In recent years, the use of feed additives has become increasingly popular in animal nutrition, prompting the search for new foods that not only meet or exceed nutritional requirements, but also have beneficial effects on animal health. These substances, called nutraceuticals, may be good candidates for animal feed supplements (15).

Among potential feed supplements, cereals, despite their high content of bioactive compounds, have not yet received the same attention as fruits and vegetables, although an increase in the consumption of cereals and related products is associated with a reduced risk of chronic diseases (19).

Grains are especially rich in polyphenols, a large and heterogeneous group of bioactive phytochemical compounds, which are known for many beneficial effects on humans and animals. For instance, several epidemiological studies have shown that polyphenols have an important role in the prevention of numerous chronic degenerative diseases, such as atherosclerosis and cardiovascular diseases (7, 24).

The protective role of polyphenols against these diseases has been attributed mainly to their antioxidant activity. In fact, in vitro studies have shown that polyphenols act as reducing agents and, together with other compounds introduced with the diet (such as vitamin C, vitamin E and carotenoids), contribute to the antioxidant potential of foods.

The family of polyphenols includes the group of flavonoids, mainly present in fruits and vegetables, which are a category of substances with high bioactivity. This group, in turn, comprises various sub-classes, including flavonols.

Lisosan G is a fermented powder of wheat (Triticum sativum) and is marketed as a nutritional supplement. In the production process, the whole grain is first...
ground to a rough powder. Then, the bran and germ are separated and collected. Water is added to moisten the mix, then selected microbial starting cultures, typically a mix of lacto-bacilli and natural yeast strains, are inoculated to initiate fermentation. The resulting dry powder is Lisosan G, which is widely used in food production for its nutritional composition, rich in vitamins, minerals, and polyunsaturated fatty acids. This product is also reported to show significant antioxidant activity in vitro (13). In vivo, Lisosan G protects against cisplatin-induced toxicity (14), and a recent paper shows that Lisosan G contributes to preventing microcirculatory dysfunction (15). The authors of these studies suggest that the protective effect of Lisosan G could be associated with the attenuation of oxidative stress and the activation of antioxidant enzymes. Lisosan G was also reported to have a hepatoprotective effect in mice affected by hepatic steatosis, by improving both histological properties and triglyceride content. These results suggest a potential therapeutic application of Lisosan G against this emerging hepatic disorder in both humans and animals (11). Recently it was demonstrated that Lisosan G can regulate not only antioxidant systems, but also the anti-inflammatory pathway. Cell treatment with this lysate causes a modulation of phase II enzymes through the activation of the Nrf2 pathway and a decrease in the H2O2-induced translocation of NF-kB into the nucleus (9).

The beneficial properties of Desmodium, a floral plant belonging to the Fabaceae family, and of Picrorhiza, a herbal plant widely used in Ayurvedic medicine, have long been known in herbalism. Desmodium is useful in restoring the correct functionality of liver cells, which, thanks to this plant, regenerate faster. Also known are its antioxidant and anti-inflammatory activities (12).

The literature suggests that Picrorhiza is a potent modulator of the immune system and presents a strong hepatoprotective action (5, 6).

In this study, we determined the concentration of certain antioxidant compounds (polyphenols, flavonoids, and flavonols) in Lisosan G and in a new product called Lisosan Reduction, a mixture containing Lisosan G and extracts of Picrorhiza and Desmodium. In addition, the antioxidant activity of these products was assessed by the ORAC assay and by a new test based on the use of erythrocytes, the CAA-RBC. Both human and canine erythrocytes were also used to evaluate the anti-hemolytic activity of the compounds under study.

**Material and methods**

**Chemicals.** All chemicals and reagents were of analytical grade. Ethanol, chloridric acid, sodium idrosside, Folin Ciocalteu reagent, gallic acid, 6-hydroxy-2,5,7,8-tetramethyl-ylchromane-2-carboxylic acid (Trolox), catechin, quercetin and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma Aldrich Inc. (St. Louis MO), whereas 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Polysciences, Inc. (Warrington PA). Lisosan G and Lisosan Reduction were supplied by Agrisan Company (Larzian, PT, Italy).

**Powder extraction.** Lisosan G (LisG) and Lisosan Reduction (LisG Reduction) were extracted in distilled water, sonicated (10 on/10 off) for 3 cycles, and centrifuged for 10 minutes at 2300 × g at 4°C. Then, the supernatant were collected and kept at 4°C in the dark.

