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Short communication

Early exposure to probiotics in a canine model of atopic dermatitis has long-term clinical and immunological effects

Rosanna Marsella^{a,*}, Domenico Santoro^b, Kim Ahrens^a

^a Department of Small Animal Clinical Sciences, University of Florida, Gainesville, FL, USA

^b Department of Veterinary Clinical Medicine, University of Illinois at Urbana-Champaign, Urbana, IL, USA

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ABSTRACT

Probiotics modulate the immune response and may have protective effects against atopic dermatitis (AD). Clinical trials using dogs with spontaneous disease are limited by confounding factors such as different diets, environments and sensitizations while a more controlled evaluation is possible using experimental models. A validated model of canine AD showed that early exposure to *Lactobacillus rhamnosus* GG (LGG) significantly decreases allergen-specific IgE and partially prevents AD in the first 6 months of life. This study is a follow-up three years after discontinuation of LGG. Clinical signs were evaluated after allergen challenge with ragweed, timothy, *Dermatophagoides farinae*. Allergen-specific IgE, IL-10 and TGF- β were measured on the 1st day of challenge, before allergen exposure. Normal dogs were included as controls. Analyses included seven dogs in the non-probiotic and nine in the probiotic litter. For clinical scores, a 2-Group $\times$ 9-Time Analysis of Variance showed significant effects of group ($p = 0.0003$, probiotic < controls), time ($p < 0.0001$) and group $\times$ time interaction ($p < 0.0001$). IL-10 for all allergens was significantly higher in the control group than probiotics-exposed dogs. Allergen-specific IgE and TGF- β did not differ between litters. Early exposure to probiotics has long-term clinical and immunological effects in this model and larger studies using dogs with spontaneous disease are needed.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory disease whose prevalence has increased in recent decades (Grize et al., 2006; Yura et al., 2011), possibly due to changes in life-style and limited exposure to beneficial bacteria that modulate the immune system (Liu, 2007; Parronchi et al., 2000). Dogs develop an almost identical disease (Griffin et al., 2001) which is also becoming more common (Nødtvedt et al., 2006), perhaps precipitated by the shared environment (Schäfer et al., 2008). Due

to the progressive nature of AD, great effort is devoted to prevention. Interestingly, in dogs an inverse relationship between endotoxin exposure and development of AD has been reported (Looringh van Beeck et al., 2011) and living in a rural environment is associated with lower risk (Meury et al., 2011) suggesting the role of life style on the development of this disease.

Probiotics are defined as living micro-organisms, which exert health benefits beyond inherent basic nutrition. Probiotics have been considered for the prevention and/or the treatment of AD (Ozdemir, 2010; Matricardi, 2010) since children from rural environments have lower prevalence of allergies (Majkowska-Wojciechowska et al., 2007). To qualify as probiotic, bacteria need to resist acid and bile, adhere to intestinal epithelial cells, persist in the digestive tract long enough to produce antimicrobial agents and modulate immune responses, and withstand technological processing since live bacteria are necessary to exert

* Corresponding author at: P.O. Box 100126, Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610-0126, USA. Tel.: +1 352 392 2226; fax: +1 352 392 6125.

E-mail address: Marsella@ufl.edu (R. Marsella).

beneficial effects. Probiotics are typically strains of Gram-positive lactobacilli or bifidobacteria and streptococci. Beneficial effects are highly dependent on the particular strain. *Lactobacillus rhamnosus strain GG* (LGG) is one of the best studied in human medicine and known to also be a probiotic in dogs (Weese and Anderson, 2002).

The protective effects of probiotics have been mixed: some authors reported beneficial effects in preventing (Rijkers et al., 2010; Kalliomäki et al., 2010) and treating allergies (Gerasimov et al., 2010), while others described inability to prevent disease (Boyle et al., 2011). The current and most accepted position is that probiotics are best used as preventative rather than treatment with prenatal administration and particular strain being the most important factors (López et al., 2010; Rijkers et al., 2010; van HOFFEN et al., 2010; Tang et al., 2010).

