Adverse food reactions in dogs due to antibiotic residues in pet food: a preliminary study

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Summary
In the last decades, adverse food reactions have increased considerably in dogs and cats. In this study we report on the possible onset of food intolerances symptoms, including otitis, diarrhoea, generalised anxiety, and dermatitis in a cohort of 8 dogs consuming commercial diets. All dogs received an organic chicken-based diet for 15 days. We performed analysis of blood biochemical parameters, kibble composition, and oxytetracycline (OTC) serum concentration before and after 15 days of organic chicken-based diet supplementation. We hypothesised that a chronic intake of contaminated food enhanced by the presence of nanoparticle aggregates might be at the base of the onset of pharmacologic or idiopathic food intolerances. At the end of the evaluation period, an overall significant reduction of otitis, diarrhoea, generalised anxiety, and dermatitis was observed. Biochemical analyses indicate a significant increase in the alkaline phosphatase, from 41 to 52.5 U/L, after 15 days (**$p < 0.01$), while a significant decrease in Gamma-glutamyl transferase and urea, from 9.37 to 6.25 U/L and from 32.13 ± 8.72 to 22.13 ± 7.8 mg/dL, respectively, was observed (*$p < 0.05$). A significant decrease, from 0.22 to 0.02 µg/mL, in mean OTC serum concentration was also observed (**$p < 0.01$). Composition analysis revealed the presence of OTC, calcium, aluminium, silicon, and phosphorous nanoparticle aggregates. Further research on a wider sample size would help to confirm the hypothesis proposed here.

Reazioni avverse al cibo in cani dovute alla presenza di residui antibiotici nel pet food: uno studio preliminare

Riassunto
Negli ultimi decenni sono aumentate considerevolmente le reazioni avverse al cibo nei cani e nei gatti. In questo studio si riportano i sintomi da intolleranza alimentare, tra cui otite, diarrea, ansia generalizzata e dermatite, in 8 cani che consumavano regolarmente diete commerciali a base di crocchette. Tutti i cani hanno ricevuto dieta commerciale a base di pollo biologico per 15 giorni misurando, all'inizio e alla fine della valutazione, i parametri emato-biochimici, la composizione delle crocchette che avevano assunto precedentemente e la concentrazione sierica di ossitetraciclina (OTC). Si è ipotizzato che l'assunzione cronica di cibo contaminato, accresciuta dalla presenza di aggregati di nanoparticelle, potrebbe essere alla base dell'insorgenza d'intolleranze alimentari farmacologiche o idiopatiche. Al termine del periodo di valutazione la sintomatologia clinica è regredita significativamente. Le analisi biochimiche hanno evidenziato un aumento significativo della fosfatasi alcalina dopo 15 giorni da 41 a 52,5 U/L (**$p < 0.01$), mentre è stata osservata una diminuzione significativa della gamma-glutamil transferasi e dell'urea, rispettivamente da 9,37 a 6,25 U/L e da $32,13 \pm 8,72$ a $22,13 \pm 7,8$ mg/dL (*$p < 0.05$). L'analisi della composizione delle

crocchette ha rivelato la presenza di OTC, calcio, alluminio, silicio, fosforo e aggregati di nanoparticelle. Ulteriori ricerche su un campione più ampio contribuirebbero a confermare l'ipotesi qui proposta.

Introduction

Adverse food reactions (AFR) are clinical responses following the ingestion of a dietary component (Cianferoni and Spergel, 2009). They can cause cutaneous and/or gastrointestinal signs in dogs and cats (Gaschen and Merchant 2011).

These reactions can be divided into toxic (food poisoning) and non-toxic, which are further subdivided into non-immune mediated (food intolerances) and immune mediated [Immunoglobulin E (IgE)-mediated, non-IgE-mediated food allergies, and celiac disease] (Ortolani and Pastorello 2006).

While toxic food reactions can occur because of the presence of a toxin in a particular foodstuff, or are a consequence of food processing, IgE-mediated food allergies represent most common adverse food reactions (Ortolani and Pastorello 2006). Conversely, non-IgE-mediated food allergies are immune reactions, which depend on antibodies (different from IgE), food and food antibodies immune complexes and even cell-mediated immunity. Food intolerances can result from enzymatic defects (β-galactosidase deficiency) (Mattar *et al.* 2012), or can arise after the intake of vasoactive amines (dopamine, histamine, norepinephrine, phenylethylamine, serotonin, and tyramine) that are present in foods (Metcalfe *et al.* 2011). Vasoactive amine histamine, degraded by microorganisms from histidine, is frequently present in high quantities in cheese, alcoholic beverages, and fermented foods (e.g. spoiled fish) (Ortolani and Pastorello 2006). Symptoms of histaminic intoxication are erythema, vasodilation, tachycardia, hypertension, migraine, vomiting, and diarrhoea, which generally resolve within a few hours (Maire *et al.* 1992). Other non-classifiable reactions are regarded as undefined (idiopathic) food intolerances and, in predisposed individuals, can be due to the presence of food additives (sulphites, nitrites, nitrates, monosodium glutamate, and some colourings and even antibiotics) (Ortolani and Pastorello 2006, Di Cerbo *et al.* 2014a).

