italy. The ILab Aries analyzer (Instrumentation Laboratory, Milan, Italy) was used to analyse serum levels of albumin and triglycerides. ROMs were evaluated on rabbit serum, using the d-ROMs test (Diacon s.r.l., Grosseto, Italy) (Cesarone et al. 1999; Benedetti et al. 2004; Pasquini et al. 2008). This test is based on the principle that oxygen-free radicals are atoms that possess one or more unpaired electrons in one of their outer orbitals; due to their extreme reactivity, these free radicals tend to react with certain organic molecules and generate ROMs. The latter are more stable than their predecessors and can therefore be quantified. In the d-ROMs test, ROMs (primarily hydroperoxides) are generated (by the Fenton reaction), in the presence of iron (released from plasma proteins by means of an acidic buffer), along with some alkoxy radicals and peroxy radicals. These radicals react with an aromatic amine, which is oxidized and converted into a pink derivative that can be quantified photometrically, at a wavelength of 550 nm (Giongo et al. 2011). The intensity of the colour developed is directly proportional to the concentration of ROMs, according to the Beer–Lambert law. The results of the d-ROMs test are expressed in arbitrary units known as ‘Carratelli Units’.  

| Table 1. Ingredients of the diet (%) and chemical composition of the diet. |
|---|---|
| Ingredients | % of inclusion | Chemical composition of the diet |
| Corn | 21.2 | Dry matter, % 90.6 |
| Barley | 18.5 | Organic matter, % 90.1 |
| Dehydrated alfalfa meal | 50.0 | Crude protein, % 17.0 |
| Soybean seed meal | 6.6 | Crude fibre, % 18.8 |
| Oil | 1.0 | Ether extract, % 4.1 |
| Lignosulphite | 1.5 | Crude ash, % 9.9 |
| Vitamin–mineral premix* | 1.2 | Nitrogen-free extract, % 50.2 |
| *Composition of the Vitamin–mineral premix (per kg of diet): Vit. A 200 IU; α-tocopheryl acetate 16 mg; Niacine 72 mg; Vit. B6 16 mg; Choline 0.48 mg; DL-methionine 600 mg; Ca 500 mg; P 920 mg; K 500 mg; Na 1 g; Mg 60 mg; Mn 1.7 mg; Cu 0.6 mg. | | Neutral detergent fibre, % 30.5 |
| Acid detergent fibre, % 21.4 | Acid detergent lignin, % 3.9 | Gross energy, MJ/kg DM 18.2 |
(1 CARR U = 0.08 mg hydrogen peroxide/ dl), according to the following formula:

\[ \text{CARR U} = F(\Delta \text{Abs/minutes}), \]

where \( F \) is a correction factor with an assigned value (approximately 9000 at 37°C, according to the results obtained with the standard), and \( (\Delta \text{Abs/minute}) \) are the mean differences of the absorbance recorded at 1, 2, and 3 min.

The serum antioxidants were evaluated by the anti-ROMs test (Diacron s.r.l., Grosseto, Italy). This method exploits the ability of antioxidants to reduce ferric iron to ferrous iron, giving rise to a red-purple colouration, which can be quantified photometrically at a wavelength of 550 nm, due to a reaction with the \( \alpha \text{-dipyridyl} \) molecule. Colour intensity increases proportionally according to the quantity of iron reduced by the antioxidants present in the sample (Giongo et al. 2011). This test enables discrimination between the concentration of the so-called ‘fast antioxidants’, determined at the start by the instrument, that is, those which are fast-acting, such as Vitamin C or Vitamin E, and the concentration of ‘slow antioxidants’, determined at a later stage by the instrument, such as thiol-SH groups and uric acid. Results are expressed in \( \mu \text{Eq of reduced iron/litre} \) using ascorbic acid as a standard, according to Giongo et al. (2011). To ensure the accuracy and sensitivity of these tests, for the analysis of d-ROMs and anti-ROMs, we used diagnostic kits and an automatic spectrophotometer, standardized and calibrated for the purpose by Diacron s.r.l., Grosseto, Italy.

The use of this automatic analyser allowed optimization of the standardization, by allowing high reproducibility and accuracy of the method and enabling the use of much lower amounts of samples and reagents compared to those required by other conventional methods.

### 2.4. Statistical analyses

In order to establish whether the differences in the means of the measures recorded from the blood samples collected at the beginning and at the end of the experimental phase were significantly different from zero, we carried out a paired difference test. Given the relatively high number (30) of individuals in the samples, a Wilcoxon signed-rank test \((\leq 0.01)\) was only performed in the presence of strong violations of the Shapiro–Wilk normality test \((\leq 0.01)\) on the differences between the groups. In the absence of strong violations of this assumption, a paired \( t \)-test \((\leq 0.01)\) was employed in the statistical analyses of the samples. All analyses were performed using \( R \) statistical analysis software, version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

To evaluate the mean \( \pm \text{SEM} \) of the productive performances, we used SPSS software package (version 11.5.1 for Windows, SPSS Inc., USA).