The immunomodulatory effects of probiotics are still not completely understood (Elmadfa et al., 2010; Ohashi et al., 2009). Proposed mechanisms include stimulation of a Th1 response and up-regulation of regulatory T cells (de Rooock et al., 2010). Modulation of IL-10 could also be important since IL-10 has an important regulatory role in allergic and inflammatory responses (Akbari et al., 2001). Some researchers reported an increase of IL-10 (Niers et al., 2005), while others reported a decrease (Meyer et al., 2007). Similarly, changes in transforming growth factor beta (TGF- β), an equally important cytokine involved in the immune-regulatory mechanisms (von der Weid et al., 2001), have been varied; some investigators report no effect after administration of LGG (Rose et al., 2010), whereas others showed an increase (Isolaauri et al., 2000).

Animal models have greatly contributed to the expansion of knowledge in the field of AD (Marsella and Olivry, 2003). A canine model has been validated (Marsella et al., 2006) and demonstrated to have the same clinical and immunological features of canine and human AD (Marsella et al., 2006; Marsella and Girolomoni, 2009). In this model, prenatal administration of LGG significantly decreased allergen-specific IgE (Marsella, 2009). Although complete prevention of AD was not demonstrated, probiotic-exposed puppies developed less severe signs (Marsella, 2009). The present study represents a follow-up of the abovementioned study three years after discontinuation of LGG to evaluate severity of clinical signs and measure allergen-specific IgE, and IL-10 and TGF- β production after allergen stimulation.

2. Material and methods

All procedures used in this study were approved by the Institutional Animal Care use Committee of the University of Florida.

2.1. Animals

2.1.1. Atopic litters

Two litters of atopic Beagles (same sire and dam) were used (Marsella, 2009). One litter had received LGG while the other had not (control). LGG was chosen because it is viable in canine intestine when administered at 50×10^9 – 500×10^9 CFU (Weese and Anderson,

2002). Ten Culturelle® (Amerifit Inc., Cromwell, CT) capsules (200×10^9 CFU) were administered to the bitch daily, starting at week three of the second pregnancy and continuing throughout lactation. The offspring from the second pregnancy (probiotic litter) received 100×10^9 CFU daily, from three weeks until 6 months of age. Both litters of puppies were sensitized to *Dermatophagoides farinae* at 3 weeks of age as described (Marsella, 2009). Briefly, sensitization was done with *D. farinae* pure culture (RMB83M, >99% pure whole bodied natural milled *D. farinae*, Greer Laboratories, Lenoir, NC). One gram of *D. farinae* culture was mixed with sterile saline to form a thick paste (400 mg/ml) and each puppy received 0.3 ml of *D. farinae* twice weekly for a total of 12 weeks. The paste was applied to small areas of untreated skin in the axillae or inguinal areas using a soft brush. At age 2 all dogs were also sensitized to ragweed, timothy and *D. farinae* from Nelco Laboratories (Deep Park, NY). Sensitization involved biweekly epicutaneous application of timothy, short ragweed and *D. farinae* allergens for a total of 4 months. Allergenic extract at 100 mg/ml, diluted to 50% in glycerine was applied with dropper vials, 2.6 ml (133 mg) to inguinal area, alternating right and left side, and right axillary area.

All dogs were on the same diet and housed in the same temperature- (18°C) and humidity-controlled (68%) facility. Dogs were housed in individual cement-floored kennels cleaned daily using high temperature and high pressure wash.

At the time of this study one litter (probiotics-exposed) was 3 years old while the other one was 4 years old (control). The probiotics-exposed litter was composed by nine dogs, three females and six males. The control group was composed by seven dogs, five females and two males.

2.1.2. Normal beagles

Ten non-atopic normal Beagles were also used as controls for blood draws (four females and six males [mean age: 4 years]) as reference values of normal cytokine levels.

All dogs were intact. None of the females were in oestrus at the time of this study and they may have been at different stages in their hormonal cycle.

2.2. Allergen challenge

Both atopic litters were challenged with allergens as previously described (Marsella et al., 2006). Briefly, a solution of 50 mg of each allergen (ragweed, timothy, *D. farinae*, Nelco Laboratories Inc., Deep Park, NY) mixed in 50% glycerol was sprayed on the floor of travelling kennels, where the dogs were allowed to spend 3 h/day, 3 days in a row.