The aim of this preliminary study is to provide new insights into idiopathic food intolerances in dogs with a particular focus on the role of antibiotics (e.g. oxytetracycline, OTC) or their metabolites as possible triggering factors. We previously demonstrated the pro-apoptotic and pro-inflammatory role of OTC bound to chicken bone, which is widely used in pet food production (Odore *et al.* 2015, Di Cerbo *et al.* 2016b). However in that study we didn't provide direct evidence of the clinical impact of OTC-contaminated pet food on the animal's health. In this study we also report the usefulness of an organic chicken-based diet supplementation in relieving AFR-related symptoms in a small cohort of dogs within 15 days.

Materials and methods

In this study we enrolled 8 client-owned indoor-housed neutered dogs with otitis, diarrhea, generalized anxiety, and dermatitis that were aroused after food ingestion. The dogs were of different breeds, sex (2 males and 6 females) and age (mean age ± standard error of mean; 3.62 ± 0.74 years) with otitis, diarrhoea, generalised anxiety, and dermatitis. We also performed complete profiles of blood biochemical parameters and kibbles (which belonged to their diets) composition.

Blood biochemical parameter evaluation

We analysed blood biochemical parameters (calcium, cholesterol, triglycerides, creatine phosphokinase, total bilirubin, albumin, alkaline phosphatase, creatinine, gamma-glutamyl transferase, glucose, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, phosphorous, total protein, and urea) using a chemistry analyser (Dimension RXL, Siemens S.p.A, Milan, Italy). Serum protein electrophoresis was examined with GENIO S device (Interlab s.r.l., Rome, Italy).

Enzyme-linked immunosorbent assay (ELISA) for serum OTC evaluation

Based on previous observations (Di Cerbo *et al.* 2014a, Mazzeranghi *et al.* 2017), 4 mL of blood was withdrawn from each dog at first visit and after 15 days of their new diet supplementation. Blood samples were analysed using an OTC-specific ELISA kit for pets (Cat. # DE – 100430, Genemed Synthesis, Inc., San Antonio, USA). Two healthy dogs were chosen as the control group. Their serum OTC concentration was far below the detection threshold.

Evaluating OTC presence in kibbles

In accordance with relevant literature (Moretti *et al.* 2016), we analysed the kibble samples with a UPLC Ultimate 3000 Dionex Thermo Fisher system coupled to an LCQ-Orbtrap (Thermo Scientific, San Jose, CA, USA) mass spectrometer. High Performance Liquid Chromatography (HPLC) analyses were run on a Poroshell 120 EC-C18 column (2.7 µm _ 100 mm, 3 mm) at 30 °C. The mobile phases were constituted with solvent A: water containing 0.1% formic acid (v/v); and solvent B: methanol. The gradient programme was 0.0 min 5% B, 0.0-1.0 min 5% B, 1.1-20.0 min 95% B, 20.1-25.0 min 95% B, 25.1-26.0 min 5% B, 26.1-30.00 min 5% B, 11.0-20.0 min 5% B; running at a flow rate of 0.25 mL min^{-1}. The injection volume was set at 5 µL. Analytes were detected with heated electrospray ionization (HESI-II) in positive mode. The optimised heated electrospray ionization (HESI-II) temperature was set at 320 °C, and the capillary temperature at 300 °C. The electrospray voltage was set at 3.00 kV. Sheath and auxiliary gases were 35 and 15 arbitrary units, respectively. Instrument calibration was performed infusing the LTQ Velos ESI positive calibration solution. The acquisition was achieved in full scan/dd-MS2. All quantitative data were calculated using the full scan data. Mass range in full scan was within m/z 150-1200. The data were acquired at a resolution of 70,000 full widths at half maximum (FWHM) (m/z 200). The mass spectrometer was controlled by Xcalibur 3.0 software (Thermo Fisher Scientific, San Jose, CA, USA).

