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BRIEF REPORT

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The synergistic effect of prebiotics, probiotics and antioxidants on dogs with chronic kidney disease

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ABSTRACT

The use of probiotics, prebiotics and antioxidants could be found beneficial for dogs with chronic kidney disease (CKD). The aim of our case-control study is to evaluate the synergistic effect of a diet integrated with a supplement containing probiotics (*Lactobacillus acidophilus*), prebiotics (fructoligosaccharides) and antioxidants (*Olea Europaea* extract) on the nutritional status and on serum and urinary parameters of dogs with CKD. A total of 30 dogs classified with IRIS CKD stage 3 were enrolled and randomly assigned to a control (CG, $n = 15$) and a treated (TG $n = 15$) group. The trial consisted in a 7-days adaptation period, followed by 90 days where animals in the TR group received the supplement, while in the CG group the placebo. No significant changes in body weight and body condition score were recorded. We recorded a significant improvement of the protein plasmatic level and a decrease in blood phosphorus, systolic pressure, BUN, proteinuria and urine protein-to-creatinine ratio throughout the trial in the TG compared to the CG group. Furthermore, the parameters related to inflammation and oxidative stress (C-reactive protein and Reactive Oxygen Metabolite- derived compound, respectively) were lower in the TG than in the CG group throughout the study. Our results showed that the supplement allows to maintain the correct nutritional status and to improve blood and kidney parameters in dogs with advance stage of CKD. This supplement could be considered as a new nutritional approach for treating this condition.

HIGHLIGHTS

- Diet supplemented with prebiotics, probiotics and antioxidants is safe for dogs with CKD.
- The synergic effect of prebiotics, probiotics and antioxidants included in the supplement under study shows the maintenance of a good nutritional status and the improvement of blood and urinary parameters in dogs with CKD.

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Supplement; prebiotics; probiotics; antioxidants; dog; CKD

Introduction

Chronic kidney disease (CKD) is a chronic degenerative disease frequently occurring in dogs (Bartges 2012). Diagnosis of CKD is made through the anamnesis (age, breed, sex, diet, and pharmacological therapy), clinical signs (polyuria, polydipsia, weight loss, decreased appetite, ulcers, dehydration, hypertension, lethargy) and laboratory findings (blood cytometric examination, serum and urinary biochemical profile and renal morphology) (Relford et al. 2016). Data obtained from animals must be then interpreted using the International Renal Interest Society (De Loor et al. 2013). Scientific studies have been highlighted an abnormal composition of the microbiota in CKD patients (Vanholder and Glorieux 2015). In fact, it has been shown that also animals affected by the disease have an increase in bacterial species subject to proteolytic fermentation, such as *Clostridium* and *Bacteroides* and a decrease in *Lactobacilli* and *Bifidobacteria* (Nallu et al. 2017). Moreover, occurring inflammation and oxidative stress have a significant detrimental effect on renal function (Irazabal and Torres 2020). The use of ingredients in the diet that have an effect directly on the renal function or through a secondary product

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mediated by the microbiome can be beneficial for patients with renal disease. Therefore, in order to maintain a correct balance of the gastrointestinal microbiota, the use of probiotics, prebiotics and antioxidants has increased in humans as well as in animals (Rose et al. 2017; Fusi et al. 2019; Bruni et al. 2020; Saettone et al. 2020). *Lactobacillus* and *Bifidobacterium* are the most widely used probiotics in veterinary medicine. In fact, it has been shown that *Lactobacillus acidophilus* stabilises the microbiota and strengthens the gastrointestinal barrier in dogs and cats by limiting the adhesion of pathogens. Prebiotic fibres (such as fructooligosaccharides) are a source of nutrition for probiotics and contribute to the beneficial bacterial proliferation of the colon by reducing intestinal inflammation and increasing the absorption of nutrients (Hall et al. 2016). In addition, scientific studies indicate that a diet supplemented with specific antioxidants (e.g. *European Olea,* Vitamin E, carotenoids, polyphenols, flavonoids) is important for limiting renal oxidative stress and the progression of CKD (Halfen et al. 2020).

The purpose of our study was to evaluate the synergistic effect of probiotics (*L. acidophilus*), prebiotics (fructoligosaccharides) and antioxidants (*Olea Europaea* extract), administered as a sole food supplement on the nutritional status and on serum and urinary parameters in dogs with CKD.

