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## Natural zeolite (chabazite/phillipsite) dietary supplementation influences faecal microbiota and oxidant status of working dogs

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### ABSTRACT

We evaluated whether chabazite/phillipsite dietary supplementation might affect the faecal microbiota, oxidant and antioxidant status of working dogs at rest undergone to a trial test. Forty English Setter dogs were involved in two replicate trials. At each replicate, dogs were divided into two homogeneous groups (10 dogs/group). During a period of 28 days, diet was supplemented (Z group) or not supplemented (C group) with chabazite/phillipsite at the dose of 5 g/head/day. On day 29, dogs were subjected to a trial test. Faecal characteristics were assessed at 0 and 29 days (within two hours from the end of the trial test). Faecal consistency was not affected by dietary supplementation ( $p > .05$ ). On day 29, *Lactobacillus* spp. and *Enterococcus* spp. counts were higher and *Enterobacteriaceae* were lower in Z than in C group ( $p < .05$ ). At day 0 and at day 29, immediately before and within one hour from the end of the trial test, blood samples were recorded to evaluate the oxidant and antioxidant status. A reduction of 40% in thiobarbituric acid reactive substances (TBARS) levels was observed in Z compared to the C group ( $p < .05$ ). Differences in nitric oxide and antioxidant enzymatic system levels were not significant ( $p > .05$ ). Our results suggest that chabazite/phillipsite dietary supplementation, improves the intestinal microbiota ecosystem and may counteract the oxidative damage caused by physical stress in hunting dogs at the beginning of the working season.

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### Introduction

In working dogs, keeping a balanced intestinal microbial ecosystem during the working season is particularly desirable (Gagne et al. 2013). Stressor exposure may impact the stability of the intestinal microbiota (Galley et al. 2014). A bidirectional neurohumoral communication system, known as the gut–brain axis, integrates the host gut and brain activities. Physical and mental stress related to exercise can affect the gastrointestinal (GI) permeability, motility, secretion and mucin production, thereby altering the habitat of resident bacteria and promoting changes in microbial composition or activity (Carabotti et al. 2015). These effects may be both due to a prolonged and an acute exercise. In short-lasting acute stressors, changes of the number of bacteria shed in the stool and of some populations of the colonic mucosa-associated microbiota are reported (Galley et al. 2014).

We know that physical inactivity reduces the whole body resistance to oxidative stress while training increases the resistance against oxidative stress, providing enhanced protection (Powers & Jackson 2008; Radak et al. 2008; Tong et al. 2012). Physical exercise increases the rate of oxygen consumption, which implies a raise of free radicals production. Due to their high reactivity, free radicals and others oxidants (e.g. nitric oxide) react with various organic molecules and cause lipid peroxidation, leading to extensive cell and tissue damage (Powers et al. 2011). An increase of thiobarbituric acid reactive substances (TBARS) plasma levels may be considered as a sign of cellular lipid oxidation and be used as a marker of oxidative status (Da Silva et al. 2013). The potential damage caused by an excess of reactive oxygen and nitrogen species is controlled by an intricate antioxidant defence system, that includes many enzymes (e.g. superoxide dismutase, glutathione peroxidase, catalase), as well as

non-enzymatic antioxidants (e.g. glutathione) (Powers & Jackson 2008).

Clay added to the diet can alter the microbial population in the gastrointestinal tract resulting in a more favourable gut microflora and modify the characteristics of the intestinal environment such as pH or oxidation state (Subramaniam & Kim 2015). *In vitro* and *in vivo* antioxidant properties of natural clays, such as zeolites have been reported (Dogliotti et al. 2012; Montinaro et al. 2013; Zarkovic et al. 2003). Some studies indicated that dietary inclusion of zeolites delayed lipid peroxidation with water-soluble peroxy radicals, and reduced the catalytic production of radicals to protect the organism (Wu et al. 2015). The zeolite structures are based on tetrahedral anionic building blocks connected through oxygen atoms and with an open structure, which can accommodate a wide variety of positive ions. Besides these characteristics, zeolites exhibit versatile absorptive, cation exchanger, dehydrating–rehydrating and catalytic properties (Passaglia & Sheppard 2001). The exchange capacity is the ability to release beneficial elements while capturing and binding others. It has been indicated as an important requirement for the biological application of zeolites (Guo et al. 2011). Among zeolites, chabazite/phillipsite has a high cation-exchange capacity (>200 mEq/100 g) (Pabalan & Bertetti 2001).

