



Evaluation on the effects of 0.1% *Peumus boldus* leaf and *Spiraea ulmaria* plant extract combination on bacterial colonization in canine atopic dermatitis: A preliminary randomized, placebo controlled, double-blinded study

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ABSTRACT

Defective skin barrier characterize canine atopic dermatitis (AD). Pyoderma is the most common complication. Herbal compounds have been suggested as alternatives to control bacterial colonization for their effect on natural antimicrobial peptides (AMPs). This study evaluated the effects of 0.1% *Peumus boldus* leaf and *Spiraea ulmaria* plant extract combination on clinical signs, bacterial colonization and AMPs secretion in atopic dogs compared to placebo.

Twenty privately-owned atopic dogs were randomly divided in 2 groups (treatment: n = 10; placebo: n = 10) and their abdomen was sprayed every 24 h for 4 weeks. Total and inguinal clinical scores (CADESI-03), manual bacterial count, and skin washes for AMPs (cBD3-like and cCath) were performed on days 0, 14 and 28. AMPs were detected using in-house, previously-validated, canine-specific ELISAs. Data were statistically analyzed and a $p < 0.05$ was considered significant.

Clinical scores and AMPs secretion did not differ significantly between the two groups at any time point. A significant reduction of the clinical scores was seen in the placebo group at 14 and 28 days ($p < 0.04$). On days 14 and 28, a reduction in the bacterial count was seen in the treated group compared with placebo ($p < 0.009$ and $p = 0.04$, respectively). Compared to baseline, a reduction in *Staphylococcus* spp. was seen in the treated group after 14 days of treatment ($p < 0.03$).

These results show the efficacy of this plant extract combination against bacterial colonization, suggesting its potential usefulness in preventing bacterial infection in atopic dogs. The influence of this compound on AMPs secretion or other mechanisms should be further evaluated.

1. Introduction

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases in dogs. Canine AD is a chronic pruritic disease requiring life-long pharmacological management, with significant costs and potential side effects (Olivry et al., 2010, 2015). The pathogenesis of AD is not completely elucidated, although a skin barrier defect has been demonstrated in both dogs and people (Santoro et al., 2015a). Skin barrier impairment, genetic and immunological alterations, and altered interaction with the cutaneous microflora have been suggested as possible causes of recurrent infections in atopic dogs (Santoro et al., 2015a).

In the past decade, an alarming increase in bacterial resistance has been documented in both dogs and people with AD (DeBoer and Marsella, 2001; Kedzierska et al., 2008). These subjects require constant use of antimicrobials with the potential risk of selecting for resistant bacterial strains (Weese et al., 2015; Pitiriga et al., 2017). For this reason, researchers are looking into alternative treatment and preventative options for bacterial infections and colonization. Under this prospective, the use of metals and plant extracts have been hypothesized as good alternative options (Higaki et al., 1997; Higaki et al., 1999; Gloor et al., 2002; Lemire et al., 2013; Oktar et al., 2015). Plant extracts have also been considered with particular interest in the

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veterinary field. In fact, very recently, plant extracts have demonstrated a positive effect on natural immune defenses with the implied potential to reduce the insurgence of skin infection in atopic dogs (Marsella et al., 2010; Olivry et al., 2010; Olivry et al., 2015; Santoro et al., 2017).

The use of *Peumus boldus* (aka Boldo) and *Spiraea (Filipendula) ulmaria* (aka Meadowsweet) have been tested in vitro on healthy and atopic canine keratinocytes showing a positive effect on production and secretion of selected antimicrobial peptides (AMPs) without stimulating an inflammatory reaction (Santoro et al., 2017). This proof-of-concept study confirmed previous larger studies on the immunological properties of these plant extracts in people (Lanthers et al., 1991; Churin et al., 2008; Drummond et al., 2013).

Thus, due to the positive immunological properties of Boldo and Meadowsweet plant extracts and the lack of inflammatory stimulation, the aim of this randomized, double blinded, placebo controlled clinical trial was to evaluate the effects of 0.1% *Peumus boldus* leaf and *Spiraea ulmaria* plant extract combination on clinical signs, bacterial colonization, and AMPs secretion in privately-owned atopic dogs. The primary outcomes were the evaluation of bacterial colonization and the effect on AMP secretion using non-invasive techniques. The secondary outcomes included the effect of this compound on clinical signs of naturally occurring AD in dogs and the possible correlation among the variables analyzed.

