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Experimental addition of *Eleutherococcus senticosus* and probiotic to the canine diet

Research Article

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Abstract: There is a current trend to support pet health through the addition of natural supplements to their diet, taking into account the high incidence of medical conditions related to their immune system and gastrointestinal tract. This study investigates effects of the plant *Eleutherococcus senticosus* as a dietary additive on faecal microbiota, faecal characteristics, blood serum biochemistry and selected parameters of cellular immunity in healthy dogs. A combination of the plant with the canine-derived probiotic strain *Lactobacillus fermentum* CCM 7421 was also evaluated. Thirty-two dogs were devided into 4 treatment groups; receiving no additive (control), dry root extract of *E. senticosus* (8 mg/kg of body weight), probiotic strain (10^8 CFU/mL, 0.1 mL/kg bw) and the combination of both additives. The trial lasted 49 days with 14 days supplementation period. Results confirm no antimicrobial effect of the plant on the probiotic abundance either *in vitro* (cultivation test) or *in vivo*. The numbers of clostridia, lactic acid bacteria and Gram-negative bacteria as well as the concentration of serum total protein, triglyceride, glucose and aspartate aminotransferase were significantly altered according to the treatment group. Leukocyte phagocytosis was significantly stimulated by the addition of probiotic while application of plant alone led to a significant decrease.

Keywords: *Probiotics • Lactobacillus fermentum • Eleutherococcus senticosus • Gut microflora • Serum biochemistry • Cellular immunity*

1. Introduction

The incidence of special health problems in dogs has shown a rapid increase in recent years. Veterinary Pet Insurance analysis reported ear infections, skin allergies and infections, gastritis and enteritis as the top five canine medical conditions of 2010 [1]. Many factors may be involved (medical drugs and vaccines, environmental pollutants, lack of exercise, excess stress, genetic factors *etc.*). However, inappropriate nutrition could probably be considered as the critical cause since the gastrointestinal (GI) system plays a central role in immune system homeostasis necessary to prevent the mentioned diseases. The importance of immune modulation at the GI level can be understood easily, considering that approximately 70% of the entire immune system is found at this site and that in the lamina propria there are about 80% of all plasma cells responsible for IgA antibody production [2,3]. Neverless, the ageing process impaires cell-mediated immunity as was observed also in dogs [4].

The use of bioactive plant species as a dietary additive alone or combined with probiotic microorganisms could be helpful in the prevention of impaired immune functions, although scientific knowledge on their effects in canine organism is scarce [5-7]. Despite that, most recent surveys show an upward trend in using phytoproducts by veterinarians (e.g. three-quarters of the veterinarians in Austria, Germany and Switzerland, [8]).

The medicinal herb *Eleutherococcus senticosus* (Rupr.& Maxim.) Maxim. (Araliaceae) is an approximately two-meter high, thorny shrub native to the far eastern areas (China, Korea, Japan, Russian Far East) where it is commonly referred to as ciwujia. This plant, known

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as an adaptogen, has been shown to have a wide range of other pharmacological effects as observed in studies in humans or mice: antistress, antioxidant, antitumor, immunostimulatory, antiinflammatory, antipyretic, hypocholesterolemic, hypoglycemic, choleretic and radioprotectant activity [9-11]. The active compounds isolated from *E. senticosus* include phenylpropanoids (e.g syringin, caffeic acid, sinapyl alcohol, coniferyl aldehyde), lignans (e.g. sesamin, syringoresinol and its glucoside), saponins (e.g. daucosterol, b-sitosterol, hederasaponin B), coumarins (e.g. isofraxidin and its glucoside), vitamins (vitamin E) and provitamins (β-carotene, [9]).

The current trend of feeding dogs by commercial food products with reduced microbial populations (heated above 100°C) results in an insufficient microbial exposure which is required for the induction of immune mechanisms. Probiotic supplementation of canine diet may provide a promising alternative to promote an effective gut defense barrier. Probiotic microorganisms display a wide range of positive effects although strain specific, but overall include a trophic action on the intestinal mucosa (SCFA, vitamin and enzymes production), competitive exclusion of enteric pathogens, production of pathogen inhibitory substances (lactic acid, bacteriocin, *etc*.), inhibition of microbial toxin action, neutralization of dietary carcinogens, antioxidant activity and immunomodulation [12,13]. Some probiotic strains reduce pathological alterations in paracellular permeability to large molecules or bacteria, degrade and modify the structure of antigen macromolecules, reduce mucus degradation, stimulate mucosal immunity and modulate the production of inflammatory mediators in the intestinal epithelium [14-16].

Since no investigation has been conducted on the influence of *E. senticosus* on the overall health status of dogs, we decided to test the effects of *E. senticosus* extract by oral application alone (ES group) and in combination with canine-derived probiotic strain *L. fermentum* CCM 7421 (ES+LF group) on the faecal microbiota, faecal characteristics, serum biochemistry and cellular immunity in healthy dogs. The study included the *L. fermentum* CCM 7421 animal group receving the probiotic strain alone (LF group).

2. Experimental Procedures

2.1. *In vitro* assay

The effect of *E. senticosus* extract on the growth of probiotic *L. fermentum* CCM 7421 strain was tested *in vitro* in order to be sure that the extract and the strain can be combined in the *in vivo* experiment. Dry root extract of *Eleutherococcus senticosus* (ethanolic extraction, dry matter 95±1%, ash 7.3±0.5%, purchased from Calendula, a.s., Nová Ľubovňa, Slovakia) was added to de Man-Rogosa-Sharpe broth (MRS, pH 5.58, Merck, Germany) in the amount of 1.0, 5.0, 10.0 or 20.0 g/L of broth. Each broth was inoculated (1% w/v) with an 18 h preculture and incubated at 37°C for 24 h aerobically. The pH and growth – CFU/mL (colony forming units) was determined on MRS agar plates (Merck) at time 0 and after 24 h of cultivation. The MRS broth inoculated with CCM 7421 strain and without extract addition served as the control.

