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Nutrigenomic activity of plant derived compounds in health and disease: Results of a dietary intervention study in dog



Sandy Sgorlon*, Bruno Stefanon, Misa Sandri, Monica Colitti

Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Via delle Scienze, 208, 33100 Udine, Italy

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ABSTRACT

The study was conducted to investigate the effects of dietary administrations of four nutraceuticals in dogs. Seventy four dogs were enrolled in the trials, 24 healthy dogs were fed with a control diet (CT) and the experimental groups received for 60 days the same diet supplemented with nutraceuticals, namely *Echinacea angustifolia* (EA, 0.10 mg/kg live weight as echinacoside; 14 dogs), *Vaccinium myrtillus* (VM, 0.20 mg/kg live weight as anthocyanidin, 13 dogs), *Curcuma longa* (CL, 6.60 mg/kg live weight as curcumin, 18 dogs with arthrosis), and *Sylibum marianum* (SM, 1.5 mg/kg live weight as silybin, 8 dogs with hepatopathy). Dogs were weighed at the beginning of study and blood samples were collected at the beginning (T0) and at the end (T60) of the study. VM significantly down regulated *TNF*, *CXCL8*, *NFKB1* and *PTGS2* and decreased plasma ceruloplasmin (CuCp). The activity of EA was evidenced by the significant decrease of *TNF* and *NFKB1* expression and CuCp levels and by the increase of plasma Zn. Administration of CL caused a significant decrease of CuCp and increase of Zn and a down regulation of *TNF*, *CXCL8*, *NFKB1* and *PTGS2*, corroborating the anti-inflammatory action of curcuminoids. After 60 days of treatment with SM, plasma ALT/GPT activity was reduced and paraoxonase was increased, supporting the antioxidant activity of silymarin, also confirmed by the significant up regulation of *SOD2*. Results indicated that nutraceutical administrations in dogs can be an interesting approach to modulate immune response in order to improve health condition of animals.

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1. Introduction

The inclusion of nutraceuticals in the diet of human, livestock and companion animals has gained popularity (Colitti and Grasso, 2014; Swanson et al., 2003) in the last decades and in the market there is a large variety of supplements or foods containing a broad type of plant extracts, pure compounds and, more in general, functional compounds. Among the other, the activity of nutraceuticals can be directed towards immune system, arthrosis, Alzheimer, obesity, ageing, oxidative stress, depression, intestinal bowel disease and intestinal bowel syndrome or metabolic syndrome (Wichtl, 2004). Often, the same nutraceutical has several positive effects, as the case of curcumin (Ara et al., 2016; Colitti et al., 2012) or resveratrol (Tomé-Carneiro et al., 2013; Woode et al., 2015). According to the EU Regulation 1831/2003 (Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003) on additives for use in animal nutrition, only the herbal extract registered in the annexes can be used for animals, and the inclusion in this list has to follow a standard protocol. In the last published list (Annex I list of additives Regulation (EC) No 1831/2003, Released 18.04.2016), natural products are registered mainly in the

technological category of sensory additives, subcategory “flavouring compounds: substances the inclusion of which in feedstuffs increases feed smell or palatability”. Nevertheless, several petfoods have claim for specific activities of natural products, but scientific evidences of their benefits are not always conclusive. To assess the activity of plant nutraceutical, researchers have published studies using laboratory animals or *in vitro* cell cultures (Farinacci et al., 2008; Farinacci et al., 2009; Pomari et al., 2013; Pomari et al. 2014; Pomari et al., 2015; Stefanon et al., 2015), and scientific evidences are also available for companion animals (Colitti et al., 2012; Comblain et al., 2015).

Plants contain an almost infinite list of compounds with pharmacological and nutraceutical properties and in the present study we concentrated on 4 purified extracts, *Vaccinium myrtillus*, *Curcuma longa*, *Echinacea angustifolia* and *Sylibum marianum*, with documented activity for specific biological functions.

Vaccinium myrtillus (VM) is a shrub belonging to *Ericaceae* family growing spontaneously in Europe, Northern Asia, Iceland, Canada and United States. Its dry and powdered fruits and leaves have been used in European traditional medicine for the treatment of many disorders. It is generally named “bilberry” or “European blueberry” to distinguish from “American blueberry”. Anthocyanins chemical group, responsible for the typical bilberry’ color, represent the main active compounds contained in this fruit (Kähkönen et al., 2003), which have a well-recognized antioxidant property. Among berries, bilberry or *Vaccinium*

* Corresponding author at: Department of Agricultural, Food, Environmental and Animal Sciences, via delle Scienze, 206, 33100 Udine, Italy.
E-mail address: sandy.sgorlon@uniud.it (S. Sgorlon).

myrtilus specie is the richest in anthocyanins, furthermore showing the most effective antioxidant activity (Nakajima et al., 2004).