2. Materials and methods

This study was a prospective, randomized, double-blinded, placebo-controlled study in privately owned atopic dogs. The study was approved by the Institutional Animal Care and Use Committee. All dogs entered the study with the owners' written informed consent.

2.1. Dogs

2.1.1. Inclusion criteria

Dogs with a previous diagnosis of non-seasonal AD were eligible to be enrolled. The diagnosis of AD was based on compatible history, and clinical signs and exclusion of other pruritic diseases according to Favrot's criteria (Favrot et al., 2010) and following recently published guidelines (Hensel et al., 2015). Additional topical therapy (e.g. shampoos or conditioners) was not permitted for the entire duration of the study.

2.1.2. Exclusion criteria

Dogs receiving glucocorticoids or other immunomodulatory drugs (e.g. cyclosporine or oclacitinib) or allergen specific immunotherapy were excluded from the study. The withdrawal period for topical, systemic and/or depot glucocorticoid treatment was two, four, and eight weeks, respectively. The withdrawal period for oclacitinib and systemic and topical calcineurin inhibitors was two weeks and eight weeks respectively. Dogs were also excluded from the study if a low number of total bacteria (< 500 colony forming units [CFUs]) or no *Staphylococci* spp. were cultured on the day of enrolment. If at any time point, a secondary infection developed and/or the severity of the disease warranted rescue therapy, the dog was withdrawn from the study and treated appropriately.

2.1.3. Intervention, randomization and masking

Each dog enrolled was randomized, using a computer software (www.randomizer.org), and allocated to a treatment or control group (ratio 1:1). The treatment consisted of a spray solution containing 0.1% *Peumus boldus* leaf and *Spiraea ulmaria* plant extract combination (Santoro et al., 2017). The control product consisted of the water base, but lacked the active ingredients. Both products were dispensed in identical bottles and were indistinguishable by appearance, smell and consistency. The clinician and all site personnel, with the exception of the treatment administrator, were blind to the treatment group

assignments, as were the owner and the laboratory personnel. No other treatments, including antibiotics and antiseptics were allowed during the study.

2.1.4. Treatment

The treatment was administered at home by the owners daily. The owners were instructed to spray glabrous areas (axillae, inguinal area, ventral thorax, medial thighs) of each dog for a total of 10 pumps, corresponding to ~20 ml of product, every 24 h for 4 weeks. Licking was prevented by distracting the dog or placing an E-collar for the time needed for the product to dry. Each dog was clinically evaluated by the same investigator (RM) on days 0, 14, and 28.

2.2. Outcome measurements

The primary objectives of the study were to evaluate the effects of the spray formulation containing a mixture of 0.1% of Boldo and Meadowsweet extracts on bacterial load and on AMPs secretion in dogs with AD. The secondary objective was to evaluate the effect of this formulation on clinical lesions measured by the Canine Atopic Dermatitis Extent and Severity Index score (CADESI-03) (Olivry et al., 2007; Olivry et al., 2008).

2.2.1. Bacterial load evaluation

A sterile compact dry swab, consisting of a sterile swab and transporting media (HyServe GmbH & Co. KG, Uffing, Germany), was rubbed for 10 s on a well-defined standardized area (15 cm²) of the ventral abdomen at each visit and plated on Columbia blood agar (BAP) and colistin and nalidixic acid (CNA) enriched plates (Remel Inc., San Diego, CA, USA). The first plate is an unselective medium for detection of both Gram+ and Gram- bacteria (BAP); the second plate (CNA) is specific for isolating Gram+ bacteria (CNA). A total of 100 µl of the transporting media and a minimum of three serial dilutions were plated for a total of 6 plates per evaluation. The dilutions were performed in order to ensure a representative number of CFUs, between 30 and 300, per plate (Markey et al., 2013). All plates were cultured at 37 °C for 24 h, and then the colonies were manually counted and identified based on characteristic morphological appearance for *Staphylococci* spp. (e.g. β-hemolysis, small white colonies, etc.). The final number of CFU/ml took into consideration the dilution factors.

Furthermore, the control and the plant extract solution were tested for direct antimicrobial activity. Briefly, isolated colonies of *Staphylococcus pseudintermedius* (ATCC 49444), *S. aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) were suspended in sterile double distilled water to achieve an optical density equal to a McFarland 0.5 standard (Sensititre Inc., Westlake, OH, USA). The bacterial suspension was plated on BAP plates and treated (1 spray at ~5 cm from the plate) with either the control or the plant extract, or no treatment at all (positive control) and incubated at 37 °C for 18–20 h.

