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Application of potential probiotic *Lactobacillus fermentum* AD1 strain in healthy dogs

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Abstract

Probiotic utilization is becoming increasingly popular in veterinary medicine. However, only few probiotic products are available commercially for use in dogs in our market. Therefore, the aim of our study was to determine the properties of new potential probiotic *Lactobacillus fermentum* AD1 strain—own canine isolate and to investigate its effect on several microbiological and biochemical parameters in healthy dogs. The strain expressed in vitro survival by pH 3.0 after 3 h (86.8%) and in the presence of 1% bile (75.4%). The AD1 strain adhered to the canine and human intestinal mucus. It was sensitive to commonly used antimicrobials. Fifteen healthy dogs were supplemented with 10^9 *L. fermentum* AD1 for 7 days. At the end of AD1 strain application, numbers of faecal lactobacilli and enterococci increased significantly in the canine faeces. Significant increase of total protein and total lipid and significant reduction of glucose in serum of dogs were noted. These data indicate that *L. fermentum* AD1 survive transit through the canine gastrointestinal tract, and populate the colon and probably increased absorption of some nutrients. Whether longer time of its application lead to the same results as well as its potential to improve immune function in dogs remains to be determined.

Keywords: Dog; Probiotic; *Lactobacillus*; Microflora; Blood parameters

1. Introduction

The importance of the intestinal microflora has attracted much interest in recent years, particularly with respect to ways in which the microbiota can be manipulated to improve health. One possible way of modulation of the intestinal microflora is using probiotics. Many definitions of probiotics has been provided; however, generally they can be defined such as microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being [1]. Appealing properties of probiotics include the ability to reduce antibiotic use, the apparently high index of safety, and the public's positive perception about 'natural' or 'alternative' therapies. Potential probiotic bacteria are classified, and generally regarded as safe [2] as opposed to antibiotics, which have a number of recognized adverse effects. Probiotic utilization is becoming increasingly popular in veterinary medicine. However, only few probiotic products are available commercially for use in dogs and cats in our market (Probican paste, Medipharm s.r.o., Czech Republic) and they contain commercial human strain such as *Enterococcus faecium* M-74. Many animals are receiving commercial human probiotics that are more widely available. Some commercial dog and cat foods also claim to contain probiotics. Incorporation of probiotics into diets may have the advantage of easy, daily administration of beneficial organisms. A variety of microorganisms, typically lactic acid bacteria such as lactobacilli, bifidobacteria, and enterococci, have been evaluated as potential probiotics [3]. Some species of yeast have also been studied [4]. Probiotics have been recommended for the treatment or prevention of many conditions mainly gastrointestinal disorders, many of which lead to diarrhoea. Four mechanisms have usually been attributed to probiotics to

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explain their protective effects: (a) antagonism through the production of antimicrobial substances [5]; (b) competition with the pathogen for adhesion sites or nutritional sources [6]; (c) immunomodulation of the host [7]; and inhibition of the production or action of bacterial toxins [8]. The first three mechanisms are ordinarily attributed to lactic acid bacteria while the last two are more specifically attributed to yeast. Only few studies concerning application of probiotic strain to dogs have been performed. Trends towards increased digestibility of protein and an increased production of lactate and reduced pH were observed in the canine ileum [9,10]. Administration of *Lactobacillus acidophilus* strain DSM 13241 to healthy dogs was associated with increased numbers of faecal lactobacilli and decreased numbers of clostridial organisms as well as with significant increases in red blood cells, hematocrit, hemoglobin concentration, neutrophils, monocytes, and serum immunoglobin G concentration [11]. Significant reduction of *Clostridium* spp. counts was achieved in canine experiment with *E. faecium* NCIB 10415 [12]. The commercial product Vitacanis (Microbiol, Brazil) containing *L. acidophilus*, *E. faecium* and *Saccharomyces cerevisiae*, developed for the prevention of intestinal disorders in dogs and cats, provided protection against the experimantal challenge with *Salmonella enterica* ser. *Typhimurium* [13]. In another study, heat-killed *E. faecalis* FK-23 augmented non-specific immune responses in healthy dogs [14].