Curcuma longa (CL), also known as turmeric, is a perennial plant of the Zingiberaceae family and the rhizome contains compounds called curcuminoids, which include curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin. Therapeutic uses suggested for curcumin refer to a huge range of biological activities, like cancer chemopreventive, antitumor and neuroprotective, primarily based on its recognized antioxidant and anti-inflammatory properties (Venugopal and Sudheer, 2007). Among the activities of CL, a strong anti-inflammatory activity has been reported in dogs (Colitti et al., 2012; Comblain et al., 2015).

Echinacea angustifolia (EA) is a perennal herb belonging to Asteraceae family, that includes 9 species, but only *E. pallida*, *E. angustifolia* and *E. purpurea* have shown pharmacological properties. American Indians were the first to use *Echinacea* species for many different ailments, including cough, sore throat and tonsillitis (Caruso and Gwaltney, 2005).

An extensive description of *Echinacea* genus was made in a study for EFSA (European Food Safety Authority) evaluating its use as additive in animal production. In particular, the report concerned *Echinacea*' chemical active constituents, pharmacology, toxicology and efficacy either in human than in veterinary studies (Franz et al., 2005). Most relevant *Echinacea* active constituents, principally found in the roots, are echinacoside, cichoric and chlorogenic acids, caffeic acid derivatives and different polysaccharides like fructans, xyloglucans and heteroxylenes. Among the 3 species, *E. angustifolia* shows the highest concentration in phytochemicals, in particular echinacoside content, absent in the others (Senchina et al., 2006; Zhai et al. 2007). Known immune modulatory effects of EA are the activation of macrophages (Barrett, 2003) and polymorphonuclear granulocytes (Farinacci et al., 2009), resulting in an increased phagocytic activity. This immune stimulation activity of EA can be used not only to cope with infectious diseases but also when stressful events temporary lowering immune defenses (Sgorlon et al., 2012).

Silybum marianum (SL), also known as milk thistle, is a biennial plant of the Asteraceae family, which can be typically found in middle-south European regions, where it grows spontaneously. The main active component of SM is silymarin, a phytocomplex comprising different flavonoids among which the most important are silybin, isosilybin, silychristin and silydianin, concentrated in fruits and seeds (Pradhan and Girish, 2006). Silymarin has several beneficial actions useful in the treatment of hepatobiliary disease, including antioxidant, anti-inflammatory, and antifibrotic properties (Trappoliere et al., 2009; Verma and Thuluvath, 2007), also in dogs (Vandeweerd et al., 2013).

The present study reports the results of a dietary intervention study which involved the phytocomplexes of VM, CL, EA and SM. Pure extracts were added to a semi moist complete diet and fed for 60 days to dogs to assess the effects on plasma biochemical parameters and target gene expression in the peripheral leukocytes.

2. Materials and methods

2.1. Dog recruitments and allocation to groups

All dogs were adult and were recruited in one shelter and in three kennels. The shelter (S0) was a private Dog Shelter under regular veterinary monitoring for clinical diseases and therapeutic protocols and the dogs were for at least from 1 year hosted in the shelter. Kennel 1 (K1) was a private kennel, with resident utility and defense working dogs. Kennel 2 (K2) was a training center, where owners regularly carried dogs for education and to reinforce physical conditions. Kennel 3 (K3) was a facility for hunting dogs breeding, with some animals regularly involved in hunting sessions. Dogs were under the control of the respective veterinarian and all data were obtained from medical records, including a standard complete physical examination performed by the

veterinarians, which were asked to follow the dogs during the study. All dogs had to fulfill the following criteria: aged >2 years, medium to large size, resident in the shelter or attending regularly the kennels for at least 1 year.

Within each site, the dogs with no evidence of clinical diseases for at least 1 year and without any therapeutic protocol for at least 6 months were considered healthy and randomly allotted to the control group (CTRL) or to *Echinacea angustifolia* (Polinacea® - EA) group. Exclusion criteria were the presence of infectious and parasitic diseases, systemic, neurological or traumatic diseases or general symptoms of intolerance/allergy to nutraceutical diets administered in this dietary intervention study. Dogs actively involved in training were allocated to *Vaccinium myrtilus* (VM) group, those with history of arthrosis were allocated to *Curcuma longa* group (CL) and those suffering from liver diseases to *Silybum marianum* group (SM). At the end, according to dogs clinical conditions and history and owners availability, a total of 74 dogs were enrolled (Table 1), 23 hosted in the shelter (S0), 14 resident in K1, 25 resident in K3 and 12 regularly attending K2. Crossbreeds were the most representative individual in S0 (18 dogs), whilst in K1 and K2 the majority of dogs were German Shepherd (11 dogs in K1 and 5 in K2) and in K3 English Setter (21 dogs). Age, breed, sex and live weight at the beginning of the study of all the dogs are reported in the Supplementary Table 1.