2.2.2. Skin washes

To evaluate secreted AMPs, the skin was washed using a sodium phosphate buffered solution, as previously reported, with few modifications (Harder et al., 2010). In brief, the skin was rinsed with 900 µl of 10 mM sodium phosphate buffer pH 7.4 containing 150 mM NaCl (skin rinsing buffer) in 2 standardized areas (2.5 cm²) using a sterile upper half of a cut off 15 ml plastic centrifuge tube to demarcate the area. Another 900 µl was repeated in 2 other areas twice for a total of 6 areas (15 cm² total area) rinsed with 2700 µl in total. The skin rinsing buffer was then concentrated using Amicon® Ultra centrifugal filters Ultracell® - 3 K and stored at -80 °C until used for competitive inhibition ELISA for canine AMPs (cBD3-like and cCath).

2.2.3. ELISAs

Protein levels of AMPs (cBD3-like and cCath) in the skin washing

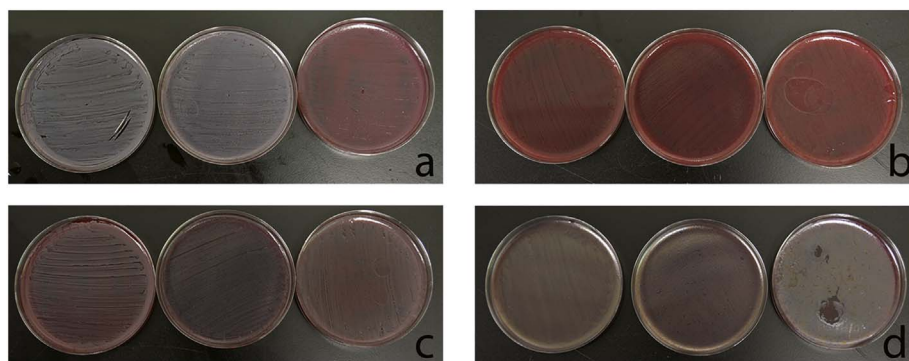


Fig. 1. Microbiological testing of blood agar plates after 20 h of incubation at 37 °C. From left to right in each panel – positive control (no treatment), control (1 spray of the control solution), treatment (1 spray of the plant extract solution). a) *Staphylococcus pseudintermedius* (ATCC 49444); b) *Escherichia coli* (ATCC 25922); c) *Staphylococcus aureus* (ATCC 29213); d) *Pseudomonas aeruginosa* (ATCC 27853). No differences are seen among the three groups for any of the bacterial strain tested.

fluids were measured using a custom-made, canine-specific competition inhibition ELISA previously validated (Santoro et al., 2013; Santoro et al., 2015b).

2.2.4. Clinical evaluation

Dogs were evaluated every 2 weeks for a total of 4 weeks for clinical signs to monitor severity of AD using a validated scoring system, the CADESI-03 (Olivry et al., 2007, 2008). Briefly, the dog's body was divided into small sections, each of which received a score based on the clinical signs evaluated. Clinical signs included diffuse erythema, erythematous macules, papules, excoriation, and alopecia. The scoring system ranged from 0 (absent) to 3 (severe) for each site and sign. The total score was calculated by adding the scores of all clinical signs and body sites and a separate score of the inguinal/abdominal area was also calculated. Both total and regional (inguinal) score were used in the statistical analysis.

2.2.5. Statistical analysis

Data were analyzed using both the intention to treat with the last value carried forward (ITT) and the per-protocol analysis. The two analyses gave very similar results and the ITT analysis was used. Mean values and 95% confidence intervals were calculated for all results. The Kolmogorov–Smirnov test of normality was used ($\alpha = 0.05$). Differences in CADESI-03 scores (total and regional) between the two groups (treatment vs control) at each time point were compared using the Student's unpaired *t*-test or the Mann–Whitney test. Differences in bacterial counts and AMP secretion between the two groups (treatment vs control) at each time point were compared using the Mann–Whitney test. A repeated measurement ANOVA was used to evaluate the over-time changes (within the group) in CADESI-03 (total and regional), bacterial counts, and AMP secretion compared to baseline. The Dunnett's or the Dunn's multiple comparison test were used as post-hoc analysis based on the distribution of each variable. Finally, Spearman correlation test was used to evaluate possible correlations among the variables of interest. *p* values of < 0.05 were considered statistically significant. Statistical analysis was done using the GraphPad Prism6 Software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Dogs