Materials and methods

Animals and study design

A group of 30 medium-sized adult owned dogs (age> 1 year) was included in the study. All dogs were diagnosed with IRIS CKD stage 3, following a four-stage scale of disease progression according to the International Renal Interest Society (IRIS) guidelines. These dogs were enrolled by the Napolivet Veterinary Clinic in Naples (Italy) and randomly assigned to two groups. Fifteen dogs ($n = 15$, 5 males, 10 females), were supplemented with the complementary feed under study (TG group) and the other ($n = 15$, 5 males and 10 females) with a placebo (CG group). Dogs with concomitant diseases (acute kidney injury, pre-renal or post-renal azotaemia, genitourinary tract inflammation or infection, urinary tract obstruction, chronic heart disease, neoplasia, hypothyroidism, diabetes) have been excluded from the study.

Supplement and diet

During the entire duration of the trial (90 days), and the 7 days of adaptation, both CG and TG dogs received a complete dry commercial diet (Royal Canin – Renal canine) twice daily. The quantity of food offered was calculated on the basis of the estimated needs according to the NRC guidelines (Council NR 2006). The same diet was maintained throughout the entire study period in both groups. The TG group received the supplement (*Renal N*, Candioli s.r.l., Italy) for 90 days. It contains *L. acidophilus* D2/CSL (30 mg/day) (Bruni et al. 2020), fructooligosaccharides (400 mg/day) and *Olea Europaea* extract (40 mg/day) (Supplementary Appendix 1) . The CG received the placebo for the same period of time (Supplementary Appendix 1).

Analysis

Body weight (BW) and Body Condition Score (BCS) were recorded at the baseline T0 (day 0), then at T30 (day 30), T60 (day 60) and T90 (day 90). The BW (kg) was measured always using the same scale (pet scale, four sensors, maximum 100 kg, d¼100 g; Momert, Duna_ujv_aros, Hungary) and at the same time of the day. BCS is an effective assessment of body fat and it is evaluated through the visual examination and palpation of the animal. Scores from 1 to 9 were assigned by the same trained veterinarian who performed the BW measurement, a score of 4 or 5 represents the ideal (FEDIAF 2020). Measurements were made simultaneously with the BW, thus avoiding excessive handling of animals. At the beginning and at the end of each visit, the blood pressure (BP) was measured by an indirect Doppler method using the radial pulse, with the dog in left lateral decubitus. During the examination, five measurements were taken and the average value recorded (WGng Vet USA Digital Blood Pressure NIBP). Blood samples were obtained by puncture of the jugular vein during each of the four scheduled veterinary cheques. The haematochemical and biochemical parameters were measured by using an haematology analyser (scil Vet abc Plus) according to the instructions of the manufacturer. Blood cell subgroups were quantified using flow cytometry. The hematochemical parameters measured were: haematocrit (HCT), haemoglobin (HG), red blood cells (RBC), white blood cells (WBC), neutrophil (N), eosinophil (EO), lymphocytes (LYM), basophiles (BA), platelets (PLTS). The biochemical parameters measured were: blood urea nitrogen (BUN), creatinine (CREA), phosphorus (P), total protein (TP), albumin (ALB), albumin/

globulin (A/G), glucose (GLU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin (BIL), cholesterol (CHOL), and the symmetric dimethylarginine (SDMA).

Venous blood gas analysis was performed immediately after blood collection with a standard analytical device in order to evaluate the ionised calcium (iCA) (An et al. 2013).

An ELISA test for dogs (Tridelta Development Limited) was performed for the analysis of C-reactive protein (CRP) (Kjelgaard-Hansen et al. 2003), while the Reactive Oxygen Metabolite- derived compound (d-ROMs) analysis was carried out using a Free-Diacron international SRL automatic analyser (Alberti et al. 2000).

Urine samples were obtained by cystocentesis with a 5 ml syringe. A sterile collection tube was inserted and analysed. Then, the samples were centrifuged (2 min at 1500 × g), the supernatant removed and stored at + 4 °C, so the urine sediment was obtained. The examination of the urinary sediments and the specific weight was performed by refractometry (Aution Micro) .

The urinary protein concentration (UP) (mg/dl) (pyrogallol red method) and the creatinine concentration (mg/dl) (Jaffè method) were evaluated in the urine within 12 hours of collection. The urine protein-to-creatinine ratio (UPC) was then calculated.

