Evaluation of Residual Antibacterial Effects on Canine Skin Surface and Hairs Following Treatment with Five Commercial Mousse Products Against Staphylococcus pseudintermedius in vitro

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EVALUATION OF RESIDUAL ANTIBACTERIAL EFFECTS ON CANINE SKIN SURFACE AND HAIRS FOLLOWING TREATMENT WITH FIVE COMMERCIAL MOUSSE PRODUCTS AGAINST STAPHYLOCOCCUS PSEUDINTERMEDIUS IN VITRO

A Thesis
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

The Department of Veterinary Clinical Sciences

by
Ch-Yen Wu
D.V.M., National Taiwan University, 2015
August 2022
This thesis work is dedicated to my family members in Taiwan, who have always been my strongest support with their unconditional love.
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ABSTRACT

Topical products are essential treatments for canine superficial bacterial folliculitis. However, studies for commercial products typically evaluate antibacterial effects on hairs rather than the skin surface. In addition, the interference from the hair length has not been evaluated in these studies. Therefore, the study objective for this study was to evaluate the residual antibacterial effects of five mousse products, (1) 2% chlorhexidine and 2% miconazole, (2) 0.05% phytosphingosine, (3) 2% salicylic acid and 10% ethyl lactate, (4) 3% chlorhexidine and 0.5% climbazole, and (5) 2% chlorhexidine and 1% ketoconazole on both the skin surface and hairs in both short- and long-haired dogs. Products were applied once on 15 short-haired and eight long-haired dogs without dermatologic disease and which had not been treated with topical or systemic antimicrobial products within the previous four weeks. Skin surface swabs and hairs were collected from the trunk before treatment, and at one hour and at two, four, seven, 10, and 14 days post-treatment. Samples were placed on Mueller–Hinton plates pre-streaked with non-antibiotic resistant *Staphylococcus pseudintermedius*. Each dog’s samples per time point were placed on the same plate. Inhibition zones were measured after 24 h of incubation. No inhibition was seen with pre-treatment samples or with products 2 and 3. In short-haired dogs, both skin swabs and hairs from areas treated with mousses 1, 4, and 5 produced post-treatment inhibition until Day 14 ($p < 0.001$). In long-haired dogs, treated hairs demonstrated inhibition until Day 14 with mousses 4 and 5 ($p < 0.001$) but only until Day 10 with mousse 1 ($p < 0.001$). However, skin swabs from mousse 1 only produced inhibition until Day 4 in long-haired dogs ($p <$
0.001. In conclusion, evaluation of hairs may overestimate bacterial inhibition on the skin surface, especially in long-haired dogs.
CHAPTER 1. INTRODUCTION

1.1. Canine Superficial Bacterial Folliculitis

Superficial bacterial folliculitis (SBF) secondary to underlying allergies is one of the most common reasons for a canine patient to be presented to a veterinarian. This condition is typically associated with *Staphylococcus pseudintermedius*; however, *Staphylococcus schleiferi*, *Staphylococcus aureus*, and other bacteria are occasionally involved.\(^1,2\) Additionally, SBF is frequently associated with pruritus. Trauma associated with patient scratching may disrupt the integrity of the skin and the natural defense mechanisms. This increases the likelihood that SBF may progress into furunculosis and cellulitis, and therefore medical intervention is often required to prevent progression of the disease.\(^3,4\)

1.2. Antibiotic Resistance and Its Increasing Prevalence

Medical intervention for SBF usually includes antibiotic use. However, in both human and veterinary medicine, antibiotic resistance has become an emerging concern. Meticillin resistance in staphylococci is typically developed by acquiring the *meca* gene located on staphylococcal cassette chromosome *mec*. This gene allows staphylococci to have an altered penicillin-binding protein (PBP2a) that has a lower affinity to all the \(\beta\)-lactam antibiotics.\(^5\) Meticillin-resistant *S. pseudintermedius* (MRSP) in dogs is frequently reported worldwide, especially in North America and Europe. Furthermore, an increasing prevalence in the same geographical area has been documented in many studies.\(^6-14\) Given that SBF is frequently associated with *S. pseudintermedius*, the increasing prevalence of MRSP has gained much attention from veterinary professionals. In addition, it is not uncommon that MRSP may also possess resistance
to two or more than two additional antimicrobial classes. These isolates are referred to as multi-drug resistant *S. pseudintermedius*, in which resistance to all but a few antibiotics, such as amikacin, linezolid, rifampicin, and vancomycin, are frequently observed.\textsuperscript{6}

To date, several factors have been identified in association with the subsequent development of antibiotic resistance. For example, recent antimicrobial use has been identified as a risk factor for clindamycin resistance in *S. pseudintermedius*.\textsuperscript{15} Moreover, the development of MRSP infection has been associated with multiple factors such as recent antimicrobial use, including history of MRSP infections, and history of hospitalization or visit to the clinic.\textsuperscript{16-18}

1.3. Topical Therapy for Canine Superficial Bacterial Folliculitis

In reaction to the increasing prevalence of antimicrobial resistance, a number of veterinary working groups have published guidelines to allow veterinarians to better understand the appropriate use of antimicrobial use and practice antimicrobial stewardship.\textsuperscript{1,6} These guidelines recommend using topical therapy as the sole treatment for SBF and any surface or superficial infections associated with MRSP unless it is not practical due to the owner or patient factors.\textsuperscript{1,6}

It has been demonstrated that topical antiseptic therapy (e.g., chlorhexidine or benzoyl peroxide shampoos) as the sole treatment can be used to improve clinical signs and may be as effective as oral amoxicillin-clavulanic acid.\textsuperscript{19-22} Antiseptics such as chlorhexidine, chlorhexidine with miconazole, and benzoyl peroxide have been recommended as preferred topical treatments for generalized SBF.\textsuperscript{1}
Other than the ingredients, the format of the topical antiseptic products also plays a crucial role in SBF treatment. Currently, multiple formats are widely available on the market, including shampoos, rinses, sprays, and mousses.

1.4. Scope of the Thesis

Among the commercial antiseptic products, mousse products have several properties that may make them superior to other treatment formats for many situations. For example, bathing can be time-consuming, labor-intensive, and difficult to perform frequently enough for optimal results. In these cases, topical mousse application between shampoos may maintain antibacterial effects on the skin surface and facilitate owner compliance.23

However, it is often difficult to determine whether the application of the mousse results in adequate distribution of the product to the skin in dogs with long or very dense coats. One difficulty inherent in evaluating the potential effectiveness of a topical product is finding an appropriate experimental model. Several studies have investigated the ability of shampoos, sprays, mousse, and wipes to inhibit bacterial growth.24-27 Most of these studies evaluated a portion of the canine hairs, as it is the easiest type of sample to obtain. However, it can be argued that this model does not adequately demonstrate the antiseptic efficacy on the skin surface itself. This may be particularly true in dogs with long, dense coats, in which penetration of the mousse to the skin surface may be suboptimal. Thus, the actual antibacterial efficacy of these products on the skin surface and the influence of the hair length on the antibacterial efficacy of these products still warrant further studies.
Therefore, the main objective of this thesis research was to use a novel variant of an established protocol to evaluate the antibacterial efficacy of mousse products against *S. pseudintermedius* on both the skin surface and the hairs of both short and long-haired dogs.
CHAPTER 2. RESIDUAL ANTIBACTERIAL EFFECTS ON CANINE SKIN SURFACE AND HAIRS FOLLOWING TREATMENT WITH THE MOUSSE PRODUCTS

2.1. Introduction

2.1.1. Background Data

Results from a study entitled “Residual antibacterial activity of canine hair treated with five mousse products against Staphylococcus pseudintermedius in vitro” by Ramos and colleagues were used as background data for our study. A summary of the study is as follows: on Day −3, all dogs were bathed with an all-purpose, non-antimicrobial shampoo (DermaLyte, Dechra Veterinary Products; Overland Park, KS, USA). On Day 0, five areas were treated with topical mousse products: four antimicrobial mousses and one non-antimicrobial control mousse. Hair samples were collected prior to treatment and one hour following treatment. Subsequent clipped samples were obtained on days 2, 4, 7, 10, and 14. Hair samples were also obtained from areas between the application sites to verify that the mousse products did not diffuse between the treatment sites.

Bacterial culture plates (Mueller–Hinton) were inoculated with S. pseudintermedius; then, 0.02 g of each hair sample was placed onto the plates. The plates were cultured for 24 hours, and then measurements were obtained from the edge of the hair bundle to the edge of the bacterial growth (“inhibition zone”).

Three of the mousse products produced significant inhibition (relative to their own pre-treatment levels) until Day 10. One mousse produced significant inhibition until Day 14. No differences were seen between the fourth test mousse and the control mousse.