Kibble samples (1 g) were extracted in a polypropylene tube, with 8 ml of ethylenediaminetetraacetic acid (EDTA) McIlvain buffer pH 4, 1 ml CH_3OH, 1 ml CH_3CN, and 10 ml H_2O. The tubes were mechanically mixed for 10 minutes, sonicated for 15 minutes, and then centrifuged for 10 minutes at 3,500 rpm. Clean up was performed on 6 ml SPE cartridge OASIS PRIME (Waters) activated with 3 ml CH_3OH and 3 ml H_2O. The supernatant (5 ml) was loaded followed by a 3 ml water rinse; OTC was eluted from the cartridge with 3 ml of CH_3OH and then concentrated to dryness in a heating block at 45 °C under a stream of nitrogen. Dry extract was reconstituted with 14% CH_3OH in water before the UPLC-Q-Orbitrap analysis.

Cell culture

We purchased K562 myelogenous leukemia cell line from American Type Culture Collection (ATCC) (LGC Standards srl, Milan, Italy). We grew K562 cells in RPMI supplemented with 10% fetal bovine serum (FBS) 100 µg/mL streptomycin, 100 U/mL penicillin, 2 mM glutamine (Euroclone Spa, Milan, Italy). The cells were cultured in a humidified incubator, which provided an atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 °C.

Determination of cell viability

Serial dilutions of powder OTC (150, 75, 35, and 15 µg/ml) or liquid OTC (350, 175, 87.5, and 40 µg/ml) were prepared in RPMI culture medium. Cell viability was assessed after 24-48-72 hours of continuous exposure with the above concentrations. A Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) was used to measure the cytotoxicity on K562 cell line. Briefly, the K562 cells were plated on 96-well plates (Euroclone, Milan, Italy) at concentrations of 5,000 or 10,000 cells/cm^2. After being exposed to the desired concentrations of the different compounds, 10 µl of CCK-solution was added to each well and incubated for a period of 2 hours at 37 °C. Finally, absorption was measured at 450 nm using a multiplate reader Multiscan FC (Thermo Scientific, USA). Dimethyl sulfoxide (DMSO) 3% was used as a toxic reference drug. Cell viability was expressed as a percentage of that of the untreated cells (control). For each concentration of tested compounds, mean values of the mean absorbance rates from four wells were calculated.

Nanoparticle detection

Nine samples of kibbles belonging to different brands were collected and analysed for the presence of inorganic foreign bodies. We hypothesised that daily intake of kibbles contaminated with antibiotic residues contributed to the onset of otitis, diarrhoea, generalised anxiety, and dermatitis in dogs. From each kibble a 1-2 cm^2 sample was detached, put on a carbon adhesive disk and then transferred to an aluminium stub to rule out the possible presence of any environmental pollutants. Samples were analysed in triplicate to check the repeatability of the analysis. The samples were observed with an Environmental Scanning Electron Microscope (ESEM-Quanta, FEI Company, The Netherlands), which was equipped with an X-ray microprobe of an Energy Dispersive System (EDS, EDAX, USA), in order to analyse the chemical composition of any possible pollutant present in the kibbles. By using the ESEM, it is possible to operate at environmental conditions, at normal room pressure, and at low vacuum, reducing the possibility of contamination and creation of artefacts.

Clinical evaluation

Dogs received veterinary inspections at the beginning of the evaluation and after 15 days. A general physical examination (inspection, auscultation, percussion, and palpation) was first performed to evaluate constitution and skeletal development, nutritional status and muscle tonicity,

sensory state, skin and subcutaneous connective tissue, the status of lymph nodes, apparent mucous, body temperature, arterial pulse, and breath. Then a deeper clinical evaluation was also performed. A differential diagnosis approach was followed for each clinical symptom. In dogs affected by diarrhoea, a coprological evaluation was performed to rule out the presence of intestinal parasites. In dogs affected by otitis, an auricular swab was performed to rule out the presence of bacteria, fungi, or mites. In the cases of dermatitis, a skin scraper ruled out the presence of any mites responsible for demodectic or sarcoptic mange; a trichological evaluation ruled out the presence of mycetes; and a skin swab ruled out the presence of bacteria. None of dogs reported any treatment with antibiotics in the last year.

The work detailed here was performed in compliance with national Italian and international regulations (Italian regulation D.L.vo 116/1992 and European Union regulation 86/609/EC) for procedures and animal care. We additionally consulted the recommendations of the CONSORT 2010 statement pertaining to randomised controlled trials (Bian and Shang 2011).

Statistical methods

Data were analysed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as the means ± standard error of the mean (SEM) and were checked for normality test using the D'Agostino-Pearson normality test. Differences in serum OTC concentration and biochemical parameters before and after 15 days of the new diet supplementation were analysed using a Wilcoxon test. A $*p < 0.05$ was considered significant.

Results

An overall significant reduction of otitis, diarrhoea, generalised anxiety, and dermatitis after the administration of the organic chicken-based diet.