### 3. Results and discussion

In our study, the rabbits were reared in standard conditions and the diets were formulated in order to satisfy the nutritional requirements of growing rabbits. The ingredients of the diet, the chemical composition, and GE of the diet are reported in Table 1. The zootechnical performances of the rabbits (initial live weight, final live weight, total feed consumption, total weight gain, and feed efficiency values) are given in Table 2. In our study, the productive performances are generally in agreement with other nutritional studies in rabbits (Alikata et al. 1992; Yalçın et al. 2003; Dal Bosco et al. 2004). To verify that the rabbits were in good nutritional status, we also evaluated serum levels of albumin and triglycerides, as they are among the most important blood parameters of animal nutritional status. Albumin levels indicate the level of proteins in the blood, while the levels of triglycerides provide us with information on the overall metabolism of nutrients. The biochemical analyses of rabbit serum are reported in Table 3. The levels of albumin and triglycerides at the beginning and at the end of the study showed means that were not significantly different (paired \( t \)-test with \( p \)-value equal to 0.356 and .886 for albumin and triglycerides, respectively). Furthermore, the values of albumin and triglycerides lay within the standard range for rabbits (Kaneko et al. 2008); this suggests that the rabbits were within physiological nutritional conditions, for both proteins and plasma lipids. The level and quality of proteins and lipids contained in the diets in fact influence plasma albumin and triglycerides; if these parameters are not in the reference range for rabbits, a dietetic imbalance could be present. Indeed, the protein–energy malnutrition status is indicated by hypoalbuminemia. Albumin is synthesized by the liver, and serum albumin is a major component of serum proteins, which sustains osmotic pressure. Serum albumin is the most common index of nutrition status (Sekine et al. 2013). Albumin concentration in serum is also influenced by many factors that are independent of nutritional factors, such as infections, trauma (by an increase in the transcapillary escape rate of albumin), hydration status (by hemodilution), liver function (by an increase in synthesis) and kidney disease (by albumin loss) (Sekine et al. 2013). Furthermore, the influence of diet on plasma triglyceride concentrations is a subject of great interest. Elevated levels of plasma triglycerides may be due to a state of dyslipidemia related to dietary imbalances. The most common cause of elevated triglyceride levels or dyslipidemia is undoubtedly over-nutrition. However, the ingestion of more calories than are needed not only increases adipose tissue, but also promotes triglyceride synthesis by the liver. Different types of fatty acids differently affect serum triglyceride levels; in fact, medium-chain saturated fatty acids have been reported to increase triglyceride levels, while polyunsaturated fatty acids

| Table 2. Productive performance (means ± SEM) of rabbits. |
|----------------------------------------------------------|
| Initial weight, g                                     | 2318 ± 7.3 |
| Final weight, g                                      | 3562 ± 10.9 |
| Total feed consumption, g                           | 7936 ± 37.4 |
| Total weight gain, g                                  | 1240 ± 12.7 |
| Feed/gain ratio, g/g                                  | 6.38 ± 0.04 |
| Mortality, %                                          | 0.0                           |

| Table 3. Biochemical analysis of rabbits’ serum.       |
|--------------------------------------------------------|
| Biochemical parameters                            Initial values (mean ± sem) | Final values (mean ± sem) |
|--------------------------------------------------------|
| Albumin, g/dl                                        3.997 ± 0.029                 | 4.007 ± 0.029                 |
| Triglycerides, mg/dl                                 128.1 ± 1.681                 | 128.033 ± 1.866               |
have been reported to reduce serum triglycerides in some patients with hypertriglyceridemia (Scott & Kurenitz 1990). During clinical examination, all the animals showed a very good state of health: the rabbits had shiny coats, absence of lesions to the eyes and ears; healthy oral cavity mucosa, teeth and paws in good condition, well-formed stools, regular urination, and lively, active behaviour. Therefore, by zootechnical, behavioural, and general clinical external evaluations, we are confident that these rabbits were all in good health during the test and free of overt inflammatory disease. Table 4 reports the descriptive values of ROMs and antioxidants in rabbit serum at the beginning and at the end of the experiment, expressed as mean ± standard error of the mean. In our study, for the levels of d-ROMs the differences in the mean values were not statistically significant (paired t-test with p-value equal to .705), or rather these values did not change during the study period. Although the d-ROMs method has been successfully tested in vitro and applied in some animal species (swine (Meineri et al. 2015), dog (Pasquini et al. 2008)) and in humans (Giongo et al. 2011), the reference values in rabbits are lacking. For example, in research on humans, the reference value of the d-ROMs test, determined on a sample of about 5000 healthy people, is between 250 and 300 CARR U (corresponding to the range between 20.00 and 24.00 mg hydrogen peroxide/dl), regardless of gender and age. However, infants have significantly lower values, while pregnant women display higher values. In humans, values greater than 300 CARR U indicate progressively increasing levels of oxidative stress. The values of d-ROMs that we found in rabbits were comparable to those reported in humans; conversely, in other animal species, the values of d-ROMs were different. Brambilla et al. (2002), for instance, considered the response to oxidative stress as an effective parameter for assessing the welfare of pigs; in their studies of pigs, the values of d-ROMs were around 550–600 CARR U. Furthermore, the normal reference value of the d-ROMs test in dogs ranged between 56.4 and 91.4 CARR U (Pasquini et al. 2008). Antioxidant parameters in healthy rabbits, under standard conditions of nutritional and zootechnical breeding, have not yet been defined in the literature. Regarding the assessment of plasma antioxidants, we found values from the anti-ROMs test (fast antioxidants, in particular vitamin E and vitamin C). For these values of anti-ROMs test as well, the differences between the means were not statistically significant (paired t-test with p-value equal to 0.941 and .636 for fast and slow antioxidants, respectively). In our study, both the values of the d-ROMs test and the anti-ROMs test remained unchanged during the test phase; as the conditions of the experimental period were similar for all animals, we calculated the confidence interval of the values at the end of the experimental period (Table 5). In humans, however, the values of anti-ROMS test were different from those that we found in rabbits. In healthy humans, values greater than 200 μeq/L are considered optimal for the first result (relating to rapid antioxidants), and values greater than 1000 μeq/L are considered optimal for the second result (relating to slow antioxidants). Values lower than these limits are indicative of a condition of oxidative stress, due to the reduced antioxidant defenses. This finding will therefore be useful to investigate the possible causes that led to the lowering of these values.