2.3. Clinical evaluation

Severity of clinical signs was scored using the canine atopic dermatitis extent and severity index scoring system (CADESI-03) (Olivry et al., 2007). Dogs were scored at baseline (before allergen exposure), 6 h after the beginning of each challenge, and daily afterwards for a total of 5 days (0, 6, 24, 30, 48, 54, 72, and 96 h after the first allergen challenge). An additional evaluation was made on day 10, seven

days after discontinuation of allergen exposure. In the scoring system, the dog's body is divided into areas and each of them receives a score for erythema, erythematous macules, papules, excoriations, and alopecia. Individual score ranges from 0 (absent) to 3 (severe) for each site and each symptom and the total scores are calculated by adding the scores of all body sites. Total scores were used in the statistical analysis.

2.4. Blood samples

2.4.1. Sample collection and processing

On day 0 of the study (before allergen challenge) 12 ml of blood were collected from all the atopic dogs and the nine normal dogs. 6 ml each were transferred to EDTA. Serum was stored at -80°C until analyzed by ELISA with Allercept® 48-allergen SE Regional Panel performed by HESKA Veterinary Diagnostic Laboratories (Loveland, CO) for the measurement of allergen-specific IgE in the samples from the atopic dogs. Results are reported in ELISA Absorption (EA) units and included *D. farinae*, short ragweed and timothy grass. Canine blood in EDTA was then processed within 1–2 h for cell culture as described by Strasser et al. (1998) with modifications. Briefly, 6 ml of undiluted room temperature blood was layered over 12 ml of ice cold Lymphoprep™ (Axis-Shield PoC, Oslo, Norway) in a 50 ml polypropylene tube and centrifuged for 30 min at $340 \times g$. The top layer above the interphase was removed and the white layer was transferred to a 15 ml tube where it was washed with Dulbecco's Phosphate Buffer Saline (D-PBS) for 10 min at $340 \times g$. The supernatant was removed and the pellet washed three more times at $300 \times g$ for 10 min with D-PBS. After last wash 2 ml cold ammonium chloride erythrocyte lysis buffer (0.15 M NH_4Cl , 0.01 M KHCO_3 , 0.0001 M EDTA, pH 7.1) was added and incubated for 10 min, then centrifuged 5 min at $200 \times g$. The cells were washed two more times in PBS at room temperature to eliminate residual platelets, and then re-suspended in RPMI/10% FBS. Cell counts were taken using a hemacytometer and assessed for viability (>95%) with trypan blue exclusion staining. Subsequent experiments by flow cytometry determined an average purity of >95% lymphocytes was isolated using this technique.

2.4.2. Cell culture set-up

Peripheral blood mononuclear cells were re-suspended to 1×10^7 cells per ml, and then 100 μl was aliquoted into five separate flat bottomed 96 well plates. Cells rested overnight in a 37°C , 5% CO_2 incubator, then 100 μl of the following were added diluted in RPMI; RPMI only, 20 $\mu\text{g}/\text{ml}$ of *D. farinae* (10,000 AU/ml) in 50% glycerol, 2 mg/ml of short ragweed (81,000 PNU/ml or 100 mg/ml), 2 mg/ml (1–10, w/v) of timothy grass (74,000 PNU/ml) allergenic extracts (Nelco Labs, Deer Park, NY), 2 $\mu\text{g}/\text{ml}$ of *E. coli* lipopolysaccharide (LPS), and phytohaemagglutinin (PHA) (Astarte Biologics, Redmond, WA). Preliminary studies had been done using glycerol only and it was found that insignificant differences existed in cell culture controls using culture media with 0.05 $\mu\text{l}/\text{well}$ of glycerol vs. control without glycerol. Based on these results and due to limitations in the amount of blood that could be obtained

from each Beagle, for the actual study it was decided not to include the glycerol as additional control and only cells culture media with and without cells were used for baseline controls. Cells were incubated for 24 h, collected, centrifuged for 5 min and culture media only transferred to eppendorf tubes that were frozen at -80°C until used in ELISA assays for IL-10 and TGF- β 1.

2.4.3. Elisa assays for canine IL-10 and TGF- β 1

ELISAs were performed according to manufacturer's protocol using Quantikine Canine IL-10 and TGF- β 1 kits (R&D Systems, Minneapolis, MN). Culture media for TGF- β 1 was diluted 1:2 in RPMI media only and activated to immunoreactive TGF- β 1, then assayed after neutralization. Culture media was undiluted for testing with IL-10. Samples were assayed in duplicate and included normalization controls for each subject's lymphocytes in culture media with no allergens. This result was then subtracted from allergen and control values. Results are presented in pg/ml.