2.2. Dietary Intervention Study

For the study, 5 diets were formulated to satisfy the nutrients requirements according to NRC (2006) indications and produced in a form of semi moist food. For the formulation, energy, protein, linoleic acid, eicosapentaenoic acid, mineral and vitamins requirements were considered. All the 5 diets contained the same ingredients with the same proportions (Table 2), the only variation being the inclusion of minerals and vitamins of the different diets according with the nutraceuticals supplementation and the specific clinical conditions. The commercial name of nutraceuticals, main active compounds, purity and daily dose (mg/kg live weight) added to the experimental diets are reported in Table 3. The nutraceuticals were purchased from Indena (Indena Spa, Milan, Italy).

From the beginning of the study, within each site dogs were fed with different commercial diets. Starting from 15 days before the beginning of the study, they were fed with the control diet (CTRL), consisting of a semi moist canned food without inclusion of nutraceuticals. After this adaptation period, each group received a different diet, as indicated in Table 1, for 60 days. The amount of diet offered was calculated on the basis of the live weight.

At the beginning (T0) and end (T60) of the study, blood samples were collected in the morning just before meal from the cephalic vein in 5 ml vacuum tubes with Li-heparin (Venoject, Terumo Europe N.V., Leuven, Belgium) and in PAXgene Blood RNA System tube (Preactix, Hombrechtikon, Switzerland), for biochemical analysis and RNA extraction, respectively.

For biochemical analysis, blood was immediately refrigerated to 6–8 °C, transported to the laboratory within 2 h and centrifuged at

Table 1
Number of dog allotted for the treatments in each of the four sites of study.

Group	S0	K1	K2	K3	Total
CTRL	7	4	6	4	21
VM	–	4	4	5	13
CL	6	3	–	9	18
EA	5	–	2	7	14
SM	5	3	–	–	8
Total	23	14	12	25	74

CTRL = control; VM = *Vaccinium myrtilus*; CL = *Curcuma longa*; EA = *Echinacea angustifolia*; SM = *Silybum marianum*.

S0 = shelter; K1 = Kennel 1; K2 = Kennel 2; K3 = Kennel 3.

Table 2

Ingredients and composition of the moist food used fed to the dogs during the study; for the treated group, extracts were included in the supplement.

Item	Unit	Value
Ingredient		
Water	% As Fed	40.0
Chicken meat	"	14.0
Rice	"	8.0
Carrot, dehydrated	"	6.0
Turkey meat	"	6.0
Fish, herring	"	5.0
Oil, sunflower	"	3.0
Venison meat	"	3.0
Pork liver	"	3.0
Goose meat	"	3.0
Supplement	"	2.5
Oat flakes	"	2.0
Potatos flakes	"	2.0
Eggs, dehydrated	"	2.0
Fish oil	"	0.5
Composition		
Water	%	60.3
Protein	%/DM	26.6
Fat	"	19.0
Crude fiber	"	2.2
Ash	"	3.5
Starch	"	35.0
Metabolizable energy	Kcal/kg DM	4200

DM = dry matter.

3000 rpm for 15 min. Plasma was aliquoted to 1.5 ml vials and frozen at -20°C until analysis. Blood collected in PAXgene blood RNA System tubes was incubated at room temperature for 2 h for RNA stabilization and then stored at -80°C until analysis.

All the procedures and sample collections were in compliance with the National legislation (Law 116/91) and internal ethical guidelines down by the University of Udine as well as Public Veterinary Service at the time the study was performed.

2.3. Biochemical and gene expression analysis

Urea, glucose, cholesterol, triglycerides, uric acid, creatin-kinase (CK), alanine transferase (ALT/GPT), aspartate transferase (AST/GOT), gamma glutamil transferase (gGT) were analysed using a Roche Cobas® 6000 analyzer with proprietary kits (F. Hoffmann-La Roche AG, Basel Switzerland). Plasma Zn was analysed with the Sentinel kit (17640H, Sentinel CH. SpA, Milan, Italy). Plasma paraoxonase (PON) activity was measured by adapting the method of Ferré et al. (2002) to the ILAB 600 and ceruloplasmin was analysed according to the Cerón and Martínez-Subiela (2004) method.

For genes expression evaluation, RNA was extracted from the PAXgene blood RNA System tubes, following the manufacturer's

Table 3

Characteristics of the nutraceuticals and daily dose (mg/kg of live weight) administered to the dogs.

Group	Latin name	Active compound		Daily dose of extract
		Name	% in the extract	
VM	<i>Vaccinium myrtillus</i>	Anthocyanidin	1	20
CL	<i>Curcuma longa</i>	Curcumin	20	33
EA	<i>Echinacea angustifolia</i>	Echinacoside	2	5
SM	<i>Sylibum marianum</i>	Sylibin	15	10

VM = diet supplemented with extracts of *Vaccinium myrtillus*; CL = diet supplemented with extracts of *Curcuma longa*; EA = diet supplemented with extracts of *Echinacea angustifolia*; SM = diet supplemented with extracts of *Sylibum marianum*.

instructions. RNA concentration was measured with a spectrophotometer (NanoDrop 1000 ThermoScientific, Wilmington, DE, USA), resulting in a purity of 1.8–1.9. RNA integrity was evaluated through the observation of 18S and 28S ribosomal bands after electrophoresis on 1% agarose gel, in the presence of GelRed (Biotinum, Aurogene, Rome, Italy) and further checked using an Agilent Bioanalyser and the RNA nano Lab chip kit (Bioanalyser Nanochip QC, USA).