These results supported the manufacturer’s claims of antimicrobial efficacy for three of the four mousse products. However, the fourth mousse did not demonstrate
significant bacterial inhibition. For the three effective mousses, the results suggest that efficacy may persist for longer than commonly used treatment intervals (typically every one to three days).

2.1.2. Follow-up Feasibility Study

One healthy dog was used for this study. One pump of mousse (DOUXO® Chlorhexidine Mousse, Ceva Animal Health LLC; Lenexa, KS, USA) was applied to the hair and skin of a healthy dog and allowed to dry for one hour. Hair samples were obtained from treated and untreated areas. Sterile culture swabs were placed tip down on treated skin and rotated back and forth for approximately 30 seconds. The same procedure was performed on untreated skin. Treated and untreated hair samples were placed on Mueller–Hinton bacterial culture plates (Remel, Thermo Fisher Scientific; Lenexa, KS, USA) that were inoculated with non-antibiotic resistant S. pseudintermedius (American Type Culture Collection 49444) (Figure 2.1, A and B, respectively) as described in the background study. The tip of the culture swabs from the treated and untreated skin (Figure 2.1, C and D, respectively) were also plated, as was a swab removed directly out of the original packaging (Figure 2.1, E).

Figure 2.1. Follow-up feasibility study: hair samples and skin swab sample tips taken one hour after applying DOUXO® Chlorhexidine Mousse on a healthy dog placed on culture plates pre-streaked with Staphylococcus pseudintermedius. (A) Hair sample from a treated area. (B) Hair sample from an untreated area. (C) Skin swab sample tip from a treated area. (D) Skin swab sample tip from an untreated area. (E) Sterile swab tip.
Results showed a visible inhibition zone around treated hairs but not untreated hairs after 24 hours of culture. A similar inhibition zone was noted around the culture tip used to swab treated skin but not from the swab used for untreated skin or the sterile control swab.

2.1.3. Objective and Hypotheses

The objective of this study was to evaluate the antibacterial effects of the following five commercial mousse products on the hairs and skin surface of both short- and long-haired dogs:

1) Chlorhexidine gluconate 2%, miconazole nitrate 2%, and tromethamine and disodium EDTA (TrizEDTA®) (MiconaHex+TrizTM Mousse, Dechra Veterinary Products; Overland Park, KS, USA)

2) Phytosphingosine HCl 0.05% (DOUXO® Calm Mousse, Ceva Animal Health LLC; Lenexa, KS, USA; control mousse)

3) Salicylic acid 2% and ethyl lactate 10% (BioSeb™ Mousse, VetBiotek; Largo, FL, USA)

4) Chlorhexidine gluconate 3%, climbazole 0.5%, and phytosphingosine salicyloyl 0.05% (DOUXO® Chlorhexidine Mousse, Ceva Animal Health LLC; Lenexa, KS, USA)

5) Chlorhexidine gluconate 2%, ketoconazole 1% and phytosphingosine salicyloyl 0.05% (Phyto CHX+KET Antiseptic Mousse, Covetrus; Dublin, OH, USA)

Based on the background data and the follow-up feasibility study, we hypothesized:
1) There will be no inhibition of bacterial growth from the control mousse (mousse 2: phytosphingosine HCl 0.05%)

2) Regardless of the hair length, mousse products will have effective distribution to skin surface and hairs, resulting in inhibition of bacterial growth

3) Mousse 4 containing chlorhexidine gluconate 3%, climbazole 0.5%, and phytosphingosine salicyloyl 0.05% will create a longer inhibition of bacterial growth than other mousse products

2.2. Materials and Methods

2.2.1. Sample Size Determination

The number of animals selected for this study was based upon the results of other similar studies evaluating the effectiveness of different topical antiseptics in the treatment of canine bacterial folliculitis. Specifically, four previously published studies with similar materials and methods as follows:

1) Ramos SJ, 2019: in this study, 15 dogs were treated with five different topical antimicrobial mousse simultaneously by applying the products to five different areas on the dog.26

2) Mesman ML, 2016: in this study, 12 dogs were treated with four different topical antimicrobial sprays simultaneously by applying the products to four different areas on the dog.25

3) Kloos I, 2013: in this study, 42 dogs were divided into four groups of 10 or 11 dogs. Each group of dogs was treated with two different antimicrobial products. Seven antimicrobial products were evaluated in this study. The
large number of dogs in the study is attributed to the testing of seven different products with the ability to only apply two products per dog.\textsuperscript{24}

4) Kwochka KW, 1991: in this study, 10 dogs were used to determine the efficacy for four different antibacterial shampoos.\textsuperscript{28}

To further reinforce the sample size selection for the present study, an online sample size calculator (glimpse.samplesizeshop.org) was used to determine subjects' numbers for a repeated measures design analysis of variance (ANOVA). Based on a power of 90\%, and hypothetical mean values and standard deviations from a prior study,\textsuperscript{26} a sample size of seven dogs to detect within-group differences and a sample size of 12 dogs to detect between-group differences (here, between any given treatment group and the control group) was determined to be appropriate.

When the sample size calculations were evaluated in light of previously reported sample sizes, it was decided that 15 dogs (20\% increase to account for possible loss to follow-up) would be sufficient to provide statistically meaningful data. For the current work, it was essential to determine whether antiseptic product could effectively cover the skin and proximal hair shafts of the dogs. However, there was a possibility that this distribution could be affected by coat length. Therefore, two treatment groups (short-haired, n=15; long-haired, n=15) were used so that the effect of hair length could be evaluated.

2.2.2. Patient Characteristics

The following inclusion criteria were used to recruit dogs for this study:

1) At least three months of age.

2) Weighed at least 20 lbs.
3) Overall good health.
4) No evidence of dermatological disease based on clinical examination by a veterinary dermatologist or veterinary dermatology resident.
5) Any dog breed or sex was accepted.

Likewise, the following exclusion criteria were used to exclude dogs from the study:
1) Any evidence of dermatologic disease.
2) Dogs that had received any systemic antimicrobials, systemic antifungals, or any topical antimicrobial therapies in the four weeks prior to enrollment.

Prohibited medications:
1) Any topical products or medications (except for those listed below).
2) Injectable, topical, or oral glucocorticoids, antibiotics or anti-fungal medications.

Prohibited Activities:
1) Baths were not allowed during the study.
2) Swimming was not allowed during the study.

Acceptable medications:
1) Heartworm prevention
2) Flea medication
3) Other medications as discussed with the investigator

Drug withdrawal times:
1) Systemic antimicrobials, systemic antifungals: 30 days
2) Topical antibiotics / antiseptics: 30 days
Criteria for removal from the study:

1) Client request

2) Development of systemic illness (e.g., vomiting, diarrhea, fever, lethargy or weight loss) regardless of whether the illness was thought to be related to the materials and procedures used in the study.

3) Development of dermal irritation at the application site or sampling sites (e.g., significant erythema, crusting, erosion, ulceration or profuse scaling) where the product was applied or sample acquired.

4) Distress or more than minor discomfort during sample collection: examples would include vocalization, more than momentary resistance to restraint, repeated attempts to evade handling, attempts to bite or harm the investigators, or significant struggling during bathing, handling, Elizabethan collar wear, clipping or sample acquisition (e.g., scratching, rolling, twisting, lunging)

5) Major lack of compliance: (1) bathing the dog or allowing them to swim prior to the last sample collection, (2) missed rechecks/samplings, and (3) repeated late rechecks/samplings

The final number of dogs in the study was 26. Dogs were divided into two groups: 18 dogs with hair averaging ≤ 2 cm in length (short-haired group), and eight dogs with hair averaging at least 4 cm in length (long-haired group).

2.2.3. Test Product Application

Three days prior to the initial application of the test products (Day −3), dogs were bathed with a general maintenance shampoo containing no active anti-bacterial
ingredients (DermaLyte, Dechra Veterinary Products; Overland Park, KS, USA) to ensure a clean coat prior to study start.

Study product blinding was performed by the senior investigator. The label on each bottle of mousse was obscured by covering it with opaque paper, and a number (1–5) was assigned to each mousse. This information was kept by the senior investigator so that any replacement bottles of mousse would be assigned the same number as previous bottles of the same product. The primary investigator (the author) was not informed of the identity of the mousses (beyond their assigned numbers) until the end of the study. The primary investigator performed all clipping, mousse application, sample acquisition, and sample processing. The primary investigator also kept a log of all the sample measurements. The senior investigator was not involved in sample acquisition or measurements. Sample "unblinding" was performed at the end of the study by simple removal of the obscuring paper.