2.2 Animals and diet

Healthy adult dogs (n=32; 18 females, 14 males) were randomly divided into four experimental groups, 8 animals in each. The age of the dogs ranging between 1-11 years (mean age 2.7±1.1) and body weights between 19.2 and 44.0 kg (mean BW 31.0±7.9 kg). They belong to the following breeds: German Shepherd n=20, Belgian Shepherd (Malinois) n=3, cross-breed n=3, Rottweiler n=2, Doberman n=2, German Shorthaired Pointer n=1, Rhodesian Ridgeback n=1. All experimental procedures were approved by the Ethic Commission of the Institute of Animal Physiology, Slovak Academy of Sciences (Košice, Slovakia). Dogs were housed individually in a whole environment, but covered, facility measuring 3.0x3.0 m with box 1.5x0.8 m (temperature, 10-15°C). They were fed and exercised individually and had access to fresh water at all times. They received a commercial, nutritionally complete, extruded dry dog food twice a day (Purina Pro Plan Dog Adult Medium Breed, Chicken & Rice formula, Netslé Purina PetCare Company, St. Louis, USA), which cointained (g/100g diet): crude protein 27.0, crude fat 17.0, crude fibre 2.0, ash 7.0, calcium 1.3, phosphorus 1.0 (sodium selenite 0.015 mg, copper sulphate 2 mg, vitamin A 2100 IU, vitamin E 17 IU, vitamin D3 160 IU, ascorbic acid 7 mg), 3580 kcal metabolizable energy/kg. Adaption period to this food was 4 weeks before experiment.

The experiment lasting 49 days was composed of a baseline (day 0), supplementation (day 1-14) and post-supplementation period (day 15-49). The animals were assigned to the following experimental groups: the control (C, n=8) without any treatment, the ES group (n=8) supplemented with dry root extract of *Eleutherococcus senticosus* (at a dose 8 mg/kg body weight), the LF group (n=8) supplemented with *L. fermentum* CCM 7421 (0.1 mL/kg BW, 10^8 CFU/mL of Ringer buffer, Merck, Germany), and the ES+LF group (n=8) fed the combination of root powder and a probiotic culture at the same doses as the previous groups. Dogs were supplemented with the appropriate additive daily

during the feeding time (for 14 days) and monitored for changes in clinical condition, vital parameters and appetite throughout the study.

2.3 Preparation of *L. fermentum* CCM 7421 for application to dogs

The probiotic characteristics of strain *L. fermentum* CCM 7421 (AD1, isolate from canine faeces) have been previously presented [7]. A rifampicin-marked strain of *L. fermentum* CCM 7421 was prepared by subsequent cultivation on MRS agar plates (Merck) supplemented with 100 µg/mL of rifampicin. The strain was then cultivated in MRS broth (Merck) at 37°C for 24 h. Cells were harvested after centrifugation (10 min at 2000x*g*) and the culture sediment was resuspended in Ringer buffer (Merck, pH 7.0) to a concentration of 10^8 CFU/mL. The solution was stable for 1 week at 4°C. Then it was replaced by a new cell culture.

2.4 Sampling procedures

Fresh faecal samples were collected at days 0 (pre-treatment), 7, 14 (treatment), 21, 28, and 49 (post-treatment period) during morning individual walking to ensure that faeces were correctly allocated to the proper animal. The determination of faecal score and pH measurement were perfomed immediately. Blood samples (from *vena cephalica antebrachii*) were collected at days 0, 14, 28, and 49 in plastic tubes containing (1) 10 UI heparin (20 µl/mL of blood) for phagocytic activity, glutathione peroxidase determination, (2) 1.5% EDTA (0.1 mL/mL of blood) for iodo-nitro-tetrazolium reductase test, and (3) without anticoagulant for determination of biochemical parameters. The dogs were not allowed access to food in the 16-hour overnight period prior to venipuncture. The blood samples for testing of biochemical parameters were centrifuged (15 min at 2500x*g*) after 30 min and sera frozen at –20°C.

2.5 Microbiological analysis

The samples of faeces (1 g) were mixed with sterile Ringer buffer (Merck, pH 7.0) and homogenized (3 min) using a stomacher (IUL, Instruments, Spain). Microbial populations were determined according to the standard microbiological method by serial dilution (10^{-1} to 10^{-7}). Aliquots of the dilutions (100 µL) were inoculated onto the following selective media: Mac Conkey agar for enumeration of *E. coli* (Becton and Dickinson, USA), Mannitol salt agar for staphylococci (Oxoid, UK), M-*Enterococcus* agar for enterococci (Becton and Dickinson), MRS agar for lactic acid bacteria (LAB, Becton and Dickinson), MRS agar with rifampicin (100 µg/mL) for *L. fermentum* CCM 7421, Pseudomonas agar (Biomark, India) for *Pseudomonas* sp., Aeromonas medium base (Oxoid) for *Aeromonas* sp., and *Clostridium difficile* agar base (Oxoid) for clostridia. Plates were incubated aerobically at 37°C for 24-48 h except for Aeromonas and Pseudomonas plates incubated at 25°C for 48-72 h. Clostridia were cultivated anaerobically (Bactron Anaerobic Chamber, Shel Lab, Sheldon Manufacturing Inc., USA, athmosphere composition 90% N_2 + 5% H_2 + 5% CO_2) at 38°C for 48 h. The results were expressed as $\log_{10}$ CFU per gram of moist faeces.