In our study, the properties and effect of new potential probiotic *Lactobacillus fermentum* AD1 strain (isolated from canine faeces) on selected faecal microflora as well as on serum levels of total protein, lipid, cholesterol, alaninaminotransferase, urea and glucose were studied in 15 healthy dogs.

2. Materials and methods

2.1. Characteristic of AD1 strain

The AD1 strain was isolated from the faeces of healthy dog—Tibetan terrier (6 years old). The sample was serially diluted in saline buffer (pH 7.0), plated on MRS agar (Becton and Dickinson, Cockeysville, MD, USA) and incubated at 37 °C for 48 h. To genotype AD1 strain, sequence analysis with specific primers of *L. fermentum* according to Walter et al. [15] HDA1 5′-ACTCCTACGGGAGGCAGCAGT-3′ and HDA2 5′- GTATTACCGCGGCT -3′ (Microsynth AG, Balgach, Swiss) was carried out. DNA was isolated by Wizard Genomic DNA Purification Kit (Promega, USA).

2.2. Sensitivity or resistance of AD1 strain to antimicrobials, low pH and bile

Antimicrobial sensitivity/resistance of the AD1 strain was tested by the standard agar disc diffusion method and following discs (Becton and Dickinson) were used: chloramphenicol (30 μg), tetracycline (30 μg), vancomycin (30 μg), erythromycin (15 μg), ampicillin (10 μg), and penicillin G (10 IU).

Resistance of the AD1 isolate to bile was tested according to Gilliland and Walker [16]. Briefly, overnight culture was inoculated (2%) into MRS broth (Becton and Dickinson) without and with 1% w/v ox-bile purified (Biomark, India) added and incubated at 37 °C. Samples were taken at 0 h and after 24 h of incubation. They were serially diluted in saline buffer (1:10, 0.85%, pH 7. 0) according to the standard microbiological method and 100 μL from the appropriate dilutions were plated on MRS agar. The total amount of strain AD1 was expressed as $\log_{10}$ of colony forming units (CFU) per mL. The results are the average of three experiments + SD.

To test survival of the isolate at low pH value, the cells of overnight culture (MRS broth, Becton and Dickinson) were harvested by centrifugation (at 2000*g* for 15 min), resuspended in 0.05 M phosphate buffer (pH 3.0), and held at 37 °C for 1, 2 and 3 h. The CFU were determined on MRS agar. The results are the average of three experiments + SD.

2.3. Mucus adhesion assay

Human intestinal mucus was isolated from the healthy part of resected colonic tissue according to Ouwehand et al. [17]. Canine mucus was prepared from canine jejunal chyme essentially as described [17,18]. Adhesion to human and canine mucus was studied on microtitre plate wells [17]. Briefly, dissolved mucus was immobilized in polystyrene microtitre plate wells (Maxisorp, Nunc, Denmark) and radioactively labelled bacteria (100 μL) were added and the wells incubated at 37 °C for 1 h. The adherent bacteria were lysed with 1% SDS in 0.1 mol/L NaOH at 60 °C for 1 h. The radioactivity of the lysed bacterial suspension was measured by liquid scintillation. The results are expressed as the average of at least three independent experiments in four parallel studies.

2.4. Preparation of AD1 strain for application to dogs

Rifampicin-marked strain of *L. fermentum* AD1 was prepared because of differentation of this strain from other lactobacilli. Rifampicin-marked AD1 strain (resistant to 100 μg/mL) was cultivated in MRS broth (Merck) at 37 °C for 24 h. Cells were harvested after centrifugation (2000*g*, 10 min) and culture sediment was resuspended in saline buffer (0.85%, pH 7.0) to concentration of 10^9 CFU/mL (OD_{600} 0.900). The solution was kept at 4 °C.