Day 0 marked the “official” beginning of the study. Narrow-width clippers were used to outline five, 5 cm² patches on the trunk of each dog. Patches A, B and C were located on the left side of the dog from cranial to caudal, respectively. Patches D and E were located on the right side of the dog from cranial to caudal, respectively. These patches outlined where each mousse was applied. Mousse application (mousses 1–5) was rotated through each patch to minimize site-associated confounding factors such as sebum quantity and differences in hair length. For example, the first dog had the products applied in the following schedule: A1, B2, C3, D4, E5, while the second dog had the products applied in this schedule: B1, C2, D3, E4, A5. All patch sections were
at least 5 cm apart from neighboring sections to ensure no cross-contamination. The mousses tested included the following:

1) Chlorhexidine gluconate 2%, miconazole nitrate 2%, and tromethamine and disodium EDTA (TrizEDTA®) (MiconaHex+Triz™ Mousse, Dechra Veterinary Products; Overland Park, KS, USA)

2) Phytosphingosine HCl 0.05% (DOUXO® Calm Mousse, Ceva Animal Health LLC; Lenexa, KS, USA; control mousse)

3) Salicylic acid 2% and ethyl lactate 10% (BioSeb™ Mousse, VetBiotek; Largo, FL, USA)

4) Chlorhexidine gluconate 3%, climbazole 0.5%, and phytosphingosine salicyloyl 0.05% (DOUXO® Chlorhexidine Mousse, Ceva Animal Health LLC; Lenexa, KS, USA)

5) Chlorhexidine gluconate 2%, ketoconazole 1% and phytosphingosine salicyloyl 0.05% (Phyto CHX+KET Antiseptic Mousse, Covetru; Dublin, OH, USA)

Since one of the mousse products (DOUXO® Calm Mousse) did not produce significant bacterial inhibition in a previous study,26 this mousse served as a control in our study. Each dog served as its own control and had all five products applied to separate patches of its coat, as mentioned above. One pump of each mousse was applied and massaged into the hair coat and onto the skin of the respective patch. Gloves were changed between the applications of each mousse. The mousses were allowed to dry for one hour, during which time the dog was required to wear an
Elizabethan collar to prevent the dogs from removing the products by licking. Dogs were not allowed to be bathed again or to swim until the end of the study.

2.2.4. Sample Acquisition

Dogs were seen on days 0, 2, 4, 7, 10, and 14 for hair collection. At each collection, a small square of hair was trimmed close to the skin in each treatment zone. The hairs were wrapped and stored in sterile gauze sponge squares until sample processing. The clipped area was gently wiped to remove any adherent hair fragments, then the tip of a standard-sized sterile swab was placed on the clipped skin surface and gently rotated back and forth for 30 seconds. A duplicate swab was obtained from an adjacent area of clipped skin. In addition, hairs and skin surfaces from between the treated sections were also sampled on days 2 and 4 to ensure the mousse products had not diffused to adjacent areas.

Each treatment area had its own clippers to ensure no cross-contamination. All clippers were cleaned after use using a toothbrush and sterile saline (to avoid being contaminated by antiseptics). Samples were collected twice on Day 0. Hair and swab samples were first collected from each area before mousse application and then again 1 hour after application. Samples were collected at each post-treatment visit.

2.2.5. Sample Processing

The collected hairs were then weighed and plated in small bundles of 0.02 g increments on Mueller–Hinton agar plates (Remel, Thermo Fisher Scientific; Lenexa, KS, USA) inoculated with a 0.5 McFarland solution containing non-antibiotic resistant *S. pseudintermedius* (American Type Culture Collection 49444). Hairs collected from dogs in group 1 (hairs ≤ 2cm in length) were plated *in toto*. Hairs collected from dogs in group
2 (hairs ≥ 4 cm in length) were cut so that the most proximal 2 cm and the most distal 2 cm were both sampled and plated in duplicate. In addition, the tip of each swab was cut and placed tip down on the inoculated plate. These plates were large enough to permit simultaneous plating of all samples per treatment area per dog per time point. Plates were incubated for 24 hours at 37°C.

After incubation, the plates were measured to determine the width of the area around the hair or swab in which bacterial growth did not occur (inhibition zone). For the hair samples, the extent of inhibition was assessed by measuring the perpendicular distance from each side of the mid-hair bundle to the edge of bacterial growth inhibition, as described in a previous study (Figure 2.2, A).26 For the skin swab samples, since it is difficult to determine the exact point where the round tip of the skin swab touches the agar, the diameters of the inhibition zone over the center of the skin swab were measured; this method followed the Kirby–Bauer disk diffusion test standards established by the American Society for Microbiology and the Clinical and Laboratory Standards Institute (Figure 2.2, B).29,30

Figure 2.2. Measurement of bacterial growth inhibition. (A) Hair sample: the perpendicular distance from each side of the hair bundle to the edge of bacterial growth inhibition was measured. (B) Skin swab tip sample: the diameters of the edge of bacterial growth inhibition perpendicular to each other were measured.
2.2.6. Analysis of Results

SPSS Statistics v28.0 for MacOS (IBM Corp.; Armonk, NY, USA) was used for statistical analysis. The minimum of the four values (two sides from duplicate samples) from each hair and swab sample was analyzed by repeated measures ANOVA. Shapiro–Wilk test was used to check normality, and it was determined that normality was met except for a few outliers. The significance threshold was set to 0.05, with all hypothesis tests two-sided. LSD post hoc tests were used for confidence interval adjustment. Independent variables were labeled as follows:

1) Time: pre-treatment, one-hour post-treatment, Day 2, Day 4, Day 7, Day 10, and Day 14

2) Hair length: short-haired and long-haired

3) Mousse: 1, 2, 3, 4, and 5

The definition of antibacterial effect was when there was a statistically significant difference between pre-treatment and a time point in the size of the inhibition zone. The last day with a significant difference was documented. For example, if a significant difference was noted between pre-treatment and one-hour post-treatment on Day 0, pre-treatment and Day 2, and pre-treatment and Day 4 but no difference between pre-treatment and Day 7, then Day 4 was documented.

2.3. Results

Three of the 18 short-haired dogs dropped out from the study: one of them developed superficial bacterial folliculitis during the study, and two of them accidentally had heavy rain dampen their hair coat. Therefore, 15 short-haired and eight long-haired dogs completed the study. For each dog over the 14-day study period, 82 skin swab
and 82 hair samples were collected. A total of 1230 skin swab samples and 1230 hair samples were collected from short-haired dogs, and 656 skin swab samples and 1312 hair samples (including proximal and distal hairs) were collected from long-haired dogs.

Measurements of the inhibition zones of skin swab samples from short-haired dogs at different time points are shown in Table 2.1 and Figure 2.3, hair samples from short-haired dogs shown in Table 2.2 and Figure 2.4, skin swab samples from long-haired dogs shown in Table 2.3 and Figure 2.5, and proximal and distal hair samples from long-haired dogs shown in Table 2.4, Figure 2.6, and Figure 2.7.
Table 2.1. Measurements of inhibition zones of skin swab samples from short-haired dogs at different time points.

<table>
<thead>
<tr>
<th>Mousse</th>
<th>Day 0 (1h)</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.34 ± 2.06 (5.74, 12.65)</td>
<td>7.72 ± 2.42 (4.24, 11.66)</td>
<td>6.43 ± 2.72 (0, 10.59)</td>
<td>4.89 ± 3.08 (0, 9.98)</td>
<td>4.1 ± 3.32 (0, 8.94)</td>
<td>3.21 ± 3.7 (0, 8.78)</td>
</tr>
<tr>
<td>2</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td>3</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td>4</td>
<td>9.97 ± 2.16 (5.78, 13.82)</td>
<td>8.38 ± 2.27 (3.9, 12.59)</td>
<td>7.86 ± 2.1 (4.66, 12.13)</td>
<td>7.84 ± 1.69 (5.4, 10.7)</td>
<td>7.15 ± 1.54 (4.69, 9.65)</td>
<td>6.08 ± 2.9 (0, 9.58)</td>
</tr>
<tr>
<td>5</td>
<td>9.92 ± 2.15 (6.67, 13.61)</td>
<td>8.58 ± 2.15 (3.85, 11.73)</td>
<td>7.59 ± 2.6 (0, 10.63)</td>
<td>6.74 ± 3.84 (0, 11.02)</td>
<td>6.08 ± 3.74 (0, 11.25)</td>
<td>4.83 ± 3.86 (0, 9.72)</td>
</tr>
</tbody>
</table>

Mean ± standard deviation (minimum, maximum) in millimeters.
Table 2.2. Measurements of inhibition zones of hair samples from short-haired dogs at different time points.