**Blood and erythrocytes.** Human blood from healthy volunteers was kindly provided by the Fondazione G. Monasterio (Pisa, Italy). Canine blood was kindly provided by the Department of Clinical Veterinary, San Piero a Grado (Pisa, Italy). Blood samples were collected into EDTA-treated tubes and centrifuged for 10 min at 2300 × g at 4°C. Plasma and buffy coat were discarded, and erythrocytes were washed twice with phosphate-buffered saline pH 7.4.

**Biochemical assays.** Total phenolic content was determined according to the colorimetric method described by Singleton et al. (21). The concentration of polyphenols was expressed as mg of gallic acid equivalents (GAE)/g of dry weight (dw). Total flavonoid content was measured by the aluminum chloride method described by Kim et al. (8), and flavonoid concentration was expressed as mg of catechin equivalent (CE)/g dw. Total flavonol content was determined by the spectrophotometric method described by Romani et al. (20), and flavonol concentration was expressed as mg of quercetin equivalent (QE)/g dw.

The total antioxidant capacity of Lisosan G and Lisosan Reduction was determined by the oxygen radical absorbance capacity (ORAC) assay, according to Ninfali et al. (18) with some modifications. Peroxyl radicals were generated by 2,2-azobis (2-amidinopropane) dihydrochloride, and fluorescein was used as probe. The fluorescence decay was read at 485 nm excitation and 514 nm emission with a VictorTM X3 Multilabel Plate Reader (Waltham, MA). In each experiment Trolox was used as standard and the ORAC values were expressed as micromoles of Trolox equivalents (TE)/100 g of sample dry weight (dw).

The antioxidant activity was also evaluated in an ex vivo system by a modified assay of cellular antioxidant activity in red blood cells (CAA-RBC) (1). Briefly, erythrocytes were diluted 1:100 in PBS pH 7.4 and incubated for 1 hour at 37°C with 15 µM 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) and the natural extracts. Later, they were washed twice in PBS to remove the excess of antioxidants, resuspended in cold PBS, and transferred in a 96-well microplate. Finally, 1.2 mM of 2'-Azobis,2-amidinopropenediylhydrochloride (AAPH) was added to the cell suspension, and fluorescence was read at 485 nm excitation and 535 nm emission with a VictorTM X3 Multilabel Plate Reader (Waltham, MA). Quercetin was used as standard. Each value was expressed in CAA units, according to Wolfe and Liu (23), as follows: CAA unit = 100 - (100 × [SA] / [CA]) × 100, where [SA] is the integrated area of the sample curve and [CA] is the integrated area of the control curve. The hemolysis was measured according to the method described by Miskacka et al. (17), using AAPH, a generator of peroxidic radicals, to induce red blood cell lysis. Briefly, an erythrocyte sus-
pension at 5% was pre-incubated with Trolox (standard) or with the natural extracts at 37°C for 1 hour, then exposed to 50 mM AAPH at 37°C for 4 hours. Finally, the samples were centrifuged for 5 minutes at 1000 × g, diluted 10 times with PBS, and the absorbance was read at 540 nm. Control (CNT) and blank refers to erythrocytes not pre-treated, but incubated with AAPH (CNT) or PBS (blank). The values reported are the percentage of hemolysis compared to the control.

Statistical analysis. Statistical analyses were performed by GraphPad Prism version 4.00 (GraphPad software, San Diego, CA). Data are expressed as mean ± standard deviation of three replications. Significant differences were evaluated by one-way ANOVA with post hoc Dunnett’s test and an unpaired t-test. A p-value lower than 0.05 was considered statistically significant.

Results and discussion

The concentrations of polyphenols, flavonoids, and flavonols, as well as the ORAC units, in Lisosan Reduction and Lisosan G are shown in Table 1.

The phytochemical compounds are present in large quantities in both products, but the quantities of polyphenols and flavonoids are double in Lisosan Reduction compared to Lisosan G (p < 0.0001), whereas the content of flavonols is less different (p < 0.005). Lisosan Reduction also showed a higher antioxidant activity measured by the ORAC assay compared to Lisosan G (p < 0.0001). The higher values of phytochemical content and antioxidant activity in Lisosan Reduction are probably due to the presence of the extracts of Desmodium and Picrorhiza. This result shows a greater effectiveness of the mixture compared to the single component.