2.5. Application of AD1 strain to dogs

L. fermentum AD1 strain was applied per os to 15 clinically healthy dogs (included nine bitches, six dogs) of various breeds (five cross-breeds, two English Cocker Spaniels, Doberman, Weimaraner, Dachshund,

two German Shorthaired Pointers, Slovenský kopov, Welsh Springer Spaniel, Český fúzač) and of various ages (from 0.5 to 3 years old) during 7 days in daily dose of 3 mL (10^9 CFU/mL of saline solution). The exact dose was verified by diluting, spreading and incubating of AD1 strain on MRS agar. Application of the strain to dogs was performed with the agreement of Ethic Commission of Institute of Animal Physiology, Slovak Academy of Sciences. All dogs were housed in environmentally enriched facilities (shelter in the area of University of Veterinary Medicine), fed and exercised individually, and had free access to fresh water at all times. The dogs were fed commercial granulated feed APORT Ideal Adult (Tekro s.r.o., Žitňany, Slovakia) once per day in daily dose 20 g/kg of body weight. Adaptation period to this food was minimally 4 weeks before experiment. Faeces and blood samples (from *vena cephalica antebrachii*) were collected before application and after 7 days of AD1 strain administration. The dogs were not allowed access to food in the 16-h overnight period prior to venipuncture. Dogs were monitored daily for changes in clinical condition, vital parameters, appetite, and faecal consistency. To test stability of AD1 strain in the digestive tract of dogs, faeces from six dogs were sampled also 6 months (weekly) after cessation of administration AD1 strain.

The samples of faeces were serially diluted in saline buffer (pH 7.0) according to the standard microbiological method and plated on the media according to ISO norms: Mac Conkey agar (Becton and Dickinson) for enumeration of *Escherichia coli* (ISO 16654), Mannitol salt agar (Becton and Dickinson) for staphylococci (ISO 6888), M-*Enterococcus* agar (Becton and Dickinson) for enterococci (NF V 04–503), MRS agar (Merck, Germany) for lactic acid bacteria—lactobacilli (ISO 15214) and MRS agar with rifampicin (100 μg/mL) for *L. fermentum* AD1. They were cultivated at 37 °C for 24–48 h. Numbers of CFU were expressed as $\log_{10}$ CFU/g. The results are given as arithmetical means + SD.

Thirty minutes after blood sampling, samples were centrifuged (3000*g* for 10 min) and sera were tested by using of BIO-LA-TEST (Lachema, a.s., Brno, Czech Republic) for total protein (TP 300), total lipid (TL 180), cholesterol (CHOL 150), glucose (GLU GOD 250), aminotransferase (ALT 360) and urea (UREA 450).

2.6. Statistical analysis

Statistical evaluation of the results was performed by the Students' *t* test with the level of significance set at $P<0.05$.

3. Results

Identification by sequencing confirmed classification of AD1 strain as *L. fermentum*. The antimicrobial susceptibility test indicated that AD1 strain was sensitive to chloramphenicol, tetracycline, vancomycin, erythromycin, ampicillin, and penicillin G. Survival of AD1 strain at pH 3.0 was found 99.9% + 0.9 after 1 h (decrease by 0.01 $\log_{10}$ CFU/mL), 94.7% + 5.1 after 2 h (decrease by 0.53 $\log_{10}$ CFU/mL) and 86.8% + 3.9 after 3 h (decrease by 1.33 $\log_{10}$ CFU/mL). *L. fermentum* AD1 strain was able to grow in the presence of 1% bile and it was found 75.4% + 1.6 of cells after 24 h of incubation (decrease by 2.38 $\log_{10}$ CFU/mL). The adhesive capacity of AD1 strain to intestinal mucus achieved 2.7% + 1.9 for human mucus and 2.1% + 1.1 for canine mucus.