As shown in Figure 1, a significant decrease in mean OTC serum concentration was observed in all dogs from an initial value of 0.22 ± 0.12 µg/mL to 0.02 ± 0.03 µg/mL after 15 days of the organic chicken-based diet supplementation.

Biochemical analyses, which are reported in Table I, indicate a significant increase in the alkaline phosphatase from an initial value of 41 ± 7.74 U/L to 52.5 ± 11.84 U/L after 15 days of the organic chicken-based diet supplementation ($**p < 0.01$). Further, we observed a significant decrease in Gamma-glutamyl transferase and urea, from an initial value of 9.37 ± 7.61 U/L to 6.25 ± 5.6 U/L and

from 32.13 ± 8.72 to 22.13 ± 7.8 mg/dL, respectively ($*p < 0.05$).

Despite the significant variations of metabolic parameters these always fell within normal species range both at the beginning and after 15 days.

As to the cytotoxicity assessment of OTC, both in the powder and liquid 20% form, results are shown in Figure 2.

OTC powder 150 µg/mL (selected on the basis of the amount of active principle that is expected to be provided to chicken during a treatment according to the antibiotic instructions) produced significantly cytotoxic results after 24 hours of incubation (83% of cell viability, $**p < 0.01$) and enhanced its cytotoxicity at 48 and 72 hours, respectively, with an overall cell viability of 67%, $***p < 0.001$ (Figure 2C). By halving the concentration to 75 µg/mL, a significant cytotoxic effect was observed only after 48 and 72 hours of incubation, with a cell viability of 82 and 76%, respectively ($****p < 0.0001$) (Figure 2E). A similar trend was observed at a concentration of 35 µg/mL, with a cell viability of 79% and 78%, respectively ($*p < 0.05$, $***p < 0.001$) (Fig 2G). At a concentration of 20 µg/ml, no cytotoxic effect was observed (Figure 2I).

OTC liquid 20% at a concentration of 350 µg/mL (chosen on the basis of the amount of antibiotic that is expected to be provided to chicken during a treatment), resulted in a significantly cytotoxic effect at 24, 48, and 72 hours of incubation, with a cell viability of 78%, 50%, and 46%, respectively ($**p < 0.001$ and $****p < 0.0001$) (Figure 2D). At a concentration of 175 µg/mL, a similar trend was observed with a cell viability of 87%, 82%, and 66% at 24, 48, and 72 hours, respectively ($*p < 0.05$, $**p < 0.01$ and $***p < 0.001$) (Figure 2F). A concentration of 87.5 µg/mL resulted in a significantly cytotoxic
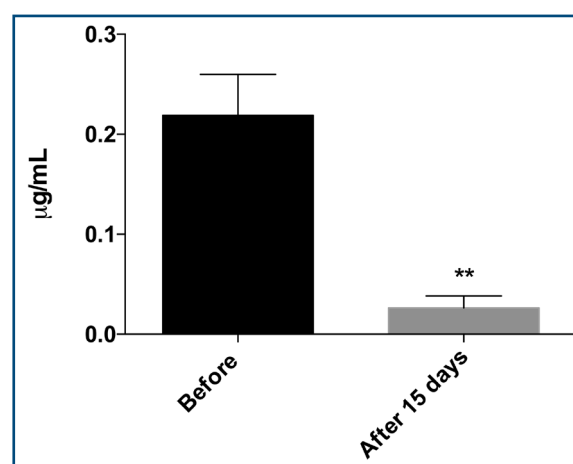
Figure 1. *Graphical representation of mean oxytetracycline serum concentration in dogs before and after 15 days of specific diet supplementation.* $**p < 0.01$.

Table I. *Biochemical parameter changes in dog sera before and after 15 days of specific diet supplementation.*

Parameter	Before		After 15 days	
	Mean	SD	Mean	SD
Albumin (g/dL)	2.97	0.09	2.68	0.24
Alkaline phosphatase (U/L)	41.0	7.74	52.5[**]	11.84
Total bilirubin	0.04	0.1	0.11	0.03
Calcium (mg/dL)	10.0	0.96	9.43	0.49
Cholesterol (mg/dL)	248.5	62.07	253.1	84.75
Creatine phosphokinase (U/L)	244.8	130.3	160.8	75.19
Creatinine (mg/dL)	0.93	0.09	0.98	0.08
Gamma-glutamyl transferase (U/L)	9.37	7.61	6.25[*]	5.6
Glucose (mg/dL)	51.75	28.58	66.63	3.66
Glutamic-oxaloacetic transaminase (U/L)	41.5	5.78	35.88	6.44
Glutamic-pyruvic transaminase (U/L)	58.0	9.38	53.75	4.62
Phosphorous (mg/dL)	4.92	0.89	4.07	1.15
Total protein (g/dL)	6.67	0.42	6.51	0.34
Triglycerides (mg/dL)	56.38	16.72	66.0	7.84
Urea (mg/dL)	32.13	8.72	22.13[*]	7.8

[*] $p < 0.05$; [**] $p < 0.01$

effect only after 72 hours of incubation, $*p < 0.05$ (Figure 2H), while at a concentration of 40 µg/mL, no cytotoxic effect was observed at any time of incubation (Figure 2J).