To our knowledge, no research to date has focussed on the use of zeolites in working dogs, in which the psychological and physical stress to which they are subjected can affect the health and well-being. Therefore, the aim of the study was to assess whether chabazite/phillipsite dietary supplementation can influence (i) the faecal microbiota and (ii) the oxidant and antioxidant status of healthy hunting dogs at rest undergone to a trial test.

## Materials and methods

The study was conducted in compliance with Italian law (Lgs. D. 26/2014) within the scope of the Directive 2010/63/EU on the protection of animals used for scientific purposes.

### Zeolites source and composition

Powdered zeolitic clay, sterilised at 200 °C for 20 min, was obtained from Verdi S.p.A (Castelnuovo di Sotto, Italy). The total zeolite content of the clay was ~73.0%, which comprised 93.2% chabazite ( $\text{Na}_{0.14}\text{K}_{1.03}\text{Ca}_{1.00}\text{Mg}_{0.17} [\text{Al}_{3.46}\text{Si}_{8.53}\text{O}_{24}] \times 9.7\text{H}_2\text{O}$ ) and 6.8% phillipsite ( $\text{Na}_{0.9}\text{Ca}_{0.5}\text{K}_{0.6} [\text{Si}_{5.2}\text{Al}_{1.28}\text{O}_{16}] \times 6\text{H}_2\text{O}$ ). No traces of clinoptilolite were found. In Table 1 the

**Table 1.** Chemical composition of natural zeolite (chabazite/phillipsite) utilised in the present study.

Chemical composition	
Moisture, g/kg	120
SiO <sub>2</sub> , g/kg	520
Al <sub>2</sub> O <sub>3</sub> , g/kg	170
TiO <sub>2</sub> , g/kg	5
Fe <sub>2</sub> O <sub>3</sub> , g/kg	36
P <sub>2</sub> O <sub>5</sub> , g/kg	3
MnO, g/kg	2
MgO, g/kg	20
CaO, g/kg	57
Na <sub>2</sub> O, g/kg	6
K <sub>2</sub> O, g/kg	61
Cation-exchange capacity, mEq/g	2.2
Bulk density, g/cm <sup>3</sup>	0.7–0.9

mineral chemical composition, the cation-exchange capacity and the bulk density (Cresswell & Hamilton 2002) of the utilised zeolite are reported.

### Animals and treatment

Forty English Setter dogs reared in the same kennel were involved in the study with two replicates separated by time (20 dogs/replicate). Inside each replication, the animals were subjected to the same environmental conditions. Mean age (years) was  $3.46 \pm 2.1$  and mean body weight (BW, kg) was  $19.02 \pm 3.2$ . The dogs were considered healthy, based on physical examination and blood biochemistry analysis and did not receive medications that are expected to alter the gut microbiota (i.e. antibiotics). The dogs were wormed one month before the start of the study. At each replicate, dogs were divided into two homogeneous groups (10 dogs/group), based on their age and weight. In both groups, sexes (5 males, 5 not pregnant females) were equally distributed. The animals were individually penned with a rest area inside ( $2.70 \times 1.40$  m) and a paddock outside ( $4.50 \times 1.40$  m). During a period of 28 days, they received a diet (Table 2) administered at about 25 g dry matter/kg of BW<sup>0.75</sup>, once a day, at 17:00 h, supplemented (Z group) or not supplemented (C group) with chabazite/phillipsite at the dose of 5 g/head/day. Free access to water was provided. For each dog, zeolite was weighed at each meal and added to the ration. Regardless of the presence or not of chabazite/phillipsite, all animals consumed the ration completely within 30 min of dosing.