Twenty dogs with non-seasonal AD were enrolled in the study (10 in each group). Of these, 3 males and 7 females were in the control group and 4 male and 6 females were in the treatment group. The average age of the dogs in the control group was 5.2 ± 2.3 years, whereas the average age in the treatment group was 6.9 ± 4.9 years. The average weight of the dogs in the control group was 21.2 ± 11.6 kg, whereas the average weight in the treatment group was 18 ± 9.3 kg. There was no difference in age ($p = 0.3$), sex ($p = 1$), or weight ($p = 0.5$) distribution between the two groups. The most common breeds were

mixed breed (10), beagles (3), German shepherd (2), Australian cattle dog (2), followed by Maltese (1), Pug (1), and Boston terrier (1). Two dogs, one for each group, were withdrawn from the study on day 14. The dog in the control group was withdrawn due to the need of systemic antibiotic for a generalized superficial pyoderma. The dog in the treatment group was withdrawn due to euthanasia for causes unrelated to the study (aggressive behavior present prior the enrollment in this study).

3.2. Primary outcome

3.2.1. Direct antimicrobial activity of the control and the plant extract solution

At 20 h post-inoculation, there was no difference in bacterial growth on either of the blood agar plates showing a lack of direct inhibitory/antibacterial activity of either the control or the plant extract combination (Fig. 1).

3.2.2. Bacterial load evaluation

The treatment group had an overall lower amount of total bacteria during the study. In particular, on day 14 there was a significant reduction in total bacterial count on both media, CNA ($p = 0.004$) and BAP ($p = 0.009$), in the treatment group ($1.6 \times 10^3 \pm 2.5 \times 10^3$ CFU/ml and $2.8 \times 10^3 \pm 4 \times 10^3$ CFU/ml, respectively) compared to the control group ($49.3 \times 10^3 \pm 93 \times 10^3$ CFU/ml and $53 \times 10^3 \pm 92.6 \times 10^3$ CFU/ml, respectively) (Fig. 2). Similarly, on day 28 there was a significant reduction in total bacterial count on the BAP media ($p = 0.04$) in the treatment group ($41.6 \times 10^3 \pm 75.5 \times 10^3$ CFU/ml) compared to the control group ($87.4 \times 10^3 \pm 101.2 \times 10^3$ CFU/ml). Furthermore, a significant reduction of *Staphylococcus* spp. was seen on both media in the treatment group after 14 days (BAP: 155 ± 285 CFU/ml; CNA: 174 ± 327 CFU/ml) compared to baseline (BAP: $4 \times 10^3 \pm 7 \times 10^3$ CFU/ml; CNA: $4.6 \times 10^3 \pm 9.5 \times 10^3$ CFU/ml) (BAP: $p = 0.02$; CNA: $p = 0.03$). Finally, no differences were seen between baseline and day 28 for any of the bacteria (total and *Staphylococcus* spp.) (Fig. 2).

3.2.3. Antimicrobial peptides (cBD3-like and cCath ELISAs)

There was not a significant difference in either of the AMPs tested between the two groups at any time point nor over-time (Fig. 3).

3.3. Secondary outcome

3.3.1. Clinical evaluation

There was no significant differences in total or regional CADESI-03 score between the two groups at any time point. However, a significant reduction of the total CADESI-03 score on day 14 (from 40 ± 20 to 28 ± 12 ; $p = 0.03$) and 28 (from 40 ± 20 to 21 ± 10 ; $p = 0.009$) compared to baseline was seen in the control group (Fig. 4). Similar results were obtained for the regional CADESI-03, in which a significant difference was only seen in the control group with a significant reduction of the score on day 28 (from 8 ± 4 to 4 ± 3 ; $p = 0.04$)