2.6 Faecal characteristics

Fresh faecal samples were visually scored (FS-faecal score) according to the following system: 1 = hard dry faeces; 2 = hard, formed stool; 3 = soft, formed, and moist stool; 4 = soft, unformed stool; 5 = watery liquid. Detection of pH (pH Meter, Hanna Instruments, USA) was performed immediatelly. Approximately 5 g of samples were stored at -20°C until analysis of dry matter (DM). Ammonia concentration was tested using an Ammonia Assay Kit (Sigma-Aldrich, USA).

2.7 Blood analysis

Biochemical parameters in blood serum were determined by colorimetric methods (Spectrophotometer UV-2550 Shimadzu, Japan) using kits (Randox Laboratories Ltd., UK) for the following parameters: total protein (TP 245), albumin (AB 362), urea (UR107), triglyceride (TR 210), cholesterol (CH 200), glucose (GL 2623), alanine aminotransferase (AL 100), aspartate aminotransferase (AS 147), calcium (CA590), inorganic phosphorus (PH 1016), glutathione peroxidase (RS 504). Phagocytic activity (PA) was tested microscopically after Pappenheim staining according the method of Šteruská [17] and expressed as percentage of phagocyte ingested yeast cells to the total number of phagocytes (100 PMNs were counted per sample). Measurement of tetrazolium reductase activity of phagocytes was performed according to the technique described by Lokaj and Oburková [18] and expressed as index of metabolic activity (IMA) based on the ratio of mean optical density (OD_{485}) of leukocyte suspension with starch (stimulated phagocytic cells) to the leukocyte suspension without the starch (negative control).

2.8 Statistical analysis

The results are expressed as the mean + standard error. Statistical analyses were performed with GraphPad Prism software (version 5.0). Data were analyzed for the the effects of treatment, time and treatment x time interaction using two-way analysis of variance (ANOVA, Bonferroni post-test) with the level of significance set at $P<0.05$.

3. Results

3.1 *In vitro* assay

The presence of *E. senticosus* extract in MRS broth in the concentrations of 0.1, 0.5, 1.0 and 2.0% only marginally affects the growth of probiotic *L. fermentum* CCM 7421 strain. We observed an inhibition by just 0.4 $\log_{10}$ CFU/mL at 2.0% concentration compared to the control (Table 1). After 24 h cultivation, no significant differences in the pH values of cultures and control were observed. In addition, the initial pH value of MRS broth was slightly decreased with increasing amount of plant extract added (acidifing effect of extract).

3.2 Microbial populations in faeces

In the canine experiment, oral supplementation with *E. senticosus* extract (ES group), *L. fermentum* CCM 7421 (LF group) and their combination (ES+LF group) resulted in certain significant effects according to the microbial genera tested in faecal samples (Table 2). The population of LAB was higher in both groups supplemented with the probiotic (LF and ES+LF) compared to the ES group with a significant difference detected at day 28 and 49 ($P<0.01$ and $P<0.05$, respectively). In addition, a non-significant decrease of LAB numbers in the LF and ES+LF group shortly after the cessation of the probiotic supplementation (day 21) was replaced again by an increase in the later post-treatment phase (day 28 and 49). The probiotic strain CCM 7421 was detected in faecal levels up to 10^6 CFU/g on average (day 7), but later the numbers decreased to 10^3-10^2 CFU/g (day 28 and 49). Thus a significant effect of time was observed ($P=0.0045$). The treatment effect ($P=0.0044$) was observed also in clostridia numbers which were significantly lower in the LF and ES+LF group in the early post-treatment period (day 21, $P<0.05$, compared to the ES). However, the lowering effect in the LF started already at the end of supplementation phase (day 14). In contrast, the ES+LF group significant reduction occurred only in the post-treatment phase (days 21 and 28). Numbers of faecal enterococci were not affected by the time or treatment ($P>0.05$). The population of *E. coli* was more abundant in the ES+LF group compared to groups with application of additives only (ES and LF group, $P<0.05$) during the supplementation phase (day 7 and 14). Numbers of other tested gram-negative genera (*Pseudomonas* sp., *Aeromonas* sp.) were similarly higher in the combined ES+LF group when compared to the ES group. The difference between these groups was significant at day 14 ($P<0.01$). An effect of time in the *Pseudomonas* population was also noted ($P=0.0065$).

3.3 Faecal characteristics

The application of *L. fermentum* CCM 7421 caused a significant decrease of faecal pH value in the dogs of LF group (Table 3). The decrease was significant compared to other treated groups, especially at day 7, 14 and 28 ($P<0.01$). In contrast, pH values in the ES and ES+LF groups tended to be higher than the control levels ($P>0.05$) between day 7 and 28. No treatment or time effect was noted ($P>0.05$) with the consistency of faeces. However, faeces from both groups fed the *E. senticosus* extract (ES, ES+LF) showed lower DM content than the LF group (day 49, $P<0.05$) and more liquid consistency also in FS in the ES+LF group (day 14, $P<0.05$). An effect of treatment ($P<0.05$) on the faecal ammonia concentrations was observed whereby samples of dogs in the ES+LF group showed lower values compared to that of the LF group (day 14, $P<0.05$; day 49, $P<0.05$). This difference in the DM base was not significant ($P>0.05$).