Figure 3 shows a 600X magnification of a kibble sample acquired with ESEM. The sample was drawn from one of the diets that had been assumed by dogs with otitis, diarrhoea, generalised anxiety, and dermatitis. The presence of amorphous nanoparticle aggregates, mainly constituted by calcium and phosphorous (particle A), and calcium, aluminium, and silicon (particle B) is clearly visible.

Mass spectrometry analyses produced clear evidence of the only presence of OTC residues in kibble samples withdrawn from diets provided to dogs with otitis, diarrhoea, generalised anxiety, and symptoms of dermatitis (Figure 4).

Discussion

Results clearly demonstrate a significant reduction of OTC concentration at the end of the evaluation period with respect to that at the beginning of the evaluation. Biochemical parameters did show some significant variation, though these always remained within normal ranges. Results relating to cytotoxicity demonstrated that both powder and liquid OTC showed a significant decrease in cell viability, which was time and concentration dependent. Food intolerance symptoms regressed with the new organic, chicken-based diet supplementation.

The unavoidable effects of OTC have been well

characterized. This antibiotic is widely used in intensive farming to prevent overcrowding and diseases associated with promiscuity, as a contaminant of meat, bone meal (20-30%), and poultry by-products, which are some of the main ingredients of pet foods (Odore *et al.* 2015, Di Cerbo *et al.* 2016b, Mazzeranghi *et al.* 2017, Maine *et al.* 2015). A high percentage of bone meal drags OTC residues that are therefore inevitably present in the kibbles of commercially available diets, and that can accumulate within a pet's body (Mazzeranghi *et al.* 2017). It is worth noting that, unfortunately, authorities do not investigate possible bone contamination, since it is not considered to be edible. We previously reported that broiler chickens treated with an oral administration of a commercially available liquid formulation of OTC 20% had a bone and muscle concentration of 1286.3 µg/kg of the antibiotic (Odore *et al.* 2015). Based on this, a bone meal with an OTC concentration of 1286.3 µg/kg would drag an approximate rate of 12 µg per 10 gr of kibbles. We also observed that after 48 hours of incubation with RPMI 1640 growth medium diluted 1:2, 1 g of the bone with 1286.3 µg/kg of OTC induced a significant apoptosis percentage in K562 cells and peripheral blood mononuclear cells (PBMCs) ($*p < 0.05$).