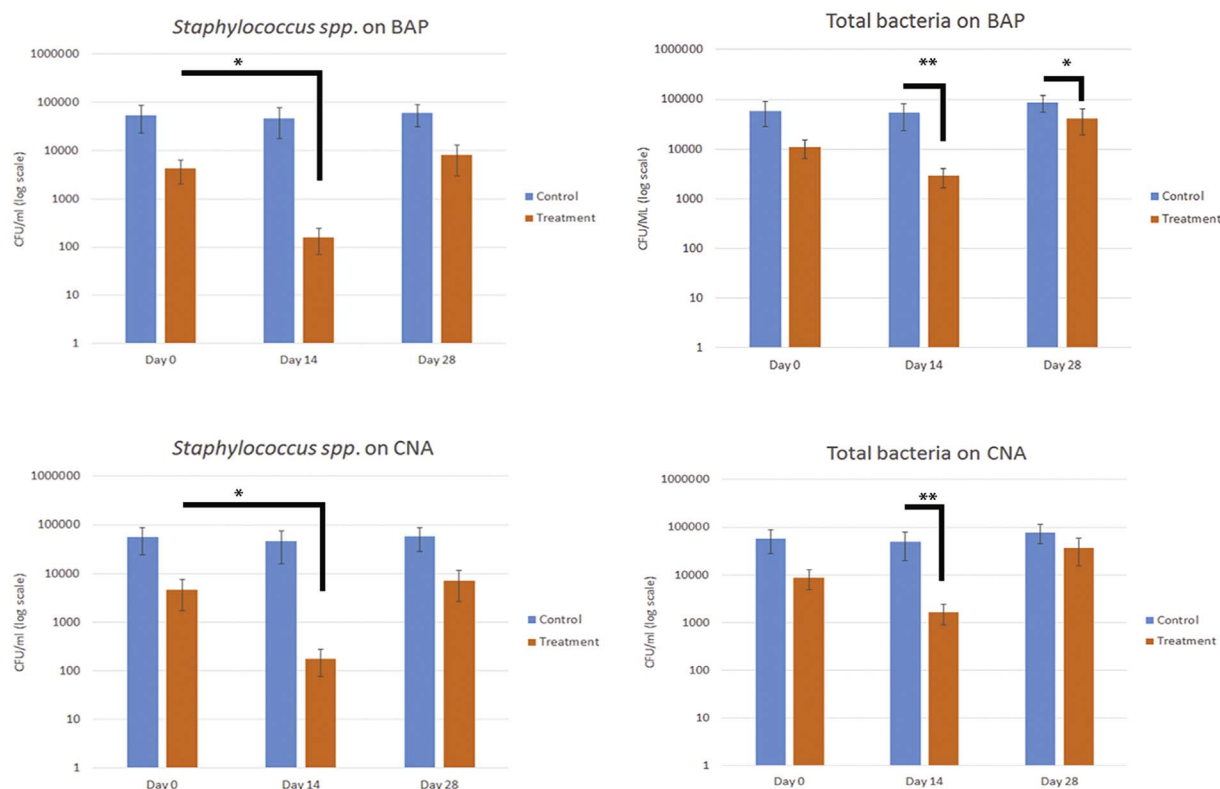


Fig. 2. Bacterial count on blood agar plate (BAP) and colistin/nalidixic acid plate (CNA). Bar: standard error. Groups were compared using Mann-Whitney test and repeated measurements ANOVA with Dunn's multiple comparison test. * $p \leq 0.05$; ** $p \leq 0.01$.

(Fig. 4).

3.3.2. Correlation among variables

A correlation matrix was performed to assess possible correlations among the different variables in each group and each time point. The analysis was done with particular focus on CADESI-03, bacterial load and AMP secretions as well as bacterial load and AMP secretion (Table S1).

3.3.2.1. Control group (Table 1). In the control group there was a moderate to strong correlation between CADESI-03 and regional CADESI-03 on day 0 ($r = 0.67$; $p = 0.04$) and 14 ($r = 0.66$; $p = 0.04$). In addition, a strong correlation was seen between bacterial counts (total and *Staphylococcus*) on both media (BAP and CAN) ($r = 0.70$ – 0.97 ; $p = 0.03$ – 0.00001) at each time point. Significant correlations were not seen between either of the CADESI-

03 scores, bacterial load or AMPs.

3.3.2.2. Treatment group (Table 1). When the treatment group was analyzed, moderate to strong correlations were seen. In particular, on day 0, strong correlations were seen between CADESI-03 and *Staphylococcus* spp. count on both media (BAP and CAN) ($r = 0.7$; $p = 0.03$ and $r = 0.71$; $p = 0.02$, respectively); regional CADESI-03 and bacterial load on both media (BAP and CNA) ($r = 0.73$; $p = 0.02$ and $r = 0.79$; $p = 0.01$, respectively); regional CADESI-03 and *Staphylococcus* spp. count on both media (BAP and CNA) ($r = 0.75$; $p = 0.02$ and $r = 0.77$; $p = 0.01$, respectively); *Staphylococcus* spp. count on BAP media and *Staphylococcus* spp. count and bacterial load on CNA media ($r = 0.98$; $p = 0.000008$ and $r = 0.87$; $p = 0.002$, respectively); bacterial load between the two media (BAP and CAN) ($r = 0.73$; $p = 0.02$); *Staphylococcus* spp. count and bacterial load on CNA media ($r = 0.87$; $p = 0.002$); finally, bacterial load on CNA media