3.4 Cellular immunity assay and biochemistry in blood

The singular application of *E. senticosus* to dogs resulted in a significant decrease of leukocyte

Concentration	Time (h)			
	Growth ($\log_{10}$ CFU/mL)		pH of culture	
(% w/v)	0	24	0	24
0.0 (control)	6.27 ± 0.12	8.09 ± 0.19	5.49 ± 0.05	3.98 ± 0.03
0.1	6.12 ± 0.30	8.26 ± 0.24	5.48 ± 0.09	3.98 ± 0.05
0.5	6.46 ± 0.10	8.13 ± 0.12	5.46 ± 0.05	3.96 ± 0.08
1.0	6.81 ± 0.04	8.56 ± 0.07	5.41 ± 0.10	3.96 ± 0.03
2.0	6.38 ± 0.08	7.81 ± 0.02	5.35 ± 0.07	3.93 ± 0.02

Table 1. *In vitro* growth of *L. fermentum* CCM 7421 in MRS broth (Merck, pH 5.58) without and with the addition of *E. senticosus* dry root extract at a concentrations of 0.1, 0.5, 1.0 and 2.0% (w/v).

Values are means of two replicates ± SEM

Microorganism	Exper. group	Time (d) 0	7	14	21	28	49	P Value Effect of treatment	Effect of time	Inter-action
Aeromonas sp.	C	5.60 ± 0.30	5.66 ± 0.24	5.68 ± 0.35	5.66 ± 0.32	5.67 ± 0.21	5.71 ± 0.14	0.0001	0.6286	0.8782
	ES	5.59 ± 0.27	5.35 ± 0.29	4.34 ± 0.48A	5.35 ± 0.64	4.60 ± 0.36	5.47 ± 0.65			
	LF	5.53 ± 0.43	5.03 ± 0.54	5.08 ± 0.26	5.39 ± 0.34	4.88 ± 0.35	5.71 ± 0.64			
	ES+LF	5.62 ± 0.53	6.24 ± 0.41	6.46 ± 0.75B	6.32 ± 0.49	6.17 ± 0.22	6.37 ± 0.45			
Clostridium-like	C	5.55 ± 0.21	5.50 ± 0.18	5.50 ± 0.32	5.56 ± 0.27	5.59 ± 0.25	5.60 ± 0.19	0.0044	0.1948	0.4636
	ES	5.60 ± 0.26	5.88 ± 0.19	5.46 ± 0.45	6.23 ± 0.21a	5.10 ± 0.75	5.04 ± 0.97			
	LF	5.62 ± 0.29	5.13 ± 0.29	3.85 ± 0.57	4.33 ± 0.42b	4.42 ± 0.50	5.32 ± 0.77			
	ES+LF	5.51 ± 0.68	5.15 ± 0.68	5.19 ± 0.73	4.25 ± 0.31b	3.76 ± 0.39	5.05 ± 0.76			
Enterococcus sp.	C	4.67 ± 0.11	4.71 ± 0.20	4.65 ± 0.17	4.73 ± 0.20	4.68 ± 0.17	4.71 ± 0.22	0.2280	0.5894	0.8825
	ES	4.86 ± 0.36	3.86 ± 0.26	4.43 ± 0.41	4.83 ± 0.36	4.34 ± 0.21	5.50 ± 0.33			
	LF	4.71 ± 0.34	4.99 ± 0.26	4.78 ± 0.34	5.04 ± 0.36	5.09 ± 0.40	5.21 ± 0.42			
	ES+LF	4.77 ± 0.75	5.17 ± 0.73	5.33 ± 0.84	5.41 ± 0.58	4.62 ± 0.39	5.08 ± 0.62			
Escherichia coli	C	5.62 ±0.18	5.66 ± 0.21	5.69 ± 0.14	5.62 ± 0.12	5.65 ± 0.26	5.70 ± 0.26	0.0006	0.4399	0.7858
	ES	5.90 ± 0.41	5.72 ± 0.32	4.50 ± 0.56a	5.52 ± 0.50	5.16 ± 0.41	5.98 ± 0.72			
	LF	5.70 ± 0.32	4.80 ± 0.42a	5.20 ± 0.25	5.31 ± 0.33	5.02 ± 0.42	5.71 ± 0.55			
	ES+LF	5.79 ± 0.68	6.78 ± 0.32b	6.27 ± 0.99b	5.97 ± 0.55	6.33 ± 0.40	6.83 ± 0.40			
LAB	C	6.46 ± 0.23	6.57 ± 0.13	6.40 ± 0.27	6.50 ± 0.20	6.41 ± 0.18	6.51 ± 0.26	<0.0001	0.1578	0.1391
	ES	6.55 ± 0.47	6.65 ± 0.37	6.82 ± 0.13	7.03 ± 0.26	5.17 ± 0.83A	6.49 ± 0.43a			
	LF	6.49 ± 0.43	7.86 ± 0.42	6.72 ± 0.39	6.55 ± 0.52	7.76 ± 0.68B	7.87 ± 0.26			
	ES+LF	6.44 ± 0.84	7.81 ± 0.81	8.03 ± 0.65	7.51 ± 0.58	7.63 ± 0.39B	8.28 ± 0.41b			
L. fermentum CCM 7421	LF	ND	5.73 ± 0.48	4.69 ± 0.56	4.00 ± 0.87	2.95 ± 0.46	2.17 ± 0.55	0.2560	0.0045	0.6356
	ES+LF	ND	5.81 ± 0.90	4.67 ± 1.30	3.82 ± 0.47	3.98 ± 0.63	3.96 ± 0.79			
Pseudomonas sp.	C	5.74 ± 0.28	5.75 ± 0.38	5.69 ± 0.21	5.73 ± 0.33	5.76 ± 0.21	5.80 ± 0.13A	0.0020	0.0065	0.2604
	ES	5.69 ± 0.36	5.95 ± 0.27	4.88 ± 0.33A	5.78 ± 0.56	5.20 ± 0.46	6.19 ± 0.56			
	LF	5.93 ± 0.38	6.45 ± 0.54	5.42 ± 0.38	5.87 ± 0.45	5.28 ± 0.52	7.64 ± 0.36B			
	ES+LF	5.86 ± 0.65	6.44 ± 0.52	6.83 ± 0.28B	6.27 ± 0.26	6.34 ± 0.21	6.89 ± 0.45			