In this study we also demonstrate the usefulness of an organic chicken-based pet food diet made with meat that is not derived from intensive farming, in relieving otitis, diarrhoea, generalised anxiety, and dermatitis in a small cohort of dogs. In fact, we observed a significant reduction of the mean OTC serum concentration in all dogs after 15 days of
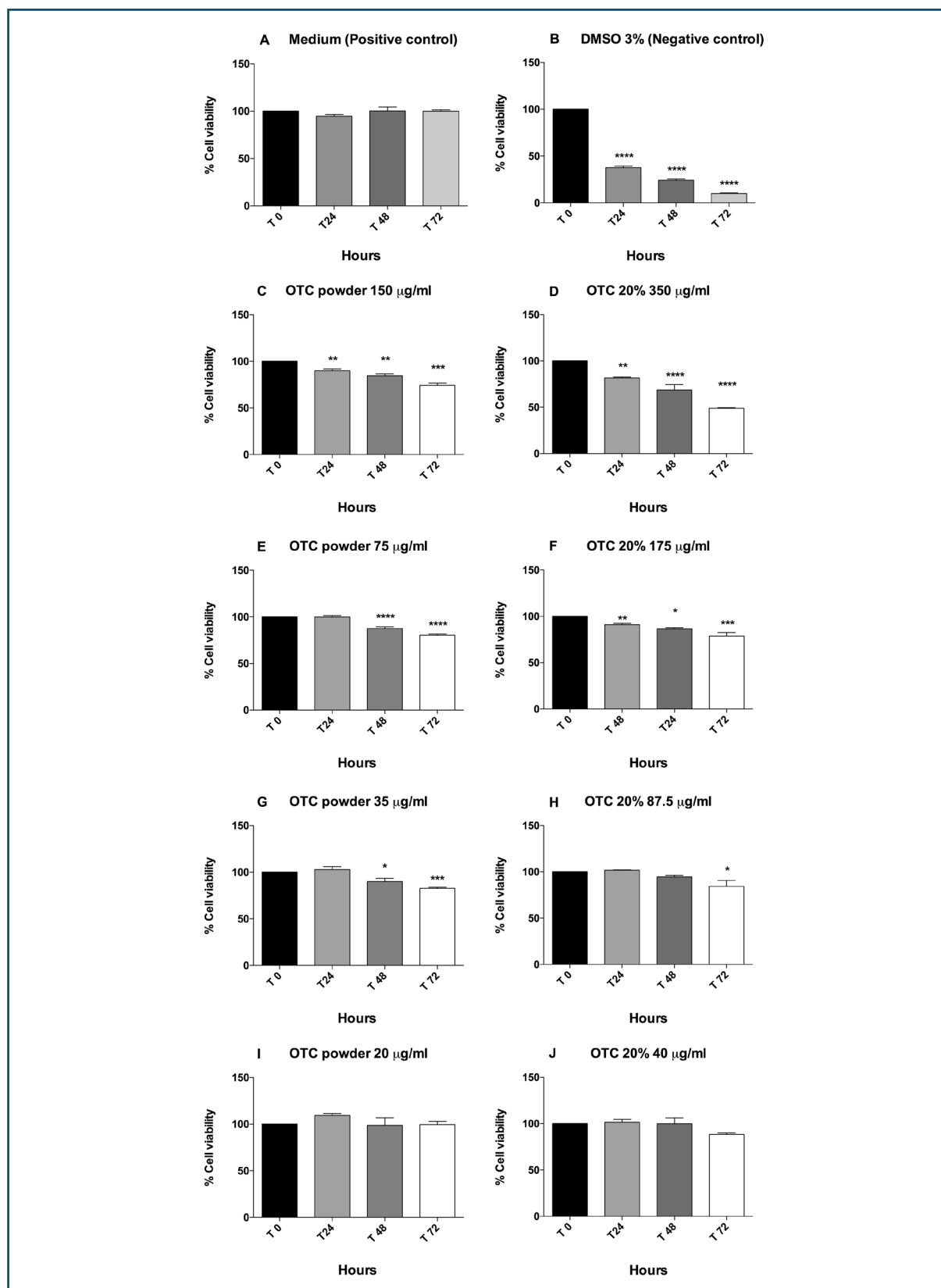
Figure 2. *Graphical representation of two different OTC formulation (powder and liquid) cytotoxicity on K562 cell line at 0, 24, 48 and 72 hours of incubation.* (**A**) Positive control (K562 cells with RPMI culture medium), (**B**) negative control (K562 cells with RPMI culture medium and 3% DMSO), (**C**) K562 cells with RPMI culture medium and OTC powder 150 µg/mL, (**D**) K562 cells with RPMI culture medium and OTC liquid 20% 350 µg/mL, (**E**) K562 cells with RPMI