Statistical analysis

The variability of each parameter recorded over time was analysed using PROC MIXED of SAS (SAS 9.4 2013).

The statistical model was built as the following:

$$y_{ijn} = \mu + G_i + A_j + GA_{ij} + e_{ijn}$$

where:

- yijn = dependent variable (BP, BW, BCS, HCT, HB, RBC, WBC, PLTS,BUN, CREA, P, TP, GLU, AST, ALP, BIL, iCA, UP, UPC, CRP, d-ROMs, SDMA.
- μ = overall mean;
- G_i = fixed effect of the i^{th} group (j = 1, 2: control, treatment);
- T_j + = fixed effect of the j^{th} recording time (j = 1, 7: 0, 30, 60, 90 days)
- GT_{ij} = fixed effect of the interaction between the ith group and jth time
- e_{ijn} = error.

Time was treated as a repeated measure and dog within groups as repeated subject. The auto-regressive covariance structure was used. Least square (LS) means were separated by pair-wise t-test using PDIFF option in SAS® with the Tukey adjustment for multiple comparisons. Significance was set at 5% level.

Results and discussion

In the present study, we combined three types of ingredients: prebiotics (Fructoligosaccharides), probiotics (*L. acidophilus* D2/CSL) and antioxidants (Olea *Europea* extract) in a single supplement to evaluate their synergic effect on the nutritional status and on serological and urinary parameters in dogs with advanced CKD.

Several researchers have studied the effectiveness of the dietary use of probiotics (living microorganisms), prebiotics (non-digestible food ingredients) and antioxidants on the balance and well-being of the microbiota in subjects with kidney diseases (Swanson et al. 2002; Fernandez-Prado et al. 2017; Koh and Rowling 2017). For example, *L. acidophilus* is a probiotic capable of modifying the intestinal microbiota and influencing the enteric inflammatory condition ((Mutsaers et al. 2013).

All dogs were in a stable condition during the whole study period and no gastro-enteric symptoms (i.e. vomiting, diarrhea) were recorded confirming the safety and tolerability of the product. No changes in the feed administration/consumption have been registered. No significant difference in BW and BCS in the two groups was found for the entire study period (Supplementary Appendix 2), thus indicating good maintenance of the nutritional conditions (value close to 5 on a BCS 1–9 scale).

We have observed an improvement in the blood and urinary parameters normally used by veterinarians to monitor the progress of the kidney disease (Wilkinson and McEwan 1991). Regarding blood count values and liver parameters (Supplementary Appendix 2), the two groups of dogs did not statistically differ, thus indicating a low probability of infection during the study and no damage of the liver function due to the supplementation further underlying the safety of the product.

The effects of adding the studied supplement on blood pressure, blood parameters and urinary biochemical profile are shown in Supplementary Appendix 2. Blood pressure at T30, T60 and T90 was significantly lower in the TG group than in the CG group ($p < .01$). We noticed that both groups were slightly hypertensive (<150 mmHg) at the time of the enrolment, but the TG group shows significant lower

blood pressure values from T30 on. Systemic hypertension is common in canine and feline CKD; reactive oxygen species (ROS) are believed to contribute to the increase in systemic blood pressure in CKD, therefore the antioxidants contained in the supplement, *Olea Europaea* extract, are able to reduce hypertension (Susalit et al. 2011).

Serum creatinine did not differ between groups at all times considered. This could be the result of a too short supplement administration time and then it would be difficult to see a significant improvement of this parameter.

SDMA remained stable and there was no difference between groups at different time intervals. It is used as a biomarker of the decline in kidney function. It has been shown that circulating levels of SDMA increase in dogs and cats with renal insufficiency (Hall et al. 2015; El-Khoury et al. 2016). In our study, SDMA tends to decrease in the TG group but the differences do not reach significance even at the end of the study ($p = .07$), indicating that the renal damage is present but not in an advanced stage yet (Hall et al. 2016).