Rifampicin-resistant mutant of AD1 strain was used in experiment in vivo. Consumption of *L. fermentum* AD1 for 7 days was not associated with any changes in clinical status of animals. The results for the population of faecal microflora before and after 7 days of application are presented in Fig. 1. Numbers of faecal lactobacilli increased significantly ($P<0.001$) during AD1 strain administration by 3.3 $\log_{10}$ CFU/g in average. Similarly, the number of enterococci in faeces was significantly ($P<0.001$) higher (by 1.6 $\log_{10}$ CFU/g). There were no significant difference in the counts of *E. coli* and *Staphylococcus* sp. between sampling at 0 and 7 days. Total counts of AD1 strain ranged between 7.0 and 8.7 $\log_{10}$ CFU/g during its application and between 3.0–5.0 $\log_{10}$ CFU/g during next 6 months after cessation of its application. The effect of *L. fermentum* AD1 on biochemical parameters in blood serum are shown in Table 1. The concentration of total protein increased in all experimental dogs and the increase achieved 12.7 g/L in average ($P<0.001$). Total lipid values were also significantly higher by 1.5 g/L in average ($P<0.01$). Glucose concentrations decreased significantly by 0.7 mmol/L in average ($P<0.01$). No significant differences in serum cholesterol, alaninaminotransferase and urea were detected.

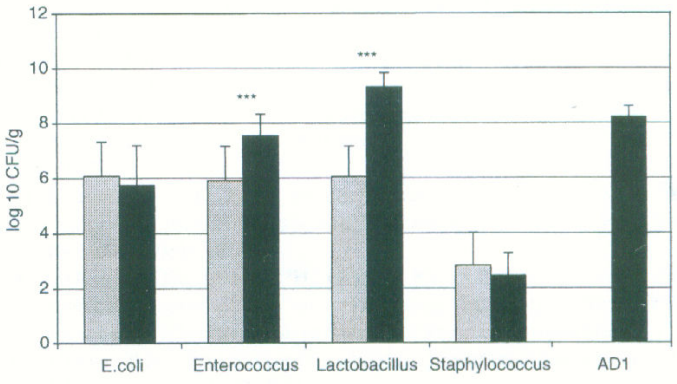

Fig. 1. Total counts of selected bacterial groups in faeces of 15 healthy dogs before and after *L. fermentum* AD1 application; ***$P<0.001$; ▩ day 0; ■ day 7. Media used: Mac Conkey agar (Becton and Dickinson, USA) for enumeration of *E. coli*, M-*Enterococcus* agar (Becton and Dickinson) for enterococci, MRS agar (Merck, Germany) for lactobacilli, Mannitol salt agar (Becton and Dickinson) for staphylococci, and MRS agar with rifampicin (100 μg/mL) for *L. fermentum* AD1.

kinds of subjects (healthy vs. hypercholesterolemic), and length of treatment periods. Bomba et al. [30] reported that *L. casei* (294/89) did not influence the concentration of blood lipids in calves and reduced cholesterol levels in the first week of application. Although, to optimize cholesterol level in the dogs is not so important—they normally have an abundance of the high-density lipoproteins [31] which allows them to eat large quantities of animal fats—*L. fermentum* AD1 had shown modulation effect on it.

It can be concluded that the addition of *L. fermentum* AD1 in the diet increased significantly the number of lactic acid bacteria in canine digestive tract, increased significantly total protein and total lipid and decreased significantly the concentration of glucose in bloodstream of dogs. Whether longer time of its application lead to the same results, remains to be determined.

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Table 1
Biochemical parameters in blood serum of dogs before and after *L. fermentum* AD1 application

Parameter	Before AD1 application		After AD1 application		*P* value
	Mean	SD	Mean	SD	
Total protein (g/L)	60.73	8.42	73.42	6.39	<0.001
Total lipid (g/L)	4.54	1.46	6.04	0.77	0.002
Cholesterol (mmol/L)	5.37	1.41	5.24	1.02	0.775
Alaninaminotransferase (μkat/L)	0.82	0.22	0.76	0.14	0.367
Urea (mmol/L)	6.46	0.73	6.42	1.00	0.901
Glucose (mmol/L)	6.63	0.43	5.98	0.55	0.002