culture medium and OTC powder 75 µg/mL, (**F**) K562 cells with RPMI culture medium and OTC liquid 20% 175 µg/mL, (**G**) K562 cells with RPMI culture medium and OTC powder 35 µg/mL, (**H**) K562 cells with RPMI culture medium and OTC liquid 20% 87.5 µg/mL, (**I**) K562 cells with RPMI culture medium and OTC powder 15 µg/mL, (**J**) K562 cells with RPMI culture medium and OTC liquid 20% 40 µg/ml (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ and ****$p < 0.0001$).
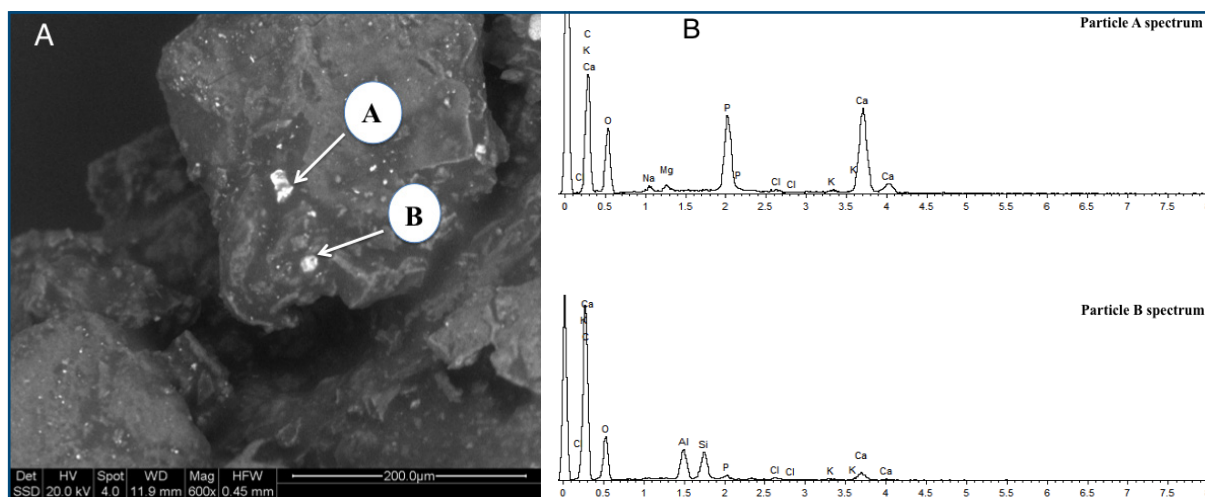
Figure 3. *Kibble sample microphotographs (A) along with its Energy Dispersive System spectrum (B) of the two nanoparticle aggregates embedded.*
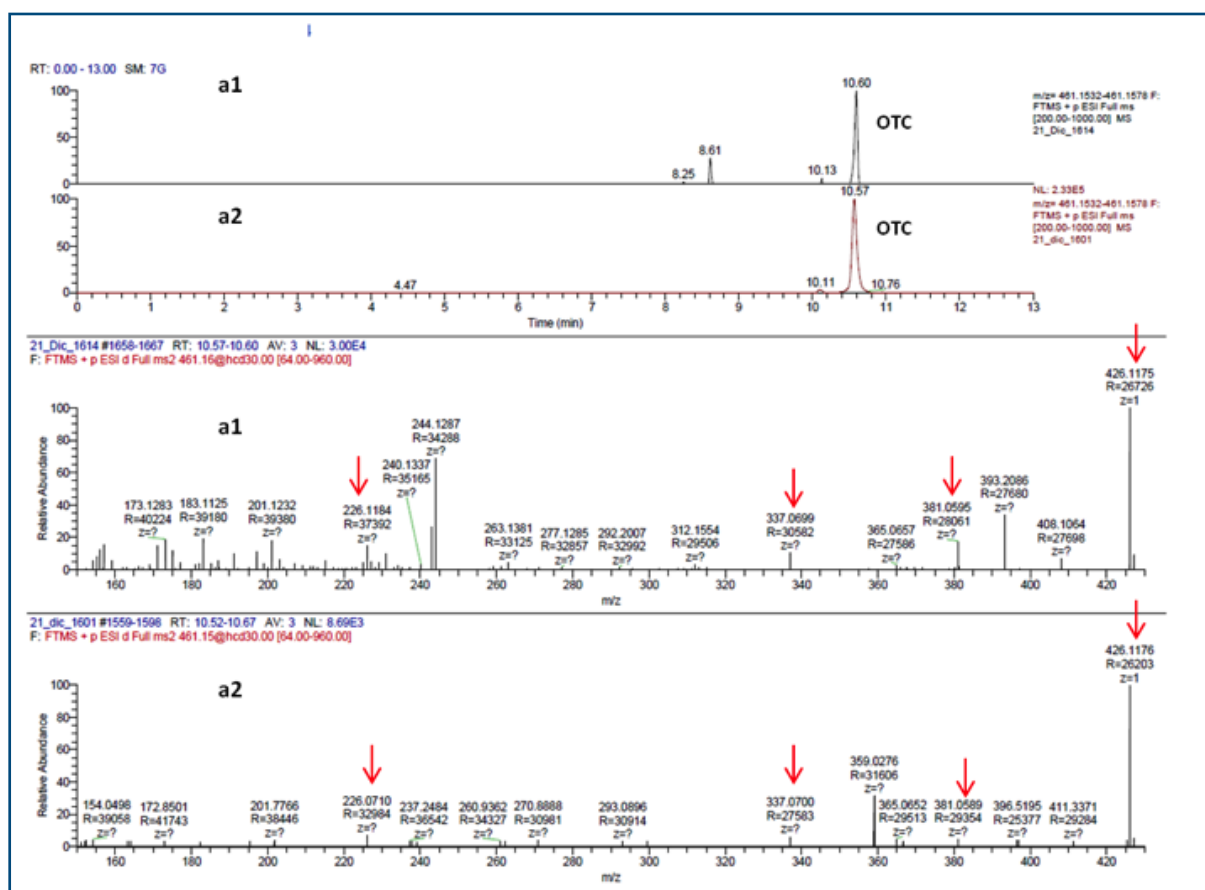
Figure 4. *Chromatograms and tandem Mass spectrometry (MS/MS) spectra of OTC in a1) pet food sample at 19.25 μg/Kg⁻¹ and in a2) reference standard at 1.0 μg/L⁻¹.*