### Trial test

On day 29, all dogs were subjected to aerobic physical activity, which consisted of a continuous gallop for

**Table 2.** Ingredients (%) and chemical composition of diet.

Ingredients	
Poultry fresh meat	26.00
Bread	12.80
Commercial extruded mixed feed	4.00
Wheat bran	3.20
Vitamin and mineral premix <sup>a</sup>	0.25
Water	53.75
Proximate analysis:	
Moisture, %	78.95
Crude protein, % DM <sup>b</sup>	24.94
Crude fat, % DM	23.51
Crude fibre, % DM	2.42
Ash, % DM	5.46
ME, MJ/kg DM	18.40

<sup>a</sup>Providing per kilogram of diet: vitamin A, 12,500 IU; vitamin D3, 400 IU; vitamin E, 50 mg; vitamin B1, 2 mg; vitamin B2, 4 mg; vitamin B6, 2 mg; vitamin B12, 0.010 mg; vitamin C, 100 mg; vitamin PP, 20 mg; vitamin K, 2 mg; Biotin, 0.2 mg; Fe, 800 mg; Cu, 15 mg; Mn, 35 mg; Se, 0.05 mg; Zn, 110 mg.

<sup>b</sup>DM: dry matter.

30 min. The trials were performed outdoor (at 10:00 h) at a mean temperature and relative humidity of  $28 \pm 5$  °C and of  $68 \pm 11\%$ , respectively.

### Samples collection

The animals were weighed at day 0 and at day 29, before the beginning of the test trial. Faeces consistency was scored by a single operator using a scale of 1 (hard) to 5 (watery), on days 0 and 29 (Grieshop et al. 2002). Individual faecal samples were collected directly from the rectum, using a sterile glove lubricated with water on day 0 and within two hours from the end of the trial test (Galley et al. 2014). The faeces were placed in sterile polyethylene bags, and immediately transported to the laboratory on ice packs. Faecal samples were processed within 3 h from the collection.

Blood samples were collected from all dogs, by cephalic venepuncture into evacuated tubes containing lithium-heparin, at day 0 (at 08:00 h) and at day 29, immediately before and within one hour from the end of the trial test (Pasquini et al. 2010). Plasma was separated by centrifugation at  $3000 \times g$  for 15 min and frozen at  $-20$  °C until analysis.

### Analysis

#### Zeolite

The mineralogical quantitative phase analysis of zeolitic powder was performed using the combined Rietveld-RIR method, which allows the determination of both crystalline and amorphous phases in a sample

(Gualtieri et al. 1999). Briefly, the combined Rietveld-RIR method is based on a procedure in which a known amount of an internal standard (10 wt% corundum NIST 674a) is added to the mixture and considered as a component itself. The refined values of the phase fractions are converted into weight fractions, and rescaled into absolute values with respect to the amount of added standard. Data sets were collected with a Philips PWI 729 Bragg–Brentano diffractometer (Philips Corp., Amsterdam, The Netherlands) in the angular range  $17$ – $120$ °  $2\theta$  using a step scan of  $0.02$ °  $2\theta$  and 10 seconds/step. Refinements of the powder spectra of the samples were performed using the GSAS software package (Larson & Von Dreele 1999).

The chemical composition and the cation-exchange capacity were determined using 2 g of material mounted on a gooch filter and slowly percolated by 1 N  $\text{NH}_4\text{Cl}$  solution at about  $50$  °C until the concentration of ions in the eluate was roughly equal to the value present as impurity in the ingoing solution or less than 0.5 ppm. Concentration of the eluted cations was accomplished by atomic absorption spectroscopy by using a Perkin Elmer 303 instrument (PerkinElmer, Milano, Italy). Ions content of the zeolite before (natural) and after the exchange with  $\text{NH}_4$  were determined in energy dispersive mode using a Philips XL 40/604 operating with vacuum of  $\sim 10$  mm Hg (Gualtieri et al. 1999).