4. Discussion

A potential probiotic strain is expected to have several desirable properties. First, the organism should be a normal inhabitant of the gastrointestinal tract of healthy dogs. In our study, AD1 strain was isolated from faeces of healthy Tibetan Terrier. Firstly, AD1 strain was identified as *Lactobacillus casei* [19]; but after sequence analysis it was alloted to the species *L. fermentum*. In order to exert health-promoting probiotic effects, it is important for the bacterial strain to survive the inhospitable environment in the animal's gastrointestinal tract. The primary barrier to microorganisms in the stomach is the gastric acid with the intensity of the inhibitory action being related to pH [20]. Key factor determining the survival of bacteria in gastric juice is the pH, but components in the gastric juice may confer some protective effect on the bacterial cell as also observed for strains of *L. acidophilus*, *Lactobacillus bulgaricus* and *Streptococcus termophilus* [21]. In present study, survival of *L. fermentum* AD1 at 3.0 after 3 h was over 80% that was also benchmark for selection of strains in study of Prasad et al. [22]. Bile resistance is important for an organism that is expected to grow in the intestinal tract. The mean intestinal bile acids concentration in human gastrointestinal tract is believed to be 0.3% w/v [23] but there is poor information about this parameter in canine intestinum. In our case, 1% w/v of bile was used and survival of strain AD1 was tested in its presence after 24 h of incubation. Survival of this strain was sufficient, although slightly lower as in acidic conditions. Adhesion to the intestinal mucus is considered a prerequisite for successful colonization and is important for immune modulation by the probiotics [17]. Although, *L. fermentum* AD1 had lower adhesion ability to human and canine intestinal mucus under in vitro conditions (e.g. commercial human strain *Lactobacillus rhamnosus* GG adhered to canine jejunal mucus in 35% in vitro; [24]) it persisted in canine gastroinstestinal tract even 6 months after cessation of its administration in healthy dogs. On the other hand, *L. rhamnosus* GG persisted in dogs only 3–5 days after cessation of its application [25]. That is, not all results under in vitro studies correlates with results achieved under in vivo conditions. Antibiotic resistance of probiotic microorganisms is an area of growing concern. It is believed, but insufficiently scientifically documented, that antimicrobial agents used for animals can promote the emergence of resistance in these bacteria. That is, it can lead to the transfer of resistance to other pathogenic bacteria through the exchange of genetic material (e.g. by plasmids, transposons) [26]. Since *L. fermentum* AD1 strain was not resistant to commonly used antimicrobials, it is safer for use in live animals, and its lack of resistance also indicates that it cannot contribute to the transfer of resistance to other microorganisms.

Application of *L. fermentum* AD1 to healthy dogs caused significant increase of lactobacilli; the counts of AD1 strain dominated. AD1 strain had the ability to survive its transit through the gastrointestinal barrier in dogs. The detection of AD1 strain in faeces during 6 months after its ingestion clearly demonstrated that AD1 strain colonized and replicated itself in the canine intestinal tract. Although, persistance should be less important than colonization during administration from clinical aspect, optimal probiotic strain should persist in digestive tract at least transiently. Count of enterococci also increased significantly; it seems that they can also well grow in the environment formed by strain AD1 (e.g. lower pH). Significant increase of serum total protein in dogs was probably due to better utilization and absorption of proteins in feed. Similarly, significantly better organic nitrogen retention and nitrogen utilization was achieved in experiment of Scheuermann [27] in growing pigs after application of Paciflor (CIP 5832) and it resulted also in increase of body mass. On the other hand, no significant differences in total protein were detected in experiment testing effect of a commercial probiotic microbial gel containing a combination of *Saccharomyces cerevisae*, *E. faecium* and *L. acidophilus* in Holstein bull calves [28]. Although, Menke [29] stated that urea concentration in blood is negatively correlated with N conversion, in our study, no changes of urea values were detected despite of higher total protein levels. In general, the most commonly tested biochemical parameters in serum are cholesterol and total lipid. *L. fermentum* AD1 had modulation effect on cholesterol level and significantly increased total lipid level. There are some controversial effects of probiotic bacteria on the cholesterol metabolism. The discrepancies in the results are the result of the use of different strains, different