A Primer3 Input software (Rozen and Skaletsky, 2000) was used to design the primer sequences encoding for actin beta (*ACTB*), mitochondrial ribosomal protein S7 (*MRPS7*), C-X-C motif chemokine ligand 8 (*CXCL8*), nuclear factor kappa B subunit 1 (*NFKB1*), mitochondrial superoxide dismutase 2 (*SOD2*), prostaglandin-endoperoxide synthase 1 (*PTGS1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), peroxisome proliferator activated receptor gamma (*PPARG*), interferon gamma (*IFNG*), nitric oxide synthase 2 (*NOS2*), BCL2 associated X protein (*BAX*), BCL2 like 1 (*BCL2L1*), B-cell CLL/lymphoma 2 (*BCL2*), nuclear receptor subfamily 3 group C member 1 (*NR3C1*), nuclear receptor subfamily 3 group C member 2 (*NR3C2*), cytochrome P450 family 11 subfamily B member 2 (*CYP11B2*), cytochrome P450 family 21 subfamily A member 2 (*CYP21A2*), cytochrome P450 family 3 subfamily A member 4 (*CYP3A4*), glutathione S-transferase alpha 3 (*GSTA3*) and Tumor Necrosis Factor alpha (*TNF*). Primers were designed on *Canis lupus familiaris* sequences, based on UniGene (NCBI; build number 13). Primers and product lengths for each gene are listed in Table 4 according to the HUGO Gene Nomenclature Committee (HUGO Gene Nomenclature Committee at the European Bioinformatics Institute <http://www.genenames.org/>).

2.4. Reverse transcription

Reverse transcriptions were performed with 400 ng of total RNA using Improm-IITM Reverse Transcriptase system (Promega, Milan, Italy). Total RNA samples with 1 μl random hexamers (0.5 $\mu\text{g}/\mu\text{l}$ MBI Fermentas, Italy) and free nuclease water to a final volume of 20 μl were incubated at 70°C for 5 min in a PTC-100TM thermocycler (MJ Research Inc. Waltham, MA USA). Then, a mix was prepared with 4 μl of Improm-II Reverse Transcriptase buffer (5X Promega, Milan Italy), 2.4 μl MgCl_2 (50 mM), 1 μl of Improm-II Reverse Transcriptase and 1 μl of dNTP (10 mM) was added to the reaction and incubated at 37°C for 90 min and finally at 94°C for 5 min.

The final cDNA concentration was assumed as 20 ng/ μl .

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

An aliquot of each cDNA samples were pooled and standard curves with serial dilution of pool were used for each gene to optimize PCR conditions and to calculate efficiency, fluorescence baseline and threshold. The qRT-PCRs were performed in triplicate form using Platinum® SYBR® Green qPCR SuperMix-UDG (InvitrogenTM, Milan Italy). For these reactions, a master mix with the following components was prepared to the indicated end concentration: 0.5 μl of cDNA, forward and reverse primers, 6.25 μl of 2X Platinum SYBR Green qPCR SuperMix-UDG and water to a total volume of 12.5 μl . cDNA concentrations and primers molarities were different for each gene and determined with standard curves analyses performed before RT-PCR reactions. The PCR amplifications were conducted applying 40 cycles (10 s at 95°C , 30 s at the specific annealing temperature, 30 s at 72°C) in a 96-well spectrofluorometric thermal cycler (CFX-96TM, BioRAD, Hercules, California). The melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected.

The expression of target genes was normalized using the *ACTB* and *MRPS7* genes and ΔCt s were calculated by the difference between Ct of genes target and the geometric mean of the two housekeeping genes. The expression level of a given target gene in each experimental group was analysed by the $2^{-\Delta\Delta\text{Ct}}$ method (Bustin et al., 2009; Livak and

Table 4
Primer pair sets and parameters used in the real time PCR analysis.