<table>
<thead>
<tr>
<th>Mousse</th>
<th>Day 0 (1 h)</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.18 ± 1.29 (4.97, 9.49)</td>
<td>5.39 ± 1.55 (3.59, 8.47)</td>
<td>4.64 ± 1.47 (1.93, 7.99)</td>
<td>3.47 ± 1.62 (1.34, 7.37)</td>
<td>3.08 ± 1.95 (0, 7)</td>
<td>2.24 ± 1.95 (0, 6.08)</td>
</tr>
<tr>
<td>2</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td>3</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td>4</td>
<td>7.46 ± 1.44 (4.59, 9.52)</td>
<td>5.89 ± 1.3 (4.25, 9.14)</td>
<td>5.37 ± 1.32 (3.62, 8.94)</td>
<td>5.13 ± 1.22 (3.21, 7.89)</td>
<td>4.55 ± 1.62 (1.51, 8.07)</td>
<td>3.98 ± 1.56 (1.32, 7.69)</td>
</tr>
<tr>
<td>5</td>
<td>7.86 ± 2.16 (4.63, 12.07)</td>
<td>6.35 ± 1.97 (3.91, 9.41)</td>
<td>5.07 ± 1.79 (1.79, 8.69)</td>
<td>4.4 ± 1.99 (2.15, 10.11)</td>
<td>3.99 ± 2.16 (1.77, 8.74)</td>
<td>3.44 ± 2.21 (0, 8.34)</td>
</tr>
</tbody>
</table>

Mean ± standard deviation (minimum, maximum) in millimeters.
Table 2.3. Measurements of inhibition zones of skin swab samples from long-haired dogs at different time points.

<table>
<thead>
<tr>
<th>Mousse</th>
<th>Day 0 (1 h)</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.25 ± 1.64 (5.49, 11.14)</td>
<td>6.61 ± 2.97 (0, 9.25)</td>
<td>3.69 ± 3.12 (0, 7.01)</td>
<td>1.23 ± 2.28 (0, 5.22)</td>
<td>0.68 ± 1.93 (0, 5.46)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td>2</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td>3</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td>4</td>
<td>8.52 ± 1.71 (6.28, 10.84)</td>
<td>8.78 ± 1.67 (6.59, 10.72)</td>
<td>6 ± 2.79 (0, 9.22)</td>
<td>4.87 ± 4.36 (0, 10.05)</td>
<td>6.03 ± 4.11 (0, 11.2)</td>
<td>2.9 ± 4.09 (0, 9.59)</td>
</tr>
<tr>
<td>5</td>
<td>9.61 ± 2.37 (5.08, 13.35)</td>
<td>8.4 ± 2.71 (4.37, 11.52)</td>
<td>6.33 ± 3.94 (0, 9.12)</td>
<td>5.7 ± 3.69 (0, 9.79)</td>
<td>3.99 ± 3.46 (0, 8.38)</td>
<td>2.56 ± 2.76 (0, 5.53)</td>
</tr>
</tbody>
</table>

Mean ± standard deviation (minimum, maximum) in millimeters.
Table 2.4. Measurements of inhibition zones of proximal and distal hair samples from long-haired dogs at different time points.

<table>
<thead>
<tr>
<th>Mousse</th>
<th>Hair</th>
<th>Day 0 (1 h)</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proximal</td>
<td>5.92 ± 1.42 (3.54, 8.51)</td>
<td>5.17 ± 1.31 (2.13, 6.32)</td>
<td>3.74 ± 1.5 (1.71, 5.46)</td>
<td>2.27 ± 1.85 (0, 5.22)</td>
<td>1.89 ± 1.95 (0, 5.08)</td>
<td>0.84 ± 1.25 (0, 3.23)</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>6.55 ± 0.96 (5.37, 8.63)</td>
<td>4.9 ± 1.23 (2.24, 6.26)</td>
<td>3.9 ± 1.85 (1.93, 6.34)</td>
<td>2.69 ± 1.51 (0, 4.81)</td>
<td>1.83 ± 1.76 (0, 5.29)</td>
<td>1.11 ± 1.35 (0, 3.16)</td>
</tr>
<tr>
<td>2</td>
<td>Proximal</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td>3</td>
<td>Proximal</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
<td>0 ± 0 (0, 0)</td>
</tr>
<tr>
<td>4</td>
<td>Proximal</td>
<td>6.99 ± 1 (5.38, 8.92)</td>
<td>5.3 ± 1.02 (2.96, 6.19)</td>
<td>4.5 ± 1.48 (1.47, 6.17)</td>
<td>4 ± 2.59 (0, 6.68)</td>
<td>3.62 ± 1.84 (0, 5.94)</td>
<td>2.78 ± 1.8 (0, 5.41)</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>6.6 ± 1.39 (4.94, 9.49)</td>
<td>4.77 ± 1.27 (1.89, 5.67)</td>
<td>4.21 ± 1.17 (2.12, 5.35)</td>
<td>3.8 ± 1.97 (0, 6.47)</td>
<td>3.05 ± 1.84 (0, 5.52)</td>
<td>2.09 ± 2.29 (0, 4.85)</td>
</tr>
<tr>
<td>5</td>
<td>Proximal</td>
<td>6.46 ± 0.97 (4.74, 7.81)</td>
<td>5.25 ± 1.15 (2.88, 6.73)</td>
<td>4.37 ± 1.76 (2.62, 7.78)</td>
<td>3.89 ± 1.8 (2.09, 7.07)</td>
<td>2.71 ± 1.46 (0, 4.88)</td>
<td>2.07 ± 1.58 (0, 5.19)</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>6.22 ± 0.9 (5.39, 8.09)</td>
<td>5.06 ± 1.43 (2.74, 6.67)</td>
<td>3.93 ± 1.52 (2.18, 6.9)</td>
<td>3.57 ± 2.19 (2.13, 5.94)</td>
<td>2.21 ± 1.59 (0, 4.68)</td>
<td>1.52 ± 1.79 (0, 4.83)</td>
</tr>
<tr>
<td>Mean ± standard deviation (minimum, maximum) in millimeters.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.3. Mean width of inhibition zones of skin swab samples from short-haired dogs at different time points.
Figure 2.4. Mean width of inhibition zones of hair samples from short-haired dogs at different time points.
Figure 2.5. Mean width of inhibition zones of skin swab samples from long-haired dogs at different time points.
Figure 2.6. Mean width of inhibition zones of proximal hair samples from long-haired dogs at different time points.
Figure 2.7. Mean width of inhibition zones of distal hair samples from long-haired dogs at different time points.
2.3.1. Within Subject Effects from Time, Hair Length, and Mousse

Significant within subject effects were noted for time \( (p < 0.001) \), hair length \( (p = 0.011) \), mousse \( (p < 0.001) \), time*hair length \( (p = 0.002) \), time*mousse \( (p < 0.001) \), and hair length*mousse \( (p = 0.006) \) on the size of the inhibition zone created by skin swab samples. In addition, significant within subject effects were also noted from time \( (p < 0.001) \), hair length \( (p = 0.012) \), mousse \( (p < 0.001) \), time*hair length \( (p = 0.009) \), time*mousse \( (p < 0.001) \), and hair length*mousse \( (p < 0.001) \) on the size of the inhibition zone created by hair samples.

2.3.2. Lack of Inhibition of Bacterial Growth in Samples from Mousse 2 (Control Mousse) and Mousse 3

Statistical analysis was performed to determine the duration of the antibacterial effect (as defined in 2.2.6), and it was found that none of the skin swab and hair samples from areas treated with either mousse 2 (control mousse, phytosphingosine HCl 0.05%) or mousse 3 (salicylic acid 2% and ethyl lactate 10%) produced a significant inhibition zone at any time point \( (p = 1.0) \), including one-hour post-treatment on Day 0.

2.3.3. Duration of Inhibition of Bacterial Growth in Samples from Short-Haired Dogs After Treatment with Mousses 1, 4, and 5

In short-haired dogs, skin swab and hair samples treated with mousses 1, 4, and 5 all produced significant inhibition of bacterial growth until Day 14 \( (all \ p < 0.001) \).

2.3.4. Duration of Inhibition of Bacterial Growth in Samples from Long-Haired Dogs

In long-haired dogs, the skin swab samples from mousses 4 and 5 both produced significant inhibition until Day 14 \( (p = 0.002 \ and \ p = 0.006, \ respectively) \). These mousse products also produced significant inhibition until Day 14 in proximal hair
samples (both $p < 0.001$) as well as distal hair samples ($p < 0.01$ and $p = 0.002$, respectively).

In comparison, the skin swab samples from mousse 1 produced significant inhibition until Day 4 ($p < 0.001$) but did not produce significant inhibition on days 7, 10, or 14 ($p = 0.164$, 0.425, and 1, respectively). The proximal hair samples from this mousse product produced significant inhibition until Day 10 ($p < 0.001$) but did not produce significant inhibition on Day 14 ($p = 0.091$).