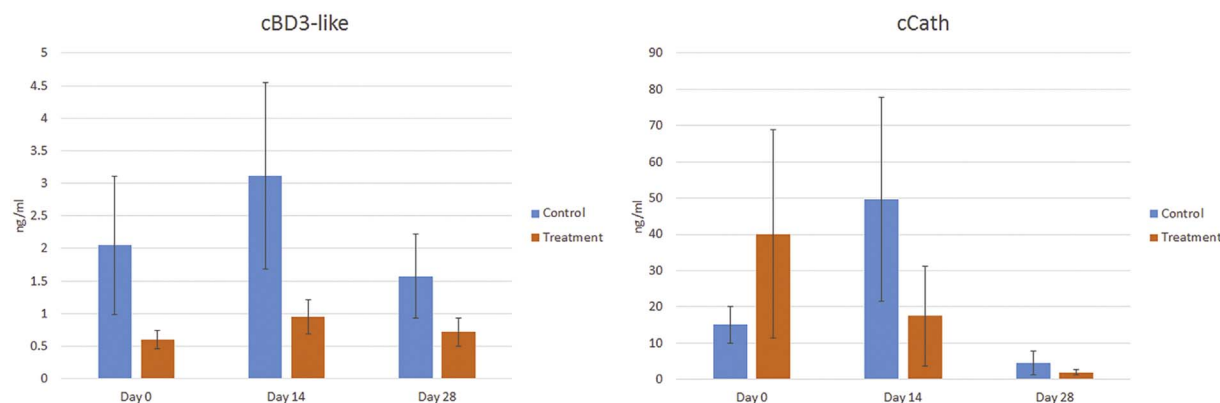


Fig. 3. Protein levels for canine β -defensin 3-like (cBD3-like) and cathelicidin (cCath) in the skin wash collected from both groups. Bar: standard error. Groups were compared using Mann-Whitney test and repeated measurements ANOVA with Dunn's multiple comparison test.

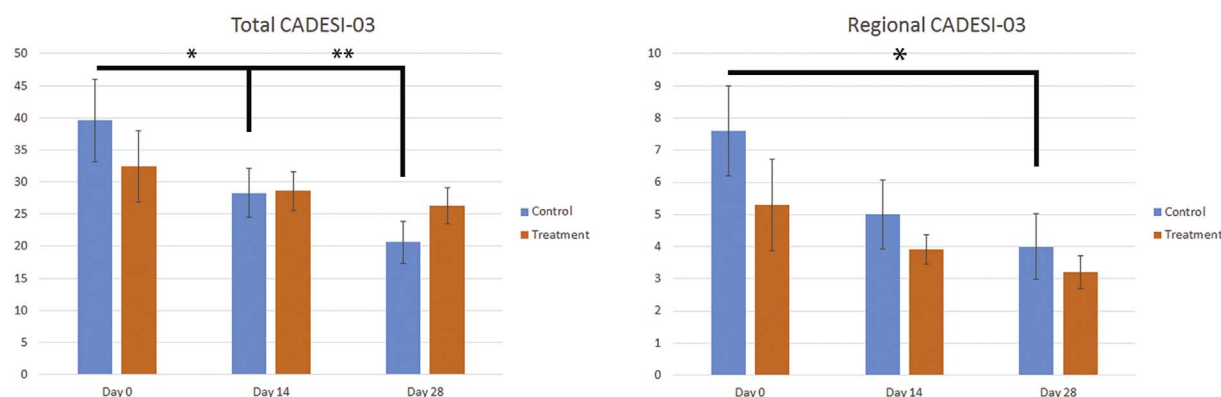


Fig. 4. Individual results for total and regional CADESI-03 scores. Bar: standard error. Groups were compared using Unpaired *t*-test or Mann-Whitney test and repeated measurements ANOVA with Dunnett's or Dunn's multiple comparison tests.

and cBD3-like secretion ($r = 0.74$; $p = 0.02$). On day 14, a moderate inverse correlation was seen between regional CADESI-03 score and *Staphylococcus* spp. count on BAP media ($r = -0.66$; $p = 0.03$); a strong correlation between *Staphylococcus* spp. count on both media ($r = 0.7$; $p = 0.03$); total bacteria on both media ($r = 0.81$; $p = 0.006$); finally, *Staphylococcus* spp. count and total bacteria on CNA ($r = 0.7$; $p = 0.03$). On day 28, a strongly negative correlation was seen between regional CADESI-03 and cCath secretion ($r = -0.75$; $p = 0.007$); total bacteria on BAP and *Staphylococcus* spp. count ($r = 0.69$; $p = 0.03$) and total bacteria on CNA ($r = 0.83$; $p = 0.004$); finally, *Staphylococcus* spp. count and total bacteria on CNA ($r = 0.85$; $p = 0.003$).