Gene	Genbank accession number	Sequence (5' → 3')	Product length, bp	Ta (°C)
<i>ACTB</i>	5597004	For: ACTGGGACGACATGGAGAAG Rev: AAGCGTACCCTCGTAGAT	280	59
<i>MRPS7</i>	73965035	For: CGCAAC CCCTATGTCATCTT Rev: CAACTTCTCTGGCAGCAGCA	201	57
<i>CXCL8</i>	415265	For: TCTGGCAGCTTTTGTCTT Rev: GGGCCACTGTCAATCACTCT	151	57
<i>NFKB1</i>	50080137	For: TACCCCGAGGTCAAATCTG Rev: CTCTGTCATTCGTGCTTCCA	237	57
<i>SOD2</i>	50978897	For: CTAAGGGTGGTGGAGAACCA Rev: AAGCGTCCCTGCTCTTATT	240	58
<i>PTGS2</i>	13641174	For: ATCCCTTCTCTGCGAAATAC Rev: CATCAGGTACAGGGGAAGA	160	57
<i>PTGS1</i>	402743309	For: TGCTCATGCGTCTGGTACTC Rev: GTCTGGCAACTGCTTCTTCC	188	57
<i>PPARG</i>	103472123	For: GGATTCTCCAGCATTTCCA Rev: GAGTTGGAAGGCTCTTCGTG	195	57
<i>IFNG</i>	50978905	For: CGGTGGTCTCTTTCTGTAG Rev: TCCCTCTACTGGTGTCTGT	198	59
<i>NOS2</i>	925115237	For: GGAGGAGCAGTACTGTTGG Rev: GTCATGAGCAAAGGCACAGA	178	57
<i>BAX</i>	27372189	For: CATGGAGTTGAGAGGATGA Rev: CCTTGAGCACCAGTTTGTCT	152	57
<i>BCL2L1</i>	50978741	For: ACCTGACATCCCAGCTTCC Rev: CGATCCGACTCACCAATACC	180	59
<i>BCL2</i>	50950156	For: ATGTGTGTGGAGAGCGTCAA Rev: CCTTCAGAGACAGCCAGGAG	188	59
<i>NR3C1</i>	730321601	For: TCTGCCTCGTGTGTCTGAC Rev: GCTTCCAAGTTTATCCCAAGC	214	58
<i>NR3C2</i>	928154864	For: GTCACCATCTGGAGAGCAT Rev: CAGCTCAAGGCAAATGATGA	233	57
<i>CYP11B2</i>	545521058	For: CCTTGTGGATGACTCTGT Rev: GGTAGTTCTGAGCACCACG	223	57
<i>CYP21A2</i>	55742761	For: GAGCACTGGTCCATCCAAAT Rev: GTGTCCTTCGAGGAGCTGT	237	56
<i>CYP3A4</i>	928136586	For: TACCTGCCTTTTGGAACTGG Rev: ACAATGGGCTTTTCAGGTTG	176	58
<i>GSTA3</i>	23452498	For: ATGCCACCTGATCAAAAAG Rev: CTTACAGCAGGGGAAGTTGG	214	57
<i>TNF</i>	167765472	For: ACCACACTCTTCTGCCTGCT Rev: CTGGTTGTCTGTCAGTCCA	219	58

For, forward; Rev, reverse; Ta, annealing temperature.

Schmittgen, 2001) where the $2^{-\Delta\Delta Ct}$ represents the difference of a given target gene between each treated group (CTRL, VM, CL, EA and SM) at T0 vs CTRL group at T0. The same analysis was done at T60. The *n*-fold expression of a given target gene was calculated as $\log(2)$ (ratio).

2.6. Statistical analysis

Statistical analysis of biochemical data were analysed using a mixed model, with fixed effect of time of sampling (T0 and T60), treatment (CTRL, VM, CL, EA and SM) and their interaction, with animal repeated within time and the random effect of site of sampling (S0, K1, K2 and K3). Estimated marginal means were calculated and reported in the tables. For expression data, a general linear model was used, with fixed effect of treatment (CTRL, VM, CL, EA and SM) and random effect of site of sampling. The SPSS (SPSS, 1997) package was used for computations and means were considered to significantly differ for $P < 0.05$.

3. Results

During the experimental period animals ingested the complete diets without refusals, allowing optimal feeding conditions and availability of nutrients. The biochemical parameters that resulted significantly different between the 5 groups or between days of sampling are reported in Table 5.

The activity of ALT/GPT was higher ($P < 0.01$) in the SM group than in the other groups and at T0 in comparison to T60 ($P < 0.01$). Effect of the time of sampling was also evident, with a significantly lower activity of the enzyme at T60 ($P < 0.01$). The interaction of the fixed effects was also highly significant ($P < 0.01$), as the ALT activity only in the SM group decreased from T0 to T60. The mean values of PON did not show significant variation between treatments, whilst significantly increased ($P < 0.01$) at T60 in comparison to T0. A significant interaction was also observed ($P < 0.01$), since in the VM group the PON values increased at T60 in comparison to T0. The CTRL and SM groups showed lower values of CuCp ($P < 0.01$) in comparison to VM, CL and EA groups, and the means at T0 were higher ($P < 0.01$) than at T60. Also for this variable, the interaction was highly significant ($P < 0.001$) and the mean values in the VM, CL and EA groups were lower at T60 than at T0. The plasma concentration of Zn was not affected by treatment, but increased from T0 to T60 ($P < 0.01$). A significant effect of the interaction was evident only in CL and EA groups, where the mean Zn values were higher ($P < 0.01$) at T60 in comparison to T0. No significant effect of time of sampling and treatments were calculated for urea, glucose, cholesterol, triglycerides, uric acid, CK, AST/GOT and gGT.