Table 2.5 summarizes the last time points with significant inhibition from skin swab and hair samples for the mousse products in short- and long-haired dogs.

Table 2.5. Last time points with significant inhibition of bacterial growth.

<table>
<thead>
<tr>
<th>Mousse</th>
<th>Short-haired dogs</th>
<th>Long-haired dogs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skin swabs</td>
<td>Hairs</td>
</tr>
<tr>
<td>1</td>
<td>Day 14</td>
<td>Day 14</td>
</tr>
<tr>
<td>2</td>
<td>Not significant</td>
<td>Not significant</td>
</tr>
<tr>
<td>3</td>
<td>Not significant</td>
<td>Not significant</td>
</tr>
<tr>
<td>4</td>
<td>Day 14</td>
<td>Day 14</td>
</tr>
<tr>
<td>5</td>
<td>Day 14</td>
<td>Day 14</td>
</tr>
</tbody>
</table>

2.3.5. Comparison of the Size of Inhibition Zones from Proximal Versus Distal Hair Samples from Long-Haired Dogs

To investigate the potential effects of hair length on mousse distribution to different portions of the hair shafts, the size of the inhibition zones from proximal versus distal hair samples was evaluated. Tests of within-subjects demonstrated that the hair
portion did not create an overall difference in the size of the inhibition zones between proximal and distal hair samples from long-haired dogs \( (p = 0.919) \).

2.3.6. Comparison of the Size of the Inhibition Zones from Skin Swab Samples from Short- versus Long-haired Dogs

To further investigate the potential effects of hair length on mousse distribution to the skin surface, the size of the inhibition zones from skin swab samples was evaluated. Tests of between-subjects demonstrated that hair length created a difference in the size of the inhibition zone \( (p = 0.001) \), and the estimated marginal mean in short-haired dogs was greater than in long-haired dogs. Therefore, skin swab samples from short-haired dogs produced bigger inhibition zones than those from long-haired dogs.

Further evaluating each mousse individually, inhibition zones were smaller in skin swab samples from long-haired dogs than from short-haired dogs treated with mousses 1 and 4 \( (p < 0.001, p = 0.022, \text{ respectively}) \). In contrast, the sizes of the inhibition zones for samples treated with mousse 5 were not different in short-haired dogs versus long-haired dogs \( (p = 0.105) \).

2.3.7. Contaminant Bacteria

Growth of contaminant bacteria surrounding the hair and skin swab samples was frequently noted at varying times from samples treated with mousses 2 and 3. However, such growth typically was not present until Day 10 or 14 from samples treated with mousses 1, 4, and 5. These bacteria frequently, but not always, created small irregular inhibition zones. They were speciated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) and identified as *Bacillus cereus* and *Enterobacter cloacae*. 
2.4. Discussion of Hypotheses

2.4.1. Hypothesis 1

Our first hypothesis was that there would be no inhibition of bacterial growth from the control mousse (mousse 2: phytosphingosine HCl 0.05%).

There was no bacterial inhibition from any of the skin swab samples from mousse 2. It can be argued that the absence of bacterial inhibition from the skin swab samples could be due to the ineffective distribution of the mousse 2 onto the skin surface; however, no antibacterial effects were noted from hair samples either. Since mousse 2 was directly applied to the hair, the absence of a response cannot be attributed to distribution, and thus we do not believe distribution was an issue for skin either. Thus, the results proved our first hypothesis that mousse 2 (control mousse, phytosphingosine HCl 0.05%) created no inhibition of bacterial growth. These results were consistent with those of a previous study.\textsuperscript{26}

Although the selection of this mousse product as a control treatment in our study was based on prior study findings, it is noteworthy that one of the ingredients (phytosphingosine) can have antibacterial effects.\textsuperscript{31,32} However, the antibacterial effect of phytosphingosine alone has never been evaluated in dogs. Furthermore, there is only one study evaluating the antimicrobial effects of a chlorhexidine–phytosphingosine shampoo.\textsuperscript{33} In the face of the known antibacterial effect of chlorhexidine, it is difficult to evaluate the antibacterial effect contributed by phytosphingosine.

Besides phytosphingosine, the effects of the product vehicle must also be considered. The vehicle of topical products can potentially impact the effectiveness of topical products via many mechanisms, including stability, solubility, viscosity,
penetration, and releasing rate of the active ingredients as well as the interactions between all the ingredients in the product.\textsuperscript{3} We consider that it is possible that the amount of the mousse applied in our study (one pump per 5 cm\textsuperscript{2} patch) was not sufficient to produce significant bacterial inhibition on the skin or hairs; however, the reason remains unclear and warrants further studies.

\subsection*{2.4.2. Hypothesis 2}

Our second hypothesis was that regardless of the hair length; mousse products would have effective distribution to skin surface and hairs, resulting in inhibition of bacterial growth.

For mousses 2 and 3, neither skin swab samples nor hair samples produced significant inhibition of bacterial growth at any time point. In contrast, for mousses 1, 4, and 5, significant inhibition of bacterial growth was noted from skin swab and hair samples from both long- and short-haired dogs at one-hour post-treatment, with residual effects persisting until Day 4 to Day 14 (Table 2.5). Based on these results, we confirmed the hypothesis that mousses 1, 4, and 5 had effective distribution to the skin surface and hairs and resulted in inhibition of bacterial growth, although not for mousses 2 or 3.

\subsection*{2.4.3. Hypothesis 3}

Our third hypothesis, which was based on the results of a previous study,\textsuperscript{26} was that mousse 4 (containing chlorhexidine gluconate 3%, climbazole 0.5%, and phytosphingosine salicyloyl 0.05%) would create a longer inhibition of bacterial growth than other mousse products.
In our study, mousses 1, 4, and 5 produced significant inhibition from the skin swab and hair samples in short-haired dogs until Day 14 (Table 2.5). In contrast, mousse 1 only produced significant inhibition from the skin swab and proximal hair samples in long-haired dogs until Day 4 and Day 10, respectively, whereas both mousses 4 and 5 still produced significant inhibition from the skin swab, proximal hair, and distal hair samples until Day 14 (Table 2.5). Therefore, while we confirmed that mousse 4 produced longer inhibition effects than mousses 1, 2, and 3, mousse 5 demonstrated the same duration of inhibition effects. Thus, our hypothesis was disproven.

It is expected that mousses 1, 4, and 5 produced significant antibacterial effects because they all contain chlorhexidine with anazole (miconazole, climbazole, and ketoconazole, respectively). Chlorhexidine has strong antibacterial activity and binds covalently to proteins in the skin, therefore, causing persistent antibacterial effects. It is one of the most commonly recommended topical treatments for superficial bacterial folliculitis and meticillin-resistant staphylococcal infections in dogs.\textsuperscript{1,6,34} In addition, since synergism against meticillin-resistant and meticillin-susceptible \textit{S. pseudintermedius} and \textit{Staphylococcus aureus} has been shown \textit{in vitro} when chlorhexidine is combined with miconazole,\textsuperscript{35} it is likely that synergism may be present in products combining chlorhexidine with other azoles. Thus, significant antibacterial effects from these products are expected.

As mentioned above in 2.4.1, we must also consider that other factors such as the vehicle or the formulation of the product may also play a crucial role in the duration of antibacterial effects, and maybe even more important than the concentration of an
active ingredient.\textsuperscript{24,36,37} For example, mousse 4 contains a higher concentration of chlorhexidine than mousse 5 (3% and 2%, respectively). Nonetheless, they both produced significant inhibition from the skin swab, proximal hair, and distal hair samples until at least Day 14 (Table 2.5). As another example, mousses 1 and 5 contain the same concentration of chlorhexidine (2%) (albeit with a different azole; miconazole 2% and ketoconazole 1%, respectively), but mousse 1 produced shorter antibacterial effects on skin swab and proximal hair samples in long-haired dogs (until Day 4 and Day 10, respectively), whereas mousse 5 produced antibacterial effects on all the samples until Day 14, regardless of hair length (Table 2.5). It is unknown whether the synergistic effect when combined with miconazole 2% in mousse 1 is weaker than when combined with ketoconazole 1% in mousse 5 as no published information is available.

In addition, mousses 1 and 5 contain differing “inactive” ingredients, making the interpretation of the antibacterial effect contribution from each ingredient difficult. For example, mousse 5 contains propylene glycol, which is an antibacterial agent as well as a solvent.\textsuperscript{3} This agent may affect the overall antibacterial effects of this product even though it is not listed as an active ingredient. Based on these findings, we also propose that the formulation likely affects the duration of antibacterial effects more than the concentration of chlorhexidine alone.