Table 2. Faecal microbial populations of control dogs (C, n=8), dogs fed *E. senticosus* (ES, n=8), *L. fermentum* CCM 7421 (LF, n=8) and the combination of both additives (ES+LF, n=8) for 14 days.

Values are means log_{10} CFU/g ± SEM. Significant results: ab P<0.05; AB<0.01. ND - not detected.

Parameter	Exper. group	Time (d)						P Value		
		0	7	14	21	28	49	Effect of treatment	Effect of time	Interaction
pH	C	6.3 ± 0.2	6.3 ± 0.1	6.3 ± 0.1	6.3 ± 0.2	6.3 ± 0.1	6.3 ± 0.1	<0.0001	0.1347	0.1223
	ES	6.2 ± 0.1	7.0 ± 0.2A	6.4 ± 0.2	6.4 ± 0.2	6.8 ± 0.2A	6.2 ± 0.3			
	LF	6.3 ± 0.2	6.0 ± 0.2B	5.8 ± 0.1A	5.7 ± 0.1	5.8 ± 0.1B	6.2 ± 0.1			
	ES+LF	6.3 ± 0.3	7.0 ± 0.2A	6.9 ± 0.3B	6.4 ± 0.2	6.8 ± 0.4A	6.4 ± 0.3			
Faecal score	C	3.2 ± 0.1	3.2 ± 0.2	3.2 ± 0.2a	3.1 ± 0.1	3.1 ± 0.1	3.2 ± 0.2	0.1448	0.7891	0.6374
	ES	3.2 ± 0.1	3.3 ± 0.2	3.1 ± 0.1A	3.3 ± 0.2	3.2 ± 0.2	3.2 ± 0.2			
	LF	3.2 ± 0.1	3.3 ± 0.1	3.2 ± 0.1a	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1			
	ES+LF	3.1 ± 0.2	3.3 ± 0.2	3.9 ± 0.3bB	3.4 ± 0.2	3.3 ± 0.2	3.3 ± 0.2			
Dry matter (%)	C	29.8 ± 1.2	NT	30.0 ± 0.9	NT	30.1 ± 1.3	30.0 ± 3.2	0.1965	0.4325	0.5865
	ES	30.5 ± 1.2	NT	29.6 ± 2.3	NT	29.2 ± 1.5	24.7 ± 1.4a			
	LF	29.0 ± 0.8	NT	33.2 ± 1.7	NT	30.4 ± 1.2	31.1 ± 1.4b			
	ES+LF	29.6 ± 2.0	NT	29.0 ± 1.2	NT	29.1 ± 2.9	28.0 ± 1.4			
Ammonia (mg/g)	C	0.71 ± 0.04	NT	0.73 ± 0.02	NT	0.74 ± 0.07	0.73 ± 0.03	0.0016	0.5663	0.5875
	ES	0.70 ± 0.04	NT	0.82 ± 0.08	NT	0.85 ± 0.11	0.64 ± 0.09			
	LF	0.71 ± 0.04	NT	0.85 ± 0.04a	NT	0.82 ± 0.06	0.86 ± 0.05a			
	ES+LF	0.68 ± 0.13	NT	0.57 ± 0.12b	NT	0.61 ± 0.10	0.53 ± 0.09b			

Table 3. Faecal characterstics of control dogs (C, n=8), dogs fed *E. senticosus* (ES, n=8), *L. fermentum* CCM 7421 (LF, n=8) and the combination of both additives (ES+LF, n=8) for 14 days.
Values are means ± SEM. Significant results: ab P<0.05; AB P<0.01; NT - not tested.

phagocytic activity (PA) compared to the control, LF and ES+LF groups (P<0.01, day 28 and 49, Figure 1). On the contrary, both probiotic groups (LF, ES+LF) showed a significant increase of PA compared to the control at day 49 (P<0.01). The treatment effect (P<0.0001) and the interaction of treatment and time (P<0.0001) on the PA parameter and also in the index of phagocytic activity (IPA) was observed. Similarly, a decrease of IPA in the ES group (day 28 and 49, P<0.01 compared to all groups) and an increase, especially in the LF group, was detected (day 49, P<0.01 compared to the control and ES group). On the other hand, respiratory burst activity of leukocytes expressed as IMA was the highest in the dogs supplemented only the *E. senticosus* extract (ES group, day 14, P<0.05 compared to the control and ES+LF, Figure 2).