#### Diet

Proximate analyses were performed according to the Commission Regulation (EC) 152/2009 laying down the methods of sampling and analysis for the official control of feed (Annex III). The predictive equation proposed by NRC (2006) for calculating metabolisable energy (ME) diet content was used.

#### Blood

Nitric oxide (NO) was assessed by measuring nitrite levels in plasma by the microplate method, based on the formation of chromophore after reaction with the Griess reagent. This was prepared fresh daily by mixing equal volumes of stock A (1% sulphanilamide, 5% phosphoric acid) and stock B (0.1% N-[naphthyl] ethylenediamine dihydrochloride) (Ding et al. 1988). After a 10 min incubation at room temperature with the Griess reagent, the absorbance was determined by Victor3 1420 multilabel counter (PerkinElmer, Milano, Italy), using a 540 nm against 620 nm filter. The absorbance at 620 nm was subtracted to eliminate the yellow interference. The standard curve was performed using serial dilutions of sodium nitrite ( $50$ – $0.39$   $\mu\text{M}$ ;

linear regression:  $y = 0.0223x + 0.102$ ;  $r = .99$ ). The inter-assay variability was less than 5%.

Superoxide dismutase (SOD) activity was determined by a SOD Determination Kit (Sigma-Aldrich, St. Louis, MO). The colorimetric assay was performed measuring formazan produced by the reaction between a water-soluble tetrazolium salt and  $O_2^-$ , produced by the reaction of an exogenous xanthine oxidase. The remaining  $O_2^-$  is an indirect hint of the endogenous SOD activity. The absorbance was determined by Victor3 1420 multilabel counter, reading at 450 nm against 620 nm. The standard curve of SOD ranged from 0.156 to 20 U/mL. The detection sensitivity was 0.3 U/mL. The interassay variability was less than 3%.

The Glutathione peroxidase activity colorimetric assay kit (BioVision Incorporated, Milpitas, CA) was used to evaluate glutathione peroxidase (GPx) activity. Briefly, GPx reduces cumene hydroperoxide while oxidising reduced glutathione to oxidised glutathione (GSSG). The generated GSSG is reduced to GSH with the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) by glutathione reductase. The decrease of NADPH, measured at 340 nm by Victor3 1420 multilabel counter, is proportional to GPx activity. The assay has a detection sensitivity  $\sim 0.5$  mU/mL of GPx. The interassay variability was less than 5%.

Thiobarbituric acid reactive substances were determined by TBARS Assay kit (Abnova GmbH, Heidelberg, Germany). The malondialdehyde (MDA)-TBA adduct, formed by the reaction of MDA and TBA under high temperature (90–100°C) and acid conditions, was measured by Victor3 1420 multilabel counter at 530 nm. As specified by the manufacturer (i.e. under the standardised conditions of the assay described in the datasheet) the dynamic range of the kit is 0–50  $\mu$ M. The interassay variability was less than 7%.

### Faeces

The homogenates of individual faecal specimens were serially diluted with both half-strength Wilkins–Chalgren Anaerobe Broth (WCAB) and Buffered Peptone Water (ThermoScientific–Oxoid, UK). Dilutions in duplicate were plated on vancomycin and bromocresol green (LAMVAB) agar (Hartemink & Rombouts, 1999) for lactobacilli, Azide maltose agar (Biolife, Italy) for enterococci and MacConkey agar (Merck, Germany) for *Enterobacteriaceae* counts.

MacConkey agar plates were incubated aerobically at 37°C for 24 and 48 h, respectively. Other media were incubated anaerobically at 37°C for 48–72 h. The taxonomy of the colonies isolated on selective media

were determined at genus and species level by API System (Bio-Merieux, Italy). Values of colony forming units (CFU) have been expressed as  $\log_{10}$ /g of faeces.