In addition, although every mousse was applied at one pump to each 5 cm\textsuperscript{2} patch, the volume of the mousse from each pump, the surface area of the mousse from each pump (smaller bubbles create a bigger surface area), the viscosity of the mousse, and the density of the hairs in each patch could have been different and thus affected the amount of mousse reaching the skin surface and likely subsequently affected the
duration of antibacterial effects. Standardization of the volume and the surface area of the mousse from each pump was not performed because the study was developed to simulate a clinical scenario where the client applies the mousse using the pump manufactured with the bottle. A rough attempt to standardize the number of hairs in contact with the mousse products was attempted by rotating each mousse through different 5 cm² patch locations; however, the hair density may still have varied in the study population.

2.5. Discussion of Additional Findings

2.5.1. Comparison of Duration of Antibacterial Effects with a Previous Study

In the current study, hair samples treated with mousses 1, 4, and 5 produced bacterial inhibition for at least 10 days. These results are similar to those reported in a previous study evaluating the same commercial mousse products.²⁶ In that study, mousse 4 was the only one that produced inhibition for 14 days, whereas in the current study, both mousses 4 and 5 produced inhibition for 14 days, regardless of hair length. The reason for the difference is unclear but may be related to the time spent massaging the products into the skin, as neither study used a specified duration of product application.

2.5.2. Differences in Antibacterial Efficacy on the Skin Surface Between Short- and Long-haired Dogs

As demonstrated in 2.3.6, skin swab samples from short-haired dogs produced bigger inhibition zones than those from long-haired dogs. Among the mousse products, the size of inhibition zones in samples treated by mousse 5 was not affected by the hair length, whereas mousses 1 and 4 produced larger inhibition zones in short-haired dogs.
than in long-haired dogs. It is possible that the amount of mousse 5 in contact with the skin surface may have been more than mousses 1 and 4 due to the differences in volume per pump, surface area of the mousse foam per pump, and viscosity of the mousse; therefore, the presence of long hair was not enough to affect the antibacterial effects from mousse 5.

2.5.3. Effects of Hair Length on Antibacterial Efficacy

Duration of antibacterial effect from mousse 1 in long-haired dogs was different between skin swab, proximal hair, and distal hair samples (until Day 4, Day 10, and Day 14, respectively) (Table 2.5), despite the lack of inhibition zone size differences between proximal and distal hair samples as shown in 2.3.5. Therefore, evaluating the hairs instead of the skin surface may overestimate the actual duration of antibacterial effects on the skin surface in long-haired dogs.

2.5.4. Lack of Antibacterial Effects of Mousse 3

Mousse 3, which contains salicylic acid 2% and ethyl lactate 10%, produced no significant inhibition of bacterial growth in any sample. This result was consistent with a previous study. However, even though this previous study did not demonstrate antibacterial effects from mousse 3, it was still reasonable to expect some antibacterial effects might be seen. The first ingredient in mousse 3, salicylic acid, is mainly known for its keratolytic activity with mild anti-pruritic and anti-inflammatory effects. It is also antibacterial and has also been shown to inhibit biofilm produced by Staphylococcus epidermidis. However, studies evaluating the antibacterial effects of this ingredient have all been performed using combination products. For example, one study evaluating antibacterial effects on S. pseudintermedius of a shampoo product (sulfur,
triclosan, and salicylic acid) found decreased colony-forming unit counts;\textsuperscript{28} one study evaluating another shampoo (sodium hypochlorite and salicylic acid) found decreased bacteria counts on skin surface cytology, decreased clinical lesion severity, and improved owner assessment scores; and another study evaluating an ear cleaner (lactic acid and salicylic acid) found 83.3\% of external ear infections due to \textit{S. pseudintermedius} resolved within two weeks.\textsuperscript{40} Since all the products evaluated in these studies contain other antibacterial ingredients, it is difficult to evaluate the specific antibacterial effects contributed by salicylic acid.

The second ingredient in mousse 3, ethyl lactate, penetrates rapidly into hair follicles and sebaceous glands and is hydrolyzed by bacterial lipases into ethanol and lactic acid.\textsuperscript{38,41} Ethanol solubilizes lipids and lactic acid and decreases the skin pH, both leading to antibacterial effects;\textsuperscript{41,42} however, both compounds are short-lived \textit{in vivo}.\textsuperscript{38} Although ethyl lactate may have antibacterial effects at high concentrations,\textsuperscript{36} studies evaluating the antibacterial effects of ethyl lactate shampoo have shown conflicting results. For example, two studies showed good clinical responses,\textsuperscript{43,44} while two studies showed questionable results or no efficacy.\textsuperscript{24,45} The ear cleaner study referenced above evaluated salicylic acid and lactic acid,\textsuperscript{40} a hydrolyzed product of ethyl lactate; however, it may not be accurate to extrapolate the antibacterial effects from lactic acid to evaluate ethyl lactate. In addition, it may not be appropriate to attribute all of the antibacterial effects to either salicylic acid or lactic acid.

Due to the lack of consistent evidence for the antibacterial effects of salicylic acid and ethyl lactate, further studies may be required to explain the absence of antibacterial effects of these ingredients in the current study.
2.5.5. Comparison of Size of the Inhibition Zones Between the Mousse Products

The size of the inhibition zones between the mousse products was not compared because it was determined to have limited value. In the Kirby–Bauer disk diffusion test, every antibiotic has a breakpoint defining susceptibility. The breakpoint is established and affected by many factors, including the diffusion rate of the antibiotic into the agar gel, which is affected by the concentration, solubility, and molecular weight of the tested antibiotic. Without information such as established breakpoints, diffusion rate, or concentration of the mousse products, the size of inhibition zones of the mousse products is not comparable to each other. Therefore, the size of inhibition zones of each mousse product was only compared to the product itself.

2.5.6. Influence of Mousse Products on Contaminant Bacteria

Contaminant bacteria were also noted in a previous study, however, only *Bacillus* spp. were documented in that study. In our study, *Bacillus cereus* and *Enterobacter cloacae* were isolated, both of which created small irregular inhibition zones different enough from the regular and large inhibition zones created by the mousse products, allowing differentiation between the two. It is not unexpected to isolate *Bacillus* spp. and *Enterobacter* spp. from canine skin because the former has been identified as normal cutaneous flora bacteria and the latter has been isolated from canine skin, respiratory tracts, and intestinal tracts.46,47

Interestingly, these bacteria were noted at varying times from samples treated with mousses 2 and 3, but typically absent until Day 10 or 14 from samples treated with mousses 1, 4, and 5. Since hair and skin swab samples treated by mousses 2 and 3 created no bacterial inhibition at any time points, it is unsurprising that the *Bacillus* spp.
and *Enterobacter* spp. were able to grow on the culture plates without hindrance. In contrast, mousses 1, 4, and 5 created significant bacterial inhibition, and the *Bacillus* spp. and *Enterobacter* spp. were only able to grow when the residual antibacterial effects decreased over the study period.

### 2.6. Limitations

A few potential limitations were identified in this study, including:

1) The number of animals: a prior sample size calculation used for this study suggested that 12 dogs would be ideal for each group (short- and long-haired dog groups). Although only eight dogs were included in the long-haired dog group due to difficulties recruiting patients in a timely manner, significant differences were detected in the expected groups, except for mousse 5; therefore, the impact of a smaller number of long-haired dogs was considered low in this study.

2) Samples collected: skin swab samples were used in this study. Although these samples may represent the skin surface, they may not replicate the real effects on the skin surface or in the hair follicles.

3) Hair density: each of the mousse products were applied to a 5 cm² patch, however, the patches were located at different anatomic areas and thus differences in hair density may have played a role in the amount of mousse distributed to the skin surface. To minimize this effect, application of the mousse was rotated between subjects. In addition, it was not feasible to standardize the hair density difference in each patient and different anatomic locations.
4) Mousse ingredients: many active and inactive ingredients were included in each mousse product, and we do not have all evidence-based information on all the interactions between the ingredients.

5) Volume and surface areas of the mousse: as previously discussed, the volume and surface areas of the mousse delivered by each pump may be different between mousse products, thus affecting the amount in contact with the skin surface.

6) Diffusion and susceptible breakpoints of mousse products: information on mousse diffusion and susceptible breakpoints are not available to determine the real antibacterial efficacy.

7) Complete canine skin microbiome: although *S. pseudintermedius*, the most common bacterium associated with canine superficial bacterial folliculitis, was used in this study to simulate a clinical scenario, the microbiome of canine skin is composed of a variety of bacteria and fungi that could contribute to pathogenicity and affect the antibacterial effect of mousse products *in vivo*.