2.4.4. Statistics

Clinical scores, allergen-specific IgE, IL-10 and TGF- β 1 were analyzed using repeated-measures analyses of variance (ANOVA). Group and time were evaluated as variable factors. At each time point, unpaired *t* tests were used for comparison between groups. All analyses were performed using the statistical software package SAS System for Windows version 9.0 (SAS Institute, Cary, NC). $p < 0.05$ was considered significant. All data are presented as mean $\pm$ standard deviation (SD), unless otherwise indicated.

3. Results and discussion

In this study, a 2-Group $\times$ 9-Time Analysis of Variance on the clinical scores showed significant effects of group ($p = 0.0003$, higher clinical scores in the non-probiotic litter), time ($p < 0.0001$) and group $\times$ time interaction ($p < 0.0001$) (Fig. 1). Allergen-specific IgE did not differ between litters for any allergen (Fig. 2). In the control litter 6/21 samples (28%) and 14/27 samples (52%) in the probiotic-exposed litter had a value < 150 EA, which is considered normal.

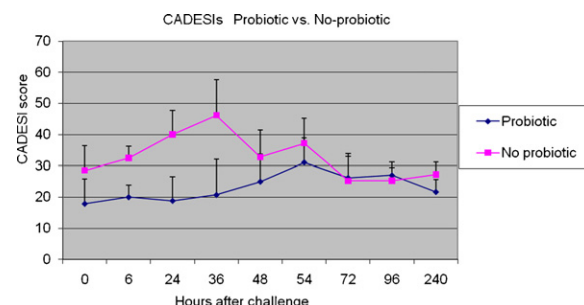


Fig. 1. Scores of clinical signs in the two litters of atopic puppies, one exposed to probiotics and the other one non exposed to probiotics (control). Clinical signs are scored using the Canine Atopic Dermatitis Severity and Extent Score Index (CADESI) and measured before (time 0) and various times (expressed in hours) after allergen exposure.

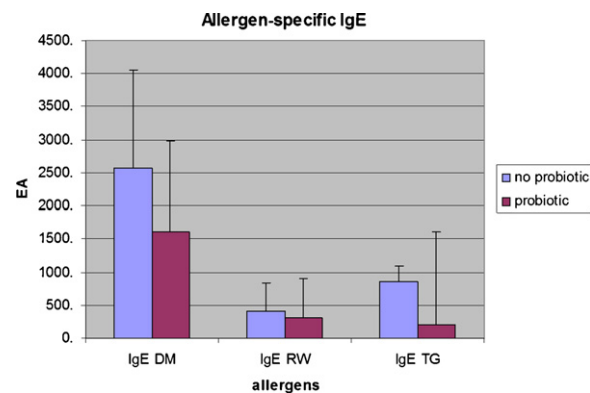


Fig. 2. Allergen-specific IgE against dust mites (DM, *D. farinae*), ragweed (RW) and timothy grass (TG) in serum samples from both litters of atopic dogs before the beginning of allergen challenge. Results are reported in ELISA Absorption (EA) units. Results < 150 EA are normal.

For IL-10 (Fig. 3), the control litter had higher IL-10 than the probiotic-exposed litter for all allergens (timothy $p=0.0035$; ragweed $p=0.0027$; *D. farinae* $p=0.011$). The normal Beagles had significantly less IL-10 than both litters of atopic Beagles ($p<0.0001$) for all allergens. Average baseline values for cells with no allergen challenge was 73.54 pg/ml for the probiotic litter, 85.92 pg/ml for the control and 31 pg/ml for the normal dogs. There was no value for RPMI/5%FBS only. For TGF- β 1 (Fig. 4) no significant differences between atopic litters were found. Average baseline values for cells with no allergen challenge was 3285 pg/ml for the probiotic litter, 2298 pg/ml for the control and 2661 pg/ml for the normal dogs. The value for RPMI/5%FBS only averaged 227 pg/ml. Atopic Beagles had significantly more TGF- β 1 after allergen stimulation with any of the allergens than normal Beagles ($p<0.001$).