### Statistical analysis

Individual data were analysed using a GLM procedure in SAS 9.4 (SAS Inc. 2012). Data were previously checked for normality. The ANOVA model included treatment (two levels: C and Z), replicate (two levels) and sex (two levels) as fixed factors. The interactions were not significant and were removed by the model.

Blood and faecal parameters at day 29 were covaried with those at day 0. Results are presented as LSM. The significance level was set at  $p \leq .05$ .

### Results and discussion

Replicate and sex were not significant factors for any parameter, so the data are discussed only on the basis of differences between treatments.

Chabazite/phillipsite did not affect BW of the dogs ( $p = .259$ ), as well as faecal consistency ( $p = .113$ ) (Table 3). This finding is not in agreement with Vondruskova et al. (2010), who report that the use of clay minerals retards the rate of digestive passage through the intestines and that their ability to absorb water results in more compact and better shaped faeces.

Changes in faecal microbial concentration were observed (Table 3). Evidences suggest that the effects of zeolites on GI microbiota do not depend on a direct action on the bacterial flora but on their ability to shift pH and buffering capacity of gastrointestinal secretions (Trckova et al. 2004).

Chabazite/phillipsite is not absorbed by the intestinal epithelium and for this reason it could affect the intestinal habitat by leveraging its high

**Table 3.** Effects of chabazite/phillipsite (Z) supplementation on faecal score and faecal microbial concentration at day 29.

Item	Group <sup>a</sup>		SEM <sup>b</sup>
	C	Z	
Dogs/replicate, n	10	10	
BW, kg	19.50	18.13	2.67
Faecal score <sup>c</sup>	2.81	2.88	0.29
<i>Lactobacillus</i> spp. <sup>d</sup> , $\log_{10}$ CFU/g of faeces	8.32 <sup>e</sup>	8.98 <sup>f</sup>	0.04
<i>Enterococcus</i> spp. <sup>d</sup> , $\log_{10}$ CFU/g of faeces	7.27 <sup>e</sup>	8.10 <sup>f</sup>	0.04
<i>Enterobacteriaceae</i> <sup>d</sup> , $\log_{10}$ CFU/g of faeces	7.27 <sup>f</sup>	6.35 <sup>e</sup>	0.06

<sup>a</sup>C = untreated group with chabazite/phillipsite, Z = treated group with chabazite/phillipsite;

<sup>b</sup>SEM, standard error of the difference of means.

<sup>c</sup>On a five points (1–5) scale.

<sup>d</sup>Samples collected *post* trial.

<sup>e,f</sup>Different letters in the same row indicate statistical difference ( $p < .05$ ).



cation-exchange capacity (Gershkovich et al. 2009; Pabalan & Bertetti 2001). According to cation-exchange capacity, natural zeolites can adsorb or detoxify bacterial toxins and thus prevent the increased intestinal permeability and the damage produced by bacterial toxins (Andronikashvili et al. 2009; Subramaniam & Kim 2015). Interaction between bacteria and the absorbents is controlled by electrostatic attraction (Rong et al. 2008). Under physiologic conditions, bacterial cell walls are negatively charged while chabazite/phillipsite structure presents mono and divalent exchangeable cations (i.e.  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{K}^+$ ) (Guo et al. 2011). On day 29, *Lactobacillus* spp. counts were higher, while *Enterobacteriaceae* spp. counts were lower in Z than C group ( $p < .05$ ). An increase of *Enterococcus* spp. concentration was found in Z compared to C group ( $p < .05$ ). The increase of *Lactobacillus* spp. counts has been associated with decreased faecal concentrations of potentially pathogenic bacteria and decreased levels of carcinogenic and putrefactive compounds in *digesta* (Grieshop et al. 2002). In addition, *Lactobacillus* species are the most probiotic lactic acid bacteria utilised and some of them have been studied to improve the health and brain function of dogs (Biagi et al. 2007; Bravo et al. 2011). Likewise, the bacteria belonging to the families of *Enterobacteriaceae* and *Enterococcus* play an imperative role in maintaining the normal digestive function of the hosts. However, an increase of enterococcal and enterobacter infections has been observed, due to the finding that they are emerging as community-acquired pathogens for their ability to develop high-level resistance to antimicrobials in dogs (Hetsa et al. 2013; Kataoka et al. 2014; Ossiprandi et al. 2008).