### 2.7. Conclusions

In conclusion, this study demonstrated significant differences between mousse products both with regards to antibacterial effects as well as the duration of antibacterial effects in dogs with different hair lengths. Mousses 2 and 3 both produced no significant bacterial inhibition either on the hairs or the skin surface. Mousses 4 and 5 both significantly inhibited *S. pseudintermedius* growth *in vitro* for at least 14 days, and mousse 1 demonstrated a shorter duration in long-haired dogs than in short-haired dogs. Hair length may affect the antibacterial effects of the mousse products on the skin;
however, the differences between the mousse products are likely due to multiple factors rather than a simple failure of distribution. In addition, evaluating hairs may overestimate antibacterial effects on the skin of long-haired dogs.
CHAPTER 3. RESIDUAL ANTIBACTERIAL EFFECTS ON PLAIN PAPER DISKS FOLLOWING TREATMENT WITH THE MOUSSE PRODUCTS

3.1. Introduction

As discussed in chapter 2, the size of the inhibition zones can be impacted by both patient factors (e.g., hair length, hair density, etc.) and compound-specific factors (e.g., stability, solubility, viscosity, penetration, etc.). Therefore, we elected to directly assess the efficacy of the mousse product without the influence of the patient factors and compound-specific factors.

A literature review was conducted to find an appropriate method to standardize the quantity of mousse being applied to the Mueller–Hinton plates. A prior study evaluated the antimicrobial activity of chlorhexidine and acetic acid/boric acid impregnated cleansing wipes by placing the wipes cut into 6 mm round disks onto the culture plates.\textsuperscript{27} This was determined to not be appropriate for this study because the mousse products are liquid; thus, we modified the method by placing the mousse-impregnated disks onto the plates. Since such disks were not commercially available at the time of the study, a literature review was conducted to search for publications describing methods of standardized application of antimicrobial agents to plain paper disks. The two methods described below were considered likely candidates for the study.

The first method was originally used for disk diffusion tests for extended-spectrum $\beta$-Lactamases against $Klebsiella$ pneumoniae, $Klebsiella$ oxytoca, $Escherichia$ coli, and $Proteus$ mirabilis.\textsuperscript{48} Briefly, 10 $\mu$L of clavulanate solution was applied to a commercially available ceftazidime disk. The disk was allowed to dry for 30 minutes, after which it was placed on the agar surface.
The second method was originally used for the detection of antibiotic and drug residues in milk and dairy products.\textsuperscript{49} Briefly, a plain paper disk was removed from the container with the forceps. While holding the disk with the forceps, 90 μL of the milk sample was measured by a micropipette. Next, the plain paper disk was gently placed on the agar surface and 90 μL of the milk sample was immediately added to the disk while holding the pipet vertical to the agar surface.

The first method was more found to be more appropriate for the present study because there was a concern that the second method could result in spillover of the mousse products onto the agar plate and falsely increase the size of the inhibition zone.

The test results of the paper disk method were used to compare with the inhibition zones created by hair samples (whole hair shafts from short-haired dogs and proximal hairs from long-haired dogs) in chapter 2.3. We hypothesized that paper disk samples would produce bigger inhibition zones than the hair samples, indicating that patient factors and compound-specific factors affect the antibacterial effects of mousse products on the skin surface.

3.2. Materials and Methods

To ensure the mousse products had sufficient contact with the Mueller–Hinton plates, we attempted to find the maximal volume of mousse products that a plain paper disk could absorb. Therefore, the following two methods were conducted and compared.

3.2.1 Disks Applied with 20 μL of Mousse Products (DT)

1) Five mL of mousse product was aspirated and placed into a clean glass test tube with a plastic disposable dropper.

2) The agent was allowed to sit for 30 minutes to allow the bubbles to burst
3) A 6-mm plain paper disk was removed from the container with flame-sterilized forceps and was placed on a clean petri dish.

4) Ten μL, 15 μL, 20 μL, and 25 μL of mousse were measured with the micropipette and applied directly to a disk. Following a few test applications, the 20 μL volume was determined as the best option because it did not create a spillover of the mousse product.

5) The disks were allowed to dry for 30 minutes.

6) The disk was gently placed on a Mueller–Hinton agar plates (Remel, Thermo Fisher Scientific; Lenexa, KS, USA) inoculated with a 0.5 McFarland solution containing non-antibiotic resistant \textit{S. pseudintermedius} (American Type Culture Collection 49444), as previously described in chapter 2.

7) The same procedure was repeated for each mousse product, and each plate was large enough to have two disks placed.

8) The plates were incubated for 24 hours at 37°C.

9) After incubation, the extent of inhibition was assessed by measuring the distance from each side of the disk to the edge of bacterial growth inhibition; therefore, each sample had two measurements.

3.2.2. Disks Soaked in Mousse Products (DS)

The second method directly soaked the disks in mousse products to be compared with the disks applied with 20 μL (DT).

1) 5 mL of mousse product was aspirated and placed into a clean glass test tube with a plastic disposable dropper.
2) The sample was held for 30 minutes to allow the bubbles in the mousse to burst.

3) A 6-mm plain paper disk was removed from the container with flame-sterilized forceps.

4) The disk was placed and soaked in the glass test tube containing the mousse product, and the sample was held for 30 minutes to allow the mousse to be absorbed.

5) 30 minutes were allowed for the mousse to be absorbed.

6) The disk was removed from the glass test tube and placed onto a clean petri dish.

7) 30 minutes were allowed for the mousse to be dry enough.

8) The disk was gently placed on the Mueller–Hinton agar plates inoculated with a 0.5 McFarland solution containing non-antibiotic resistant *S. pseudintermedius* (American Type Culture Collection 49444), as previously described in chapter 2.

9) The same procedure was repeated for each mousse product. Each plate was large enough to have two disks.

10) The plates were incubated for 24 hours at 37°C.

11) After incubation, the extent of inhibition was assessed by measuring the distance from each side of the disk to the edge of bacterial growth inhibition; therefore, each sample had two measurements.
3.2.3. Analysis of Results

SPSS Statistics v28.0 for MacOS (IBM Corp.; Armonk, NY, USA) was used for statistical analysis. A one-way ANOVA was used to evaluate the sizes of the inhibition zones created by disks applied with 20 μL of mousse products (DT, chapter 3.2), disks soaked in mousse products (DS, chapter 3.2.2), and hair samples (whole hair shafts from short-haired dogs and proximal hairs from long-haired dogs, chapter 2.3). LSD post hoc tests were used when equal variances were assumed. The significance threshold was set to 0.05, with the hypothesis test two-sided.

3.3. Results

In mousse 1, 2, 4, and 5, both DT and DS samples produced clear and smooth inhibition zones, while in mousse 3, both DT and DS samples produced a concentric “target-like” zone of bacterial inhibition as shown in Figure 3.1.

Figure 3.1. A paper disk treated with mousse 3 demonstrated a target zone composed of four concentric rings. A complete inhibition zone (CI, red) closest to the paper disk was followed by a partial inhibition zone (PI-1, purple), followed by a non-inhibition zone (NI, green), and finally followed by another partial inhibition zone (PI-2, yellow).
The target zone was composed of four concentric rings including a complete inhibition zone (CI, red) closest to the paper disk, a partial inhibition zone (PI-1, purple), a non-inhibition zone (NI, green), and another partial inhibition zone (PI-2, yellow). Only the CI width was measured and documented as suggested by the standard from the American Society for Microbiology and the Clinical and Laboratory Standards Institute.\textsuperscript{29,30}

Measurements of the inhibition zones of disks applied with 20 μL of mousse products, disks soaked in mousse products, and hair samples are summarized in Table 3.1.

Table 3.1. Measurements of inhibition zones of disks applied with 20 μL of mousse products, disks soaked in mousse products, and hair samples.

<table>
<thead>
<tr>
<th>Mousse</th>
<th>Disks Applied with 20 μL of Mousse Products (DT)</th>
<th>Disks Soaked in Mousse Products (DS)</th>
<th>Hair samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.16 ± 0.71 (10.31, 11.98)</td>
<td>10.5 ± 0.25 (10.25, 10.84)</td>
<td>6.74 ± 1.44  (3.54, 9.49)</td>
</tr>
<tr>
<td>2</td>
<td>4.55 ± 0.25 (4.34, 4.90)</td>
<td>4.56 ± 0.21 (4.31, 4.78)</td>
<td>0 ± 0        (0, 0)</td>
</tr>
<tr>
<td>3</td>
<td>1.34 ± 0.14 (1.17, 1.46)</td>
<td>1.74 ± 0.15 (1.53, 1.9)</td>
<td>0 ± 0        (0, 0)</td>
</tr>
<tr>
<td>4</td>
<td>10.17 ± 0.21 (10.02, 10.46)</td>
<td>10.38 ± 0.2  (10.13, 10.59)</td>
<td>7.3 ± 1.3    (4.59, 9.52)</td>
</tr>
<tr>
<td>5</td>
<td>9.31 ± 0.35 (8.98, 9.62)</td>
<td>9.52 ± 0.21 (9.28, 9.77)</td>
<td>7.37 ± 1.93  (4.63, 12.07)</td>
</tr>
</tbody>
</table>

Mean ± standard deviation (minimum, maximum) in millimeters.
No difference was noted in the size of inhibition zones between DT and DS \((p = 0.976)\). Both DT and DS samples produced bigger inhibition zones than the hair samples \((both \ p < 0.001)\).