4. Discussion

The main finding of this preliminary study was the significant reduction in total bacteria counts on days 14 and 28 for the treatment group compared to the control group. In addition, a significant reduction in the staphylococcal count on day 14 compared to baseline in the treatment group was also present. Furthermore, analyzing the bacterial data as a percentage of *Staphylococcus* spp. in the total bacterial population in BAP, although not significant, a marked decrease in

Staphylococcus spp. was seen in the treatment group on day 14 (from 37% to 11%) and 28 (from 37% to 15%) (Dunn's multiple comparisons – $p = 0.07$ on day 14 and $p = 0.1$ on day 28). These data suggest that *Staphylococcus* spp. may be more susceptible to the treatment than other bacteria. Interestingly, a significant difference was not seen between baseline and day 28. This could be due to many reasons; one of those could be decrease owner compliance and/or the mandatory lack of shampooing. The effect of the lack of shampooing could have determined an increase of bacterial load due to accumulation of cellular debris and skin secretions that may have facilitated the bacterial growth in both groups. In addition, further studies are needed to confirm the positive antimicrobial effects shown in this preliminary study. A long-term study in atopic dogs with recurrent skin infections would be useful to show the potential preventative effect of this compound.

It is also worth noting that although a decrease in bacterial load was present during the study, a direct antibacterial action by the suspension was not evident in the *in vitro* experiment of this study for any of the ATCC strain tested. These results suggest an indirect activity of the tested product on the bacterial load possibly increasing the natural defenses as suggested in a previous *in vitro* study (Santoro et al., 2017). To test this hypothesis, skin wash was collected at each time point and

Table 1
Summary of the correlations among the variables analyzed.

	Parameter A	Parameter B	r value	p value
Control group day 0	CADESI-03	CADESI-03 regional	0.67	0.04
	Bacterial counts* BAP	Bacterial counts* CNA	0.95	0.0001
Control group day 14	CADESI-03	CADESI-03 regional	0.66	0.04
	Bacterial counts* BAP	Bacterial counts* CNA	0.97	0.00002
Control group day 28	Bacterial counts* BAP	Bacterial counts* CNA	0.84	0.004
Treatment group day 0	CADESI-03	<i>Staphylococcus</i> count BAP	0.7	0.03
	CADESI-03	<i>Staphylococcus</i> count CNA	0.71	0.02
	CADESI-03 regional	Total bacteria count BAP	0.73	0.02
	CADESI-03 regional	Total bacteria count CNA	0.79	0.01
	CADESI-03 regional	<i>Staphylococcus</i> count BAP	0.75	0.02
	CADESI-03 regional	<i>Staphylococcus</i> count CNA	0.77	0.01
	<i>Staphylococcus</i> count BAP	<i>Staphylococcus</i> count CNA	0.98	0.000008
	<i>Staphylococcus</i> count BAP	Total bacteria count CNA	0.87	0.002
	Total bacteria count BAP	Total bacteria count CNA	0.73	0.02
	<i>Staphylococcus</i> count CNA	Total bacteria count CNA	0.87	0.002
	Total bacteria count CNA	cBD3-like	0.74	0.02
Treatment group day 14	CADESI-03 regional	<i>Staphylococcus</i> count BAP	-0.66	0.03
	<i>Staphylococcus</i> count BAP	<i>Staphylococcus</i> count CNA	0.7	0.03
	Total bacteria count BAP	Total bacteria count CNA	0.81	0.006
	<i>Staphylococcus</i> count CNA	Total bacteria count CNA	0.7	0.03
Treatment group day 28	CADESI-03 regional	cCath	-0.75	0.007
	Total bacteria count BAP	<i>Staphylococcus</i> count CNA	0.69	0.03
	Total bacteria count BAP	Total bacteria count CNA	0.83	0.004
	<i>Staphylococcus</i> count CNA	Total bacteria count CNA	0.85	0.003

CADESI-03: Canine Atopic Dermatitis Extent and Severity Index; *(total and *Staphylococcus*); cBD3-like: canine β -defensin 3-like; cCath: Canine cathelicidin.