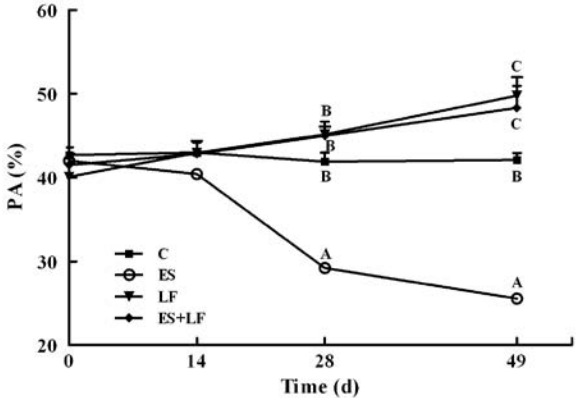

Figure 1. Phagocytic activity of PMNs leukocytes in blood samples of the control (C, n=8), dogs fed *E. senticosus* (ES group, n=8), *L. fermentum* CCM 7421 (LF, n=8) and their combination (ES+LF group, n=8) for 14 days. Results are expressed as mean percentage ± SEM. Significant results: different letters P<0.01.

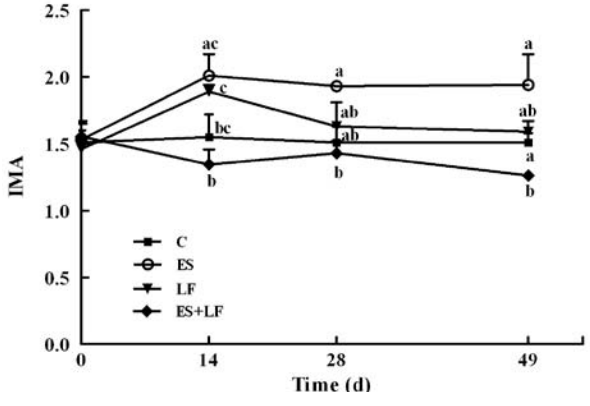

Figure 2. Respiratory burst activity of leukocytes in blood samples of the control (C, n=8), dogs supplemented with *E. senticosus* (ES group, n=8). *L. fermentum* CCM 7421 (LF group, n=8) and the combination of both additives (ES+LF, n=8) for 14 days. Results are expressed as index of metabolic activity (mean ± SEM). Significant result: different letters P<0.05.

According to our results, from the analysis of biochemical parameters in blood serum of dogs (Table 4), treatment effects on the concentration of total protein, triglyceride, glucose (with time effect) and aspartate aminotransferase were detected. Total protein was detected to be significantly higher in the ES+LF group compared to the LF or ES group (P<0.01). The concentration of urea was found to be higher in the LF group but only compared to the ES+LF group at day 28 (P<0.05) corresponding with a higher level of albumin in the LF group at the same collection day (P>0.05). The level of triglyceride decreased in the ES group (day 14-49). The concentration of glucose increased significantly in the LF group during the treatment (day 14, P<0.05) and also in the post-treatment phase compared to the control (day 28, 49, P<0.05). A lower level of aspartate aminotransferase was observed in both groups of dogs supplemented with *E. senticosus* extract compared to the control and LF group in the post-treatment period (ES, day 28, ES+LF group, day 49, P<0.05). Other parameters tested (albumin, cholesterol, alanine aminotransferase, glutathione peroxidase, calcium and phosphor) were not observed to change significantly (data not shown).

4. Discussion

E. senticosus does not belong to the group of plants with high antimicrobial potency, e.g. *Origanum vulgare*, *Thymus vulgaris, Satureja hortensis etc*. [19,20]. However, it contains chiisanogenin – a compound with broad but moderate inhibitory activities towards Gram-positive (staphylococci, *Bacillus cereus*) and also Gram-negative bacteria (*E. coli*, *Samonella* sp., *Proteus* sp., [21]). The poor antimicrobial effect of *E. senticosus* extract was reported also by Haviarová *et al*. [22] who assessed 330 bacterial indicators from which only 2.1% (staphylococci) were sensitive to the extract. The present *in vitro* assay shows that it is possible to combine *E. senticosus* extract with probiotic strain *L. fermentum* CCM 7421 since almost no growth inhibition was detected up to 2% (w/v) concentration in MRS broth. Dietary supplementation of dogs with *L. fermentum* CCM 7421 and *E. senticosus* extract (at a dose 8 mg/kg BW) did not negatively affect the colonisation and survival of the probiotic. The faecal persistance of the probiotic after cessation of application of the combined substances was similar, as in our previous experiments when the strain had been applied alone [7]. Microbiological analysis of faeces demonstrated no negative impact of *E. senticosus* extract administration on canine faecal microbiota in the