Thus, a beneficial and sustained effect in decreasing severity of clinical signs despite active allergen challenge was found in this canine model of AD 3 years after discontinuation of LGG. This protective effect of LGG is similar to previous results in children at high risk of AD (Kalliomäki

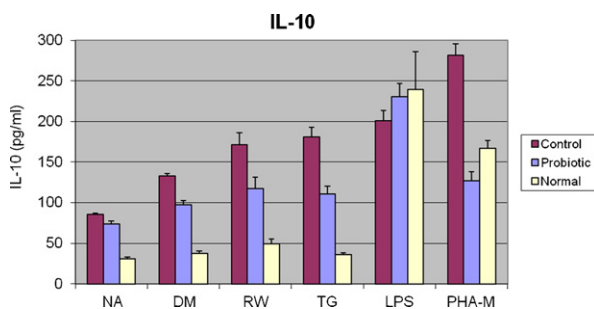


Fig. 3. Results of IL-10 production by peripheral blood mononuclear cells in atopic (probiotic exposed and not) and normal beagles after allergen incubation (*D. farinae* [DM], ragweed [RW] and timothy grass [TG]). Samples were assayed in duplicate and included controls for each subject's lymphocytes incubated with no allergen (NA) and culture media. Controls included lipopolysaccharide (LPS) and phytohaemagglutinin (PHA). Results are presented in pg/ml. Data represent mean $\pm$ SD for Control ($n=7$), Probiotic ($n=9$) and Normal ($n=10$) groups of dogs.

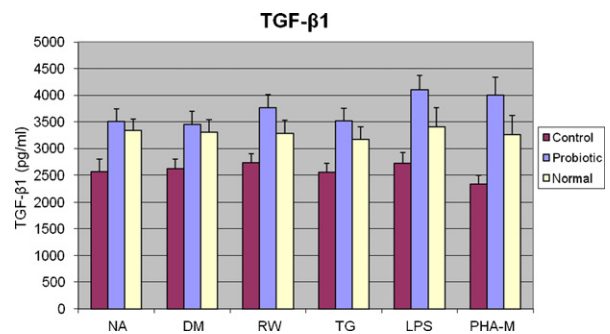


Fig. 4. Results of TGF- β 1 production in atopic (probiotic exposed and not) and normal beagles after allergen incubation (*D. farinae* [DM], ragweed [RW] and timothy grass [TG]). Samples were assayed in duplicate and included normalization controls for each subject's lymphocytes incubated with no allergen and culture media. Controls included lipopolysaccharide (LPS) from *E. coli* and phytohaemagglutinin (PHA). Results are presented in pg/ml. Data represent mean $\pm$ SD for control ($n=7$), probiotic ($n=9$) and normal ($n=10$) groups of dogs.

et al., 2001). Where a reduction of 50% the incidence of eczema at 2 and 4 years of age (Kalliomäki et al., 2003) was described. Similarly to Kalliomäki studies (2003), we also found no difference in sensitization overtime. Thus, while the probiotics do not protect from sensitization, the beneficial effect on clinical signs is persistent.

In the atopic Beagles of this study LGG decreased IL-10 compared to the control Beagles and brought the probiotic-exposed atopic litter closer to the levels observed in the normal Beagles. This result is similar to what reported by Meyer et al. (2007) and suggest that in our dogs IL-10 behaved more like a Th2 rather than an anti-inflammatory cytokine.

The lack of significant changes in TGF- β found in our study is similar to previous reports in the literature (Betsi et al., 2008; Isolauri et al., 2000; Meyer et al., 2007; Rose et al., 2010). A limitation of our study, which will be addressed in future studies, is that we did not measure T regulatory cells but simply measured TGF- β 1. Additional limitations of this study are that the two litters of atopic dogs had different ages. However, coming from the same mother and having the mother being exposed to probiotic prenatally the difference in age was not avoidable. This could have only been avoided using a different mother, which would have introduced another type of limitation (different genetic predisposition). Additionally, the two atopic litters were not sex matched and there was a significant switch in male/female ratio between the two litters. Unfortunately, this could not be controlled by breeding the same parents. Although the number of dogs included in this study is limited, an important strength was to be able to control for genetics, environment, diet, and number of sensitizations.

In summary, in this follow-up study in our canine model, a positive clinical effect was found after oral administration of LGG in highly predisposed atopic Beagles. Although no changes in TGF- β 1 were found, a significantly lower production of IL-10 was evident in PBMC from probiotics-exposed dogs after allergen stimulation. We conclude that this model could be beneficial to aid in the evaluation of the immune mechanisms involved

with probiotics administration and analyze the impact of genetic background. Additional studies with larger number of dogs are warranted to better assess the protective effects of probiotics against development of allergies.

Conflict of interest

None to declare.

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