The *n*-fold values for the genes that resulted significantly different between the 5 groups are reported in Table 6. In comparison to the CTRL group, a significant ($P < 0.05$) down regulation of *TNF* was observed in VM, CL and EA groups; instead the mean value of *TNF* in SM group was not different from the other groups. The same significant differences between groups were reported for *NFKB1* ($P < 0.01$); instead,

Table 5

Estimated means for the biochemical parameters measured in the plasma of dogs at the beginning (T0) and at the end (T60) of the dietary intervention study to assess the effect of nutraceutical administrations.

Item	ALT		PON	CuCp	Zn			
	U/l	mg/l						
Treatment								
CTRL	45.8	B	134.7	NS	1.23	B	26.0	NS
VM	35.9	B	133.7	NS	1.67	A	24.8	NS
CL	39.2	B	139.3	NS	1.78	A	23.2	NS
EA	42.9	B	134.6	NS	1.54	A	27.9	NS
SM	77.0	A	118.6	NS	1.20	B	24.7	NS
Day of sampling								
T0	54.7	A	126.2	B	1.72	A	23.0	B
T60	41.6	B	138.1	A	1.25	B	27.6	A
Interaction								
CTRL – T0	48.7	B	130.0	CD	1.22	CD	25.7	B
CTRL – T60	42.9	BC	139.5	ABC	1.24	CD	26.4	B
VM – T0	36.9	C	114.6	E	1.86	B	24.7	BC
VM – T60	35.0	C	152.7	A	1.48	C	24.9	BC
CL – T0	39.7	BC	145.5	AB	2.22	A	19.4	C
CL – T60	38.7	BC	133.1	BC	1.33	CD	26.9	B
EA – T0	43.6	BC	128.9	CDE	1.98	AB	21.5	BC
EA – T60	42.3	BC	140.3	ABC	1.11	D	34.2	A
SM – T0	104.7	A	111.8	E	1.33	CD	23.8	BC
SM – T60	49.3	B	125.4	CDE	1.08	D	25.7	B
Effects, sig of P								
Treatment	***	NS	***	NS	***	NS	***	NS
Day of sampling	***	***	***	***	***	***	***	***
Interaction	***	***	***	***	***	***	***	***
Random effect of site	NS	NS	NS	NS	NS	NS	NS	NS
MSE	297.5	468.0	0.16	68.52				

CTRL = control group; VM = diet supplemented with extracts of *Vaccinium myrtillus*; CL = diet supplemented with extracts of *Curcuma longa*; EA = diet supplemented with extracts of *Echinacea angustifolia*; SM = diet supplemented with extracts of *Sylibum marianum*; ALT = alanine transferase; PON = paraoxonase; CuCp = ceruloplasmin; Zn = zinc.

***P < 0.001; **P < 0.01; A, B, C, D, E on the same column denote significant differences for P < 0.001 or P < 0.01; NS: not significant.

CXCL8 expression was significantly (P < 0.05) down regulated in VM and CL groups and up regulated in EA group in comparison to CTRL group. The relative gene expression of PTGS2 in VM and CL groups was significantly down regulated (P < 0.01) in comparison to EA and CTRL groups. The n-fold values in SM group significantly differ only from CL group. An up-regulation of SOD2 was evident in VM, CL and SM groups (P < 0.05) in comparison to CTRL group and the EA group was significantly different only from CL and SM groups. For all the measured gene expressions, no significant effect of site of sampling was observed.

Table 6

Gene expressions in the white blood cells of dogs at the end (T60) of the dietary intervention study to assess the effect of nutraceutical administrations. Values are reported as log₂(n-fold) in comparison to the beginning of the study (T0).

Treatment	TNF Mean	se		CXCL8 Mean	se		NFKB1 Mean	se		PTGS2 Mean	se		SOD2 Mean	se	
CTRL	0.58	0.32	a	0.14	0.46	b	0.06	0.34	A	0.32	0.41	A	0.23	0.40	c
VM	-0.60	0.38	b	-1.86	0.54	c	-1.62	0.39	B	-1.72	0.48	BC	1.47	0.47	ab
CL	-1.02	0.34	b	-1.96	0.46	c	-2.47	0.35	B	-2.12	0.43	C	1.64	0.42	a
EA	-0.79	0.39	b	2.68	0.54	a	-1.62	0.41	B	0.46	0.50	A	0.59	0.48	bc
SM	-0.05	0.52	ab	-0.45	0.71	bc	-0.20	0.54	AB	-0.16	0.66	AB	2.21	0.64	a
Effect															
Treatment	*			*			***			***			*		
Site	NS			NS			NS			NS			NS		

CTRL = control group; VM = diet supplemented with extracts of *Vaccinium myrtillus*; CL = diet supplemented with extracts of *Curcuma longa*; EA = diet supplemented with extracts of *Echinacea angustifolia*; SM = diet supplemented with extracts of *Sylibum marianum*.