Parameter		Exper. group	Time (d) 0	14	28	49	P Value ET[a]	Et[b]	I[c]
TP	g/L	C	65.2 ± 2.1	65.0 ± 1.8	65.3 ± 1.3	64.9 ± 2.1	0.0044	0.9592	0.5902
		ES	65.0 ± 2.4	61.0 ± 2.2A	61.3 ± 2.6	61.9 ± 3.0			
		LF	65.1 ± 1.2	63.8 ± 1.4A	64.9 ± 1.9	64.1 ± 1.6			
		ES+LF	64.9 ± 2.8	71.7 ± 2.0B	66.8 ± 2.4	68.3 ± 2.7			
ALB	g/L	C	37.2 ± 0.9	37.1 ± 1.1	37.0 ± 1.0	37.1 ± 1.2	0.7350	0.9976	0.9992
		ES	37.3 ± 1.9	36.0 ± 1.1	36.9 ± 0.7	36.7 ± 1.4			
		LF	37.1 ± 0.9	37.9 ± 0.8	38.6 ± 0.9	38.1 ± 0.9			
		ES+LF	37.3 ± 2.8	37.4 ± 1.2	36.5 ± 3.3	37.4 ± 2.2			
UREA	mmol/L	C	7.70 ± 0.61	7.74 ± 0.86	7.76 ± 0.50	7.72 ± 0.68	0.1016	0.9464	0.9324
		ES	7.58 ± 0.68	7.67 ± 0.55	7.68 ± 0.60	7.64 ± 0.58			
		LF	7.77 ± 0.57	7.86 ± 0.41	8.23 ± 0.52a	7.59 ± 0.41			
		ES+LF	7.60 ± 1.06	6.93 ± 0.76	5.98 ± 0.45b	6.90 ± 0.37			
TRIG	mmol/L	C	0.89 ± 0.12	0.90 ± 0.09	0.90 ± 0.06	0.92 ± 0.16	0.0123	0.6675	0.8323
		ES	0.89 ± 0.06	0.62 ± 0.05	0.60 ± 0.12	0.51 ± 0.08			
		LF	0.91 ± 0.07	0.94 ± 0.13	0.98 ± 0.16	0.83 ± 0.13			
		ES+LF	0.90 ± 0.23	1.04 ± 0.14	0.84 ± 0.18	0.90 ± 0.18			
GLU	mmol/L	C	4.58 ± 0.15	4.68 ± 0.12A	4.61 ± 0.22a	4.60 ± 0.15a	<0.0001	0.0290	0.2610
		ES	4.55 ± 0.28	4.85 ± 0.49a	5.40 ± 0.37	5.27 ± 0.42			
		LF	4.61 ± 0.31	5.99 ± 0.38Bb	5.75 ± 0.31Bb	5.66 ± 0.16b			
		ES+LF	4.40 ± 0.32	4.31 ± 0.09A	4.49 ± 0.23A	4.69 ± 0.12			
AST	ukat/L	C	0.21 ± 0.01	0.22 ± 0.02	0.21 ± 0.01a	0.21 ± 0.03a	0.0016	0.1783	0.1470
		ES	0.22 ± 0.06	0.15 ± 0.02	0.10 ± 0.02b	0.19 ± 0.03			
		LF	0.22 ± 0.02	0.21 ± 0.02	0.22 ± 0.03a	0.23 ± 0.03A			
		ES+LF	0.21 ± 0.02	0.14 ± 0.03	0.17 ± 0.04	0.10 ± 0.02Bb			

Table 4. Biochemical parameters in blood serum of control dogs (C, n=8), dogs fed *E. senticosus* (ES, n=8), *L. fermentum* CCM 7421 (LF, n=8) and the combination of both additives (ES+LF, n=8) for 14 days.

TP - total protein; ALB - albumin; TRIG - triglyceride; GLU - glucose; AST - aspartate aminotransferase.
Values are means ± SEM. Significant results: ab P<0.05; AB P<0.01; a Effect of treatment, b Effect of time; c Interaction.

dose range tested. Rather the opposite was observed: some regulative effects on abundance of gram-negative bacteria (detected mainly in the *E. senticosus* alone supplemented group) with no main effects on beneficial lactic acid bacteria (LAB) populations. Faecal numbers of LAB followed by probiotic application were detected to be significantly higher in both probiotic groups (LF, ES+LF) compared to the plant extract alone application (ES group). This agrees with a significant increase of lactobacilli observed in our canine study testing of 7-days application of *L. fermentum* CCM 7421 [7]. There is a lack of information on the pro/antimicrobial properties of *E. senticosus* studied under *in vivo* conditions, except for the experiment of Fang *et al*. [23] with weaned piglets which also indicated the potential of *E. senticosus* extract supplementation (0.1% of diet) to regulate the intestinal microbiota composition. The reduction of staphylococcal population in faeces of rabbits after 3-weeks supplementation of diet with *E. senticosus* extract (0.015% of diet) was noted in our previous experiments [24]. The possible mechanism of *E. senticosus* regulative effects on microbiota could be of an immunological nature, for instance, by a modulation of lymphocytes, cytokines and antibodies which can mount an inhibitory defence against a wide range of bacteria [25]. The decrease in the numbers of clostridia detected in the LF group is a frequently observed result in canine studies after probiotic application [26,27]. However there are few studies that specifically investigate the mechanisms of *Clostridium* sp. colonisation resistance. Probiotics have been shown to block the attachment sites for clostridia or

their toxins and may directly destroy pathogenic toxins (e.g. toxin A or B of *C. difficile*; [28]). The stimulation of immune function (e.g. increase of IgA levels) is a further possible mechanism of anti-clostridial effect of probiotics [28]. A slight (significant only to ES group at day 14) but longer-lasting (day 7-49) increase of *Aeromonas* sp. and *Pseudomonas* sp. populations in the combined group might lead to an increased risk of translocation and infection, such as ear and skin infections, pneumonia or septicemia, more likely in immunocompromised animals.

Whereas the faecal microbiota was balanced, a no less important faecal characteristic – consistency - remained suboptimal throughout the study, except for the probiotic group (LF). The results revealed visually (FS) more liquid consistency in the ES+LF group (day 14, $P<0.05$), however a trend for lower DM content was observed in the ES group (day 49, $P<0.05$ compared to the LF group). The dose of *E. senticosus* extract or the length of its administration to dogs might be lower/shorter from this viewpoint, but it remains to be tested. The concentration of faecal ammonia was partially higher only in the probiotic LF group at day 14 and 49 and may be connected with the lower faecal water content (higher DM), compared to the other treatments, as well as to the lower faecal pH that reduces ammonia absorption by its protonic dissociation (the formation of the less diffusible NH_4^+ compared to the diffusible NH_3, [29]).