the organic chicken-based diet supplementation. This trend is consistent with what Mazzeranghi and colleagues report, where 18 cats significantly improved their cutaneous adverse food reactions symptoms as the OTC serum concentration decreased (Mazzeranghi *et al.* 2017).

We have additionally previously reported the potential of other diet supplementation in relieving clinical symptoms in dogs suffering from chronic otitis externa (Di Cerbo *et al.* 2016a), chronic halitosis (Di Cerbo *et al.*, 2015), Leishmania (Cortese *et al.* 2015), atopic dermatitis, and gastrointestinal disturbs (dehydration, appetite loss, regurgitation,

emesis, abdominal pain, flatulence, borborygma, diarrhoea, weight loss, stool consistency, blood, and mucus present in the stool) (Di Cerbo *et al.* 2014b), cognitive impairment (Sechi *et al.* 2015), and keratoconjunctivitis sicca (Destefanis *et al.* 2016).

Findings that indicate the dog serum OTC at only 0.22 mg/mL – below the cytotoxic concentration measured *in vitro* – is not responsible of the reported symptoms. However, the serum concentration did not reflect the complete pharmacokinetic of OTC, which can accumulate in different organs. Moreover, OTC is able to stimulate the immune system even at low concentrations as we demonstrated in a previous study (Di Cerbo *et al.* 2016).

We also investigated the presence of OTC in the kibbles belonging to the diets provided to dogs that developed otitis, diarrhoea, generalised anxiety, and dermatitis, therefore ruling out the presence of any other antibiotic and tetracycline family member.

It was possible to observe the only presence of OTC at a mean concentration of 19.0 ± 3.7 µg/kg^{-1} close to the value, which did not result to be cytotoxic *in vitro* (20 µg/mL). This might explain the onset of symptoms following the ingestion of contaminated food.

Further microscopic investigation that we carried out highlighted the presence of nanoparticle aggregates embedded in the kibbles. The EDS spectrum of the nanoparticles aggregate A revealed the presence of calcium and phosphorous as the main constituents. These might be ascribed to the presence of bone meal, which have previously been recognised as one of the main ingredients of pet food (Di Cerbo *et al.* 2016b, Guidetti *et al.* 2016, Palmieri *et al.* 2014, Sechi *et al.* 2017, Mazzeranghi *et al.* 2017, Odore *et al.* 2015, Maine *et al.* 2015). Intriguingly, EDS spectrum of the nanoparticles aggregate B revealed the presence of silicon, which is typical of food processing procedures that use it as an additive to prevent caking in powdered products (Winkler *et al.* 2016). Although many *in vivo* studies report the almost complete safety of synthetic amorphous silica (SAS), none of these studies examine local effects on the lymphoid tissue of the gastrointestinal mucosa (Rutter and Shott, Winkler *et al.* 2016). Nanoparticles, including those of silica, are known to penetrate the gut-associated lymphoid tissue, impairing tolerance towards food constituents (Sass *et al.* 1990, des Rieux *et al.* 2006, Awaad *et al.* 2012, Powell *et al.* 2015) and triggering immune-mediated conditions that carry the hallmarks of inflammatory bowel disease (IBD)

(Zolnik *et al.* 2010). Moreover, a recent work from Sighinolfi and colleagues demonstrates the inability of some nanoparticles to execute neoplastic transformation rather their trend to enhance tumour progression (Sighinolfi *et al.* 2016).

An overall improvement in diarrhoea was observed after 48 hours of the organic chicken-based diet supplementation. Conversely, dermatitis and otitis took longer periods – from 48 hours to 15 days – to completely restore, and were accompanied by a consequent reduction of general anxiety, which was probably linked to an overall chronic inflammatory status (Sechi *et al.* 2017, Parashar and Udayabanu 2016). In light of these observations, it is conceivable to hypothesise that a chronic intake of contaminated (e.g. OTC) food can induce a chronic inflammatory status, enhanced also by the presence of nanoparticle aggregates, fostering gut immunity and microbiota impairment and consequently bringing on symptoms such as otitis, diarrhoea, dermatitis, and generalised anxiety (Mayer 2011).

Our study provides clinical evidence of the presence of a plethora of symptoms that may occur in dogs fed on commercially available pet food diets where OTC is present. Nevertheless, we cannot rule out the possible combined pro-inflammatory activity exerted by nanoparticles, whose presence is almost unavoidable during the overall production process. Given the small cohort of dogs that were considered in this study, further research with a larger sample size along with well-detailed immunological profiles are needed to support these preliminary observations.

Acknowledgments

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