3.4. Discussion

3.4.1 Comparison of DT and DS methods

The two methods, applying 20 \(\mu\)L to the disk (DT) and directly soaking the disks (DS), showed no difference in the size of inhibition zones, indicating 20 \(\mu\)L is likely the maximal amount a plain paper disk can absorb to ensure sufficient contact with the Mueller–Hinton agar without spillover to the agar. Therefore, both methods were considered appropriate to evaluate the direct effects of the mousse products.

3.4.2 Hypothesis and Relevant Discussion

The results also proved our hypothesis that paper disk samples (both DT and DS) produced bigger inhibition zones than the hair samples, indicating the patient factors and compound-specific factors decreased the antibacterial effects of mousse products on the skin surface, as previously discussed in chapter 2.4. Of note, in mousse 5, although a difference was noted in the size of inhibition zones between DT and hair samples, the \(p\)-value was close to insignificance \((p = 0.047)\). In addition, as previously shown in chapters 2.3.6 and 2.5.2, in skin swab samples treated by mousse 5, the size of inhibition zones was not different in short-haired dogs versus long-haired dogs. Putting these together could imply that the distribution of mousse 5 was likely less affected by these factors.
3.4.3 Inhibition Zones Produced by Samples Treated with Mousses 2 and 3

Interestingly, in contrast to the lack of bacterial inhibition from skin swab and hair samples treated by mousses 2 and 3 (as described in CHAPTER 2), bacterial inhibition was noted from the DT and DS samples treated by these two products. It is possible that the patient factors and compound-specific factors decreased the antibacterial effects, so the antibacterial effects could only be seen when the mousses had direct contact (DT and DS) with the Mueller–Hinton plates. Considering the clinical scenario, it is possible that applying a large amount of these mousse products to the skin to ensure sufficient contact with the skin surface may overcome the effects from patient factors and compound-specific factors.

Of note, mousse 2 was able to produce bacterial inhibition in DT and DS samples. This product was selected to be a negative control in CHAPTER 2 based on a previous study demonstrating no significant bacterial inhibition from this product.26 The results in CHAPTER 2 were consistent with the previous study; however, the active ingredient, phytosphingosine HCl 0.05%, has been reported to have antibacterial effects.31,32 Based on the bacterial inhibition noted from DT and DS samples, we propose that this product is not ideal as a control treatment for future studies.

3.4.4 The “Target-like” Zone Produced by DT and DS Samples Treated with Mousse 3

As shown in Figure 3.1, a special type of concentric inhibition zones, which is target-shaped, was noted in DT and DS samples treated by mousse 3. The target zone was composed of four concentric zones including a complete inhibition zone (CI) closest to the paper disk, a partial inhibition zone (PI-1), a non-inhibition zone (NI), and another partial inhibition zone (PI-2).
A “target-like” zone of two concentric rings has been noted in inducibly cefoxitin-resistant *Macrococcus*-like organism: an outer complete inhibition zone at minimum inhibitory concentration (MIC) surrounding an inner non-inhibition zone at supra-MIC levels.\(^{50}\) An even more similar phenomenon has been documented in a study evaluating triclosan.\(^{51}\) A “target-like” zone of three concentric rings was noted from the triclosan-treated disk on the plate pre-streaked with *Enterococcus faecalis*. It was proposed that a concentration-dependent resistance to triclosan was induced by activating multi-drug efflux system: a very high concentration of triclosan inhibited bacterial growth, followed by a lower concentration that activated the multi-drug efflux system allowing bacterial growth, followed by a lower concentration that was not enough to activate the efflux therefore bacterial inhibition was seen, and finally followed by minimal concentration or none of triclosan therefore no bacterial inhibition. This may explain the findings in our study: the concentration of mousse 3 ingredients (salicylic acid and ethyl lactate) at CI was high enough to completely inhibit bacterial growth, followed by the concentration at PI-1 that partially inhibited bacterial growth, the concentration at NI that induced resistance, and finally the concentration at PI-2 that was not able to induce resistance but able to partially inhibit bacterial growth.

The “target-like” zone phenomenon in Kirby–Bauer tests is conceptually similar to the skip wells phenomenon in broth microdilution tests in which no bacterial growth is seen in wells with lower antimicrobial concentrations although growth did occur at higher concentrations. The skip wells phenomenon may indicate hetero-resistance or selection of resistant mutants and has been documented in broth microdilution tests of polymyxin B for *Enterobacter* spp., *Pseudomonas* spp., *Acinetobacter* spp., and colistin.

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for \textit{Enterobacter} spp.\textsuperscript{52-56} When all of these factors are considered together, we propose that the “target-like” zone shown in our study is most likely due to hetero-resistance or selection of mutant bacteria, possibly induced by the concentration gradient of salicylic acid and/or ethyl lactate in mousse 3.

\section*{3.5. Conclusions}

In conclusion, patient factors (e.g., hair length, hair density, etc.) and compound-specific factors (e.g., stability, solubility, viscosity, penetration, etc.) resulted in decreased antibacterial effects of the mousse products on the skin surface when compared to antibacterial effects on the hairs. Mousses 2 and 3 demonstrated antibacterial effects when they were directly in contact with treatment target.
CHAPTER 4. FINAL DISCUSSION AND CONCLUSIONS

In the tested mousse products, mousse 4 (DOUXO® Chlorhexidine Mousse, Ceva Animal Health LLC; Lenexa, KS, USA), which contains chlorhexidine gluconate 3%, climbazole 0.5%, and phytosphingosine salicyloyl 0.05%, and mousse 5 (Phyto CHX+KET Antiseptic Mousse, Covetrus; Dublin, OH, USA), which contains chlorhexidine gluconate 2%, ketoconazole 1% and phytosphingosine salicyloyl 0.05%, both produced inhibition of *S. pseudintermedius* growth on the skin and hairs for at least 14 days regardless of hair length. These results indicate that these mousses penetrate the skin surface and have persistent antibacterial effects. In contrast, mousse 1 (MiconaHex+Triz™ Mousse, Dechra Veterinary Products; Overland Park, KS, USA), which contains chlorhexidine gluconate 2%, miconazole nitrate 2%, and tromethamine and disodium EDTA (TrizEDTA®), produced shorter duration inhibition on the skin and hairs in long-haired compared to short-haired dogs; mousse 2 (DOUXO® Calm Mousse, Ceva Animal Health LLC; Lenexa, KS, USA; control mousse), which contains phytosphingosine HCl 0.05%, and mousse 3 (BioSeb™ Mousse, VetBiotek; Largo, FL, USA), which contains salicylic acid 2% and ethyl lactate 10%, had no bacterial inhibition on the hairs or the skin surface. The lack of antibacterial effects was likely due to the effects from patient factors (e.g., hair length, hair density, organic materials on the hairs, etc.) and compound-specific factors (e.g., stability, solubility, viscosity, penetration, etc.); however, these products may still demonstrate antibacterial effects when a large amount is applied and allowed sufficient contact with the skin surface. We also concluded that evaluating hairs may over-estimate actual antibacterial effects on the skin surface, especially in long-haired dogs.
REFERENCE LIST


VITA

Chi-Yen Wu is the son of Mrs. Yu-Chin Tsai and Mr. Ming-Chien Wu. He was born and raised in Chiayi City, Taiwan alongside his elder brother, Cheng-Han Wu. He obtained his Doctor of Veterinary Medicine degree from the National Taiwan University (NTU) in 2015. Following graduation, he completed a small animal rotating internship at the NTU Veterinary Hospital in 2017. He was accepted into the Louisiana State University School of Veterinary Medicine (LSU SVM) to complete the Program for the Assessment of Veterinary Education Equivalence in 2018. Throughout the year at the LSU SVM, his passion for veterinary dermatology became evident. He was offered a veterinary dermatology internship followed by a joint veterinary dermatology residency and a Master of Science program at the LSU SVM. He is anticipated to graduate with a Master of Science degree in Biomedical and Veterinary Medical Sciences (Veterinary Clinical Sciences) in August 2022 and take the board certifying examination of the American College of Veterinary Dermatology in November 2023. Upon completing his master’s program and residency training, he expects to dedicate himself to veterinary dermatology in clinics, research, and education in the United States, Taiwan, and worldwide.