**Table 4.** Effects of chabazite/phillipsite (Z) supplementation on BW and on oxidant/antioxidant parameters at day 29.

Item	Group <sup>a</sup>		SEM <sup>b</sup>
	C	Z	
Dogs/replicate, n	10	10	
NO, $\mu\text{mol/L}$			
Pre trial	4.69	5.03	1.33
Post trial	4.75	4.17	1.21
SOD, U/mL			
Pre trial	9.00	10.23	4.36
Post trial	8.73	11.05	5.01
GPx, mU/mL			
Pre trial	76.00	82.90	5.00
Post trial	77.09	81.73	4.86
TBARS, nmol/mL			
Pre trial	2.28	2.60	0.99
Post trial	3.03 <sup>d</sup>	1.84 <sup>c</sup>	0.97

<sup>a</sup>C: untreated group with chabazite/phillipsite; Z: treated group with chabazite/phillipsite.

<sup>b</sup>SEM: standard error of the difference of means.

<sup>c,d</sup>Different letters in the same row indicate statistical difference ( $p < .05$ ).

Chabazite/phillipsite indirectly affect the systemic oxidative system during its transit through the GI tract. Gut is a stress-signalling organ and it is a key source of reactive oxygen species (Bhattacharyya et al. 2014). Despite the intestinal protective barrier provided by the epithelial layer, pathogens can cause inflammation by activating the polymorphonuclear neutrophils and macrophages to produce inflammatory cytokines and other mediators that further contribute to oxidative stress (Chen et al. 1998). Several GI pathological conditions arise in part from oxidative stress (Maloy & Powrie 2011).

It has been suggested that zeolites can trap the free radicals in their complex structure, inactivating and eliminating them (Zarkovic et al. 2003). In humans undergoing regular aerobic exercise training, zeolite supplementation has beneficially affected intestinal barrier integrity without modify redox markers in the blood (Lamprecht et al. 2015). In our study, we observed a non-significant reduction of NO (−12%) and an increase of SOD (+27%) and GPx (+6%) plasma levels in response to the trial test (Table 4) in the Z group compared to the C group. It has been reported that the overproduction of nitric oxide and the activities of the antioxidant enzymatic systems are dependent on various factors, such as intensity and duration of exercise (Hogg & Kalyanaraman 1999). Therefore, it is conceivable to assume that the intensity and duration of exercise performed has not been sufficient to enable the achievement of NO toxic concentrations for cells and the activation of antioxidant enzymatic systems. Conversely, TBARS values were lower by 40% in Z than in the C group ( $p = .021$ ). This finding is consistent with what a previous study observed in broiler chickens supplemented with zeolites, where a reduction of lipid peroxidation markers was observed in the serum, liver, and intestinal mucosa (Wu et al. 2015).

## Conclusions

In conclusion, natural zeolites chabazite/phillipsite dietary supplementation can be an interesting tool to improve the intestinal microbiota in working dogs, such as hunting dogs. Moreover, by maintaining the stability of the intestinal microbiota, they can help to prevent the lipid peroxidation caused by physical stress in untrained subjects. Future studies will be needed to clarify which mechanisms mediate the observed chabazite/phillipsite effects and to assess its employment during an exhaustive aerobic exercise or in moments in which the dogs are subjected to

physiological stress like gestation, lactation and weaning.

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## Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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