analyzed for AMP concentration. Unfortunately, due to the lack of reagents available, only cBD3-like and cCath were tested, which showed a lack of difference in AMP secretion in either group at any time point. Possible explanations for this discordance between the lack of difference in AMP secretion and a reduction of bacterial load could be due to the high variability of the data associated with a low number of dogs enrolled (low statistical power) as well as the possibility of the stimulation on AMP not tested in this study (e.g. cBD103). In fact, it is important to highlight that the AMPs tested in this study only represent a minimal component of the complex AMP diversity present on the canine skin. Thus, the lack of increased secretion of cBD3-like and cCath may not completely reflect the activity of the compound tested on AMPs in dogs. Another explanation is that although the gene expression is increased (Santoro et al., 2017); this is not followed by an increase in protein secretion. This theory is in agreement with a recent study in people with AD showing a lack of increased secretion of AMPs (BD3/103) due to a defect in the lamellar bodies' secretion system (Kisich et al., 2008). If this defect is also true in dogs, which is presently unknown, it may explain the lack of effect of the plant extract compound on AMPs in this study. It would have been extremely interesting to test the secretion of cBD103 in the present study; however, this was not possible due to the lack of specific ELISAs available. Finally, another possibility would be a potential interaction between inactive compounds in the plant extracts and keratinocytes or cutaneous microflora. This interaction may lead to the activation, by keratinocytes or the cutaneous microflora, of inactive compounds in the plant extracts leading to a decrease in bacterial burden.

Although a reduction in the CADESI-03 score was present in both groups, this was only significantly different from day 0 in the control group. The lack of reduction in total and regional CADESI-03 score in the treatment group, although unexpected, is explainable. One reason could simply be a higher variability in the treatment group compared to the control group associated with the mild severity of the lesions and the low number of dogs enrolled (low statistical power). Another reason could be the lack of any pro- or anti-inflammatory properties of this compound on canine atopic keratinocytes (Santoro et al., 2017). Since the CADESI-03 is a measurement of the cutaneous inflammation, a lack of anti-inflammatory properties could result in a lack of CADESI-03 modification. Thus, the addition of an anti-inflammatory component to the mixture tested could induce a more clinically evident reduction in the CADESI-03 score. Dogs in this study were selected based on the initial bacterial load independently from the CADESI-03 score, although only mild to moderate cases were enrolled. These criteria were based on the necessity to recover a high number of bacteria and Staphylococci to have a more conclusive statistical analysis (Markey et al., 2013). The decision to enroll only mild to moderate cases was based on the necessity to avoid other topical and/or systemic medications during the trial. Thus, it is possible that with different inclusion criteria (e.g. more severe cases or a longer trial) a potential beneficial clinical effect of this compound could be evident.

Finally, a correlation matrix analysis was performed comparing clinical score with bacterial load and AMP secretions at each time point. Interestingly, a shift in the correlation panel was evident between the two groups. In particular, in the treatment group there was a strong negative correlation between regional CADESI-03 and cCath at the end of the study. The reason for this correlation is unclear. It is possible that the reduction in regional CADESI-03 would be a consequence of increase in cCath production and decrease in bacterial load. Similarly interesting was the strong positive correlation between total bacteria and cBD3-like secretion on day 0. As for cCath a complete explanation for this is not known, however, it could indicate a baseline response of the skin to bacteria present on the skin. Why more correlations between AMPs and bacteria were not evident at the end of this study, in either group, is not clear at the moment and more studies are needed to explain such correlations.

5. Conclusions

In conclusion, the clinical and bacteriological results of this preliminary study show a positive effect of the 0.1% Boldo and Meadowsweet plant extract combination on the bacterial burden, in particular against *Staphylococcus* spp. bacteria within the first 14 days of treatment. The clinical score was invariant and this could be due to the lack of anti-inflammatory property of the tested product. The amount of AMPs (cBD3-like and cCath) was invariant between the two groups and over-time, showing a lack of direct effect of the tested compound on the secretion of such AMPs. However, due to the lack of direct antimicrobial activity of the compound on standard bacterial strains, the data of this study suggest a potential effect of this compound on other immune defense mechanisms. Thus, more studies are needed to better clarify the effect of this compound on natural immune defenses in dogs. In addition, due to the potential transporting system for AMPs, it would be interesting to test the same combination in a product that rebalanced the skin lipid secretion to see if that would be associated with a higher secretion of AMPs in dogs.

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Conflict of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.rvsc.2018.02.006>.

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