The separated tests (fast and slow antioxidants) provide clearer indications than measuring the total antioxidant capacity of plasma (Meineri et al. 2015). In our work, we chose to evaluate the parameters of oxidative stress under physiological conditions in the rabbit, using the d-ROMs test and anti-ROMs test, which both have an easy-to-use, rapid, cheap, and reliable methodology.

### 4. Conclusions

Evaluation of the plasma oxidative status for different animal species could become an important parameter of the health conditions of animals (Vassalle 2009). Until now, the available techniques to estimate the value of the oxidative state, for example, evaluating plasma levels of malondialdehyde, and plasma levels of superoxide dismutase or glutathione peroxidase, have presented some technical characteristics that limited their use to specialized research laboratories (Griendling & Fitzgerald 2003; Ridker et al. 2004). Recently, faster, cheaper, reliable, and easier techniques have been developed and proposed, such as d-ROM and anti-ROM tests (Ridker et al. 2004; Vassalle & Andreassi 2004). These tests can also be implemented with automated analysers, allowing the simultaneous execution of a large number of samples in a short time, avoiding handling of samples, and further reducing the sources of variability compared to manual protocols (Vassalle 2009). Evaluation of oxidative stress can be applied to various applications of veterinary interest by highlighting negative events that could affect the welfare of the animal at a farm level. In fact, in the assessment of animal welfare, the increase of ROMs, which is not counterbalanced by an adequate antioxidant response, may reflect a stress exposure due, for example, to poor transport conditions (long journeys, overcrowding, high temperatures, and thirst) (Vassalle & Andreassi 2004; Vassalle 2009). Moreover, the parameters of oxidative stress can act as biomarkers for detecting illicit hormonal treatments, which affect the safety and quality of animal products for human

### Table 4. Values of reactive oxygen metabolites (d-ROMs test, CARR U) and antioxidant activity (fast antioxidants and slow antioxidants, anti-ROMs test, μEq of reduced iron/litre) in rabbit serum at the beginning and at the end of the experimental period.

| Biochemical parameters | Initial values (mean ± sem) | Final values (mean ± sem) |
|------------------------|-----------------------------|---------------------------|
| Reactive oxygen metabolites (d-ROMs test) | 299.9 ± 6.1 | 296.8 ± 6.4 |
| Fast antioxidants (anti-ROMs test) | 85.2 ± 2.1 | 85.4 ± 2.7 |
| Slow antioxidants (anti-ROMs test) | 594.7 ± 4.2 | 597.2 ± 4.7 |

### Table 5. Confidence intervals for the studied parameters.

| Parameters | (CI) lower values | (CI) upper values |
|------------|------------------|------------------|
| Reactive oxygen metabolites (d-ROMs test, CARR U) | 283.991 | 309.543 |
| Fast antioxidants (anti-ROMs test, μEq of reduced iron/litre) | 80.037 | 90.762 |
| Slow antioxidants (anti-ROMs test, μEq of reduced iron/litre) | 590.122 | 604.211 |
| Albumin, g/dl | 3.964 | 4.066 |
| Triglycerides, mg/dl | 124.319 | 131.747 |

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consumption. In conclusion, we have evaluated the parameters of plasma oxidative status in rabbits under physiological conditions; thus providing an introduction to the knowledge of these parameters in rabbits, as has been carried out by other authors in different animal species. Further investigations are required for longer periods, in different rearing conditions and in more complex physiological states (pregnancy and weaning), or in rabbits affected by diseases in which these parameters of oxidative stress could be elevated.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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