TNF = tumor necrosis factor alpha; CXCL8 = C-X-C motif chemokine ligand 8; NFKB1 = nuclear factor kappa B subunit 1; PTGS2 = prostaglandin-endoperoxide synthase 2; SOD2 = mitochondrial superoxide dismutase 2.

*P < 0.05; ***P < 0.001; a, b, c on the same column denote significant differences for P < 0.05; A, B, C on the same column denote significant differences for P < 0.01. NS: not significant.

The effect of treatments was not significant for PTGS1, PPARG, IFNG, NOS2, BAX, BCL2L1, BCL2, NR3C1, NR3C2, CYP11B2, CYP21A2, CYP3A4 and GSTA3 expressions.

4. Discussion

Research with dogs has some critical points, since it is not easy to find large number of individuals homogeneous for breed, age, sex, feeding regimes and environmental conditions within one location. Another aspect is the need to reduce the number of animals for experiments (3R Regulation, Directive 2010/63/EU), a common problem for all the animals, but especially in the case of companion animals. For these reasons, often the study with companion animals is more an observational study, in which the variables are not under complete control for the ethical and logistic issues reported above. In the present study, to control the residual variability we used a multicenter randomized design, by allocating a control group in each of the 4 locations and applying a mixed model including the random effect of the locations. Due to the very limited number of studies published about the use of these compounds in dogs, and to the limitations imposed by ethical constraints to perform bioavailability study and dose response experiments, the amount of extracts administered to the animals corresponded to the dosage recommended by the supplier (INDENA, Milan, Italy) for humans. Moreover, SM and CL were provided in form of phytosome complex, to enhance bioavailability of active compounds (Filburn et al., 2007; Kidd, 2009).

Anthocyanins chemical group, responsible for the typical bilberry' color, represents the main active compound contained in this fruit, which have a well-recognized antioxidant property (Kähkönen et al., 2003). Among berries, bilberry or *Vaccinium myrtillus* specie is the richest in anthocyanins, furthermore showing the most effective antioxidant activity (Nakajima et al., 2004). A wide range of bilberry extract dosages has been used in humans (Monograph, 2001) and the only dietary intervention study in Alaskan Husky dogs only reported a daily supplementation of 20 g of raw blueberries (*Vaccinium angustifolium*) for 2 months (Dunlap et al., 2006). No indication of active compounds concentration was reported, but the supplementation resulted effective in blood antioxidant activity after dog exercise. The significant increase of PON and the up regulation of SOD2 observed in the present study support the antioxidant effect of bilberry extract, as also reported in human (Nile and Park, 2014). Besides to the antioxidant activity, bilberry also demonstrated anti-inflammatory properties in macrophages, down regulating the expression of IL1b, PTGS2, TNF, CCL22 (Chen et al., 2008) and NFKB (Lee et al., 2014). This anti-inflammatory activity is confirmed by the significant down regulation of TNF, CXCL8, NFKB1 and PTGS2 and by the significant decrease of the positive acute phase protein CuCp.

In vitro and in vivo animal studies have demonstrated the ability of various Echinacea preparations to enhance the activities of immune

cells. Activity was found for hydrophilic and lipophilic extracts and fractions (Franz et al., 2005). Echinacea immunomodulatory activity displays 2 main routes, being the first the modulation of immunocompetent cells and the second a cellular anti-inflammatory activity, especially during bacterial or viral infections (Hudson et al., 2011).

In dogs, the only study on immune modulatory effect of Echinacea showed that dietary treatment with this plant extract caused an increase of phagocytic activity and IgM (Torkan et al., 2015). However, the authors did not report any information about the species of Echinacea and the part of the plant used in the trial. Furthermore, the placebo and the experimental groups before treatment showed significantly different means for these variables. Studies in humans (Guiotto et al., 2008) demonstrated a dose dependent down regulation of *Echinacea purpurea* on pro-inflammatory cytokines *CXCL8*, *IL6*, *IL10* and *TNF*. A significant upregulation of *IL8* subsequent to treatment with Polinacea® was reported in neutrophils by Farinacci et al. (2009) and in humans by Dapas et al. (2014). The chemokine *IL8* was significantly upregulated also in *ex-vivo* stimulated human blood cells obtained from volunteers orally treated with *Echinacea purpurea* (Ritchie et al., 2011). In the present study, the administration of Polinacea® led to an upregulation of *CXCL8*, which is a strong chemoattractant and angiogenic factor. This result suggests a positive role of EA in the stimulation of the immune system promoting neutrophil activation possibly through the up regulation of *CXCL8* mRNA levels. The anti-inflammatory activity of EA, at least in healthy dog, is confirmed in the present study, by the significant down regulation of *TNF* and *NFKB1* and by the significant decrease of the positive acute phase protein CuCp and by the increase of plasma Zn levels. As reported by Mariani et al. (2006), circulating Zn levels are inversely correlated to *IL6*, *CXCL8* and *TNF*.