On the other hand, BUN was significantly lower in the TG group than in the CG group at T30, T60 and T90 ($p < .01$). BUN value is an important marker of renal failure as it represents the concentration of non-protein nitrogenous compounds (i.e. urea) in the blood. High level of azotaemia in the blood, as described by Krawiec (1996), can cause a range of clinical symptoms in dogs such as loss of appetite, nausea, vomiting, anorexia and gastrointestinal disturbances (Krawiec 1996). Interestingly, in our study the BUN values decreased in the TG group compared to the CG group. This result confirms the efficacy of the supplement, especially the prebiotic and probiotic components, in reducing the intestinal absorption of nitrogen waste derived from protein catabolism. Regarding the urinalysis (Supplementary Appendix 2), we registered several significant changes at T90 when the UP was significantly lower in the TG group than in the CG group ($p < .01$). The UPC was significantly lower in the TG group compared to the CG group at T30, T60 and T90 ($p < .01$). Similarly, the UPC was significantly lower in the TG group from T30 on. These results show an improvement in the kidney function of the subjects in the TR group.

The hepatic parameters (AST, ALP, BIL) are shown in Supplementary Appendix 2 and no significant differences ($p > .05$) between the two groups in the time period considered were found.

Regarding CRP and d-ROMs (Supplementary Appendix 2), at the end of the study (T90) the CRP was significantly lower ($p < .01$) in the TG group than in the CG group. Circulating bacterial DNA, derived from the gut due to increased intestinal permeability was detected in patients with CKD and correlated with an increase in plasma CRP (Stenvinkel 2005). Our study shows that dogs that received supplementation (TG) have a significant reduction ($p < .01$) in the oxidative stress parameter (d-ROMs) value compared to the CG group from T30.

The data confirms some previous observations made by Bulger and colleagues (Bulger et al. 1979) according to which probiotics, prebiotics and antioxidants are able to reduce inflammation and to influence the renal function through the mediation of the microbiome. In dogs, dietary antioxidant supplementation has been found associated with improved renal function (Brown 2008). Recently, the *Olea Europaea* extract has been studied because of its antioxidant benefits mainly due to the high number of phenolic compounds present in the olive tree. Olive leaves contain phenolic compounds (oleuropein, hydroxytyrosol, verbascoside, apyx-nin-7-glucoside and luteolin-7-glucoside) with antioxidant, antihypertensive, hypoglycaemic and hypocholesterolemic effects (Halfen et al. 2020). In our study, these antioxidants may have reduced the blood values of d-ROMs related to oxidative stress.

Regarding the haematological parameters (Supplementary Appendix 2), we found higher values for serum proteins (TP) at T60 and T90 in the TG group compared to the CG group; this result is very relevant because animals with CKD in poor nutritional condition have a shorter survival (Rudinsky et al. 2018). Thus, a good nutritional management is very important to ensure a long-term prognosis for sick animals and to avoid the occurrence of cachexia or sarcopenia (Freeman 2012).

P was higher in the TG group than in CT group at T60 and T90 ($p < .01$). We found a significant reduction in blood phosphorus in the TG group compared to the CG group at T60 and T90, this is an interesting result but of difficult interpretation based on the fact that no effect on P was expected given the type of supplement administered. As the kidney play a crucial role in maintaining P balance, the significant reduction in blood P in the TG group may be due to an improvement of the kidney condition. In dogs with renal insufficiency the P is not eliminated via the urine and its level in the blood is higher than in healthy dogs.

GLU and iCA remained stable in the two groups through the study period, therefore the subjects under

study do not present any disturbance in sugar and calcium metabolism.

Conclusion

Our study shows that the use of probiotics, prebiotics and antioxidants allows to maintain a good nutritional status and to improve blood and urinary parameters in dogs with advanced CKD. The administration of our supplement could be considered a new nutritional approach for the management of canine CKD. Further studies conducted for a longer period of time and on a larger group of dogs would be beneficial to strength our findings. Further laboratory tests, such as microbiota evaluation, faecal nitrogen dosage or other metabolites of bacterial metabolism would be useful to better understand the effect of the supplement on intestinal balance and consequently its influence on the kidney disease.

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Ethical approval

The experimental study was approved by the Ethical Committee of the Department of Veterinary Science-Unito (protocol n. 4/2021) according to the guidelines of the Based on national, EU and international legislation on the protection of animal welfare and good veterinary practices in the breeding, maintenance and treatment of animals used in research activities in accordance with Legislative Decree 6.4.2006, n. 193 Implementation of Directive 2004/28/EC containing the Community code of veterinary medicines, art. 69.

Disclosure statement

One of the authors is an employee of the Candioli Pharma S.r.l. One of the authors is a scientific consultant for the Candioli Pharma S.r.l. Candioli Pharma S.r.l. may be affected by the research reported.

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