Furthermore, we studied the effects of used additives on the activation of blood macrophages which are known to participate in the immunological response by phagocytosis, antigen presentation and the production of cytokines, reactive oxygen species and nitrogen species involved in the destruction of pathogens [30]. Many investigators have demonstrated two contrary views on the immunomodulatory effects of *E. senticosus*: the stimulation [22,31,32] and the supression [33] of immune responses. It seems that the differing doses of adaptogen may play an important role in the immunomodulation effect, e.g. significant increase of phagocytosis were noted only at a dose rate of 0.030% extract in the diet compared to a non-significant increase at a dose rate of 0.015% in our rabbits experiments [22]. On the other hand, a lot of probiotic strains have an immunostimulation effect after their interaction with the M cells in Peyer´s patches, with gut epithelial cells and with the associated immune cells. After contact with these cells, the release of cytokines is induced to up- or down-regulate the immune response [34]. The results of Donnet-Hughes *et al*. [35] suggest that a minimum daily dose of 10^9 viable *L. johnsonii* La1 was required to significantly modulate phagocytosis and respiratory burst activity and that faecal persistence of the strain may not be a prerequisite for this form of immune reactivity. These authors detected significantly greater PA even 4 weeks after viable probiotic bacteria were no longer in the faeces. In our study, a significant increase of PA was detected 2 ($P<0.001$) and 5 ($P<0.01$) weeks after cessation of probiotic application, but the probiotic strain still persisted in certain levels in faeces. A significant increase of PA ($P<0.05$) after 4-days application of *L. fermentum* CCM 7421 strain was observed in our previous study using newborn Japanese quail [36]. The immunostimulation of the respiratory burst of the sole phagocytes is a strain-dependent characteristic of probiotics and mostly only long-term probiotic treatments have lead to significant results [37]. However, in our study a significant increase of respiratory burst activity ($P<0.05$) was noted only in the group treated with *E. senticosus* extract alone. To stimulate the immune status of dogs is of great importance in relation to the current alarming occurence of immunodeficiency caused by various factors such as aging, overvaccination, unproper nutrition (e.g. mycotoxins), certain drugs, stress *etc*. Interestingly, according to this study, unlike the probiotic the extract of *E. senticosus* seems to be an inappropriate additive to activate phagocytes in dogs.

In blood serum, the concentration of total protein was detected to be higher in dogs consuming only the combination of *E. senticosus* and *L. fermentum* CCM 7421 for 2 weeks (at day 14, $P<0.01$). Kong *et al*. [38] reported a significant increase of the serum contents and an apparent ileal digestibility of most amino acids tested in weaned piglets supplemented with *E. senticosus* extract (0.1% of diet). Although protein metabolism and nitrogen retention was shown to support also some probiotic strains in animals [39] including strain CCM 7421 but after a shorter supplementation period [7,36], no increase was detected in the LF group in this study. The possible reason of the specifity to increase total protein might be connected with differences in the microflora abundance (e.g. proteolytic genera) detected among treatments leading also to a different ammonia content in faeces and serum concentrations of urea. An effect of probiotic on serum protein level often depends on the length of its administration, on the initial serum protein concetrations (individual regulative effect of probiotic without changes in the average for the whole experiment) as well as on age of animals (growth support in the young age). Serum albumin concentration, a measure of nutritional status that tends to fall in many illnesses, was the highest, although non-significantly, in dogs supplemented with the probiotic alone (LF group, $P>0.05$). Whereas protein anabolism was stimulated after application of the probiotic and

E. senticosus combination, saccharide metabolism was supported especially in the probiotic group through the production of SCFA (the only group with a decrease of faecal pH, data on SCFA analysis not shown). An important effect of probiotics is activation of hepatic gluconeogenesis through lactic acid production [40]. An increase of glucose concentration in the LF group (day 14, $P<0.05$) can be explained by conversion of lactic acid to pyruvic acid and then to glucose. The glucose lowering effect of *E. senticosus* (syringin), observed in a few studies, appears to be related with the increase of insulin secretion [41] but was not indicated in our experiment. The decrease of triglyceride concentration was observed in the *E. senticosus* group of dogs during the treatment and also in the post-treatment phase. Several human studies deal with the influence of *E. senticosus* active components on serum lipid profile. Szolomicki *et al*. [41] observed a significant decrease of triglyceride, total cholesterol, LDL cholesterol and free fatty acids in healthy volunteers after 30 days treatment. Other authors detected a similar decrease of LDL cholesterol ($P<0.001$) whereas total cholesterol and triglyceride were not changed in blood serum of postmenopausal women supplemented with leaf extract for 6 months [10]. Although an inhibitory activity towards pancreatic lipase showed *E. senticosus* compounds [42], more detailed studies are needed to establish the exact mechanism of hypolipaemic action. Hepatoprotective effects are ascribed to certain *E. senticosus* components (e.g. glycoprotein) which are able to decrease elevated levels of alanine aminotransferase and aspartate aminotranseferase [43]. In our experiment, a significant decrease of aspartate aminotransferase compared to the control was noted in both groups supplemented with the herbal extract (ES, ES+LF) while the concentration of alanine aminotrasferase was not changed significantly.

In conclusion, the supplementation of dogs with *E. senticosus* at a dose 8 mg/kg BW for 14 days did not affect the growth of canine probiotic strain *L. fermentum* CCM 7421 and therefore the combination of both additives is possible. However, it is necessary to establish the right dose and duration of the *E. senticosus* administration since faeces was found to be of a more liquid consistency in the experimental group with the addition of this plant. It remains to be determined if the supressive impact of *E. senticosus* on the cellular immunity parameter (PA) by the plant could have the same beneficial effects as observed in human studies. Our study also shows that the combination of substances does not necessarily lead to the accumulation of the positive effects of these substances when such are applied separately, but could also lead to the abolishment or to the opposite effects expected.

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