The antioxidant activity of Lisosan G and Lisosan Reduction was also evaluated in an ex vivo system by the modified assay CAA-RBC. Canine and human erythrocytes were isolated from peripheral blood and treated with the two compounds. The results of this test showed a more pronounced antioxidant effect of Lisosan Reduction compared to Lisosan G in both human and canine samples, with greater effectiveness in human erythrocytes. Figure 1 shows the results, expressed in CAA units, for CAA-RBC in human erythrocytes treated with Lisosan Reduction and Lisosan G, which are respectively 44.5 ± 4.1 and 39.2 ± 6 CAA units, compared to 90.5 ± 3.8 of the standard. In canine erythrocytes (Fig. 2), the extracts showed a weak antioxidant effect, with 5.7 ± 0.55 CAA units after treatment with Lisosan G, and 11.1 ± 0.45 CAA units after treatment with Lisosan Reduction, compared to 86.6 ± 0.15 units of the standard.

The anti-hemolytic effects of Lisosan G and Lisosan Reduction were evaluated in human and canine erythrocytes exposed to AAPH, a generator of peroxidic radicals. The hemolysis assay showed a more marked effect of the mixture compared to Lisosan G in all the samples – even in this case, the efficacy of the compounds was higher in human erythrocytes. The pre-treatment with Lisosan G and Lisosan Reduction caused a significant inhibition of hemolysis in all the samples, compared to the control treated only with AAPH (Fig. 3

<table>
<thead>
<tr>
<th>Polyphenols (mg GAE/g dw)</th>
<th>Flavonoids (mg CE/g dw)</th>
<th>Flavonols (mg QE/g dw)</th>
<th>ORAC (µmol TE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LisG</td>
<td>3.73 ± 0.1</td>
<td>2.43 ± 0.17</td>
<td>2.65 ± 0.13</td>
</tr>
<tr>
<td>LisG Reduction</td>
<td>8.39 ± 0.22****</td>
<td>9.3 ± 0.06****</td>
<td>3.16 ± 0.22*</td>
</tr>
</tbody>
</table>

Explanation: **** = p < 0.0001; * = p < 0.05 vs LisG

Fig. 1. Cellular antioxidant activity in human red blood cells (CAA-RBC). Mean of 3 independent experiments ± SD

Fig. 2. Cellular antioxidant activity in canine red blood cells (CAA-RBC). Mean of 3 independent experiments ± SD
and 4). As shown in Figure 3, in human erythrocytes, Lisosan Reduction exerts the maximum anti-hemolytic effect, comparable to that of the standard (Trolox), and is significantly more effective than Lisosan G (p < 0.001). In canine samples (Fig. 4), the mixture and the Lisosan G showed the same anti-hemolytic action, comparable with that observed after treatment with the standard. A blank was used to evaluate the spontaneous haemolysis of the sample. These results suggest that both human and canine erythrocytes are good models for testing the antioxidant capacity of individual compounds and mixtures.

The different results observed in the erythrocytes of dogs and humans, may be due to differences in the structure and strength of the cell membrane. In this regard, it has been reported that the membranes of red blood cells of different species show differences in the percentage of fatty acids, which are related to the diet and enzymes synthesized, and may change with the age of the subjects (3).

In recent years, research in human and veterinary field has focused on food and feed safety, as well as on welfare. New feeding strategies based on feed additives have been implemented in order to improve not only animal health but also the quality of animal products (16). Many studies on rabbits showed the beneficial influence of dietary supplementation with plant extracts on biochemical parameters of blood (22), immune response (2), as well as meat quality and production (10). Our findings show that Lisosan G and, in particular, Lisosan Reduction are rich in several bioactive compounds, such as polyphenols and especially flavonoids and flavonols. In addition, both the lysate and the mixture show high antioxidant activity, which was evaluated in vitro by the ORAC assay and ex-vivo by the CAA-RBC assay in human and canine erythrocytes.

In view of these results, Lisosan G and Lisosan Reduction can be recommended as feedstuff additives that improve animal health by increasing endogenous antioxidant defenses. It would also be interesting to evaluate the effect of such supplementation on the quality of meat and other animal derivatives.

Many diseases, including those that afflict the canine species, are strongly influenced by the oxidative status of the organism, both in their development and progression. The enhancement of antioxidant defenses through dietary strategies may be of great benefit to the health of man and his best friend.

References


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