Therapeutic uses suggested for curcumin refer to a huge range of biological activities, like cancer chemopreventive, antitumor and neuroprotective, primarily based on its recognized antioxidant and anti-inflammatory properties (Oliveira et al., 2015). Curcumin is known to protect cell membranes against the peroxidative damage caused by Reactive Oxygen Species (ROS) produced during cellular breathing. Lipid peroxidation is known to be a free-radical-mediated chain reaction leading to cell membrane damages but primarily to oxidative stress, which can result in severe metabolic dysfunctions, included inflammation (Venugopal and Sudheer, 2007). The antioxidant activity of curcumin has been observed in neutrophil cells during inflammatory activation with PMA (phorbol myristate acetate), with a suppression of superoxides production and inhibition of cell adhesion, events associated with the setting up of the inflammatory response (Farinacci et al., 2009). In the present study, the CL dogs at T0 showed a significant higher CuCp and lower Zn concentrations in comparison to CTRL, supporting the diagnosis of arthritis. The anti-inflammatory activity of curcumin is confirmed, since after 60 days of CL administration, the mean values of these parameters were similar to the CTRL group. The significant down regulation of *TNF*, *CXCL8*, *NFKB1* and *PTGS2* observed after 60 days of CL supplementation corroborates the effectiveness of curcuminoids in reducing the inflammatory process. These results agree with those of Colitti et al. (2012) obtained in dogs affected by osteoarthritis supplemented with curcumin or a NSAIDS PTGS2 selective inhibitor. In that study, dogs received daily 4 mg or 5 mg/kg body weight of curcumin or NSAIDS respectively, for 20 days. As expected both molecules significantly down regulated *TNF* gene expression, but only curcumin acted as a suppressor of genes related to macrophages proliferation. Moreover, CL up regulated the I κ B kinase enzymatic complex, responsible for the *NFKB* inactivation by trapping it in the cytoplasm of cells. The clinical outcome of the 18 dogs after 60 days of CL was considered in terms of absence of pain to palpation, voluntary activity, gait and stiffening of the leg, in comparison to T0. Considered the limited number of subjects, no statistical analysis was carried out, but interestingly for 10 dogs symptoms were reduced and in 2 disappeared; no anti-inflammatory drugs were required in the period of the study. These clinical data confirm those previously reported

(Colitti et al. 2012) using the same standardized extract of *Curcuma longa*.

Silymarin has several beneficial actions useful in the treatment of hepatobiliary disease, including antioxidant, anti-inflammatory, and antifibrotic properties (Pradhan and Girish, 2006; Verma and Thuluvath, 2007). In an extensive clinical review on Silymarin's properties Wellington and Jarvis (2001) explained which are the different mechanisms involved in its biological activity. They indicated the increasing in the synthesis of ribosomal RNA (rRNA) species through stimulation of polymerase I and rRNA transcription (thereby increasing the synthetic rate of structural and functional proteins), the blockage of the uptake of toxins such as α -amanitin from *Amanita phalloides* (the deathcap mushroom), and the protection of the cell membrane from osmotic stress and radical-induced damage. In the present study, the group of dogs allotted to SM treatment suffered from liver diseases as also confirmed by higher plasma ALT activity at T0 (Table 5). The significant reduction of this enzyme activity at T60 is consistent with the well-known liver protectant activity, which was observed also by Fehér et al. (2015) and Liu et al. (2013). According to Kidd (2009), another important function of SM is the antioxidant activity, conserving glutathione in liver cells and protecting cell membranes against reactive oxygen species. PON-1 is an important serum enzyme synthesized by the liver involved in lipid metabolism, membrane stability and control of oxidative stress (Holvoet, 2008; Varzi et al., 2007). Studies in dogs infected with ehrlichiosis (Karnezi et al., 2016) and with induced endotoxiemia (Tvarijonavičiute et al., 2012) have reported a reduction of PON activity and an increase of positive acute phase protein. The significant increase of PON after SM administration, observed in the present study, supports the antioxidant activity of silymarin that is corroborated by the significant up regulation of mitochondrial *SOD2*. This is one of the main physiological defense mechanisms against free radicals, which is subject to depletion during a period of toxic substance overload (Holvoet, 2008). It was reported that *in vitro* incubation with silymarin in a concentration equivalent to the usual therapeutic dosage markedly increased the SOD in lymphocytes of patients with alcoholic cirrhosis (Abenavoli et al., 2010). However, the limited number of dogs in this group does not allow to draw conclusions from a clinical point of view, but biochemical and molecular outcomes suggest the usefulness of this nutraceuticals in the hepatopathies.

In conclusion, present data indicated that the nutraceutical administration in dogs can be an interesting approach to modulate immune response in order to improve health condition of animals. In particular, VM and CL showed a significant anti-inflammatory activity and, in addition, VM displayed antioxidant properties. The results of EA confirm the known immune system modulation. SM showed significant antioxidant and liver protectant activities. On the basis of these preliminary results the tested compounds can be claimed for their biological activity in dogs, in petfood formulation.

Supplementary data to this article can be found online at doi:10.1016/j.rvsc.2016.10.005.

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